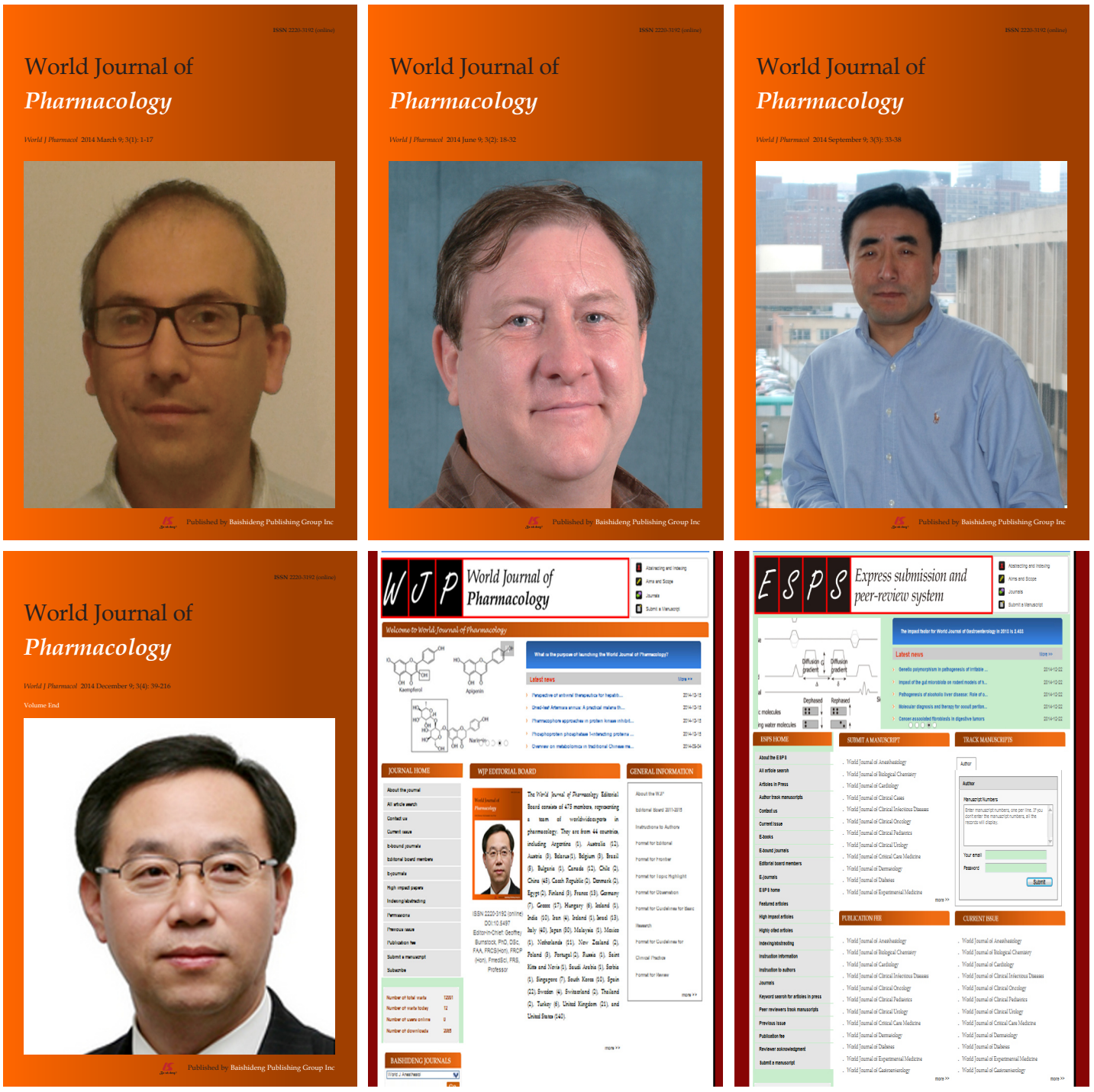
ISSN 2220-3192 (online)

World Journal of *Pharmacology*

2014 Bound Volume 3 Issue 1-4: 1-216



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WJP World Journal of Pharmacology

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ISSN 2220-3192 (online)

World Journal of *Pharmacology*

World J Pharmacol 2014 March 9; 3(1): 1-17

Published by Baishideng Publishing Group Inc

WJP

World Journal of Pharmacology

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Quarterly Volume 3 Number 1 March 9, 2014

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World Journal of Pharmacology

ISSN
ISSN 2220-3192 (online)

LAUNCH DATE
February 9, 2012

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PUBLICATION DATE
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World J Pharmacol 2014 March 9; 3(1): 1-17
ISSN 2220-3192 (online)
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Implantable (Bio)sensors as new tools for wireless monitoring of brain neurochemistry in real time

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Author contributions: Farina D and Calia G contributed to acetylcholine biosensor and references reorganization; Alvau MD and Puggioni G contributed to glucose and lactate biosensors; Bazzu G and Migheli R contributed to dopamine, norepinephrine, and serotonin microsensors; Rocchitta G contributed to glutamate biosensor, ascorbic acid microsensor, ethanol biosensor, and biotelemetry; Sechi O contributed to oxygen and nitric oxide microsensors and ethanol biosensor; Desole MS and Serra PA contributed to title, abstract, introduction, conclusion, references reorganization, and manuscript overview; Farina D and Alvau MD equally contributed to this study.

Supported by The Regione autonoma della Sardegna (fund P. O. R. SARDEGNA F. S. E. 2007-2013-Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l. 3. 1)

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Received: January 15, 2014 Revised: March 3, 2014
Accepted: March 6, 2014
Published online: March 9, 2014

Abstract

Implantable electrochemical microsensors are characterized by high sensitivity, while amperometric biosensors are very selective in virtue of the biological detecting element. Each sensor, specific for every neurochemical species, is a miniaturized high-technology device resulting from the combination of several factors: electrode material, shielding polymers, applied electrochemical technique, and in the case of biosensors, biological sensing material, stabilizers, and entrapping chemical nets. In this paper, we summarize the available technology for the *in vivo* electrochemical monitoring of neurotransmitters (dopamine, norepinephrine, serotonin, acetylcholine, and glutamate), bioenergetic substrates (glucose, lactate, and oxygen), neuromodulators (ascorbic acid and nitric oxide), and exogenous molecules such as ethanol. We also describe the most represented biotelemetric technologies in order to wirelessly transmit the signals of the above-listed neurochemicals. Implantable (Bio)sensors, integrated into miniaturized telemetry systems, represent a new generation of analytical tools that could be used for studying the brain's physiology and pathophysiology and the effects of different drugs (or toxic chemicals such as ethanol) on neurochemical systems.

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Key words: Electrochemical microsensors; Amperometric biosensors; Neurotransmitters; Bioenergetic substrates; Wireless biotelemetric technologies

Core tip: Electrochemical microsensors and amperometric biosensors arouse enormous scientific interest because of their low-cost technology and because they guarantee real-time monitoring of changes of the most important brain compounds. In conjunction with miniaturized telemetric devices, the electrochemical sensors, allow the neurochemical monitoring of extracellular space of discrete brain regions in awake, untethered animals for days or weeks. This new scientific approach opens new frontiers for studying the physiological and physiopathological pathways in wild-type animals and in genetic models of the most widespread neurodegenerative diseases.

Farina D, Alvau MD, Puggioni G, Calia G, Bazzu G, Migheli R, Sechi O, Rocchitta G, Desole MS, Serra PA. Implantable (Bio)sensors as new tools for wireless monitoring of brain neurochemistry in real time. *World J Pharmacol* 2014; 3(1): 1-17

Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i1/1.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i1.1

INTRODUCTION

The identification, observation, and quantification of extracellular biomolecules in the central nervous system (CNS) is a field of growing interest for studying the brain in physiological conditions and for identifying neurochemical changes during neurological diseases. The study of neurochemistry in real time is very important in preclinical (and recently also in clinical) research and for developing new therapeutic strategies for many neuropsychiatric diseases, such as schizophrenia, depression, epilepsy, multiple sclerosis, and neurodegenerative diseases (*i.e.*, Parkinson's and Alzheimer's diseases), and also for neural conditions that deeply influence individual and social behavior such as addiction.

For decades, the extracellular neurochemistry of the CNS has been studied using *in vivo* microdialysis. Microdialysis is a minimally invasive technique suitable for measuring low-molecular-weight compounds in the extracellular compartment of several organs, tissues, or specific brain regions[1]. The microdialysis idea originated in the 1970s with the aim of implanting a hollow dialysis fiber (microdialysis probe) into a tissue for simulating the role of a blood capillary and recovering molecules from the extracellular compartment to highlight their regional changes in concentration[2,3]. When implanted in the brain, the microdialysis probe is perfused with an appropriate Ringer solution (that mimics the composition of the extracellular space fluid) so that neurochemicals are able to diffuse down their concentration gradients out of the probe. The recovered microdialysis samples are analyzed using different analytical methods. The poor temporal resolution and the need to have an available expensive analytical laboratory (for analyzing microdialysis samples) represent the major limitations of this technique.

In recent decades, implantable electrochemical sensors and biosensors have been emerging because of their versatility, their multiple applications, and most of all, their high spatial and temporal resolution[4-6]. In particular, implantable amperometric sensors have been proven to be very sensitive so as to allow the detection of very low concentrations of the studied analytes[5]. The basic idea of implantable electrochemical sensors is to "concentrate" an entire analytical laboratory "on the tip of a pin" without the need of an expensive analytical apparatus or of a dedicated laboratory.

In the past years, despite their high sensitivity, the main limitation for the use of electrochemical sensors was related to their poor selectivity. Recently, the development of new sensing materials and new shielding polymers and, mainly, the introduction of biological elements such as molecular recognition sites have allowed overcoming this limitation in a large part.

Today, each sensor, specific for every neurochemical species, is a miniaturized high-technology device resulting from the combination of several factors: electrode material, shielding polymers, applied electrochemical technique, and in the case of biosensors, biological sensing material, stabilizers, and entrapping chemical nets.

The dimensions of implantable electrochemical sensors vary from a few micrometers (5-10) up to 125 μm (always lower than those of a microdialysis probe, around 220 μm), and their sensing surface can be increased without increasing their invasiveness using new nanomaterials (*i.e.*, carbon nanotubes); this process is often indicated as "nanostructuration" or simply "nano-on-micro". But one of the most exciting perspectives, for future development and applications, is to combine implantable sensors with miniaturized electronic devices in order to transmit neurochemical signals at a distance so that awake animals are allowed to be totally free to move[4-6].

In this study, we highlight the state-of-art of electrochemical microsensors and biosensors, already used in preclinical research for recording neurochemical changes, suitable to be integrated in biotelemetry systems for the wireless monitoring of brain neurochemistry.

IMPLANTABLE (BIO)SENSORS

We have chosen to describe the available technology for the *in vivo* electrochemical monitoring of neurotransmitters (dopamine, norepinephrine, serotonin, acetylcholine, and glutamate), bioenergetic substrates (glucose, lactate, and oxygen), neuromodulators (ascorbic acid and nitric oxide), and exogenous molecules such as ethanol. In the next section, we also describe the most represented biotelemetric technologies to combine with the sensors in order to wirelessly transmit the signals of the above-listed neurochemicals.

Dopamine, Norepinephrine, and Serotonin

Brain neurotransmitters such as the tyrosine derivatives dopamine, norepinephrine and the neuroactive tryptophan derivative serotonin have been implicated in the neurochemistry and physiology of mental diseases and neurological disorders.

Catecholamine biosynthesis is a common pathway from tyrosine[7], where the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine by tyrosine hydroxylase is the rate-limiting step. Dopamine, a catechol-like neurotransmitter derived by L-3,4-dihydroxyphenylalanine decarboxylation, is actively involved in reward pathways[8,9] and in cognitive functions[10]. Its metabolism mainly occurs by reaction with monoamine oxidase and catechol-O-methyltransferase with the formation of dihydroxyphenylacetic acid , homovanillic acid, and 3-methoxytyramine. Neuronal death of catecholaminergic cells in the substantia nigra, with a consequent significant reduction of dopamine levels[11] as well as dihydroxyphenylacetic acid, homovanillic acid[12] and

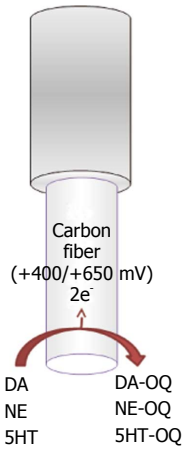

Figure 1 Schematic representation of the carbon-based microsensor used for detecting dopamine, norepinephrine, and 5-hydroxytryptamine in the central nervous system of awake, freely moving animals. DA: Dopamine; NE: Norepinephrine; 5TH: 5-Hydroxytryptamine, serotonin; DA-OQ: DA-derived orthoquinones; NE-OQ: NE-derived orthoquinones; 5HT-OQ: 5HT-derived orthoquinones.

3-methoxytyramine[13] in the striatum is a hallmark in Parkinson's disease[1]. On the other hand, an increase in dopaminergic levels is involved in the etiopathogenesis of schizophrenia[14,15].

Formed by β-hydroxylation of dopamine, norepinephrine plays multiple roles as a hormone and a neurotransmitter. Norepinephrine is involved in directly increasing heart rate, suppressing neuroinflammation[16], and triggering the glycogenolysis and the release of glucose from energy stores[17], and along with serotonin, it is implicated in depression and anxiety disorders[18]. Moreover, the serotonergic system is also implicated in several neuroregulatory processes such as stress, aggression, pain, sleep, appetite, reproduction, circadian rhythm, and cardiovascular and respiratory functions[19].

All of these compounds are electrochemically active, show a similar 2-electron oxidation reaction with similar peak potentials at physiological pH, and can be directly detected by electrochemical oxidation of the molecule[20].

$$\text{DA (Ox)} \rightarrow \text{DA-QUINONE} + 2e^- + 2H^+ \quad (1)$$

$$\text{NE (Ox)} \rightarrow \text{NE-QUINONE} + 2e^- + 2H^+ \quad (2)$$

$$\text{SEROTONIN (Ox)} \rightarrow \text{SEROTONIN-QUINONE} + 2e^- + 2H^+ \quad (3)$$

The electroactive neurotransmitters can be directly detected *in vitro* and *in vivo* using different electrochemical techniques (Figure 1) such as constant potential amperometry (CPA)[21], chronoamperometry[22,23], differential pulse voltammetry (DPV)[24], and fast-scan cyclic voltammetry (FSCV)[8,25-27]. Different microelectrodes for voltammetric recordings in the CNS are available, such as carbon paste microelectrodes, where carbon powder is mixed with silicon oil[10]; epoxy carbon microelectrodes, where epoxy resin is mixed with carbon paste; and carbon fiber, gold, and platinum (Pt) microelectrodes[20].

Along carbon-fiber microelectrodes, FSCV is the most common technique used for dopamine, norepinephrine and serotonin *in vivo* monitoring.

Carbon-fiber microelectrodes (Figure 1) are made by inserting a carbon fiber (outer diameter ranging between 5 and 30 μm, most commonly about 7 μm) into a glass capillary, which is pulled with a pipette puller and sealed by epoxy resin with 25 to 100 μm of the fiber protruding from the glass. The final geometry of the electrode, cylindrical[28] or disk shaped[29], is obtained by cutting or polishing the protruding carbon fiber[30]. Because of their dimension, carbon-fiber microelectrodes minimize distortion caused by ohmic drop, and then, coupled with a minimal tissue damages when implanted into the brain, they are suitable for high-temporal-resolution measurements[28]. In addition, a 7 μm carbon fiber does not stimulate glial reaction[25], in agreement with the evidence that probes that are less than 12 μm in diameter are not encapsulated as demonstrated by previous studies[31]. FSCV is a technique with high resolution and selectivity, where the potential applied to the microsensor is cycled between the reduction and the oxidation peaks of the analyte of interest[20]. For dopamine and norepinephrine recordings, a scan rate in a triangle fashion at 400 V/s is applied. The potential of the carbon-fiber microelectrode is ramped linearly from -400 mV *vs* Ag/AgCl to +1.3 V and back and held at -400 mV between scans[32]. To obtain the 5HT recording, an N-waveform scan rate is used, in which the applied potential is scanned first from 0 mV to +1200 mV then to -600 mV and back to 0 *vs* Ag/AgCl[27]. Typically, the waveform is applied for 10 ms, and voltammetric scans are repeated at 100 ms intervals. During the anodic sweep, the catecholamine (dopamine and/or norepinephrine) and serotonin present at the electrode surface are oxidized into corresponding orthoquinone and then reduced back at the original form during the cathodic sweep. The number of molecules that undergo electrolysis is directly proportional to the measured current[21]. The peak positions during oxidation and the reduction sweep as well as the peak shape can be used to distinguish different analytes[33].

Using fast-scan cyclic voltammetry, dopamine, norepinephrine, and serotonin have been shown a similar oxidation peak at approximately +650 mV *vs* Ag/AgCl[33-35] and a single reduction peak around -200 mV for dopamine and norepinephrine or Wdouble reduction peaks around 0 and -500 mV *vs* Ag/AgCl for serotonin[27].

Because they are virtually identical, voltammograms alone cannot be used to distinguish dopamine and norepinephrine[36], but histology and pharmacology, such as the use of dopamine drugs (raclopride, GBR 12909), can aid in this distinction even in simultaneous measurements with FSCV[37]. Ascorbic acid is the main electroactive interference molecule in the extracellular fluid (ECF) of the brain for electrochemical measurements. Ascorbic acid is 10^4-10^6 times higher than the concentrations of catecholamines in the ECF of the brain, and its concentration is approximately 0.5 mmol/L[37,38]. The carbon-fiber microsensor selectivity for catecholamines can be enhanced

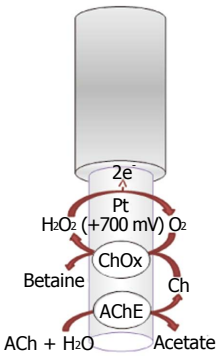

Figure 2 Schematic representation of the platinum-based biosensor used for detecting acetylcholine in the brain of awake, freely moving animals. ACh: Acetylcholine; Ch: Choline; ChOx: Choline oxidase; AChE: Acetylcholinesterase.

by applying on fibers a negatively charged resin (Nafion) able to concentrate cations such as dopamine on the active surface of the sensor and, at the same time, to repel anions such as ascorbic acid and dihydroxyphenylacetic acid[22,39].

Although carbon-fiber microelectrodes are the most used sensors for dopamine and norepinephrine for *in vivo* recording, new strategies are developed to monitor catecholamines real time in the brain.

As recently suggested by Njagi *et al*[40], an amperometric biosensor can be fabricated deposing an enzyme, such as tyrosinase, onto the surface of a carbon-fiber electrode. The enzyme immobilized in a biocompatible matrix and with a final diameter of about 100 μm provides an alternative to FSCV for *in vivo* monitoring of dopamine[40].

Acetylcholine

The neurotransmitter acetylcholine and its metabolite choline play a critical role in various functions of the CNS[41]. The concentration of acetylcholine in the ECF of the brain is 0.1-6 nmol/L[42]; the abnormalities in their concentrations are related to several neural diseases[43]. In particular, it is involved in learning and memory formation[44], in the development and maintenance of addiction[45], and in neurodegenerative disorders such as Alzheimer's disease[46] and Parkinson's disease[47,48]; dysregulation of cholinergic transmission is correlated to cognitive alterations such as those manifested in Alzheimer's disease[49]. Furthermore, organophosphorus (OP) and carbamate pesticides and neurotoxic compounds are capable to inhibit the acetylcholinesterase enzyme (AChE), which is responsible of the hydrolysis of acetylcholine[50].

Therefore, the *in vivo* determination of acetylcholine and choline is important because a rapid and an effective method for simultaneous determination of levels of acetylcholine and choline is needed for the characterization of cholinergic transmission in normal and pathological physiology[51,52]. The most common methods developed for the simultaneous determination of acetylcholine and choline require a conversion into more easily detectable compounds[52].

A lot of strategies have been used to obtain selective detection for acetylcholine and choline with biosensors. Among all acetylcholinesterase-based biosensors, amperometric acetylcholinesterase/choline oxidase (ChOx) biosensor is especially performing because of its potential high sensitivity, reproducibility, and excellent selectivity for *in vivo* simultaneous determination of neurotransmitters; these devices are usable for *in situ* determination of choline and acetylcholine and have been implanted in rat brain[51]. The working mechanism of acetylcholinesterase (Figure 2) is based on the following biochemical reaction[53]:

$$ACh + H_2O \rightarrow Ch + acetate \quad (4)$$

While the choline, in the presence of oxygen, is oxidized by choline oxidase, forming hydrogen peroxide (H_2O_2), which can be easily oxidized onto electrode surface:

$$Ch + H_2O + 2O_2 \rightarrow betaine + 2H_2O_2 \quad (5)$$

The oxidation current of hydrogen peroxide can be used for the evaluation of acetylcholine, choline, and acetylcholinesterase activity. Acetylcholine signal is attenuated by acetylcholinesterase inhibitors such as neostigmine or physostigmine[54,55]. The enzymes acetylcholinesterase and choline oxidase are immobilized on the solid electrode surface such as platinum-iridium (Pt/Ir)[51,56] (Figure 2) or carbon fibers[57]. In order to prevent signal of interferents, different shielding strategies are currently used different. For example, ascorbate oxidase (AAO) is used to minimize interference from ascorbic acid, which is present in relatively high concentrations in the brain ECF[58]; polymeric films are also used onto the sensor surface that limit the access of potential interferences due to electrostatic repulsion (*e.g.*, Nafion) and nonconducting polymers [*e.g.*, poly-(phenylenediamines) (PPD)] that restrict the permeability of small organic molecules (*e.g.*, major interferences ascorbate and urate) while retaining a high permeability to small species such as hydrogen peroxide[59]. The acetylcholinesterase/choline oxidase layer is trapped onto the surface electrode by the cross-linking of amino groups of the enzymes with glutaraldehyde[51]. Moreover, the enzyme layer also includes bovine serum albumin (BSA) that provides stabilization of the enzyme activity in the immobilized state[51].

Hence, the amperometric sensors for acetylcholine and choline are successfully applied and provide a useful tool to analyze basic mechanisms of cholinergic physiology in normal and pathological conditions and those involved in the activity of pharmacological cholinergic drugs.

Glutamate and ascorbic acid

Even if glutamate is a nonessential amino acid, it has been shown to be the most abundant in the brain. As fully described, glutamate represents the most important excitatory neurotransmitter. In plasma, glutamate concentrations reach 50-100 μmol/L while in the whole brain, they are 10-12 mmol/L, but we must take into account that glutamate reaches only 0.5-2.0 μmol/L in ECFs[60].

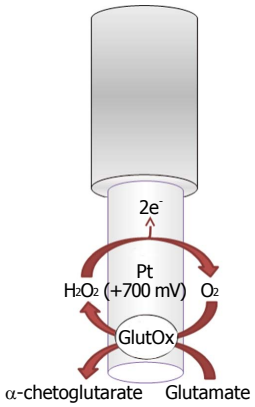

Figure 3 Scheme of glutamate biosensor. The transducer is made of a platinum (Pt) wire that immobilizes the glutamate oxidase (GluOx) enzyme that selectively transforms glutamate in alpha-ketoglutarate, producing H_2O_2 that is then oxidized on the Pt surface.

Glutamate is well known to be involved in most phases of normal brain functions such as memory and learning, cognition, cell migration, differentiation, and death; but at the same time, it is known to play important roles as a highly toxic endogenous excitotoxin[61]. Recently, some authors have highlighted its involvement not only in the development of the CNS, particularly related to neuronal survival, growth, and differentiation, but also in the development of several circuits[62]. In this regard, for example, it has been widely shown that low glutamate levels during neurogenesis may have a key role in the development of schizophrenia[63], and high glutamate levels can also interfere with astroglial proliferation and neuronal differentiation[61]. Glutamate has been of particular importance because of its possible involvement in neurodegenerative diseases such as amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, and others. In fact, the chronic overexcitation of neurons, stimulated by glutamate, is a newer concept that has linked glutamate excitotoxicity to neurodegeneration in amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and Alzheimer's dementia[64].

The importance of glutamate has generated a strong interest in the development of several tools for the detection of this amino acid. Different methods have been developed to determine glutamate, including optical methods, patch clamp, and microdialysis[65], but also including fluorometric, chromatographic, or spectrophotometric techniques, which, however, have some intrinsic limitations, such as being time-consuming, requiring pretreatment of the sample, being labor intensive, and requiring skilled handling. Nowadays, electrochemical methods are considered as one of the most promising approaches because of easiness, high spatial resolution, high sensitivity, and specificity[66]. From the neurochemical point of view, a wide range of amperometric biosensor designs, based mainly on glutamate oxidase enzyme loading [GluOx; molecular weight, 140 kDa; solution Michaelis constant (KM), 0.21 mmol/L in neutral buffer; pI, 6.2], have been developed[67-75].

The aim of monitoring brain glutamate using amperometric biosensors, however, is very challenging, mainly because the baseline ECF concentration of glutamate is estimated to be $\leqslant 5$ μmol/L[76-103].

Glutamate oxidase-based biosensors (Figure 3) exploit the capability of the oxidase to selectively convert L-glutamate as follows:

$$\text{L-glutamate} + H_2O + \text{GluOx/FAD} \rightarrow$$
$$\alpha\text{-ketoglutarate} + NH_3 + \text{GluOx/FADH}_2 \quad (6)$$

$$\text{GluOx/FADH}_2 + O_2 \rightarrow \text{GluOx/FAD} + H_2O_2 \quad (7)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (8)$$

The byproduct hydrogen peroxide is then oxidized, on the transducer surface, by applying a positive potential generating a current flow directly proportional to the glutamate concentrations.

Pt generally is the electrode material of choice for electrooxidation of hydrogen peroxide[77,78]. Various strategies are as well realized in order to shield the biosensor from electroactive interfering substances that usually occur in ECF: first of all, ascorbic acid, through the electrochemical deposition of polymers[68-74]; the use of anionic substances such as Nafion[68,70,79]; or the coimmobilization of the ascorbate oxidase enzyme[75].

The amperometric biosensors have been proven to be interesting devices for *in vivo* measurement of glutamate concentrations and also for their response time, which has been estimated to be about a few seconds[73,74], making these biosensors suitable for the study of the rapid changes in the concentrations of glutamate both in physiological conditions or during pharmacological treatments.

Ascorbic acid is a water-soluble vitamin. It is widely known for its role as an antioxidant, but it is as much recognized as a cofactor in several enzymatic reactions, including those concerning the synthesis of catecholamines, carnitine, or cholesterol[80].

Because humans are lacking the enzyme L-gulono-1,4-lactone oxidase, they cannot synthesize ascorbate, so they, therefore, have efficient machineries for both absorption and recycling of this vitamin[81]. Among them is the transporter sodium-dependent vitamin C transporter-1 (SVCT1) involved in the body homeostasis of ascorbic acid, and the transporter SVCT2 that is necessary for the defense of active cells against oxidative stress[82]. Even the ubiquitous GLUT-type glutamate transporters play a key role in the homeostasis of this vitamin inasmuch as they are involved in the uptake of dehydroascorbate, the oxidized form of ascorbate, in order to be recycled to ascorbate[83].

In the CNS, ascorbic acid is an essential micronutrient, and although the entire brain concentrations are between 1 and 2 mmol/L, the neuronal concentrations have been evaluated to be as high as 10 mmol/L, whereas concentrations in glial cells are about 1 mmol/L[84,85]. At the same time, the ascorbate concentrations present in brain ECF have been estimated to comprise between 200 and 400 μmol/L[81].

Those findings suggest not only that ascorbate has a

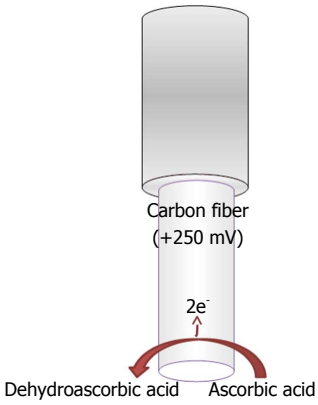

Figure 4 Scheme of AA sensor used in constant potential amperometry. In this representation, the transducer is made of a carbon fiber. The AA is oxidized by applying mild potentials (+250 mV or less) needed for oxidizing the AA to dehydroascorbic acid.

significant role in normal neuronal physiology but also that, given the structural characteristics as an electron donor and free-radical scavenger, it has assumed its role as a neuroprotective molecule and as an important component of the neuronal antioxidant pool[81].

Neurons and glia are able to interact with each other in order to conserve CNS ascorbate, using the mechanism of heteroexchange in which ascorbate release is related principally to glutamate uptake[86,87].

Ascorbic acid is easily oxidized in the following manner (Figure 4)

$$\text{L-ascorbic acid} \rightarrow \text{Dehydroascorbic acid} + 2e^- + 2H^+ \quad (9)$$

by applying a mild anodic potential[4] at the transducer surface (Figure 4), when a constant potential is applied, and generating a current flow directly proportional to the ascorbate concentrations.

For ascorbate *in vivo* monitoring, the transducer is typically made of composite materials of carbon such as carbon paste[87,88] or fibers[89] and multiwalled carbon nanotube (MWNT)-modified carbon fibers[90].

The transducer surface is sometimes modified for excluding electroactive interfering species such as positive catecholamines, so the electrode modification is carried out by the deposition of overoxidized poly (1,2-phenylenediamine)[89].

Cyclic voltammetry (CV)[89,90], square-wave voltammetry[89], and differential pulse voltammetry[91] have been used for *in vivo* measurements of ascorbic acid in the brain of animal models. The latter methods have been proven to be the most sensitive for sensing and biosensing because they change the potential pulsing from one potential to another in a relatively short range of time, different to what happens for the CV where the potential is constantly modified in a linear way[92].

Constant potential voltammetric techniques have also been used for *in vivo* monitoring of ascorbic acid in the brain by applying mild positive potentials such as +120 mV *vs* Ag/AgCl, when this is the implanted reference electrode (RE)[4], or +250 mV when the implanted RE is Ag^+[93].

All the applied techniques have confirmed what was found with other methods that the ascorbate concentrations present in neuronal extracellular spaces are close to 500 μmol/L, emphasizing the reliability and specificity of the reading of the ascorbic acid sensors.

Glucose and lactate

Glucose, a main nutrient in the brain[94], is the most important factor for its energetic metabolism[95-98] and is actively involved in ATP synthesis; it is an important modulator of memory in multiple tasks and improves memory in patients with Alzheimer's disease and Down's syndrome[99,100].

Lactate is another important molecule involved in brain energetic metabolism as energetic substrate for neurons[96] or product of glycolysis under anaerobic condition[94,97].

For a long time, lactate production in the brain was viewed as a lack of oxygen, as the lack of an aerobic oxidation process, or as a mismatch between glycolytic and oxidative rates, but it has recently been identified as an alternative food to glucose[97,100,101].

Contemporary studies in the amount of glucose and lactate in the brain are significant both in physiological conditions and in the presence of disease[102-104].

The recognition and quantification levels of glucose and lactate are possible by using innovative devices such as biosensors constituted by an electric transducer and a biological component such as enzymes; for example, glucose oxidase (GOx), L-lactate oxidase (LOx), or L-lactate dehydrogenase (LDH) is commonly used in the design, respectively, of glucose and lactate amperometric biosensors and their exploiting simple enzymatic reactions and relatively easy sensor design configuration[105]. In particular, amperometric methods have been widely used in glucose and lactate sensing. The biochemical reactions, in presence of oxygen, occurring at glucose and lactate biosensors are as follows[5,106,107]:

$$\beta\text{-D-Glucose} + FAD^+\text{-GOx} \rightarrow \text{D-Glucono-}\delta\text{-Lactone} + FADH_2\text{-GOx} \quad (10)$$

$$FADH_2\text{-GOx} + O_2 \rightarrow FAD^+\text{-GOx} + H_2O_2$$

$$\text{L-Lactate} + FAD^+\text{-LOx} \rightarrow \text{Pyruvate} + FADH_2\text{-LOx} \quad (11)$$

$$LOx + O_2 \rightarrow FAD^+\text{-LOx} + H_2O_2$$

$$\text{L-Lactate} + NAD^+\text{-LDH} \rightarrow \text{Pyruvate} + \text{NADH-LDH} \quad (12)$$

In the electrochemical biosensor (Figures 5 and 6), the hydrogen peroxide byproduct from oxidase enzymes is directly proportional to the quantity of substrate glucose or lactate transformed by the enzymes as shown below in equation (8)[4].

Many studies of neuronal applying biosensors in experimental models *in vivo* are present in literature[108]. These studies show different types of biosensor designs, made with several transducer materials. Biosensors are mainly composed of noble metals, such as gold and/or

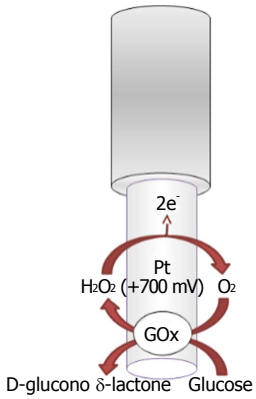

Figure 5 Schematic representation of the platinum-based biosensor used for detecting extracellular glucose in the central nervous system of freely moving animals. The immobilized glucose oxidase (GOx) selectively transforms glucose in D-gloconolactone in the presence of molecular O_2 and generates H_2O_2 that is promptly oxidized on platinum surface.

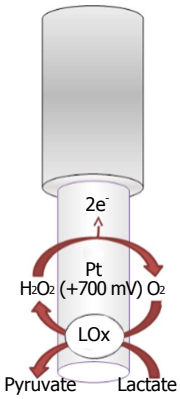

Figure 6 Schematic representation of the platinum-based biosensors for detecting extracellular lactate in the central nervous system of awake animals. In the presence of O_2, the immobilized enzyme [lactate oxidase (LOx)] selectively converts the substrate (lactate) in the corresponding product (pyruvate) and generates H_2O_2 that is oxidized on the Pt surface.

Pt, although recently, other systems use conductive carbon based materials.

A new approach for the simultaneous detection of brain glucose and lactate in real time is reached by the use of a biotelemetric device fixed on the head of the animal[109-111].

In a previous study[6], O-phenylenediamine (OPD) monomers were electrodeposited onto a Pt/Ir cylinder electrode (diameter, 125 μm) surface. The next step was to immobilize GOx, stabilized with polyethylenimine (PEI), by immersing the transducer in the BSA solution and after in the glutaraldehyde solution (GTA). The lactate biosensor was initially made in the same way by changing the oxidase enzyme, but substituting the BSA/GTA with a final layer of polyurethane (PU)[6] for increasing the linear region. CPA was used, fixing the applied potential for hydrogen peroxide oxidation at +700 mV *vs* Ag/AgCl RE.

There are numerous problems with this approach because it is necessary to apply a high potential to detect hydrogen peroxide (+700 mV)[112,113] and the concentration of oxygen can change in the region in which the biosensor is implanted and the resulting current is not directly correlated with the extracellular concentrations of lactate[113-115].

Furthermore, the presence of interfering electroactive species in the tissues and the reactions of biopolymerization are needed to be considered[116,117]. In the nineties, to solve these problems, Karyakin proposed to modify the transduction element using carbon compounds coated with a thin film of Prussian blue (PB), Fe4 [Fe(CN)6]3[113,114,118-121].

After the introduction of PB in the field of biosensors were formulated different materials as supports and methodologies of deposition to improve its electrocatalytic properties and stability[122]. In recent years, some research groups have worked on glucose and lactate microbiosensors based on PB electrodes made of carbon fiber (CFE) modified to detect enzyme-generated hydrogen peroxide low applied potential (0 mV).

Afterward, the enzyme stabilizer PEI was added to improve the performance of the enzyme[122], and GOx and LOx were subsequently immobilized. In order to avoid signal of interferents, OPD was electrodeposited[122]. For the first time, a glucose and lactate microbiosensor, based on PB-modified CFE, is able to detect physiological changes in molecular levels at a low applied potential in the CNS[123].

Moreover, the ultrasmall biosensor size is apposite for *in vivo* neuroscience studies. In contrast, the first generation of microbiosensor transducers based on noble metals have high dimensions (diameter, approximately 100 μm) even if they have been used successfully over the last few decades for the monitoring of neurochemical species[116]. Consequently, the use of carbon-fiber microbiosensors (diameter, approximately 10 μm), modified with PB, seems to be more suitable for use in these studies because it reduces brain damage during insertion[124] and provides an even higher temporal resolution, allowing the real-time correlation with animal behavior[125].

Oxygen and nitric oxide

Oxygen and endogenous nitric oxide are gaseous molecules playing a pivotal role in mediating important biological processes yet are involved in very distinct aspects of organism physiology. Oxygen is indispensable for animal life; an adequate tissue oxygen content, delivered by hemoglobin through the bloodstream, is fundamental to supply cellular metabolic demands, as oxygen is involved in energy production as well as in aerobic cellular metabolism[126].

In contrast, an insufficient oxygen concentration in tissues leads to hypoxia, a severe altered condition in which low oxygen availability prevents aerobic metabolism and oxidative phosphorylation in the cell, yielding to impoverishment of high-energy compounds such as ATP and, lastly, inducing cellular dysfunction and death[127,128].

Though oxygen is a crucial substrate for cellular

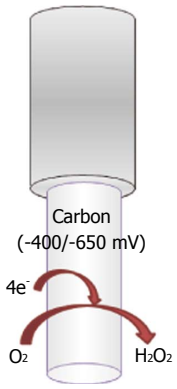

Figure 7 Schematic representation of the carbon-based sensor used for detecting the molecular O_2 dissolved in the extracellular space of the brain of freely moving animals. The O_2 is reduced on the carbon surface at low potentials and converted to water in a one- or two-step reaction (see text).

functions, it also provokes damage because of the toxicity of oxygen-derived reactive species (ROS), such as hydrogen peroxide, singlet oxygen, hydroxyl radicals, and superoxide anion[129]. ROS free radicals attack lipids, proteins, DNA, and RNA and expose cells to oxidative stress, which has been demonstrated to be involved in the pathogenesis of several neurodegenerative diseases[129,130].

Endogenous nitric oxide is a gaseous signaling molecule released in low concentration (tens of nanomoles to low micromoles), characterized by possessing a lifetime of a few seconds[131], as nitric oxide is a highly reactive free-radical species. Nitric oxide production mainly involves the enzymes NO-synthases, which catalyze nitric oxide formation as a byproduct of the reduction of the amino acid L-arginine into l-citrulline[132,133]. Nitric oxide acts as a transitory paracrine and autocrine signaling molecule, by activating the soluble guanylyl cyclase, increasing cellular cyclic guanosine monophosphate (c)[134].

Since its discovery in 1987[135-137], when first nitric oxide was recognized as being involved in the physiological actions of endothelium-derived relaxing factor, mediating vasodilatation, the knowledge of the important role that nitric oxide plays in physiopathology and pharmacology exponentially increased. In fact, further studies revealed how nitric oxide actions are implicated in the cardiovascular system, in the immune response[138], as well as in the nervous systems, mediating neurotransmission[131,139]. Furthermore, nitric oxide is a mediator of both antitumor and antimicrobial activities[140].

Otherwise, the disruption of nitric oxide production seems to be involved in diseases such as atherosclerosis[141], hypertension, cerebral and coronary vasospasm, and ischemia-reperfusion injury. In fact, nitric oxide is attacked by ROS, specifically by superoxide anion, forming peroxynitrite, which generates further reactive nitrogen species (RNS) such as nitrogen dioxide and dinitrogen trioxide. Like ROS, RNS damage lipids, proteins, and other macromolecules, thus also contributing to the onset of diabetes and neurodegenerative diseases[141-143].

The detection of oxygen and nitric oxide tension in the brain has been studied *in vivo*, providing critical information about the physiopathology and pharmacological implications of these molecules.

A wide variety of O_2-sensitive microsensors have been developed. Electrochemical devices exploiting amperometric techniques of detection, such as CPA, differential-pulse amperometry (DPA), CV, and fast-scan voltammetry (FCV), allow the reliable direct reduction of oxygen. Carbon paste and noble metal transducers are the most commonly diffused. Reactions involved in the electrochemical reduction of oxygen at the electrode's surface can occur *via* two mechanisms: a single-step reaction yields to detectable intermediates (Figure 7):

$$\text{oxygen} + 4H^+ + 4e^- \rightarrow 2H_2O \quad (13)$$

In the second mechanism, two-step O_2 reduction forms H_2O_2 as measurable intermediate:

$$\text{oxygen} + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (14)$$

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \quad (15)$$

Changes after physiological stimulations or pharmacological treatments were recorded in the extracellular space of the striatum, by using optic microfibers, assessing that oxygen concentration is about 50 μmol/L[143].

Electrochemical oxygen microelectrodes using CPA at a noble metal transducer bare, such as gold or Pt, allowed the long-term monitoring of oxygen subcutaneous and venous dynamics[144,145].

Nevertheless, several groups preferred to use carbon-paste electrodes (CPEs) because of their longer *in vivo* stability, less surface fouling[146], and quite easy manufacture[147] (Figure 7). Venton *et al*[148] used the FCV technique in a study in which dissolved oxygen was measured in the rat caudate-putamen, by using 5 μm Nafion-coated carbon fibers with a subsecond time resolution. FCV was used also in a study that targeted oxygen levels in the striatum of primates during reward delivery. In this case, the diameter of the carbon fibers ranged from 12 to 33 μm[149].

Lowry *et al*[101,150,151] largely used carbon paste-based miniaturized electrodes in an experimental session in which the effects of anesthesia were studied *in vivo*, as well as the effects of hypoxia and hyperoxia on brain energy metabolism in the striatum[147-149]. Changes in oxygen at CPEs were usually monitored by using the DPA technique[151,152]. Two equally sized cathodic pulses were applied: the first from a resting potential at -150 to -350 mV, corresponding to the foot of the reduction wave for oxygen, and the second, which corresponds to the peak of the reduction wave, from -350 to -550 mV.

In addition, oxygen microsensors were used by Finnerty *et al*[153] in real-time monitoring of oxygen levels in an animal model of schizophrenia, coupled with the use of a glucose biosensor and an nitric oxide microsensor. Oxygen reduction at CPEs has been widely detected also *via* CPA[152]. For example, by applying a constant cathodic potential of -650 mV *vs* a saturated calomel RE, oxygen reduction was recorded in real time in the hippocampus of freely moving rats[115].

Furthermore, CPEs of 200 μm in diameter were implanted in the dorsal and the ventral hippocampus of rats

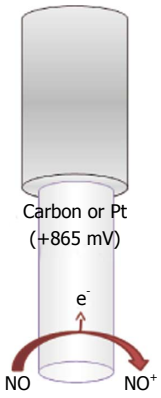

Figure 8 Schematic representation of the more widely used sensor for detecting NO in the brain of freely moving animals. The NO is directly oxidized on a carbon (or platinum) surface to NO^+. This sensor is particularly sensitive to electroactive interferences in virtue of the very high oxidation potentials.

to investigate spatial processing and anxiety. Even in this case, the applied potential was -650 mV *vs* a silver wire REF[154]. The CPA technique was also used by Bazzu *et al*[110] to monitor striatal oxygen levels in a telemetric *in vivo* study. Working electrodes, consisting of miniaturized conical-shaped epoxy-carbon electrodes (180 μm), allowed oxygen detection by fixing the reduction potential at -400 mV *vs* Ag/AgCl REF.

Recently, oxygen amperometry was applied to a behavioral study of reward processing in the rat nucleus accumbens. CPEs (200 μm in diameter) were used by applying a constant potential of -650 mV *vs* a silver wire REF to reduce oxygen. Data showed similar results to those obtained in human fMRI studies, confirming how oxygen amperometry is a powerful technique for the measurement of brain function[155].

In the attempts of monitoring the concentration of the unstable nitric oxide molecule *in vivo* and to test nitric oxide donor drugs, several microsensors have been developed since the 1990s[156]. The majority exploits electrochemical amperometric techniques to directly detect nitric oxide. Commonly, an oxidant potential is applied (higher than +850 mV *vs* Ag/AgCl), in view of the fact that nitric oxide and oxygen reduction potential are very close, so oxygen interferes with nitric oxide measurement (at nitric oxide-reducing potentials) (Figure 8).

Basically, a double reaction occurs at the transducer's face, usually carbon fiber or noble metals[157-161], involving the formation of NO^+, which is further converted into nitrite (Figure 8):

$$NO - e^- \rightarrow NO^+ \quad (16)$$

$$NO^+ + OH^- \rightarrow HNO_2 \rightarrow H^+ + NO_2^- \quad (17)$$

Otherwise, metalloporphyrin-modified sensors[162-164] are also largely used:

$$Fe(II) + NO \rightarrow Fe(II) - NO \quad (18)$$

$$Fe(II) - NO + H^+ + e^- \rightarrow Fe(II) - HNO \quad (19)$$

$$Fe(II) - HNO + 2H^+ + 2\,e^- \rightarrow Fe(II) + H_2NOH \quad (20)$$

Because of the enormous interest kindled by the wide range of actions of nitric oxide, several *in vivo* experiments were conducted to monitor nitric oxide release on different tissues[165-168]. Friedemann *et al*[169] developed an electrochemical electrode using carbon fiber as a transducer, coated with Nafion and further electropolymerized with OPD. Nitric oxide was quantified amperometrically using differential pulse voltammetry[169].

Wu *et al*[170,171] research group conducted several experiments in which physiological nitric oxide actions on a cat' s brain were investigated. Nitric oxide concentration was measured in real-time using voltammetry techniques, implanting Nafion-/porphyrin-/OPD-coated carbon-fiber electrodes. A highly sensitive and selective NO electrode was used to measure the nitric oxide concentration in a rat hippocampus[172]. In addition, an electrochemical nitric oxide microbiosensor based on cytochrome C, immobilized onto a functionalized conducting polymer layer, was implanted in the striatum. Nafion was used for its shielding properties toward interference electroactive molecules present in the brain, chiefly ascorbic acid[173]. Brown *et al*[174] and Finnerty *et al*[175] obtained a simple and useful design by modifying a Pt sensor with multicoated Nafion layers. This electrochemical sensor was successfully implanted in the striatum of freely moving rats, allowing the real-time nitric oxide at Nafion-coated Pt. Santos *et al*[176] recently developed an electrochemical biomimetic sensor based on nanocomposite hemin-based microelectrode, measuring exogenous NO in the rat hippocampus *in vivo* using CV.

Ethanol

In the last decades, ethanol has become the most widespread psychotropic toxic substance in Western countries because it is widely legally accepted and also because it is available at a low cost. Acute, subacute and chronic exposure to ethanol may have important effects on the CNS, therefore it becomes significative to monitor ethanol kinetic and its effects on the brain using the most appropriate techniques[177]. The main effects of ethanol consumption cause significant effects on the CNS, principally enhancing the action of the neurotransmitter GABA and generating disinhibition, ataxia, and sedation[178]. Subchronic exposure to ethanol enhances the dopamine neurotransmission in the mesolimbic system[179,180] and increases dopamine levels in the nucleus accumbens[181], playing an important role as a "rewarding" molecule[182-184].

Recently, implantable electrochemical biosensors have been developed for monitoring the real-time changes of ethanol concentrations in the brain ECFs of freely moving animals (Figure 9). As previously described for other implantable biosensors, the ethanol biosensor exploits the presence of an enzyme, the alcohol oxidase, to selectively quantify ethanol using the production of a directly oxidizable byproduct (hydrogen peroxide), electrochemically detectable on the surface of a Pt transducer[185,186]. The main characteristic of this biosensor is its capability of monitoring ethanol changes second by second and over

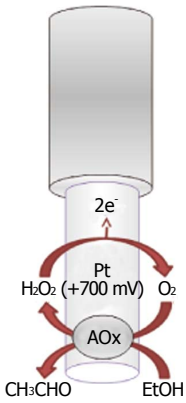

Figure 9 Schematic representation of the biosensor for the detection of exogenous ethanol in the brain of freely moving animals. EtOH: Ethanol; CH_3CHO: Acetaldehyde; AOx: Alcohol oxidase.

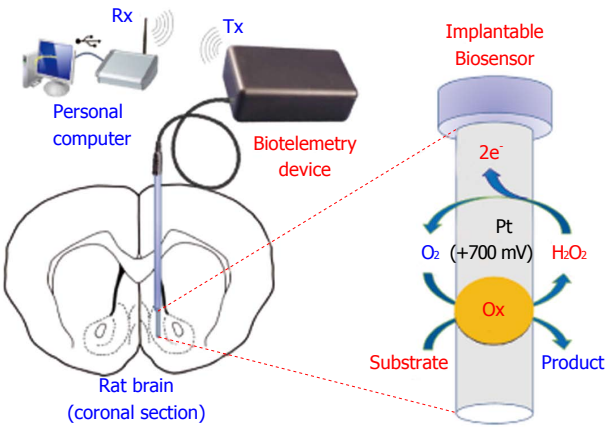

Figure 10 Schematic representation of the biotelemetry system, connected to a constant potential amperometry-based amperometric biosensor, for the real-time monitoring of brain neurochemistry in freely moving animals. Ox: Oxidase enzyme.

a period of two weeks. This neurochemical tool has been proven to be successful, especially when associated with a miniaturized telemetric system (see next paragraph). According to the results of previous studies[177,185,186], the ethanol biosensor has been demonstrated to be a reliable device for the short-time monitoring of exogenous ethanol in the CNS, and it could be used for studying ethanol pharmacokinetics during addiction and the real-time effect of drugs on ethanol levels in the CNS.

BIOTELEMETRY

Biotelemetry has be defined as the recording of physiological parameters by uni- or bidirectional electromagnetic signals[6,187], or more simply, it represents a variety of techniques intended for real-time monitoring of physiological parameters. Innovative biotelemetry systems (Figure 10) have been developed for studying brain neurochemistry[188], in particular for monitoring CNS dopamine in freely moving animals[189-191] and, more recently, in humans[192]. The wireless detection of dopamine requires complex waveform generation and high-resolution synchronization; indeed, as previously shown, FSCV allows the redox detection of dopamine up to ten times per second[189-191]. Also chronoamperometry and differential pulse voltammetry techniques have been demonstrated to work in conjunction with telemetric devices[158,193-196]; the resulting systems are very complex, not easily miniaturizable, and difficult to use in small rodents. On the contrary, non-pulsed techniques, such as CPA, free the microcontroller unit (MCU) from high-density calculations, allowing an increase in the number of implantable sensors and facilitating the miniaturization of the electronics[109,197]. The battery-powered biotelemetric device (Figure 10), composed of an amperometric module, an MCU, and a transmitter, polarizes the sensors and sends sensor data to a receiving unit connected to a PC. The system electronics exhibits low power consumption, high stability, and good linear response[3]. A CPA-based biotelemetry device may be easily interfaced with amperometric microsensors and biosensors[6,109,197] and leave enough MCU computing power available for other tasks such as motion detection using inertial physical sensors. Indeed, in a previous study, we described this new approach with the simultaneous detection of brain glucose, lactate, and movements in real time using a biotelemetric device fixed to the head of a freely moving rat[6].

COMPARISON BETWEEN VOLTAMMETRY AND MICRODIALYSIS

Although voltammetric techniques have been widely used in last decades, microdialysis still remains the "gold standard" for *in vivo* neurochemical study of the brain extracellular compartment. The advantages in using this technique include the possibility of measuring several neurochemicals at the same time with high sensitivity and very high selectivity, providing a more complete picture of the ECF. Its invasiveness, associated with low temporal resolution, and the necessity of using connecting tubes to carry out the experiments do not make it particularly suitable for monitoring fast neurochemical changes and do not allow the application of wireless techniques. As an alternative, electrochemical sensors are increasingly-used tools to study the neurochemical modifications in the ECF. The main characteristics of these devices are represented by very low invasiveness (carbon fibers in particular), when compared with microdialysis probes, and, most of all, their capability of monitoring variations of analytes in seconds or fractions. Furthermore, some electrochemical sensors have been demonstrated to be effective for weeks or months when implanted in the brain and, as described in this review, they are the optimal candidates for wireless detection. The Table 1 summarizes the principal characteristics of the main techniques indicated in this review.

CONCLUSION

Implantable (Bio)sensors, integrated into miniaturized telemetry systems, represent a new generation of analytical tools for studying brain neurochemistry of awake, freely

Table 1 Principal characteristics of the main techniques indicated in this review and used for *in vivo* monitoring of brain neurochemistry

Characteristics of the technique	Technique				
	Electrochemical techniques (voltammetry)				Microdialysis
	CPA	CA	DPV	FSCV	
Brain invasiveness	+	+	+	+	++
Selectivity	+	+	++	++	+++
Sensitivity	++	++	+	+	+++
Concentration range	nmol/L-mmol/L	nmol/L-mmol/L	nmol/L-mmol/L	nmol/L-mmol/L	fmol/L-mmol/L
Temporal resolution	++++	+++	++	+++	+
Spatial resolution	++	+++	++	+++	+
Monitoring period	d/wk	d/wk	d/wk	d/wk	h/d
Untethered detection	++	+	+	+	-

CPA: Constant potential amperometry; CA: Chronoamperometry; DPV: Differential pulse voltammetry; FSCV: Fast-scan cyclic voltammetry.

moving animals in real time. This approach, based on simple and inexpensive components, could be used as a rapid and reliable model for studying the physiology, the pathophysiology, and the effects of different drugs (or toxic compounds such as ethanol) on brain neurochemical systems.

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World J Pharmacol 2014 March 9; 3(1): I-V
ISSN 2220-3192 (online)
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World Journal of Pharmacology

ISSN

ISSN 2220-3192 (online)

Launch date

February 9, 2012

Frequency

Quarterly

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ISSN 2220-3192 (online)

World Journal of *Pharmacology*

World J Pharmacol 2014 June 9; 3(2): 18-32

Published by Baishideng Publishing Group Inc

WJP

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NAME OF JOURNAL
World Journal of Pharmacology

ISSN
ISSN 2220-3192 (online)

LAUNCH DATE
February 9, 2012

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REVIEW

Arsenic exposure decreases rhythmic contractions of vascular tone through sodium transporters and K^+ channels

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Received: April 5, 2014 Revised: June 4, 2014
Accepted: June 5, 2014
Published online: June 9, 2014

Abstract

Arsenic-contaminated drinking water is a public health problem in countries such as Taiwan, Bangladesh, United States, Mexico, Argentina, and Chile. The chronic ingestion of arsenic-contaminated drinking water increases the risk for ischemic heart disease, cerebrovascular disease, and prevalence of hypertension. Although toxic arsenic effects are controversial, there is evidence that a high concentration of arsenic may induce hypertension through increase in vascular tone and resistance. Vascular tone is regulated by the rhythmic contractions of the blood vessels, generated by calcium oscillations in the cytosol of vascular smooth muscle cells. To regulate the cytosolic calcium oscillations, the membrane oscillator model involves the participation of Ca^{2+} channels, calcium-activated K^+ channels, Na^+/Ca^{2+} exchange, plasma membrane Ca^{2+}-ATPase, and the Na^+/K^+-ATPase. However, little is known about the role of K^+ uptake by sodium transporters [Na^+/K^+-ATPase or Na^+-K^+-$2Cl^-$ (NKCC1)] on the rhythmic contractions. Vascular rhythmic contractions, or vasomotion are a local mechanism to regulate vascular resistance and blood flow. Since vascular rhythmic contractions of blood vessels are involved in modulating the vascular resistance, the blood flow, and the systemic pressure, we suggest a model explaining the participation of the sodium pump and NKCC1 co-transporter in low dose arsenic exposure effects on vasomotion and vascular dysfunction.

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Key words: Arsenic; Vasomotion; Na^+/K^+-ATPase; Na^+-K^+-$2Cl^-$; K^+ channels; Nitric oxide; Prostaglandin; Vascular

Core tip: Vascular tone is regulated in part by cytosolic calcium oscillations. Arsenic can induce an increase in vascular tone and resistance. We suggest a model explaining the participation of the sodium pump and Na^+-K^+-$2Cl^-$ co-transporter in low dose arsenic exposure effects on vasomotion and vascular dysfunction.

Palacios J, Nwokocha CR, Cifuentes F. Arsenic exposure decreases rhythmic contractions of vascular tone through sodium transporters and K^+ channels. *World J Pharmacol* 2014; 3(2): 18-23 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i2/18.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i2.18

INTRODUCTION

Arsenic toxicity is a global environmental health problem. The toxicity of this metalloid has been observed in various countries, including Taiwan[1], Bangladesh[2], Mexico[3], United States[4], Hungary[5], Argentina[6], and Chile[7]. Volcanic emission is one of the natural sources of arsenic, and individuals are majorly exposed through contaminated drinking water[8]. Smelting companies are also an important source of individual and population exposure to these kinds of heavy metals contamination. Contamination has been reported in Russia[9], United States[10], Mexico[11], Peru[12], and Chile[13]. There are few

studies showing that Chinese workers in copper smelter, steel or iron have high levels of total arsenic in urine (50 g/g creatinine). These studies include those reported for Fushun city[14], Yunnan province[15], and Fuxin city[16].

CHRONIC ARSENIC EXPOSURE AND VASCULAR DISEASES

There are epidemiologic studies that showed an association between chronic arsenic exposure and vascular diseases[17,18]. In fact, the ingestion of the arsenic-contaminated drinking water produced an increased risk for ischemic heart disease, cerebrovascular disease, and peripheral vascular resistance[19]. Other studies report positive associations between chronic arsenic exposure in drinking water, and the prevalence of hypertension[20-24].

Currently, arsenic effects on systemic blood pressure are controversial[25,26]. However, there is ample evidence that arsenic exposure mainly increases the vascular peripheral resistance[19,27], which defines the difficulty to blood flow through the blood vessels, particularly the small arteries.

Vascular rhythmic contractions, or vasomotion, are local mechanisms that regulate the vascular resistance and blood flow[28-30]. For instance, an increase in the amplitude of the rhythmic contractions cause an increased blood flow because the vascular resistance is reduced[31]. Since vascular rhythmic contractions of blood vessels are involved in modulating the vascular resistance, the blood flow, and the systemic pressure[28,29], the effects of chronic low dose exposures to arsenic on vascular rhythmic contractions becomes of great interest.

VASCULAR RHYTHMIC CONTRACTIONS

Vascular rhythmic contractions may be considered as a compensatory mechanism to preserve the perfusion of tissues[31], especially in patients with hypertension[32,33] or ischemia[34]. The mechanisms of the vascular rhythmic contractions may account for 3 states of contraction in blood vessels with different levels of calcium. These include small, medium, and tonic contraction, but only the medium concentrations produce rhythmic contractions[35]. The changes of vascular tone are generated by calcium oscillations in the cytosol of vascular smooth muscle cells[36]. To regulate the cytosolic calcium oscillations, the membrane oscillator model considers that activity of Ca^{2+} channels, calcium-activated K^+ channels, Na^+/Ca^{2+} exchange, plasma membrane Ca^{2+}-ATPase, and the Na^+/K^+-ATPase, voltage-dependent calcium channel, and transient receptor potential channel are essential for maintaining calcium oscillations[37].

ROLE OF NA^+/K^+-ATPASE AND NA^+-K^+-$2CL^-$ COTRANSPORTER ON RHYTHMIC CONTRACTIONS

Little is known about the role of K^+ uptake through Na^+/K^+-ATPase and Na^+-K^+-$2Cl^-$ (NKCC1) on the rhythmic contractions. Na^+/K^+-ATPase and NKCC1 cotransporter are responsible for the major K^+ uptake in vascular smooth muscle cells[38-40]. Recent reports demonstrates that rhythmic contractions were associated with tonic and phasic responses, the tonic dependent on $[Ca^{2+}]i$ and the phasic on potassium efflux (through K^+ channels) and potassium uptake[41,42].

Na^+/K^+-ATPase is responsible for the electrochemical gradient of sodium and potassium ions, it also plays a vital role in the regulations of ionic homeostasis in tissues and cells. In vascular smooth muscle cells, Na^+/K^+-ATPase plays a major role in the regulation of vascular tone[43,44], an increase in Na^+/K^+-ATPase activity leads to hyperpolarization and relaxation of smooth muscle[45], while its inhibition blunts rhythmic contractions in vascular smooth muscle cells[46].

It was postulated that the inhibition of K_{ATP} channels reduces extracellular K^+ and Na^+/K^+-ATPase activity, increases intracellular calcium concentration *via* Na^+/Ca^{2+} exchanger, uncouples vascular smooth muscle cells *via* gap junctions, and eliminates vascular rhythmic contractions[47,48]. Also, the inhibition of inward-rectifier K^+ channels (Kir) decrease Na^+/K^+-ATPase activity in vascular smooth muscle cells[49]. It is important to remember that the Na^+/K^+-ATPase participates in relaxation of vascular smooth muscle cells through K^+ channels. For instance, Na^+/K^+-ATPase is involved in K^+-induced vasodilatation of hamster cremasteric arterioles[50], and vasodilation in the human forearm[51]. When K^+ (1 to 15 mmol/L) accumulates in the extracellular space, Na^+/K^+-ATPase activity increases efflux of potassium through Kir. This leads to hyperpolarization and vasodilatation of the vascular smooth muscle cells[49,52]. In contrast, the opening of calcium-activated K^+ channels inhibits the Na^+/K^+-ATPase function[53,54], and vascular rhythmic contractions[28].

NKCC1 is an obligatory symport system with an apparent stoichiometry of 1:1:2 sodium, potassium and chloride ratios respectively. Although the co-transporter is bidirectional in resting vascular smooth muscle cells, the sum of the electrochemical gradients for the three transported ion species determines net influx[55].

Evidence for the role of NKCC1 co-transporter on vascular rhythmic contractions is scanty, but it is worthy of note that the inward current of Cl^- decreases rhythmic contractions by increasing vasoconstriction[47]. NKCC1 is responsible in part to keep intracellular Cl^- concentration above the electrochemical equilibrium[56] as such helping to maintain the electrochemical gradient and cellular reactivity. Phenylephrine-induced stimulation of NKCC1 increases intracellular Cl^- concentration, depolarize vascular smooth muscle cells[57], open L-type calcium channels[58] and produce vasoconstriction. In the vascular oscillator model[59], the release of intracellular Ca^{2+} from the reticulum stimulates the inward current of Cl^- *via* the calcium-activated Cl^- channel[60] and cyclic guanosine monophosphate (cGMP)-activated Ca^{2+}-dependent Cl^- channels[61]. This leads to membrane depolarization, opening

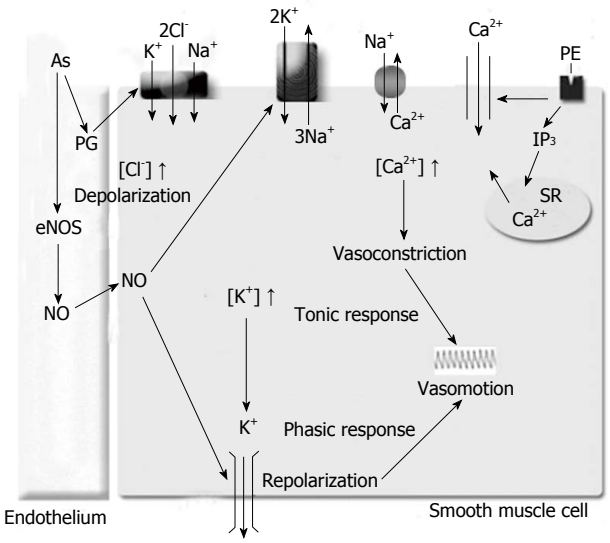

Figure 1 Putative model of arsenic effect on vasomotion phenomenon in blood vessels. The figure shows the stimulation of the Na^+/K^+-ATPase by endothelial nitric oxide (NO) and stimulation of the Na^+-K^+-$2Cl^-$ cotransporter by endothelial prostaglandins (PG). Arsenic would reduce NO bioavailability or would increase PG level, both of them would produce an increase in vasoconstriction or a decrease in the repolarization of the cell membrane, respectively, and then would reduce vasomotion. PE: Phenylephrine; As: Arsenic; eNOS: Endothelial nitric oxide synthase; SR: Sarcoplasmic reticulum.

L-type calcium channels and reduction in the oscillations of vascular tone. Therefore these findings suggest that the cotransporter NKCC1 would be responsible, in part, for vasoconstriction by chloride.

EFFECT OF ARSENIC ON VASCULAR RHYTHMIC CONTRACTIONS

Vascular rhythmic contractions are dependent in part on endothelial nitric oxide (NO)[46], but there are few studies showing that the arsenic reduces vasomotion (vascular rhythmic contractions) by decreasing the NO bioavailability[62].

It is well established that heavy metals such as arsenic induce increases in vascular resistance by inducing vascular endothelial dysfunction (VED)[62,63]. VED consists of a reduction in endothelium-dependent vasorelaxation caused by a decrease in the release of endothelial NO[64]. Arsenic-induced VED is caused in part by oxidative stress.

Oxidative stress from pollutants like arsenic causes an increase in the reactive oxygen species, this leads to a modification of amino acids of proteins, mainly sulfur-containing amino acids methionine and cysteine[65]. Arsenic causes oxidative stress through peroxynitrite generation in aortic endothelial cells, producing loss of biological activity in enzymes and proteins[66,67]. In this context we had shown that chronic arsenic exposure in drinking water reduced acetylcholine-induced relaxation in female rat aorta[68], impairment of the endothelial nitric oxide synthase activity and decreasing of endothelial NO production[69,70].

NO is reported to activates Na^+/K^+-ATPase function[71], we observed that acetylcholine and sodium nitroprusside (SNP) induces activation of Na^+/K^+-ATPase activity, and SNP effect is abolished by inhibition of PKG (KT-5823)[72]. Cogolludo *et al*[73] (2001) showed that SNP activates Na^+/K^+-ATPase in mesenteric piglet's arteries while Tamaoki *et al*[74] (1997) found that cGMP activates Na^+/K^+-ATPase in pulmonary artery smooth muscle cells.

Since arsenic decreases the NO bioavailability[62], and the NO increases Na^+/K^+-ATPase function[71] which enhances the vascular rhythmic contractions, we may suggest that arsenic decreases the vascular rhythmic contractions by Na^+/K^+-ATPase function (Figure 1). Similar conclusions would be expected with the Kir channel, as Chen *et al*[75] (2010) demonstrated that arsenic trioxide produces down-regulation of Kir channel in cardiomyocytes of rats, and the Kir channel function increases Na^+/K^+-ATPase activity[49].

Although the endothelial NO does not affect NKCC1 co-transporter function[76], the endothelial prostaglandins increase NKCC1 activity thereby enhancing the contractile response to agonist in rat aorta[77-80]. Moreover, the endothelial prostaglandins increase agonist-induced rhythmic contractions in rat aorta[81], rat mesenteric artery[82], and arterioles of the cheek pouch of male hamsters[42]. Furthermore, arsenic increases the cyclooxygenase-2 (COX-2) protein in aortic endothelial cells[67], COX-2 in HUVEC[83], and enhances COX-1 and COX-2 activities in hind paw muscle of male rats[84]. Therefore, as a result of the prostaglandins effect on the vascular contractility through NKCC1 described above, arsenic might increase the vascular rhythmic contractions by NKCC1 co-transporter function.

The major toxic species of arsenic used in several studies are arsenite (trivalent inorganic arsenic, *i.e.,* arsenic trioxide) or arsenate (pentavalent inorganic arsenic). Although the concentration of arsenate in drinking water is higher than those of arsenite, toxic effects of arsenate have not been properly documented. Arsenate is mainly metabolized by organisms as monomethylarsonic acid and dimethylarsinic acid, which significantly are not toxic[85]. However, this theory of the methylation of inorganic arsenic as a detoxification process has been revised[86] as other trivalent methylated species with higher toxicity have been reported[87]. Possibly, the biological effect of arsenate is mainly by reduction to arsenite[88].

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P- Reviewer: Chen F, Sandow SL **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i2.24

World J Pharmacol 2014 June 9; 3(2): 24-32
ISSN 2220-3192 (online)
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MINIREVIEWS

Pharmacological management of neuropathic pain in patients with vestibular schwannomas: Experience of the Atlantic Lateral Skull Base Clinic

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Received: April 29, 2014 Revised: May 31, 2014
Accepted: June 5, 2014
Published online: June 9, 2014

Abstract

Neuropathic pain is chronic pain generated by disorders of the peripheral and central nervous system, including skull base tumours. A skull base tumour can be any type of tumour that forms in the skull base, and this includes vestibular schwannomas which arise from the sheath of the inner ear vestibulocochlear nerve (eighth cranial nerve). Growth of the tumour, surgical resection, and/or stereotactic radiotherapy may result in compression and/or irritation of the fifth cranial nerve (trigeminal nerve) resulting in facial pain and/or numbness. Non-trigeminal afferent input may contribute to the wide constellation of symptoms seen in orofacial pain patients. The purpose of this report was to develop a decision tool to guide the recognition and treatment of neuropathic pain in this specialized population. Recommendations for treatment are based on evidence presented in Canadian and international neuropathic treatment guidelines. Algorithms are included for assessment and treatment of adult patients with agents that are recognized to have analgesic efficacy within the broad context of neuropathic pain.

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Key words: Acoustic neuroma; Stereotactic radiotherapy; Tricyclic antidepressants; Serotonin-norepinephrine reuptake inhibitors; Calcium channel modulators; Tramadol; Opioids

Core tip: The complexity of managing trigeminal neuralgia and neuropathic pain conditions among patients with skull base tumors requires a simple albeit comprehensive treatment algorithm that can be employed effectively by general practitioners, surgeons and other primary care prescribers in acute care or ambulatory clinical settings. We describe a simple treatment algorithm formulated on recommended best practice and based on clinical experience. It is intended to guide treatment, facilitate management and evaluation of outcome data (self-reported pain, quality of life measures) to elucidate the use of standardized approaches to pain management in patients with skull base etiology.

Hebb ALO, Sawynok J, Bance M, Walling S, Chisholm K, Morris DP. Pharmacological management of neuropathic pain in patients with vestibular schwannomas: Experience of the Atlantic

Lateral Skull Base Clinic. *World J Pharmacol* 2014; 3(2): 24-32 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i2/24.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i2.24

INTRODUCTION

This report considers pharmacological approaches to be used within the skull base tumour health community. Its intent is to offer assistance in treating patients who present with neuropathic pain (NP) in the context of orofacial, head and neck pain, including trigeminal neuralgia (TN), which is the most common craniofacial pain syndrome that is neuropathic in origin. The impetus for assembling the information stems from the experiences of practitioners within an interdisciplinary clinic who consistently rely on formularies and consultation with colleagues for advice in treating neuralgia and NP. The document offers suggested algorithms for assessment and treatment of patients with agents that are recognized to have analgesic efficacy within the broader context of NP. It makes the distinction between generalized NP[1] and NP due to skull base tumours affecting the head, neck, face and glossopharyngeal region[2,3], but also recognizes commonalities in mechanisms underlying various forms of NP[1].

The Atlantic Lateral Skull Base Clinic

Vestibular schwannomas (VS) are slow growing, benign neoplasms that may be life threatening due to compression of central structures. Extra-axial tumours arising from the Schwann cell sheath of the vestibular or cochlear nerve (8th cranial nerve) are referred to as acoustic neuromas or, more appropriately, VS. Clinical diagnosis of a VS suggests an incidence of 0.7-1 per 100000 people, although incidental (*i.e.*, non-clinically relevant) discovery of VS suggests an incidence as high as 2 per 10000 people[4]. In some cases, VS are associated with NP and TN, often secondary to tumour compression or stereotactic radiation treatment affecting cranial nerves within the cerebellopontine angle (CPA).

The Atlantic Lateral Skull Base Clinic provides coordinated care through Neurootology (Division of Otolaryngology), Neurosurgery and the Stereotactic Radiotherapy Group to patients with unilateral or bilateral VS, other CPA tumours, as well as lesions of the petrous apex and jugular foramen. This program is unique in Canada, offering a single centre, multidisciplinary approach for lateral skull base lesions. The Atlantic Lateral Skull Base Clinic serves a population of more than 2 million people in a catchment area that includes Newfoundland, Prince Edward Island, New Brunswick and Nova Scotia[5]. Treatment options for skull base tumours include monitoring clinical progression, surgery, stereotactic radiation therapy (SRT), balance and hearing rehabilitation. An interdisciplinary clinic provides an ideal environment in which to identify and intervene in the treatment and management of NP affecting the head and neck region. The focus of this work is on clinical experiences within our clinic. With low incidence, it will be very unlikely there will ever be randomized clinical trials that provide direct guidance for pain management in patients with VS. In that event, one must rely on extrapolation, and what we report is implementation of that approach and what it looks like in clinic.

Disease background

NP is a chronic pain state that is initiated by peripheral and central nervous system injury caused by trauma, inflammation, infection or metabolic disease, and includes conditions such as distal polyneuropathy due to diabetes [diabetic neuropathy (DN)] and post-herpetic neuralgia (PHN)[1]. The Canadian Pain Society (CPS) estimates (based on 8.2% chronic NP prevalence in the general population) that 1 million Canadians live with NP[6-8]. Neuropathic pain interferes with activities of daily living and work performance, impairs mood, decreases quality of living and generates three-fold increases in health care costs relative to matched controls[9]. NP conditions involve spontaneous (paroxysmal or ongoing) and stimulus-evoked (*e.g.*, mechanical and thermal) symptoms; continuous or intermittent spontaneous pain is frequently described as burning, stabbing, shooting or shock-like; stimulus-evoked pain includes allodynia (pain in response to non-painful stimulation, extreme sensitivity to touch) and hyperalgesia (enhanced response to painful stimuli); NP can also involve tingling and numbness[1,6,9].

TN involves irritation or compression of the 5th cranial nerve (trigeminal nerve), which evokes paroxysmal episodic stabbing pain of the facial area. Classically, pain is described as a sharp, shooting, electric shock-like, unilateral pain with acute onset and termination in distribution of the trigeminal nerve; this usually involves the V2 (maxillary) and V3 (mandibular) divisions but is rare in the V1 (ocular) division[10]. TN has an incidence of 4-28 per 100000 person years[11,12]. It can arise due to vascular alterations, non-vascular lesions, or tumours and other skull base abnormalities which exert pressure on the trigeminal nerve located in the CPA. Trigeminal NP is more continuous, and is characterized as burning, aching, throbbing[10]. VS, or acoustic neuromas, are the most frequent CPA tumour to cause TN-like symptoms[13]. Non-trigeminal nociceptive input, concurrent with induced masticatory responses (*i.e.*, hypoglossal, spinal accessory, facial, glossopharyngeal and vagal motor centers), may contribute to the wide constellation of symptoms seen in orofacial pain patients[14]. The distinction between TN and trigeminal NP is important, as there are different treatment recommendations for each[1,10].

Current algorithms

Treatment guidelines and decision rules improve patient outcomes. Recent literature providing strategies for the treatment of NP include the consensus statement and guidelines from the CPS[6], as well as the Neuropathic Pain Special Interest Group of the International Associa-

tion for the Study of Pain[15,16]. It needs to be emphasized that most recommendations have been derived from studies on PHN and DN. The European Federation of Neurological Societies (EFNS) Task Force included an approach to the management of NP pain associated with damage to the trigeminal nerve[17].

There remains a paucity of information on how to assess, diagnose and treat pain in patients with VS or other skull base tumours, pain that appears to be of neuropathic origin and typically resides within the head, face, neck and glossopharyngeal area. In order to provide guidance relating to this specialized patient population, we constructed an algorithm for NP and craniofacial pain, including TN, based on Canadian contemporary standards of care and existing NP treatment algorithms. Recommendations consider pharmaceutical agents with evidence of efficacy in neuropathic pain, patient tolerability of the dose range expected to be needed, and actual therapeutic efficacy observed within our clinical practice. To date, there has not been a published tool that provides a clearly-defined algorithm for the assessment and treatment of NP in patients with skull base tumours. It is intended that this report provides guidance to primary care practitioners treating NP in patients with skull base tumours, using specific drugs or combinations of drugs, to improve outcomes in clinical practice with respect to patient self-reports of pain and quality of life.

CHARACTERIZATION OF PAIN ASSOCIATED WITH SKULL BASE TUMOURS

Skull base tumours involve the proliferation of abnormal cells in the part of the brain that meets the base of the skull. Symptoms of skull base tumours may include twitching, paralysis or facial pain. Craniofacial NP disorders include neuropathies, neuromas and neuralgias. Although significant interpractitioner and institutional variability exists, facial neuropathy and trigeminal nerve disturbances are relatively uncommon in comparison with unilateral hearing loss, tinnitus and vertigo or even idiopathic headache[18,19]. Even if uncommon, when a patient presents with what appears to be NP, the origin of the pain and appropriate treatment actions may be more difficult to determine than the existence of pain. At the very least, the challenge is to identify where the pain is coming from and to distinguish it from idiopathic headaches. Headaches are present in 50%-60% of patients with unilateral VS at the time of diagnosis[4,20]. Clinically, headaches that are unresponsive to over-the-counter analgesics may be a subtle cue that the pain originates from compression of the cranial nerves by the tumour[21]. It has been reported that 3% to 45% of patients with CPA VS experience facial paresis, facial neuropathy and trigeminal nerve disturbances (hypoesthesia, paresthesia, and neuralgia) due to compression by the tumour on the ipsilateral[13,22], and less commonly, on contralateral[23] cranial nerves; up to 93% are at risk secondary to irradiation treatment[24-27].

The incidence of pain following SRT treatment is common. It has been estimated that 93% of lesions treated with SRT leave the patient at risk of radiation-induced TN[26]. Other centers report that trigeminal symptoms occur in $\geqslant$ 3% of patients whose tumours approach the level of the trigeminal nerve[25]. It is also important to distinguish NP from pain associated with hydrocephalus which may occur following tumour irradiation[19]. As such, patients presenting with pain should be referred for a magnetic resonance imaging (MRI) or computerized tomography scan. It is evident, given the diversity of pain mechanisms and individual patient responses that no single drug works for all NP states. In this respect, successful management of pain syndromes first necessitates accurate assessment.

PHARMACOLOGICAL MANAGEMENT OF NEUROPATHIC PAIN

Assessment of pain and associated symptoms is necessary for diagnosis and management of NP. The self-report version of the Leeds Assessment of Neuropathic Symptoms and Signs scale and the French Neuropathic Pain Group clinician-administered questionnaire called DN4 may be used, based on their high sensitivity and selectivity[1,28,29]. The use of these tools is meant to complement but not replace clinical judgment.

Current treatment guidelines provide an evidence-based approach to the treatment of NP. Treatment guidelines have been developed based on data collected from randomized controlled clinical trials of anticonvulsants (carbamazepine, oxcarbazepine), tricyclic antidepressants (TCAs) (amitriptyline, nortriptyline), serotonin-norepinephrine reuptake inhibitors (SNRIs) (duloxetine, venlafaxine), calcium channel ligands (gabapentin, pregabalin), local anesthetics (5% lidocaine patch), opioids (morphine, methadone) and opioid-like hybrid drugs (tramadol)[6,16,17]. It should be mentioned that if the 5% lidocaine patch is unavailable, a lidocaine gel formulation or compounded cream may be substituted, although limited efficacy in non-post-herpetic pain has been reported[30]. A general overview of our suggested algorithm for the management of NP in our clinic is presented in Figures 1 and 2.

The algorithms presented below are based on published clinical guidelines to simplify the management and evaluation of NP in patients with lateral skull base tumours. Suggested first, second and third line agents [determined by efficacy, indicated by number-needed-to-treat (NNT), and patient tolerability as indicated by number-needed-to-harm (NNH), or side-effects] are listed in Figure 3. NNT and NNH vary according to the etiology of the pain and reference consulted[31,32]. Algorithms for individual pharmacological agents include initial starting doses, titration doses, temporal intervals and maximal dosing schedules[33]. In addition, TCAs, SNRIs, gabapentinoids, opioid analgesics and tramadol must all be used

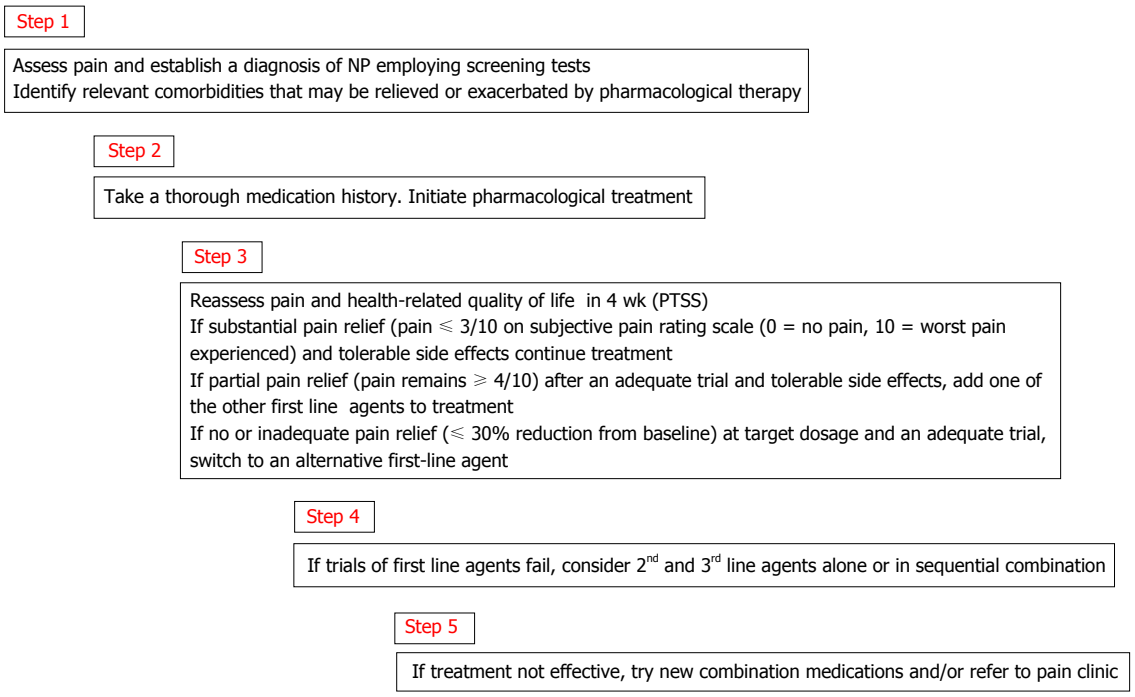

Figure 1 Assessment and management of neuropathic pain. NP: Neuropathic pain; PTSS: Pain treatment satisfaction scale.

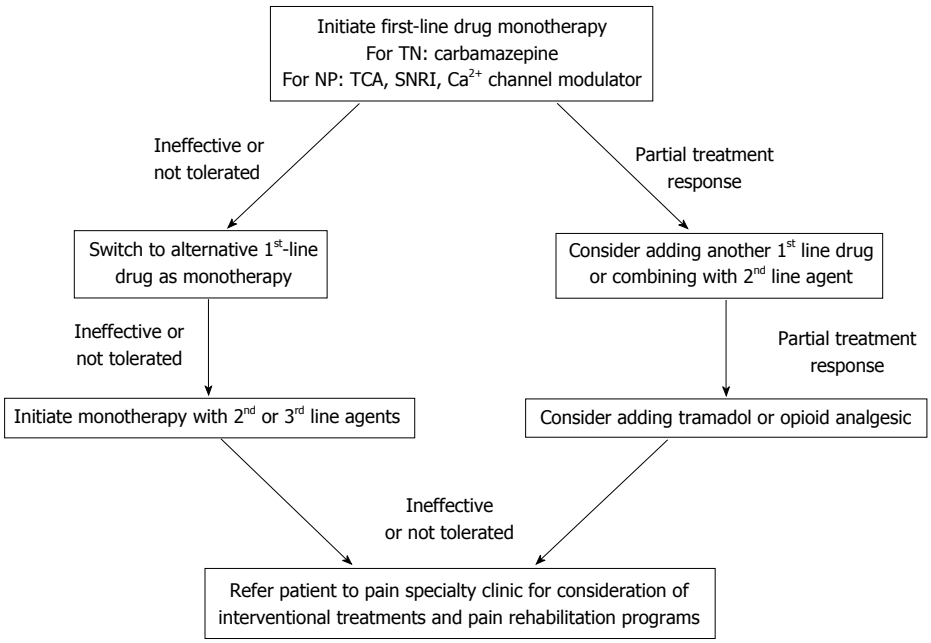

Figure 2 Management of neuropathic pain or trigeminal neuralgia in the Atlantic Lateral Skull Base Clinic. NP: Neuropathic pain; TN: Trigeminal neuralgia; SNRI: Serotonin-norepinephrine reuptake inhibitor.

with caution in elderly patients because of the risk of falls and cognitive impairment[15,16]. Recommendations are also made regarding the duration of adequate trials at maximum tolerated dosages to evaluate the impact on self-reported pain.

Initial and subsequent agents

For management of classical TN (characterized by paroxysmal, unilateral pain), carbamazepine is the first choice, but otherwise it is not used; this agent has a NNT around 1.9 with virtually complete pain relief[6,10,34-36] (Table 1). Oxcarbazepine can, and should, be substituted for carbamazepine if there is an unacceptable side effect profile[10,34-36] (Table 1).

For management of NP, there are several TCAs available, but amitriptyline and nortriptyline are commonly used, and exhibit NNT values of 1.3-3.6[32,37-39]; NNH values are 3-6 for minor, and 14-28 for major, harm. The analgesic properties of TCAs are independent of their antidepressant effects, and several mechanisms, in

Table 1 Summary of drugs used for management of trigeminal neuralgia and neuropathic pain

Drug	Action	Dosing	Common side effects[1]
CBZ	Blocks Na^+ and Ca^{2+} channels	300-1000 mg; 100 mg BID initially, increase by 200 mg weekly Adequate trial 8-12 wk, 2 wk at maximal dose	Drowsiness, ataxia, headaches, nausea, vomiting, constipation, blurred vision, rash Drug interactions Taper doses when discontinuing
OXC	Keto derivative of CBZ, same actions	Equivalent efficacy to CBZ; 300-2400 mg; 300 mg BID initially, increase by 600 mg weekly Adequate trial 8-12 wk, 2 wk at maximal dose	Improved tolerability compared to CBZ Vertigo, fatigue, dizziness, nausea, hyponatraemia in high doses No major drug interactions Taper doses when discontinuing
TCAs or tricyclic antidepressants: nortriptyline, amitriptyline	Block NA and 5-HT reuptake, block Na^+ channels, interact with several neurotransmitter systems	Nortriptyline 10 mg (elderly) or 25 mg (adult) at bedtime; increase dose by 10 or 25 mg every 3-7 d; up to 75-100 mg daily Adequate trial 6-8 wk, 2 wk at maximal dose Amitriptyline doses similar	Nortriptyline is better tolerated than amitriptyline Dry mouth, constipation, blurred vision, sedation, orthostatic hypotension Taper doses when discontinuing
SNRIs or serotonin-noradrenaline reuptake inhibitors: Duloxetine, Venlafaxine	Similar actions to TCAs, but fewer interactions with receptor systems	Duloxetine: 60 mg/d, increase to 120 mg after 1 wk Venlafaxine: 75 mg/d, increase to 225 mg over 3 wk Adequate trial 4-6 wk, 2 wk at maximum dose	Headache, nausea, dry mouth, sleepiness, fatigue, constipation, dizziness, decreased appetite, and increased sweating. Taper doses when discontinuing Drowsiness, dizziness, weakness, feeling nervous, tinnitus, increased sweating, blurred vision, dry mouth, changes in appetite or weight, facial flushing, mild nausea, constipation, sexual side-effects Taper doses over 7-10 d when discontinuing
Gabapentin	Ca^{2+} channel modulator	100-300 mg TID, increase dose every 1-7 d; maximum dose 3600 mg daily Adequate trial 3-8 wk titration, 2-8 wk at maximal dose	Dizziness, sedation, weight gain, weakness, tiredness, nausea, diarrhea, constipation, blurred vision, headache, breast swelling, dry mouth, fatigue, myalgia, loss of balance or coordination Taper doses when discontinuing
Pregabalin	Ca^{2+} channel modulator	50 mg OD or 25 mg BID, double dose each week; maximum daily dose 600 mg Adequate trial is 4 wk at maximal dose	Dizziness, drowsiness, loss of balance or coordination, problems with memory or concentration, anxiety, depersonalization, hypertonia, hypesthesia, decreased libido, nystagmus, paresthesia, twitching, breast swelling, tremors, dry mouth, constipation Taper doses when discontinuing (minimum of one week)
Tramadol	Inhibits NA and 5-HT reuptake, binds opioid receptors	50 mg BID, increase by 50-100 mg daily in divided doses over 3-7 d as tolerated; 400 mg is maximum dose (300 mg in elderly) Adequate trial is 4 wk at maximum dose	Dizziness, spinning sensation, constipation, upset stomach, headache, drowsiness, feeling nervous or anxious
Morphine	Interacts with mu opioid receptors in spinal cord and brain, regulates synaptic activity in pain pathways	10-15 mg q4h or prn (equianalgesic doses for other opioids); after 1-2 wk, convert to long-acting opioid (*e.g.*, CR hydromorphone) Titration allows for dose escalation Adequate trial is 4-6 wk	Sedation, pruritus, constipation, diarrhea, weight loss, nausea, vomiting, stomach pain, loss of appetite, flushing (warmth, redness, tingling), headache, dizziness, spinning sensation, memory problems, sleep problems (insomnia), strange dreams Taper doses when discontinuing

[1]These are not a complete list of side effects and others may occur. CBZ: Carbamazepine; 5-HT: Serotonin; NA: Noradrenaline; OXC: Oxcarbazepine; q4h: Every four hours; BID: Twice a day; TCA: Tricyclic antidepressant; SNRI: Serotonin-norepinephrine reuptake inhibitors; prn: Pro re nata; TID: Three times a day; OD: Once daily; CR: Continuous release.

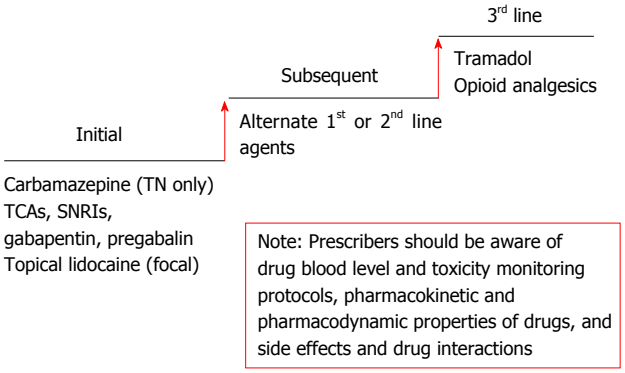

Figure 3 Treatment algorithm for trigeminal neuralgia or neuropathic craniofacial pain. TN: Trigeminal neuralgia; SNRIs: Serotonin-norepinephrine reuptake inhibitors; TCA: Tricyclic antidepressant.

addition to blockage of serotonin and norepinephrine reuptake, are involved in their actions[40]. It should be emphasized that caution must be employed with the use of TCAs in older patients because of anticholinergic adverse effects, sedation, risk of falls, and risk of cardiac toxicity. Nortriptyline, a metabolite of amitriptyline with similar pharmacological effectiveness reflected in similar NNT values[6,37,39], has a lower incidence of adverse effects compared to amitriptyline[32,41]. The lowest effective dose of TCA should be used in NP patients, avoiding patients with ischemic heart disease or increased risk of cardiac death[15] (Table 1). Where available, lidocaine medicated plaster (NNT = 4.4, NNH = 29), a topical formulation, can be considered first-line treatment if the pain is focal and there are tolerability issues for oral for-

mulations[16,37].

The EFNS and Neuropathic Pain Special Interest Group (NeuPSIG) recommend the two SNRIs, duloxetine and venlafaxine, as first-line options, while Canadian guidelines consider these second-line options for treatment of NP. Duloxetine (NNT = 5-6; NNH = 7-9 minor, 13-15 major) has primarily been examined in DN, while venlafaxine (NNT = 3-5, NNH = 7-9 minor, 16 major) has been examined in a broader range of NP conditions[31,37-39] (Table 1). Venlafaxine may be employed in combination with gabapentin to increase its efficacy[16,30,42,43].

The Consensus Statement and Guidelines from the Canadian Pain Society list gabapentin and pregabalin as first-line agents in the treatment of NP[6]. Most of the literature and guidelines for NP are based on PHN and DN and may not be applicable to all NP conditions[9,15]. Gabapentin (NNT = 4.1-6.4, NNH = 3.7) and pregabalin (NNT = 3.3-6.0, NNH = 3.7-8.8) (Table 1)[32,37,38] can be considered as first- or second-line agents for management of orofacial NP associated with skull base tumours. The EFNS Task Force has identified the usefulness of combination therapy, including TCA-gabapentin for the management of NP and TN[17] (Figure 2).

Gabapentin and pregabalin decrease the release of several neurotransmitters involved in pain through binding to the α2-δ subunit of voltage-gated calcium channels, synaptic γ-aminobutyric acid modulation and synaptogenesis[44]. Side effects of gabapentin include dry mouth, dizziness, gastrointestinal disturbances and cognitive impairment. There is some evidence that the efficacy of gabapentin is increased in painful DN, when combined with venlafaxine or morphine[31,45]. Combination of gabapentin with nortriptyline was superior to either drug alone, and combination of gabapentin with opioids has shown increased efficacy for the treatment of TN and NP[17] (Figure 2).

Third-line agents

Tramadol, a hybrid drug with SNRI and μ-opioid agonist properties, has a NNT = 3.4-4.9, and a NNH = 7.7; it should not be combined with TCAs due to increased risk of serotonin syndrome[32,37] (Table 1). Opioids are generally safe if titrated slowly[46]. While there is some evidence to support opioids in NP[15,32,37,39], opioids as primary therapy are not always effective, and combination therapy may be needed[31,42,44]. The analgesic efficacy of a combination of morphine and gabapentin was increased compared to each individual agent in patients with PHN and painful DN; however, while the maximal tolerated doses were lower in combination therapy, there was report of increased adverse effects[16].

There are many liabilities and controversies surrounding the use of opioids in those with chronic non-cancer pain[28,46]. For treating NP with opioids we follow recommendations of the 2010 National Opioid Use Guideline Group[46]; referral to a chronic pain service is indicated when combination therapies involve primary and adjuvant treatment beyond the common agents listed.

Other agents

Although beyond the scope of this document and better reserved for pain specialty clinics, interventional procedures, compounded drugs (such as carbamazepine, gabapentin, antidepressants, lidocaine) delivered as topical formulations for the orofacial region[47], invasive techniques (may include intravenous lidocaine)[6] or intradermal botulinum toxin[16] may be considered. Several recent case series reports and a controlled study support the efficacy and safety of botulinum toxin for TN[48-50]. Future treatment guidelines will position these options within current schemes.

CASE EXAMPLES

Two case examples of the presentation and treatment of neuropathic pain resulting from VS are outlined below.

Case 1

Presentation and diagnosis: RG, a 72-year-old retired fireman, presented to the Atlantic Lateral Skull Base Clinic in August 2010 with progressive right-sided hearing loss and tinnitus over 1-2 years with a new presentation of numbness and tingling on the right side of his face including the tip of his tongue. MRI revealed a right-sided cerebellar pontine angle tumour (25 mm × 27 mm) in the axial plane with mass effect on the brainstem consistent with a vestibular schwannoma/acoustic neuroma. RG opted for stereotactic radiation therapy (SRT) over surgery or a more conservative "wait and scan" approach.

Symptoms prior to treatment: In December 2010, prior to SRT therapy, lidocaine hydrochloride swish and spit was ineffective in controlling symptoms. In January 2011, he reported exacerbation of symptoms on the right side of his face, dysesthesia rather than numbness, constant burning involving the tip of the tongue and the bottom of the tongue on the right hand side; symptoms were exacerbated by eating. He had lost 30 pounds due to decreased food intake. Pregabalin 75 mg twice a day and dexamethasone 4 mg every morning with breakfast was prescribed. Over the next month, pregabalin was increased to 150 mg twice a day as he noted some early improvement. The pregabalin dose was again increased to 300 mg and RG gained 3 pounds.

Treatment and follow up: SRT began in February 2011. RG noticed an immediate improvement in his tinnitus. In March 2011, gabapentin replaced pregabalin and was increased to 1800 mg/d while dexamethasone weaning began. RG experienced hypotension in response to the increase in gabapentin and the dose was decreased to 300 mg three times a day. SRT was completed in April 2011. At this time, RG reported increased facial pain, so gabapentin was increased to 1200 and then 1500 mg/d. RG was not reporting any relief from symptoms, and numbness and right-sided trigeminal neuralgia persisted. In October 2011, he was weaned off gabapentin, and amitriptyline was prescribed for facial pain 10 mg every night, and increased to 20 mg after 1 wk. In November

2011, he reported side effects which included sleepiness and nausea. His family doctor changed the prescription to nortriptyline 10 mg for 5 d, then 20 mg/d for 5 d, and then 30 mg/d, with a plan to increase this again in the next 5 d to 50 mg/d, his current dose in February 2012. He has improved facial pain and reports improvements in eating and tasting food. He still has some paresthesia and dysesthesia in the right side of his face in the trigeminal distribution in all 3 branches. His vestibular schwannoma has shrunk by 0.5 cm.

Case 2

Presentation and diagnosis: DB aged 71, presented to the Atlantic Lateral Skull Base Clinic in March 2008 reporting a recent history of loud noises prompting headaches. She subsequently had an audiogram which showed slight decrease in useful hearing on the right side compared to the left. An MRI (November 2007) revealed a small right-sided acoustic neuroma (18 mm × 9 mm in the axial plane). General ears, nose and throat exam was normal, other than the slight decrease in useful hearing; no tinnitus or vertigo was reported. The Unterberger stepping test revealed a moderate pulling to the right; cerebellar and oculomotor function was normal. Her facial nerve was completely normal with no facial weakness, facial twitching or facial numbness. She remained stable for a year.

Symptoms prior to treatment: In July 2009, her hearing deteriorated, to 60% of normal, and she was experiencing disequilibrium. MRI revealed slight growth in the neuroma (20 mm in longest dimension, 13 mm perpendicular to the petrous apex). A subsequent MRI in November 2009 showed further growth (20 mm × 15 mm).

Treatment and follow up: DB was referred for SRT, and this was completed June 2010. MRI in January 2011 revealed central cystic changes in tumour composition consistent with SRT. DB reported no new clinical symptoms other than further decrease in hearing on the right side.

In March 2011, new neurological symptoms emerged. Initially she described the right side of her tongue as heavy. She began to have some right-sided painful secondary paresthesia on her face in cheek area, upper and lower lips and tongue [maxillary (V2) and mandibular (V3) divisions of the trigeminal nerve] that she describes as a persistent burning sensation that is made worse by eating. She also described discomfort in her right eye and right-sided facial twitching. MRI in March 2011 showed swelling of the tumour in the right CPA consistent with SRT-induced changes. She was started on gabapentin 300 mg once daily for one week, increasing the dose to twice daily the second week, and thrice daily for the third week if tolerated.

In November of 2011 she returned. Gabapentin 300 mg once daily reduced her symptoms; however, it was associated with intolerable constipation and myalgia in the upper arms. She was weaned off gabapentin and her constipation and myalgia dissipated. The right-sided acoustic neuroma had further decreased in size, approximately 18 mm × 12 mm (May 2013) compared to 22 mm × 16 mm (April 2012), yet she continued to experience persistent chronic orofacial pain. While it is possible that DB would be refractory to other pharmacologic interventions, she has declined further interventions, despite the pain and effect on her quality of life.

CONCLUSION

The present report describes evidence for application of pain management strategies in patients with VS. A decision tool and treatment algorithm is presented to facilitate evaluation and management of patients with NP resulting from skull base disorders. A pharmacological algorithm, with primary and adjuvant treatment, summarizes pharmaceutical choice and management of NP and TN in patients with VS and can be made available in the clinic for quick review by the treating physicians. This instrument is intended to guide treatment of neuropathic/trigeminal pain in patients in acute care or ambulatory clinical settings. Essential to the comprehensive management of patients is evaluation of the effects of the intervention on quality-of-life and patient satisfaction with pain management. Patient satisfaction with pain management, using the Pain Treatment Satisfaction Scale[51], as well as the American Pain Society Satisfaction Survey, was influenced by effectiveness of medication on pain severity, independent of initial pain intensity, and by communication[52]. Comparison of outcome data (self-reported pain and quality of life) will elucidate the use of standardized approaches to managing pain among patients with specific skull base etiology. The purpose of the present treatment algorithm was to develop a common scheme that may be utilized by beginning practitioners for treating this relatively uncommon, but clinically challenging, condition.

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P- Reviewer: Gottschalk A, Noll-Hussong M, Tao F
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ISSN 2220-3192 (online)

World Journal of *Pharmacology*

World J Pharmacol 2014 September 9; 3(3): 33-38

Published by Baishideng Publishing Group Inc

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World Journal of Pharmacology

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Quarterly Volume 3 Number 3 September 9, 2014

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World Journal of Pharmacology
Volume 3 Number 3 September 9, 2014

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NAME OF JOURNAL
World Journal of Pharmacology

ISSN
ISSN 2220-3192 (online)

LAUNCH DATE
February 9, 2012

FREQUENCY
Quarterly

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Telephone: +1-925-223-8242
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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
September 9, 2014

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DOI: 10.5497/wjp.v3.i3.33

World J Pharmacol 2014 September 9; 3(3): 33-38
ISSN 2220-3192 (online)
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Overview on metabolomics in traditional Chinese medicine

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Supported by National Natural Science Foundation of China, Nos. 81173500, 81373930, 81302905, 81102556, and 81202639; National Key Technology Research and Development Program of the Ministry of Science and Technology of China, Nos. 2011BAI03B03, 2011BAI03B06, and 2011BAI03B08; National Key Subject of Drug Innovation, No. 2009ZX09502-005; and Foundation of Heilongjiang University of Chinese Medicine, No. 201209
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Received: March 26, 2014 Revised: July 2, 2014
Accepted: July 27, 2014
Published online: September 9, 2014

Abstract

Metabolomics has been widely used in the modern research of traditional Chinese medicine (TCM). At the same time, the world is increasingly concerned about TCM, and many studies have been conducted to investigate different aspects of TCM. Among these studies, metabolomic approach has been implemented to facilitate TCM development. The current methods for TCM research are diverse, including nuclear magnetic resonance, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry. Using these techniques, some advantageous results have been obtained in the studies of TCM, such as diagnosis and treatment, quality control, and mechanisms of action. It is believed that the further development of metabolomic analytical techniques is beneficial to the modernization of TCM. This review summarizes potential applications of metabolomics in the area of TCM. Guidelines for good practice for the application of metabolomics in TCM research are also proposed, and the special role of metabolomics in TCM is highlighted.

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Key words: Traditional Chinese medicine; Metabolomics; Metabolite; Biomarker; Liquid chromatography-mass spectrometry

Core tip: Traditional Chinese medicine (TCM) has been used for thousands of years to treat or prevent diseases. Actual value of TCM has not been fully recognized worldwide due to the lack of scientific approaches. Metabolomics has become a hot topic in TCM research. Metabolomics is the best method to fit the holistic concept of TCM, and it can not only interpret the essence of syndrome but also elucidate the scientific connotation of prescription. This combination of TCM with metabolomics in modern health care systems may lead to a revolution in TCM therapy.

Qiu S, Zhang AH, Sun H, Yan GL, Wang XJ. Overview on metabolomics in traditional Chinese medicine. *World J Pharmacol* 2014; 3(3): 33-38 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i3/33.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i3.33

INTRODUCTION

Traditional Chinese medicine (TCM) has been important in health protection and disease control in East Asia for thousands of years. Now, it is getting more and more popular in the whole world for improving health conditions of human beings and preventing or healing diseases. Moreover it shows some advantages in early intervention, individuation medicine and combination therapies. TCM has a distinctive feature, *i.e.*, its systems

theory, which includes the holistic view and the dialectical view. It takes the human body as a whole from the key concepts of "qi, blood, yin-yang, viscera (Zang-Fu), and meridian and channel"[1]. TCM is also a natural combination of philosophy and ancient science disciplines. The overall concept and differential treatment are the most basic features. Chinese herbal medicine treats a disease by regulating and mobilizing the whole body rather than just regulating a single factor. However, the development of TCM is restricted in the world. The lack of scientific and technologic approaches makes TCM face serious challenges and suffer from inadequate modern research.

Metabolomics is a new subject concerned with the comprehensive characterization of the small molecule metabolites in biological systems. It can distinguish between diseased and non-diseased status information through the assessment of global metabolic profiles in approximative biofluids and biomarker discovery[2]. Studying the metabolome can highlight changes in networks and pathways and provide advice to physiological and pathological states[3]. New dimensions are adding to the field of metabolomics by developments in new technology, flux analysis and biochemical modeling[4-6]. The holistic analyses in the context of metabolomics will give the current essay and the foreseeable developments some relatively clear conclusions[7].

TCM recognizes the human body by system discrimination and in a cybernetic way. TCM can be characterized as holistic, emphasizing the integrity of the human body. It also pays close attention to the relationship between human and their social and natural environment[8]. Metabolomics is a promising way for research of TCM and opens a new way for using metabolomic platform to resolve TCM issues[9]. The metabolomic platforms provide a way to extend the understanding of the mechanisms of action of TCM formulae and the analysis of Chinese herbals, TCM syndromes, mineral medicine, and acupuncture[10]. They offer a useful tool to identify biomarkers and provide a new method for studying the efficacies and mechanisms of TCM in treating diseases[11]. Conclusively, metabolomics has become a key to resolving special TCM issues. Here, we give an overview of the applications of metabolomic approaches in research of TCM in recent years.

METABOLOMICS IN DIAGNOSIS AND TREATMENT IN TCM

The superiority and soul of TCM are diagnosis and treatment, and syndrome is the basic concept of the theory. The accuracy of syndrome differentiation determines the effectiveness of TCM treatment. The metabolomic technologies have been used in objectively differentiating syndromes and exploring their biological mechanisms by studying the functional activities of the human body from a system-wide perspective. It will impact our understanding of the theory behind the evidence-based Chinese medicine[12]. Lu *et al*[13] performed the overall biological characterization of the urine of psoriasis patients with Blood Stasis Syndrome. Simultaneously, they investigated the therapeutic metabolomic mechanism of the Optimized Yinxieling formula. The findings enhanced the understanding of the metabolic influence in Blood Stasis Syndrome in psoriasis patients and the mechanism of action of optimized Yinxieling[13]. In addition, that study demonstrated that metabolomics was a powerful tool in diagnosis and treatment of primary dysmenorrhea by providing information on changes in metabolites and endocrinal, neural and immune pathways. Xiang-Fu-Si-Wu Formula intervention can affect some significant perturbations in sphingolipid metabolism, glycerophospholipid metabolism and steroid hormone biosynthesis to make the metabolic discrepancy return to the normal level[14].

The personalized diagnosis in TCM can help to distinguish different types of diabetics. Metabolomics provides biomarkers for disease subtypes as a potential platform**.** It has been proved that combining metabolomics with TCM diagnosis can reveal metabolic characteristics for pre-diabetic subtypes[15]. Xu *et al*[16] used the orthogonal signal correction-partial least squares method to confirm the existence of metabolite differences among different TCM syndromes. Additionally, a new method has been developed to distinguish the difference between healthy controls and patients with TCM deficiency syndromes by uncorrelated linear discriminant analysis. It provides important information assisting TCM clinical diagnosis[16]. Metabolomics has the potential to become a diagnostic tool for diseases and provide a new way to understand pathophysiologic mechanisms. Metabolic pathways including alanine and aspartate were found to be disturbed in jaundice syndrome patients. Using this method, 44 marker metabolites have been identified to distinguish patients with jaundice syndrome from matched healthy controls[17].

METABOLOMICS IN ACUPUNCTUROLOGY

Acupuncture, as an alternative and complementary therapy, has been used for disease treatment and prevention in TCM[18]. But, the underlying mechanism of acupuncture is unclear, which precludes its widespread use. Metabolomics is similar to acupuncturology in terms of dynamic changes and comprehensiveness[19]. The high-throughput metabolomics can identify potential factors for acupuncture effects and provide valuable information towards understanding therapeutic mechanisms. Wang *et al*[17] assessed the acupuncture treatment at the "Zusanli" acupoint *via* marker metabolites, based on the perturbed signatures and pathways after acupuncture[20,21].

Many studies show the potential of an NMR-based metabolomic approach in the research of biological effects of acupuncture. It was used to investigate the metabolic change of plasma before and after electro-acupuncture in senescence-prone mice, providing a method to assess the effects of acupuncture and to understand the

underlying mechanism in neurodegenerative diseases[22]. Wu *et al*[23] have shown that acupuncture demonstrates its therapeutic effects in the relief of functional dyspepsia symptoms. After treatment, the levels of leucine/isoleucine, lactate and glucose in patients significantly changed and lipid levels slightly changed towards those of the healthy controls[23]. Acupuncture could make the metabolite network recover. An UPLC-MS-based metabolomic method has been developed to investigate the biological effect of acupuncture in acute gouty arthritis and to understand the underlying mechanism[24].

APPLICATION OF METABOLOMICS TO MATERIAL FOUNDATION

Metabolic profiling is benefit to screening active components in medicinal plants. Li *et al*[25] have used metabolomics to find metabolites with antitussive and expectorant activities. It has been shown that chlorogenic acid, 3,5-dicaffeoylquinic acid, and rutin may be closely associated with the antitussive and expectorant activities[25]. Wang *et al*[26] established a UPLC/MS method for analyzing the chemical constituents after oral administration of Yinchenhao Tang (YCHT), which was used for treatment of jaundice syndrome. Forty-five compounds *in vitro* and 21 compounds *in vivo* were detected[26,27]. The three components of YCHT are Artemisia annua L., Gardenia jasminoids Ellis, and Rheum Palmatum L., whose major active ingredients are 6,7-dimethylesculetin (D), geniposide (G), and rhein (R), respectively. The D/G/R combination had a more robust synergistic effect than any one or two of the three individual compounds by acting upon multiple target proteins[28].

ACTION MECHANISM RESEARCH

Action mechanisms of most of Chinese medicines are difficult to determine. In an attempt to address the benefits of Chinese medicine using current biomedical approaches, we regard the metabolomics technology as a powerful tool. Metabolomic techniques are promising for identifying biomarkers, clarifying mechanisms of disease, and highlighting insights into drug discovery. Zhao *et al*[29] identified 19 metabolites as potential biomarkers of chronic kidney disease, and 10 biomarkers returned to the control levels in Poria cocos-treated groups. Furthermore, they found that topical treatment with Poria cocos intervenes some primary metabolic pathways[29]. Scoparone has a potential effect against carbon tetrachloride-induced liver injury through regulating multiple perturbed pathways to the normal state[30]. The dried root of Kansui (*Euphorbia kansui L.*), an effective TCM, has been researched by NMR analysis. It provides new clues to the toxicity of Kansui from a systematic and holistic view[31].

Modified Sinisan can have an effect on liver injury through partially regulating the perturbed pathways, such as phenylalanine metabolism, tyrosine and tryptophan biosynthesis, tryptophan metabolism, retinol metabolism, and tyrosine metabolism[32]. Gou *et al*[33] investigated the effect of Xia Yu Xue Decoction on liver fibrosis by a urinary metabolomic method, based on gas chromatography coupled with GC/MS. It was suggested that the mechanism of action of Xia Yu Xue Decoction may affect ten potential biomarkers associated with microflora metabolism[33]. Chen *et al*[34] have studied the therapeutic mechanism of a traditional Chinese medicine Jiu Wei Qiang Huo decoction effects against H1N1-induced pneumonia by a metabolomic approach. The findings provided a systematic view and a basis for understanding of prevention and treatment[34].

POTENTIAL OF METABOLOMICS FOR STUDING CHINESE MEDICAL FORMULAE

Chinmedomics, defined as "elucidating the therapeutic and synergistic properties and metabolism of traditional Chinese medical formulae (chinmediformulae) and related metabolic analysis by modern techniques", has recently showed potential in evaluating TCM[35]. It supplies a way to translate chinmediformulae into practices. TCM therapy will be revolutionized by the way, which combines chinmedomics with chinmediformulae in modern health care systems[36]. A developed and validated UPLC-MS/MS method has been used to test the plasma pharmacokinetics, tissue distribution and excretion of schisandrin (the main component of shengmaisan) in rats after oral administration of shengmaisan. This method can be used to investigate the *in vivo* behaviors of the TCM components in formulae[37]. The pathogenic mechanism of yinhuang syndrome was investigated by a metabolomics method, which had identified 19 biomarkers for the progression of the yinhuang syndrome[38].

METABONOMICS IS A ROAD TO QUALITY CONTROL OF TCM

The quality control of Chinese medicine, referring to the TCM preparations comprising more than one herb, is challenging due to their extreme chemical complexity. A chemical fingerprint technique for quality control has been established for identifying herbs from different origins. Fingerprinting analysis could provide a platform to identify herbs from different origins by GC-MS, which is beneficial to quality control. In this way, Longae rhizome samples have been indentified as the characteristic components for distinguishing these samples of various geographical origins, which is good for quality control[39]. A practical quality control method for A. Radix has been set up by recognizing GC-based metabolic markers. It identified sorbitol and a glucose/4-aminobutyric acid combination as bio-markers for discriminating species and cultivation area[40].

Twelve active components in a methanol extract of Weichang'an pill were simultaneously determined using the HPLC-DAD-ESI-MS/MS technique[41]. Wang *et al*[42]

have separated and determined 18 major active ingredients of Banxia Xiexin decoction in order to achieve quality control by UPLC-MS/MS. A fingerprint profile of Niuhuang Shangqing pill has been established and 190 compounds was characterized by HPLC/qTOF-MS. It is a significant method to implement and provides a potential approach to achieve the holistic quality control of complex TCM preparations[43].

METABONOMICS PROVIDES INSIGHTS INTO THE GLOBAL ISSUES OF TCM TOXICOLOGY

Metabolomics has showed potential to improve the discovery of biomarkers for detection of toxicity. Dong *et al*[44] utilized global metabolomics to find 17 metabolites, which were regarded as phenotypic biomarkers for toxicity of Chuan Wu. Additionally, the mechanisms of Fuzi's toxicity and potential tissue-specific biomarkers for the toxicity have been explored by matabolomics. Significant changes of 14 lipid metabolites were considered the potential biomarkers for toxicity of Fuzi[45].

In TCM, a principle called "Jun-Chen-Zuo-Shi" may be used to formulate a herbal formula that can mitigate the toxicity of the main ingredient. NMR-based metabolomics approach has been used to research the toxicity of realgar after being counterbalanced by other TCMs in a TCM prescription named Niuhuang Jiedu Tablet. The counterbalanced realgar in Niuhuang Jiedu Tablet was more secure and much less toxic[46]. A TCM Paozhi approach can increase potency and reduce toxicity. An RPLC-Q-TOF/MS method based on metabolomic analysis has been explored to help improve the understanding of the transformation mechanisms underlying Paozhi. Twenty-two key biomarkers responding to detoxifying actions of Paozhi were identified[47]. Sun *et al*[48] also proved that metabolomic method greatly contributes to the investigation of processed Fuzi and provides useful information for the potential activity and toxicity of processed Fuzi.

CONCLUSION

Metabolomics is a modern technology in the post-genome era and has been being used widely in modern Chinese medicine[49,50]. Metabolomics reflects the function of organisms from terminal symptoms of metabolic network and can help understand metabolic changes of a complete system caused by interventions in the holistic context. Its character is consistent with the whole thinking of TCM, and it may be beneficial to provide an opportunity to scientifically express the meaning of evidence-based Chinese medicine. It shows potential in both TCM research and drug discovery. Metabolomic applications in TCM field related to drug development from natural sources and drug discovery aim at raising the potential of metabolomics in reducing the gap between TCM and modern drug discovery, and highlight the key role of biomarkers for drug discovery and development of traditional oriental medicine. It is expected that current metabolomic technologies can impel the development of TCM, especially in the understanding of the concept of Chinmedomics. Currently, systems biology is in accordance with the holistic concept and practices of TCM and will help to understand the mechanisms of TCM. As one part of "Omic", metabolomics, playing an import role in systems biology, has a non-selective approach and can thus lead to the identification of all the metabolites. Metabolomics should be devoted to establishing and improving its own databases, linking other genomics together to solve the problems of TCM, in order to enhance its self-worth in the field of TCM research. Overall, incorporation of metabolomics technologies into TCM can make it possible to study the mechanism of TCM.

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P- Reviewer: Tang WF, Wang XP **S- Editor**: Ji FF
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ISSN 2220-3192 (online)

World Journal of *Pharmacology*

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Volume End

Published by Baishideng Publishing Group Inc

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NAME OF JOURNAL
World Journal of Pharmacology

ISSN
ISSN 2220-3192 (online)

LAUNCH DATE
February 9, 2012

FREQUENCY
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PUBLICATION DATE
December 9, 2014

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DOI: 10.5497/wjp.v3.i4.39

World J Pharmacol 2014 December 9; 3(4): 39-55
ISSN 2220-3192 (online)
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REVIEW

Dried-leaf *Artemisia annua*: A practical malaria therapeutic for developing countries?

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Supported by Worcester Polytechnic Institute and University of Massachusetts Center for Clinical and Translational Science partially; partially by Award Number NIH-R15AT008277-01 from the National Center for Complementary and Alternative Medicine
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Received: June 25, 2014 Revised: September 9, 2014
Accepted: October 1, 2014
Published online: December 9, 2014

Abstract

Artemisinin from the plant *Artemisia annua* (*A. annua*) L., and used as artemisinin combination therapy (ACT), is the current best therapeutic for treating malaria, a disease that hits children and adults especially in developing countries. Traditionally, *A. annua* was used by the Chinese as a tea to treat "fever". More recently, investigators have shown that tea infusions and oral consumption of the dried leaves of the plant have prophylactic and therapeutic efficacy. The presence of a complex matrix of chemicals within the leaves seems to enhance both the bioavailability and efficacy of artemisinin. Although about 1000-fold less potent than artemisinin in their antiplasmodial activity, these plant chemicals are mainly small molecules that include other artemisinic compounds, terpenes (mainly mono and sesqui), flavonoids, and polyphenolic acids. In addition, polysaccharide constituents of *A. annua* may enhance bioavailability of artemisinin. Rodent pharmacokinetics showed longer $T_{½}$ and T_{max} and greater C_{max} and AUC in *Plasmodium chabaudi*-infected mice treated with *A. annua* dried leaves than in healthy mice. Pharmacokinetics of deoxyartemisinin, a liver metabolite of artemisinin, was more inhibited in infected than in healthy mice. In healthy mice, artemisinin serum levels were > 40-fold greater in dried leaf fed mice than those fed with pure artemisinin. Human trial data showed that when delivered as dried leaves, 40-fold less artemisinin was required to obtain a therapeutic response compared to pure artemisinin. ACTs are still unaffordable for many malaria patients, and cost estimates for *A. annua* dried leaf tablet production are orders of magnitude less than for ACT, despite improvements in the production capacity. Considering that for > 2000 years this plant was used in traditional Chinese medicine for treatment of fever with no apparent appearance of artemisinin drug resistance, the evidence argues for inclusion of affordable *A. annua* dried leaf tablets into the arsenal of drugs to combat malaria and other artemisinin-susceptible diseases.

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Key words: Malaria; Infectious disease; *Artemisia annua*; Artemisinin; Combination therapy; Artemisinin combination therapy

Core tip: Artemisinin, extracted from the plant *Artemisia annua* (*A. annua*) L., and artemisinin derivatives are the current best antimalarial therapeutics and are delivered as artemisinin combination therapy (ACT). Availability and cost are problematic for the developing world where malaria is endemic. Oral consumption of *A. annua* dried leaves is more effective than the pure drug. A tea infusion of the leaves has prophylactic effects. Cost of producing and delivering the tea and *A. annua* dried leaf tablets is much more affordable than ACT.

Weathers PJ, Towler M, Hassanali A, Lutgen P, Engeu PO. Dried-leaf *Artemisia annua*: A practical malaria therapeutic for developing countries? *World J Pharmacol* 2014; 3(4): 39-55 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/39.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.39

INTRODUCTION

Nearly three billion people are affected by malaria with almost a million deaths annually, especially in Africa and amongst children[1]. Currently extracted from *Artemisia annua* (*A. annua*) L., artemisinin (Figure 1) is delivered in concert with another antimalarial drug [artemisinin combination therapy (ACT)] as the preferred treatment to slow emergence of drug resistance. Despite these efforts, artemisinin resistance is appearing[2] and persistent and/or asymptomatic malaria may also be playing a role in disease transmission[3-5]. Moreover, for developing countries ACT is costly and the supply is inadequate[6-9].

Artemisinin is a sesquiterpene lactone that is produced and stored in the glandular trichomes that are mainly on the leaves and floral buds of *A. annua*, a GRAS medicinal herb[10-12]. The plant also produces > 40 flavonoids[13], many polyphenols, and a variety of other terpenes including mono-, sesqui-, di-, and triterpenes[14]. As discussed later, many of these have weak antimalarial activity, and, based on transcriptome analyses, many also seem to be produced and/or stored in the glandular trichomes that also contain artemisinin[15].

We and others proposed direct consumption of *A. annua* either as a tea infusion[16-19] or by oral consumption of the leaves[20-24]. In contrast to the oral consumption of pure artemisinin, we showed that the presence of plant material significantly enhanced appearance of artemisinin in the serum of healthy and *Plasmodium chabaudi*-infected mice[22]. Because of the plethora of mild antimalarial compounds naturally present in the dried leaves of the plant, we have termed this orally consumed dried leaf therapeutic plant-based artemisinin combination therapy, or pACT. These whole plant approaches are similar to the more than 2000 year traditional use of the plant by the Chinese[25].

To produce a therapeutically effective drug using a complex material like a medicinal plant requires that a number of key factors be met: the medicinal herbal product must be therapeutically effective; levels of key chemical components in the herb must be verifiably consistent; production must also be cost effective. Here we summarize and update our recent review[26] on the effects of *A. annua* on malaria and further discuss the bioavailability and therapeutic efficacy of pACT and how such an herbal drug could inexpensively be produced with a consistent dose.

PROPHYLACTIC USE OF *A. ANNUA*

Tea infusion, its chemistry, and in vitro studies

Until recently, there have been, to our knowledge, few well-controlled studies examining extraction, recovery, and stability of artemisinin and other compounds in *A. annua* tea infusion. A systematic study of preparations of *A. annua* therapeutic tea infusion was performed by van der Kooy *et al*[27] and showed that nearly 93% of available artemisinin was extracted from dried *A. annua* leaves, but only under certain conditions. Best preparation method was: 9 g DW leaves/L, for 5 min at 100 ℃. Subsequent storage of the tea infusion at room temperature showed that artemisinin concentration was stable for > 24 h, important for malaria-endemic locations where there is no refrigeration. Artemisinin water solubility is approximately 50 mg/L[27], so the amount of artemisinin recovered from hot water tea infusions is reasonable. Other studies using the same extraction protocol also measured extraction and stability of artemisinin and some key flavonoids in the tea. Artemisinin was found to be stable at room temperature for up to 48 h[28]; however, some flavonoids were poorly extracted and not stable at room temperature[29].

Carbonara *et al*[28] detected an assortment of phenolics, including 0.06 mg/g DW cirsilineol, in an *A. annua* tea infusion prepared at about a 4-10 fold higher proportion (approximately 38 g DW/L) than that proposed as optimal (9 g DW/L) by van der Kooy *et al*[27]. Most of the measured phenolics in the tea remained constant at room temperature for 48 h post-infusion. More recently, Suberu *et al*[19] identified milligram amounts of phenolic acids, flavonoids, and sesquiterpenes in a liter of *A. annua* tea, all of which demonstrated IC_{50} values in the micromolar or less range (Table 1). Indeed, the IC_{50} of the tea infusion itself was 7.6 and 2.9 nmol/L for the chloroquine (CQ)-sensitive HB3 and CQ-insensitive Dd2 strains of *P. falciparum*, respectively, and better than artemisinin alone suggesting synergism of constituents in the tea mixture. Clearly if a tea infusion is to be a therapeutic option, it must be consistently and reliably prepared and ingested. As suggested by van der Kooy *et al*[27], ideally a liter of tea infusion would be prepared daily and consumed in equal aliquots of about 250 mL over 24 h for several days.

Tea infusion clinical trials

Ogwang *et al*[30,31] tested *Artemisia* tea as a prophylaxis against malaria in 132 adult farm workers, aged 18-60 years, for 12 mo in a randomized clinical trial in Uganda. Tea infusion was consumed once a week at 2.5 g dried leaves per adult infusion dose with 55-100 mg artemisinin/L. Malaria was tracked for 9 mo while adverse clinical effects were tracked for 12 mo. Among those who used *Artemisia* tea there were 80% fewer fever-related hospital visits. Indeed, some patients reported using *A. annua* tea for > 7 years with no incidence of malaria and no serious adverse events. Although this study suggested that once weekly consumption of *A. annua* tea infusion may offer prophylactic protection, there were no children or elderly in the study, so additional clinical trials need to be conducted with different populations and age groups. Authors argued that since a single weekly dose was effec-

Table 1 Antimalarial compounds in *Artemisia annua vs* falciparum malaria

Compound	Compound IC_{50} (μmol/L)	Compound + artemisinin IC_{50} (nmol/L)	Ref.
Terpenes			
Artemisinin	0.033 0.022, 0.023[1]	Not applicable	Liu *et al*[52]
Artemisinic acid	77.8, 61.6[1]	No numerical value provided; response depended on concentration of compound tested with artemisinin	Suberu *et al*[19]
Arteannuin B	3.2, 4.8[1]		
Dihydroartemisinic acid	21.1, 17.7[1]		
Nerolidol	9[4]	Interaction with artemisinin not yet tested	van Zyl *et al*[55]
α-pinene	1[4]		
1,8-cineole (eucalyptol)	70[4]		
Limonene	533[4]		
Phenolic acids			
Chlorogenic acid	69.4, 61.4[1]	No numerical value provided; response depended on concentration of compound tested with artemisinin	Suberu *et al*[19]
Rosmarinic acid	65.1, 65.0[1]		
Flavonoids			
Artemetin	26	26	Liu *et al*[52]
Casticin	24	26	
Cirsilineol	23	22.5	
Chrysoplenol-D	32	15	
Chrysoplenetin	36	16	
Eupatorin	65	30	
Isovitexin	72.5, 48.1[1]	Interaction with artemisinin not yet tested	Suberu *et al*[19]
Luteolin	11, 12[2]		Lehane *et al*[54]
Kaempferol	33, 25[2]		
Myricetin	40, 76[2]		
Quercetin	15, 14[2], 14.7, 4.11, 2.94[3]		Ganesh *et al*[58]
Rutin	7.1, 3.5, 10.38[3]		

[1]Against CQ-sensitive HB3 and CQ-resistant Dd2 strains, respectively; [2]Against CQ-sensitive 3D7 and CQ-resistant 7G8 strains, respectively; [3]Against fresh Bangladeshi isolates, CQ-sensitive 3D7, and CQ-resistant K1 strains, respectively; [4]Against CQ-resistant FCR-3. CQ: Chloroquine.

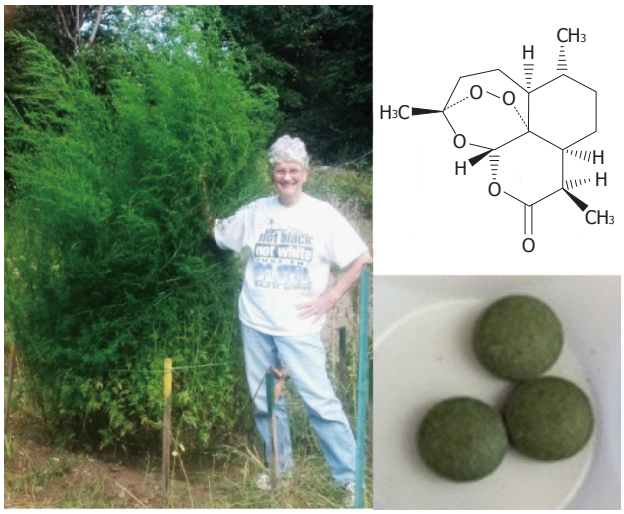

Figure 1 *Artemisia annua* (single clone of *Artemisia annua* cultivar at approximately 2 m height at floral bud formation), artemisinin and plant-based artemisinin combination therapy tablets.

tive, compounds other than artemisinin may have played the prophylactic role since artemisinin itself has short plasma half-life.

THERAPEUTIC USE OF *A. ANNUA*

Tea infusion

Reports on the efficacy of *A. annua* (cv. Artemis) tea on human malaria patients by Mueller *et al*[17,32] and Blanke *et al*[33] yielded at times conflicting results. Their tea infusions contained 47-94 mg artemisinin/L, but recrudescence was much lower in the quinine-treated control group, so parasite reappearance in the tea-treated patients was ascribed to recrudescence and not re-infection[17]. In the Blanke *et al*[33] trial that included a placebo tea, recrudescence was consistently lower in the tea patients than in those treated with 500 mg pure artemisinin. More recently, however, De Donno *et al*[34] showed that 5 g dried leaves in one liter of *A. annua* tea infusion was effective against both CQ-resistant (W2) and CQ-sensitive (D10) strains of *P. falciparum* with IC_{50} values of 5.60 nmol/L and 7.08 nmol/L, respectively, results also consistent with those of Suberu *et al*[19] as already highlighted. These latter *in vitro* studies suggested that tea should be efficacious, so why the discrepancy with the earlier human trials? Preparation methodology is crucial for preserving as much biochemical integrity of the plant as possible[27]. The more recent *in vitro* studies likely used more consistently prepared tea infusions than the earlier human trials, so variations in chemical composition of the infusions and in the plant source material could explain the different responses.

The argument that tea is a monotherapy is unsubstantiated considering the now well-established chemical complexity and related antiplasmodial activity of tea infusions of *A. annua* and its components. Although data from therapeutic tea trials in animals and in humans cor-

Table 2 Kenyan human trial data[20] for orally delivered dried leaf *Artemisia annua* (plant-based artemisinin combination therapy)

pACT (dried leaf *A. annua* tablets, ea 500 mg, 3.7 mg artemisinin/tablet)					
Artemisinin dose (mg)		No. of patients	Leaf DW (g)		% Recrudescence
Day 1	Days 2-6		Day 1	Days 2-6	
7.4 × 2	3.7 × 2	12	2	1	25
11.1 × 2	7.4 × 2	12	3	2	9.1
14.8 × 2	11.1 × 2	12	4	3	16.7
18.5 × 2	14.8 × 2	12	5	4	9.1
Compare to orally delivered pure artemisinin[39]					
Day 1	Day 2-7				
500 × 2	500	227	NA		24

A. annua: *Artemisia annua*; pACT: Plant-based artemisinin combination therapy; NA: Not available.

relate well, unfortunately, they do not support use of *A. annua* tea for treating malaria because animal and human data are comparably negative, the artemisinin dose is not easily controlled, and other potentially synergistic components in the tea are not readily controlled or extracted. Nevertheless, use of the tea could play a role in malaria prophylaxis to reduce incidence of malaria in different communities, or in temporary relief from malaria, mainly in prevention of coma or "to buy time" to enable an infected person from a rural area to travel to a hospital or clinic stocked with ACT.

Dried leaf A. annua - pACT

Recently, Elfawal *et al*[23] measured parasitemia in mice infected with *P. chabaudi* that were fed two different doses (0.6 or 3.0 mg artemisinin; 24 and 120 mg/kg) of either pure artemisinin in mouse chow or as pACT. Artemisinin delivered *via* pACT was at least five times more effective, and with a longer lasting response, than pure artemisinin in reducing parasitemia. Excluding artemisinin there are > 600 phytochemicals that have been identified in *Artemisia annua*[35], but there is currently a lack of information on the chemistry, effect of the preparation method (harvesting, drying, storage, *etc.*), and overall bioavailability of these chemicals[36].

Clinical trials using dried leaf *A. annua* are scarce in the scientific literature and few, other than those in Democratic Republic of Congo by Mueller *et al*[17,32], are published. Despite the fact that WHO does not encourage either whole plant or tea infusion clinical trials[37], some African universities have been conducting their own trials, many of which have not been published nor results assessed by polymerase chain reaction (PCR) as later done for clinical trials with ACTs (personal comm from C. Kasongo to P. Lutgen). Many of these trials used *A. annua* infusions, and compared to controls or even other antimalarial drugs, *e.g.*, artesunate-amodiaquine, showed significantly greater sensitivity of the infusion with fewer late therapeutic failures. For example, in Democratic Republic of Congo, 54 malaria-infected volunteers were treated for 10 d with capsules containing powdered leaves of *A. annua*. Each patient was given 15 g dried leaves containing 15 mg of artemisinin (artemisinin content in leaves = 0.1%[38]). After 2 d all were free of fever and 51 (or 94%) were parasite free after 10 d.

In a study aimed at preventing severe post-operative malaria at Bangui, Central Africa, powdered leaves of *A. annua* were administered in capsules to 25 patients, 22 of them children aged 1-16 years[24]. Treatment duration ranged from 3-4 d with a dose of 0.4-0.5 g/d of *A. annua* dried leaves (0.1% artemisinin leaf content) delivering 0.4-0.5 mg/d artemisinin. In spite of the very low administered daily dose of artemisinin, average parasitemia dropped by 62% in the patients with an added benefit of a strong antinociceptive response, especially beneficial to post-operative patients.

The most clinically definitive study to date of pACT efficacy was conducted at the International Centre of Insect Physiology and Ecology (ICIPE) Mbita Field campus, Suba District, in Western Kenya. This was a collaborative project between ICIPE and Kenya Medical Research Institute[20] (Table 2[39]) and was an open-label, non-randomized clinical trial mainly targeted to assess efficacy, safety, and tolerance of increasing doses of pACT delivered as tablets. The tablets were made by a Tanzania-based NGO, Natural Uwemba System for Health, from a hybrid of *A. annua* grown in the Tanzania highlands (2000-2200 m altitude). Leaves were harvested just before flowering, dried for approximately 3 wk under shade, then crushed, powdered, homogenized, and pressed into 500 mg tablets under ambient temperature. Tablets were robust with no excipient required. Using HPLC with diode array detector, analysis of hexane extracts of randomly selected batches of 100 tablets showed artemisinin content of the tablets was consistent at 0.74% ± 0.06% (*i.e.*, approximately 3.7 mg per tablet).

The four cohorts of the trial each had 12 consenting patients aged 15-56 years (average 23.42) with *P. falciparum* malaria. Based on Giemsa-stained blood smears counted against 200 wbc, parasitemia was 0.02%-4% and hemoglobin levels > 8 mg/dL. Each cohort received one of four increasing numbers of *A. annua* tablets, ranging from 2-5 tablets twice on day 1, followed by 1-4 tablets twice daily for the next 5 d (Table 2). A week following the treatments, three patients scattered throughout different cohorts showed re-appearance of parasites in blood smears; however, all doses were effective in clinical and parasitological regression of malaria, with 9%-20% recrudescence at day 28 and no measurable toxicity.

Compared to the usual large pure artemisinin doses of 1000 mg on day 1 followed by 500 mg on each of days 2-7 that were administered to 227 malaria patients[39], patients treated with pACT had generally better therapeutic outcomes (Table 2). The measured pACT cure rate also was comparable to or exceeded other results using pure artemisinin[40,41], and similar levels of artemisinin (artesunate, artemether, *etc.*[42]). Furthermore, the positive therapeutic response using pACT appeared somewhat independent of dose beyond the second level of dose test-

ed (Table 2[20]). Although oral doses used in the ICIPE[20] trials were far less than any tea studies, levels of recrudescence were much lower than tea and often better than in studies using pure artemisinin[39] (Table 2). Indeed, about 100 total mg of total artemisinin delivered *via* pACT for a full malaria treatment yielded a better recrudescence rate than the 4000 mg of pure artemisinin used by Giao *et al*[39] (Table 2). This 40-fold difference correlates well with the early pharmacokinetic studies by Weathers *et al*[21] that showed 45-fold enhanced bioavailability of the drug when delivered as pACT.

These results suggest that the natural phytochemical blend in pACT is important especially when orally administered as tablets. The results are also consistent with a study in China on mice infected with *P. berghei*, which compared the effects of pure artemisinin with crude *A. annua* extracts[43], and the studies by Elfawal *et al*[23] and Weathers *et al*[22]. In all three studies the administered products had comparable levels of artemisinin, but crude preparations and pACT were at least 3.5 times more effective in reducing parasitemia than pure artemisinin, suggesting a synergistic role for non-artemisinin constituents in the extracts and orally consumed dried leaves.

COMPARATIVE PHARMACOKINETICS AND BIOAVAILABILITY

Orally delivered artemisinin

When given orally or rectally, dihydroartemisinin showed higher bioavailability in humans than artemisinin in an early pharmacokinetic study by Zhao *et al*[44]. The C_{max}, T_{max}, and $T_{1/2}$ for orally delivered dihydroartemisinin were 0.13-0.71 mg/L, 1.33 h, approximately 1.6 h, respectively; for pure artemisinin they were 0.09 mg/L, 1.5 h, and 2.27 h, respectively. Alin *et al*[45] compared orally delivered artemisinin and artemisinin-mefloquine combination therapy for treatment of *P. falciparum* malaria. Infected and uninfected patients had similar pharmacokinetic parameters. After a single dose, bioavailability of artemisinin was not altered. Interestingly, pharmacokinetics were similar when comparing treatment failures with successes, suggesting that studies that only measure artemisinin pharmacokinetics were inadequate for predicting therapeutic success[45]. Ilet *et al*[46] also reviewed artemisinin pharmacokinetics in patients with falciparum malaria and reported a dose of 9.1 mg/kg, which was comparable to that of Alin *et al*[45]. C_{max} and T_{max} values did not differ much from those reported by Alin *et al*[45].

In the Ilet *et al*[46] review of pharmacokinetic parameters of artemisinin and its derivatives, oral pure artemisinin doses ranged from about 6-11 mg kg/L in healthy subjects and C_{max} was 0.15-0.39 mg/L. Dose seemed to have no major effect. An earlier study by Ashton *et al*[47] compared increasing artemisinin doses of 250, 500, and 1000 mg per person and both C_{max} and $T_{1/2}$ showed dose-dependent increases of 0.21, 0.45, and 0.79 mg/L, and 1.38, 2.0, and 2.8 h, respectively, but T_{max} remained relatively constant at 2.3-2.8 h.

Diet is an important consideration for any orally delivered drug, and when Dien *et al*[48] compared artemisinin oral doses given with and without food, C_{max} values were similar between subjects who fasted and those who did not. Food consumption along with artemisinin did not seem to affect artemisinin absorption. In contrast, a later rodent study by Weathers *et al*[21] observed that when artemisinin was consumed as part of a complex plant material, pACT, approximately 45-fold more drug entered the serum of mice than orally administered pure drug. Similarly, when pure artemisinin was fed to mice, it was not detectable in the serum after 60 min. However, artemisinin was detected in the serum when consumed in conjunction with mouse chow, which consists of a variety of plant materials including soy, oats, wheat, alfalfa, beet pulp, corn, *etc*[22].

In a study by Ashton *et al*[49], artemisinin at 9.1 mg/kg was given daily for 7 d, and measurements taken on days 1, 4, 7, and 21. On day 1 plasma C_{max} and $T_{1/2}$ were similar and comparable to data from other studies using a similar dose. On day 4 and 7, however, C_{max} decreased, while $T_{1/2}$ increased, indicating that although artemisinin was delivered daily for 7 d, it was either not readily absorbed or it degraded after the first dose. After the third dose, C_{max} fell from 0.31 to 0.11 mg/L, and $T_{1/2}$ increased from 3.0 to 4.8 h. These results suggested that either artemisinin was metabolized or accumulated elsewhere in the body.

In the liver, cytochrome P450 (CYP450) enzymes metabolize artemisinin to deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and a metabolite named "crystal 7"[50]. Extended artemisinin dosing may not be beneficial as shown by Svensson *et al*[50] using human liver microsomes where activity of CYP450s, CYP2B6 in particular, correlated with decreasing artemisinin serum levels. In intermittent dosing studied by Ashton *et al*[49], the P450 levels were allowed to decline for 14 d before delivery of another dose, and C_{max} rose from 0.11 to 0.20 mg/L, and $T_{1/2}$ decreased from 4.8 to 2.7 h. Generally, maximum concentration of artemisinin in the body increased with increasing doses with $T_{1/2}$ ranging from about 1.4-4.8 h for reported trials using oral pure artemisinin. Thus, increased and extended artemisinin treatment may reduce recrudescence.

Tea infusion delivered artemisinin

Other than Räth *et al*[16], there are few reports on the pharmacokinetics of tea infusion artemisinin delivered in humans. In the Räth *et al*[16] study, artemisinin C_{max} was 0.24 mg/L at 0.6 h post consumption. Tea infusion containing 94.5 mg artemisinin had a C_{max} equivalent to a dose of 250 mg pure artemisinin, but at a significantly shorter T_{max}, 0.6 h *vs* 2.8 h[47]. Compared to pure artemisinin, the shorter half-life of artemisinin in the tea infusion may account for the observed higher recrudescence. Although tea-delivered artemisinin seemed more bioavailable, its shorter $T_{1/2}$ of 0.9 h compared with about 2 h for pure artemisinin, suggested that more than two doses per day may be more beneficial; indeed, four doses a day were

recommended.

The unacceptably high recrudescence rates in clinical tea infusion trials were attributed to low plasma concentrations, almost 40% lower than that for traditional doses (500 mg per person of 60 kg or 8.3 mg artemisinin/kg) of pure artemisinin. Although not specified, tea trial doses have been estimated at about 1.5 mg/kg, close to the 1.1 mg/kg dose of pure artemisinin used by Zhao *et al*[44], which is far below the 8.3 mg/kg that is traditionally accepted as pharmacologically effective. Nevertheless, the C_{max} of 0.24 mg/L artemisinin for the tea dose is nearly twice that for pure artemisinin (C_{max} = 0.13 mg/L) as measured by Zhao *et al*[44]. *A. annua* tea also showed potent antiplasmodial activity against 40 field isolates of *P. falciparum* collected in Pikine, Senegal (mean IC_{50} 0.095 μg/mL[51]).

Dried leaf (pACT) delivered artemisinin

There are as yet no pharmacokinetic studies of pACT in humans. In a small PK study of healthy mice fed artemisinin there was about 45-fold more artemisinin delivered *via* pACT than when delivered as the pure drug[21]. More recently, pharmacokinetics of artemisinin and one of its liver metabolites, deoxyartemisinin, were compared over 120 min in healthy and *P. chabaudi*-infected mice treated with dried *A. annua* leaves at a 100 mg/kg body weight dose of artemisinin[22]. In pACT-treated healthy mice, the first order elimination rate constant for artemisinin was estimated to be 0.80/h, corresponding to a $T_{1/2}$ of 51.6 min. C_{max} and T_{max} were 4.33 mg/L and 60 min, respectively. The AUC was 299.5 μg·min/mL. The first order absorption rate constant was estimated at 1.39/h. In contrast, the AUC for pACT-treated infected mice was greater at 435.6 μg·min/mL. Serum levels of artemisinin in the infected mice continued to increase over the 120 min of the study period. As a result, the elimination half-life, $T_{1/2}$ could not be determined, so C_{max} and T_{max} could only be estimated at ≥ 6.64 mg/L and ≥ 120 min, respectively. Nevertheless, both C_{max} and T_{max} of artemisinin were greater in infected than in healthy mice.

Generally, artemisinin concentrations decreased with a concomitant rise in deoxyartemisinin levels only in healthy subjects[22]. In contrast, artemisinin levels in infected mice continued to rise over the study period whilst deoxyartemisinin levels fell and then leveled, so infection seemed to retard the capacity of the mice to process artemisinin into deoxyartemisinin over the two-hour period. Many compounds in *A. annua* inhibit *P. falciparum*[52-55] and CYP34A[56]. At the high (100 mg/kg) dose used in the study, nearly equal amounts of artemisinin and deoxyartemisinin were measured in the serum, indicating that an excessive dose of artemisinin was used.

The presence of plant material affected artemisinin pharmacokinetics. At 60 min no artemisinin was detected in serum of mice fed pure artemisinin at 100 mg/kg body weight. When plant material was present, however, as mouse chow or *A. annua* pACT, artemisinin level in the serum rose to 2.44 and 4.32 μg/mL, respectively, demonstrating that the presence of plant material, even mouse chow, had a major positive impact on the appearance of artemisinin in the blood[22]. To our knowledge, these are the only data available on pharmacokinetics for orally delivered *A. annua* in animals or humans.

NON-ARTEMISININ THERAPEUTIC COMPOUNDS IN *A. ANNUA*

Flavonoids

A. annua is rich in essential oils, coumarins, polyphenols, polysaccharides, saponins, terpenes, and flavonoids. The levels of flavonoids and other compounds in *A. annua* change with developmental growth stage, with some being highest during full bloom[57]. There are > 40 flavonoids[13], and at least 11, including artemetin, casticin, chrysoplenetin, chrysoplenol-D, cirsilineol, eupatorin, kaempferol, luteolin, myricetin, quercetin, and rutin, are reported to have weak therapeutic efficacy against falciparum malaria (Table 1[52-54,58]). Some of these flavonoids were shown to improve the IC_{50} of artemisinin against *P. falciparum in vitro* by as much as 50%, suggesting synergy (Table 1[52]). Elford *et al*[53] also showed that while casticin [5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one] showed synergism with artemisinin, it did not synergize with chloroquine, suggesting a different interactive mechanism. Combining casticin with artemisinin inhibited parasite-mediated transport systems that control influx of myo-inositol and L-glutamine in malaria-infected erythrocytes. These apparent synergistic actions between flavonoids and artemisinin suggest that flavonoids are likely to be important for efficacious use of *A. annua* consumed either as whole dried leaves or as tea.

Many flavonoids have antiplasmodial effects and inhibit *P. falciparum* growth in liver cells *in vitro* as reported for dietary flavonoids[54]. To our knowledge, there are no reports on pharmacokinetics of *A. annua* delivered flavonoids. Some flavonoids are reported to have long plasma half-lives; *e.g.*, quercetin, found in *A. annua* and most fruits, has a plasma half-life of 27 h[59]. Quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one], also found in garlic, inhibits parasite growth with differential activity against different strains of *Plasmodium* (Table 1[54,58]). Rutin, which is a rutinose [α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose] glycoside of quercetin, showed similar results, suggesting that the sugar moiety did not significantly affect antimalarial activity (Table 1[58]). Flavonoids are known to persist in the body for > 5 d; this may explain the once a week dose inducing a prophylactic effect from *A. annua* tea infusion that was reported by Ogwang *et al*[30,31]. Many dietary flavonoids inhibit *Plasmodium* growth *in vitro*, but amounts in the diets are reportedly insufficient to offer protection against malaria[54]. Plants such as *A. annua* with high concentrations of flavonoids (*e.g.*, up to 0.6%) may, however, work in concert with artemisinin to prevent malaria when consumed regularly.

The flavone luteolin [2-(3,4-dihydroxyphenyl)-5,7-

dihydroxy-4-chromenone] comprises up to 0.0023% DW in *Artemisia*[14] and has been used for a variety of ailments including cough, diarrhea, dysentery, diabetes, cancer, and malaria. Although luteolin has an IC_{50} value around 11 μmol/L[54] and is one of the more active antiplasmodial flavonoids found in *A. annua*, one cannot compare its role between studies as indicated by Ganesh *et al*[58] (see Table 1). The antimalarial response of different flavonoids seems to be affected by the strain of *Plasmodium* being tested. Luteolin also prevents completion of a full intra-erythrocytic cycle by inhibiting progression of parasite growth beyond the young trophozoite stage. The mechanism of this antiplasmodial activity seems to be related to the inhibition of parasite fatty acid biosynthesis. These lipids are required by the parasite to detoxify heme into hemozoin[60]. Independent of the human host, apicomplexan parasites use a fatty acid biosynthetic pathway. Enzymes in the pathway, like the NADPH-dependent *b*-ketoacyl-ACP reductase (FabG), are potential antimalarial targets. Among 30 flavonoids studied, luteolin and quercetin had the lowest IC_{50} values for the inhibition of these enzymes and also showed *in vitro* activity in the sub-micromolar range against multiple strains of *P. falciparum*[60].

Isovitexin {5,7-dihydroxy-2-(4-hydroxyphenyl)-6-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]chromen-4-one} is another flavone, the 6-*C*-glucoside of apigenin, that was found in *A. annua* tea infusion at > 100 mg/L with micromolar antiplasmodial activity (Table 1[19,28]). Isovitexin inhibits lipid peroxidation and xanthine oxidase activity and protects cells from ROS damage with an overall LD_{50} > 400 μmol/L[61].

Terpenes

Limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is part of the "cineole cassette" that includes 1,8-cineole (eucalyptol), limonene, myrcene, α-pinene, β-pinene, sabinene, and α-terpineol[62]; many of these affect particular stages of *Plasmodium* species. For example, limonene is often present at 7 mg/kg in *A. annua*[14] and inhibits isoprenoid biosynthesis in *Plasmodium*[63] and development at the ring and trophozoite stages[64]. Eucalyptol affects the trophozoite stage[65]. Limonene also arrests protein isoprenylation in *P. falciparum*, halting parasite development within 48 h of treatment[64]. The IC_{50} against *in vitro Plasmodium* in these trials was 2.27 mmol/L, more than twice the IC_{50} of 533 μmol/L measured by van Zyl *et al*[55]. Limonene and its metabolites remain in the plasma for at least 48 h[66], so the pharmacokinetics is favorable, which is important for elimination of gametocytes and malaria transmission.

The volatile monoterpene α-pinene (4,6,6-trimethylbicyclo[3.1.1]hept-3-ene) is present in the plant at levels up to 0.05% of dry weight[14]; it has an IC_{50} of 1.2 μmol/L, in the range of quinine at 0.29 μmol/L[55]. Eucalyptol (1,8-cineole) may comprise up to 30% [0.24%-0.42% (V/DW)] of the essential oil in *A. annua*[67] and is a strong inhibitor of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8[68]. Both chloroquine-resistant and chloroquine-sensitive *Plasmodium* strains are affected at the early trophozoite stage[65].

Eucalyptol (1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane) is also volatile and rapidly enters the blood when delivered either as an inhalant or orally[69,70]. At an IC_{50} of 0.02 mg/mL and low toxicity (LD_{50} of approximately 25 mg/mL), either oral or inhalation delivery is reasonable[65,71]. Indeed eucalyptol concentrations can reach 15 μg/mL in 60 min[69] suggesting its possible use as an antimalarial inhalant.

Artemisia ketone (3,3,6-trimethyl-1,5-heptadien-4-one), a major constituent of some cultivars of *A. annua*, has barely been studied. Other ketones like curcumin[72] have been implicated as inhibitors of β-hematin synthesis, so artemisia ketone may play a similar role and affect hemozoin formation. Although hemoglobin is required for *Plasmodium* survival and multiplication in merozoites inside the red blood cell, it leaves toxic debris like heme. The parasite subsequently oxidizes Fe^{2+} in heme to Fe^{3+} forming hematin, a nontoxic insoluble polymeric crystal called β-hematin (also known as hemozoin), which also inhibits cell-mediated immunity against the parasite. Water extracts of *A. annua* inhibit hemozoin synthesis[73].

Essential oils often contain a large amount of monoterpenes that may enhance the antimalarial effect of artesunate and even reverse the observed resistance of *P. berghei* against artesunate[74]. Monoterpenes tend to be higher in the pre-flowering phase of *A. annua*[75], but are drastically reduced by high drying temperatures or drying in the sun[13,76] and, of particular concern, during compression of dried leaves into tablets[77]. Although monoterpenes have some antimalarial potential, most are rather volatile and thus they may be therapeutically less important than the nonvolatile flavonoids, phenolic acids, and higher molecular weight sesquiterpenes.

Unlike α-pinene and eucalyptol, camphor (1,7,7-trim ethylbicyclo[2.2.1]heptan-2-one) has no reported antimalarial activity, but it may comprise as much as 43.5% of the essential oil of *A. annua*[78]. Considering camphor is less volatile than either eucalyptol or α-pinene (melting points of 204 ℃, 176 ℃, and 155 ℃, and flash points of 54 ℃, 49 ℃, and 33 ℃, respectively), it may instead play a role in enhanced transport of hydrophobic molecules like artemisinin from pACT across the intestinal wall into the bloodstream[21,22]. Camphor may also affect thymocyte viability and aid in developing malaria immunity through production of T-cells[79]. At 50 μg/mL, camphor increased viability of cultured thymocytes[80].

The sesquiterpene nerolidol (3,7,11-trimethyl-1,6,10-dodecatrien-3-ol) has an IC_{50} of 0.99 μmol/L and arrests development of the intraerythrocytic stages of the parasite (Table 1[55]). Indians of the Amazon basin in Brazil treated malaria using the vapors of the leaves of *Viola surinamensis*; nerolidol was identified as the active constituent leading to 100% growth inhibition at the schizont stage[81]. Nerolidol levels vary with the cultivar tested, with one of the highest values found in plants from Ethiopia[82]. There is a greater concentration of this sesquiterpene in stems

than leaves of *A. annua*[83].

Other sesquiterpenes found in the artemisinin biosynthetic pathway were only recently shown to have antiplasmodial activity at μmol/L levels, similar to that of other compounds found in the plant (Table 1[19]). These artemisinic compounds were extracted into *A. annua* tea infusions and showed varying interactions with artemisinin depending on their relative concentrations and the target parasite strain. For example, arteannuin B showed an additive interaction with artemisinin against the CQ-sensitive *Plasmodium* HB3 strain, while against the CQ-insensitive Dd2 strain the interaction was synergistic.

Phenolic acids

Rosmarinic ((2"R")-2-[[(2"E")-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxyphenyl) propanoic acid) and chlorogenic ((1*S*,3*R*,4*R*,5*R*)-3-{[(2*Z*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid) acids are strong antioxidants found in a wide variety of *A. annua* cultivars[56]. In Caco-2 studies, these acids significantly inhibited activity of CYP3A4, one of the hepatic P450s responsible for metabolism of artemisinin to deoxyartemisinin, an inactive form of the drug[50]. These and other phenolic acids are present in *A. annua* tea infusion[19]. Both phenolic acids have an IC_{50} of about 65 μmol/L (Table 1) and also significantly reduced secretion of cytokines IL-6 and IL-8, and thus enhanced antimalarial activity while reducing inflammation[56].

Other compounds often found in A. annua and that may affect pACT efficacy

Although polysaccharides in other medicinal plants have been more extensively studied, they seem to have been rather overlooked in *A. annua*, probably because most *Artemisia* extracts are obtained using organic solvents and polysaccharides are only soluble in water. Polysaccharides extracted from *Artemisia iwayomogi* showed hydroxyl radical scavenging activity three times stronger than glutathione or caffeic acid, and ROS inhibition was twice as strong as ascorbic acid[84]. In *A. iwayomogi*, more polysaccharides were found in stems than in leaves and their solubility was also higher from stem than from leaf tissue[84].

The combination of polysaccharides with lipophilic molecules like artemisinin may lead to a higher bioavailability of the antimalarial constituents when delivered *via A. annua,* which may explain the lower effective therapeutic dose against malaria observed for pACT than for pure artemisinin[20,23,26]. Indeed, Han[85] showed that ginseng polysaccharides had preventive and curative antimalarial activities and synergized with artesunate in malaria-infected mice. Sulfated polysaccharides inhibited the *in vitro* invasion of merozoites into erythrocytes and interfered with merozoite surface protein[86-88]. Heparin and other sulfated polysaccharides have been shown to inhibit blood-stage growth of plasmodium[89,90]. Some sulfated polysaccharides inhibited the formation of rosettes between infected red blood cells (iRBC) and uninfected RBCs, as well as adhesion of iRBCs to placental chondroitin sulfate A, which is linked to severe disease outcome in pregnancy-associated malaria[91].

Saponins, common in many plants, have an important role in human and animal nutrition and are reportedly present in *A. annua*, but only as measured in alcoholic extracts using the nonquantitative foaming test[92,93] (Weathers, unpublished). These soap-like amphiphilic (lypo- and hydro-philic) bioactive compounds are mainly produced by plants. Recently, there has been interest in the clinical use of saponins as chemotherapeutic agents[94], and as adjuvants for vaccines[95]. At very low doses saponins are efficient, have hemolytic properties, produce 40-50 Å pores in erythrocyte membranes, and modulate the sodium pump and ATPase[96]. Saponins also have a hypoglycemic effect mainly by inhibiting intestinal permeability and absorption of glucose and may therefore inhibit the growth of *P. falciparum,* which needs glucose to grow[97]. Better identification, quantification, and investigation into the role of saponins in pACT efficacy are warranted.

The coumarin, scopoletin (7-hydroxy-6-methoxychromen-2-one), also known for its antinociceptive properties[98,99], is commonly found in most *Artemisia* species at, for example, about 0.2% (w/w) in a Luxembourg cultivar. Known for its anti-oxidant, hepatoprotective, and anti-inflammatory activities, scopoletin scavenging capacity for hydroxyl radical, DPPH, superoxide anion, hydrogen peroxide, and Fe^{2+} chelating activity is almost at the level of α-tocopherol (Vitamin E)[100].

Although not antiplasmodial, scopoletin inhibits TNF-α, IL-6, and IL-8 at millimolar concentrations, and is thus likely one of the major anti-inflammatory and antipyretic constituents of *A. annua*[101]. Coumarins can activate lymphocytes, thereby stimulating immunological functions[102]. Indeed, scopoletin induced cell proliferation in normal lymphocytes with an immunomodulatory effect[101]. In uninfected erythrocytes internal Na concentration is much lower than external concentration, but the K concentration is higher; in infected blood cells this situation is drastically reversed[103]. Scopoletin significantly stimulated erythrocyte membrane ATPases at 0.1 μmol/L, in particular Na-K-ATPase *vs* Ca-ATPase or Mg-ATPase[104], so scopoletin may affect malaria infection. A significant hormetic effect was also noticed; stimulation was higher at scopoletin concentrations of 10 μg/mL than at 1 or at 100 μg/mL. In addition scopoletin also inhibited ADP-platelet aggregation at a range of 0.1 to 5 μmol/L and improved blood rheology[105].

Scopoletin may also affect the interaction between malaria and uric acid. Cyclical fevers and high levels of inflammation characterize malaria and this likely aids parasite clearance. Excessive and persistent inflammation, on the other hand, can lead to severe malaria[106]. In the cytoplasm of their parasitophorous vacuole, *Plasmodium*-infected erythrocytes contain uric acid precipitates that are released upon erythrocyte rupture. Uric acid precipitates are mediators for inflammatory cytokines IL-6, IL-8, and are considered a danger signal for innate immu-

pACT:
Dried leaves → Milling and homogenization → AN → Assay

eAN based on de Vries *et al*[108]:
Dried leaves → Milling and homogenization → Extraction at 30 ℃-40 ℃ → Crystallization → Filtration/washing → Decolorization → Crystallization → Filtration → Drying at 55 ℃-60 ℃ → AN → Assay at steps along the way → Chemical conversion to artemether or artesunate

Figure 2 Comparison between plant-based artemisinin combination therapy production and extracted artemisinin from dry harvested leaves to product ready either for packaging (plant-based artemisinin combination therapy) or conversion to artemether or artesunate (extracted artemisinin). AN: Artemisinin; eAN: Extracted artemisinin; pACT: Plant-based artemisinin combination therapy.

nity. Uric acid is also the causative agent in gout. These precipitates could offer a novel molecular target for anti-inflammatory therapies in malaria. Scopoletin inhibits the activity of xanthine oxidase in hyperuricemic mice after peritoneal administration, and this hypouremic effect is fast and dose-dependent[107].

Toxicology

Although many of the compounds in *A. annua* have not been tested for their toxicity in, a survey of available MSDS data showed that the LD_{50} levels for orally administered compounds in rodents ranged from about 160 mg/kg for quercetin to > 8000 mg/kg for nerolidol. The artemisinin LD_{50} measured *via* oral dose in a mouse was 4228 mg/kg. Therefore, at the estimated amounts of dried leaves of pACT that may be orally consumed by a malaria patient, most of the compounds reported thus far in *A. annua* are at concentrations that are orders of magnitude below their LD_{50} toxicity values.

Toxicology of the dried leaf tablets used in the Kenyan human trial measured the following components: serum levels of urea, serum proteins, creatinine, γ-glutaryl transferase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, or alkaline phosphatase levels, hemoglobin, and pre- and post-electrocardiograms[20]. Compared to levels prior to treatment with pACT, there was no significant change post-treatment.

PRODUCTION CONSIDERATIONS

Production comparisons with traditional extraction

Because production costs are usually closely held secrets, there are few cost estimates that are publicly available to compare pACT production with extracted artemisinin. However, costs can be estimated from a study by de Vries *et al*[108] where they reported a 1 kg recovery of artemisinin from *A. annua* containing 0.6% artemisinin. Downstream processing costs and product losses increase with increasing number of unit operations (unit ops), a fact often not generally appreciated[109]. Indeed for biotechnology processes, recovery can be anywhere from 9%-51%[110]. As an example, if each step of a 4 step process is 95% efficient, then the overall process has a final efficiency of about 81%, while a single step process at 95% efficiency has a 95% overall recovery. The described process steps for extracted artemisinin (eAN) *vs* pACT-AN are shown in Figure 2. From the point of harvested dried leaves to material ready for packaging or conversion to the delivered drug (*e.g.*, artesunate or artemether), pACT has one unit op and eAN has eight[108]. Extraction solvents and other chemicals are clearly no longer part of the cost. Because there is one *vs* eight unit ops for eAN and at least two of the eAN unit ops involve significant amounts of heat, pACT energy cost is significantly reduced by at least 90%. Costs for labor, interest, depreciation, and maintenance are all also affected by the number of unit ops[109], so we estimated that with seven fewer unit op steps those costs would reduce by approximately 88%. Although better extraction processes may be in play[111], using the de Vries *et al*[108] analysis our estimate of cost reduction for producing pACT is about 30% less than the cost of producing eAN. Data provided by de Vries *et al*[108] was based on 0.6% artemisinin content, so if a higher producing cultivar was harvested, costs would drop proportionately. Moreover, cost drops again because with pACT there is no need to convert artemisinin to artesunate or artemether; those conversions were necessary because they have higher bioavailability than pure artemisinin, which is not an issue with pACT[21,22].

The de Vries *et al*[108] process cost estimation focuses on a production yield of 1 kg of artemisinin from 500 kg dried leaves, so per Giao *et al*[39] that amount of pure artemisinin would treat only 250 patients. Based on the data shown in Table 3 from Kenyan or WPI *A. annua* at 0.7 and 1.4% artemisinin, 15 and 7.5 g DW leaves, respectively, are required for a total adult pACT treatment; so from 500 kg leaves, 33300 and 66600 patients could be treated, respectively. This represents more than a 130-fold increase in patients treated compared to pure artemisinin with proportionate reduction in price.

A. annua dry leaf production varies around the globe. "In East Africa yields average 2.5 T/ha (range = 0.75-4.2)..."[112]. Based on our field trials[113], the reported average *A. annua* leaf production in E. Africa[112], and the doses used in the Kenyan human trial[20], one can estimate the amount of dry leaf production, and depending on the amount of artemisinin in the biomass, estimate possible number of adult patients that could be treated with pACT (Table 3).

Current ACT drugs vs pACT

Using the dosing information obtained from the Kenyan human malaria trial[20], each adult needs about 100 mg artemisinin total over 6 d for a malaria treatment, so for *A. annua* leaves with 0.7% artemisinin, 15 g of dried leaves would be needed for a 6 d treatment course. At 2 ton of dried leaves harvested per hectare, 127260 adult patients could be treated for malaria (Table 3). For leaves

Table 3 Estimated numbers of adult patients treatable from plant-based artemisinin combination therapy[1]

For *A. annua* cultivar containing	Number of patients treated at various dry leaf tonnage			
	2 T/ha[2]	3 T/ha	4 T/ha[3]	5 T/ha
0.7% artemisinin/g DW (Kenyan cultivar)	127260	190890	254520	318150
1.4% artemisinin/g DW (WPI cultivar)	254520	381780	509040	636300

[1]Assumptions: each adult needs 100 mg artemisinin (AN) over 6 d for a cure; at 0.7% and 1.4% AN that is approximately 15 and 7.5 g DW leaves, respectively, for a single adult total malaria treatment; [2]Below the average of 2.5 T/ha reported for all of East Africa; [3]Equal to the maximum obtained growing *A. annua* SAM in the Stow, MA, United States field trials. *A. annua*: *Artemisia annua*.

Table 4 Estimated number of patient treatments by current artemisinin combination therapy *vs* plant-based artemisinin combination therapy

Combination therapy drug	Adult treatments per ton of artemisinin
AL[1]	1.76 million
AS/AQ[1]	2.5 million
pACT leaves with 0.7% artemisinin[2]	8.6 million

[1]http://www.rollbackmalaria.org/partnership/wg/wgprocurementsupply/docs/psmwg_ppACT-API.pdf, p.2 [cited May 27, 2014]; [2]Assumes a 6 day treatment with pACT, with each patient receiving 15 g dried leaves per full malaria treatment for leaves with 0.7% artemisinin. To obtain an amount of artemisinin equal to 1 T of the extracted drug, one would have to harvest 142.8 tons of dried *A. annua* leaves containing 0.7% artemisinin. AL: Artemether/lumefantrine; AS/AQ: Artesunate/amodiaquine; pACT: Plant-based artemisinin combination therapy.

containing 1.4% artemisinin, only 7.5 g of dried leaves are required, so from a hectare of land producing 2 tons of leaves twice as many patients could be treated (Table 3). Clearly choosing cultivars that have higher levels of artemisinin in their leafy biomass will dramatically increase the number of patients that can be treated from 1 ha.

According to Roll Back Malaria, from one ton of purified artemisinin current ACT therapy can provide 1.76 million adult malaria treatments using artemether/lumefantrine, and 2.5 million adult treatments using artesunate/amodiaquine[114] (Table 4). Using the same one ton artemisinin equivalent, but delivering the drug *via* pACT with 0.7% artemisinin content, one would have harvested about 142.8 tons of dried *A. annua* leaves. Assuming 15 g dried leaves per patient from the dosing data in the Kenyan human malaria trial (Table 2[20]), 8.64 million adult patients could be treated, about a four-fold increase over either of the current ACT drugs. The actual cost of pACT, therefore, mainly depends on the cost of the dried leaves and their artemisinin content.

As yet unpublished data from the Rich and Weathers labs demonstrated that pACT prevents emergence of artemisinin drug resistance; the plant itself seems to function as its own ACT (pACT). This would obviate the need for inclusion of a co-drug as used in currently administered ACTs. The co-drug costs at least as much as the artemisinic portion of the drug[6]. Consequently, elimination of the added co-drug could result in at least an additional 50% reduction in cost, so that the final pACT cost reduction is conservatively estimated to be far below that of a current course of ACT therapy.

Considering that *A. annua* is nontoxic and safe to consume orally, dose may not have to be adjusted for children. On the other hand, the leaves taste bitter, so masking the taste, perhaps with sugar, should help with pediatric treatment. Our recent simulated digestion study showed that adding table sugar (sucrose) to pACT did not significantly alter the amount of artemisinin released after digestion, with the added benefit of doubling the amount of flavonoids released[115].

Comparison with emerging artemisinin sources or other newer antimalarial drugs

There are at least three other emerging antimalarial therapeutic technologies: synthetic artemisinin[116], semi-synthetic artemisinin (SSA) production from genetically engineered microbes[117], and a single dose drug, OZ439[118]. In early 2013, Sanofi/PATH Drug Development Programme, announced they would have the capacity to produce up to 60 MT of SSA in 2014 at about $400/kg, depending on quantity; Sanofi now has WHO prequalification for its SSA[119]. Although not much cheaper than the current price of about $550/kg[120], supply would be more or less unlimited. Despite what might seem as an advantage to large amounts of SSA production, there are also some serious disadvantages, and comparison of some advantages and disadvantages for each of these new synthetic antimalarial drugs and pACT is noted in Table 5.

QUALITY ASSURANCE CONSIDERATIONS

Agricultural quality

The traditional and least costly method for cultivating *A. annua* uses seeds and in developing countries farmers prefer to save seeds from one growing season to the next. However, seed generated plants of *A. annua* will vary widely from generation to generation even with high quality starting stock (see review by Ferreira *et al*[10]). Stem cuttings of *A. annua* readily root in about two weeks, so clonal propagation *via* rooted cutting is recommended to eliminate this variability. Although this method of propagation is not cost effective for large plantations, it would work for a few hectares or for controlled environment agriculture. Given the large numbers of patients that could be treated from growing just a few hectares of *A. annua* (Table 3), clonal propagation by rooted stem

Table 5 Comparison of emerging antimalarial therapeutic technologies with plant-based artemisinin combination therapy

Technology	Advantages	Disadvantages
Synthetic AN[116]	Fully synthetic method giving AN = compound	Requires co-drug to obviate emergence of AN drug resistance
	Lowers AN cost compared to extraction	Not yet in production
		Needs sophisticated process
		Likely all under Western control
		Challenging patient compliance due to multiday dosing
Semi-synthetic AN[117]	Semi-synthetic method giving authentic AN	Requires co-drug to obviate emergence of AN drug resistance
	Lowers AN cost compared to extraction	Production began *via* Sanofi
		Needs sophisticated process
		Likely all under Western control
		Challenging patient compliance due to multiday dosing
OZ439[118]	Single dose cure insures patient compliance	Requires co-drug to obviate emergence of AN drug resistance
	In successful Phase 2 trials	Not yet in production
	Mechanism of action not the same as AN	Needs sophisticated process
	Probably low cost due to full synthesis	Likely all under Western control
pACT[20-24]	Has its own in planta co-drug to obviate emergence of AN drug resistance	Not yet in production
	Very low cost	Likely to meet push back from pharmaceutical industry
	Very consistent product	Challenging patient compliance due to multiday dosing
	Can be used to treat other diseases	
	Can be locally owned, produced, managed, and distributed	

AN: Artemisinin.

cuttings is recommended. Since pACT therapy involves the direct consumption of the dried leaves of the plant, harvested leaf material must be kept clean, which is easiest to do in controlled environment agriculture and following Good Agricultural Procedures[121], particularly as applied to fresh produce[122]. However, controlled agriculture would probably result in loss of agricultural jobs, a concern to be assessed locally. Alternatively, great care must be taken during field harvest and post-harvest storage, so as not to affect the quality of the product. WHO has established good agricultural practices specifically for *A. annua* for purposes of artemisinin extraction[123], for general medicinal plants[124], and to minimize contamination of herbal medicines[125].

Chemical consistency and quantification

To deliver a reliable dose of therapeutics to a patient, the dried leaves of harvested *A. annua* must have a reliable and consistent composition. Clonal propagation provides the required consistency. Recently we showed that of 10 crops harvested from vegetative and early flowering plants grown over three years under diverse conditions in the lab, field, and home garden, the artemisinin content of a single clone of *A. annua* (SAM) was 1.38% ± 0.26% (w/w)[77]. Thus, despite variations in culture and environmental conditions, a consistent level of the main therapeutic constituent can be achieved. Moreover, the content of harvested leaves is certainly not a guarantee of finished product, *e.g.*, compressed leaf tablets. Analyses by Weathers *et al*[77] showed that although artemisinin content was very stable after tablet compression, other constituents vaied significantly. For example, although flavonoids increased with tablet compression, the more volatile monoterpenes decreased substantially. Thus, it is critical to monitor the composition profile of both incoming harvested material as well as the final product.

Complex and expensive analytical procedures have been used to analyze the many products found in *A. annua*, but they are not necessary to measure and assure product quality. Artemisinin is easily extracted and then can be quantified using a variety of thin layer chromatography (TLC) methods and visualized with *p*-anisaldehyde stain[126,127]. Other key constituents like the flavonoids are also readily separated using TLC and visualized under either UV ± $AlCl_3$ reagent[128]. Total flavonoids also can be quantified using inexpensive visible spectroscopy *via* the $AlCl_3$ method with quercetin used as an inexpensive standard. To our knowledge no inexpensive, reliable spectrophotometric assay is available to measure artemisinin in complex plant extracts.

SOCIOECONOMIC BENEFITS

Other diseases

Artemisinin and its derivatives are also effective against a number of viruses[129], a variety of human cancer cell lines[130-133], and several neglected tropical diseases including schistosomiasis[134], leishmaniasis[135,136], trypanosomiasis[137], and some livestock diseases[133,138].

Although they rank below malaria in terms of public health importance, schistosomiasis, leishmania, and trypanosomiasis result in estimated annual infections of about 240 million, 1.3 million (0.3 visceral and 1.0 cutaneous), and 30000, respectively[139]. These diseases along with many others respond to treatment with artemisinins. Although the IC_{50} is about 1000-fold greater than for *Plasmodium* sp., the greater apparent bioavailability of artemisinin *via* oral pACT[20-22] would likely reduce the amount of drug required for treatment. At present, pACT has not been tested *in vivo* for diseases other than

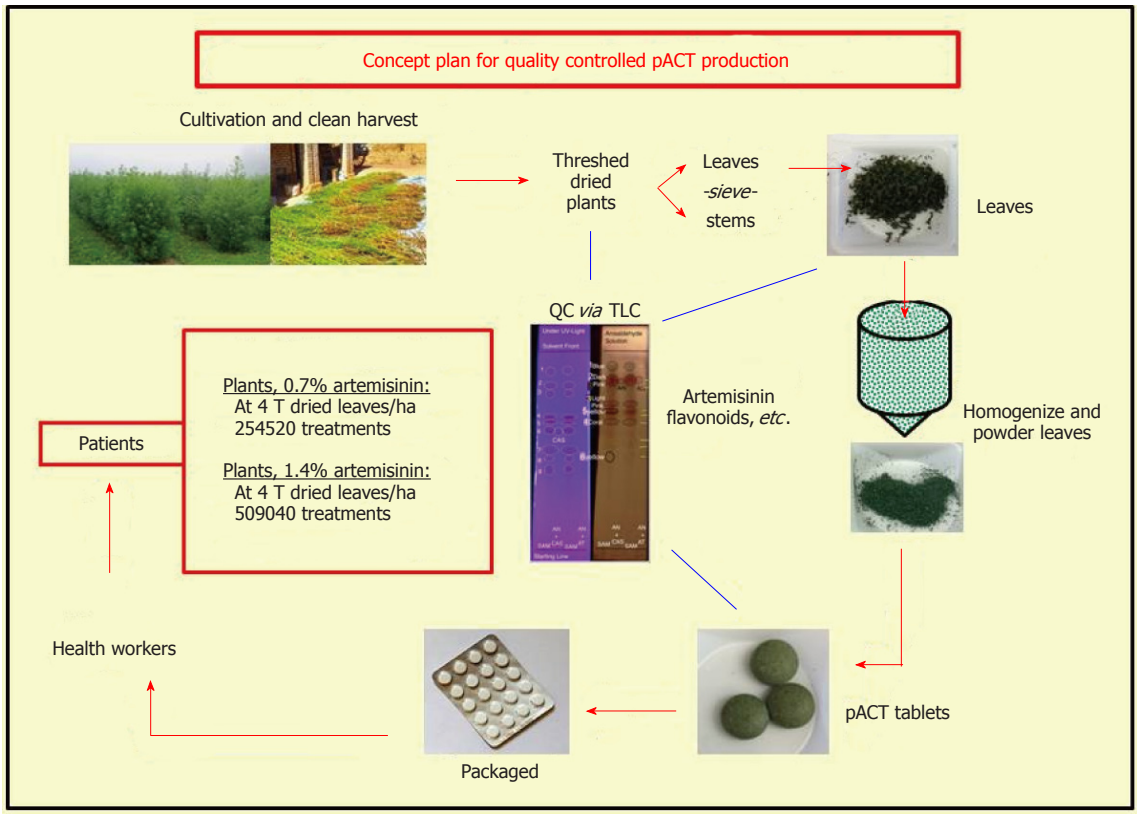

Figure 3 Overall scheme for plant-based artemisinin combination therapy production. pACT: Plant-based artemisinin combination therapy; TLC: Thin layer chromatography.

malaria.

Malaria treatment is further complicated for Human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients. Malaria and HIV co-infection represents a major health burden in Africa mainly because it is now "well established that HIV infection results in a higher incidence and more severe manifestations of malaria"[140]. With a weakened immune system, AIDS patients are more susceptible to malaria and also respond slower to malaria therapy[140-142]. Furthermore, in a meta-analysis by Tusting *et al*[143], socioeconomic development strongly correlated with better malaria therapeutic outcomes. Recently, *A. annua* has demonstrated anti HIV activity[126,144] and thus oral consumption of the dried leaves of this herb will not only treat malaria, but should also enhance the well-being of HIV/AIDS patients.

Agriculture, jobs and self-determination

A. annua is grown in more than 75 countries[145]. In 2011 about 163 MT of artemisinin were extracted from plantations and small stakeholder farms mainly located in China, Vietnam, and Eastern Africa including Madagascar; value was about $550/kg[120]. With the advent of the production of semi synthetic artemisinin by Sanofi, 60 MT were projected for 2014 with an anticipated price of about $400/kg[119]. As this new source of artemisinin becomes available, the Netherlands Royal Tropical Institute projected that the market for natural *Artemisia* will significantly destabilize, undermining the security of farmers. The Tropical Institute was further concerned that "pharmaceutical companies will accumulate control and power over the production process; *Artemisia* producers will lose a source of income; and local production, extraction and (possibly) manufacturing of ACT in regions where malaria is prevalent will shift to the main production sites of Western pharmaceutical companies", disrupting the fragile economics of these already impoverished countries[120]. The average small stakeholder crop area is about 0.2 ha in China and Africa[120], so while implementation of pACT may not require as much agricultural land as for extracted artemisinin, it could still help provide small stakeholders with a source of income. We have estimated that localized micro manufacturing plants could be constructed for < $50000 USD, and produce quality-controlled pACT tablets with readily verifiable contents. Our overall approach, schematically illustrated in Figure 3, leads to local control of malaria and possibly other artemisinin susceptible diseases while also improving the socioeconomic status of the populations.

CONCLUSION

Evidence is mounting for the therapeutic efficacy of the use of dried leaves of *A. annua*, pACT, to treat malaria and possibly other diseases. The complex mixture of antiparasitic compounds in the plant seems to account for its therapeutic activity with animal and human trials

supporting this claim. It is also clear that the cost of using pACT is a fraction of that for any other current or emerging antimalarial therapeutic. Likewise, the recent evidence of persistent and/or asymptomatic malaria suggests that a more prophylactic approach to malaria using pACT or even *A. annua* tea may be warranted. Considering that for > 2000 years this plant was used in traditional Chinese medicine for treatment of fever with no apparent appearance of artemisinin drug resistance, taken together the cumulative evidence argues for inclusion of pACT into the arsenal of drugs to combat malaria, and very likely, other diseases.

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P- Reviewer: Masocha W **S- Editor**: Ji FF **L- Editor**: A
E- Editor: Lu YJ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i4.56

World J Pharmacol 2014 December 9; 3(4): 56-71
ISSN 2220-3192 (online)
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REVIEW

Asthma in pregnancy

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Received: July 1, 2014 Revised: August 1, 2014
Accepted: September 16, 2014
Published online: December 9, 2014

Abstract

Asthma affects approximately 8% of women during pregnancy. Pregnancy results in a variable course for asthma control, likely contributed to by physiological changes affecting the respiratory, immune, and hormonal systems. While asthma during pregnancy has been associated with an increased risk of maternal and fetal complications including malformations, available data also suggest that active asthma management and monitoring can decrease the risk of adverse outcomes. The diagnosis, disease classification, and goals for asthma management in the pregnant woman are the same as for nonpregnant patients. However, evidence shows that pregnant asthmatics are more likely to be undertreated, resulting in asthma exacerbations occurring in approximately one third and hospitalization in one tenth of patients. Pharmacotherapeutic management of asthma exacerbations in pregnant patients follows standard treatment guidelines. In contrast, the principles of asthma maintenance therapy are slightly modified in the pregnant patient. Patients and practitioners may avoid use of asthma medications due to concern for a risk of fetal complications and malformations. A variable amount of information is available regarding the risk of a given asthma medication to cause adverse fetal outcomes, and it is preferable to use an inhaled product. Nevertheless, based on available data, the majority of asthma medications are regarded as safe for use during pregnancy. And, any increased risk to either the mother or fetus from medication use appears to be small compared to that associated with poor asthma control.

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Key words: Asthma; Pregnancy; Fetal outcomes; Maternal outcomes; Management of asthma; Pharmacotherapy

Core tip: This comprehensive review of the impact of asthma during pregnancy provides information regarding proposed pathophysiological alterations and fetal and maternal outcomes associated with asthma during pregnancy. In addition, we outline the treatment of acute exacerbations and the maintenance management of asthma throughout pregnancy, including specific information on the various classes of medication used to treat asthma.

Blackburn HK, Allington DR, Procacci KA, Rivey MP. Asthma in pregnancy. *World J Pharmacol* 2014; 3(4): 56-71 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/56.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.56

INTRODUCTION

Asthma is a common condition affecting approximately 8% of pregnant women[1]. Epidemiological evidence demonstrates that the course of asthma during pregnancy is variable and unpredictable, with approximately one-third of women experiencing an improvement, one-third experiencing a worsening, and one-third having no

changes in asthma symptoms[2,3]. It is also apparent that poor control of maternal asthma leads to increased risk of adverse maternal and fetal outcomes. Because of these risks and the unpredictable course of asthma symptoms, it is especially important to provide appropriate monitoring and management of the asthmatic patient throughout pregnancy. This review will discuss the physiologic changes associated with asthma during pregnancy, asthma control and its effect on pregnancy outcomes, and the management of asthma in pregnancy.

PHYSIOLOGICAL CHANGES AND ASTHMA IN PREGNANCY

The relationship between the changes in body physiology as pregnancy progresses and the physiological processes driving asthma symptoms is not well understood, but it is evident that the relationship is bidirectional and complex[3-5]. It is thought that changes including alterations in pulmonary physiology, maternal immune function and hormonal balance contribute to the unpredictable course of asthma during pregnancy.

Pulmonary changes

As pregnancy progresses, the uterus expands and causes elevation of the diaphragm by 4-5 centimeters, resulting in a decrease in lung functional residual capacity (FRC) of 10%-25%. However, the decrease in FRC does not typically result in significant changes to forced vital capacity, peak expiratory flow rate, or forced expiratory volume in 1 second (FEV1). Minute ventilation (VE) may be elevated as much as 50% by the third trimester of pregnancy as a result of progesterone-driven increases in tidal volume and respiratory rate[6]. Concomitantly, oxygen consumption can increase up to 35%[7]. Respiratory alkalosis occurs as a result of the increase in VE but is compensated for by increased renal excretion of bicarbonate. Typical arterial blood gas values in pregnancy are altered only slightly from the nonpregnant state, with a normal pH of 7.40-7.45 and pCO_2 of 28-32 mmHg[7-9].

Due to the pulmonary changes during pregnancy, dyspnea is common and manifests as shortness of breath with rest or mild exertion. The pulmonary changes are often magnified in an asthmatic patient, and may contribute to the perception of changing symptoms during pregnancy[6].

Immunologic changes

Physiological immunosuppression is characteristic of pregnancy and results in feto-maternal tolerance required for completion of a normal gestation[10]. Changes in immune characteristics during pregnancy include a shift in the helper T cell (Th1)/Th2 ratio toward a Th2-predominant immune state and an increase in regulatory T cells (Tregs) that work to suppress activation of effector T cells and natural killer cells. This immune deviation is thought to prevent Th1-induced fetal rejection as paternally originated antigens are expressed during development[10]. A number of immune-mediated disease states can be affected by this Th2-predominant shift during pregnancy. For example, rheumatoid arthritis is a Th1-mediated disease that goes into remission during pregnancy in the majority of patients[11]. Asthma, on the other hand, has traditionally been categorized as a Th2-predominant disease state, with allergic Th2-type inflammation leading to airway hyperresponsiveness in patients. Evidence suggests that the pregnancy-associated Th2 immunological shift leads to worsening of the Th2-driven manifestations of asthma[10,12,13].

Immune changes in pregnant asthmatic women have not been well elucidated but recent studies have helped to better characterize the interplay of immunologic processes associated with pregnancy and asthma. Results of several studies provide evidence that exaggerated Th2 responses in pregnant women with uncontrolled asthma contribute to worsening of maternal symptoms, as well as low birth weight in neonates[14,15]. In contrast, no differences in the Th1/Th2 ratio were observed between healthy pregnant women and pregnant women with well-controlled asthma, suggesting that pregnancy and asthma do not have additive effects in terms of Th2 prevalence if asthma is well-controlled with medication therapy[15,16].

The relative number of peripheral Treg cells has been found to be lower in asthmatic compared to healthy pregnant women. It is thought that a decrease in Treg cells results in decreased suppression of the effects of pro-inflammatory Th17 cells and may contribute both to worsening of symptoms as well as increased likelihood of poor fetal outcomes in asthmatic patients[16]. Increased numbers of pro-inflammatory Th17 cells, have been observed in pregnant asthmatic women, and are hypothesized to contribute to impaired intrauterine growth (Figure 1)[3]. Continued research is needed to further characterize the complex and bidirectional relationships between T-cell subpopulations and the immunologic processes of asthma, in order to understand their role and importance in asthma during pregnancy.

Another factor associated with immunological changes of pregnancy relates to women's changing susceptibility to respiratory pathogens. The pregnancy-associated decrease in cell-mediated immunity is also known to make pregnant women more susceptible to viral respiratory infections, a common precipitating factor in asthma exacerbations during pregnancy[17,18].

Hormonal changes

As a pregnancy progresses, concentrations of circulating maternal hormones increase to varying degrees. As such, inter-individual variations in hormonal changes could contribute to the unpredictable course seen in maternal asthma. Pregnancy is associated with an increase in serum free cortisol, a hormone with endogenous anti-inflammatory activity which can improve asthma[19]. Evidence also suggests that levels of sex steroids including estrogen and progesterone can affect asthma symptoms. Changes

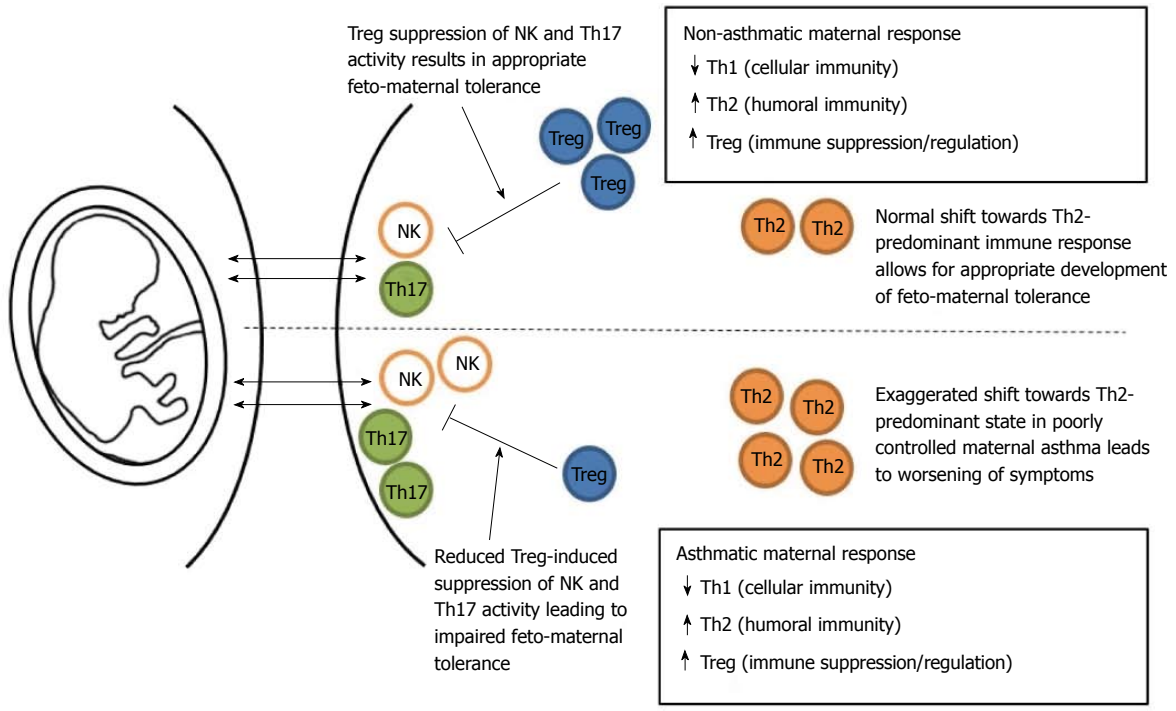

Figure 1 Immunology of Asthma in pregnancy. NK: Natural killer; Treg: Regulatory T cells.

in asthma symptoms are known to occur throughout the menstrual cycle, with up to 40% of females experiencing premenstrual asthma worsening during the follicular phase when progesterone and estrogen levels are normally low[20-23].

Estrogen levels have been shown to be correlated with the quantity of peripheral Tregs, suggesting an interrelationship between the hormonal and immunological effects of pregnancy. Arruvito *et al*[24] observed a strong correlation between increasing estradiol levels in fertile women and the percentage of Tregs found in the total number of $CD4^+$ T lymphocytes. An elevation of estrogen during the third trimester has been associated with increased bronchial mucus production and airway edema, thereby increasing symptoms of asthma[6,25]. Increased progesterone levels during pregnancy can result in a multitude of effects as a potent smooth muscle relaxant. While smooth muscle relaxation in the lungs would likely improve asthma symptoms, relaxation of the smooth muscle controlling the esophageal sphincter could result in increased gastroesophageal reflux, a condition known to exacerbate the symptoms of asthma[26].

Additionally, serum progesterone levels have been shown to exert effects on the regulation of β2-adrenoreceptors in female asthmatics, which could in turn effect changes in asthma symptoms and medication response. In a study of seven asthmatic females receiving exogenous progesterone during the follicular phase of their menstrual cycle, a significant decrease in lymphocyte β2-adrenoreceptors was determined when compared to baseline, with a trend towards decreased responsiveness to exogenously administered isoproterenol. Results of this study suggest that downregulation of β2-adrenoreceptors in response to increases in serum progesterone may result in loss of responsiveness to both endogenous catecholamines and exogenously administered beta-agonists[27].

Available evidence also suggests there may be inherent differences in the course of asthma during pregnancy based on fetal sex, with a worsening of maternal asthma more likely in the presence of a female fetus[28-31]. In a study of pregnant women with asthma, there was a significantly increased dose requirement of inhaled corticosteroid (ICS) and a significant rise in circulating monocytes that progressed throughout the pregnancy in women with a female compared to those carrying a male fetus[29]. It has been proposed that factors such as differences in hormone or protein production between male and female fetuses account for the variability in maternal asthma severity and treatment response, although currently there is no specific evidence to support this hypothesis.

ASTHMA CONTROL AND PREGNANCY OUTCOMES

Maternal outcomes

Compared to pregnant women without asthma, asthma during pregnancy is consistently associated with higher rates of preeclampsia, pregnancy-induced hypertension, transient hypertension of pregnancy, gestational diabetes, placenta previa or placental abruption, premature labor or delivery, cesarean section and postpartum hemorrhage[32-37]. Results from a recent retrospective cohort study conducted in the United States from 2002-2008 of obstetric complications among women with asthma (n = 17044) compared to women without asthma (n =

206468) found asthma was associated with increased rates of preeclampsia [adjusted odds ratio (aOR = 1.14; 95%CI: 1.06-1.22), superimposed hypertension (aOR 1.34; 95%CI: 1.15-1.56), placenta previa (aOR = 1.30; 95%CI: 1.08-1.56), placental abruption (aOR = 1.22; 95%CI: 1.09-1.36), preterm premature rupture of membranes (aOR = 1.18; 95%CI: 1.07-1.30), gestational diabetes (aOR = 1.11; 95%CI: 1.03-1.19), maternal hemorrhage (aOR = 1.09; 95%CI: 1.03-1.16), breech presentation (aOR = 1.13; 95%CI: 1.05-1.22), prelabor cesarean delivery (aOR = 1.16; 95%CI: 1.09-1.23) and maternal intensive care unit admission (aOR = 1.34; 95%CI: 1.04-1.72)[38]. Blais *et al*[39] reported women with asthma have a higher rate of spontaneous abortion (OR = 1.41; 95%CI: 1.33-1.49) but a lower rate of induced abortion (OR = 0.92; 0.88-0.97); neither finding was influenced by baseline asthma severity. Actively managing asthma during pregnancy has resulted in reducing risks of certain maternal outcomes to non-significant levels such as preterm delivery and low birth weight but without effect on pre-eclampsia[40].

Fetal outcomes

Fetal complications associated with maternal asthma include premature delivery (< 37 wk), small for gestational age (SGA, defined as < 10% percentile for matched age), low birth weight (LBW, defined as < 2500 gm), intrauterine growth restriction, mortality, and congenital malformations. Asthma during pregnancy has been associated with increased rates of neonatal sepsis and hospitalization in some studies but results are inconsistent[33,41,42]. Clifton *et al*[30] completed a meta-analysis of literature describing perinatal outcomes in women with asthma. The meta-analysis included data from 11 prospective and 15 retrospective cohort studies with total subject populations exceeding 1500000. Subgroup analysis was conducted to compare outcomes of prospective *vs* retrospective studies, as well as outcomes of studies with active asthma management as compared to those without active asthma management. Maternal asthma without active management was associated with increased rates of preterm delivery [risk ratio (RR) = 1.41; 95%CI: 1.22-1.61], pre-eclampsia (RR = 1.54; 95%CI: 1.32-1.81), LBW (RR = 1.46; 95%CI: 1.22-1.75), and SGA (RR = 1.22; 95%CI: 1.14-1.31). Studies in the meta-analysis that included active asthma management reduced the relative risk of preterm labor and delivery to non-significant levels[40].

Maternal asthma is associated with increased risk of fetal malformations but the magnitude of the risk appears to be only slightly greater than risk associated in non-asthmatic pregnant women. Tata *et al*[43] performed a case-controlled study using 5124 fetal cases of major congenital malformations compared with over 30000 matched controls. Results indicated the malformation risk due to maternal asthma was marginally greater (aOR = 1.10; 95%CI: 1.01 to 1.20) than that in controls and that the risk could be modified by asthma therapy in the year before and during pregnancy[44]. Additionally, a meta-analysis and systematic review of maternal asthma and its influence on fetal outcomes was recently conducted by Murphy and colleagues. When compared to control groups consisting of pregnant women without asthma, maternal asthma was associated with a significantly increased RR for neonatal sepsis (RR = 2.27; 95%CI: 1.12-4.58), hospitalization (RR = 1.50; 95%CI: 1.03-2.20) and perinatal mortality (RR = 1.25; 95%CI: 1.05-1.50). Investigators also found an overall increased risk of congenital malformations (RR = 1.11; 95%CI: 1.02-1.21) but the increased risk was only significant in the subcategory of retrospective cohort studies that did not include active asthma management[44].

MANAGEMENT OF ASTHMA IN PREGNANCY

The goals of therapy and principles of management of asthma in pregnant women are similar to those for non-pregnant women[9]. However, pregnant women are more likely to be undertreated by physicians for a given level of asthma severity[45,46]. Evidence has also shown that pregnant asthma patients often avoid medications they believe are potentially harmful to the fetus. Rocklin *et al*[36] tracked the prescription claims data in a cohort of 112171 pregnant women who were enrolled in the Tennessee Medicaid program. Subjects significantly decreased ($P \leqslant 0.0005$) their use of asthma medications during the 5th-13th weeks of pregnancy. Utilization rates during the first trimester declined by 23% for ICS prescriptions, 13% for short-acting B2-agonists (SABA) prescriptions and 54% for rescue corticosteroid prescriptions[47].

It is known that critical fetal organ development occurs predominately during the first trimester, and available data suggest that first trimester asthma exacerbations in the mother lead to higher rates of malformation in the offspring. Infants born to mothers with a first trimester asthma exacerbation, compared to those without, were more likely to suffer at least one malformation affecting 9.2% of infants (aOR = 1.48 with 95%CI: 1.04-2.09) and a major malformation affecting 6.0% of infants (aOR = 1.32 with 95%CI: 0.86-2.04)[48]. Such evidence highlights the need for careful management of maintenance therapy in pregnant asthmatics, and suggests the value of a multidisciplinary approach to assessment and treatment of asthma to promote appropriate therapy and medication adherence.

Diagnosis and classification of asthma

The diagnosis of asthma in a pregnant patient is the same as in the nonpregnant population, ideally with confirmation by spirometry showing at least partially reversible obstruction of the airways. If a pregnant patient presents with symptoms of new-onset asthma without spirometric confirmation of diagnosis, they should be treated with appropriate asthma therapy only after other diagnoses are excluded. Differential diagnoses associated with new-

onset dyspnea during pregnancy include physiologic dyspnea of pregnancy, pulmonary embolism, amniotic fluid embolism, pneumonia or bronchitis, GERD, and/or vocal cord dysfunction[49,50]. It should be noted that testing of bronchial hyperresponsiveness with methacholine challenge is contraindicated during pregnancy due to a lack of safety data[49].

Evidence clearly shows that the course of asthma is unpredictable as the pregnancy progresses[17,51]. While baseline asthma severity, the frequency of asthma exacerbations, and the level of asthma control in prior pregnancies have been used as predictors for asthma outcomes during pregnancy, monitoring and assessment of patients with all levels of asthma severity is important. In a study of 1739 women, patients' asthma severity was classified at the onset of pregnancy based on standard criteria including FEV1, symptoms and rescue inhaler use. It was found that 13%, 16%, and 52% of women classified as having mild, moderate, and severe asthma, respectively, suffered at least one asthma exacerbation during pregnancy, suggesting a correlation between baseline asthma severity and the risk of an exacerbation during pregnancy. However, results of the study also demonstrated that 30% of patients initially classified with mild asthma progressed to moderate or severe disease throughout the course of pregnancy, while 23% of patients categorized as having baseline moderate or severe asthma improved to the mild disease category[51]. Results of this study and other studies (Table 1) illustrate the unpredictable nature of asthma in pregnancy and emphasize the ongoing need for monitoring of asthma symptoms regardless of initial asthma severity. Generally, a patient's asthma course and severity will revert to their pre-pregnancy status approximately 90 d postpartum[52].

Acute asthma exacerbation management

Most women with asthma complete their pregnancy without incident. But data also indicate 20%-36% of patients will experience an asthma exacerbation, 9%-11% will require hospitalization and some will need ICU management and rarely (< 1%), intubation[9,38,51]. Exacerbations during pregnancy most commonly occur during the 25th-36th week of pregnancy with fewer episodes occurring during labor and the peripartum period[53-55]. In addition, evidence suggests that pregnant black women with asthma are more likely to experience and require medical care for exacerbations[9].

In the Emergency Department (ED), the pregnant asthmatic patient should receive a thorough physical examination, spirometry or peak flow meter assessment and arterial blood gas evaluation. Assessment of maternal oxygen saturation *via* pulse oximetry should be conducted to ensure oxygen saturation is maintained at or above 95%. Spirometry or peak flow meter results can be compared to the patient's baseline measurements or their predicted personal best. Arterial blood gas results usually demonstrate a compensated respiratory alkalosis common to pregnancy. As such, an otherwise normal value for arterial pCO_2 (pCO_2 of 40 mmHg) in some cases may signal relative hypercapnia and could be an indicator of respiratory fatigue in the pregnant patient[5].

The health status of the fetus must also be ascertained. Specific recommendations for fetal assessment are determined by the stage of pregnancy but a biophysical profile that combines ultrasound and a non-stress test is routine. These tests are used to measure amniotic fluid volume and fetal heart rate, muscle tone, breathing episodes and gross movements[9,56].

Recommended initial medical treatment for an acute asthma exacerbation in a pregnant woman presenting to the ED follows standard treatment guidelines. In addition to oxygen supplementation, inhaled albuterol every 20 min up to three doses in the first hour is recommended. If the exacerbation is severe, 500 μg of inhaled ipratropium bromide can supplement albuterol administrations. Oral or intravenous corticosteroids are recommended for individuals with inadequate response to bronchodilator therapy, for individuals who have required multiple short courses of steroids throughout their pregnancy or for those receiving systemic corticosteroids at time of presentation to the ED[5,9]. If the pregnant patient responds favorably to the bronchodilators and/or corticosteroids, generally within 4 h of presentation to the ED, she may be discharged. On discharge from the ED, a short 5-10 d course of oral prednisone given at 40-80 mg as a single or divided daily doses is recommended to prevent asthma relapses[5,9].

Alternatively, hospitalization is recommended if a maternal oxygenation saturation of 95% or greater cannot be maintained on room air after appropriate medication administration, if FEV1 or PEF measurements are persistently less than 70% despite therapy, or if fetal distress is evident. Subcutaneous or intravenous terbutaline can be utilized on a case-by-case basis if inhaled SABA have been maximized whereas systemic epinephrine should be avoided[9]. Life threatening asthma episodes are characterized by significant maternal hypoxemia (PaO_2 < 60 mmHg), hypercapnia ($PaCO_2$ > 40), respiratory acidosis, maternal respiratory fatigue and/or fetal distress. Intubation and mechanical ventilation can be required in these life-threatening circumstances and on rare occasion, delivery of the newborn by cesarean section is indicated[9,56].

Maintenance therapy

Due to the increased risk of adverse pregnancy outcomes associated with poor asthma control, optimal management of maternal asthma through optimization of maintenance therapy becomes especially important. Although evidence exists to support the safety of most major classes of medications used for asthma management during pregnancy, patients and providers often remain apprehensive about the use of any drug therapy. Unfortunately, the consequence of decreasing or discontinuing asthma medications during pregnancy is an increase in the likelihood of poor asthma control and its associated risks to both mother and fetus. Recent research has high-

Table 1 Pregnancy associated asthma morbidity by severity classification

	Mild asthma *n* (%)		Moderate asthma *n* (%)		Severe asthma *n* (%)	
	Schatz *et al*[51] (*n* = 873)	Murphy *et al*[55] (*n* = 63)	Schatz *et al*[51] (*n* = 814)	Murphy *et al*[55] (*n* = 34)	Schatz *et al*[51] (*n* = 52)	Murphy *et al*[55] (*n* = 49)
Asthma Exacerbation	110 (12.6)	5 (8)	209 (25.7)[b]	16 (47)	27 (51.9)[d]	32 (65)
Unscheduled physician or ED presentation	99 (11.3)	4 (6.3)	157 (19.3)[b]	14 (41)	19 (36.5)[b]	20 (41)
Oral corticosteroid use	19 (2.2)	0 (0)	71 (8.7)[b]	4 (11.8)	20 (38.5)[b]	19 (38.8)
Hospitalization	20 (2.3)	2 (3.2)	55 (6.8)[b]	1 (2.9)	14 (26.9)[b]	9 (18.4)

[b]$P < 0.0001$, [d]$P < 0.001$ *vs* preceding severity group (moderate to mild; severe to moderate). ED: Emergency Department.

lighted the importance of a multidisciplinary approach to patient education and management of maternal asthma by involving physicians, pharmacists, midwives and others associated with perinatal care, in order to ensure appropriate treatment and promote patient adherence[57,58].

Regular visits to evaluate asthma control are recommended throughout pregnancy in all patients regardless of disease severity. If there is any indication that maternal asthma symptoms are worsening, more frequent monitoring would be indicated. Disease evaluations should include objective assessment of lung function with the use of spirometry or peak flow meter, as well as assessment of symptoms using a validated questionnaire such as the Asthma Control Test or Asthma Control Questionnaire (ACQ)[59,60]. More recent research from a double-blind, parallel-group, controlled trial focused on the use of the fraction of exhaled nitric oxide (FeNO) as a marker of airway inflammation in asthma during pregnancy. Results showed a 50% reduction in asthma exacerbations using a treatment algorithm guided by FeNO compared to that guided by symptom assessment[60]. These early results are encouraging but the use of FeNO is not yet widely available, and its use has yet to be included in guidelines as a standard of care.

As in nonpregnant patients, the pharmacological treatment of asthma should be implemented in a stepwise fashion using current guidelines, with "step-up" therapy indicated if the patient is not adequately controlled with current therapy. Prior to medication regimen changes, issues such as poor medication adherence, improper inhaler technique, and other conditions associated with worsening dyspnea including pneumonia, pulmonary embolism or amniotic embolism, should be assessed[5,50].

There are, however, several exceptions to current asthma treatment guidelines in pregnant women. First, when a step-up in controller medication is indicated, an ICS should be initially trialed in preference to a combination ICS/long-acting bronchodilator (LABA) product due to the safety concerns associated with LABAs that will be discussed below (Table 2). Second, current asthma guidelines recommend step-down therapy to be considered in non-pregnant patients who are well controlled on a regimen for a minimum of three months[50]. In contrast, maintenance therapy should not be altered or escalated during pregnancy in asthmatics who are well controlled since fetal risks associated with the loss of disease control outweigh the benefits associated with a reduction in maintenance therapy[59,61].

Education

Patient education is an important component of appropriate management in any patient with asthma. Several studies have highlighted the importance of asthma education during pregnancy, using strategies to provide information to patients about the disease and its treatment, as well as improving medication adherence[55,57]. One recent study by Lim and colleagues showed that 70% of surveyed women were unaware of the risks associated with poor asthma control, while 32% discontinued or changed medications during pregnancy without discussing the changes with a healthcare professional[57]. Asthma education can directly address these issues and promote improved outcomes.

Key education topics for patients should include general information about asthma, potential complications and their relationship to pregnancy, proper use of inhaler devices, appropriate self-monitoring, adherence to medications, and optimal control of environmental factors. A written asthma action plan should be established to assist patients with self-monitoring and treatment in response to asthma control based on symptoms and/or peak flow monitoring. The plan should be developed in coordination with a healthcare provider and communicated to all those involved in the treatment of the patient. Patients should receive follow-up education and assessment of medication adherence and inhaler technique at every visit. The use of regular education and monitoring through a multidisciplinary team approach has been shown to significantly decrease ACQ scores when compared to groups receiving usual asthma care without education[50,57-59].

Nonpharmacologic measures and immunizations

Nonpharmacologic approaches can improve asthma symptoms while decreasing the use of "as needed" medication, thereby minimizing any associated maternal or fetal risk. The identification and avoidance or removal of indoor and outdoor environmental asthma "triggers" may greatly reduce the risk of asthma exacerbation. Common triggers including mold, dust, animal dander, cockroaches, pollens, and perfumes are often impossible to avoid completely but minimization of exposure is a treatment goal. Furthermore, smoking cessation and/or avoidance

Table 2 Stepwise approach to asthma therapy in pregnant and nonpregnant patients[49,60,62]

	Step	Preferred therapy in nonpregnant patients	Preferred therapy in pregnant patients	Alternative therapy in pregnant patients
Intermittent asthma	1	SABA, as needed[1]	SABA, as needed[1]	N/A
Persistent asthma	2	Low-dose ICS	Low-dose ICS	LTRA
	3	Low-dose ICS + LABA, or medium-dose ICS	Medium-dose ICS	LTRA
	4	Medium-dose ICS +LABA	Low-dose ICS + LABA	Medium-dose ICS, or high-dose ICS, or low-dose ICS + LABA + LTRA
	5	High-dose ICS + LABA	Medium-dose ICS +LABA, or high-dose ICS + LABA	LTRA + theophylline
	6	High-dose ICS + LABA + oral corticosteroid	High-dose ICS + LABA + oral corticosteroids	Omalizumab

[1]SABA should be included as quick-acting rescue medication to be used as needed in all patients. SABA: Short-acting beta-agonist; LABA: Long-acting beta-agonist; ICS: Inhaled corticosteroid.

of secondhand smoke always should be incorporated to treatment plans of pregnant asthmatic patients[50,62].

Immunization against influenza is strongly recommended in both pregnant and postpartum patients with asthma, as influenza is more likely to cause severe illness in these populations, leading to serious disease exacerbations that pose risk to maternal and fetal wellbeing. Pregnant women should receive an inactivated influenza vaccine by injection while postpartum women who are breastfeeding may receive either the live or attenuated vaccine given *via* the intranasal route or by injection. Recommendations regarding administration of pneumococcal vaccine in asthmatic patients vary between countries, and some controversy regarding the effectiveness of pneumococcal vaccination in asthma exists. Although no evidence of maternal or fetal harm has been demonstrated following administration of the pneumococcal vaccine (PPSV23) during pregnancy, providers should make every effort to vaccinate women with asthma prior to pregnancy. Pneumococcal vaccine is not, however, contraindicated in breastfeeding[63-65].

Management of comorbid conditions

While the use of allergen immunotherapy is known to be effective for improving asthma symptoms in patients with allergies, anaphylaxis is the greatest risk accompanying allergen injections in the asthmatic patient and has the potential to result in maternal and/or fetal death. The risk is especially high earlier in the course of immunotherapy when allergen doses are being increased. Consideration of the benefits and risks of allergen immunotherapy generally favors continuation of the treatment if a patient has reached a maintenance or near-maintenance dose without adverse reactions prior to a pregnancy. However, initiation of allergen immunotherapy during pregnancy is not recommended[50].

As in nonpregnant patients, rhinitis and gastroesophageal reflux may lead to exacerbation of asthma symptoms during pregnancy. Management of these comorbid conditions should be considered an integral part of patient care because pregnancy can result in physiological alterations that lead to worsening of the conditions. Details of the management of these conditions during pregnancy are beyond the scope of this review; however, the interested reader is referred to references for further information[66-71].

ASTHMA MEDICATIONS USED DURING PREGNANCY

Data from three studies describing the association of congenital malformations with maternal asthma medication use have been recently published[44,72,73]. The National Birth Defects Prevention Study by Källén *et al*[73] included 2853 infants with one or more specific malformations compared to a control group of 6726 unaffected infants. Mothers of cases and controls were contacted by telephone and asked to describe their medication use beginning one month prior to and through their third month of pregnancy. Other potential risk factors such as tobacco and alcohol use, co-morbid chronic diseases and exposures at home and work were also solicited. Congenital malformations included esophageal atresia, small intestinal atresia, anorectal atresia, limb deficiencies, diaphragmatic hernia, omphalocele, or neural tube defects. Significant associations were determined for omphalocele with both bronchodilators (SABA and/or LABA use) and anti-inflammatory medication use (aOR = 4.13; 95%CI: 1.43-11.95), isolated anorectal atresia with anti-inflammatory use (aOR = 2.12; 95%CI: 1.09-4.12) and isolated esophageal atresia with bronchodilator use (aOR = 2.39; 95%CI: 1.23-4.66). No other positive associations with other birth defects were determined[72].

Cydulka *et al*[45] conducted a systematic review and meta-analysis of the literature concerning the association of maternal asthma disease management with the risk of congenital malformations. Data from 12 cohort studies (four prospective and eight retrospective studies) of women with asthma stratified according to disease severity, exacerbation history, corticosteroid use, or bronchodilator use were included in the analysis. In accordance with other studies, maternal asthma was associated with a significantly increased risk of malformations for the en-

tire group (RR = 1.11; 95%CI: 1.02-1.21) but no increase was observed in the subset of patients in the prospective studies with active asthma medication management. While the presence of asthma was associated with an overall increased risk of congenital malformations, significant associations were not found for any specific factors related to asthma including maternal asthma exacerbation history, bronchodilator use, or ICS use[44].

Mendola *et al*[38] used data from the Swedish Medical Birth Register for the period 1996-2011 to investigate the risk of congenital malformations in infants born to women who had received medications for asthma during early pregnancy. Maternal drug use information was obtained from midwife interview records of patients during the first perinatal care appointment that typically occurred during the 10th-12th wk of pregnancy. The data spanned a 15-year timeframe with over 1.5 million births, including those of 44772 (2.9%) patients who received asthma medications from at least one of the following medication classes: inhaled adrenergics (SABA and/or LABA), ICS, anticholinergics, anti-allergics, xanthines, and leukotriene receptor antagonists. Women receiving antiasthmatic medications were compared to women who did not receive a drug from the listed classes, with adjustments made for year of delivery, maternal age, parity, smoking, and BMI. Those receiving antiasthmatic drugs were further stratified into specific medication classes.

Results indicated the OR for bearing an infant with a major congenital malformation was 1.09 (95%CI: 1.03-1.15) for women receiving any antiasthmatic medication *vs* those with no exposure to asthma medications. Median cleft palate (but not cleft lip/palate) with an OR of 1.45 (95%CI: 1.06-1.98), cardiovascular defects with an OR of 1.13 (95%CI: 1.04-1.23), and pyloric stenosis with an OR of 1.42 (95%CI: 1.06-1.91) were determined to be significantly increased malformations in infants born to mothers who took asthma medications. Risk estimates for the associations of the number of different asthma medications taken by the mother with a major malformation were significant for use of medication from a single group 1.11 (95%CI: 1.04-1.19) and use of medications from three or more groups 1.18 (95%CI: 1.01-1.38). In regard to specific medication classes, significantly increased odds or risk ratio OR/RR were found for the use of SABA (OR/RR = 1.10; 95%CI: 1.04-1.10) and ICS (OR/RR = 1.08; 95%CI: 1.01-1.16). However, there was no examination of asthma severity and its potential links to fetal outcomes in this study. Furthermore, as with all of these recently published studies, increased congenital risks could not be linked to specific, individual medications within a given medication group[73].

β2-agonists

SABAs are effective bronchodilators for quick-acting relief of asthma symptoms and are generally considered safe for use during pregnancy and breastfeeding. While SABA use was associated with a small increased risk of congenital malformation in some of the large studies described above, most studies evaluating maternal SABA use during pregnancy have not shown significant increases in adverse maternal or fetal outcomes associated with drug use[6,74,75]. In other studies that did show significant increases in adverse events potentially correlated with SABA use during pregnancy, reference groups of healthy non-asthmatic women or mixed asthmatic plus non-asthmatic women were used, making it impossible to discern if observed adverse outcomes were attributable to medication use or the disease[73,74,76-80]. Results of a single population-based case-control study of 511 pregnant women with asthma demonstrated an increased risk of congenital malformations with fenoterol use, but no association with other SABA use[79]. Due to the preponderance of evidence supporting the safety of SABA use in pregnancy, the drugs should be used according to guidelines for the quick-relief of asthma symptoms.

There is limited data regarding the safety of LABAs during pregnancy. In a population-based retrospective cohort study of β-2 agonist use in pregnancy, Eltonsy and colleagues observed a nonsignificant trend for an increased risk of major congenital malformations in infants of women who used LABAs during the first trimester. In the same study, SABA use was not associated with any increased risk of malformations[74]. On further analysis of those using a LABA in the first trimester (*n* = 165), investigators found that while there was no significant increase in all major malformations (defined as malformations that were life-threatening, caused major cosmetic defects, or resulted in at least one hospitalization within the first year of life), there were significant increased risks for the subtype of major cardiac malformations (aOR = 2.38, 95%CI: 1.11-5.10), genital organ malformations (aOR = 6.84, 95%CI: 2.58-18.10) and major "other and unspecified congenital malformations" (aOR = 3.97, 95%CI: 1.29-12.20)[75]. The authors of the study offered explanations for the observed trend of adverse outcomes associated with LABA use beyond that of a true causal relationship. First, while the investigators attempted to correct for asthma severity in the analysis of the data, it was possible that there was residual confounding of results by asthma disease severity. Second, specific interactions between concurrent LABA and steroid use have been identified including effects on protein kinase A (PKA) and ligand-independent activation of glucocorticoid receptors[81]. Because LABAs were used concomitantly with ICS for asthma in this study, authors suggest that observed increases in fetal malformations might be due to an effect of LABA use on steroid function, leading to potentiation of steroid-associated adverse effects[75].

Other studies examining LABA use have not found significant association between LABA use and major fetal malformations, and evidence supporting differences in the safety profiles of individual LABAs is lacking[75,77,80-83]. A recent study by Cossette *et al*[84] failed to find any statistically significant differences in low birth weight or preterm birth for infants of mothers who had used sal-

meterol *vs* formoterol during pregnancy.

Due to a lack of robust evidence for the safety of LABA use in pregnancy, the medications should only be used if asthma control cannot be achieved using medium-dose steroids in addition to SABAs. As previously noted, this recommendation is a deviation from asthma guidelines for nonpregnant patients (Table 2). Maternal plasma concentrations of inhaled LABAs have been shown to be undetectable or minimal, however, and the use of the agents is not considered a contraindication to breastfeeding[85].

Inhaled and systemic corticosteroids

Due to potent and predictable anti-inflammatory effects, ICS form the foundation of maintenance therapy in patients with persistent asthma. As a drug class, ICS have generally been shown to decrease the risk of asthma exacerbations among pregnant women, with no increased rate in adverse maternal or fetal outcomes[86-88]. Systemic absorption of an ICS is typically very low, with data demonstrating very low to undetectable plasma concentrations of triamcinolone, fluticasone, ciclesonide and beclomethasone after inhalation[89]. Inhaled budesonide has approximately 39% bioavailability, but results of studies of inhaled budesonide in lactation demonstrated a negligible amount transferred to the breastfeeding infant[90]. Additionally, similar incidences of adverse pregnancy outcomes were observed in a randomized, controlled trial comparing the use of inhaled budesonide *vs* placebo in pregnant women with asthma[90]. Information from other studies of ICS use in asthmatic patients during pregnancy provide similar evidence indicating no significant increased risk for neonatal adverse events including oral clefts, cardiac defects, spina bifida and other congenital malformations beyond those expected in the general population[88,91-93].

Concerns regarding the safety of corticosteroids in pregnancy have been specifically addressed in a number of studies. Data from most studies support the safety of ICS use for asthma during pregnancy[55,76,88,93-96]. However, in a retrospective cohort study of 817 asthmatic women during pregnancy, Lim *et al*[97] found statistically significant increases in the rates of pregnancy-induced hypertension (OR = 1.7, 95%CI: 1.0-2.9) and neonatal hyperbilirubinemia (OR = 1.9, 95%CI: 1.1-3.4) associated with the use of ICS or oral corticosteroid as compared to those using no medication. It is important to note, however, that outcomes associated with oral and inhaled corticosteroids were combined in this analysis, rather than independently assessed. Additionally, the authors cite the inability to distinguish well-controlled from uncontrolled asthma as an important limitation to this study[96].

Comparative studies of various ICS and commonly used dosages are somewhat lacking. All ICS except budesonide are classified as pregnancy category C by the United States Federal Drug Administration (FDA). Budesonide was moved to category B based on evidence of its safety from Dombrowski *et al*[93]; however, no specific data exists to suggest that other ICS are less safe for use during pregnancy. The United States National Heart Lung and Blood Institute guidelines of 2008 state that budesonide is the preferred ICS during pregnancy, but states that other ICS may be continued[58,59]. More recent global guidelines do not distinguish a preferred ICS for treatment of asthma during pregnancy, consistent with evidence from studies that has not shown significant differences in adverse maternal or fetal outcomes between patients using an ICS with beclomethasone, budesonide or fluticasone during pregnancy[50,77,88].

Few studies contain information regarding dosage ranges for the ICS used, making it difficult to determine if any risk of fetal adverse effects is dose-related[98]. Investigators in several studies did detect trends towards increased rates of SGA infants and increased congenital malformations with increasing doses of ICS, but the differences did not reach statistical significance and authors could not rule out confounding of results due to asthma severity[98,99]. In summary, there is no compelling evidence to substantiate a correlation between the use of an ICS during pregnancy and an increased risk of adverse infant outcomes; currently, these agents should be used when necessary to maintain asthma control.

Systemic corticosteroids should be reserved for use in acute exacerbations for all asthma patients or in those patients unable to achieve disease control using other agents. In contrast to ICS, oral corticosteroids have been associated with a higher incidence of maternal adverse effects, including preeclampsia and gestational diabetes[94,96,100,101]. A meta-analysis and systematic literature review conducted by Murphy *et al*[44] could not rule out the possibility of increased malformation risk associated with maternal oral corticosteroid use during a critical period for fetal lip and palate closure. The suggestion is based on data from case control studies that have indicated cleft lip and or cleft palate may not only be associated with maternal asthma but also with exposure to first-trimester oral corticosteroids[101,102]. As with other asthma therapies, the benefits associated with gaining control of severe uncontrolled asthma symptoms often outweigh the risks of adverse events associated with systemic steroid use. Nevertheless, systemic corticosteroids should be used judiciously in pregnancy and patients should be closely monitored for adverse effects.

Leukotriene receptor antagonists

Leukotrienes are potent mediators in the signaling pathways of allergic inflammation and thus play a central role in the pathophysiology of asthma. Leukotriene antagonists (LTRAs) function to reduce inflammation through this pathway and can reduce asthma exacerbations and improve lung function in persistent asthma. Few studies have been conducted to analyze the effects of this medication class exclusively and large, well-designed studies of LTRA use in pregnancy are lacking.

Limited data from available studies have shown conflicting results regarding maternal and fetal adverse out-

comes associated with the use of LTRAs[103-108]. A study of 180 pregnant asthmatics examined the effects of montelukast exposure compared to two separate groups of pregnant women: 180 disease-matched controls using inhalers but with no exposure to LTRAs and 180 age-matched healthy controls with no known teratogen exposure. Investigators found that in asthmatic women who used montelukast during the first trimester of pregnancy, there were significantly increased rates of infant LBW, preterm delivery and fetal distress when compared to healthy non-asthmatic maternal controls. Although only 47.4% of pregnant women taking montelukast in the first trimester continued the medication throughout pregnancy, a subgroup analysis of these patients demonstrated no significant difference in rates of fetal distress or preterm delivery when compared to asthmatic controls. This finding suggested a protective effect of montelukast likely due to improved asthma control throughout pregnancy. Moreover, investigators found no significant differences in adverse effects in pregnant women with asthma exposed to LTRAs compared to disease-matched asthmatic maternal controls[108].

In contrast, a previously published study generated results that indicated a nonsignificant increase in the rate of malformations in 96 asthma patients who received a LTRA throughout pregnancy as compared to 122 pregnant asthmatics who received SABA monotherapy. Malformations were observed in 5.95% of LTRA compared to 3.9% of SABA users, respectively ($P = 0.524$). Notably, the study had important limitations in addition to a relatively small sample size. The women taking LTRAs also were exposed during pregnancy to other asthma medications including SABAs, ICS, and oral corticosteroids. Also, LTRA use was associated with an increased patient baseline asthma severity which was not adjusted for in this study, and could account for the increased rate of malformations[104]. Further studies are needed in order to determine the safety of LTRA agents during pregnancy and/or lactation, but available data suggest that if necessary, montelukast would be the preferred LTRA due to a greater amount of evidence supporting its safety and a safer lactation profile.

Theophylline

Theophylline is a drug with mild bronchial anti-inflammatory effects[105]. While it is not a preferred agent in the treatment of asthma due to prevalent adverse effects, drug-drug interactions and the need for monitoring of serum concentrations, theophylline may be beneficial in selected patients. In a prospective study, 153 women with asthma including 85 receiving theophylline were followed throughout the course of their pregnancy. Results of the study demonstrated a significantly reduced risk of preeclampsia in patients treated with compared to those not receiving theophylline. Investigators suggested that theophylline's ability to increase cAMP levels and thereby reduce vascular reactivity and platelet aggregation may result in the decreased incidence of preeclampsia[106].

A subsequent study compared theophylline with inhaled beclomethasone therapy in 398 pregnant females with mild or moderate persistent asthma. No significant differences in adverse obstetric outcomes including preeclampsia, preterm delivery and oligohydramnos were detected between patient groups. Additionally, there was no significant difference in the number of asthma exacerbations between the two groups, although there was a significant increase in the proportion of women with a FEV1 less than 80% of predicted among the theophylline group[107]. Available evidence suggests that use of theophylline in pregnancy is likely safe; the drug is currently classified as a category C medication by the United States FDA[85,108].

Mast-cell stabilizers

Mast-cell stabilizers prevent mast-cell release of histamine and other inflammatory mediators during allergic response. Although they are not commonly compared to other asthma medications, they are considered effective second-line agents for asthma control. Very few studies have evaluated the use of mast-cell stabilizers for asthma during pregnancy and major limitations of available studies include small patient sample size, concurrent use of other medications, and comparison of treatment groups to healthy, non-asthmatic controls. Nevertheless, cromolyns are considered safe for use during pregnancy due to limited systemic bioavailability, and could be an appropriate adjunctive therapy in some patients[86,109].

Omalizumab

Omalizumab is a recombinant monoclonal anti-IgE therapy that works by binding and neutralizing the effects of IgE in basophils and mast cells, thereby preventing downstream allergic inflammation. The biologic therapy is reserved for patients with moderate to severe persistent asthma who are unable to be controlled by medium- to high-dose ICS plus LABA therapy. As a relatively new therapy, evidence for safety of omalizumab use in pregnancy is very limited. Currently, the Xolair® Pregnancy Registry (EXPECT) is collecting data for an ongoing observational study designed to monitor outcomes in women exposed to omalizumab during the time period starting eight weeks prior to conception and continuing throughout the pregnancy. Of the 128 known outcomes from preliminary data in this registry, there were 119 live births, with 117 singletons and two pairs of twins for a total of 121 infants. Of these infants, 16% were premature (gestational age less than 37 wk) and 7% had a birth weight less than 2.5 kg. The rate of major birth defects was 4%, with observed defects including patent foramen ovale, cutaneous mastocytosis, hemangioma, hypospadias, and bilateral renal pelvis dilation[110]. It is important to note that this agent is typically reserved for patients with moderate to severe asthma, and thus, it is difficult for effects related to omalizumab use to be differentiated from effects due to disease severity. Currently, the United States FDA has classified this agent as pregnancy cat-

Table 3 Asthma medications in pregnancy and lactation

Medication	United States FDA pregnancy category[1]	Australian Drug Evaluation Committee pregnancy category[2]	German pregnancy risk category[3]	Lactation[85]
Inhaled corticosteroids				
Beclomethasone	C	B3	Group 3	Unknown
Budesonide	B	A	Group 3	Unknown
Ciclesonide	C	B3	--	Unknown
Fluticasone	C	B3	Group 5	Unknown
Mometasone	C	B3	Group 5	Unknown
Short-acting β-agonists				
Albuterol	C	A	--	Likely safe
Levalbuterol	C	A	--	Unknown
Terbutaline	C	A	--	Likely safe
Long-acting β-agonists				
Formoterol	C	B3	Group 4	Unknown
Salmeterol	C	B3	Group 5	Unknown
Leukotriene inhibitors				
Montelukast	B	B1	Group 5	Likely safe
Zafirlukast	B	B1	--	Possibly unsafe
Zileuton	C	--	--	Likely safe
Mast-cell stabilizers				
Nedocromil	B	B1	Group 4	Unknown
Cromolyn	B	A	Group 1	Unknown
Systemic corticosteroids				
Dexamethasone	C	A	--	Likely safe
Methylprednisolone	C	A	Group 3	Likely safe
Prednisone	C	A	Group 3	Likely safe
Theophylline	C	A	--	Likely safe
Omalizumab	B	B1	Group 4	Unknown

[1]United States Federal Drug Association pregnancy categories[111]: Category A: Adequate and well-controlled studies have failed to demonstrate a risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters); Category B: Animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women; Category C: Animal reproduction studies have shown an adverse effect on the fetus and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks; Category D: There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks; Category X: Studies in animals or humans have demonstrated fetal abnormalities and/or there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience, and the risks involved in use of the drug in pregnant women clearly outweigh potential benefits; [2]Australian Drug Evaluation Committee pregnancy categories[111]: Category A: Drugs which have been taken by a large number of pregnant women and women of childbearing age without any proven increase in the frequency of malformations or other direct or indirect harmful effects on the fetus having been observed; Category B1: Drugs that have been taken by only a limited number of pregnant women and women of childbearing age, without an increase in the frequency of malformation or other direct or indirect harmful effects on the human fetus having been observed. Studies in animals have not shown evidence of an increased occurrence of fetal damage; Category B2: Drugs that have been taken by only a limited number of pregnant women and women of childbearing age, without an increase in the frequency of malformation or other direct or indirect harmful effects on the human fetus having been observed. Studies in animals are inadequate or may be lacking, but available data show no evidence of an increased occurrence of fetal damage; Category B3: Drugs that have been taken by only a limited number of pregnant women and women of childbearing age, without an increase in the frequency of malformation or other direct or indirect harmful effects on the human fetus having been observed. Studies in animals have shown evidence of an increased occurrence of fetal damage, the significance of which is considered uncertain in humans; Category C: Drugs that, owing to their pharmacological effects, have caused or may be suspected of causing harmful effects on the human fetus or neonate without causing malformations. These effects may be reversible; Category D: Drugs that have caused are suspected to have caused or may be expected to cause an increased incidence of human fetal malformations or irreversible damage. These drugs may also have adverse pharmacological effects; Category X: Drugs that have such a high risk of causing permanent damage to the fetus that they should not be used in pregnancy or when there is a possibility of pregnancy; [3]German pregnancy risk categories[113]: Group 1: Extensive human tests and animal studies have not shown the drug to be embryotoxic/teratogenic; Group 2: Extensive human tests of the drug have not shown the drug to be embryotoxic/teratogenic; Group 3: Extensive human tests of the drug have not shown the drug to be embryotoxic/teratogenic. However, the drug appears to be embryotoxic/teratogenic in animals; Group 4: No adequate and well-controlled studies of the drug's effects on humans are available. Animal studies have shown no embryotoxic/teratogenic effects; Group 5: No adequate and well-controlled studies of the drug's effects on humans are available; Group 6: No adequate and well-controlled studies of the drug's effects on humans are available. Animal studies have shown embryotoxic/teratogenic effects"; Group 7: There is a risk that the drug is embryotoxic/teratogenic in humans, at least in the first trimester; Group 8: There is a risk that the drug is toxic to fetuses throughout the second and third trimesters; Group 9: There is a risk that the drug causes prenatal complications or abnormalities; Group 10: There is a risk that the drug causes hormone specific action on the human fetus; Group 11: There is a known risk that the drug is a mutagen/carcinogen.

egory B based on evidence from animal studies (Table 2). Due to the small amount of human safety data, appropriate risk-benefit analysis should be undertaken before use in pregnancy.

DISCUSSION

Despite large amounts of data related to the influence of asthma and its treatment on maternal and fetal outcomes,

there are a number of limitations to these studies. Many early studies evaluating the influence of asthma control on maternal and fetal outcomes failed to assess, or poorly defined baseline asthma severity as well as the frequency, timing and severity of asthma exacerbations during pregnancy. However, data are available from other studies that have corrected for race, smoking status, age of mother, mean gestational age at enrollment, baseline asthma severity classification, severity of asthma exacerbations and other important covariates[33,35,42,111]. Based on existing information, it is clear that poor maternal asthma control has serious implications for both maternal and fetal health.

While congenital malformations are believed to be caused by a variety of factors associated with maternal asthma during pregnancy, establishing an association between maternal asthma effects and neonatal congenital malformations has also been challenging. Small sample sizes, varying study designs (case-control *vs* cohort), the timing of maternal study enrollment, a lack of correction for multiple testing and other confounders are routinely cited as key limitations[43,44,73]. Additionally, separating the impact of maternal asthma from effects caused by asthma medications on resultant fetal malformations is a daunting task. Given the low overall rate of congenital malformations in the general population (3%), a power analysis indicates that nearly 12000 women with asthma would be needed to detect a relatively small 15% increase for a major congenital malformation, given an alpha level of significance of 0.05 and a beta of 0.80[73]. Generally, data from large studies support a small increased risk of malformations from asthma medication use, although this risk is difficult to delineate from confounding factors including asthma severity, asthma control during pregnancy, fetal hypoxia at birth or simply chance[44,73,73]. Further studies that control for these confounding factors are required in order to truly separate the effects of disease *vs* the effects of medication use in pregnancy.

Although patients may express concerns regarding possible fetal adverse effects related to medication use, the majority of medications used for asthma maintenance therapy are regarded as safe (Table 3). In 2008, the United States FDA proposed the elimination of current pregnancy categories A, B, C, D, and X in favor of more detailed information for drug safety in pregnancy in lactation. The new format of pregnancy and lactation labeling aims to provide improved information for risk analysis and patient counseling on package inserts for all drugs. With its final version currently undergoing review and clearance, these changes are expected to improve the data available for the sometimes difficult clinical decision-making regarding the use of prescription drugs during pregnancy and lactation[112,113].

Most evidence indicates that improved maternal and fetal outcomes are correlated with improved asthma control with medications during pregnancy, suggesting the greatest adverse fetal risks are associated with poor asthma control[48]. Outcomes can be further improved through appropriate disease monitoring and management, patient education, and optimization of nonpharmacologic interventions to improve asthma control[50,57-59].

CONCLUSION

As a common condition in pregnancy, asthma can have a significant impact on both maternal and fetal health. Frequent monitoring and optimization of both pharmacological and nonpharmacological modalities are crucial to maintaining asthma control throughout pregnancy. Asthma management should also focus on education to promote patient understanding of the risks associated with uncontrolled asthma, avoidance of asthma triggers, proper inhaler technique and appropriate adherence to asthma therapy. Although some patients and providers will be concerned about the use of asthma medications during pregnancy, evidence shows the greatest risk of adverse maternal and perinatal outcomes is associated with uncontrolled asthma, and that the benefits of maintaining asthma control outweigh the risks associated with medication use.

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P- Reviewer: Dong L, Lee SH **S- Editor**: Ji FF **L- Editor**: A
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DOI: 10.5497/wjp.v3.i4.72

World J Pharmacol 2014 December 9; 3(4): 72-85
ISSN 2220-3192 (online)
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REVIEW

Targeted approaches for HER2 breast cancer therapy: News from nanomedicine?

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Received: July 28, 2014 Revised: August 29, 2014
Accepted: September 23, 2014
Published online: December 9, 2014

Abstract

About 30% of human breast cancers are human epidermal growth factor receptor 2 (HER2)$^+$. This particular biological portrait is characterized by the overexpression of HER2 receptor with the subsequent deregulation of downstream pathways, which control cellular survival and proliferation. The most effective treatment for HER2$^+$ cancer is represented by therapy with HER2-targeted agents. Anti-HER2 therapy dramatically improves clinical outcomes, although it shows some limitations in achieving a proper treatment. These drawbacks of HER2-targeted therapy may be overcome with the development of HER2-targeted drug delivery nanodevices. These nanoparticles possess an internal three-dimensional compartimentalization, which allows to combine the specific target recognition with their capability to act as a drug reservoir for the selective delivery of chemotherapics to tumor sites. Moreover, nanoparticles useful in photothermal ablation or in photodynamic therapy have been functionalized in order to match specificity in tumor cell recognition and suitable chemical properties. Here, we summarize the state of the art concerning the HER2$^+$ breast cancer and anti-HER2 therapy, in particular deepening the contribution of the nanomedicine. Description of preclinical studies performed with HER2-targeted nanoparticles for HER2$^+$ breast cancer therapy will be preceded by an overview on HER2-targeting molecules and nano-conjugation strategies. Further investigation will be necessary to introduce these nano-drugs in clinical practice; however promising results encourage an upcoming translation of this research for the next future.

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Key words: Human epidermal growth factor receptor 2; Human epidermal growth factor receptor 2$^+$-breast cancer; Nanomedicine; Nanoparticle; Targeted-therapy

Core tip: About 30% of human breast cancers are characterized by the overexpression of human epidermal growth factor receptor 2 (HER2) receptor, which determines the deregulation of cell survival and proliferation pathways. The HER2-targeted therapy is the most effective treatment, despite some related limitations, which could be bypassed with the development of nanoparticles for HER2-targeted drug delivery, photothermal ablation or photodynamic therapy. Here, we describe HER2$^+$ breast cancer features and anti-HER2 therapy, and focus on the contribution of nanomedicine in this context, by reporting HER2-targeted nanoparticles under preclinical investigations. Promising results suggest upcoming clinical application of these nanocompounds in the next future.

Mazzucchelli S, Truffi M, Fiandra L, Sorrentino L, Corsi F. Targeted approaches for HER2 breast cancer therapy: News from nanomedicine? *World J Pharmacol* 2014; 3(4): 72-85 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/72.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.72

INTRODUCTION

The erythroblastic leukemia viral oncogene homolog (ErbB) family of receptors and associated pathways main-

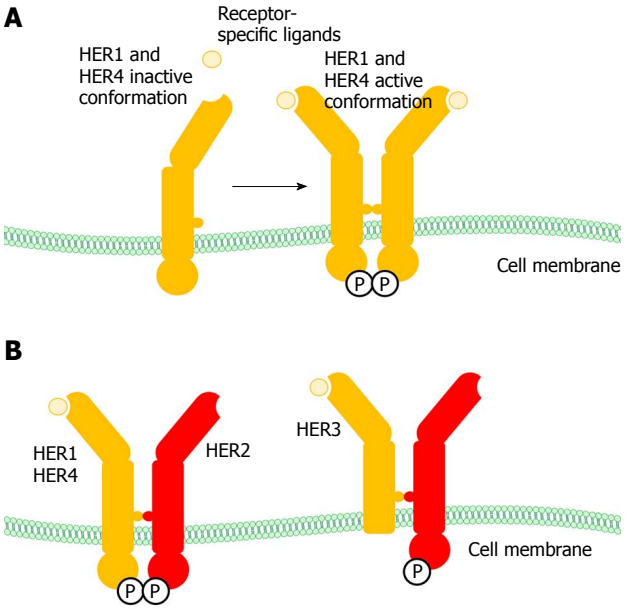

Figure 1 Characteristic features of ErbB receptors family. A: Schematic representation of HER1 and HER4 conformational change upon ligand interaction; B: Schematic representation of HER2 heterodimers. HER: Human epidermal growth factor receptor 2; ErbB: erythroblastic leukemia viral oncogene homolog.

ly regulate cell survival and proliferation. They include many actors, which cross-talk with each other and which may have overlapping functions. The typical redundancy of robust physiological processes, including the ErbB pathways, is employed by normal cells to guarantee their survival. However, it may also be highly dangerous during the early stages of tumor development, since it contributes to increase the proliferative potential of cancer cells[1]. Actually, the main goal in clinical practice is to selectively kill the tumor cell before it can acquire the capability of metastasizing and to reduce the onset of severe side effects related to chemotherapics. Based on this rationale, several nanotechnological devices have been developed to target delivery of chemotherapeutic agents toward cancer cells in order to minimize their toxic effects on healthy tissues while increasing the antitumor efficacy[2]. Although the number of nanoparticle strategies developed for drug delivery is increasing rapidly, they can be classified into two major groups: (1) particles containing organic molecules; and (2) particles that use inorganic elements, usually metals, as a core.

In this review, we focus on the ErbB receptor human epidermal growth factor receptor 2 (HER2) with the aim to summarize the state of the art of $HER2^+$ breast cancer and related targeted therapy. In particular, we wish to explore the contribution of nanotechnology in the development of HER2-targeted nanoparticles for therapeutic purpose.

HER2 AND THE ErbB FAMILY OF PROTEINS

HER2 is a cell membrane-bound tyrosine kinase receptor that is overexpressed in 20%-30% of breast cancer in humans. It belongs to the ErbB family of proteins, consisting of four different membrane receptors: epidermal growth factor receptor 1 (EGFR, HER1, ErbB1), 2 (HER2, ErbB2), 3 (HER3, ErbB3) and 4 (HER4, ErbB4)[3]. Each receptor includes an extracellular domain recognized and bound by the ligand, an α-helical transmembrane portion and an intracellular tyrosine-kinase domain[4]. Within the ErbB family there are also 13 polypeptide ligands that share the conserved epidermal growth factor (EGF) domain. The EGF family of polypeptides specifically binds the ErbB receptor and generally include three classes of proteins. The first one contains several EGFR ligands such as EGF, transforming growth factor (TGF)-α, amphiregulin and Epigen. The second group is constituted of β-cellulin, heparin binding EGF and epiregulin, which display dual specificity toward EGFR and HER4. The last group contains neuregulins (NRGs), which are divided into two subclasses depending on recognition of HER3 and HER4 (NRG-1 and NRG-2) or HER4 only (NRG-3 and NRG-4)[5]. Generally, ErbB receptors take on an inactive conformation. The binding of the physiological ligand determines and stabilizes a conformational change that makes the dimerization domain within the extracellular portion accessible to other receptors of the family (Figure 1A). The receptor dimerization is essential for ErbB function and for activating the downstream cascade of signal transduction. Dimerization can take place between two different ErbB receptors (heterodimerization) or between two identical ErbB molecules (homodimerization). The receptor dimerization causes transactivation of the tyrosine-kinase domain by phosphorylation, so that each receptor activates its partner[4]. In the ErbB family, only HER3 and HER2 are non-autonomous: the first one does not have intrinsic kinase activity since it is unable to bind adenosine triphosphate (ATP), whereas HER2 is an orphan receptor, since it lacks a physiological ligand[3,4].

PHYSIOLOGICAL MECHANISM OF ACTION: FROM THE RECEPTOR TO THE PATHWAYS

Since it is an orphan receptor, HER2 is always in a constitutively active conformation, which exposes the dimerization domain to other receptors of the ErbB family. Therefore, HER2 cannot homodimerize and needs to be activated by heterodimerization with ligand-activated HER1, HER3 or HER4 (Figure 1B)[3]. After dimerization, the cross-phosphorylation of dimer partner creates docking sites for the engagement of downstream signaling actors. Depending on the type of ligand and the type of ErbB receptor recruited by HER2, different adaptor proteins are engaged and different pathways are activated[4]. Two key pathways are activated by HER2: the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K)/Akt pathway, which promote proliferation and cell survival, respectively. The activation of the MAPK pathway is due to

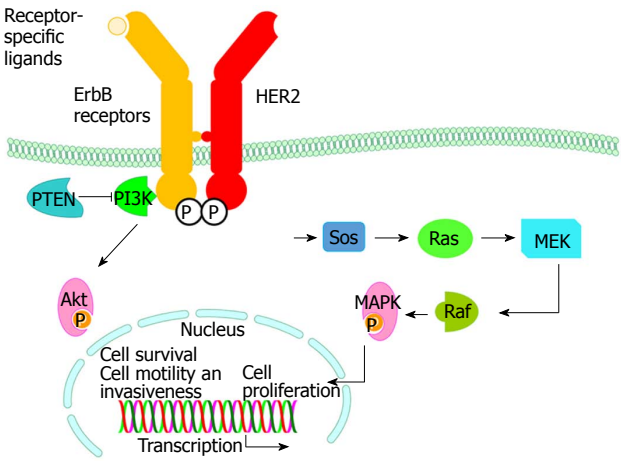

Figure 2 Schematic representation of HER2 physiological pathways. Ras: Rat sarcoma protein; Sos: Son of sevenless; PI3K: Phosphoinositide 3-kinase; MAPK: Mitogen-activated protein kinase; HER: Human epidermal growth factor receptor; ErbB: Erythroblastic leukemia viral oncogene homolog.

the recruitment and activation of the rat sarcoma protein (Ras) by the transducer molecule son of sevenless, which is in turn activated by growth-factor-receptor-bound-2 previously activated by Src-Homology-2 containing (Shc). Shc is activated upon interaction with the phosphorylated tyrosine residues within the HER2 intracellular domain. Activation of Ras kinase triggers the activation of the MAPK signaling cascade, which includes the phosphorylation of Raf, MEK and MAPK. Upon phosphorylation by MEK, MAPK translocates into the nucleus, where it regulates the transcription of genes involved in angiogenesis, proliferation and cell cycle control (Figure 2).

Differently, the PI3K pathway is activated through interaction of the phosphorylated serine or threonine residues of the receptor with the PI3K or with one of its transducer proteins, such the ubiquitin ligase Cbl. The activation of PI3K leads to the conversion of Phosphatidyl inositol 2 (PI) into PI3, with subsequent activation by phosphorylation of the Akt kinase. Once phosphorylated, Akt interacts with several transcription factors involved in cell cycle control, suppression of apoptosis and cell survival, such as mTOR, p27, nuclear factor-κB, glycogen synthase kinase-3β, and modulates their activation/inhibition[4,6]. The formation of PI3 is antagonized by the phosphatase PTEN, which acts reverting PI3 in PI2. Interestingly, HER2 is also able to translocate into the nucleus, where it interacts with the cyclooxygenase-2 promoter and directly activates the transcription of specific HER2-dependent genes (Figure 2)[7].

MOLECULAR FEATURES AND PATHOGENESIS OF HER2$^+$ BREAST CANCERS

HER2$^+$ breast cancer is characterized by HER2 overexpression due to Her2 gene amplification or aneuploidy in more than 90% of cases[8]. In addition to gene amplification and aneuploidy, HER2 overexpression may derive from transcriptional deregulation involving *cis*-acting enhancer elements near Her2 promoter or overexpression of transcription factors that bind this region[9]. As a result of HER2 overexpression, many intracellular signaling proteins and physiological pathways are activated[1]. Moreover, the negative regulatory loops usually active in normal cells are impaired, further contributing to pathology onset[10]. Frequently, in HER2$^+$ breast cancer the deregulation of the PI3K/Akt pathway takes place. Indeed, the PI3K activity is maintained high by the preferred interaction of HER2 with HER3. HER3 has impaired kinase activity and is unable to form homodimers but it contains six docking sites for the PI3K interaction that makes it the major PI3K activating receptor of the ErbB family. HER3 is the preferred partner of HER2 and the HER3/HER2 dimer functions as an oncogenic unit[5]. The activation of PI3K leads to the phosphorylation and subsequent activation of Akt, which determines, among others, many important downstream effects in the oncogenic process, such as the downregulation of cyclin D1 and p27, which increase tumor cell proliferation and survival[11]. Another typical outcome of HER2 overexpression is the hyperactivation of the MAPK pathway that results in the transcription of genes that drive cell proliferation and migration, thus conferring to tumor cells poor differentiation, invasiveness and metastatic behavior[1,11].

Generally, HER2 overexpression is also combined with increased angiogenesis, since HER2 is able to modulate the balance between pro- and anti-angiogenic factors. In particular, high HER2 expression has been related to high levels of the pro-angiogenic molecules VEGF, IL-8 and angiopoietin-2[11].

It has to be noted that HER2 extracellular portion is subjected to metalloproteinase cleavage, which generates a kinase-active p95 fragment. At present, it is unknown whether this activated fragment undergoes nuclear translocation and regulates HER2-dependent genes expression[12]. Moreover, decreased levels of phosphatase expression (*e.g.*, PTEN), increased expression of ErbB receptor partners and/or their ligands[12,13], cross-talk with other tyrosine-kinases (*e.g.*, IGF-IR) are alternative mechanisms leading to HER2 hyperactivation even in absence of HER2 overexpression[1,3,8,14].

CLINICAL FEATURES OF HER2$^+$ BREAST CANCER

As widely stated in literature, breast cancer is a heterogeneous disease and includes various subsets with distinct biological portraits. HER2$^+$ breast cancer is characterized by a poor clinical outcome when anti-HER2 therapy is not administered. Notoriously, HER2 overexpression is related to lower hormonal receptor (HR) positivity, higher index of mitosis, and frequent p53 mutations. Clinical implications of these features include shorter metastasis-free and overall survival[15]. A retrospective

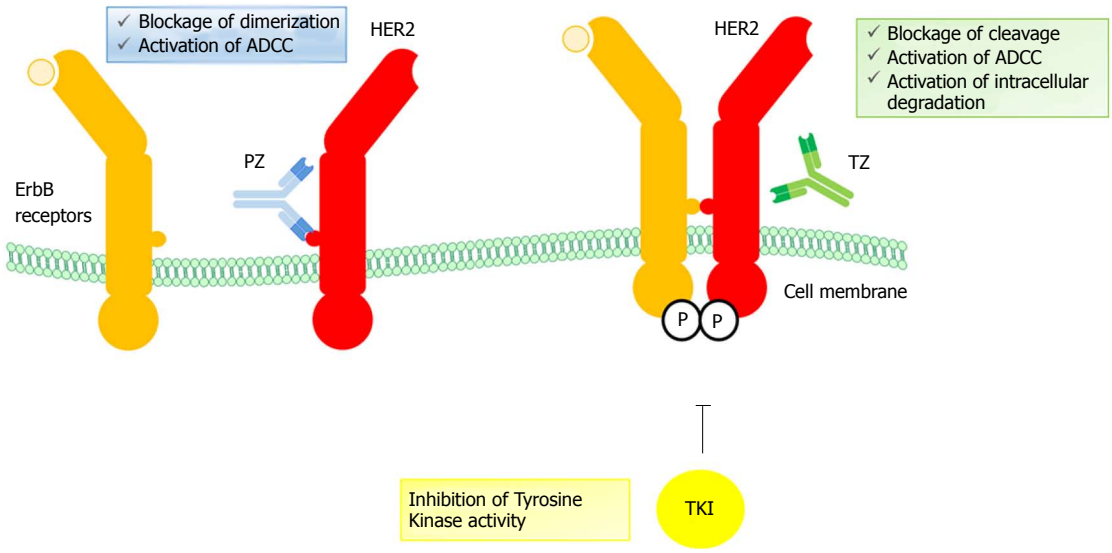

Figure 3 Schematic representation of mechanism of action of HER2-targeted drugs. PZ recognizes an epitope within the HER2 dimerization domain, thus preventing interaction with other activated ErbB receptors. Moreover PZ recruits natural killer cells, which mediate ADCC. TKI act on HER2 tyrosine kinase activity, by blocking intracellular signaling. TZ binds the juxtamembrane portion of HER2, thus preventing receptor cleavage and stimulating ADCC response and receptor degradation after endocytosis of the HER2-TZ complex. PZ: Pertuzumab; TZ: Trastuzumab; TKI: Tyrosine kinase inhibitors; ADCC: Antibody dependent cellular cytotoxicity; HER: Human epidermal growth factor receptor; ErbB: erythroblastic leukemia viral oncogene homolog.

study on 892 breast cancer patients showed a significantly higher frequency of distant metastases for $HER2^+$ tumors, together with a lower 9-year disease free survival and a lower 7-year overall survival compared to the other subgroups[16]. However, the clinical behavior of $HER2^+$ cancers may also depend on HR status[17]. A recent prospective cohort study was conducted on 3394 $HER2^+$ breast tumors: among these, HR^- cancers more likely presented higher T stage (T3 to T4 in 17% *vs* 10%, $P < 0.001$), nodal involvement (52% *vs* 45%, $P < 0.001$), and higher histologic grade (81% *vs* 60%, $P < 0.001$)[18]. Interestingly, $HER2^+/HR^-$ cancers were associated with more frequent brain recurrences (OR = 1.75, $P = 0.033$), and less frequent bone metastases as a first distant recurrence (OR = 0.53, $P = 0.005$), thus indicating a more aggressive disease. Therefore, $HER2^+$ cancers may be divided into two distinct clinical entities based on HR status, although further studies are needed.

MANAGEMENT OF $HER2^+$ BREAST CANCER

Among the different therapeutic strategies employed for $HER2^+$ breast cancer, Trastuzumab (TZ) is the most widely used agent. TZ is a humanized monoclonal antibody developed starting from the murine antibody 4D5 and constituted of two antigen-binding sites that recognize the juxtamembrane portion of HER2 receptor. It functions by blocking the downstream signaling activity of HER2, thus causing cell cycle arrest and reduced angiogenesis[4]. TZ inhibits the PI3K survival pathway by increasing PTEN membrane localization and activity, with resulting inhibition of proliferation[14]. The inhibition of PI3K signaling may also result from HER2 internalization and degradation upon TZ interaction. However, it is still under debate whether HER2 may effectively be downregulated by TZ or not[19,20]. Moreover, there is evidence that TZ-HER2 interaction activates the immunological response mediated by the antibody-dependent cellular cytotoxicity (ADCC), through recruitment of natural killer (NK) cells. NK cells express on their surface the Fcγ receptor IIIa, which recognizes and binds the Fc domain of TZ[1,4,12]. In addition, the interaction of TZ with HER2 prevents the proteolytic cleavage of HER2 extracellular domain, its serum-release and the production of the truncated p95 kinase active fragment by masking the cleavage site to metalloproteinase (Figure 3)[12,13]. At present, it is still unknown if TZ can act directly on HER2 intracellular partners[14,21]; however TZ likely inhibits signaling downstream HER2-HER1 heterodimers[4]. Finally, TZ treatment blocks the cell cycle in the G_1 phase, leading to reduced proliferation. This event is coupled to reduced expression of proteins involved in the sequestration of the cyclin-dependent kinase inhibitor p27KIP1, including cyclin D1. This results in increased expression of p27KIP1 protein, which causes cell cycle arrest in S phase[1,12].

The significant improvement in overall survival and disease free survival achieved with TZ in $HER2^+$ breast cancer may be considered a paradigm of the importance of targeted therapy in clinical practice, since TZ-based chemotherapy regimens have changed the clinical course of the disease. A phase Ⅱ randomized clinical trial on $HER2^+$ metastatic breast cancers showed that the addition of TZ resulted in a significantly improved overall response rate (61% *vs* 34%, $P = 0.0002$), and overall survival (31.2 mo *vs* 22.7 mo, $P = 0.0325$)[22]. In a recent Cochrane systematic review on a total of 1497 patients in which TZ was administered in combination with chemotherapy,

the efficacy of TZ was confirmed, with an improved overall survival (HR = 0.82, $P = 0.004$), progression-free survival (HR = 0.61, $P < 0.00001$), and overall response rate (RR = 1.58, $P < 0.00001$), although an increased risk in congestive heart failure was evident[23]. Because of the great efficacy as a first-line and adjuvant treatment, TZ has been successfully introduced also in the neoadjuvant setting. Interestingly, the first phase Ⅲ randomized trial on neoadjuvant TZ was prematurely stopped due to the evident superiority of TZ-based chemotherapy[24]. Other more extensive trials, such as GeparQuattro and NOAH, demonstrated similar results, with a substantial improvement of pathologic complete response rates and 5-year event-free survival in the TZ arm[25,26].

Besides TZ, other antibodies against HER2 are currently under investigation. In particular, Pertuzumab (PZ) is a humanized monoclonal antibody that recognizes an epitope within the HER2 dimerization domain. It is able to inhibit heregulin-induced activation of HER2 phosphorylation and cell growth. Differently from TZ, PZ blocks the heterodimerization of HER2 with HER3, which is extremely relevant in tumorigenesis. However, PZ is not able to prevent the formation of EGFR-HER2 dimers, thus limiting its therapeutic efficacy[27,28]. As observed for TZ, PZ efficacy is also mediated by the recruitment of the ADCC system (Figure 3). Because of its capability to inhibit HER2 dimerization with HER3, PZ has been approved by FDA for clinical use in association with TZ, thus helping to overcome resistance to anti-HER2 treatment. This approval has been obtained on the basis of a phase Ⅲ Clinical Evaluation of PZ and TZ (CLEOPATRA) trial, in which placebo plus TZ plus docetaxel was compared to PZ plus TZ plus docetaxel for first-line treatment of 808 $HER2^+$ metastatic breast cancer[4]. Median progression-free survival was significantly higher in PZ group (18.5 mo *vs* 12.4 mo), and preliminary analysis showed also a favorable trend about overall survival. Currently other studies are investigating the role of PZ in $HER2^+$ breast cancer in progression under TZ treatment, confirming a synergistic role between the two antibodies[29].

In the last ten years, an antibody-drug conjugate, named T-DM1 (Genentech), has been developed, and it is constituted by a TZ molecule conjugated with the anti-microtubule agent DM1. TZ-DM1 recognizes HER2, is internalized and release DM-1 into the cytoplasm of $HER2^+$ cells[30]. In February 2013, T-DM1 was approved by FDA for treatment of metastatic $HER2^+$ breast cancer previously treated with TZ and taxanes. The efficacy of T-DM1 in this setting has been assessed in comparison with Lapatinib on 991 patients, with an overall survival of 30.9 mo *vs* 25.1 mo ($P < 0.001$): in particular, T-DM1 was associated with a higher objective response rate (43.6% *vs* 30.8%, $P < 0.001$) and a lower toxicity profile[31].

Another class of biological drugs for HER2-targeted therapy is represented by tyrosine-kinase inhibitors (TKI). They are small molecules that bind the ATP binding site of ErbB receptors, and prevent the activation of both PI3K and MAPK signaling pathways, thus increasing apoptosis and reducing proliferation (Figure 3)[4]. Among them, the most clinically advanced is Lapatinib, a dual inhibitor of HER2 and EGFR[32]. Lapatinib has the advantage to act also on p95 activated fragment of HER2, which strongly correlates with poor prognosis[33,34]. Lapatinib has gained a great interest for breast cancer treatment mainly for two reasons: its orally available formulation and its efficacy in the treatment of TZ-resistant metastatic HER2-positive breast cancer, with a reduction in risk of death by 26%[35]. Moreover, Lapatinib has been recently studied in the neoadjuvant setting in association with TZ (NeoALTTO Trial), with a pathologic complete response of 51.3% *vs* 29.5% with TZ alone[36]. Other TKIs, such as HKI-272, ARRY-334543 and BIBW-2992, are under clinical investigation for breast cancer[4].

Finally, some inhibitors of the Heat Shock Protein 90 (Hsp90) have been developed for breast cancer therapy. Indeed, Hsp90 has a role in controlling the stability of nascent and mature forms of HER2. Inhibition of its activity results in HER2 ubiquitination and subsequent proteasomal degradation, thus blocking HER2 downstream signaling pathway[3]. A phase II trial has been conducted on 31 patients with $HER2^+$ breast cancer in progression after TZ treatment, subsequently treated with the Hsp90 inhibitor tanespimycin: the objective response rate was 22% with a progression-free survival of 6 mo, therefore demonstrating the efficacy of the drug against this subset of breast cancer[37]. However, tanespimycin has been suspended for further clinical studies, and other novel Hsp90 inhibitors are currently studied.

ONSET OF RESISTANCE

Frequently, $HER2^+$ cancers develop resistance to HER2-targeted therapies[38]. In particular, the development of resistance toward the widely used TZ has been extensively examined. Generally, resistance to TZ occurs because of three different mechanisms: (1) epitope masking; (2) upregulation of HER2 signaling; and (3) alterations of the immune response[39]. As regards to epitope masking, two candidates have been identified: Mucin 4 (MUC4) and the CD44/hyaluronan polymer complex. MUC4 is an O-glycosylated membrane-associated protein, which is upregulated in TZ-resistant JIMT-1 cells. Binding of TZ to HER2 was reduced in JIMT-1, while it was restored after knockdown of MUC4[40]. A similar result was observed with the CD44/hyaluronan polymer complex, where knockdown of CD44 or chemical inhibition of hyaluronan synthesis restored TZ-HER2 recognition in JIMT-1 cells. In both cases, the TZ-resistance is probably due to the steric hindrance of the complex that prevents TZ binding and internalization, without altering HER2 signaling[41]. Upregulation of HER2-signaling is another mechanism found to bypass TZ hurdle. It results from the overexpression of some ErBb family members and the subsequent increase in heterodimer formation. Indeed, in presence of an excess of ErbB ligands the

resulting heterodimers drive cells towards proliferation and inhibition of apoptosis, thus interfering with TZ action[42]. However, the HER2/HER1 complex may also undergo antibody-induced internalization, ubiquitination, and proteolysis, that disable its transforming activity[1]. Moreover, up to 30% of HER2$^+$ breast cancers express p95, an amino-terminal truncated form of HER2. Since p95 is a constitutively active kinase lacking the TZ binding site, it is able to confer TZ resistance[43]. In this situation, treatment with PZ or with one of TKIs may replace responsiveness to anti-HER2 therapy[44]. Another mechanism to bypass the TZ-mediated blockade of HER2 signaling is the activation of downstream effectors by alternative routes, *e.g., via* the insulin-like growth factor 1 receptor (IGF-1R) or c-Met, often overexpressed in TZ-resistant cells, and able to hyperactivate the PI3K/Akt pathway. Treatments with inhibitors of IGF-1R or c-Met may restore TZ sensitivity[8,45]. Decreased expression of the Akt inhibitor PTEN is another crucial factor in TZ resistance. TZ upregulates the microRNA miRNA-21, a physiological inhibitor of PTEN phosphatase[46]. The reduction of PTEN expression maintains Akt active, and diminishes TZ efficacy[14]. Moreover, the hyperactivity of the PI3K pathway causes epigenetic changes, which result in the inhibition of FoxO, the transcription of antiapoptotic genes[47] and the downregulation of p27KIP1[44]. Finally, the alteration of the immune response may cause TZ resistance in tumor cells. It is well known that TZ treatment induces ATCC, which triggers tumor cell death[48]. It exists a FcγRIIIa polymorphism, which makes it less effective at inducing ATCC. This mechanism of resistance is common to both TZ and PZ treatments[49].

Besides these three main mechanisms, other minor ones are involved in TZ resistance and have to be taken into account. These concern the discovery of HER2 mutants with modulated receptor activities, and subsequent more aggressive tumor phenotype[16], and the increased activation of Notch receptors upon TZ or Lapatinib treatment, which contributes to the development of resistance[50,51].

To overcome the previously described mechanisms of resistance to TZ a new agent against HER2$^+$ cancers, called Neratinib, is being investigated for clinical use. Neratinib is a pan-HER irreversible TKI, also available for oral administration, and ErbB2 mutations were found to be sensitive to Neratinib in some preclinical studies[52]. In a phase Ⅱ trial on patients with or without previous treatment with TZ, Neratinib was administered daily at 240 mg dosage. The 16-wk progression-free survival rates was 59% for patients with prior TZ and 78% for the other group of patients and the most common adverse event was diarrhea[53]. Interestingly, Neratinib was recently administered in combination with weekly paclitaxel and TZ in a phase I trial on metastatic HER2$^+$ positive cancers previously treated with TZ, Lapatinib or T-DM1, with an objective response in 38% of patients and a median time to progression of 3.7 mo, therefore suggesting that dual anti-HER blockade with Neratinib and TZ may be more effective than single-agent inhibition[54]. Therefore Neratinib represents a promising tool for HER2$^+$ TZ-resistant breast tumors, and a phase Ⅲ trial comparing Neratinib plus Capecitabine and Lapatinib plus Capecitabine in metastatic HER2$^+$ breast cancer is ongoing[55].

THERAPEUTIC IMPROVEMENT FROM NANOTECHNOLOGY

Over the last thirty years, we all have witnessed the great development of nanotechnologies. In particular, nanomedicine has shown promising scenarios for clinical practice with the development of more effective, less toxic and smart therapeutics[56]. The novel field of nano-oncology was created and several nanodevices for tumors treatment have been developed in order to overcome limitations of conventional therapies. Indeed, chemotherapy lacks of selectivity toward tumor cells and therefore it is highly toxic toward healthy tissues. It has limited accessibility to the tumor tissues, and requires high doses to be efficient[2]. Moreover, conventional chemotherapics are usually unable to cross biological barriers, thus bearing limited efficacy at several metastatic sites[57]. Nanoparticles possess physical and chemical properties suitable for molecular and cellular interactions, partially due to their high surface-to-volume ratio. Moreover, their capability to form internal 3D nanostructures gives them the appropriate flexibility to be exploited as drug delivery devices able to overcome biological barriers, and to transport hydrophobic and poorly water-soluble drugs. Nanoparticles can be designed to have a large therapeutic payload and to be applied in combinatorial therapy since they can accommodate multiple drugs. Moreover, nanoparticles protect embedded drugs, thus allowing in certain cases to overcome drug resistance, which is crucial for effectiveness of cancer treatment. Finally, nanoparticle surface can be engineered with antibodies, peptides or other biologically active molecules in order to achieve a selective targeting of tumor malignancies[58].

In the next paragraphs, we will overview the ligands and the conjugation employed for the development of HER2-targeted nanoparticles, and we will report some nanotechnological approaches for the targeted-therapy of HER2$^+$ breast cancer.

HER2-TARGETED LIGANDS FOR NANOPARTICLES BIOENGINEERING

An active targeting strategy relies on the coupling of a targeting moiety to the surface of nanoparticles, thus providing specific binding to cancer biomarkers overexpressed at the target site. Such a targeting mechanism increases specific recognition of tumor cells and internalization of the nanocomplex through receptor-mediated endocytosis[59-61]. The influence of a targeting molecule on the pharmacokinetics, biodistribution and tumor accumulation of nanoparticles depends on several

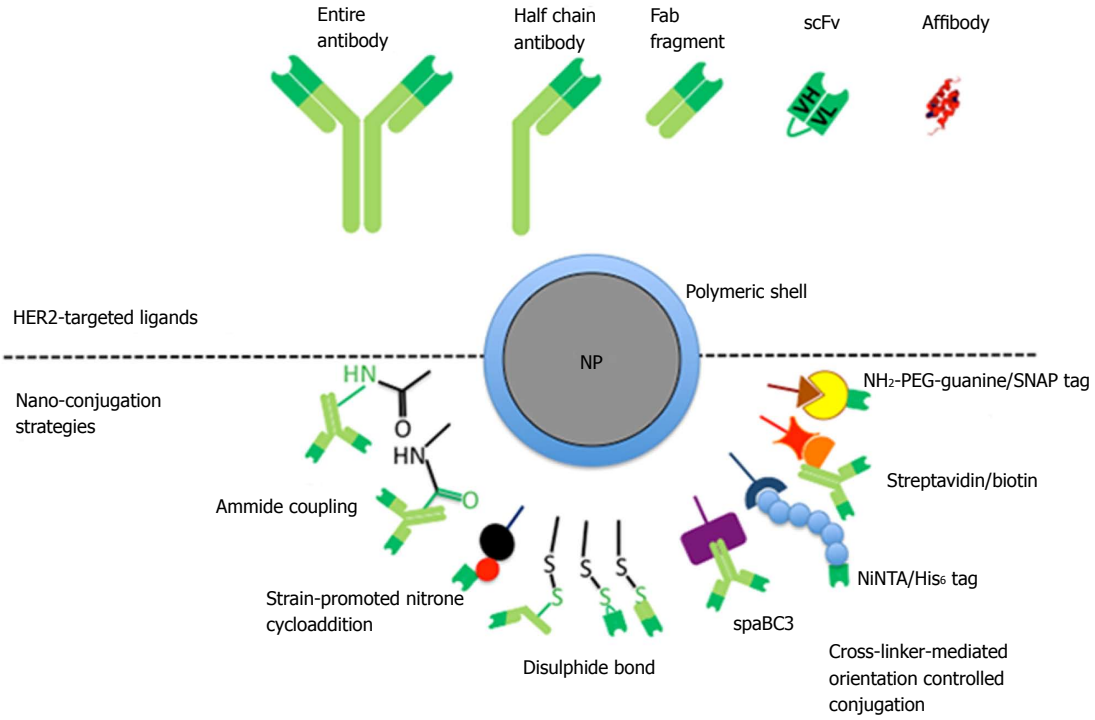

Figure 4 Schematic representation of HER2-targeting ligands and conjugation strategies employed for NPs functionalization. HER: Human epidermal growth factor receptor; scFv: Single-chain fragment variable antibodies.

factors, including the nature of the ligand, its density on the surface of the nanoparticle and its activity[62]. Targeting moieties exploited for nanoparticle functionalization include peptides, proteins, oligonucleotides, aptamers, carbohydrates, lipids, and other biologically active molecules. Among them monoclonal antibodies and antibody-derived ligands, are widely used (Figure 4). Indeed, antibody-based therapy has received wide attention because of its stability to selectively target tumor cells through receptor-specific interactions[63-65]. The selective tumor targeting capability of antibodies can be exploited in nanotechnology by covalently coupling antibodies directed against HER2 to the surface of colloidal nanoparticles, thus achieving higher cellular uptake and improved antitumor efficacy of nanoformulated drugs. In the context of HER2-targeted nano-therapy the most studied example of nanoparticle-conjugated ligand is TZ, a humanized monoclonal antibody already used as single agent after chemotherapy or in combination with chemotherapy in HER2-overexpressing metastatic breast cancer treatment. TZ has been used as a targeting moiety to be conjugated onto the surface of different nanoparticles, including quantum dots, magnetic and gold nanoparticles, in order to achieve selective recognition of $HER2^+$ tumor cells for both imaging and therapeutic applications, with promising results obtained in preclinical studies on $HER2^+$ breast cancer-bearing animal models. Different kinds of TZ-functionalized nanoparticles have been extensively reported[1,66]. However, conjugating entire antibodies onto nanoparticles may lead to increased immunogenicity of the resulting nano-compound and reduced circulation time and tissue penetration[64]. Recombinant antibodies with small size have been developed in order to overcome such problems. Nano-conjugation of the half-chain of the monoclonal antibody TZ dramatically improves the intracellular trafficking and the long-term stability of the nano-compound in both *in vitro* and *in vivo* settings[67]. Anti-HER2 Fab fragment of the monoclonal antibody TZ has also been shown to enhance tumor cell uptake resulted from HER2-mediated internalization of HER2-targeted liposomes[68,69]. Innovative and intriguing ligands, which have provided promising results, are single-chain fragment variable antibodies (scFv), variable V_H and V_L regions of antibodies connected through a synthetic loop[63]. Anti-HER2 scFv immobilized onto the surface of magnetic nanoparticles has proved to be highly effective in selectively targeting $HER2^+$-breast cancer cells, and has shown faster cellular interaction and incorporation of nanoparticles when compared to entire TZ ligand[67]. A number of nanoparticles have also been functionalized with HER2 affibody molecules, small proteins mimicking the active portion of the Fab region of TZ[70,71]. Nanoparticle-affibody conjugates have shown highly specific targeting and efficiency toward $HER2^+$-breast cancer, thus representing another promising class of targeting ligands with simple, robust, and precise structure and high affinity.

Besides antibodies and antibody-derived ligands, other active molecules directed toward HER2 have been proposed as interesting ligands for nano-formulation. In particular, Lapatinib is a dual inhibitor of the tyrosine kinase receptors EGFR and HER2 used to treat advanced breast cancers, and its poor water solubility has been overcome by conjugation with lipoprotein-like nanopar-

ticles (LTNPs). Such nano-compounds could be taken up by breast tumor cells by endosomes through clathrin-dependent pinocytosis and macropinocytosis, with subsequent escape from endosomes to the cytoplasm. Within tumor cells, LTNPs induce a significant cell arrest at G_0/G_1 phase compared with equal concentrations of classical lapatinib. They also could passively accumulate into the tumor *in vivo via* the enhanced permeability and retention effect where they induce elevate anti-tumor activity[72,73].

NANO-CONJUGATION OF HER2-TARGETED LIGANDS

When designing nano-devices for targeted treatment, a crucial issue concerns the optimization of functionalization strategies to achieve an efficient and specific targeting. The structural features of a nano-compound may affect its biological functions; hence many efforts have been focused on development of new strategies for nanoparticle surface bioengineering (Figure 4). In particular, fine control of positioning, spatial orientation and conservation of the activity of targeting biomolecules have reveled essential for the generation of nano-compounds with well-defined and reproducible properties[74,75]. Reliable conjugation strategies include physical adsorption and formation of covalent chemical connections, often through coupling with appropriate crosslinkers[75,76]. Physical adsorption is usually related to protein ligands destabilization. Moreover, ligand orientation, number of immobilized molecules and bond stability are completely out of control. Instead the covalent coupling between the ligand and the nanoparticle gives some advantages in terms of stability of the ligand conjugation and versatility of the conjugation strategy. Indeed, chemical properties of nanoparticles have sometimes to be modulated with different functionalities depending on the functional groups found on the targeting ligands. Frequently superficial amino and carboxylic groups on the surface of nanoparticles are employed for amide coupling, thus obtaining covalent binding between the ligand and the biocompatible polymers coating the nanoparticles surface. Cysteine residues have also been found as preferred conjugation sites on proteins in general, and further exploited for HER2-targeted ligands bioconjugation. Such cysteines, either naturally present in the polypeptide sequence or introduced at specific positions by site-directed mutagenesis in case of recombinant ligands, can be activated with reducing agents and used to form disulfide bonds with properly modified nanoparticles surface[77,78]. Traditionally, poly ethylene glycol (PEG) or poly ethylene oxide molecules are used to coat nanoparticles surface in order to reduce eventual aspecific interactions of the nanoparticle with the cells and function as spacer. Anti-HER2 antibodies have been conjugated to PEGylated nanoparticles, by covalent attachment to superficial amino and carboxylic groups[79-81]. Polyvynil-pyrrolidone (PVP) and poly-D,L-lactic-co-glycolic acid (PLGA) are other clinically safe polymers used to coat nanoparticles, which can interact with a variety of agents[82]. Vivek *et al*[83] have developed TZ-conjugated PVP-PLGA nanoparticles for targeted delivery of drugs to HER2-overexpressing breast cancer cells.

Optimization of nanoparticle functionalization has lead to the development of smart conjugation techniques, which allow fine-tuning of the orientation of the targeting biomolecules, in order to maintain and/or further exploit the targeting capability and the therapeutic efficacy of HER2-directed ligands[75,84]. In several cases both TZ and the nanoparticle surface have been modified with heterobifunctional linkers, such as N-succinimidyl-3-(2-pyridyldithio)propionate, succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate and succinimidyl iodoacetate, commercially available and widely used crosslinkers for bioconjugation. They allow to determine the exact number of reactive amines on the nanoparticle surface, thus widely contributing in controlling ligand density onto the surface of the resulting nano-compound[7,84-86]. A classical approach, although not applicable in medicine, is based on the strength of streptavidin-biotin complex. Basically biothinylated TZ reacts with streptavidin-modified nanoparticles, thus generating HER2-targeted nano-compounds[87]. Another smart conjugation strategy consists in taking advantage of spaBC3, a monodomain variant of protein A, a natural peptide linker endowed with high affinity for IgGs. It has been used to bind iron oxide and gold nanoparticles for tight TZ immobilization through the Fc fragment, thus achieving an optimal presentation of the target-directed Fab fragments and keeping full binding capacity of the bound antibody[88]. In several cases the use of a protein biolinker is suited for a controlled site-specific conjugation of HER2-targeted ligands and it also contribute in stabilizing nanoparticle while producing.

Recombinant ligands offer an extremely desirable versatility in terms of nanoparticle conjugation, since their polypeptide sequence can be easily genetically engineered leading to generation of useful functionalities for nanoparticles conjugation. Anti HER2 scFv were modified inserting a His-tag in N-terminal position leading to conjugation of NiNTA functionalized nanoparticles. Otherwise, the mutation of a serine residue with a cysteine within the V_H-V_L linker region or the insertion of a N-terminal serine have been probed to nanoparticle conjugation through disulphide bridges formation nanoparticles or nitrone cycloaddition[78]. These different chemical immobilization strategies of anti-HER2 scFv have been developed and tested, thus leading to multiple scFv specific and uniform orientations on the surface of nanoparticles and demonstrated subsequent effect on the targeting efficiency of the nano-compound. Another recently explored bioconjugation approach exploits the genetic fusion between the scFv module and a small enzyme (*i.e.*, SNAP tag), which works as "capture" unit. Nanoparticles have been functionalized with a suicide inhibitor of the enzyme, allowing covalent, irreversible and specific immobilization of the scFv on the nanoparticle

Table 1 Nanoparticles for breast cancer therapy

Nanoparticle	Delivered active molecule	Activity on HER2 + breast cancer cells or tumors
TZ-polymeric NPs[90]	DOX	nuclear drug delivery and apoptotic effect (*in vitro* study)
TZ-PLGA-PEG NPs[91]	DTX	HER2 specific targeting, cellular internalization and cytotoxic activity (*in vitro* study)
anti-HER2 affibody-PLA-PEG-Mal NPs[70]	Pxtl	HER2 specific targeting, cellular internalization and cytotoxic activity (*in vitro* study)
rhuMAbHER2 (Fab')-PLGA NPs[92]	PE38KDEL	HER2 specific targeting, cellular internalization and cytotoxic activity (*in vitro* study); anti-tumor activity (*in vivo* study)
scFv-PEG-PLA NPs[96]	*siPlk1*	cellular internalization, Plk1 silencing, apoptotic effect (*in vitro* study); *siPlk1* accumulation in tumors and anti-tumor activity (*in vivo* study)
Herceptin-PLA-TPGS+TPGS-COOH NPs[97]	DTX/IOs	cellular internalization and cytotoxic activity (*in vitro* study)
anti HER2-PEG–gold NPs[98]	phthalocyanine	HER2 specific cytotoxic activity (*in vitro* study)

TZ: Trastuzumab; DOX: Doxorubicin; DTX: Docetaxel; PLGA: Poly-D,L-lactic-co-glycolic acid; PEG: Poly ethylene glycol; IOs: Iron oxides; HER2: Human epidermal growth factor receptor 2.

surface. In this case the immobilized molecules are fully active since the bioconjugation reaction takes place in mild conditions without affecting scFv stability[89].

HER2-TARGETED NANOPARTICLES FOR BREAST CANCER THERAPY

In breast cancer therapy, many studies have been devoted to the development of HER2-targeted nanodevices as delivery system for chemotherapics (anthracyclines and taxanes) or other molecules exerting an anti-tumor effect (Table 1). Most of them are mainly focused on the development and characterization of bioengineered NPs for $HER2^+$ breast cancer cells targeting, and have demonstrated their cytotoxic effect *in vitro*. In 2009, Shi and collaborators developed an amphiphilic copolymeric NP, where surface furan groups were used to bind, by a simple diels-alder coupling chemistry, both an anti-HER2 antibody and the chemotherapeutic doxorubicin (DOX)[90]. The DOX-conjugated immuno-NPs were able to efficiently deliver DOX into the cytoplasm, and then into the nucleus of $HER2^+$ breast cancer cells, where DOX exerts its function. This intracellular DOX accumulation was significantly higher than that measured when DOX was delivered by non-functionalized NPs. These results demonstrated, for the first time, the great prospective of a surface-conjugation strategy for the development of nanoformulated DOX, which proved to be more efficient than the conventional encapsulation for the nuclear delivery of this drug. The enhanced HER2-mediated intracellular uptake of DOX also resulted in increased apoptosis of $HER2^+$ breast cancer cells, when compared to non-functionalized DOX-NPs.

In 2011, Koopaei *et al*[91] developed a copolymeric immuno-nanocarrier for the active delivery of docetaxel (DTX) to human breast cancer cells. DTX was encapsulated in PLGA-PEG nanoparticles functionalized with TZ. A fast and over time sustained release of DTX from NPs was first observed *in vitro*, together with a specific interaction of DTX-PLGA-TZ with $HER2^+$breast cancer cells. Cytotoxicity of HER2-targeted DTX-PLGA was compared with that of free DTX and non-specifically targeted nanoformulates. The greatest cytotoxic effect was obtained with the immune NPs as results of their specific interaction with HER2 receptors on cancer cell surface.

Another taxane commonly used in clinical practice, the paclitaxel (Ptxl), has been nanoformulated to be actively delivered to breast cancer cells. An interesting study of Alexis and colleagues[70] addressed the numerous drawbacks of the antibody-based approach for an efficient drug delivery to tumors, mainly related to the large hydrodynamic size of the ligand. Here, it was sponsored the use of an anti-HER2 affibody, which shows several advantages in comparison to the entire monoclonal antibody: (1) smaller size (15 kDa *vs* 150 kDa); (2) considerable distance between the functional end group and the conjugation site; and (3) high *in vitro* and *in vivo* stability. Copolymeric NPs conjugated to the anti-HER2 affibody (NPs-Affb) efficiently bound $HER2^+$ cancer cells and were internalized. The cytotoxic effect of Ptxl encapsulated into targeted NP-Affb was then evaluated in comparison to that of nude NPs, non-targeted NPs (Pxtl), NPs-Affb and free Pxtl. A significant decrease of cells viability was observed both with free Pxtl and non-targeted NPs (Pxtl) after 2 h, but a further significant decrease in cell viability was obtained with NP-Affb (Pxtl).

Despite the conspicuous literature about the *in vitro* therapeutic potential of nanostructured chemotherapics, only few researchers have assessed the efficacy of biofunctionalized nanodevices *in vivo*. In 2009, Gao and collaborators decided to encapsulate the anti-cancer *Pseudomonas* exotoxin A (PE)-based immunotoxin into PLGA nanoparticles[92]. In particular, PE38KDEL, a 38 kDa mutant form of PE, was loaded into PLGA nanoparticles targeting HER2 (PE-NP-HER), where the anti-HER2 portion was represented by a Fab' fragment of a humanized anti-HER2 monoclonal antibody (rhuMAbHER2). Once assessed that the integrity and the potent activity of PE38KDEL were maintained after encapsulation in PLGA particles, *in vitro* interaction of PE-NP-HER with $HER2^+$ breast cancer was compared to that obtained with

HER2-negative cells and the cytotoxic effect on the two cell types was also evaluated. PE-NP-HER were exclusively internalized by HER2$^+$ cells and a strong cytotoxicity occurred specifically in these cells. *In vivo* toxicity studies were performed upon intravenous injection of PE-NP-HER, PE-NP, PE-HER and PE38KDEL in mice. A 3-fold lower LD_{50} (mg/Kg) and no influence on hepatic functionality were observed for PLGA-loaded PE, compared to non-encapsulated toxins. A dose-dependent inhibition of tumor growth was observed in mice injected both with PE-HER and PE-NP-HER, even though a 2-fold higher dose of the PE-HER was necessary to obtain the same effects of the nanoformulated immunotoxin.

A recent nanotechological approach proposes the employment of NPs for the delivery of small interfering RNA (siRNA)[93-95]. In the current year, it has been developed a nanocarrier for the delivery of siRNA targeting the gene encoding polo-like kinase 1 (Plk1)[96]. The si*Plk1* was encapsulated in a PEG-PLA shell functionalized with the anti-HER2 scFv (ScFv$_{Her2}$-NP$_{siPlk1}$), to exert an active targeting of HER2$^+$ breast cancer. ScFv$_{Her2}$-NP$_{siPlk1}$ were efficiently internalized by cancer cells and promoted Plk1 silencing, inducing tumor cell apoptosis. Nanocomplex-mediated accumulation of si*Plk1* in HER2$^+$ breast tumors was also observed *in vivo*, in parallel with a dose-dependent anti-tumor efficacy: ScFv$_{Her2}$-NP$_{siPlk1}$ significantly increased the inhibition of tumor growth, when compared to non-targeted NP$_{siPlk1}$, and allowed to reduce the active dose of injected siRNA.

Nanotechnology has found a great application also in thermal therapy where gold or magnetic NPs have proved to be very useful in triggering ablation of cancer cells. In 2012, Mi and colleagues identified a multimodal strategy for breast cancer treatment, where the chemotherapy DTX was formulated with a PLA-tocopheryl-PEG-succinate and carboxyl group-terminated TPGS (TPGS-COOH) copolymer, containing iron oxides (IOs) for hyperthermia therapy[97]. TPGS-COOH molecules were conjugated with TZ for HER2 targeting. The *in vitro* therapeutic efficiency of these multimodal NPs was tested on HER2$^+$ breast cancer cells. A stronger cytotoxic activity was observed on cells incubated with TZ-IO-NPs under the exposure to an alternating current field, or with TZ-DTX-NPs, in comparison to the corresponding non-targeted NPs.

In photodynamic therapy of cancer, irradiation with visible and/or near-infrared light induces the activation of photosensitiser drugs, able to generate reactive oxygen species and trigger apoptotic or necrotic response of target cells, thus leading to cell death. Stuchinskaya and collaborators developed a PEG-gold NP conjugated to the phthalocyanine and functionalized with an anti-HER2 antibody on PEG chains. Upon red laser irradiation a strong cytotoxic effect of these NPs was observed on HER2$^+$ cells and not on HER2-negative cells[98].

CONCLUSION

About 30% of breast cancers are associated with HER2 receptor overexpression, which strongly correlates with a poor prognosis. Indeed, HER2 regulates several highly redundant pathways involved in cellular survival and proliferation, which are deregulated in HER2$^+$ cancer. At present, conventional therapy with biological drugs, such as Trastuzumab, Pertuzumab or Lapatinib, has provided satisfactory results, although still shows some limitations in achieving a proper treatment. In this context, the development of HER2-targeted nanoparticles exploited as drug delivery systems may overcome these drawbacks. Specific HER2 ligands have been conjugated on the surface of nanoparticles, thus providing a specific recognition of HER2$^+$ cancer cells. Their specific target recognition is combined with the nanoparticles capability to act as a drug reservoir for a selective delivery to tumor sites. In addition, therapeutic efficiency can be reached also by combining targeting molecules with nanoparticle useful for photothermal ablation. In this review we have extensively analysed HER2$^+$ breast cancer features and related targeted therapy, particularly underlining the precious contribution that nanomedicine may provide. Moreover, we have described various molecules used to target HER2 and related nano-conjugation strategies, and provided a detailed overview of preclinical studies performed with HER2-targeted nanoparticles developed for cancer therapy. Further investigations and synergic collaborations between nanotechnologists and physicians will hopefully allow to achieve the introduction of these nano-drugs in clinical.

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P- Reviewers: Langdon S, Zhang H **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i4.86

World J Pharmacol 2014 December 9; 3(4): 86-96
ISSN 2220-3192 (online)
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REVIEW

Telomerase activity: An attractive target for cancer therapeutics

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Received: July 28, 2014 Revised: October 1, 2014
Accepted: October 28, 2014
Published online: December 9, 2014

Abstract

Telomeres are non-coding tandem repeats of 1000-2000 TTAGGG nucleotide DNA sequences on the 3′ termini of human chromosomes where they serve as protective "caps" from degradation and loss of genes. The "cap" at the end of chromosome required to protect its integrity is a 150-200 nucleotide-long single stranded G-rich 3′ overhang that forms two higher order structures, a T-loop with Sheltering complex, or a G-quadruplex complex. Telomerase is a human ribonucleoprotein reverse transcriptase that continually added single stranded TTAGGG DNA sequences onto the single strand 3′ of telomere in the 5′ to 3′ direction. Telomerase activity is detected in male germ line cells, proliferative cells of renewal tissues, some adult pluripotent stem cells, embryonic cells, but in most somatic cells is not detected. Re-expression or up-regulation of telomerase in tumours cells is considered as a critical step in cell tumorigenesis and telomerase is widely considered as a tumour marker and a target for anticancer drugs. Different approaches have been used in anticancer therapeutics targeting telomerase. Telomerase inhibitors can block directly Human TElomerase Reverse Transcriptase (hTERT) or Human TElomerase RNA telomerase subunits activity, or G-quadruplex and Sheltering complex components, shortening telomeres and inhibiting cell proliferation. Telomerase can become an immune target and GV1001, Vx-001, I540 are the most widespread vaccines used with encouraging results. Another method is to use hTERT promoter to drive suicide gene expression or to control a lytic virus replication. Recently telomerase activity was used to activate pro-drugs such as Acycloguanosyl 5′-thymidyltriphosphate, a synthetic ACV-derived molecule when it is activated by telomerase it does not require any virus or host active immune response to induce suicide gene therapy. Advantage of all these therapies is that target only neoplastic cells without any effects in normal cells, avoiding toxicity and adverse effects of the current chemotherapy. However, as not all the approaches are equally efficient, further studies will be necessary.

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Key words: Human telomerase reverse transcriptase; Immunotherapy; Suicide gene therapy; Acycloguanosyl 5′-thymidyltriphosphate; Telomerase inhibition

Core tip: One of the hallmark of cancer is the replicative immortality of tumor cells guaranteed by telomerase activity that counteracts progressive telomere shortening during cellular replication: this makes telomerase a tumor marker and a target for anticancer drugs. In this review we summarize and update the most recent innovative studies and results on the different strategies that consider telomerase as a target for cancer therapy. In particular, we try to point out the advantages and the potentialities of some innovative approaches, compared to other, equally promising, but that need further investigations.

Picariello L, Grappone C, Polvani S, Galli A. Telomerase activity: An attractive target for cancer therapeutics. *World J Pharmacol* 2014; 3(4): 86-96 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/86.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.86

TELOMERES, TELOMERASE AND CANCER

Telomeres are non-coding tandem repeats of 1000-2000 TTAGGG nucleotide DNA sequences on the 3' termini of human chromosomes[1-3] where they serve as protective "caps" from degradation and loss of genes. In this way cells can discriminate between double strand breaks and natural chromosome ends[4,5]. In human somatic cells, telomeres become critically short after successive cell divisions (number of divisions depending on the length of their telomeres), cells stop division and replicative senescence occurs[6]. As a consequence, telomeres can reach a critical length that is no longer suitable to assemble into T-loop: this triggers a localized DNA damage response and p53-mediated cell cycle arrest[7-9]. However, cells that have inactivated the p53-pathway cell cycle checkpoint, are able to continue dividing, bypassing senescence, loosing telomeric sequence with each division[9,10] and reach a "crisis" stage[11,12]. In this way telomeres become so short that cannot protect chromosome ends, so that they fuse together to produce a dicentric chromosome, inducing an increase aneuploidy and genomic instability that finally will lead to p53-independent apoptosis[13,14]. Bypassing crisis rarely occurs in human cells (1 in 10^{-6} in epithelial cells and 1 in 10^{-7} in human fibroblasts) and this leads to cell immortality and cancer cell progression, characterized by capability to continue to proliferate without limits.

The "cap" at the end of chromosome required to protect its integrity is a 150-200 nucleotide-long single stranded G-rich 3' overhang that forms two higher order structures, a T-loop with Sheltering complex, or a G-quadruplex complex. Sheltering complex is represented by six proteins (TRF1 and TRF2, POT1, TPP1, TIN2, RAP1) responsible for maintaining the T-loop structure. G-quadruplex is stabilized with BRACO19, RHS4 and telomestatin proteins. Sheltering complex with T-loop, G-quadruplex and its stabilizers can lock the telomeric 3' overhang and block telomerase from accessing telomeres[15] (Figure 1).

Telomerase is a human ribonucleoprotein reverse transcriptase that continually adds single stranded TTAGGG DNA sequences onto the single strand 3' of telomere in the 5' to 3' direction and translocates to the new terminus[16,17]. This cycle goes on as far as telomerase dissociates from telomere[18,19]. Telomerase is composed of two main subunits: the catalytic protein Human TElomerase Reverse Transcriptase (hTERT) and the ribonucleoprotein template Human TElomerase RNA (hTER)[15-17]. In particular hTER consists of 451 nucleotides of which only nucleotides 46 through 56 (5'-CUAACCCUAAC-3') represent a template for new telomeric added DNA sequences (Figure 2).

Many proteins associated to the core components hTERT and hTER are required and are necessary for stability regulation, recruitment and activity of the holoenzime[20]. hTER is expressed in all human cells, as well as normal and tumour cells, so telomerase activity is limited by of hTERT expression, whereas is present[21,22].

Telomerase activity is detected in proliferative cells of renewal tissues, in some adult pluripotent stem cells, male germ line cells, embryonic cells, but not in most somatic cells[23]. However, telomerase activity is found in almost all human cancer cell lines and in about 85%-90% of primary tumours[24]. In fact, one of the hallmark of cancer is the replicative immortality and so the ability to endlessly growth is synonymous of telomerase reverse transcriptase reactivation. Up-regulation or re-expression of telomerase in tumour cells is considered as a critical step in cell tumorigenesis and telomerase is widely considered as a tumour marker and a target for anticancer drugs. Progressive telomere shortening during cellular replication is counteracted by telomerase activity[1,25].

One of the advantages of anticancer therapies targeting telomerase is that the telomeres of highly proliferating cancer cells are shorter (5 kb) compared to that in normal somatic cells and stem cells (10-20 kb) that have not yet reached critical lengths as a result of aging[26,27]. The difference in telomerase activity and telomere lengths in normal and cancer cells leads to a more selective therapeutics cytotoxicity on cancer cells and a minimal impact on normal cells with a limitation of collateral effects that can be evaluated[28].

TELOMERASE INHIBITION AS A THERAPY

Telomerase inhibitors can be employed as a selective anticancer therapy, disrupting telomerase-positive cancer cells replicative capacity[29].

To target telomerase in cancer treatment we can find two types of approaches: the first one is blocking directly telomerase hTERT or hTER subunits activity, with consequent shortening of telomeres leading to the arrest of cell replication. The second approach is to block telomerase by an indirect method, targeting G-quadruplex stabilizers or Sheltering complex components with the consequence of preventing telomerase interaction with telomeres or binding of proteins associated with telomerase; this leads to telomere uncapping and cell apoptosis[30].

Antisense olignucleotides-targeting hTER

One of the most recent strategy for a direct telomerase enzymatic inhibition, is the use of antisense oligonucleotides inhibitors. These molecules are complementary to the 11-base template region of telomerase (hTER) and can be used to block the translation of sense RNA. In order to hybridize the hTER-template the antisense oligonucleotides must get to the hTER region without being degraded by nucleases. For this reason the challenge for this kind of drugs is both access and stability. To better get its target, antisense oligonucleotides have been modified and significantly improved in the past years.

Currently GRN163L (Imetelstat®) is one of the first generation most promising telomerase inhibitor targeting hTER used in cancer treatment; it is a lipid modified

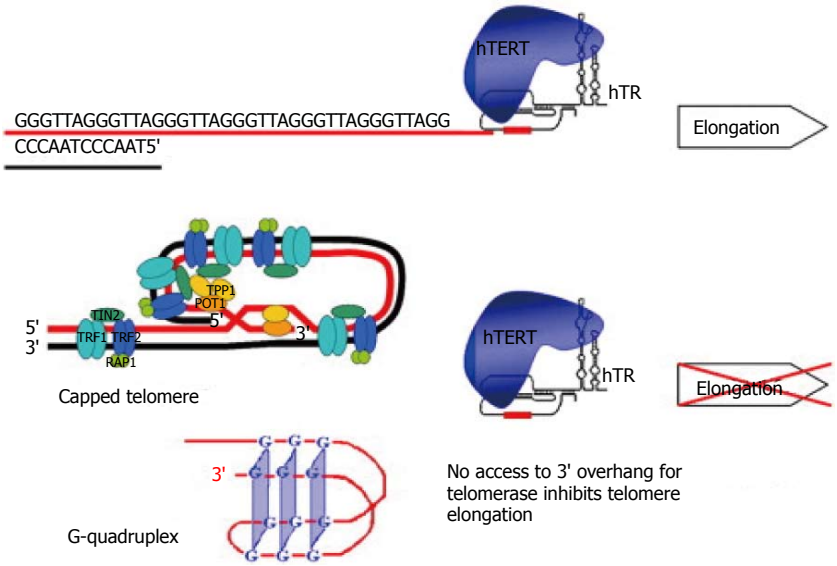

Figure 1 Impact of open or capped telomere structure on the telomerase activity. (Reproduced with permission from Philippi C, Loretz B, Schaefer UF, Lehr CM. J Control Release 2010; 146: 228-240. Copyright Clearance Center, Inc.). hTERT: Human TElomerase Reverse Transcriptase.

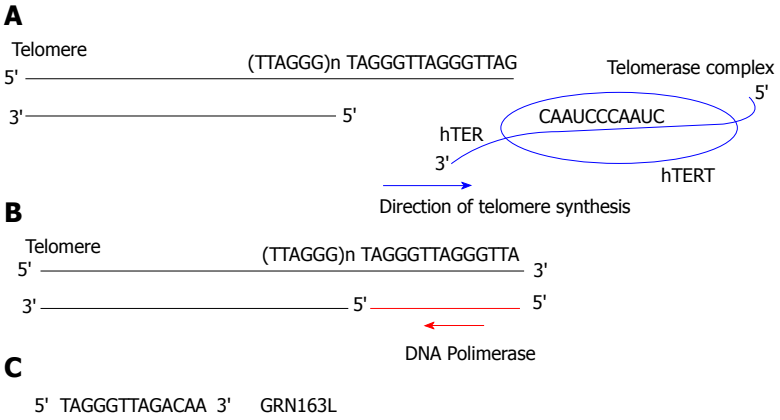

Figure 2 Telomeres an telomerase complex. (TTAGG)n sequences form a 3'-overhang on the 3' end of chromosome. Telomerase is composed by hTERT and hTER subunits; hTER is the RNA template for DNA synthesis to add new telomeric TTAGG sequences on 3'-overhang; B: DNA polymerase completes the lagging strand; C: GRN163L sequence complementary. hTERT: Human TElomerase Reverse Transcriptase; hTER: Human TElomerase RNA.

version of GRN163, a 13-mer oligonucleotide N3'-P5'-thio-phosphoramidate, that required a lipid carrier molecule and a lipid-base transfection agent to adequately enter tissue and cellular membranes[31,32]. On the contrary, GRN163L with a covalently bound lipophylic palmitoyl (C16) group linked to its 5'-thio-phosphate[33] is lipid soluble, and shows an higher drug availability and bio-distribution, without any lipid carrier supply[32]. GRN163L in part overlaps the hTER template region by binding with high affinity and specificity at its active site, acting as competitive telomerase inhibitor and causing a total enzyme inhibition[32,33] (Figure 2).

The GRN163L inhibitory effect on telomerase activity has been evaluated in different cancer cell lines[34] and its effects were evident as well as "*in vitro*" and "*in vivo*" models; in fact, long term treatment with GRN163L reduced cell viability in cancer cells derived from bladder[33] glioblastoma[35], multiple myeloma[36], Barrett's adenocarcinoma[37], as well as breast[38,39], lung[40], liver cancer[41] and prostate[42].

Recently, the effects of GRN163L have been tested on a panel of ten pancreatic cancer cell lines, and the results indicated that the inhibitory effect of the drug was maintained also after its removal[43]: in fact, only three weeks after the GRN163L removal, a telomerase recovery was observed, but the enzyme was less processive. This suggests that to maintain continuous telomerase inhibition and to reduce side effects risk after a pharmacological treatment of a patient with GRN163L, a maintenance dose given once every other week might be sufficient. However, the reversible effects of Imetelstat have been also previously demonstrated on rat mesenchymal stem cells[44].

A combined treatment where homologous recombination and telomerase inhibition are associated, causes a significant increase in telomeres attrition, relative to each treatment alone, leading to senescence and apoptosis in Barrett's adenocarcinoma[45].

Tamakawa *et al*[46] showed that the DNA damage induced in S/G_2 phase of the cell cycle, by genotoxic stimulus was potentiated by the telomerase inhibition induced by GRN163L in breast and colorectal cancer cells[46].

In previous studies, synergies between GRN163L and various anticancer treatments such as microtubule inhibition, inhibition of oncogenic signals and ionizing radiation, were considered to be dependent on longer-term changes associated with chromatin status[47] and telomere length[48].

Telomere shortening induced by telomerase inhibitors would affect the self-renewal properties of cancer stem cells (CSCs), normally not responding to standard chemotherapy, but capable of inducing initiation and currency

in different hematologic and solid tumours[49,50].

Many studies showed that CSCs can represent the Imetelstat target in different cancers[35,42,51], and that a telomere shortening-independent as well as dependent Imetelstat mechanism of action on CSCs subpopulation, can be suggested[52,53]. The effect of Imetelstat was evaluated on both the bulk cancer cells and putative CSCs of breast and pancreatic cancer cell lines. The *in vitro* treatment inhibited telomerase activity, cell growth, self renewal in bulk cancer cells and putative CSCs, with a consequent reduced cancer engraftment in nude mice[52]; in particular an increased sensitivity of CSCs to Imetelstat did not correlate with differences between telomerase activity expression levels or telomere length of CSCs and bulk tumour cells suggesting a telomere shortening- independent mechanism of action for the Imetelstat effects on CSCs subpopulation.

All these studies support the hypothesis that conventional therapies often fail to target CSCs while the use of telomerase inhibitor could have the potential role for more durable clinical response in many tumors, reducing relapse recurrence.

Imetelstat is currently in phase Ⅱ clinical development for breast cancer, non-small cell lung carcinoma, multiple myeloma, and other tumor types[30].

Inhibitors targeting hTERT: BIBR1532

BIBR1532 [2-(E-3-naphtalen-2-yl-but-2-enylylamino]-benzoic acid] is actually a promising hTERT inhibitor among the few TERT inhibitors developed. BIBR1532 is a small synthetic non-nucleic compound that linking hTERT in its active site, inhibits telomerase in a non-competitive manner: BIBR1532 does not cause chain termination events but rather leads to an overall reduction in the number of added TTAGGG repeats[54]; in particular the drug could act translocating the enzyme-DNA-substrate complex, or favouring the DNA substrate disjunction from the enzyme during the copy of the template[55].

In the last few years, different studies showed that BIBR1532 treatment induced telomerase activity reduction with consequent cell growth arrest in different human cancer cell lines[54,56-60], without affecting normal stem cells[61]. In addition telomeres targeting might represent a valid strategy for the re-sensitization of chemoresistant chondrosarcomas[56], and a rapid induction of a high level telomere dysfunction appears to be a crucial parameter for the development of future telomerase-based therapeutic[62]. However, although some human squamous cell carcinoma cell lines are resistant to telomerase inhibition[63] some works suggest that a valid strategy for the treatment of both drug-resistant and drug-sensitive cancers may be pharmacological telomerase inhibition in combination therapy[64-66].

IMMUNOTHERAPY FOR TELOMERASE EXPRESSING CANCER

As previously described, nearly all cancer cells over-express functional active telomerase, and hTERT-specific epitopes are expressed on tumour cells, but not on normal cells. In this way, telomerase become an immune target, and can be eradicated by the stimulation of the immune system with specific vaccines. Telomerase-target immunotherapy sensitizes immune cells against tumor cells expressing hTERT peptides as surface antigens[67]. The consequent expansion of telomerase-specific CD8+ cytotoxic T lymphocytes is directed to target and kill telomerase positive cancer cells[68,69].

Recently, multiple peptides are known to induce hTERT-specific immune responses[68] and several vaccine strategies are being developed and used: among these GV1001, Vx-001, I540, are the most widespread therapeutic approaches. As almost all human tumor-associated antigens are self-proteins, their specific T cells are often tolerated: this is the major problem of cancer immunotherapy. For this reason, overcoming tumor-specific self-tolerance is a principal goal in cancer immunotherapy.

Self-tolerance is commonly directed against "dominant" (high affinity for HLA) but not against "cryptic" (low affinity for HLA) peptides[70,71], so the simplest way to circumvent tolerance is to use these cryptic peptides[72] as for example Vx-001 (9-mer cryptic TERT 572 peptide) that was developed as tumour-associated antigen of hTERT to induce cytotoxic T lymphocyte responses[73,74].

Immunological response associated with extended survival were evident in patients with advanced non-small-cell lung cancer treated with Vx-001 vaccine (TERT572Y peptide)[74]; in patients with various types of chemo-resistant advanced solid tumours (stages Ⅲ and Ⅳ) the vaccination with Vx-001 stimulates TERT572-specific reactive T cells in a great number of patients independently of the disease stage or clinical status before vaccination and a late immune response correlated with longer survival was induced[73,75].

State of the art of clinical trials using anti-telomerase cancer immunotherapy is encouraging. In fact, vaccines are tested in breast, lung, melanoma, prostate, and pancreatic cancer[76-82] and these trials have widely induced a specific immune response against hTERT positive cancer cells. Encouraging results have been also obtained in patients with advanced melanoma, where immunity to hTERT has been safely generated[83]. The combination of cancer vaccination with chemotherapy showed that temozolomide and GV1001 induced immune and clinical response in 78% of stage Ⅳ melanoma patients, that developed long-term T-cell memory and survived more than those rapidly losing their responses[84]. Vaccination with GV1001 was well tolerated and immunized the great part of non-small cell lung cancer patients establishing durable T-cell memory[85]. However, GV1001 vaccination was not effective in cutaneous T cell lymphoma patients, raising concerns about also its safety[86]. The survival data indicated that patients with non-resectable pancreatic cancer treated with GV1001 showed that immune response correlated with an extended survival, suggesting that the vaccine could be the new goal for pancreatic cancer patients treatment and encouraging further clinical

studies[82]. On the contrary, in patients with advanced and metastatic pancreatic cancer the use of GV1001 telomerase vaccination in combination with chemotherapy, induced a weak and transient immune response and did not improve overall survival[80,81]. Likewise, a low dose cyclophosphamide treatment in combination with GV1001 vaccination in patients with advanced hepatocellular carcinoma did not show antitumor efficacy[87]. Further studies and new strategies are needed to analyze and to enhance the immune response effect of telomerase vaccination during chemotherapy, in patients with both pancreatic and hepatocellular cancers.

Vaccination with autologous dendritic cells transfected with hTERT mRNA (GRNVAC1) represents another anticancer approach that induced immunological response in human. Immunotherapy targeting the hTERT subunit of telomerase has been demonstrated to induce an important immune responses in cancer patients after vaccination with single hTERT peptides, while vaccination with dendritic cells transfected with hTERT mRNA has a key role in inducing efficient immune responses to multiple hTERT epitopes. In this way this kind of therapy can be an attractive approach to more efficient immunotherapy[88-90].

TELOMERASE-EXPRESSING CELLS AS TARGET OF ONCOLYTIC VIRUSES

Recently has been shown that the use of hTERT promoter to drive the expression of a suicide gene and/or control the replication of a lytic virus, can be a successful approach to target cancer cells.

To drive the expression of a suicide gene, the expression of a pro-apoptotic protein, like TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) or pro-drug-activating enzyme[91-96] is controlled by the hTERT promoter, generally active in cancer cells expressing telomerase. These cells are injected with viruses carrying the suicide gene and then killed by a toxin derived from the administration of a pro-drug activated by the pro-drug-activating enzyme.

A second clinical approach, is to use the hTERT promoter to control the replication of a lytic virus. Oncolytic effects on tumors can be mediated by oncolytic viruses, tumor selective viruses genetically modified and engineered to replicate in and kill only cancer cells. For this purpose, the *E1* gene expresses viral proteins E1A and E1B necessary for adenovirus replication, but the modified virus can replicate only in cells which express telomerase if gene itself is redesigned to be controlled by the hTERT promoter[97-100]. One such virus is telomelysin (OBP-301) that in pre-clinical studies targets selectively only telomerase-expressing cells.

The modified viruses induce cytolysis in several kinds of human cancer cell lines in which can replicate; when human lung, prostate or liver cancer cells were used in xenotransplantation models, intratumoral injection of the virus reduced tumor growth and improved mice survival[97-100].

The potential role of oncolytic virotherapy has recently been demonstrated to be a promising strategy in the management of human gastrointestinal cancer[101]. Studies about OBP-301 have been shown that it mediates the effective *in vivo* purging of metastatic tumor cells from regional lymph nodes and moreover it co-operates to optimize treatment of human gastrointestinal malignancies[102]. Moreover, telomerase-specific oncolytic viruses is a potential treatment of human squamous cell carcinoma of head and neck[103], while in pancreatic cancer the combination therapy with gemcitabine has been tried, exhibiting enhanced cytotoxic effects both "*in vitro*" and "*in vivo*"[104]. In addition, preclinical study showed that OBP-301 can be used for treatment of human hepatocellular carcinoma and that its tumor-killing activity persists after multiple injections[105].

Data regarding combination therapy with OBP-301 and chemotherapeutic agents are preliminary but encouraging[106]. In particular Boozari *et al*[107] showed that the combination of intratumoural virotherapy with an antitumoural vaccine, could represent a promising immunotherapeutic strategy against hepatocellular carcinoma and metastasis.

TELOMERASE CANONICAL ACTIVITY AS A THERAPY

Recent studies revealed that telomerase canonical activity can be exploited for therapeutic purpose.

The evidence that telomerase is expressed in almost all tumor cells, preventing telomeres shortening by continually adding single stranded TTAGGG DNA sequences, prompted us to develop a thymidine analogue pro-drug, acycloguanosyl 5'-thymidyltriphosphate (ACV-TP-T) (Figure 3). This molecule is a synthetic ACV modification that is metabolized by telomerase, and this reaction releases the active form of acyclovir able to reduce pancreatic and hepatocellular carcinoma cells growth as well as "*in vitro*" and "*in vivo*"[108,109].

ACV is a nucleoside analogue acting as a DNA chain terminator that could be used in the suicide gene therapy[110]. ACV or the ACV analogue ganciclovir[110,111] when used as antiviral agent needs a first phosphorylation to ACV monophosphate by herpes virus thymidine kinase (TK) carried by wild-type herpes virus or, in the suicide gene therapy, by engineered adenovirus (Figure 3), then cellular kinases perform the two remaining phosphorylation to obtain the ACV triphosphate. This active metabolite is incorporated into DNA during its replication causing DNA chain termination.

On the contrary, ACV-TP-T, may be metabolized by telomerase that incorporates thymidine in replicating telomeres and releases ACV diphosphate. This process skips the viral TK phosphorylation, allowing the cellular kinases to go on with further phosphorylation to obtain the active drug[108,109]. The results showed that after activation of ACV-TP-T by telomerase, cell proliferation is significantly

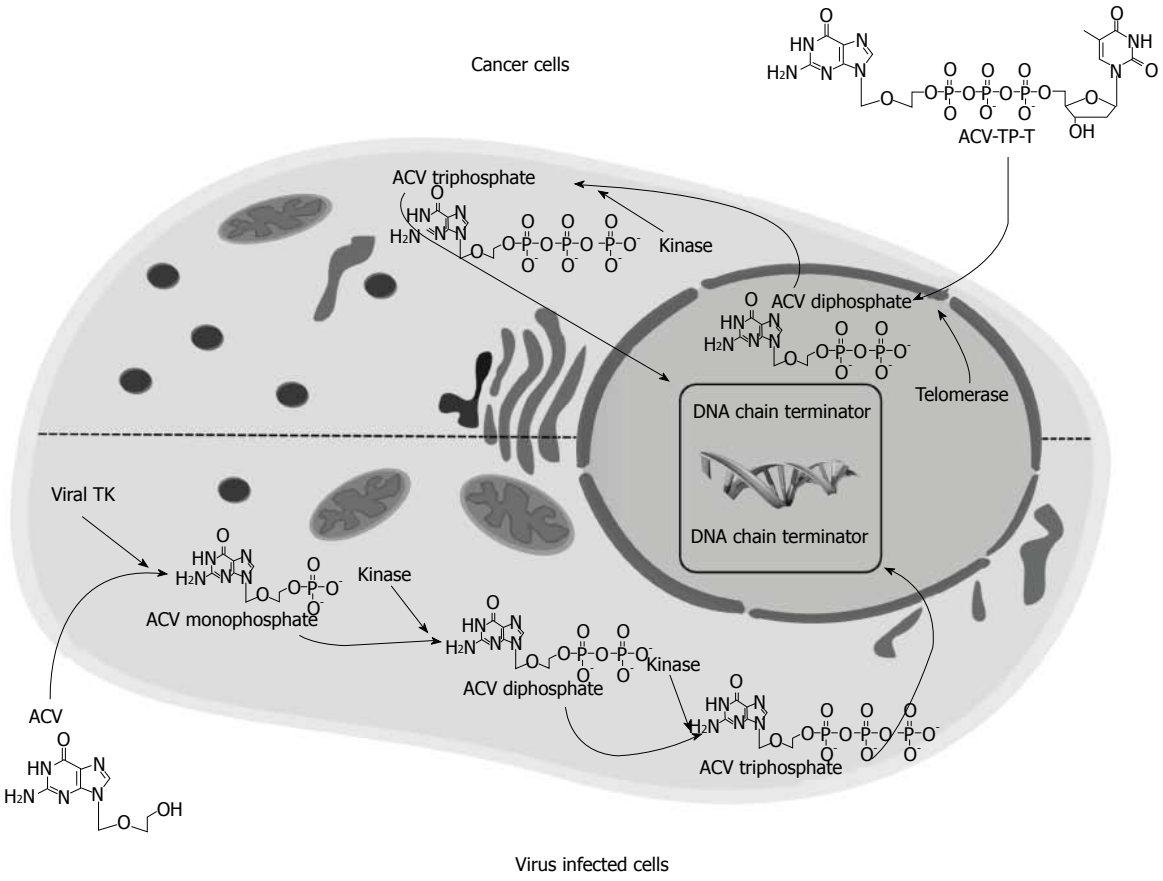

Figure 3 Structure and schematic mode of action of Acycloguanosyl 5'-thymidyltriphosphate in comparison with ACV. For activation, ACV requires to be phosphorylated to ACV monophosphate by viral TK carried either by wildtype herpes virus or, in the suicide gene therapy, engineered adenovirus. ACV monophosphate is then further phosphorylated by cellular kinases to the triphosphated active form. Conversely, ACV-TP-T is substrate of telomerase that incorporates the thymidine in the replicating telomeres and directly release ACV diphosphate skipping the viral TK phosphorylation step. (Reproduced with permission from Ref [108]. Copyright 2011 AGA Institute). ACV-TP-T: Acycloguanosyl 5'-thymidyltriphosphate; TK: Thymidine kinase.

reduced and apoptosis is increased in different human pancreatic adenocarcinoma cell lines. High and low telomerase activity is related with low and high IC_{50} of the drug, respectively. On the other hand, the cytosine-containing pro-drug ACV-TP-dC, which is not a telomerase substrate, is not able to reduce pancreatic cancer cell proliferation. Moreover, ACV-TP-T administration increases apoptosis, reduces growth, proliferation and vascularization of pancreatic xenograft tumors in mice[108].

Analogue results were obtained in human and murine hepatocellular carcinoma cell lines and in transgenic and orthotopic murine models of hepatic cancers[109]. Furthermore, in orthotopic syngenic mice, ACV-TP-T has been used alone or in combination with the approved standard of care, Sorafenib, a multikinase inhibitor. Combination therapy showed a synergistic effect between Sorafenib and ACV-TP-T.

Advantages of this strategy are evident. Despite recent improvements in suicide gene therapy, the application of adenovirus-mediated therapy is limited by many factors: the low and transient expression levels of the transgene[110,112,113], the induction of immune response in the host[108], and a late carcinogenesis[112]. In addition ethical concerns regarding the use of virus in patients[112,113] could be a limitation.

The use of telomerase promoter[114] and the introduction of conditionally replication-competent adenovirus[115] only partially overcome the above mentioned disadvantages. Moreover, the immunotherapy based on vaccination for telomerase[84] relies on the induction of an active immune response that often is deregulated in the oncology treated patients[116].

In this contest, the use of ACV-TP-T represents a new therapeutic strategy that exploits the enzymatic activity of telomerase. This approach is efficient only in neoplastic cells without any effects in normal cells, it avoids the toxicity and the adverse effects of the current chemotherapy, and finally, it does not require the use of any viruses or an active immune response of the host.

As a paradox in this contest telomerase switches from being a target of anticancer therapy, to an integral part of the therapy. Preliminary evidences suggest the possible use of ACV-TP-T molecule for the treatment of other tumors characterized by high telomerase expression and activity such as ovarian and adrenocortical cancers.

NON CANONICAL EFFECTS OF TELOMERASE

Telomerase activation may have both telomere-dependent

and telomere-independent implications for cancer progression: in particular, telomerase reverse transcriptase may exert some biological functions independently of its telomere maintenance enzymatic activity.

Different studies support a role of telomerase in some telomere-independent activities in cancer progression; nevertheless, apart from its role in telomere maintenance, the molecular mechanism by which telomerase promotes cancer is still not fully understood. Zhou *et al*[117] showed that hTER regulated vascular endothelial growth factor (VEGF) expression at the transcriptional level, independently of telomerase activity[117]; previous studies reported that VEGF induced hTERT expression and activity in normal[118] and cancer cells[119]. All these results suggested a positive feedback regulation that could contribute to a mutual and collaborative function of VEGF and telomerase in cancer progression.

Wu *et al*[120] in a recent review focused on various signaling pathways and genes involved in the feedback regulation of TERT. The expression of numerous genes involved in different cellular processes, as well as cell cycle and cellular signaling, could be regulated by TERT, indicating that telomerase is both an effector and a regulator in carcinoma. However, the mechanisms underlying the interaction between TERT and its target genes are still not completely understood.

Ghosh *et al*[121] suggested a functional interplay between TERT and nuclear factor (NF-κB) signaling, further reinforced by the observation that telomerase over expression resulted in enhanced expression of NF-κB target genes, whereas telomerase null mice were refractory to NF-κB activation; in addition, it seems that also hTER could regulate the expression of some NF-κB target genes. The function of hTER in gene expression regulation is not clear, in fact, hTERT can form complexes with or without hTER[122].

hTERT could be involved also in a negative feedback loop system with pRb/E2F pathway in cancer, as well as in a positive feedback loop with Wnt/β-catenin signalling, or in multiple interactions with phosphoinositide 3 kinase/Akt pathway[120]. In addition, Liu *et al*[123] demonstrated a potential role of hTERT in epithelial mesenchymal transition.

Although the mechanisms underlying the interaction between TERT and its target genes are still not completely understood, all the above observations, strengthen the idea that telomerase non-telomeric functions could be used as a new therapeutic target for cancer.

CONCLUSION

Although recent and ongoing results support an important role for telomerase targeting therapeutics in cancer treatment, additional preclinical and clinical trials are necessary to improve some of these strategies.

In fact, if difficulties with dendritic cells derivation will be easily overcome[124], vaccination with dendritic cells transfected with hTERT mRNA could potentially become an attractive approach to a more potent immunotherapy. In addition, further studies are necessary to enhance the effects of telomerase vaccination in combination with intratumoral virotherapy and with standard chemotherapeutic agents.

On the contrary, beside more promising approach offered by GRN163L that seems to target also CSC, BIBR1532 could be preferred therapy if used also in combination with standard chemotherapy for the treatment of drug-resistant cancers.

Finally, ACV-TP-T use is very promising and deserves further studies. In fact, preclinical evidences showed that this new pro-drug may be considered for treatment of hepatocellular and pancreatic carcinoma, as well as of other tumors characterized by high telomerase expression and activity.

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P- Reviewer: Majumdar APN, Paraskevas KI **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5497/wjp.v3.i4.97

World J Pharmacol 2014 December 9; 3(4): 97-109
ISSN 2220-3192 (online)
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REVIEW

Patents on antivirulence therapies

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Supported by Instituto de Salud Carlos III FEDER, Spanish Network for the Research in Infectious Diseases, No. REIPI RD12/0015; by the Spanish Ministry of Health and FEDER funding, No. FIS PI10/00056-PI13/02390 (to Tomás M) and PI12/00552 (to Bou G); and by the Miguel Servet Programme (C.H.U.A. Coruña and ISCIII) (to Tomás M)
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Received: June 28, 2014 Revised: October 30, 2014
Accepted: November 7, 2014
Published online: December 9, 2014

Abstract

Antivirulence therapy inhibits bacterial virulence factors, thus preventing the development of infection without affecting bacterial growth. The development of new antibiotics is complicated by the increasing incidence of antibiotic resistance in pathogenic bacteria. Antivirulence therapy is a promising alternative to traditional antibiotic therapy for the treatment of infectious disease, either alone or in combination with antibiotic treatment. In this review, we consider patents concerning inhibition of several bacterial virulence factors: adhesion/colonization, secretion systems, cellular signalling systems and antimicrobial resistance mechanisms. Finally, we emphasize the importance of analyzing new targets and/or molecules in this field and of considering possible resistance mechanisms.

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Key words: Patents; Quorum sensing; Adhesion; Bacterial secretory systems; Resistance

Core tip: Antimicrobial resistance in nosocomial pathogens has increased dramatically in recent years. The development of new molecules, therapies and/or new combinations for the eradication of these pathogens is therefore imperative. A new line of research in this area is called "Antivirulence Therapy".

López M, Barbosa B, Gato E, Bou G, Tomás M. Patents on antivirulence therapies. *World J Pharmacol* 2014; 3(4): 97-109 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/97.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.97

INTRODUCTION

Microbial virulence is the ability of a microbe to cause disease. Antivirulence therapies are constituted on inhibition of bacterial virulence and do not influence bacterial growth. Bacteria appreciate their environment and, once in the host they respond by starting a plan determined for the activation of virulence factors. Hence, antivirulence strategies have the ability to interfere in the recognition of the host signals that alarm the bacteria localized in the place of infection and/or that activate specific virulence factors implicated to development of the infection. If the development of virulence factors is prevented, the bacteria will be less able to colonize. Moreover, this tactic will not directly kill bacteria, so initially the evolutionary pressure for the development of resistant strains would be lower than with conventional antibiotics[1]. Inhibition of the following systems enables interruption of the process of bacterial infection: toxin production, adhesion and colonization, bacterial secretory systems, cell-to-cell signalling pathways, and antibiotic resistance mechanisms, such as efflux pumps (multidrug resistance) (Figure 1)[2]. In this review, we provide details of patents concerning the inhibition of each of these mechanisms, except for toxin production, which is specific to certain pathogens such as *Bacillus anthracis* (which causes anthrax) and *Clostridium spp.* (which causes gangrene)[1].

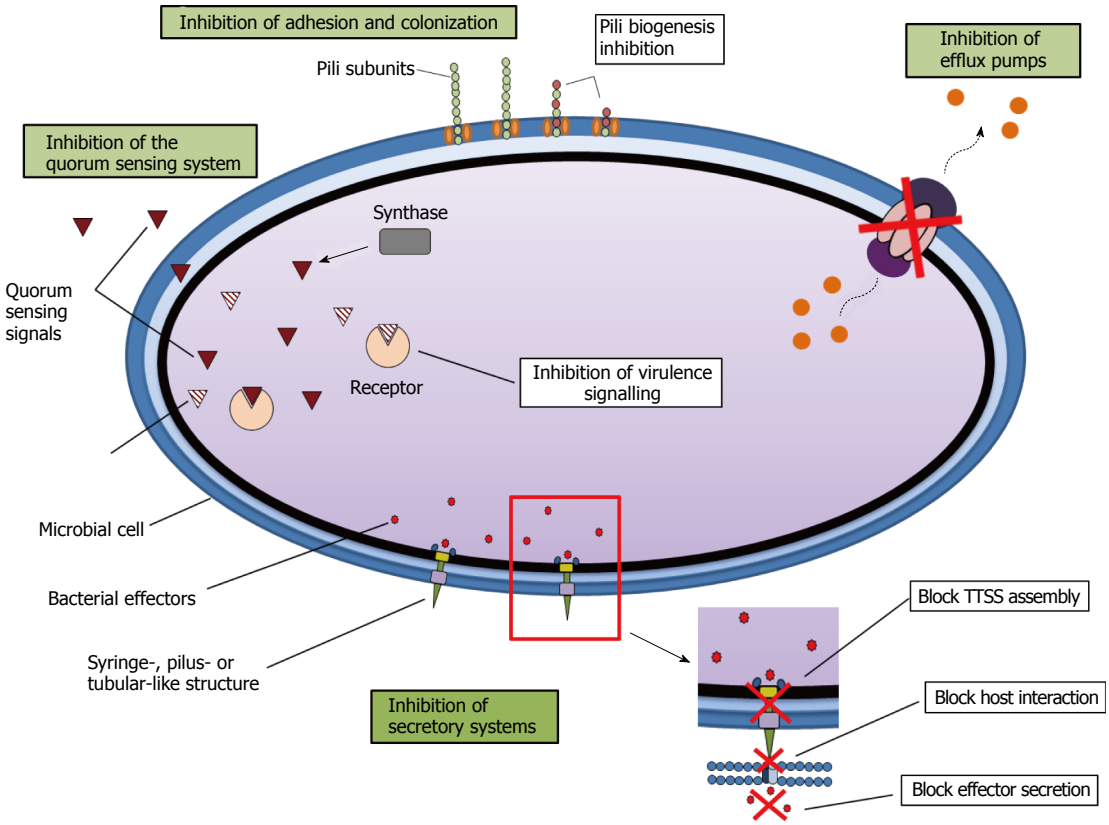

Figure 1 Anti-virulence strategies to combat bacteria-mediated disease (quorum quenchers). TTSS: Type Ⅲ secretion system.

ADHESION AND COLONIZATION MECHANISMS

Microorganisms adhere to host cells in order to colonize the host and begin infection. The majority of the bacteria own a determined host interval and will only infect hosts that express specific receptors for bacterial adhesion traits on their cell surface. Besides, once inside the host, bacteria will only infect the cells (tissue) that have the adequate receptor. Attachment of bacteria to a host cell is a complicated process managed by adhesin on the bacteria and the receptor on the cell. However, adherence is frequently only the first step in the infection course, that besides implicates internalization, deeper tissue penetration and likely systemic spread. Bacteria have different kinds of elements for adhere to the host surface, including- but not only-pili, fimbriae and in some cases flagella[3]. Adhesion can be inhibited by the following strategies: (1) prevention of adhesion complex assembly [as in the pili of uropathogenic *Escherichia coli* (*E. coli*)], for which the compounds bicyclic 2-pyridones (pilicides)[4] and Virstatin[5] have been developed; and (2) prevention of elongation and formation of a functional pilus.

A search carried out in patent databases[6,7] revealed a total of 26 patent applications related to strategies that interfere with adhesion and colonization mechanisms (Table 1). These include the use of probiotics such as *Lactobacillus reuteri*, *Bifidobacterium infantis*, *Bifidobacterium lactis*, *Lactobacillus acidophilus* and *Lactobacillus casei*, for inhibition of Candida colonization, and also *Enterococcus faecium* LJS-01, which displays a strong capacity to adhere to intestinal epithelial cells and also good antimicrobial activity. The following proteins have also been described: decorin binding protein, which prevents colonization by *Borrelia*; collagen binding protein, isolated from *Staphylococcus aureus*; C3 binding polypeptide, isolated from *Streptococcus agalactiae*; novel fluorinated linker compounds; and Zn-releasing calcium phosphate. Finally, the following targets have been identified for vaccine development: capsular polysaccharide, EtpA flagellin and pyruvate-ferredoxin oxidireductase adhesin protein.

BACTERIAL SECRETORY SYSTEMS

Many bacteria have a specialized excretory system that resembles a syringe through which bacterial toxins (effector proteins) are injected into the host cell. These systems work by imitating host proteins, thus altering the signalling pathways and enabling development of the disease[8]. Three different secretion systems are implicated in the translocation of bacterial effectors into host cells, Ⅲ, Ⅳ and Ⅵ[9].

The type Ⅲ secretion system (TTSS) comprises some proteins that form a spire-like construction through which the bacteria inject the effector proteins from the bacterial cytoplasm to the cytoplasm of eukaryotic host cells. These secreted effector proteins often modify signal transduction in the host cells to improve microbial survival, invasion or attachment[10,11]. The type Ⅳ secretory

Table 1 Patents concerning the inhibition of bacterial adhesion and colonization

Patent title	Description	Application date	Inventors	Publication number
Capsular polysaccharide adhesion antigen preparation, purification and use	General method for preparing pure capsular exopolysaccharide adhesins strains of adhesin coagulase-negative staphylococci to produce vaccines	1994	Pier Gerald B	US5980910 (A)
Method for inhibiting microbial adhesion on surfaces	A method for inhibiting microbial adhesion on surfaces in contact with an aqueous system is disclosed and involves adding a treatment comprising an alkyl sulfosuccinate surfactant to the system	1995	Wright J Barry; Michalopoulos Daniel L	US5512186
Method and apparatus for preventing adhesion and colonization of bacteria in medical devices	Activation of compounding photochemicals for preventing and eliminating adherence and colonization of bacteria	1996	Prescott Marvin A	WO9806340 (A1)
Decorin binding protein compositions and methods of use	DNA segments encoding these proteins and anti-(decorin binding protein) antibodies for use in the prevention of *Borrelia* colonization in an animal	1996	Guo Betty P; Hoeoek Magnus; Hanson Mark	WO9727301 (A1)
Collagen binding protein compositions and methods of use	Disclosed are the cna gene and cna-derived nucleic acid segments from Staphylococcus aur. Also disclosed are Col Binding Protein (CBP) for use in the treatment of pathological infections, and in particular, for use in the prevention of bacterial adhesion to Col	1997	Hoeek Magnus; Patti Joseph M; House-Pompeo Karen; Sthanam Narayana; Symersky Jindrich	US6288214 (B1)
Surfactants for reducing bacterial adhesion onto surfaces	Inhibiting microbial colonization (ethylene oxide units) of a surface in contact with an aqueous system	1997	Donlan Rodney M; Elliot David L; Kapp Nancy J; Wiatr Christopher L; Rey Paula	US6039965 (A)
Composition of treatment of Candidiasis	Inhibition of adhesion of *Candida* colonization by using probiotics (*Lactobacillus reuteri*, *Bifidobacterium infantis*, *Bifidobacterium lactis*, *Lactobacillus acidophilus* not viable and non-viable *Lactobacillus casei*)	1998	Dohnlek Margaret H; Wagner Robert Doug; Balish Edward; Hilty Milo D	WO9917788 (A1)
Antimicrobial adhesion surface	The invention provides an implantable medical device with a hydrophilic coating to limit *in vivo* colonization of bacteria and fungi	1999	Zhong Samuel P	US6468649 (B1)
Anti-bacterial compounds directed against pilus biogenesis, adhesion and activity; co-crystals of pilus subunits and methods of use thereof	The invention relates to novel compounds that mimic a chaperone G1 beta-strand or an amino terminal motif of a pilus subunit	2000	Hultgren Scott J; Sauer Frederic G; Waksman Gabriel; Fuetterer Klaus; Choudhury Devapriya; Knight Stefan D; Barnhart Michelle	US7041465 (B1)
C3 binding polypeptide of *Streptococcus agalactiae* group b-Streptococcus	The invention involves the identification of a human complement C3 binding polypeptide and the nucleic acid that encodes the polypeptide from *Streptococcus agalactiae*	2000	Smith Beverly L; Ferrieri Patricia	US6582950 (B1)
Compounds directed against pilus biogenesis and activity in pathogenic bacteria, methods and compositions for synthesis thereof	Novel fluorinated linker compounds and methods of synthesis are provided. Methods for using the fluorinated linker compounds in methods of solid-phase synthesis of the N-substituted amino acid compounds are also disclosed (inhibiting or preventing the formation of a pilus chaperone-subunit complex)	2000	Kilhlberg Jan; Larsson Andreas; Svensson Anette; Fex Tomas; Hultgren Scott J; Pinkner Jerry	WO2001020995
DbpA compositions and methods of use	The DBP gene and decorin protein compositions of *Borrelia burgdorferi* are disclosed The DBP protein and antigenic epitopes derived from them are contemplated for use in preventing bacterial adhesion to decorin	2000	Guo Betty P; Hook Magnus	US6312907
Composition and method for controlling microbial adhesion and biofilm formation of surfaces	The invention describes how coating of surfaces with an extract, particularly a fish extract, can significantly reduce microbial adhesion, attachment, colonization and biofilm formation on surfaces	2003	Gram Lone Kirsten; Vogel Birtefonnesbech; Bagge-Ravn Dorthe	WO03092382 (A1)
Packaged antimicrobial medical device and method of preparing same	An antimicrobial suture assembly (halogenated hydroxyl ethers, acyloxydiphenyl ethers, and combinations thereof) to substantially inhibit bacterial colonization	2004	Scalzo Howard; Fischer Jerome A; Rothenburger Stephen	US2004220614 (A1)
Sealing material	A sealing material is presented (fluoropolymer layer, a reinforcing layer and an adhesive) to hinder growth and colonization of bacteria	2004	Patel Malay; Napolitano Michael; Hanrahan James R; Chu Chaokang	US2005250398 (A1)

Zn-releasing calcium phosphate (Zn-CaP) compounds for antimicrobial coating on orthodontic appliances and dental implants	Compositions of Zn-releasing calcium phosphate (Zn-CaP) compounds for use as anti-bacterial coatings for orthodontic brackets and dental implants	2006	Legeros Racquel Z; Legeros John P; Park Jae Hyun; Mijares Dindo	WO2007022211 (A2)
Composition for the administration of biologically active principles in gynaecological and rectal conditions and uses thereof	The invention relates to a composition for the administration of biologically active substances in gynaecological and rectal conditions, as well as the uses of said composition	2007	Strozzi Gianpaolo; Mogna Luca	US2010092440 (A1)
Enhanced treatments to kill or debilitate pathogenic microorganisms of a mammalian body	The novel treatments involve the use of anti-adhesive polysaccharide molecules to abolish or reduce the adhesion of *Helicobacter pylori*	2008	Nifantiev Nikolay; Wieland Gerhard D	US2011245198 (A1)
Non-leaching surface-active film compositions for microbial adhesion prevention	Coating (surfactant) useful to prevent bacterial colonization on a variety of surface including surfaces of medical devices	2008	Gruening Rainer; Qu Xin; Merritt Karen; Chen Paul N; Falevich Vitaly	MX2008009326 (A)
Prevention and treatment of Gram-negative, flagellated bacterial infections	EtpA which binds to the conserved region of the flagellin protein located at the tip of the flagella in Gram-negative bacteria (development vaccine)	2008	Fleckenstein James M	US2011206694 (A1)
Method for coating medical device[1]	Method for coating a medical device to prevent bacterial adhesion, colonization and device-associated infection (isocyanate-terminated polymer)	2010	Stopek Joshua	JP2011019902 (A)
Novel *Enterococcus faecium* LJS-01 and its use as a probiotic[1]	*Enterococcus faecium* LJS-01 shows good antimicrobial activity and strong capacity to adhere to intestinal epithelial cells	2010	Lin Chuen-FU; Wu Cheng-Nan; Lu Cheng-Hsiung; Hsu Wei-Li; Chiou Ming-Tang	TW201143631 (A)
Method for detecting colonization characteristic of lactobacillus in gastrointestinal tract on basis of green fluorescent protein[1]	The invention relates to a method for detecting the colonization characteristic of lactobacillus in the gastrointestinal tract on the basis of green fluorescent protein	2011	Yanping Wang; Jingrui Wang; Jinju Wang	CN102604877 (A)
Prevention of bacterial adhesion[1]	Prevention of adhesion of microorganisms on hard surfaces by the semi-permanent modification thereof during the cleaning process. A cleaning agent that contains surface-active polymers is used to prevent the bacterial colonization of hard surfaces	2011	Veith Birgit; Weide Mirko; Corbellini Francesca; Giesen Brigitte; Stumpe Stefan; Breves Roland; Barreleiro Paula; Karten Stefan; Bockmuehl Dirkl; Meier Frank	WO2012010700 (A1)
Pyruvate-ferredoxin oxidoreductase (PFO) adhesive protein as a target for inhibiting the adherence of *Trichomonas vaginalis* and as a diagnosis and vaccinal target for trichomoniasis[1]	Novel function of PFO upon participating in the cytoadherence of the *Trichomonas vaginalis* parasite to the hosting cell. The present invention enables development of vaccines for preventing the adhesion (and therefore the colonization) of parasites to the vaginal mucosa	2011	Verastegui Rossana Arroyo	MX2011011361 (A)
Vacuum assisted percutaneous appliance[1]	This device is stabilized by fibroblast in-growth and inhibits bacterial colonization	2012	Kantrowitz Allen B; Mortin Chris; Wadsworth JR Daniel C	US2013006186 (A1)

[1]Those published from 2010 onwards are highlighted.

system is utilized to transfer bacterial DNA or bacterial effector proteins to eukaryotic cells. This system also forms a duct between the bacterial and eukaryotic cell cytoplasm. It is a pilus-like structure rather than a spire construction[9]. Type VI secretion systems form tubular construction; however, exactly how these systems assemble and give effector proteins into the eukaryotic host cells stays in great measure unknown[12].

Although a lot of various types of TTSS are known, there are a restricted number of manners of inhibiting them: (1) prevention of assembly of the TTSS; (2) inhibition of interplay with the eukaryotic host cells; and (3) inhibition of secretion of the effector proteins.

Three components that are capable of inhibiting bacterial secretion systems have been reported[13]: (1) inhibitors of the type Ⅲ secretion systems such as acylated hydrazones of salicylaldehydes in *Chlamydia* and *Shigella* infections; (2) 2-amino-5-arylidene thiazolidinone in *Salmonella*, *Pseudomonas* and *Yersinia* infections; and (3) dirylacrylonitrile, which inhibits sortase A and has shown *in vitro* activity against *S. aureus*.

A search of the patent database (up to April 2014) revealed 22 patents involving inhibition of the proteins related to secretion systems (Table 2). All of these are based on methods that describe how to identify inhibitors and target proteins of bacterial secretion systems. Two proteins groups are associated with these secretion systems: Inc and HpaB group proteins.

CELL-TO-CELL SIGNALLING: QUORUM SENSING

Cell-cell communication, or quorum sensing (QS), is a

Table 2 Patents concerning the inhibition of bacterial secretion systems

Patent title	Description	Application date	Inventors	Publication number
Method for screening for inhibitors and activators of type Ⅲ secretion machinery in *Gram-negative* bacteria	This invention relates to mutant strains of Gram-negative bacteria that constitutively secrete proteins *via* the type Ⅲ secretion machinery and to methods of identifying molecules that are able to activate or inhibit secretion in wild-type strains of Gram-negative bacteria	2001	Demers Brigitte; Sansonetti Philippe; Parsot Claude	US6696249 (B1)
Method of detecting substance inhibiting type Ⅲ secretion mechanism of bacterium and the function of secretory protein thereof	A method whereby a substance specifically inhibiting the type Ⅲ secretion mechanism and the function of a type III secretory protein secreted therefrom can be detected in large amounts within a short period of time without depending on any animal infection experiments	2001	Omura Satoshi; Abe Akio	KR1020020086208
Secreted *Chlamydia* polypeptides and method for identifying such polypeptides by their secretion by a type Ⅲ secretion pathway of a *Gram-negative* bacteria	The present invention uses a heterologous secretion system, namely a type Ⅲ system, to investigate whether some *Chlamydia* proteins, especially Inc proteins and other proteins exhibiting a similar hydropathy profile, might be secreted and demonstrates that these hybrid proteins are secreted by the type Ⅲ secretion system of *Shigella flexneri*	2003	Subtil Agathe; Parsot Claude; Dautry-Varsat Alice	US2004131624 (A1)
Bacterial system for protein transport in eukaryotic cells	Development of a system for the targeted transport of proteins into eukaryotic cells by using a type Ⅲ secretion system and bacteria strains that are mutated in hpaB or homogenous genes. The inventive bacterial system is used to transport bacterial proteins into eukaryotic cells, in order to influence or modify cellular processes such as gene expression, growth, development and defence/resistance mechanisms	2005	Bonas Ulla; Buettner Daniela	WO2005085417 (A2)
Methods of identifying modulators of bacterial type Ⅲ protein secretion system	Provides methods for identifying inhibitors or activators of bacterial type Ⅲ protein secretion system by using a recombinant beta-lactamase that can be secreted by a type Ⅲ protein secretion system. The assay could be easily adapted to a high throughput mode to allow daily screening of several tens of thousands compounds	2005	Goldschmidt Raul; Loeloff Michael	WO2005113791 (A2)
Pharmaceutical composition for the treatment of bacterial infections and sepsis	The invention involves a pharmaceutical composition comprising at least one glycogen synthase kinase 3 β; inhibitor, at least one Rho-kinase inhibitor, and an optional adequate pharmaceutical carrier for producing a drug for the preventive or therapeutic treatment of bacterial infectious diseases by synergistically increasing synthesis and secretion of type II. A secretory phospholipase A2 into the bloodstream so as to boost the body's inherent resistance to infections	2005	Menschikowski Mario; Hagelgans Albert; Siegert Gabriele	WO2005120475
Pyridone compounds as inhibitors of bacterial type Ⅲ protein secretion systems	Provides compounds that inhibit type Ⅲ protein secretion useful for the treatment and prevention of bacterial infections, particularly those caused by *Gram-negative* bacteria, and methods for their use	2005	Li Xiaobing	US2005256137 (A1)
Methods for stimulating an immune response using bacterial antigen delivery system	Provides methods for stimulating and/or increasing an immune response against tumor antigens through the use of the type Ⅲ secretion system of bacteria. The invention also relates to the preparation of antigen presenting cells from peripheral blood mononuclear cells by using bacteria with a type Ⅲ secretion system	2006	Old Lloyd J; Ritter Gerd; Nishikawa Hiroyoshi; Gnjatic Sacha; Galan Jorge E	US2009324651 (A1)
Screening system for inhibitors and activators of type Ⅲ secretion machinery in *Gram-negative* bacteria	Provides a screening system (comprising inhibitors and activators of type Ⅲ secretion machinery) that directly transfers pathogenic proteins of *Gram-negative* bacteria into a host cell to identify substances capable of activating or inhibiting the secretion of type Ⅲ protein secretion system	2006	Hwang In Gyu; Moon Jae Sun; Kim Sung Uk	KR20080051240 (A)
Application of bovine lactoferrin for preparing a medicinal agent for inhibition of bacteria growth	The invention refers to a new application of bovine lactoferrin for preparing a medicinal agent for inhibiting bacteria growth. The bovine lactoferrin inhibits the growth of bacterial pathogens expressing the type Ⅲ secretory system	2007	Makmakhon Robert Dzh; Kliari Tomas; Ochoa Tereza	RU2007140789 (A)
Bacterial secretion system and uses	-	2007	Gey Van Pittius Nicolaas Claudius; Warren Robin Mark; Van Helden Paul David	ZA200706520 (A)
Biopolymer and protein production using type Ⅲ secretion systems of *Gram-negative* bacteria	Provides proteins, polynucleotide, expression cassette, vector and bacterium compositions for obtaining proteins of interest by expression of same in Gram-negative bacteria with a type Ⅲ secretion system. Also provides uses for the proteins obtained in the manufacture of isolated proteins and pharmaceutical compositions	2007	Voigt Christopher Ashby; Widmaier Daniel Matthew	WO2008019183 (A2)

Use of the *Pseudomonas syringae* effector protein HopU1 related to its ability to ADP-ribosylate eukaryotic RNA binding proteins	The invention provides novel methods for modulation of the innate immune response of a plant to infection caused by *Pseudomonas syringae*, which injects effector proteins into host cells *via* a type Ⅲ protein secretion system. Also provides methods for enhancing or suppressing the innate immune response of the plant	2007	Alfano James R; Fu Zheng Qing; Elthon Thomas E	WO2008042026 (A2)
Method and means for preventing and inhibiting type Ⅲ secretion in infections caused by Gram-negative bacteria	Discloses a means of decreasing bacterial virulence in a mammal or in a plant by inhibition of the type Ⅲ secretion system at concentrations that do not prevent or substantially reduce bacterial growth. Also disclosed are a therapeutic method and a pharmaceutical composition	2008	Elofsson Mikael	US2010099674 (A1)
Carboplatin compound inhibiting secretion system of phytopathogenic Gram-negative bacteria and biocontrol agent of plant diseases with this compound	Provides an agent for preventing plant diseases, containing carboplatin compounds, to selectively suppress secretion system related to plant pathogenicity	2009	-	KR20110048335 (A)
Inhibition of quorum sensing-mediated processes in bacteria	Provides methods for identifying molecules that can be used to positively and negatively manipulate quorum-sensing-mediated communication to control bacterial behavior. Methods of inhibiting quorum sensing-mediated activity in Gram-negative bacteria are provided wherein the activity is pathogenicity, bioluminescence, siderophore production, type Ⅲ secretion, or metalloprotease production	2009	Bassler Bonnie; Swem Lee	US2011123586 (A1)
Type Ⅲ secretion inhibitors, analogs and uses thereof	The invention relates to compounds and compositions useful for inhibiting type Ⅲ secretion systems in pathogenic bacteria, such as Yersinia pestis, and uses of such inhibitors in the treatment and prevention of disease	2009	Goguen Jon; Pan Ning; Lee Kyungae	US2011034463 (A1)
5-substituted-2-imino-thiazolidinone compounds and their use as inhibitors of bacterial infection[1]	Provides a method for inhibiting Gram-negative bacterial pathogenesis, a method of screening for compounds that inhibit type Ⅲ secretion in *Gram-negative* bacteria, and compounds that inhibit type Ⅲ secretion in *Gram-negative* bacteria	2010	Felise Heather B; Miller Samuel I; Kline Toni	US2011039849 (A1)
Methods for Identifying Inhibitors of the type Ⅲ Secretion System[1]	Provides a method for determining whether a test compound can inhibit the function of the type Ⅲ secretion system. The method identifies drug candidates that are highly specific anti-bacterial agents for treating diseases caused by Gram-negative bacteria with a T3SS	2010	Marlovits Thomas C; Radics Julia; Schmied Wolfgang	US2013130283 (A1)
Attenuated *Salmonella* inducible secretory expression oral vaccine presentation system and application there of[1]	The invention comprises an attenuated salmonella inducible secretory expression oral vaccine presentation system containing an antigen expression carrier. The system is controlled by a promoter induced by a microenvironment in an antigen presenting cell and excreted by induction of a bacteria excretion signal, and it uses the attenuated salmonella as the host of the antigen expression carrier	2011	Zichun Hua; Guo Chen	CN102335421 (A)
Bacterial mediated delivery of nuclear protein into pluripotent and differentiated cells[1]	A modified *Pseudomonas aeruginosa* type Ⅲ secretion system has been developed that efficiently delivers selected proteins into a host cell	2011	Jin Shouguang; Bichsel Candace	WO2012012605 (A2)
Inhibitors of bacterial type Ⅲ secretion system[1]	Discloses organic compounds showing the ability to inhibit effector toxin secretion or translocation mediated by bacterial type Ⅲ secretion systems. These inhibitor compounds are useful for combating infections by *Gram-negative* bacteria with such type Ⅲ secretion systems	2012	Moir Donald T.; Aiello Daniel; Peet Norton P; Williams John D; Torhan Matthew	US2014142134 (A1)

[1]Those published from 2010 onwards are highlighted.

widespread phenomenon in bacteria that is used to co-ordinate gene expression between local populations[13]. Bacterial populations can use QS communication to coordinate the execution of important biological functions, many of which are involved in pathogen virulence, *e.g.*, biofilm formation, extracellular polysaccharide production, host colonization, motility, bioluminescence, transfer of plasmids by conjugation, and biosynthesis of antibiotics and siderophores.

All QS systems utilize small, secreted signalling molecules known as autoinducers (AIs): (1) AI-1 molecules are N-acyl-homoserine lactones (AHLs); (2) AI-2 molecules are heterocyclic furanosyl-borates; (3) AI-3 signals are catecholamines and finally; and (4) AI-4 signals are cyclic peptides. Some other QS signals go beyond these classes, *e.g.*, *Pseudomonas* quinolone signal and diffusible signal factor. New molecules will undoubtedly be discovered as the study of QS expands to species of bacteria yet to be investigated.

Targeting bacterial virulence (quorum quenchers, Figure 1) is an alternative focusing to antimicrobial therapy that offers a hopeful opportunity to inhibit pathogenesis and its consequences without producing immediately the death the target bacterium. Bacterial virulence factors have been shown to be potential targets for drug design and therapeutic intervention for Gram-negative pathogens[1]. Numerous quorum sensing inhibitors have been reported in the literature[1,2].

In 2012, Romero *et al*[14] published an article about patents concerning quorum quenching (QQ) (*i.e.*, the

mechanisms that cause the inactivation of QS communication systems)[15].

A search of patent databases (up to August 2011) revealed a total of 45 applications related to strategies for interfering with QS systems as a method of fighting microbial infections. Following the bias in the literature, the vast majority of the patented technologies based on the inhibition of QS mechanisms target AHL signals, whereas only 5 out of 45 patent cases are based on the inhibition of AI-2 signals and only 4 are based on the inhibition of peptide-based QS signals from Gram-positive bacteria. In this review, a search of more recent reports (up to April 2014) was conducted, revealing 32 patents concerning the inhibition of QS systems (Table 3). QQ occurs in *Lysobacter enzymogenes*, *Shewanella piezotolerans*, *Bacillus pumillus*, *Tenacibaculum discolor* (cect 7426) and novel alpha-proteobacteria. The molecules involved in QQ distinguish inhibitors of the AI-2 signals (triazol derivates, furan compounds and phosphorylated, branched dihydroxy-pentane-dione) and inhibitors of AHLs [(oxododecanoyl)-L-homoserine lactone and bicyclic furanones with low toxicity]. Furanones, which are naturally occurring compounds, appear to be the most widely studied QQ compounds. These compounds are toxic to Artemia and rotifers, which will limit their use in humans[15]. However, the use of C-30, a synthetic furanone, at non toxic concentrations, significantly reduced the pathogenicity of *Vibrio anguillarum* in rainbow trout[16]. Other patented compounds involved in QQ include honaucin A, 2-methylthiopyrrolidines, lovastatin and hydroxytirosol. Finally, one enzyme (OLB-26) is known to be involved in QQ.

MECHANISMS OF RESISTANCE: MULTIDRUG RESISTANCE PUMPS

Multidrug resistance (MDR) efflux pumps have multiple functions in natural microbial ecosystems. In clinical environments, MDR pumps are implicated in the following: (1) resistance to antimicrobial compounds localized on mucosal surfaces (colonization factor)[17,18]; (2) efflux virulence factors[19]; (3) QS-regulated expression of virulence traits[20]; and (4) antibiotic resistance, which is a code element in patients under treatment[21]. All of these roles are important for the survival, colonization and pathogenic outcome of virulent bacteria in clinical environments. In nonclinical environments, MDR pumps may be associated to resistance to heavy metals[22] and organic solvents[23] (plant colonization factor). Bacterial efflux pumps are classified into five families according to their composition, number of transmembrane spanning regions, energy sources and substrates: the resistance-nodulation-division (RND) family, the major facilitator superfamily, the adenosine triphosphate-binding cassette superfamily, the small multidrug resistance family, and the multidrug and toxic compound extrusion family[17,18].

Several MDR pump inhibitors were published[24]. We consider two examples of RND inhibitors pumps: (1) 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-β-naphthylamide. These act as inhibitors of RND efflux pumps and virulence traits in *Vibrio cholerae*, such as the cholera toxin and the toxin-coregulated pilus[25], and were suggested as a suitable tool for the treatment of cholera infections; and (2) Of 12 trifluoromethyl ketone compounds tested, 6 proved to be effective inhibitors of the quorum-sensing response by *Chromobacterium violaceum* 026, as well as inhibitors of the RND efflux pumps of CV026 and *E. coli*. This result is of clinical applicability and may be used for the prevention of QS responses of infecting bacteria[26].

We found 22 patents related to the inhibition of the MDR efflux in patent databases up to April 2014 (Table 4). Most of these are screening methods for microbial efflux pump inhibitors. Moreover, pump inhibitors are described as potentiators of the action of antiseptics, disinfectants and antimicrobial agents such as tigecycline. Finally, polyamine molecules are inhibitors of bacterial efflux pumps that could be used in combination with other drugs such as antibiotics, as well as pharmaceutical compositions thereof.

FUTURE PROSPECTS: RESISTANCE TO ANTIVIRULENCE COMPOUNDS

Although antivirulence therapies are novel in the field of treatment of infectious diseases, several studies involving clinical strains have demonstrated the development of mechanisms of resistance, especially to Quorum Quenching compounds[27]. *Vibrio cholerae* strains that are resistant to virstatin have also been described; the mechanisms whereby these strains colonize ther hosts are independent of the elaboration of the toxin co-regulated pilus[28].

The first evidence that cells develop resistance to QQ compounds has been reported by Maeda *et al*[29] (published ahead of print in 2011). These authors worked with a concentration of brominated furanone C-30 [the gold standard for QQ compounds, and which is a synthetic brominated furanone 4-bromo-5-(bromomethylene)-2(5*H*)-furanone] that did not influence growth in rich medium (*i.e.*, it did not inhibit growth) and utilized both transposon mutagenesis and spontaneous mutants to detect resistant bacteria.

The mechanism of this resistance was overexpression of the MexAB-OprM multidrug resistance operon due to mutations in the gene repressors *mexR* and *nalC*, resulting in efflux of the compound C-30. This quorum quenching compound showed a reduced capacity to decrease some QS-controlled virulence traits and phenotypes in the *mexR* mutant, and the pathogenicity of the *mexR* mutant against the nematode *Caenorhabditis elegans* was not decrease by the inclusion of C-30. Importantly, these authors also worked with cells from cystic fibrosis patients (Liverpool epidemic strain 12142) with *mexR* and *nalC* mutations[30] to demonstrate that, even in the absence of the QS inhibitor, cells develop resistance to quorum quenching compounds in the pathogenic state when

Table 3 Patents concerning the inhibition of quorum sensing systems

Patent title	Description	Application date	Inventors	Publication number
Anti-inflammatory and quorum sensing inhibition compounds and methods of making and using them	This invention generally relates to novel compositions based on a structure designated as "Honaucin A", including Honaucin A variants and analogs, and pharmaceutical compositions, liposomes and nanoparticles comprising them, and methods of making and using them	2011	Gerwick William H; Gerwick Lena; Choi Huykjae; Villa Francisco A; Smith Jennifer; Rowley David C	WO2011153502 (A2)
Composition for oral use	A method for suppressing dental caries by regulating biofilm formation by the bacteria that cause dental caries instead of controlling these bacteria	2011	Tsugane Takanori; Saeki Yoji	EP2620160
Conjugates of acyl homoserine lactone and catalase a from *Pseudomonas aeruginosa*	The present invention relates to the acyl homoserine lactone N-3-(oxododecanoyl)-L-homoserine lactone or butyryl L-homoserine lactone and *Pseudomona aeruginosa* KatA protein, or an antigenic portion conjugate thereof, used to treat *Pseudomona aeruginosa* infections by limiting biofilm formation and inhibiting a range of quorum-sensing dependent virulence factors	2011	Kyd Jennelle M; Cooley Margaret	WO/2012/083382
Enzyme bag containing quorum quenching enzyme immobilized silica for inhibiting biofilm formation and membrane bioreactor system for water treatment system using the bag	This invention relates to an enzyme bag containing silica-immobilized enzyme for inhibiting biofilm formation. A membrane bioreactor system for water treatment using the bag is provided for stable implementation of filtering operations over a long period of time by improving the performance of operational processes	2011	Lee Chung Hak; Yang Cheon Seok; Lee Jung Kee; Han Jong Yun; Lee Chung Hak; Yang Cheon Seoket; Lee Jung Kee; Han Jong Yun	KR20120134724 (A)
Fluidizable carrier with biofilm formation-inhibiting microorganisms immobilized therein and membrane water treatment apparatus using the same	This invention relates to a biofilm formation-inhibiting microorganism immobilized fluidizable carrier in which a biofilm formation-inhibiting microorganism is fixed therein and a membrane water treatment apparatus including the same are provided to increase a membrane cleaning period	2011	Lee Chung Hak; Kim Sang Ryoung; Lee Jung Kee	KR20130034935 (A)
Methods of disrupting quorum sensing to affect microbial population cell density	The invention relates to the modulation of quorum sensing mechanisms in a microorganism for the purpose of exploiting the fermentation capabilities of the microorganism	2011	Marrs Barry; Swalla Brian M	US2011124522 (A1)
Quorum-sensing signal molecular preparation and application thereof in tobacco waste treatment	The invention relates to the field of environmental biotechnology, in particular to preparation of a quorum sensing signal molecule and application in processing tobacco waste	2011	Meizhen Wang; Hongzhen He; Huajun Feng; Xin Zheng; Dongsheng Shen; Zhenmei LV; Hang Min	CN102392051 (A)
Synthetic analogs of bacterial quorum sensors	The invention relates to synthetic analogs of bacterial quorum sensing molecules, and methods of use of these	2011	Iyer Rashi; Ganguly Kumkum; Silks Louis A	US2012071430 (A1)
System and method for reversing the antibiotic tolerance of bacterial persister cells	The present invention relates to antibiotics and, more particularly, to a system and method for decreasing the tolerance of bacterial persister cells to antibiotics	2011	Ren Dacheng; Pan Jiachuan	EP2603576
Triazole compounds as well as preparation method and application thereof	The present invention relates to triazole derivatives, the preparation method and as the autoinducer-2 (AI-2) quorum sensing inhibitors, belonging to anti-AI-2 quorum sensing type drug technology	2011	Minyong Li; Lvpei Du; Peng Zhu	CN102219753 (A)
Use of a novel alpha-proteobacteria for quorum quenching	The invention (concerning the fields of biology, molecular biology, and aquaculture) specifically relates to a new a-proteobacteria capable of degrading/V-acyl-homoserine lactones (AHLs) for control of bacterial infectious diseases and prevention of biofilm formation	2011	Otero Casal Ana María; Romero Bernardez Manuel	WO2011154585 (A1)
2-methylthiopyrrolidines and their use for modulating bacterial quorum sensing	Formula (I) compounds are disclosed and their use in inhibiting quorum sensing in bacteria is reported	2012	Malladi Venkata L; Schneper Lisa; Sobczak Adam J; Mathee Kalai; Wnuk Stanislaw F	WO/2012/174511
Compositions for regulating or modulating quorum sensing in bacteria, methods of using the compounds, and methods of regulating or modulating quorum sensing in bacteria	The report encompasses compounds and compositions that are useful as specific AI-2 antagonists for the control of bacterial quorum sensing and methods for inhibiting or attenuating microbial virulence, biofilm formation and drug resistance	2012	Wang Binghe; Ni Nantin; Wamg Junfeng; Lu Chung-Dar; Chou Han-Ting; Li Minyong; Zheng Shilong; Cheng Yunfeg; Peng Hanjing	EP2529793 (A2)
Construction and application of unmarked *Lysobacter enzymogenes* engineering strain capable of preventing plant bacteriosis	The invention (within the field of microbial genetic engineering), specifically relates to a plant bacterial disease that can prevent dissolving enzyme production strains of *Bacillus* unmarked engineering construction and application	2012	Liu Fengquan; Qian Guoliang	CN102943061 (A)

Furan compound and preparation method and application of furan compound	The invention relates to furan derivatives, the method of preparation and as the AI-2 quorum sensing inhibitors, are anti-AI-2 type of quorum sensing	2012	Minyong Li; Peng Zhu	CN102603683 (A)
Method for increasing output of microbial lovastatin based on quorum sensing mechanism	The invention relates to the pharmaceutical raw material fermentation industry, in particular to a method of increasing microbial production of lovastatin	2012	Li Haoming	CN102925509 (A)
Method for quickly identifying food-borne pathogen bacterial biofilm formation inhibitor	The invention relates to the field of food microbiology control technology, in particular to the rapid identification of an inhibitor of foodborne bacterial biofilm formation	2012	Wenyan Zhang; Hongmei Zhang; Zhihua Tao; Wenyuan Zhou	CN102706821 (A)
Phosphorylated and branched dihydroxy-pentane-ione analogs as quorum sensing inhibitors in bacteria	The invention provides compositions and methods for modulating quorum sensing in microbes and can be used in prophylactic methods or therapy for bacterial infections and for reduction of biofilms. The compounds are AI-2 analogs and as such have structures similar to 4,5-dihydroxy-2,3-pentanedione that can act as agonists/antagonists of quorum sensing	2012	Sintim Herman; Bentley William E; Roy Yarnika; Smith Jacqueline	US2012294900 (A1)
Preparation method of imprinted polymer of bacterial quorum sensing signal molecule AI-1	The present invention relates to bacterial quorum sensing signal molecules A1-1 imprinted polymer preparation	2012	Xin Li; Ling Wang	CN102604010 (A)
Probiotics for biological control against *Vibrio* sp.	The invention relates to probiotics for biological control against Vibrio sp., and in particular, to a newly isolated bacillus strain that degrades quorum-sensing signal molecules of the pathogenic bacteria *Vibrio* sp., and inhibits biofilm formation	2012	Yang Si Yong; Woo Seo Hyung; Kang In Hye; Im Hyun Jung	WO2012105805
Quorum sensing inhibitor against a pathogenic microorganism, and an antibacterial composition using the same	A quorum sensing inhibitor and an antibacterial composition using the same are provided to suppress quorum sensing between bacteria, and to prevent or treat infection or diseases	2012	Undescribed	KR101243696
Shewanella piezotolerans 34# and application thereof to algae inhibition	The invention relates to the field of biotechnology, in particular to a marine bacterium Shewanella and the inhibition of algal growth	2012	Zhou Jin; Yin Peng	CN103173383 (A)
Simple method for testing disease resistance of pathogenic bacteria quorum-quenching gene prokaryotic expression product	The invention relates to a prokaryotic expression product of disease resistance testing methods, in particular to test pathogen populations prokaryotic expression product quenching effect of a simple disease, and belongs to the field of gene function identification techniques	2012	Ouyang Lejun; Huang Zhenchi; Zeng Fuhua; Li Limei; Li Heng	CN102972220 (A)
Use of quorum sensing inhibitors and biofilm dispersing agents for controlling biofilm-associated implantable medical device related infections	The invention generally relates to implantable medical devices and, more specifically, to the use of quorum sensing inhibitors and/or biofilm dispersing agents to control biofilm-associated infections related to the use of implantable medical devices	2012	Samade Richard; Dinesh Prashant; Nabutovsky Yelena; Bornzin Gene A; Poore John W; Karicherla Annapurna; Dalal Nirav	US2014005605 (A1)
Bacillus pumillus microbial preparation with quorum sensing system inhibiting effect	The invention relates to *Bacillus pumillus* with a quorum sensing system inhibiting effect. The invention has the advantages that Chromobacterium violaceum is used for screening out a bacterial strain F3-1. It can be used to produce a microbial preparation capable of preventing and treating aquatic bacterial diseases	2013	Song Zengfu; Fan Bin; Chen Biao	CN103525723 (A)
Bicyclic furanones with low toxicity for microbial control	The invention relates to a class of bicyclic brominated furanone structures with reduced toxicity and high activity for inhibiting biofilm formation and quorum sensing by microbes	2013	Luk Yan-Yeung; Yang Sijie	US2013197077 (A1)
Method for detecting quorum sensing quenching bacterial strain	The invention relates to a method for detecting a quorum sensing quenching bacterial strain. The method comprises the addition of a bacterial strain to be detected in a PIPES (1,4-piperazinediethan esulfonic acid) buffer solution of pH 6	2013	Zhang Xiaohua; Tang Kaihao; Shi Xiaochong; Zhang Yunhui	CN103215342 (A)
Quorum-quenching enzyme OLB-26, and coding gene and application thereof	The invention relates to a quorum sensing quenching enzyme 0LB-26 and its coding gene and application	2013	Zhou Zhigang; Zhang Meichao; Yang Yalin; Xu Li; He Suxu; Li Qing; Yu Qiang	CN103275949 (A)
Targeted enzymatic degradation of quorum-sensing peptides	The present invention generally relates to the fields of microbiology and wound care. More particularly, it concerns methods and compositions for inhibiting biofilms in wounds and on medical devices	2013	Alarcon Rodolfo; Mcnulty Amy K	US20130253382
Use of ellagitannins as inhibitors of bacterial quorum sensing	Materials and methods for the inhibition of bacterial QS are described. Methods of treating bacterial infections by administration of one or more ellagitannins in amounts effective for inhibiting bacterial QS are also provided	2013	Athee Kalai; Adonizio Allison L; Ausubel Frederick; Clardy Jon; Bennett Bradley; Downum Kelsey	US2013317094 (A1)
Use of hydroxytirosol and derivatives thereof as quorum quenchers	The quorum quenching activity of formula (I) or (II) compounds, such as hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), 3,4-dihydroxyphenylacetic acid (DOPAC) and derivatives thereof are described	2013	Au Ón Calles David; Allende Prieto Ana; Fábregas Casal Jaime; Gómez-Acebo Gullón Eduardo	WO2014060581 (A1)
Use of the cect 7426 strain for generating quorum quenching of the autoinducer-2 signal (ai-2)	The invention relates to the use of a bacterial strain of the species Tenacibaculum discolor in the control of infectious diseases and for inhibiting biofilm formation caused by bacteria, through the inhibition of quorum sensing signals type AI -2. The invention applies to the field of molecular biology	2013	Otero Casal, Ana María; Romero Bernárdez Manuel	WO2014057151 (A1)

Table 4 Patents concerning the inhibition of multidrug resistance systems

Patent title	Description	Application date	Inventors	Publication number
Method for screening for non-tetracycline efflux pump inhibitors	Provides screening methods for inhibitors of microbial efflux pumps and pharmaceutical compositions containing such efflux pump inhibitors as well as methods for treating microbial infections by use of these compositions	1995	Trias; Joaquim Chamberland; Suzanne Hecker; Scott J Lee; Ving J	US5989832 A
Efflux pump inhibitors	Provides screening methods for inhibitors of microbial efflux pumps and pharmaceutical compositions containing such efflux pump inhibitors. Also provides methods for treating microbial infections using those compositions	1996	Trias Joaquim; Hecker Scott J; Chamberland Suzanne; Lee Ving J	CA2217865
Methods and compositions for reducing bacterial tolerance of disinfectants and organic solvents	Methods and compositions useful for manipulating bacterial resistance to non-antibiotic antibacterial compositions, disinfectants and organic solvents, and for rendering bacterial cells susceptible to non-antibiotic antibacterial compositions	1997	Levy Stuart B	WO9917607 (A2)
Methods and compositions for reducing bacterial tolerance to antibacterials, disinfectants and organic solvents	Methods and compositions useful for manipulating bacterial resistance to non-antibiotic antibacterial compositions, disinfectants and organic solvents, and methods for rendering bacterial cells susceptible to non-antibiotic antibacterial compositions	1997	Levy Stuart B	US6068972 (A)
Inhibitors of cellular efflux pumps of microbes	Describes compounds that are inhibitors of efflux pump in bacteria. Also describes methods of preparing and using such compounds and the pharmaceutical compositions that include such compounds	2001	De Souza Noel John; Patel Mahesh Vithalbhai; Gupte Shrikant V; Upadhyay Dilip J; Shukla Milind Chintaman; Chaturvedi Nishith C; Bhawsar Satish B; Nair Sheela Chandresekharan; Jafri Mohammed A; Khorakiwala Habil Fakhruddin	US2002177559 (A1)
Drug discovery and increased potency of antiseptics and disinfectants based on high extracellular pH, the disablement of cellular efflux pumps, and the unexpected synergism therebetween	Methods for increasing the therapeutic potency of amphipathic compounds, *e.g.*, antiseptics and disinfectants, and the exploitation of these discoveries in the screening of small molecules, and libraries thereof, for biological activity in prokaryotes and eukaryotes	2002	Lewis Kim; Hsieh Peichung	US2003118541 (A1)
Methods to study and mechanisms of biofilm-related antibiotic resistance	Discloses various genes that encode proteins shown to play a role in microbial resistance of an organism in a biofilm and homologs thereof. Describes methods of identifying a compound that modulates microbial resistance of an organism in a biofilm and also genes encoding proteins that play a role in biofilm resistance	2002	O'Toole George A; Mah Thien-Fah	US2003166030 (A1)
Minicell-based gene therapy	Provides compositions and methods for the production of achromosomal and anucleate cells useful for applications such as diagnostic and therapeutic uses, as well as research tools and agents for drug discovery	2002	Sabbadini Roger A; Berkley Neil; Surber Mark W	US2003199088 (A1)
Potentiators of antibacterial activity	The invention relates to compounds that potentiate the activity of antibacterials, compositions useful in treating bacterial infection, and related methods. Also relates to a method of inhibiting bacterial efflux of an antibiotic, thereby increasing its efficacy	2004	Grossman Trudy H	US20040887719 20040709
Substituted polyamines as inhibitors of bacterial efflux pumps	Methods of treating bacterial infections, including those caused by multidrug resistant bacteria, by using polyamine efflux pump inhibiting compounds, optionally in combination with other drugs such as antibiotics. The pharmaceutical compositions of the polyamines are also reported	2004	Nelson Mark L; Alekshun Michael N	US2004204378 (A1)
Essential and important genes of *Pseudomonas aeruginosa* and the use thereof to design or identify antibacterial agents	Database of candidate essential genes in *Pseudomonas aeruginosa*, and other important genes that, when mutated, produce a growth attenuated phenotype. The invention includes methods for confirming the need for or importance of candidate genes, methods for using those genes to screen for new antibacterial drugs, the antibacterial agents identified using the disclosed methods, and also methods of using the same for treating and preventing *Pseudomonas* infection	2005	Bruce Kim F; Warrener Paul; Hou Kevin	US2007196829 (A1)
Method for increasing the susceptibility of peptide deformylase inhibitors by using efflux pump inhibitors	Provides methods and compositions for increasing the susceptibility of PDF inhibitors against Gram-negative organisms by using efflux pump inhibitors	2005	Dean Charles Richard; Ryder Neil Stewart	CA2569681

Products and methods for *in vivo* secretion of monatin	Provides products and methods for the *in vivo* production of monatin sweetener. The products include microorganisms that are genetically modified to secrete or to improve secretion of monatin, to produce monatin, and to both secrete/ improve secretion and produce monatin	2006	Laplaza Jose; Anderson James C; Desouza Mervyn L; Hicks Paula M; Kollmann Sherry R	WO2006113897
Rhamnose-inducible expression systems and methods	Describes rhamnose-inducible expression constructs which may include at least one rhamnose-inducible regulatory element expressing a regulatory protein and one promoter that is inducible by the regulatory protein. An open reading frame expressing a protein of interest may be placed under control of the promoter. Also describes optimized Shine-Dalgarno sequences for use with the promoter	2006	Surber Mark W	US2007122881 (A1)
Enhancement of tigecycline potency using efflux pump inhibitors	Discloses Efflux Pump Inhibitor (EPI) compounds that can be co-administered with antimicrobial agents for the treatment of infections caused by drug resistant pathogens and methods of treatment and pharmaceutical compositions for co-administering tigecylcine with an EPI	2007	Glinka Tomasz; Bostian Keith; Lomovskaya Olga; Surber Mark; Sun Dongxu	US20070832626 20070801
Method or agent for inhibiting the function of efflux pump of *Pseudomonas aeruginosa*	Discloses a method comprised of modifying any amino acid residue selected from 100th to 109th and 311th to 320th amino acid residues in an amino acid sequence for mature OprM protein, as well as an agent for inhibiting the function of an efflux pump of *Pseudomonas aeruginosa* with good efficiency. Further disclosed is a method for screening the agent	2007	Yoshihara Eisaku; Inoko Hidatoshi	CA2641988
Method or agent for inhibiting the function of efflux pump of *Pseudomonas aeruginosa*	Provides a method for inhibiting the function of the drug efflux pump of *Pseudomonas aeruginosa*, comprising modification of any amino acid residue selected from 100th to 109th or 311th to 320th amino acid residues in the amino acid sequence of mature OprM protein. Also reports an agent with such inhibitory effect, as well as a screening method	2007	Yoshihara Eisaku; Inoko Hidatoshi	WO2007/091395
Microbial production of aromatic acids	Method for the microbial production of aromatic acids from a fermentable carbon substrate using a host cell capable of producing said aromatic acid, and comprising an efflux pump for said aromatic acid. A preferred host cell comprises a member of the proton-dependent resistance/nodulation/cell division (RND) family of efflux pumps	2007	Wery Jan	US2007259409 (A1)
Near-infrared electromagnetic modification of cellular steady-state membrane potentials	Discloses systems and methods for applying near-infrared optical energies and dosimetries to alter the bioenergetic steady-state trans-membrane and mitochondrial potentials (DeltaPsi-steady) of all irradiated cells through an optical depolarization effect. This membrane depolarization provides the ability to potentiate antimicrobial, antifungal and/or antineoplastic drugs against only targeted undesirable cells	2007	Bornstein Eric	US2008139992
In vivo gene sensors	Describes methods and compositions for the detection of target genes that permit the selective expression of an effector gene in those cells expressing the target gene, thus selectively targeting these cells for treatment or elimination. The methods and compositions described may also permit the selective expression of an agent such as a therapeutic gene product, in a targeted population of cells	2009	Collins James J; Lu Timothy Kuan-Ta	WO2009/137136
Methods of reducing microbial resistance to drugs	Provides methods of treating infection, screening for inhibitors of AcrAB-like efflux pumps, and enhancing antimicrobial activity of drugs. Pharmaceutical composition comprising an inhibitor of an AcrAB-like efflux pump and an antimicrobial agent are also provided	2009	Oethinger Margaret; Levy Stuart B	US2009298873
Minicells displaying antibodies or derivatives thereof and comprising biologically active compounds[1]	Minicells are used to deliver biologically active compounds, including polypeptides, nucleic acids, small molecules, drug molecules, and chemotherapeutic agents. In some cases, the minicell displays ligands or binding moieties that target the minicell to a desired host cell	2011	Sabbadini Roger A; Berkley Neil; Surber Mark W; Klepper Robert	US20070725196 20070316

[1]Those published from 2010 onwards are highlighted.

coexisted with the pressures of antibiotic treatment; so, antimicrobial treatment can induce to resistance to QQ compounds.

Intensified efforts are needed to establish whether resistance may develop to other QQ compounds, as is the case of lactonase or acylase enzymes in connection with AHL autoinducers.

CONCLUSION

Antimicrobial resistance in nosocomial pathogens is increasing worldwide. Mortality rates of patients infected with drug-resistant pathogens have increased by approximately 50% in recent years. Hospitals have become breeding grounds for extremely resistant pathogens, exacerbating the risk associated with hospitalization. These pathogens are extremely resistant to last line antimicrobials. If the current trend continues, the beginning of a "post-antibiotic era" is predicted. Development of novel antibiotics has almost totally ceased, at least against Gram-negative bacteria, and the prospects for a reversal of this trend are bleak. It is therefore imperative to de-

velop new molecules, therapies and/or new combinations of these for the eradication of resistant pathogens. In this review, we discuss some examples of patented molecules that act by inhibiting different bacterial virulence mechanisms (adhesion/colonization and quorum sensing mechanisms, and secretory and efflux pump systems) and which open the way to studying potential new treatments for infections caused by multiresistant bacteria.

Novel targets and molecules must be discovered for antivirulence therapies, taking into account the possible development of resistance mechanisms. Further study of combinations of these compounds with other antimicrobials for the treatment of infectious diseases is also important.

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P- Reviewer: Galgoczy L, Hays J, Zhang ZM **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5497/wjp.v3.i4.110

World J Pharmacol 2014 December 9; 3(4): 110-119
ISSN 2220-3192 (online)
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REVIEW

Harnessing pharmacological knowledge for personalized medicine and pharmacotyping: Challenges and lessons learned

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Received: June 12, 2014 Revised: October 17, 2014
Accepted: October 28, 2014
Published online: December 9, 2014

Abstract

The contribution of the genetic make-up to an individual's capacity has long been recognized in modern pharmacology as a crucial factor leading to therapy inefficiency and toxicity, negatively impacting the economic burden of healthcare and restricting the monitoring of diseases. In practical terms, and in order for drug prescription to be improved toward meeting the personalized medicine concept in drug delivery, the maximum clinical outcome for most, if not all, patients must be achieved, *i.e.*, pharmacotyping. Such a direction although promising and of high expectation from the society, it is however hardly to be afforded for healthcare worldwide. To overcome any existed hurdles, this means that practical clinical utility of personalized medicine decisions have to be documented and validated in the clinical setting. The latter implies for drug delivery the efficient implementation of previously gained *in vivo* pharmacology experience with pharmacogenomics knowledge. As an approach to work faster and in a more productive way, the elaboration of advanced physiologically based pharmacokinetics models is discussed. And in better clarifying this topic, the example of tamoxifen is thoroughly presented. Overall, pharmacotyping represents a major challenge in modern therapeutics for which pharmacologists need to work in successfully fulfilling this task.

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Key words: Pharmacology; Pharmacogenomics; Personalized medicine; Pharmacokinetics; Pharmacodynamics; Pharmacotyping; Translational medicine; Drug delivery; Education; Curricula

Core tip: Drug prescription in order to be improved, the drug delivery process needs to confront the challenges of genomics knowledge translation to ensure the maximum clinical outcome for most, if not all, patients, *i.e.*, achieving pharmacotyping. The practical clinical utility of personalized medicine decisions needs to be documented and validated in the clinical setting. Physiologically based pharmacokinetic models represent an approach by which the faster and more efficient implementation of pharmacogenomics knowledge in evidence-based medicine could be achieved. Pharmacotyping represents a major challenge in modern therapeutics for which pharmacologists needs to work both in academia and research in successfully fulfilling this task.

Vizirianakis IS. Harnessing pharmacological knowledge for personalized medicine and pharmacotyping: Challenges and lessons learned. *World J Pharmacol* 2014; 3(4): 110-119 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/110.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.110

INTRODUCTION

Unanimously nowadays, nanotechnology and nanomedicine in parallel with pharmacology and pharmacogenomics (PGx) contribute knowledge and methodologies permitting individualized treatment decisions to enter

in everyday clinical practice. The personalized medicine concept along with the interdisciplinary efforts needed to achieve the desired practical clinical utility for personalized medicine decisions worldwide is extensively and thoroughly described elsewhere[1]. The latter also implies that PGx by bridging pharmacology with genetics/genomics provides additional advantage for translational medicine to positively impact drug development and delivery outcomes. This means that the molecular etiology of drug response variability, by clinically assessing the genetic factors that contribute to pharmacokinetics (PK) and pharmacodynamics (PD), is now considered an integral part of modern pharmacology and therapeutics. As a consequence, the drug administration has been changed allowing for pharmacotyping (PTx) to emerge in the prescription process *e.g.*, the individual patient (personalized) specific medicine selection and administration scheme, as proposed earlier[1-4]. From a historical point of view, pharmacogenetics as a term has been introduced by Friedrich Vogel (1959), whereas the first example of pharmacogenetics described ever is flavism disorder by Pythagoras (580-500 BC; ancient Greek mathematician). By following-up chronologically until nowadays such pharmacogenetics-related scientific breakthroughs for pharmacology, it is obvious that these focused efforts have been successful by efficiently translating multidisciplinary-based experimental data that enabled pharmacological improvements both in research and the clinical setting (for such a detailed chronological description of pharmacogenetics/PGx breakthroughs see elsewhere[1]). However from the experience gained thus far, it needs considerable effort and, more importantly, to invent focused as well as interdisciplinary-oriented "smart and sophisticated" experimental approaches to move all the way through establishing personalized medicine decisions of broader practical clinical utility. The molecular etiology of illness pathophysiology and the elucidation of genetic factors contributing to pharmacological profiles of drugs in the body are hardly experimentally approachable, especially in being thoroughly understood for all marketed therapeutics. Moreover, the interplay of genes with therapeutics implies that their interaction is also modulating drug delivery outcomes, since the mutational status of genes (gene polymorphism) and drug-regulated gene expression profiles contribute to drug response variability (Figure 1). The latter, it leads to patient phenotypic (pharmacological) response modulation, or alternatively into pharmacological response heterogeneity. Complementary, in trying to minimize the emergence of drug response variation amongst population and in order to achieve improved profiles of administered drugs worldwide, a new interdisciplinary infrastructure needs to be created and integrated in clinical practice[4]. The latter, will also help in adjusting the regulatory environment to improve drug development productivity by minimizing the emergence of adverse drug reactions (ADRs), avoiding drug interactions and thus finally improving the clinical outcome.

Moreover, by considering the issue of education in pharmacy and medicine, the better training in pharmacology will be achieved through the development of new curricula aiming to advance skills of medical and pharmacy students in implementing *in vivo* pharmacology experience with PGx information. But how this task could be attainable and productive? Already, academics in relevant disciplines confront with obstacles in trying to integrate knowledge from PGx and personalized medicine concepts into teaching curricula and enrich the skills of students toward better handling modern therapeutics issues of practical clinical utility. The previously well-established background bridge between pharmacology and other disciplines (*e.g.*, physiology/pathophysiology, chemistry) created in order future practitioners to understand drug behavior and actions in the body is being expanded by incorporating translational information extracted (*e.g.,* biochemical, biological, molecular) including that from bioinformatics and also material sciences and nanotechnology. Unanimously, the molecular approaches applied to predict and/or assess the behavior of therapeutics in the living organisms enrich the knowledge of students and also strengthen their capacity in drug prescription for better dosage-scheme selection of administered medicines in the clinical setting. And for sure, the better education by covering the concept of PGx as well as personalized medicine will be the maximum positive impact for both academics involved and healthcare practitioners would happen; the latter, however, further necessitates the proper adjustments in academia to restructure and organize relevant innovative medical and pharmacy curricula worldwide[4-6].

DEVELOPMENT OF ADVANCED PK/PD MODELS TO IMPLEMENT MOLECULAR PHARMACOLOGY FOR ENRICHING TRANSLATIONAL MEDICINE CAPACITY IN DRUG DELIVERY

Nowadays, it has been evidenced that mechanism-based PK/PD modeling has been a necessity in modern pharmacology toward speeding up early achievements in drug discovery and ensuring improved efficacy and safety profiles of candidate molecules before their final clinical development. The latter, implies that improved prediction capabilities of crucial drug-related parameters can be documented by extrapolating *in vitro* experimentation data into *in vivo* clinical variables across species[7,8]. Importantly, however, PK/PD modeling in order to contribute greatest benefits at the preclinical and the clinical era, it also needs to be embraced across regulatory bodies and pharmaceutical industrial sector, as well as the educational process in academia[1,9]. The recent advancement of genomic medicine and systems pharmacology, however, forms the baselines for multidisciplinary translational approaches by crossing the borders between molecular pharmacology with pathophysiology,

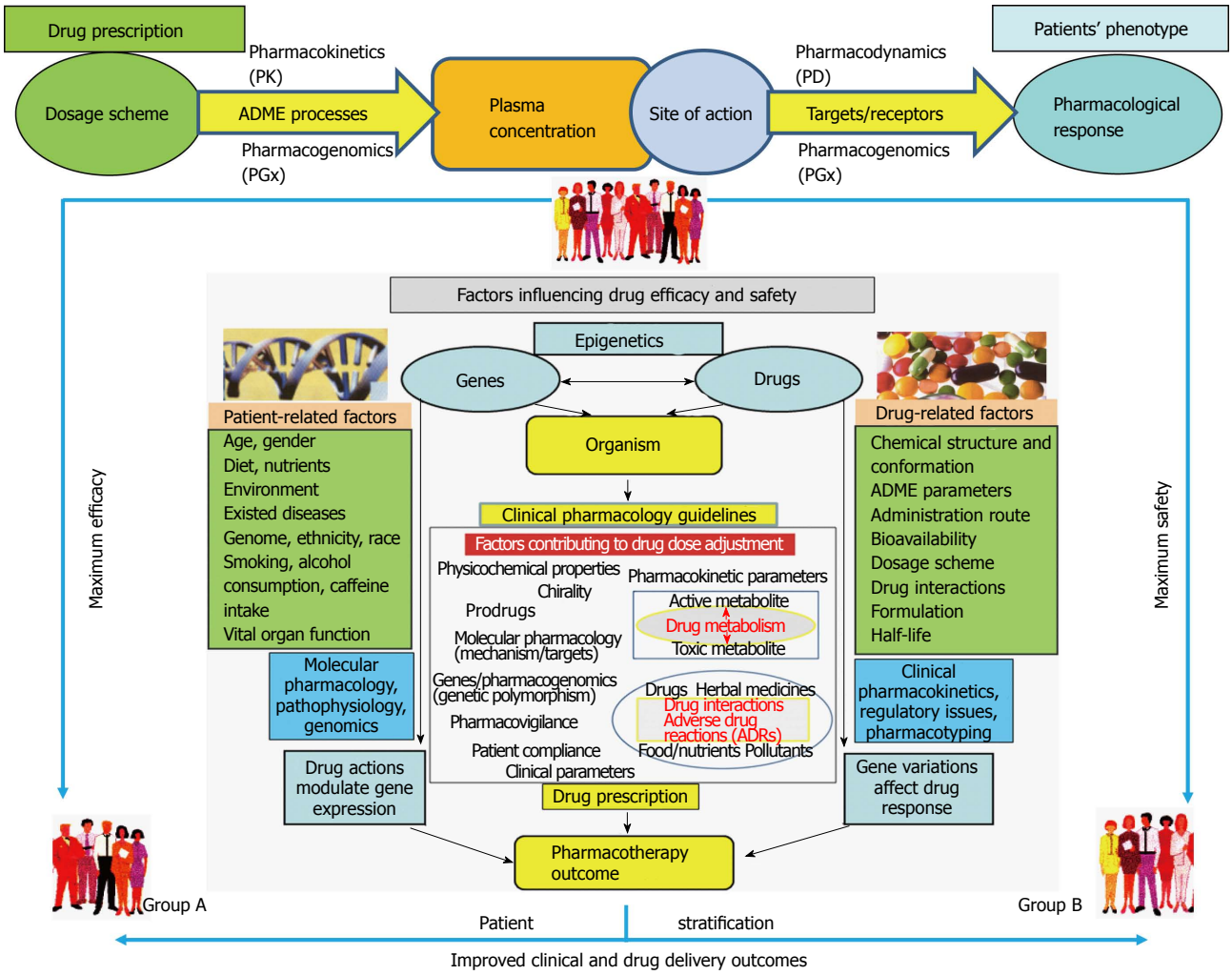

Figure 1 A roadmap of pharmacological response stages to efficiently address the pharmacotyping concepts in drug prescription. The processes and the factors related to pharmacological effects along with the scientific environment contributing to drug delivery outcomes in terms of efficacy, safety are depicted above. The need for enriching pharmacological knowledge to advance personalized medicine decisions in the clinical setting through drug dosage scheme adjustment (*i.e.*, PTx) is exemplified. The *in vivo* pharmacology experience gained thus far and it already appears in the drug regulatory environment is stressfully demanded to be empowered by pharmacogenomics knowledge in terms of PD/PK drug parameters assessment methodologies, the clinical pharmacology guidelines development and the prescription process. Complementary to this, the development of information-based workflow platforms in clinical practice incorporating algorithms to assess the efficient translation of clinical, biological, genomic and chemical information is also eagerly expected. Such a direction of major pharmacological importance permits the maximum efficacy and safety outcomes to be reached in a timely and worldwide basis for everyday healthcare (see text for more details). PTx: Pharmacotyping; PGx: Pharmacogenomics; PK: Pharmacokinetics; PD: Pharmacodynamics.

clinical sciences and genomics. In particular, systems pharmacology aims to understand the effects of drugs including ADRs in terms of pharmacological targets and within the molecular networks context that evidently has been allowing the integration of the systems biology-level in understanding the behavior of drugs in the body[10]. This direction could be proven helpful especially for complex and multifactorial illnesses where the more thorough elucidation of their molecular pathophysiology is needed; complementary, such task in turn it represents a crucial prerequisite parameter upon attempting to improve pharmacotherapy outcomes in these diseases. By enabling network analyses of interactions mediated both pathophysiological and PD/PK drug responses through the different organization levels, (from the molecular level through organ and tissue into finally the entire organism), in an integrated approach, the faster and more cost-affordable manner to empower the practical clinical utility of personalized medicine decisions will be clearly achieved[1,11-14].

It is evident, that the improved translational medicine capacity means the successful adjustment of clinical pharmacology guidelines toward personalized medicine concepts. Complementary to this point, the issue on how the already gained *in vivo* pharmacology experience can be adjusted by being enriched with relevant systems pharmacology approaches and methodologies clearly emerges in a way that the implementation in real time with the PGx knowledge could happen[15-17]. Alternatively, what it has been already established through the previously gained experience of *in vivo* pharmacology approaches, is the fact that drug pharmacological responses are evidenced by two dynamic processes being interrelated, that of PD and PK. PD describes what medicines do to the body (*i.e.*, drug-

receptor interactions), whereas PK is associated with what an organism does to therapeutics (*i.e.*, absorption, distribution, metabolism, excretion; ADME processes). As a consequence, a question then rises; on how in the new drug delivery era, maximum benefits could be ensured for all patients in terms of drug efficacy and safety? The latter task can be fulfilled only if the molecular mechanisms underlying PD/PK drug effects could decipher issues addressing either the emergence of idiosyncratic (genetic/genomic) toxic effects and ADRs in a given individual, or the involvement of environmental and epigenetic factors[1,18,19] (Figure 1). The application of predictive bioinformatic approaches and computational methodologies in evaluating PK and PD profiles of drugs represents an established approach, especially the last few decades, throughout the drug development as well as delivery processes[20]. *In silico* methods use and application of technologies to enhance the predictive capacity to ultimately improve productivity and drug delivery safety and efficacy profiles is now considered a major advancement[20-23]. In parallel, specific information-based workflow computerized healthcare systems are being developed to contribute in the exploitation of knowledge coming from interdisciplinary resources with an affordable for the end-user manner[24]. The latter, also implies the proper application of the translated knowledge into information standing types being capable to be simultaneously used in everyday healthcare approaches upon illness prognosis, diagnosis and administration of therapeutics (Figure 1).

Undoubtedly, the development of suitable translational medicine-enriched clinical pharmacology guidelines for providing instructions upon drug prescription (*e.g.*, dosage scheme adjustment, disease prognosis/diagnosis profile improvement) will efficiently facilitate the successful implementation of PGx concepts into everyday clinical practice. The latter, also refers to the information systems applied in routine patient care. Moreover importantly as it has been proposed recently, by working within this direction the maximum benefits from both nanomedicine and personalized medicine efforts is expected to happen empowering clinical outcome through the advent of personalized nanomedicine concepts at both the research and the clinical setting[25]. Complementary to this, personalized medicine is paving the way toward broader practical clinical utility of translational advancements, thus contributing toward PTx in drug prescription as well as medicine and pharmacy in general. The latter, refers to the development of clinically-applied algorithms in drug prescription regarding the genetic variables being able to affect PK and PD behavior of marketed therapeutics. The use of information-based systems into everyday healthcare has clearly shown the need of developing such unified information systems with adherence to various healthcare environments worldwide (Figure 1). To this end, the more successful development of quantitative PGx models for translation medicine is being achieved then the best benefits in PTx-based drug delivery from genetically-guided drug dose adjustment is expected[1,26,27]. In fulfilling this task of practical clinical utility, the close collaboration of clinicians with pharmacologists will pace PTx in drug prescription in a faster and more efficient manner.

HARNESSING PHARMACOLOGICAL AND TOXICOLOGICAL GENOMICS KNOWLEDGE FOR ADVANCING THE SKILLS OF FUTURE HEALTHCARE PROFESSIONALS TO IMPLEMENT PTX CONCEPTS IN DRUG PRESCRIPTION

The use of PD/PK tools implemented with biostatistics approaches in courses related to pharmacology, model-based drug development as well as predictive modeling and simulation upon pharmacological assessment empower the teaching process and ensure greatest benefits for researchers, educators, as well as students. Alternatively, in order to achieve this task for strengthening students' knowledge and skills in clinical and molecular pharmacology, PGx expertise as well as personalized medicine decision-making means of being capable to simultaneously: (1) assess and predict clinically relevant drug interactions, thus minimizing ADRs emergence risk; and (2) advance the profiles of drugs in terms of efficacy and safety for individual patients by inter-correlating clinical data, drug properties and genetic/genomic characteristics[1-6]. Moving forward in this manner for education, future healthcare practitioners will be instructed on how to more efficiently and in real time apply personalized medicine approaches in clinical practice, a fact that impose health and societal benefits in general. In addition, the successful implementation of systems pharmacology and pathophysiology approaches with *in vivo* pharmacology experience better ensures both productivity and clinical outcome for innovative molecularly-targeted therapeutics, "smart/genius" drug delivery systems, translational medicine efforts, as well as nanomedicine applications (Figure 1).

Although PGx advancements contribute clinically relevant genomics knowledge, it is also obvious however, that modern pharmacology is gaining major benefits in experimenting with new sophisticated technological methodologies in drug development and delivery era. By projecting such changes that are expected to happen for therapeutics in the near future, it is important mainly for pharmacologists in academia to be actively engaged in providing their students with strong background and skills of *in vivo* pharmacology enriched with PGx knowledge. Since the latter represents a dynamic knowledge module and an ever changing scientific environment, it means that personalized medicine concepts must be taught to allow therapeutic decisions in real time and for all pharmacological drug classes. In such case, young healthcare practitioners will be trained in getting capable for individualized prescription of drug dosage schemes,

thus minimizing the risk for toxicity, the emergence of interactions and ADRs in clinical practice. It is thus crucial for pharmacologists to prioritize the steps and the process needed to be considered in pharmacology curricula, as well as to set-up a pharmacology-focused roadmap for achieving broader utility of personalized medicine decisions.

Nowadays, laboratory medicine techniques have received major impact from genomics methodologies and experimentation. The availability of methodologies and tools allowing the simultaneous assessment of various source data of clinical relevance (*e.g.*, drug-related, genomics-focused, clinical measurements) clearly contribute toward individualized therapeutic decisions in routine healthcare. Moving forward and in order to improve pharmacology-related productivity and clinical outcome issues, this means that the creation of platforms where the pharmacological assessment and the clinical exploitation of PK/PD-related molecular targets is happening throughout the drug discovery and development process; for example, the improvement of PK/PD behavior of the designed molecularly-targeted therapeutics in the body will be better served and secured[28]. Moreover, the beneficial implementation of functional mapping framework in pharmacology by smoothly addressing PGx data integration into PK/PD processes will be greatly benefited, as proposed[29]. In that case, the already applied PK/PD-related mathematical models could be effectively coupled with PGx data referring to specific pharmacology-focused molecular networks and signaling pathways. The interdisciplinary nature of the framework and infrastructure needed represents, however, tedious and long-standing processes that obviously also rely on elucidation of illness etiology and drug behavior profiles. Besides, the establishment of selective molecular biomarkers with broader clinical validity and utility for most, if not all, pathological disorders and marketed therapeutics have to be clearly addressed. Moreover upon formulating personalized medicine decisions, the developed genomics-related methodology profiling (*e.g.*, genome-wide linkage analysis including proteomics-, genotyping-, gene array-, transcriptomics- and/or metabolomics-related data) must exhibit broader advantages in laboratory medicine applications for all patients worldwide[28-33]. The latter, means that the capability of molecular diagnostics to help addressing routine therapeutic decisions in real time is the most desirable goal nowadays for PTx and personalized medicine concepts.

ESTABLISHMENT OF NETWORK AND SYSTEMS PHARMACOLOGY METHODOLOGIES IN ENRICHING *IN VIVO* PHARMACOLOGY EXPERIENCE TOWARD FORMULATING PTX-BASED CLINICAL PHARMACOLOGY GUIDELINES

Unanimously, the availability of predictive tools to effectively address issues related to safety and efficacy profiles of therapeutics within the body represent a major task toward improving productivity and clinical outcomes of drug candidate molecules. Especially by considering the whole drug discovery and development process, that need is even more stressful at preclinical-clinical phase of development; such capacity in predicting safety and efficacy therapeutic outcomes very early is considered crucial toward establishing focused personalized medicine decisions of broader clinical utility. To do so, the implementation of clinical pharmacology guidelines has to be achieved through the knowledge coming from the use of cost-affordable PGx molecular diagnostics[3,34]. For example, the organization and development of evidence-based PGx guidelines in clinical practice represents an obstacle hindering translational medicine efforts in drug discovery and development from bench to bedside[35-37]. To this end and although various issues (*e.g.*, reimbursement, social, cost-benefit and ethical) should be simultaneously addressed, specific efforts for formulating PGx guidelines for dose recommendation schemes in specific pharmacological drug classes has been initiated and proposed[38-43].

The development and application of physiologically based pharmacokinetic (PBPK) modeling for key PK-related processes have been greatly appreciated in drug delivery and development era. The PBPK models capacity also permits to assess, predict and evaluate in a quantitative basis the potential clinical effect of drug interactions along with any impact related to disease status, genetic make-up, environmental factors and/or drug formulation properties[44,45]. Such an effort is presented in more detail elsewhere[34]. As far as the PGx data exploitation is concerned, it is crucial for example to understand that only in the circumstances where the genetic variation represents the rate-limiting PK/PD step it would be possible to directly inter-correlate such molecular knowledge with the predictability profile in drug plasma concentration; and then beneficial for the practical utility of personalized medicine to proceed toward adjusting dosage scheme for individual patients based on their genetic variation (Figure 1). Such a direction upon drug prescription, in order not to be restricted in clinical pharmacology guidelines have to also effectively integrate and address issues related to the PGx concepts, the drug interactions knowledge, as well as the emergence of ADRs[46]. For having PTx success, this relies on the ability to use drug interactions knowledge being efficiently inter-correlated with PGx knowledge for genes mediated the PK/PD behavior of therapeutics. Moving ahead, such direction necessitates a structure where suitably constructed PGx models assembling large cohort studies will be established to better serve: (1) the interdisciplinary data assessment; (2) the dissemination and broader clinical utility of personal genetic information upon illness risk prevention; and also (3) the use of PTx-based concepts upon medicines prescription[1,34]. Moreover, the importance of having in these scientific attempts expert pharmacologists to participate is crucial,

since pharmacologists would be capable to verify: (1) the enrichment of *in vivo* pharmacology experience; (2) the efficient translation of PGx information to implement therapeutics decisions; and last, but not least, (3) the adjustment of drug dosage schemes, thus making personalized medicine decisions to benefit routine clinical practice, or alternatively, to achieve PTx for individual patient populations, if not all patients.

PBPK MODELING APPROACHES TO IMPLEMENT *IN VIVO* PHARMACOLOGY EXPERIENCE WITH PGX KNOWLEDGE FOR ENSURING PTX IN CANCER THERAPY: THE CASE OF TAMOXIFEN

PGx of anticancer drugs is now considered an integral part of cancer therapy[47]. Indeed, a number of predictive PGx biomarkers to assess the safety and efficacy clinical profiles of individual marketed anticancer drugs has been validated by drug regulatory agencies (*e.g.*, the FDA and EMA) and are shown in Table 1. As mentioned above, however, the development of PBPK models implemented with systems pharmacology approaches, (assessing predictive PGx biomarkers), represents a platform where in real time the assessment of both patient-related and drug-related factors can be intercorrelated to achieve maximum efficacy and safety outcome for individual populations, *i.e.,* PTx (Figure 1). Alternatively, the latter means the elaboration of a multidisciplinary environment in order both the assessment of drug interactions and PGx data to be effectively incorporated to guide drug prescription. To better clarify this issue by analyzing the complexity existed and the hurdles needed to be overcome, the example of tamoxifen and serotonin reuptake inhibitors (SSRIs) will be further considered.

Accumulated evidence over the previous years have clearly postulated the contribution of genetic polymorphic variants of CYP2C19 and CYP2D6 (drug metabolizing enzymes) to the pharmacological response of psychotropic drugs in clinical practice[48-50]. To this end, a specific guideline for psychiatrists providing practical recommendations upon the prescription of psychotropic drugs based both on clinical drug-related as well as CYP2D6 and CYP2C19 PGx data for individual patient populations has been proposed. Although, the broader clinical applicability of such instructions is still elusive, however, the improvement of PK and PD profile toward achieving personalized medicine decisions for psychotropic drug coincides with the capacity to simultaneously assess *CYP* genes variants in routine healthcare[51-54].

The fact that metabolism of psychotropic drugs including antidepressants represents a rate-limiting step in their pharmacological profile means that specific CYP polymorphic variant forms (*e.g.*, CYP2D6) contribute either to toxicity and/or drug inefficiency in specific individual populations. Moreover, since some antidepressants are also CYP2D6 inhibitors, clinically-relevant drug interactions are expected upon their co-administration with other drugs whose pharmacological activity is based on CYP2D6 function like tamoxifen[55]. The clinical efficacy of tamoxifen varies widely among breast cancer women depending on their CYP2D6 genotype. Tamoxifen is a pro-drug which means that its active metabolite 4-hydroxytamoxifen and endoxifen being produced through the function of CYP2D6 mediates the pharmacological anti-estrogen action in the body. At the same time, co-administration of SSRIs antidepressants had previously been a common routine clinical practice and prescribed to treat hot flashes in women who take tamoxifen[56-61]. But at what extent, however, would be clinically validated the predictive capacity in dosage scheme to ensure tamoxifen efficacy (active metabolites plasma concentration) and safety (toxicity, *i.e.*, hot flashes) based on CYP2D6 function affected by genotypes and SSRIs inhibitory behavior? Moving ahead, it has been proven that either women exhibiting polymorphic null-activity for CYP2D6 (PMs; CYP2D6 poor-metabolizers), or patients under tamoxifen chemotherapy co-prescribed with potent CYP2D6 inhibitors (*e.g.*, antidepressant drugs fluoxetine and paroxetine) show a greater risk of breast cancer recurrence and mortality due to decreased levels of active tamoxifen metabolites formed in their organism. That knowledge now proposes that personalized dosage schemes of tamoxifen administration to individual or population of patients are achieved through: (1) for CYP2D6 PM women by avoiding the co-prescription of tamoxifen with SSRIs or other medicines acting as potent CYP2D6 inhibitors; (2) Alternatively, similar improvement can be achieved through the proper dose adjustment of tamoxifen, or alternatively by switching into another hormonal therapy drug class (*e.g.*, aromatase inhibitors); and (3) For breast cancer patients exhibiting normal CYP2D6 metabolism (phenotype of CYP2D6 extensive metabolizers) by selecting the co-administration of an antidepressant that exhibits no inhibitor activity for CYP2D6 (*e.g.*, venlafaxine which represents a weak CYP2D6 inhibitor). But even in this case, the clinical effectiveness and the cost-effectiveness of CYP2D6 genotyping for the management of women with breast cancer treated with tamoxifen still needs to be validated[62]. However, having this knowledge in mind one can further consider the possibility of developing advanced PBPK models in order to: (1) more thoroughly exploit clinical, pharmacological and PGx data of drugs; (2) develop proper algorithms to implement drug prescription; and (3) to facilitate new drug development productivity through predicting PD/PK behavior and reduce attrition rates in potential drug candidate molecules. Recently, the successful PBPK model development for tamoxifen delivery and for the evaluation of PK of patients with cancer clearly shows the dynamics of such scientific approaches[63,64]. And more importantly this dynamics of PBPK modeling is further strengthened from research efforts reshaping the field of PD/PK modeling by enhancing the capacity to efficiently predict drug

Table 1 Genes used as predictive pharmacogenomics biomarkers to assess the safety and efficacy clinical profiles of individual marketed anticancer drugs[1,2]

Drug	Gene	Safety/efficacy profile
Anastozole	*ER*	Lower efficacy; no response in cancer patients with tumor *ER*-negative expression
Capecitabine	*DPYD*	Lower safety; ADRs; Orodigestive neutropenia
Cetuximab	*EGFR*	Lower efficacy; no response in cancer patients with tumor *EGFR*-negative expression
	K-RAS	Lower efficacy; no response in cancer patients with tumor specific *K-RAS* mutations
Cisplatin	*TPMT*	Lower safety; ADRs; cytotoxicity associated with hearing loss in children
Crizotinib	*ALK*	Efficacy; indication only in patients bearing *ALK* gene rearrangement positive tumors (*EML4-ALK* translocation)
Dabrafenib	*BRAF*	Efficacy; indicated only in melanoma patients with BRAF V600E mutation
	G6PD	Safety; ADRs; toxicity in G6PD deficient patients
Dasatinib	*Ph+*	Efficacy; indicated only for Ph^+ tumors
Erlotinib	*EGFR*	Lower efficacy; no response in cancer patients with tumor *EGFR*-negative expression
Everolimus	*Her2/Neu*	Efficacy; indicated in HER2 protein overexpression negative in breast cancer women
	ER	Efficacy; indicated for breast cancer women bearing ER positive tumors ($ESR1^+$)
Exemestane	*ER*	Lower efficacy; no response in cancer patients with tumor *ER*-negative expression
Imatinib	*Ph+*	Efficacy; indicated only for Ph^+ tumors
	PDGFR	Efficacy; indicated in myelodysplastic- myeloproliferative syndromes with *PGFRR* gene rearrangements
	FIP1L1-PDGFRA	Efficacy; assessment of *FIP1L1-PDGFRA* translocation -fusion kinase in tumors
	c-kit	Lower efficacy; no response in cancer patients with absence of tumor activating *c-Kit* mutations
Irinotecan	*UGT1A1*	Lower safety; ADRs; diarrhea, increased risk for severe neutropenia in high doses of irinotecan
Lapatinib	*Her2/Neu*	Efficacy; indicated for over-expressing *Her2/Neu* advanced or metastatic breast cancer
Letrozole	*ER*	Lower efficacy; no response in cancer patients with tumor *ER*-negative expression
Nilotinib	*Ph+*	Efficacy; indicated only for Ph^+ tumors
	UGT1A1	Safety; increased risk of hyperbilirubinemia in patients with UGT1A1*28 genotype
6-Mercaptopurine	*TPMT*	Lower safety; ADRs; neutropenia
Panitumumab	*EGFR*	Lower efficacy; no response in cancer patients with tumor *EGFR*-negative expression
	K-RAS	Lower efficacy; no response in cancer patients with tumor specific *K-RAS* mutations
Pertuzumab	*HER2/Neu*	Efficacy; indicated only for $HER2/Neu^+$ breast cancer
Tamoxifen	*ER*	Lower efficacy; no response in cancer patients with tumor *ER*-negative expression
	CYP2D6	Lower efficacy; loss of therapeutic benefit for PMs and/or upon co-administration with CYP2D6 inhibitors; lower plasma levels of active metabolite endoxifen achieved
	FV	Safety; ADRs; risk for venous thromboembolism in breast cancer women also bearing factor V Leiden (FLV) mutations
	F2	Safety; ADRs; risk for venous thromboembolism in breast cancer women also bearing factor II (prothrombin) mutations
Thioguanine	*TPMT*	Lower safety; ADRs; Neutropenia
Trastuzumab	*HER2/Neu*	Lower efficacy; no response in cancer patients with tumor *HER2/Neu*-negative expression
Vemurafenib	*BRAF*	Efficacy; indicated only in melanoma patients whose tumors has a mutation at amino acid 600 of the B-raf protein (V600E and/or V600K BRAF mutations)

[1]See also the table of PGx biomarkers in drug labeling at the FDA that can be accessed at: http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm (Accessed on June 27, 2014); [2]Additional data can be seen in "The Pharmacogenomics Knowledge Base (PharmGKB) at: https://www.pharmgkb.org/ (Accessed on June 29, 2013). ADRs: Adverse drug reactions; G6PD: Glucose-6-phosphate dehydrogenase deficiency; PM: Poor metabolizers: ER: Estrogen receptor; TMTP: Thiopurine methyltransferase gene; DPYD: Dihydropyrimidine dehydrogenase gene; Ph: Philadelphia chromosome; FV: Factor V; F2: Factor II (prothrombin).

effects in the body[65-68]. This is an example for PTx on how the pharmacological knowledge of drug interactions covering both clinical and biochemical knowledge can be efficiently inter-correlated with PGx information of genes mediating the PK/PD behavior of therapeutics to improve delivered medicines clinical outcomes. The need for well-educated physicians and pharmacists, the proper clinical pharmacology/PGx guidelines development and adjustment, as well as healthcare infrastructure organization equipped with suitable clinically-validated technological methodologies is now, more than ever, stressful and demanding.

The analysis presented above for tamoxifen and SSRIs imply that the broader clinical utility of personalized medicine as well as PTx will be also strengthened by developing pharmacology-focused functional mapping frameworks for most, if not all, specific pharmacological drug classes. In such a case, additional benefits for translational medicine will be gained; alternatively, this refers to the successful implementation of PD/PK data and the new drug development environment with PGx knowledge[34]. Such modeling approaches clearly strengthen healthcare efforts toward the establishment of PTx as a new drug prescription "philosophy" in drug delivery. The latter, means that the practical utility of genomics information is conceptually exploited to ensure maximum safety and efficacy profiles. This way toward achieving PTx represents a very complex task that is clearly documented in the case of tamoxifen prescription where the CYP2D6 pharmacogenomics assessment by healthcare decision makers well documented the steps still needed to be addressed[69,70]. In the meantime, however, the

proper education of healthcare professionals has to be adjusted to fulfill expectations for the PTx roadmap in personalized medicine. Importantly, to highlight education needs and also to facilitate the teaching process in the revised curricula of various professionals engaged in this topic, a recently edited book volume has been organized and released as a first attempt to fill the gap in terms of the multidisciplinary perspective for personalized medicine[1].

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P- Reviewer: Cepeda C, Lee TM **S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i4.120

World J Pharmacol 2014 December 9; 3(4): 120-139
ISSN 2220-3192 (online)
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REVIEW

Phosphoprotein phosphatase 1-interacting proteins as therapeutic targets in prostate cancer

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Author contributions: Felgueiras J and Fardilha M contributed to this paper.
Supported by Fundação para a Ciência e Tecnologia (FCT) (PTDC/QUI-BIQ/118492/2010) and Fundo Europeu de Desenvolvimento Regional (FEDER) (FCOMP-01-0124-FEDER-020895), Portugal
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Received: June 29, 2014 Revised: September 1, 2014
Accepted: September 23, 2014
Published online: December 9, 2014

Abstract

Prostate cancer is a major public health concern worldwide, being one of the most prevalent cancers in men. Great improvements have been made both in terms of early diagnosis and therapeutics. However, there is still an urgent need for reliable biomarkers that could overcome the lack of cancer-specificity of prostate-specific antigen, as well as alternative therapeutic targets for advanced metastatic cases. Reversible phosphorylation of proteins is a post-translational modification critical to the regulation of numerous cellular processes. Phosphoprotein phosphatase 1 (PPP1) is a major serine/threonine phosphatase, whose specificity is determined by its interacting proteins. These interactors can be PPP1 substrates, regulators, or even both. Deregulation of this protein-protein interaction network alters cell dynamics and underlies the development of several cancer hallmarks. Therefore, the identification of PPP1 interactome in specific cellular context is of crucial importance. The knowledge on PPP1 complexes in prostate cancer remains scarce, with only 4 holoenzymes characterized in human prostate cancer models. However, an increasing number of PPP1 interactors have been identified as expressed in human prostate tissue, including the tumor suppressors TP53 and RB1. Efforts should be made in order to identify the role of such proteins in prostate carcinogenesis, since only 26 have yet well-recognized roles. Here, we revise literature and human protein databases to provide an in-depth knowledge on the biological significance of PPP1 complexes in human prostate carcinogenesis and their potential use as therapeutic targets for the development of new therapies for prostate cancer.

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Key words: Prostate cancer; Reversible phosphorylation; Phosphoprotein phosphatase 1; Phosphoprotein phosphatase 1-interacting proteins; Protein complexes; Therapeutic targets

Core tip: Protein kinases and phosphatases are challenging and valuable therapeutic targets for cancer. Here, we revise the relevance of phosphoprotein phosphatase 1 and its interactors for prostate carcinogenesis. Although only 4 complexes are characterized in human prostate cancer models, 81 additional interactors are expressed in human prostate tissue and, at least, 29 of which are involved in prostate carcinogenesis. This complex network has promising roles in the development of new therapies for prostate cancer. Therefore, efforts should be made in order to characterize their biological significance in prostate carcinogenesis.

Felgueiras J, Fardilha M. Phosphoprotein phosphatase 1-interacting proteins as therapeutic targets in prostate cancer. *World J Pharmacol* 2014; 3(4): 120-139 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/120.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.120

INTRODUCTION

Prostate cancer (PCa) is the second most commonly diagnosed non-cutaneous cancer and a leading cause of cancer-related death in men worldwide[1]. In spite of the recent advances in early diagnosis and therapeutic management of the disease, prognoses are still poor once the disease progresses to castration-resistant and metastasizes, mainly to bone[2,3]. The urgent need for a panel of reliable biomarkers that could overcome the lack of cancer-specificity of prostate-specific antigen (PSA), as well as alternative therapeutic targets is challenging the scientific community[4].

Reversible phosphorylation of proteins regulates more than 70% of all eukaryotic cellular processes[5]. Phosphorylation at serine (Ser) and threonine (Thr) residues is accomplished by protein Ser/Thr kinases (PSTKs) and reversed by protein Ser/Thr phosphatases (PSTPs)[6]. Deregulation of the counterbalanced action between PSTKs and PSTPs is frequently associated with system-wide disruption of signal transduction and malignant transformation of cells[7,8]. For this reason, emergent studies have been focused on large-scale examination of PCa phosphoproteome[9-12]. Androgen receptor (AR), for instance, is a phosphoprotein vital to the development and progression of PCa that presents, at least, 15 Ser and Thr phosphorylated residues. Phosphorylation of such residues modulates the transcriptional activity, subcellular localization, and stability of the AR[13].

The mammalian genome encodes considerably more PSTKs than PSTPs-nearly 10 to 1[14]. Hence, the success of the protein reversible phosphorylation system depends on the ability of PSTPs to form stable complexes with other proteins, giving rise to a huge number of distinct holoenzymes. This is particularly true for phosphoprotein phosphatase 1 (PPP1), a major PSTP of eukaryotic cells that controls a myriad of processes: glycogen metabolism, muscle contraction, RNA splicing, apoptosis, protein synthesis, cell cycle, among others[15-18]. PPP1 exhibits an effective catalytic machinery, but lacks substrate specificity. Therefore, a number of regulatory subunits, also known as PPP1-interacting proteins (PIPs), have been associated with the spatiotemporal regulation of PPP1 activity[19]. Given the key roles of PIPs, efforts have been made to characterize PPP1 interactomes in human tissues and to identify disease relevant PIPs[20-24].

In contrast to PSTKs, whose therapeutic benefits have been largely explored, PSTPs had been considered not "drug-targetable" for years and, thus, remain understudied[25,26]. In the case of PPP1, this vision is changing due to the increasing number of PPP1 holoenzymes that have been described, and which seem to be attractive targets for the development of new therapies[27]. In fact, pharmaceutical companies are being encouraged to pursue approaches that aim the inhibition or activation of PPP1 holoenzymes.

Here, we revise literature and human protein databases to provide an in-depth knowledge on the relevance of PIPs, expressed in human prostate tissue, for prostate carcinogenesis. Moreover, we address the biological significance of their interaction with PPP1 and consider their potential use as therapeutic targets for the management of human PCa.

RESEARCH

A comprehensive literature search of studies involving human samples or human cell lines was performed to identify articles on PPP1 and its interactors in human PCa. The Pubmed database was searched until May 2014 using the Medical Subject Heading (MeSH) whenever possible-for terms not included in MeSH (*e.g.*, "PP1-interacting protein" or "PP1 interactor") a basic Pubmed search was employed instead. MeSH terms included: ("protein phosphatase 1" or "(PIP abbreviation) protein, human") and ("prostate" or "prostatic neoplasms"). Reference lists of included studies and review articles were manually searched. The search was restricted to English-language literature.

For the sake of completeness, databases were reviewed: TissueNet and HIPPIE were used to identify additional PIPs expressed in human prostate tissue; BioGPS and The Human Protein Atlas were used to assess mRNA and protein expression levels, respectively; Gene Ontology Consortium was used to identify the biological processes in which proteins are involved; and, ScanProsite was used to identify PPP1 binding motifs for each PIP.

OVERVIEW OF PHOSPHOPROTEIN PHOSPHATASE 1 STRUCTURE

PPP1 is one of the most conserved proteins in eukaryotic species[28,29]. In mammals, three genes encode the catalytic isoforms of the enzyme-PPP1CA, PPP1CB, and PPP1CC-which are ubiquitously expressed. Additionally, *PPP1CC* gene can generate two splice variants-PPP1CC1 and PPP1CC2-with the latter one being testis-enriched. This catalytic core is analogous, both in terms of structure and mechanism of action, to all members of the phosphoprotein phosphatase superfamily[30]. The major divergences among PPP1 isoforms are found at NH_2- and COOH-terminal sequences[15]. Interestingly, the N-terminal was shown to influence the properties of the active site and, consequently, the function of the enzyme and its sensitivity to inhibitors[31,32].

PPP1 catalytic isoforms are not found freely in cells. PPP1 catalytic subunit (PPP1C) interacts with diverse regulatory subunits, known as PIPs, thus enabling the formation of distinct PPP1 multimeric holoenzymes. The nature of the relationship between PPP1 and PIPs greatly varies: (1) PIPs can be substrates for PPP1, with their functions being directly controlled through dephosphorylation by PPP1; (2) PIPs can determine the substrate specificity of PPP1 by either targeting PPP1C to specific subcellular compartments or enhancing/suppressing PPP1C activity towards different substrates; and

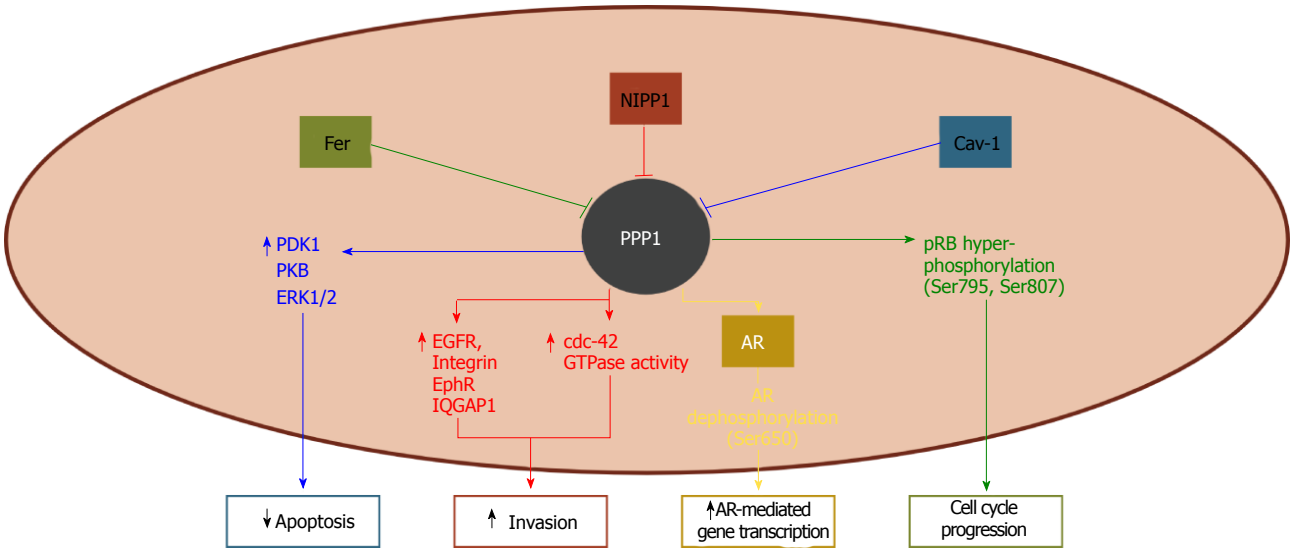

Figure 1 Phosphoprotein phosphatase-1 complexes described in human prostate cancer. Phosphoprotein phosphatase-1 (PPP1) dephosphorylates pRb at Ser795 and Ser807 and contributes to its hypophosphorylated and activated state. The activity of Fer tyrosine kinase leads to the inhibition of PPP1 and consequent hyperphosphorylation of pRb, which culminate in poor G_1-S transition control. The inhibition of PPP1 by the nuclear inhibitor of protein phosphatase 1 (NIPP1) increases the expression of epidermal growth factor receptor (EGFR), integrin, epherin receptor (EphR), and Ras GTPase-activating-like protein IQGAP1, as well as enhances the activity of cdc-42 GTPase. This, in turn, promotes invasiveness of tumor cells. Caveolin-1 (Cav-1) inhibits PPP1 and potentiates the activity of phosphoinositide-dependent kinase-1 (PDK1), protein kinase B (PKB), and extracellular signal-regulated kinase 1/2 (ERK1/2), increasing cell survival. PPP1 specifically dephosphorylates androgen receptor (AR) at Ser650, thus inhibiting AR nuclear export and enhancing AR-mediated gene transcription.

(3) some PIPs are simultaneously substrates and regulators of PPP1[15,19]. More than 200 holoenzymes have already been identified and characterized, but thousand more remain unknown[15,33].

PPP1 is not able to recognize a consensus sequence near the phosphorylated residue of its phosphotarget. Instead, PPP1C binding is mostly mediated by a short sequence (usually four to eight residues long), remote from the active site, commonly referred to as docking motif[19]. A number of novel PPP1 binding sites have been mapped in PIPs, such as SILK and MyPhoNE, but the RVxF motif (x = any amino acid except proline) is still the most frequent, described for more than 70% of PIPs[34,35]. It was also reported that some PIPs display isoform-specificity, suggesting that they possess isoform-specific docking motifs with putative location at the N- or C-terminal[19].

PHOSPHOPROTEIN PHOSPHATASE 1 COMPLEXES CHARACTERIZED IN HUMAN PROSTATE CANCER

Imbalances in the protein phosphorylation system strongly contribute to carcinogenesis. In addition to the constitutive activation of oncogenic protein kinases, there is also evidence that the gain and loss of phosphorylation sites in relevant signaling proteins occur in human cancers[36,37].

PPP1 has been shown to take part of various complexes that control cancer hallmarks[24,38]. However, whether the role of PPP1 is pro- or anti-cancer largely depends on its interacting partners and cellular context. For instance, the interaction between PPP1CA and tensin1 impairs the migration and invasion of cancer cells, and the interaction between PPP1CC and hScrib downregulates extracellular signal-regulated kinase (ERK) signaling, thus suppressing oncogene-induced transformation of primary rodent cells[39,40]. On the other hand, the interplay between PPP1 and transforming growth factor-β (TGFβ) drives malignant transformation of premalignant oral lesion cells[41].

The knowledge on the involvement of PPP1 complexes in human PCa remains scarce. Few complexes have actually been characterized and even those are not fully understood; nonetheless, such complexes seem to have central roles in prostate carcinogenesis (Figure 1).

Androgen receptor/Phosphoprotein phosphatase 1

AR plays a key role in the development of PCa and, accordingly, androgen deprivation therapy is the standard hormonal treatment for the disease[42,43]. AR is regulated by phosphorylation at multiple Ser residues and PPP1CA was shown to specifically reverse phosphorylation at Ser650[44]. Since phosphoSer650 mediates AR nuclear export, PPP1CA dephosphorylation increases the stability and the transcriptional activity of the AR (Figure 1)[44,45]. Besides being a substrate for PPP1, AR may also regulate the phosphatase activity by targeting it to chromatin, where PPP1 can modulate transcription and splicing events[44].

Nuclear inhibitor of protein phosphatase 1 / Phosphoprotein phosphatase 1

Nuclear inhibitor of protein phosphatase 1 (NIPP1) is a ubiquitously expressed scaffold protein that was firstly identified as a PPP1 inhibitor[46]. The interaction between NIPP1 and PPP1 was shown to orientate cell migration

by regulating the expression of integrin and growth factor receptors, and the activity of Cdc42 GTPase (Figure 1). Genetic disruption of this complex decreases the directional migration of PC-3 cells and impairs their migratory potential[47].

Fer tyrosine kinase /Phosphoprotein phosphatase 1

Fer tyrosine kinase is highly expressed in human malignant prostate tissues compared to normal or benign tissues, which suggests its involvement in PCa progression[48]. Fer interacts with signal transducer and activator of transcription 3 (STAT3) and phosphorylates AR at Tyr223, thus contributing to interleukin-6 (IL-6)-mediated AR activation and cell growth[49,50]. Downregulation of Fer results in the activation of PPP1CA and consequent hypo-phosphorylation and activation of retinoblastoma protein (RB1), which, in turn, leads to cells arrest at the G_0/G_1 phase (Figure 1)[51]. Accordingly, downregulation of Fer impairs the proliferation of PCa cells and their ability to form colonies in soft agar [48].

Caveolin-1 /Phosphoprotein phosphatase 1

Caveolin-1 (Cav-1) is overexpressed in human PCa and correlates positively with Gleason score, thus being suggested as a potential prognostic marker[52-55]. It has also been proposed as biomarker to monitor the response to treatments with dasatinib and sunitinib[56]. Cav-1-mediated cell survival depends on its interaction with and inhibition of PPP1 (and also PPP2), leading to the increased activity of phosphoinositide-dependent kinase-1, v-akt murine thymoma viral oncogene homolog (AKT), and ERK1/2 (Figure 1)[57].

ADDITIONAL PHOSPHOPROTEIN PHOSPHATASE 1-INTERACTING PROTEINS EXPRESSED IN HUMAN PROSTATE TISSUE

In spite of the limited number of PPP1 complexes experimentally characterized in human PCa models, several additional PIPs have already been identified as expressed in human prostate tissue. The human prostate proteome includes a total of 81 hitherto experimentally detected PIPs (Table 1)[58,59], but many more may remain unknown. None of the interactors is prostate-specific; however, 28 are highly expressed in prostate tissue, namely BAD, BCL2, CCND1, CUEDC2, GABARAPL2, HCFC1, HDAC1, HDAC10, HEYL, IKBKB, LMTK2, MAP1LC3B, MYC, NOM1, PPP1R3D, PPP1R7, PPP1R11, PPP1R13B, PPP1R37, RB1, RRP1B, RYR2, SH2D4A, STAM, STAU1, SYTL2, TRIM28, and ZFYVE9 (Table 1)[60].

Of the interactions identified, 67 were described for a specific PPP1 isoform, while 6 seem to be common to all isoforms (Table 1)[58,59]. In the vast majority, binding to PPP1 is assured *via* RVxF motif, although less described SILK, MyPhoNE, RARA, and other motifs (*e.g.,* apoptotic signature motifs and inhibitor-2 degenerate motif) are also present in some PIPs (Table 1).

INTERACTORS OF PHOSPHOPROTEIN PHOSPHATASE 1 IN PROSTATE CARCINOGENESIS: VALUABLE TOOLS FOR CANCER MANAGEMENT

PIPs expressed in human prostate tissue are key mediators of several signaling pathways and cellular processes, such as apoptosis, transcription, cell cycle, development/differentiation, and immunology/inflammation.

In the context of human prostate carcinogenesis, only 31 of these proteins have well-recognized functions. In this section, we revise the contribution of these PIPs to prostate carcinogenesis, focusing on studies involving human tissue samples or cell lines.

Apoptotic protease-activating factor 1

Apoptotic protease-activating factor 1 (APAF1) is responsible for the cleavage of procaspase-9 and mitochondria-mediated activation of caspases-9, being a major effector of apoptosis[61].

An alternative splicing product of APAF1, known as APAF1-ALT, was found in LNCaP cells. APAF1-ALT exhibits a defective pro-apoptotic function and its expression was shown to be increased under infective conditions. Therefore, this spliced form may be particularly involved in inflammation and carcinogenesis, since it compromises the apoptotic pathway[62].

Resveratrol, sulforaphane, and vitamin D3 exert their tumor suppressive functions through changes in the gene that encodes for APAF1, at least in part[63-65]. APAF1 apoptosome is also involved in malignant cells-selective induction of apoptosis by apoptin[66].

Ataxia telangiectasia mutated kinase

Ataxia telangiectasia mutated (ATM) is a ubiquitously expressed Ser/Thr kinase with a wide spectrum of downstream targets involved in cell-cycle control, DNA repair after radiation-induced damage, and apoptosis[67].

The expression levels of ATM are similar or higher in PCa samples compared to normal prostate tissue; however, its activation is higher in precursor stages of prostate tumorigenesis, like PIN[68,69].

Variants of the *ATM* gene have been associated with the risk of PCa development, and might be useful predictive markers of adverse responses to radiotherapy[70-72]. ATM maintains telomeres' length and mediates tumor surveillance[68,69,73].

Downregulation of ATM increases LNCaP, DU-145, and PC-3 cells' sensitivity to radiation-induced apoptosis[74-77]. The molecular events that arose from ATM inhibition include increased mitotic index, augmented expression of E2F transcription factor and proliferating cell nuclear antigen, and inhibition of G_2 arrest in response

Table 1 Interactors of phosphoprotein phosphatase-1 expressed in human prostate tissue

PIP	Uniprot ID	Biological processes	PPP1 specificity	PPP1 binding motif
AKAP11	Q9UKA4	Intracellular signal transduction	PPP1CB, PPP1CC	RVxF, other motifs
APAF1	O14727	Apoptosis	PPP1CA	RVxF, other motifs
ATM	Q13315	Cell cycle; response to DNA damage; protein phosphorylation	PPP1CA	RVxF, SILK
AXIN1	O15169	Intracellular signal transduction; apoptosis; regulation of protein phosphorylation; transcription	PPP1CA	-
BAD[1]	Q92934	Apoptosis	PPP1CA	other motifs
BCL2[1]	P10415	Apoptosis; response to DNA damage; transmembrane transport	PPP1CA, PPP1CB	RVxF, other motifs
BCL2L2	Q92843	Apoptosis	PPP1CA	RVxF, other motifs
BRCA1	P38398	Cell cycle; DNA repair; lipid metabolism	PPP1CA, PPP1CB, PPP1CC	RVxF, other motifs
CCND1[1]	P24385	Cell cycle; response to DNA damage; transcription	PPP1CB	-
CCND3	P30281	Cell cycle; intracellular signal transduction; protein phosphorylation	PPP1CB	-
CDC5L	Q99459	Cell cycle; transcription; mRNA splicing	PPP1CA	RVxF
CDC34	P49427	Cell cycle; protein ubiquitination; intracellular signal transduction	PPP1CB	other motifs
CDK2	P24941	Cell cycle; DNA repair; meiosis; mitosis; intracellular signal transduction; cell proliferation	PPP1CA	-
CDK4	P11802	Cell cycle; protein phosphorylation; cell proliferation	PPP1CA	-
CSRNP2	Q9H175	Apoptosis; transcription; protein phosphorylation	PPP1CA	RVxF, SILK
CUEDC2[1]	Q9H467	Intracellular signal transduction	PPP1CA	-
CUL1	Q13616	Host-virus interaction; intracellular signal transduction	PPP1CA	-
EED	O75530	Transcription	PPP1CA	RVxF
EIF2AK2	P19525	Transcription; immunity; host-virus interaction	PPP1CA	other motifs
GABARAP	O95166	Apoptosis; autophagy; transport	PPP1CC	other motifs
GABARAPL2[1]	P60520	Autophagy; transport	PPP1CC	-
GRB2	P62993	Host-virus interaction	PPP1CB	RVxF
GSK3B	P49841	Carbohydrate metabolism; differentiation; intracellular signal transduction	PPP1CA	-
HCFC1[1]	P51610	Cell cycle; host-virus interaction	PPP1CA	RVxF, RARA
HDAC1[1]	Q13547	Transcription; host-virus interaction; biological rhythms	PPP1CC	RVxF
HDAC6	Q9UBN7	Transcription; autophagy	PPP1CC	RVxF
HDAC8	Q9BY41	Transcription	PPP1CC	-
HDAC10[1]	Q969S8	Transcription	PPP1CC	-
HEYL[1]	Q9NQ87	Transcription; intracellular signal transduction	PPP1CA	RVxF
HSPA8	P11142	Host-virus interaction; mRNA processing; transcription	PPP1CA	other motifs
IKBKB[1]	O14920	Intracellular signal transduction	PPP1CA	other motifs
IKBKG	Q9Y6K9	Transcription; host-virus interaction	PPP1CB, PPP1CC	RARA
LMTK2[1]	Q8IWU2	Protein phosphorylation; intracellular transport; receptor recycling	PPP1CA	RVxF, other motifs
MAP1LC3A	Q9H492	Autophagy; intracellular signal transduction	PPP1CC	other motifs
MAP1LC3B[1]	Q9GZQ8	Autophagy; intracellular signal transduction	PPP1CC	-
MAP3K3	Q99759	Intracellular signal transduction; protein phosphorylation	PPP1CA, PPP1CC	RVxF
MAX	P61244	Transcription	PPP1CA, PPP1CB	-
MDM4	O15151	Cell cycle; cell proliferation; apoptosis; response to DNA damage and hypoxia; protein stabilization; protein complex assembly	PPP1CA, PPP1CB, PPP1CC	-
MPHOSPH10	O00566	Ribosome biogenesis; RNA processing	PPP1CA	RVxF
MYC[1]	P01106	Transcription	PPP1CA	RVxF
NCL	P19338	Transcription; angiogenesis	PPP1CB	other motifs
NCOR1	O75376	Transcription	PPP1CA, PPP1CB, PPP1CC	RVxF, other motifs
NOC2L	Q9Y3T9	Apoptosis; transcription	PPP1CA	RVxF
NOM1[1]	Q5C9Z4	Targets PPP1CA to the nucleolus	PPP1CA	RVxF, SILK
PAK6	Q9NQU5	Transcription; protein phosphorylation; cytoskeleton organization; apoptosis	-	Other motifs
PLCL2	Q9UPR0	Intracellular signal transduction; lipid metabolic process	PPP1CA	RVxF
PPP1R2	P41236	Regulation of phosphoprotein phosphatase activity; regulation of signal transduction; carbohydrate and glycogen metabolism	PPP1CB, PPP1CC	SILK, other motifs
PPP1R3B	Q86XI6	Carbohydrate and glycogen metabolism	PPP1CA	RVxF
PPP1R3D[1]	O95685	Regulation of protein dephosphorylation; carbohydrate and glycogen metabolism	PPP1CC	RVxF
PPP1R7[1]	Q15435	Regulation of protein dephosphorylation; regulation of catalytic activity	PPP1CB	other motifs
PPP1R10	Q96QC0	Regulation of catalytic activity; transcription; protein import into nucleus	PPP1CA	RVxF
PPP1R11[1]	O60927	Regulation of catalytic activity	PPP1CB	RVxF
PPP1R12A	O14974	Regulation of catalytic activity; intracellular transport; cell cycle; regulation of cell adhesion; protein dephosphorylation; intracellular signal transduction	PPP1CB	RVxF, MyPhoNE
PPP1R13B[1]	Q96KQ4	Cell cycle; apoptosis	PPP1CA	RVxF
PPP1R14B	Q96C90	Regulation of phosphorylation; regulation of catalytic activity	PPP1CC	RVxF
PPP1R15A	O75807	Apoptosis; regulation of translation; stress response	PPP1CA, PPP1CB, PPP1CC	RVxF, RARA
PPP1R15B	Q5SWA1	Regulation of translation; stress response; dephosphorylation	PPP1CA	RVxF, other motifs
PPP1R26	Q5T8A7	Regulation of phosphatase activity	PPP1CA	RVxF

PPP1R37[1]	O75864	Regulation of phosphatase activity	PPP1CA	RVxF
PTEN	P60484	Lipid metabolism; apoptosis; neurogenesis	PPP1CA	RVxF
PTK2	Q05397	Angiogenesis	PPP1CB	SILK
RB1[1]	P06400	Transcription; cell cycle; host-virus interaction	PPP1CA	RVxF, SILK, other motifs
RIPK3	Q9Y572	Necrosis	PPP1CB, PPP1CC	RVxF
RPAP2	Q8IXW5	Transcription	PPP1CA	RVxF
RPAP3	Q9H6T3	Alternative splicing; polymorphism	PPP1CA	other motifs
RRP1B[1]	Q14684	Regulation of phosphatase activity; RNA processing	PPP1CA	RVxF, other motifs
RUVBL2	Q9Y230	DNA repair; growth regulation; transcription	PPP1CA	-
RYR21	Q92736	Intracellular transport	PPP1CA, PPP1CB, PPP1CC	RVxF, other motifs
SF3A2	Q15428	mRNA processing and splicing	PPP1CA	-
SH2D4A[1]	Q9H788	Regulation of phosphatase activity	PPP1CB	RVxF, MyPhoNE
SKP1	P63208	Intracellular signal transduction	PPP1CA	RVxF
SMARCB1	Q12824	Transcription; cell cycle; host-virus interaction; neurogenesis	PPP1CA, PPP1CB, PPP1CC	RVxF
SPRED1	Q7Z699	Regulation of protein phosphorylation; regulation of protein deacetylation; response to DNA damage; development	PPP1CA	RVxF
STAM[1]	Q92783	Protein transport	PPP1CA	RVxF
STAU1[1]	O95793	Intracellular mRNA localization	PPP1CA	RVxF, other motifs
SYTL2[1]	Q9HCH5	Intracellular transport; exocytosis; regulation of phosphatase activity	PPP1CA	RVxF, SILK, other motifs
TMEM33	P57088	-	PPP1CB	-
TP53	P04637	Apoptosis; cell cycle; host-virus interaction; necrosis; transcription	PPP1CA	-
TP53BP2	Q13625	Intracellular signal transduction; apoptosis; cell cycle; embryo development; heart development; response to ionizing radiation	PPP1CA, PPP1CC	RVxF, other motifs
TRIM28[1]	Q13263	Transcription; DNA repair; protein ubiquitination; protein sumoylation; protein oligomerization; gene expression; epithelial to mesenchymal transition; innate immune response; regulation of viral release from host cell	PPP1CA, PPP1CB, PPP1CC	RVxF
TUSC3	Q13454	Intracellular transport	PPP1CA	RVxF, other motifs
USF1	P22415	Transcription	PPP1CC	RVxF, other motifs
ZFYVE9[1]	O95405	Intracellular signal transduction	PPP1CA, PPP1CB, PPP1CC	RVxF, other motifs
ZFYVE16	Q7Z3T8	Intracellular signal transduction; regulation of endocytosis; protein targeting	PPP1CA	RVxF, other motifs

[1]Highly expressed in human prostate (criteria selection: prostate mRNA expression higher than the mean mRNA expression taking into account all tissues analyzed). Other motifs include apoptotic signature motifs and inhibitor-2 degenerate motif. PPP1: Phosphoprotein phosphatase-1; ATM: Ataxia telangiectasia mutated; GSK3B: Glycogen synthase kinase-3 β; SMARCB1: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1; BRCA1: Breast cancer type 1 susceptibility protein.

to DNA damage[78]. Therefore, *ATM* gene therapy and the use of ATM inhibitors have been explored as adjuvants to radiation therapy in PCa.

Axis inhibition protein 1

Axis inhibition protein 1 (AXIN1) is a tumor suppressor that integrates the β-catenin destruction complex, along with adenomatous polyposis coli protein and glycogen synthase kinase-3 β (GSK3B).

Wnt/β-catenin signaling pathway has been extensively explored due to its impact on development, proliferation, and tumorigenesis[79]. Mutations in the signaling mediators of such system are reported in several types of cancer. For instance, 7 variations in the DNA sequence of axin-1 were found in specimens with abnormal β-catenin immunohistochemistry and 4 different polymorphisms were observed in LNCaP, DU145, PC-3, 22Rv1, and P69SV40T cell lines, as well as in the sublines M12, M2182, M2205[80].

Bcl-2 family members: BCL2, BCL2L2 and BAD

Members of the Bcl-2 family of proteins are pivotal regulators of apoptosis. Bcl-2 (BCL2) and Bcl-2-like protein 2 (BCL2L2) are anti-apoptotic proteins, while Bcl-2 antagonist of cell death (BAD) has proapoptotic functions[81].

The expression of BCL2 is not observed in normal prostate epithelial cells, but is found in PIN and increases in advanced PCa (further details in Catz *et al*[82]). Higher BCL2 expression is also found in patients that underwent radiotherapy before surgery than those who received surgical treatment as first choice[83]. BCL2 upregulation is required for the acquisition of castration-resistance, in part by suppressing TGFβ and dihydrotestosterone-mediated induction of caspase-1 expression and activation[84,85].

In conformity with BCL2, the expression of BAD is found elevated in highly proliferative states, in spite of not being helpful in the discrimination between benign and malignant prostate tissues[86]. The overexpression of proapoptotic proteins in highly proliferative states seems paradoxical since cancer cells normally take advantage of the molecular machinery to evade apoptosis. However, BAD overexpression was shown to stimulate PCa cells proliferation and enhance tumor growth[87]. On the other hand, overexpression of BAD in LNCaP cells, which are resistant to tumor necrosis factor-related apoptosis-

inducing ligand (TRAIL)-induced apoptosis, renders the cells sensitive to TRAIL effects[88].

Several studies have reported the great value of targeting apoptotic molecules in order to increase the sensitivity to apoptosis-inducing agents. Polygene therapy and other combinatorial approaches are receiving increased attention due to their effectiveness[89]. Since BCL2 is associated with increased resistance to androgen deprivation in LNCaP cells, a number of approaches aim to decrease its expression and phosphorylation state[90,91]. The targeting of BCL2 has been explored not only in already settled castration-resistant cases, but also to delay the progression to this advanced state[92,93]. In the case of BCL2L2, it was shown to be a target of miR-205-modulated chemosensitivity[94]. Pharmacological interventions targeting BAD intent to increase its expression and decrease its phosphorylation state[95-97].

Breast cancer type 1 susceptibility protein

Breast cancer type 1 susceptibility protein (BRCA1) has long been described as a tumor suppressor that regulates gene transcription and DNA damage repair[98]. In spite of the relevance of BRCA1 mutations in other types of cancers, evidences of their association with PCa development have been inconsistent and at times contradictory. While some studies point to their irrelevance in PCa development, others state that carriers of BRCA1 mutations have more aggressive phenotype and are more prone to develop distant metastasis[99-102].

The expression profile of BRCA1 during PCa progression is also very heterogeneous, although it tends to be higher in PCa compared to normal prostate epithelium[103,104]. Some works actually suggest that its expression correlates with increased tumor proliferative index and development of lethal cancer, being, therefore, considered a potential prognostic marker[105].

The mode of action of BRCA1 in PCa is complex and seeks clarification[102]. BRCA1 mediates apoptosis, cell-cycle arrest, and the response to doxorubicin treatment in PC-3 cells by targeting a wide variety of genes (*e.g.*, *CCND1, BLM, BRCA2, DDB2, FEN1, H3F3B, CCNB2, MAD2L1,* and *GADD153*)[106]. It was also shown to negatively regulate the transcription of insulin-like growth factor I receptor in an AR-dependent manner[104].

The use of anticancer drugs that inhibit poly ADP-ribose polymerase (PARP), such as niraparib and olaparib, has demonstrated efficacy in PCa patients with BRCA1 mutations[107,108].

Cyclins D1 and D3

Cyclins are key mediators of the cell cycle. Cyclin D1 (CCND1) is scarcely found in non-neoplastic tissues, but its levels are increased in the majority of localized tumors, where distinct subcellular localizations are observed according to tumor grading[109]. Likewise, CCND3 displays higher expression in PCa than in BPH and its expression correlates positively with PSA serum levels[110].

In addition to the roles of cyclins in the regulation cell cycle, CCND1/3 interact with AR. CCND1 suppresses the activity of the AR either directly, with the main mediator being the repressor domain of CCND1, or indirectly *via* histone deacetylases[111]. In this fashion, CCND1 differentially regulates the expression of several androgen-sensitive genes-it represses some genes, such as *KLK3/PSA*, while induces the transcription of others, as *CDC6* and *MCM2*. Further effects of CCND1 include alteration of transcription factor-chromatin interactions, restraining of TGFβ, Snail, Twist, and Goosecoid signaling pathways, enhancement of *Wnt* and *ES* gene expression, and enlargement of a prostate stem cell population[112,113]. The association between CCND3 and the AR represses ligand-dependent activation through cyclin-dependent kinase 4 (CDK4)-independent mechanisms and appeases androgen-dependent proliferation[114].

CCND1 has been proposed as a prognostic marker for poor clinical outcome in PCa biochemical-free recurrence. A number of strategies targets CCND1, including miR-153 and perhaps miR-449a, piperine, and L-mimosine, with the effect of the latter being only observed in PC-3 cells[115-118].

Cyclin-dependent kinases 2 and 4

CDK family of proteins regulates cell cycle progression and is involved in AR-mediated cell proliferation. CDK2 mRNA levels decrease after castration, increase after testosterone propionate treatment, and are expressed at high levels in recurrent human xenograft CWR22 tumors[119]. The expression of CDK2 and CDK4 is up-regulated within hours of androgen treatment; nevertheless, castration-resistant PC-3 cells, which do not respond to androgen stimulation, show constitutively high basal expression of both kinases[120].

The activity of CDK2 kinase is stimulated by androgen[119,120]. Increased CDK2 activity correlates with PCa cells insensitivity to TGF-β1, while even modest depletion of CDK2 in LNCaP cells results in strong growth repression[121,122].

CDK4 protein expression was not found elevated in localized prostate tumors, but its overexpression overcome 3,9-dihydroxy-2-prenylcoumestan-induced G_0/G_1 arrest in castration-resistant cells[123].

Decreased expression and inhibition of the activity of CDK2 and CDK4 are observed upon treatment with anti-proliferative agents, such as resveratrol, BZL 101, and inositol hexaphosphate[124-126]. As phosphorylation of CDK2 on Thr160 is essential for the kinase activity, the manipulation of this phospho-residue has also been analyzed[127].

GSK3B

The multifunctional GSK3B exhibits potent tumor suppressor qualities and is upregulated in many types of tumor, including PCa. The expression pattern of GSK3B differs between normal prostate and PCa cells - nuclear GSK3B is higher in normal prostate, whereas cytoplasmic GSK3B is higher in PCa. Increased GSK3B cytoplasmic

levels might determine PCa development and progression due to their correlation with high Ki-67 labeling index, low apoptotic index by TUNEL, high levels of AR and phosphorylated AKT, extracapsular extension, high Gleason score, lymph node metastasis, and biochemical recurrence-free survival[128].

GSK3B mediates both estrogen and AR signaling (for details see Mulholland *et al*[129]). GSK3B phosphorylates AR and represses AR-mediated transcription and growth[130,131]. GSK3B/AR complex, which locates within the cytoplasm and nucleus, contributes to AR stability, nuclear translocation, and consequent modulation of PCa cells' response to androgen[132]. The signaling pathway AKT/GSK3B is also involved in nuclear factor α (NFα)-induced epithelial–mesenchymal transition (EMT) in PC-3 cells by contributing to Snail stability[133]. On the other hand, suppression of GSK3B sensitizes PCa cells to TRAIL-induced apoptosis, which might suggest its involvement during resistance acquisition[134]. The suppression of GSK3B expression or phosphorylation state has also demonstrated positive results in inhibiting proliferation of PCa cells[135,136].

AKT/GSK3B signaling pathway has been targeted by a number of antiproliferative and apoptosis-induced agents, namely thiazolidenediones and isoflavone, as well as agents that impair cell migration and invasion, such as fenretinide[137-139].

Histone deacetylases-1, -6 and -8

Histone deacetylases (HDACs) are a large family of enzymes that regulates the nucleosomal histone acetylation. Members of HDACs' family are divided into four classes (classes Ⅰ-Ⅳ), according to their homology with yeast proteins, with class Ⅱ being further subdivided into class Ⅱa and Ⅱb. HDAC1 and -8 belong to the class Ⅰ histone deacetylases, whereas HDAC6 belongs to the class Ⅱb. All members of class I were found to be deregulated in many types of cancers (for review see[140]).

HDAC1 is expressed in normal prostate tissues, where it locates exclusively in the nucleus, cancer precursor lesions, and PCa, and its expression was shown to be lower in stromal cells[141,142]. Conversely, HDAC8 is primarily found in the cytoplasm of stromal cells[142].

HDAC1 expression levels correlate with tumor dedifferentiation, high Gleason score, high pT stage, and high biochemical recurrence rates[141,143]. HDAC1 is a major repressor of AR and E-cadherin, thereby regulating AR-transcriptional activity, cell proliferation and motility, and invasion[144-146].

HDAC6 deacetylates and activates HSP90 chaperone protein, which, in turn, binds to AR[147]. Indeed, HDAC6 regulates AR hypersensitivity to androgens, nuclear localization, and attenuation of its degradation[147,148]. HDAC6 might establish important interactions with other proteins since its decrease is also observed in PC-3 cells, which are castration-resistant[149,150].

The use of HDAC inhibitors in the prevention and treatment of cancer has become an area of intense research. The repression of HDAC1 expression by miR-449a induces growth arrest in PCa and its inhibition by maspin prevents pathologic gene silencing, increasing tumor cell's sensitivity to drug-induced apoptosis[151,152]. The deacetylase activity of HDAC6 decreases after sulforaphane treatment and it might be responsible for the selective effects of this agent in both hormone-sensitive and castration-resistant cells, while normal cells remain intact[149,150].

Heyl

Heyl is a member of the hairy/enhancer-of-split-related with YRPW-like motif family of transcriptional repressors. Of the three members of the referred protein family, Heyl is the more potent AR corepressor and reduces the growth of LNCaP cells. The repression of AR activity by Heyl occurs through HDAC1/2-independent mechanisms. Heyl was shown to be excluded from the nucleus in malignant cells but not in benign tissue, thus nuclear exclusion of the protein might be involved in tumor progression[153].

Inhibitor of nuclear factor-κB kinase subunit beta and gamma

Inhibitor of nuclear factor-κB (NF-κB) kinase subunit β and γ (IKBKB and IKBKG, respectively) are involved in the activation of NF-κB[154], a transcription factor that regulates cell growth, apoptosis, inflammation, angiogenesis, and metastasis (for review see[155]).

Studies on human prostate cell lines have not revealed significant differences between primary prostate cells, hormone-sensitive, and castration-resistant PCa cells[156]. However, IKBKB expression is higher in PCa tissue than in benign non-atrophic and atrophic glands[157].

The effects of sulforaphane and phenethyl isothiocyanate are mediated, at least in part, through the inhibition of IKBKB phosphorylation[158]. The loss of IKBKB and IKBKG are also involved in proteasome inhibitors-induced apoptosis[159].

Lemur tyrosine kinase 2

Lemur tyrosine kinase 2 (LMTK2) is a Ser/Thr transmembrane protein kinase mainly involved in endosomal membrane trafficking[160]. In LNCaP cells, this function is achieved, at least in part, by the interaction with myosin VI and consequent recruitment of this protein to the surface of endosomes[161]. Interestingly, the gene that encodes for LMTK2 is one of the novel common alleles associated with PCa[162]. *LMTK2* is underexpressed in PCa tissue compared to non-malignant BPH tissue due to alterations in intron 9; however, the mechanism by which this alteration leads to the increased risk of PCa is not properly understood[163]. LMTK2 functions depend on its interaction with other proteins, which includes CDK/P35 complex and PPP1, besides the already mentioned myosin VI[164,165].

MYC family of proteins: MYC and MAX

MYC deregulation is a well-established mechanism in car-

cinogenesis[166]. The role of MYC in PCa, nevertheless, is not fully understood. MYC overexpression is frequently observed in PCa, which can be partially explained by locus amplification, mainly in advanced cancers[167]. MYC stabilizes the length of telomeres and is required for EMT[168,169]. MYC and MAX interact with and regulate the AR[170].

MYC amplification status and its overexpression have been suggested as a valuable prognostic tool[171,172]. The existence of a panel of markers that encompass MYC, PTEN, and Ki67 shown benefits in predicting progression-free survival in men receiving adjuvant docetaxel after prostatectomy[173]. Cells that exhibit resistance to treatment with docetaxel have constitutive activation of MYC signaling[174].

Nucleolin

Nucleolin (NCL) is an abundant nucleolar phosphoprotein involved in various stages of ribosome synthesis. The expression and phosphorylation of NCL are extremely sensitive to androgens-with both decreasing following androgen deprivation. Thus, the control of NCL expression and phosphorylation by androgens may be an important nucleolar control mechanisms involved in the growth of prostate cells[175]. NCL can also be found in the cell surface, where it may function as a hepatocyte growth factor receptor. Cell surface NCL was shown to be upregulated during PCa progression[176].

Nuclear corepressor 1

Nuclear corepressor 1 (NCOR1), an AR co-repressor, is overexpressed in PCa cell lines compared to normal prostate cells. NCOR1 expression is confined to the S phase of cell cycle; therefore, during this time NCOR1 represses the expression of AR target genes[177]. In PCa cells, the activity of NCOR1 is positively regulated by protein kinase A (PKA)[178].

The increased expression and activity of NCOR1 impair peroxisome proliferator activated receptor α/γ-mediated expression of key target genes, such as *CDKN1A* and *TGFBRAP1*, thus contributing to the loss of ligands antiproliferative responsiveness in PCa cells[179]. NCOR1 might also be important during the process of castration-resistant acquisition[180].

Serine/threonine-protein kinase PAK 6

The expression of PAK6 is increased in primary and metastatic PCa, and correlates with cells' sensitivity to androgens[181,182]. PAK6 co-localizes with AR in the cytoplasm of normal prostate epithelium and translocates into the nucleus in malignant phenotypes, where it represses both AR- and ER-mediated gene transcription[181,183,184]. It was also shown that PAK6 phosphorylates the AR at Ser578, promoting the association of AR-E3 ligase murine double minute-2 (Mdm2) and guiding AR degradation[185].

The knockdown of PAK6 impairs PCa growth and improves chemosensitivity of docetaxel and sensitivity to radiation[186,187].

Phosphatase and tensin homolog

Phosphatase and tensin homolog (PTEN) is a dual specificity phosphatase and a recognized tumor suppressor. Inactivation of *PTEN* has been associated with many different types of cancer, including PCa, and assumes preponderant roles (further details on[188,189]). A number of molecules have been recently shown to contribute to PTEN downregulation, including lamin A/C and a subset of microRNAs (*e.g.*, miR-19b, miR-23b, miR-26a, miR-92a, and miR-153)[117,190,191]. Loss of PTEN determines PCa progression through several downstream effectors and signaling pathways, including PI3K/AKT, BIM1, CXCL12/CXCR4, and PDGF D/β-PDGFR[192-195]. The loss of PTEN is also associated with increased risk of capsular penetration[196]. Interestingly, it was recently shown that PTEN is incorporated in the cargo of exosomes prevenient from cancer cells, but not in those derived from non-malignant cells. Exosomes are able to transfer PTEN to other cells, which in turn recover the tumor-suppressor activity[197].

Recent evidences support the usefulness of PTEN in PCa management. Blood exosomes of PCa patients contain PTEN, contrarily to the exosomes isolated from normal subjects, which may indicate exosomal PTEN as a putative diagnostic tool[197]. The loss of cytoplasmic PTEN was shown to accurately distinct intraductal carcinoma from prostatic intraepithelial neoplasia, since the latter does not manifest PTEN loss at all[198]. PTEN status might also be useful in the prognostic evaluation of men with localized PCa[199,200].

Moreover, a phase Ⅱ clinical trial reported that the activity of PTEN determines the improvement of progression-free survival and is potentially required for the efficacy of cetuximab in metastatic castration-resistant PCa[201]. PTEN expression is enhanced by resveratrol-mediated AR inhibition[202].

Protein tyrosine kinase 2

Protein tyrosine kinase 2 (PTK2) regulates adhesion and motility of cells. Its upregulation and activation was observed in localized and castration-resistant PCa, in spite of being more evident in the latter case[203,204]. The complexes that PTK2 forms with paxillin and p50csk are mainly observed in metastatic PCa and contribute to the metastatic behavior[204]. PTK2 is also involved in the migration and invasion mediated by IL-8 and CXCL13-CXCR5[205,206].

Treatments with FTY720 and the combinatorial therapy with curcumin and methylseleninic acid compromises PTK2 activity, and PTK2 inhibition was shown to delay the progression of PCa[203,207]. PTK2 is also a target of genistein-mediated morphologic changes[208].

Tripartite motif-containing protein 28

Tripartite motif-containing protein 28 (TRIM28) is a substrate of ATM kinase involved in the maintenance of chromatin in condensed states[209]. The expression of TRIM28 is observed in prostate cancer lines, despite being lower in castration-resistant cell lines[210]. TRIM28 was recently identified as an activator of the AR and is also

involved in the response of prostate cells to DNA damage[210,211].

Tumor suppressor pathways: RB1 and TP53

Retinoblastoma-associated protein (RB1) and cellular tumor antigen p53 (TP53) are major tumor suppressors whose functions in PCa have been broadly explored. Loss of RB1 and TP53 is strictly associated with AR misregulation and progression to castration-resistant disease. Both their mechanism and their potential roles in managing PCa are extensively revised in Aparicio *et al*[212], Dean *et al*[213] and Lee *et al*[214].

Ryanodine receptor 2

Ryanodine receptor 2 (RYR2) is expressed in PWR-1E non-tumor cells, as well as in LNCaP and DU145 PCa cells, with the latter registering the lowest expression[215,216]. RYR2 mobilizes Ca^{2+} from intracellular stores, which is essential to the regulation of apoptosis[215].

SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1

SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 (SMARCB1) is a core subunit of the SWI/SNF family of nucleosome-remodeling complexes[217]. In aggressive PCa, the expression of SWI/SNF target genes is impaired by the binding to SChLAP1, which was shown to be aberrantly upregulated[218].

BIOLOGICAL SIGNIFICANCE OF INTERACTIONS: PPP1 REGULATORS OR SUBSTRATES?

The relationship between PPP1 and the majority of the PIPs here referred, as well as possible alterations in the dynamics of such complexes during prostate carcinogenesis, require further elucidation.

Some of the PIPs are already characterized as PPP1 regulators, substrates, or both (Figure 2). For a significant number of PIPs identified in protein-protein interaction screenings, nevertheless, the functional significance of their interaction with PPP1 remains poorly understood. Therefore, efforts should be made in order to understand PPP1 interaction with CCND1, CCND3, CDC5L, CDC34, CDK4, EED, EIF2AK2, GABARAP, GABARAPL2, GRB2, HDAC8, HDAC10, HSPA8, HEYL, MAX, NCL, IKBKB, IKBKG, MAP1LC3A, MAP1LC3B, MAP3K3, MPHOSPH10, MYC, NCOR1, NOC2L, PAK6, PLCL2, PPP1R3B, PPP1R12A, PPP1R13B, PTEN, RIPK3, RPAP2, RPAP3, RRP1B, RUVBL2, SF3A2, SH2D4A, SKP1, SPRED1, STAM, STAU1, SYTL2, and USF1.

In other cases, the biological significance of the complex is partially known, although it is not established whether the PIP is the regulator or the substrate (or even both). For instance, HDAC6 directly binds to PPP1 and the complex controls microtubule dynamics by maintaining α-tubulin in a deacetylated state, but the exact mechanism remains poorly understood[219].

Phosphoprotein phosphatase 1 regulators

AKAP11 acts as a targeting subunit of PPP1 and it can also inhibit the phosphatase activity[220,221]. BCL2L2 and CUEDC2 target PPP1 to protein complexes: BCL2L2 recruits PPP1 to BAD, forming a complex that is involved in the control of apoptosis[222]; and, CUEDC2 targets PPP1 to IKK, thereby promoting the dephosphorylation and inactivation of the kinase[223]. NOM1 acts as a PPP1 nucleolar targeting subunit, PPP1R10 targets PPP1 to the nucleus, and PPP1R15A targets PPP1 to the endoplasmic reticulum[224-227]. PPP1R10/PPP1 holoenzyme is known to regulate chromosome decondensation and apoptosis in response to cellular stresses[226,228].

PPP1C positive regulators include ATM, GSK3B, and SMARCB1. In response to ionizing radiation, PPP1 is dephosphorylated and activated by ATM[229]. ATM-mediated activation of PPP1 could occur, at least, *via* two mechanisms: (1) phosphorylation of I-2 and consequent dissociation of the complex I-2/PPP1; or, (2) dephosphorylation of PPP1C at Thr320 to amplify its activity[230]. As a result, PPP1 dephosphorylates HDAC1, leading to the dissociation of the HDAC1-PPP1-Rb complex[231,232]. In similar way to ATM, GSK3B activates PPP1 *via* phosphorylation of I-2 and consequent disruption of the I-2/PPP1 complex[233]. SMARCB1 forms a tricomplex with PPP1R15A and PPP1, and weakly stimulates PPP1 activity[234].

AKAP11, BRCA1, CDK2, LMTK2, HCFC1, PPP1R7, PPP1R11, and TP53BP2 inhibit the activity of PPP1[229,235-239].

Phosphoprotein phosphatase 1 substrates

PPP1C is a key regulator of the two major tumor suppressors: it inhibits TP53 and activates RB1 (further details on[24]). The apoptotic process is strictly controlled by reversible phosphorylation[240]. The phosphorylation of APAF1 by the 90-kDa ribosomal S6 kinase (RSK) compromises the formation of the apoptosome, impairs cells' sensitivity to cytochrome c, and inhibits apoptosis. PPP1CA was shown to reverse the RSK-mediated phosphorylation of APAF1, thus enhancing its pro-apoptotic activities[241]. BAD is also dephosphorylated by PPP1CA in a dependent way of the anti-apoptotic members BCL2, BCL2L2, and BCL-XL[222,242,243]. While BAD overexpression provides proliferative advantage to tumor cells, BAD dephosphorylation increases their sensitivity to apoptosis[87].

PPP1 exerts a positive control on Wnt signaling through dephosphorylation of AXIN1. As a result, β-catenin destruction complex dissociates, the free phospho-β-catenin accumulates in the cytoplasm, and the transcriptional activity of β-catenin is promoted[244].

In addition of being regulators, BRCA1 and GSK3B are also substrates for PPP1C. PPP1C dephosphorylates BRCA1 and enhances its DNA repair function[235,245,246]. GSK3B is also dephosphorylated and disinhibited by PPP1-mediated dephosphorylation[247].

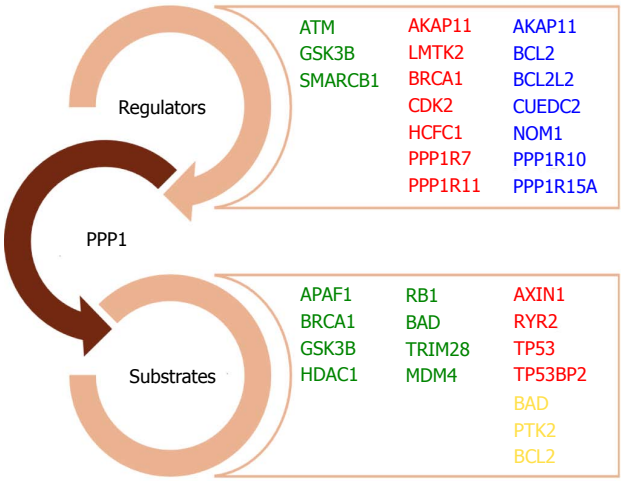

Figure 2 Phosphoprotein phosphatase 1 interacting proteins can be regulators, substrate or both. Green represents positive regulation of phosphoprotein phosphatase 1 (PPP1) (in case of regulators) or PPP1-mediated upregulation of substrates. Red represents negative regulation of PPP1 (in case of regulators) or PPP1-mediated downregulation of substrates. Blue characterizes PPP1-interacting proteins (PIPs) exhibiting PPP1 targeting ability. Yellow corresponds to proteins that are identified as substrates, but whose interaction with PPP1 are not fully understood. ATM: Ataxia telangiectasia mutated; GSK3B: Glycogen synthase kinase-3 β; SMARCB1: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1; BRCA1: Breast cancer type 1 susceptibility protein.

HDAC1 activity is promoted through dephosphorylation of Ser133 by PPP1, which leads to dissociation of HDAC1-PP1-Rb complex, and consequent increase of HDAC1 activity[232,248]. Similarly, HDAC6 and -8, which can be phosphorylated, might also be PPP1 substrates, although this fact has not been confirmed yet.

RYRs are regulated through reversible phosphorylation, and PPP1 reverts PKA-mediated phosphorylation and activation of RYR2[249-251]. PPP1 dephosphorylates TRIM28 (Ser824), enhancing its sumoylation state, and MDM4 (Ser367), enhancing its stability and leading to the consequent inhibition of TP53 activity[252]. PPP1 also reverts the phosphorylation of PTK2 and BCL2[253-255].

CHALLENGE OF TARGETING PPP1 ACTIVITY

Since PPP1 is a Ser/Thr phosphatase with major roles in several pathological processes, the manipulation of its activity is a valuable therapeutic tool that had been misjudged for years. PPP1 activity could be manipulated through direct or indirect inhibition of the catalytic site (for review see[27]).

The dissociation of PPP1 complexes through the targeting of PIPs is challenging and might overcome the problems that arise from direct inhibition of PPP1C. However, this area remains understudied and only two complexes are currently being targeted: PPP1C/HDAC and PPP1C/PPP1R15A. Trichostatin A disrupts PPP1C/HDAC and is used in the treatment of glioblastoma and PCa cells. LBH589, an inhibitor of HDAC, was also shown to be able to dissociate this complex[256]. As a consequence, AKT is dephosphorylated and its activity decreases. PPP1C/PPP1R15A complex is disrupted upon salubrinal treatment, thereby dephosphorylating eIF2α[257,258].

The increasing number of PPP1 docking motifs identified offers excellent opportunities for targeting specific complexes. In fact, the docking motif found in Bad has inspired the designing of a peptide that interferes with PPP1/BAD complex and is able to induce cell death[259]. Also, the PPP1 docking motif R/Kx(0,1)V/IxFxxR/KxR/K, a new PPP1C-dependent apoptotic signature, might be a useful tool for drug design[260].

The disruption or enhancement of several other complexes might contribute to the enhancement of PCa management, enabling more efficient therapies for advanced castration-resistant PCa. Therefore, the identification of PPP1 complexes in human prostate and their characterization in prostate carcinogenesis is imperative for the search of new therapeutic targets.

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P- Reviewer: Choi CY, Moens U **S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i4.140

World J Pharmacol 2014 December 9; 3(4): 140-152
ISSN 2220-3192 (online)
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REVIEW

Potential ability of xanthophylls to prevent obesity-associated cancer

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Supported by National Cancer Center Research and Development Fund No. 25-A-15; and by The Research Grant of the Princess Takamatsu Cancer Research Fund
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Received: June 27, 2014 Revised: October 2, 2014
Accepted: October 31, 2014
Published online: December 9, 2014

Abstract

Obesity-associated cancers, including colon cancer and breast cancer, are increasing in Asian countries with Westernized lifestyles as exemplified by reduced physical activity and increased fat/sugar consumption. An excessive accumulation of visceral adipose tissue causes insulin resistance, dyslipidemia and adipocytokine imbalance, and these factors are suggested to be involved in cancer promotion. To prevent obesity-associated cancers, researcher attention is increasing on the so-called "functional foods". In addition, new approaches to cancer control are in high demand, and using "functional foods" as supplemental or adjuvant agents in chemotherapy is thought to be a promising approach. One of these functional ingredients is xanthophylls, which are natural fat-soluble pigments found in fruits, vegetables, algae and other plants. Xanthophylls belong to the carotenoid class and have structures containing oxygen. Some studies have revealed that xanthophylls improve the inflammation status, serum triglyceride levels, blood pressure levels and liver function test values. Furthermore, recent studies show that xanthophylls possess high anti-cancer, anti-diabetic, anti-obesity and anti-oxidant properties. In this review, we highlight the recent findings for five xanthophylls, namely astaxanthin, β-cryptoxanthin, fucoxanthin, neoxanthin and zeaxanthin/lutein, and their relevance to cancer prevention.

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Key words: Cancer prevention; Xanthophylls

Core tip: Xanthophylls belong to the class of carotenoids, and are natural fat-soluble pigments found in fruits, vegetables, algae and so on. It has been shown that the versatile functions of xanthophylls have great potential for the prevention of metabolic syndrome and cancers. Xanthophylls have proved safety, and several xanthophylls provide other health benefits, including improvement of inflammation, dyslipidemia, hypertension and liver function. These findings indicate that xanthophylls could be useful to prevent obesity-associated cancer.

Terasaki M, Mutoh M, Fujii G, Takahashi M, Ishigamori R, Masuda S. Potential ability of xanthophylls to prevent obesity-associated cancer. *World J Pharmacol* 2014; 3(4): 140-152 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/140.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.140

INTRODUCTION

Obesity has recently attracted much interest as a risk factor for several cancers, such as breast cancer and colorectal cancer[1,2]. Both metabolic syndrome that is characterized by obesity, hyperlipidemia, type 2 diabetes and hypertension

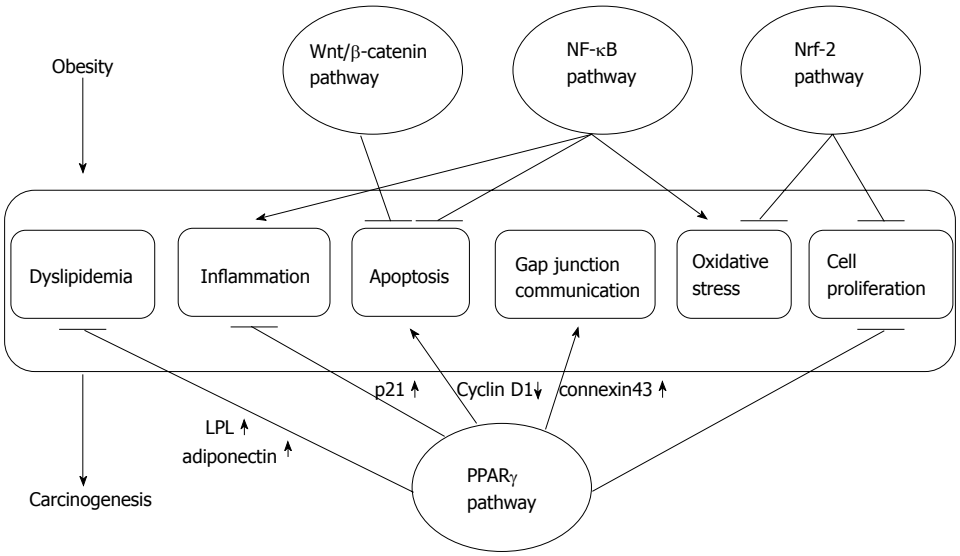

Figure 1 Possible mechanisms for obesity-associated cancer prevention. LPL: Lipoprotein lipase; NF-κB: Nuclear factor kappa B; Nrf2: Nuclear factor-erythroid 2 related factor 2; PPAR: Peroxisome proliferator-activated receptor.

Table 1 Obesity-associated cancers

Type of cancer
Breast (postmenopausal)
Colorectum
Endometrium
Esophagus
Gallbladder
Kidney
Pancreas
Thyroid

and obesity-associated cancers (Table 1) are extremely common in Western countries, and they are currently increasing in Eastern countries, including Japan. The factors linking obesity and cancer are becoming apparent, and they are insulin resistance, dyslipidemia and a subsequent adipocytokine imbalance (Figure 1)[1,2]. Carotenoid intake is reported to be inversely associated with obesity and with the risk of many cancers[3-6].

Carotenoids are fat-soluble pigments found in fruits, vegetables, algae and other plants. Humans cannot synthesize carotenoids, and we should therefore consume them as part of our diet. Carotenoids belong to the tetraterpenoid category, and they can be divided into xanthophylls and carotenes according to whether the structure contains oxygen or not. Carotenoids with structures containing oxygen are xanthophylls. As the name indicates, the color of xanthophylls is usually yellow, and they are usually lipophilic because of the long unsaturated aliphatic chain in their structure.

Because conventional chemotherapy has failed to reduce the mortality rates of common cancers, including obesity-associated cancers, new approaches to controlling the development of cancer are in great demand[7]. One approach is the use of functional foods/plant-derived agents as supplemental or adjuvant agents in chemotherapy[8,9]. Another approach is chemoprevention for the control of cancer development[8,9]. In both methods, using xanthophylls seems to be an attractive approach. As shown in this review, xanthophylls provided health benefits, such as improvements in inflammation, dyslipidemia, hypertension and liver function. Moreover, the biological significance of xanthophylls as important candidates for the chemoprevention of cancer is becoming clearer, and the safety of xanthophylls has been affirmed, as described in this review. Another candidate called - carotene is the most abundant dietary carotenoid, and long-term supplementation with this compound has been shown to be ineffective for cancer chemoprevention in several recent large-scale intervention trials[10-12].

In this review, we focus on recent findings for five xanthophylls as follows: astaxanthin, β-cryptoxanthin, fucoxanthin, neoxanthin and zeaxanthin/lutein, and their relevance to cancer prevention (Figure 2).

ASTAXANTHIN

Distribution and nature of astaxanthin

Astaxanthin (AX) is a natural fat-soluble red pigment and belongs to the xanthophyll subclass of carotenoids. Dietary sources of AX are eggs of salmon and trout, skin of red sea bream, crabs, shrimps and lobsters. AX is synthesized in microalgae (*Chlorella zofingiensis, Chlorococcum* and *Haematococcus pluvialis*). Krills (*Euphausia superba*) feed on the microalgae and in turn are fed upon by fishes. The microalga, *H. pluvialis,* is the main source of natural AX and is able to accumulate up to 4%AX on dry weight basis[13-15]. AX extracted from *H. pluviali* is used as a food dye in many countries. AX exists in stereoisomers and geometric isomers. *H. pluvialis* biosynthesizes the (3*S*, 3'*S*)-isomer, meanwhile *P. rhodozyma* biosynthesizes the (3*R*, 3'*R*)-isomer. AX has two hydroxyl groups and is able to react with fatty acids and proteins. AX is found as free, mono- and di-ester forms in organisms[13].

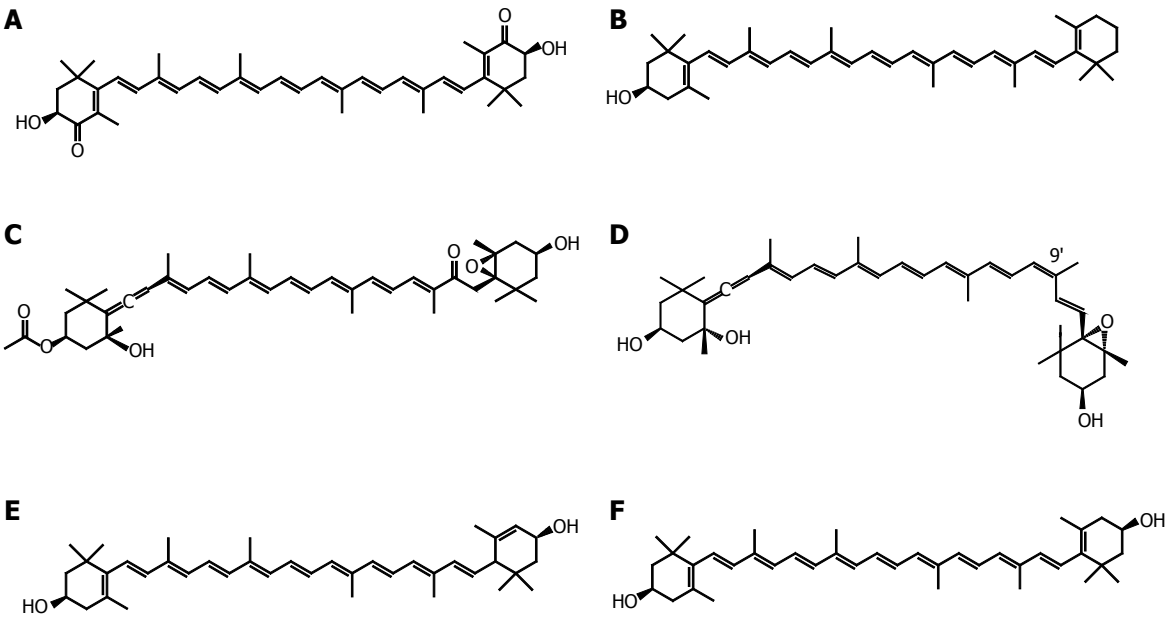

Figure 2 Structure of xanthophylls. A: Astaxanthin; B: β-cryptoxanthin; C: Fucoxanthin; D: 9'-cis-neoxanthin; E: Lutein; F: Zeaxanthin.

AX can take to transverse cell membrane orientation, and shows strong antioxidative activity[13,15]. After oral administration of AX, AX changes to all-*E*, 9*Z*-, 13*Z*-geometrical isomers and 3*R*,3'*R*-, 3*R*,3'*S* meso-, 3*S*,3'*S*-optical isomers, all of which can be detected in human blood[16].

Safety profile

Many experimental and clinical studies have demonstrated the safety of AX[13,17]. In a subchronic toxicity study in rats, feeding AX-rich microalgae biomass corresponding to doses of 465 and 557 mg AX/kg per day for 90 d in male and female rats, respectively, revealed no adverse events[18]. A randomized, double-blind, placebo-controlled study has demonstrated that it is safe to administrate 6 mg/d AX in healthy adults for 8 wk[19], and a significant decrease of triglycerides and increase of adiponectin and high density lipoprotein cholesterol in participants with mild dyslipidemia by administration of AX at doses of 12 mg/d and 18 mg/d for 12 wk[20].

Preclinical studies and anti-cancer mechanisms

Oxidative stress and inflammation are closely related to carcinogenesis (Figure 1), and many antioxidants, including carotenoids have been demonstrated to decrease cancer development in experimental animal models[14]. There are papers on preventive effects of AX on urinary bladder[21], oral[22,23], and colorectal[24,25] carcinogenesis. In mouse urinary bladder cancer induced by N-butyl-N(4-hydroxybutyl)nitrosamine (OH-BBN), AX administration at a dose of 50 ppm in water for 20 wk after OH-BBN exposure for 20 wk resulted in a decrease in the incidences of precancerous lesions and bladder cancer[21]. In rat oral carcinogenesis induced by 4-nitroquinoline 1-oxide (4-NQO), the incidence of oral precancerous lesions in rats treated with 20 ppm 4-NQO and 100 ppm AX was smaller compared to those of the non-treatment group, and oral neoplasms did not observed in rats fed AX among the 4-NQO exposure[22]. In these studies, AX decreased cell growth activity in the non-cancerous epithelial tissues of 4-NQO-exposed animals[21,22]. AX has also been demonstrated to show preventive effects in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis *via* nuclear factor-erythroid 2 related factor 2 (Nrf2) activation[23]. Moreover, AX has been shown to inhibit nuclear factor kappa B (NF-κB) and Wnt/β-catenin signaling pathways[26]. Related to colorectal carcinogenesis, AX at 500 ppm in diet significantly decreased the development of aberrant crypt foci (ACF) and the incidence and multiplicity of colorectal tumors induced by azoxymethane (AOM)[24]. AX at 200 ppm in diet also suppressed mucosal ulcers induced by dextran sulfate sodium (DSS), and development of dysplastic ACF and colonic adenocarcinoma induced by both treatment of DSS and AOM[25]. In addition, AX reduced the number and size of aflatoxin B1-induced liver preneoplastic foci in rats[27]. Growth of WAZ-2T cells, mammary tumor cells, inoculated into the mice mammary fat pad was also inhibited by AX at 100 ppm or 400 ppm in diet[28]. Lipid peroxidation activity in tumors was reduced in tumors treated with 400 ppm[28]. AX markedly attenuated the promotion of hepatic metastasis of P815 mastocytoma cells in a syngenic graft model under restraint stress[29]. In *in vitro* cell culture systems, AX suppressed invasion of rat ascites hepatoma AH109A cells[30]. AX inhibited cell proliferation and decreased cell viability of leukemia K562 cells *via* induction of apoptosis along with up-regulation of peroxisome proliferator-activated receptor (PPAR)γ and p21, and down-regulation

of cyclin D1 [31]. Induction of connexin 43, gap junction protein, through activation of PPARγ is suggested to be one of the anti-tumor mechanisms of AX[32,33]. Up-regulation of the Nrf2 pathway is also involved in antioxidant activity of AX[23,31,34], and may improve mitochondrial function[35]. However, the role of Nrf2 activation in anti-tumorigenesis is controversial. The oncogenic *K-ras* gene induces Nrf2 expression, and detoxification of reactive oxygen species promotes tumor growth[36]. Deficiency of Nrf2 has been reported to increase induction of tumors in urethane-induced mouse lung carcinogenesis, but reduce the number of malignant tumors harboring activated mutation in the *K-ras* gene, indicating that Nrf2 prevents initiation but accelerates progression under the activation of the K-ras signaling pathway[37]. Indeed, there is a report that effects of AX differ at the stages of initiation and the stage of promotion in mammary tumors. AX fed before tumor initiation delayed mammary tumor growth and modulated immune response, but AX supplementation after tumor initiation resulted in more rapid tumor growth[38]. Thus, use of antioxidants for cancer prevention is considered to be useful at the time before tumor initiation, but more caution is required in using them after the stage accompanied with activated K-ras signaling.

Clinical studies

A randomized, double-blind, placebo-controlled study has demonstrated that AX reduces oxidation of fatty acids[39], decreases oxidative stress markers[40] and inflammation[41], and improves dyslipidemia[20] and age-related dysfunction of eyes[42,43] and brain[44]. However, human cancer prevention studies using AX have not yet been reported.

β-CRYPTOXANTHIN

Distribution and nature of β-cryptoxanthin

β-cryptoxanthin (β-CRX) is one of the naturally occurring carotenoid pigments, and is also classified as a xanthophyll. Its unique character is that it is found in specific fruits and vegetables such as mango, papaya, loquat, Japanese persimmon, peach, sweet red peppers and citrus fruits of the mandarin family[45,46]. Satsuma mandarin, *Citrus unshiu*, is one of the most β-CRX rich fruits in Japan. The content of β-CRX in *C. unshiu* reaches several mg/100g wet weight. The level of β-CRX in Valencia orange is very low and grapefruit has been found to be devoid of it.

In the human body, β-CRX is easily converted to vitamin A (retinol) and is therefore considered as a provitamin A. It is also known that β-CRX might be easily absorbed[47], and is accumulated in various organs[48]. Moreover, it can be stored for several months in the human body[49]. Serum β-CRX concentration could be around 96 μg/dL[50]. It is also reported that β-CRX concentration in Japanese mother's milk and serum are nearly parallel with their intake of the Satsuma mandarin, and are higher than other countries[51,52].

Epidemiologic studies

Many epidemiological studies showed the intake of β-CRX was significantly associated with reduced risks of type 2 diabetes [relative risk (RR) = 0.58][53] and rheumatoid arthritis (RR = 0.59)[54]. β-CRX supplementation significantly decreased cigarette smoke-induced lung squamous metaplasia and inflammation[55]. Regarding cancer risk, several observational epidemiologic studies suggest that β-CRX could potentially prevent cancer development. The demonstrated cancer risks for lung, esophageal and bladder were 0.76 (RR), 0.16 [odds ratio (OR)] and 0.74 (RR), respectively, comparing the highest to lowest quintile of intake[56-58]. A greater intake of β-CRX was also inversely associated with developing undetermined cervical atypical squamous cells (OR = 0.4)[59]. Interestingly, the serum level of β-CRX is lower in the patients of liver cancer than that in healthy counterparts[60]. These results suggest that a high serum β-CRX concentration or intake of β-CRX is beneficial to human health.

Safety profile

The scientific panel on additives and products or substances used in animal feed (FEEDAP) panel members considered β-CRX to appear not to be mutagenic and show no clastogenic activity[61]. In subchronic studies, The FEEDAP panel could not find any adverse effects[61]. Also an acceptable daily intake has not been determined[61]. Previously, we have reported the chemoprevention effect of β-CRX against chemically-induced bladder carcinogenesis in ICR mice[62]. Mice were fed with 1, 5 and 25 ppm of β-CRX for 24 wk, and no clinical signs of toxicity and poor condition, low survival or histopathological changes were found[62]. Many epidemiological studies[53-60,63-68] indicated that administration of β-CRX is safe for human health.

Preclinical studies and anti-cancer mechanisms

Various functions of β-CRX have been reported recently. β-CRX is an antioxidant phytochemical and may help prevent oxidative damage[69]. Thus, it is believed that β-CRX has health benefits for people with risk of chronic diseases.

Numerous possible mechanisms for the anti-carcinogenic potential of β-CRX have been proposed. These include the antioxidant function that is associated with the enhancement of DNA repair[55,69], suppression of efficacy of key proinflammatory cytokine expression, such as tumor necrosis factor-α[55] and an apoptotic induction effect[70]. Also, β-CRX is known to stimulate the expression of the *RB* gene (a tumor-suppressor gene) and *p73* gene (a *p53*-related gene)[71] and reduce the expression of NF-κB and activator protein-1 (AP-1), that induces numerous genes including inflammation, cell proliferation, and apoptosis[55]. These mechanisms indicate that β-CRX may be a promising chemopreventive agent against cancer. Indeed, β-CRX exerts an anti-tumor promoter action *in vitro*[72] and

inhibits chemically induced carcinogenesis *in vivo*[62,71,73,74]. Previously, we investigated the effects of β-CRX extracted from *C. unshiu* oranges on OH-BBN-induced urinary bladder carcinogenesis in male ICR mice[62]. OH-BBN-exposed mice were fed with 1, 5 and 25 ppm of β-CRX for 24 wk starting 1 wk after the cessation of OH-BBN exposure. Feeding with β-CRX decreased the incidence and multiplicity of precancerous and cancerous urinary bladder lesions. Especially, 25 ppm β-CRX markedly reduced the occurrence of bladder cancer. Meanwhile, β-CRX is also reported to reduce mouse skin[71], mouse lung[74] and rat colon[71] carcinogenesis. In our report, β-CRX lowered ratios of cyclin D1-positive cell in various urinary bladder lesions, meaning that reduction in the incidence of precancerous and cancerous urinary bladder lesions is due to reduced cell cycle progression[62].

Clinical studies

The efficacy of β-CRX supplementation on obesity have been investigated[75]. Seventeen postmenopausal obese women were provided 200 mL of a beverage containing β-CRX (1.56 mg/serving and 4.7 mg/d) for 3 wk[75]. As a result, the levels of serum β-CRX were significantly elevated from 0.28 (initial period) to 1.15 mg/mL, and high molecular weight-adiponectin was also elevated from 9.8 to 11.1 mg/mL[75]. At the end of the study, the levels of serum triglyceride ($P = 0.057$) and total plasminogen activator inhibitor-1 (PAI-1) ($P = 0.052$) tended to decrease. Nishino *et al*[60] reported an intervention study where β-CRX-rich mandarin orange juice (3 mg β-CRX in 80 mL) was provided for 12 wk to obese men or obese men with elevated serum γ-glutamyl transpeptidase (γGTP) levels[60]. After drinking β-CRX for 12 wk, body weight ($P < 0.001$), BMI ($P < 0.001$) and β-GTP levels ($P < 0.005$) were decreased.

An intervention trial regarding prevention of liver cancer has also been reported[60]. Viral hepatitis with cirrhosis patients were randomly assigned into two groups in the trial. The treatment group was administered mandarin orange juice enriched with β-CRX and with the carotenoids mixture (lycopene, β-carotene and α-carotene). Patients in the control group were administered a carotenoids mixture alone. At year 2.5, cumulative incidence of liver cancer/hepatocellular carcinoma development in the mandarin orange juice group was lower than that of the carotenoids mixture alone group ($P = 0.05$). The combinational use of natural carotenoids containing β-CRX might be valuable for the prevention of liver cancer in hepatitis virus infected patients with cirrhosis.

FUCOXANTHIN

Distribution and nature of fucoxanthin

Brown seaweeds include *Undaria pinnatifida* (wakame), *Hizikia fusiforme* (hijiki), *Laminaria japonica* (ma-kombu) and *Sargassum fulvellum*. The Japanese have been estimated to intake wakame at 1 g/d[76]. Brown seaweeds are known to contain many bioactive components, *i.e.*, fucoxanthin (FX), fucoidan, vitamins, minerals, dietary fibers, proteins, ω-3 polyunsaturated fatty acids (PUFAs), polysaccharides, other carotenoids and various functional polyphenols. Fucoidan is a sulfated polysaccharide that is one of the major bioactive components in seaweed[77,78], but we would like to focus on FX in this review. FX is a xanthophyll belonging to non-provitamin A carotenoids, constructed with an unusual allenic bond, an epoxide group, and a conjugated carbonyl group in a polyene chain[79]. Some reports demonstrated that the FX content of *U. pinnatifida* is approximately 1.0-3.0 mg/g dry weight through one life cycle[80,81]. It has been proven that mice convert FX into keto-carotenoids by oxidation of the secondary hydroxyl groups (FX + H_2O → FuOH; FuOH + NAD^+ → amarouciaxanthin A + NADH)[79]. On the other hand, oral administration of kombu extract containing FX in humans revealed that the FuOH and the *cis*-isomer of FuOH could be found in the serum, detected by HPLC[82].

Safety profile

FX has been proved to be safe with no side effects by single (1000 or 2000 mg/kg BW) and repeated (500 or 1000 mg/kg BW for 30 d) oral dose toxicity studies in male and female mice[83]. In the repeated doses study, histological examination of the gonadal tissues, kidneys, liver and spleen revealed no abnormal changes[83]. In rats, 13-wk oral subchronic toxicity studies suggested that more than 2000 mg/kg BW of microalgal FX oil induce the 50% lethal[84].

Preclinical studies and anti-cancer mechanisms

Many studies suggested FX possesses anti-cancer potential, especially shown in colon cancer cell lines (Caco-2, DLD-1 and HT-29), liver cell lines (HepG2), prostate cancer cell lines (DU 145, LNCaP and PC-3) and urinary bladder[85-88]. The main biomolecules involved in anticancer mechanism is assumed to be the biomolecules related to apoptosis and cell cycle[89,90] and those may associate with antioxidant activity through their free radical scavenging action[91]. Moreover, inhibition of PI3K/Akt and NF-κB signals were reported in human cervical and breast cancer cells, respectively[92,93].

Its metabolite fucoxanthinol (FuOH) also has inhibitory effects on cancer cell growth[94,95], and 1,2-dimethylhydrazine-induced formation of colonic ACF in mice and AOM/DSS-induced colon carcinogenesis[25,96]. To find new cancer prevention approaches, we investigated the combination effect of FuOH and 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$), and found inhibition of cell viability and induction of apoptosis in DLD-1 and HT-29 cells[97]. Down-regulation of PPARγ and NF-κB p52 were suggested to be involved in the inhibition of cell viability due to the combination of FuOH and 1α,25(OH)$_2$D$_3$. It has been shown that activation of PPARγ suppresses intestinal polyp development in *Apc*-mutant mice and AOM-induced colonic ACF development in obese KK-*A*^{y} mice[98,99].

Clinical studies

FX has been reported to provide health benefits in humans, such as improvement of obesity, reduction of inflammation, healthy triglyceride levels, and improvements in blood pressure levels[100,101].

After daily intake of *U. pinnatifid*a, FuOH is detectable in human plasma[82]. Although metabolites of FX could be measured as a marker of exposure, effects of FX or FuOH in human carcinogenesis have not been reported to date. From the aspect of obesity-associated cancer, we here introduce one study that has been conducted to assess the effects of FX supplementation on weight loss. FX supplementation on obese patients with nonalcoholic fatty liver disease results in the improvement of liver inflammatory markers, such as alanine aminotransferase, aspartate aminotransferase, C-reactive protein, γ-glutamyltransferase (γGT, GGT)[101]. Of note, it has been demonstrated that increased of GGT plasma levels are associated with an increased risk of pancreatic cancer[102,103], nevertheless GGT has no causative role itself.

It is also interesting to mention that intake of 5 g/d *U. pinnatifid*a stimulated a significant 50% reduction in urinary urokinase-like plasminogen activator receptor (uPAR) proteins in postmenopausal women. uPAR, is the membrane receptor for uPA, responsible for extracellular membrane proteins degradation and PAI-1, responsible for the inhibition of plasminogen activation[104]. Generally, uPAR is known to be higher in postmenopausal women as well as in breast cancer patients[105]. Moreover, it has been reported that uPA and/or PAI-1 is positively correlated with poor prognosis in patients with breast cancer, *i.e.*, correlation with cancer metastatic potential[106,107]. Thus, uPA, PAI-1and uPAR might be used as prognostic markers for breast cancer[108], and FX may reduce such a tumor marker.

NEOXANTHIN

Distribution and nature of Neoxanthin

Neoxanthin (NX), a non-provitamin A carotenoid, has an unusual allenic bond and a 5,6-monoepoxide as well as FX. NX is widely present in terrestrial and marine biota and the occurrence of two geometric *cis*/*trans* isomers is known to be species dependent[109-111]. The 9'-*cis* form of NX (9'-*cis* NX) is mainly localized and used in the photosynthetic organs of spinach leaves and marine algae such as *Euglenophyta*. It is also used as a precursor of abscisic acid, a plant hormone[112,113]. Whereas the all-*trans* form of NX is predominant in the petals of globeflower and yellow rose, this xanthophyll is not involved in the photosynthetic system[111,114]. We mainly obtain the 9'-*cis* NX from leafy green vegetables. Fresh spinach contains 9'-*cis* NX around 5 mg/100 g in fresh leaf[110]. It has been estimated that 9'-*cis* NX exists at 0.95 μmol/L in digested fluid (9 L/d), when we ingest 100 g/d spinach.

The 5,6-monoepoxide moiety in 9'-*cis* NX is easily isomerized to 5,8-epoxide under the acidic conditions of the stomach and generates almost equal amounts of (8'-*R*/*S*)-neochrome[115,116]. After a 1-wk spinach intervention (3 mg 9'-*cis* NX/day), highly hydrophilic xanthophylls of 9'-*cis* NX and (8'-*R* and 8'-*S*)-neochromes appeared at a very low level in human plasma (about 1 nmol/L)[117]. It is known that the uptake of various carotenoids by human colon cancer cells (Caco-2 cells) positively correlates with the lipophilicity of the carotenoids[118]. The highly hydrophilic xanthophylls such as NX, FX and violaxanthin could be detected slightly in human plasma, when we intake purified forms and food matrices[79,101,117,119-121]. Because of the poor intestinal absorption of NX, a considerable amount of ingested 9'-*cis* NX and (8'-*R* and 8'-*S*)-neochrome would be delivered to the colon, and even if absorbed in the small intestine, they would be metabolized easily.

Preclinical studies and anti-cancer mechanisms

It has been reported that both 9'-*cis* NX and all-*trans* NX possess strong potential of cell growth inhibition and apoptosis induction in human prostate cancer cells[87,94,115,122], human colon cancer[122-124], mouse melanoma[122] and mouse embryonic mesenchymal cells[125]. In addition, several researchers have reported that 9'-*cis* NX, all-*trans* NX and (8'*R*/*S*)-neochrome have cancer preventive effects[126], and also anti-tumor promoter functions[70]. Moreover, induction of cell cycle arrest[115], anti-oxidant properties[127] and anti-obesity properties[128] have been reported. Recently, we additionally demonstrated that 9'-*cis* NX rapidly accumulated in the mitochondria, caused mitochondria $\Delta\Psi$ loss and thereafter the release of cytochrome *c* and production of apoptosis-inducing factor in human colon cancer cells[123]. It is regrettable that there is little information about the anti-cancer mechanisms of dietary NX in mammals, except for that described above.

Safety profile and clinical studies

No safety profile and clinical studies have been reported on 9'-*cis*NX, any -*trans* NX and (8'*R*/*S*)-neochromes. However, epidemiological data show that higher intake of fruits and vegetables, rich in highly hydrophilic epoxyxanthophylls such as NX, is associated with a lower risk of colorectal cancer[129,130]. Further studies are required to elucidate the clinical beneficial properties of NX.

ZEAXANTHIN/LUTEIN

Distribution and nature of zeaxanthin / lutein

Zeaxanthin (ZX) and lutein also belong to the xanthophyll family. Their unique character is that they are the only carotenoid among more than 600 species of carotenoid existing in eye tissue, especially in the retina[131]. Lutein can be photochemically transformed to meso-ZX. They are stereoisomer of each other, differ by the location of a double bond. Lutein is abundant in egg yolk, and in dark-green leafy vegetables, such as broccoli, brussels sprouts, kale and spinach[132]. In the human body, lutein is distributed at the skin, breasts, cervix uteri, and also found in serum in high amounts. Serum lutein and

ZX levels are reported to be around 180 and 20 ng/mL, respectively[133]. They are assumed to play a critical role in ocular health because they act as strong anti-oxidants and filtered out high-energy blue light[134]. Of note, no correlation between plasma concentrations of lutein/zeaxanthin and BMI or insulin resistance has been reported[135].

Epidemiologic studies

In many papers, target organs for lutein are reported to be the eyeballs, the skin and the heart. Regarding ocular conditions, age-related macular degeneration, cataracts, and retina pigmentosa have been reported to have some correlation with lutein. Lutein also possesses a preventive function of cardiovascular diseases/stroke[131,134,136,137].

Regarding lung cancer, some epidemiologic studies state lutein has an important cancer preventive function[4,14]. A ten-year study of 120000 United States people revealed that lung cancer incidence was significantly reduced in those who ingested a high amount of total carotenoids, including lutein and ZX[138]. Similar relationships were found in Fijians, when compared to the other South Pacific islands' people. Fijians intake 25 mg lutein daily on an average (200 g dark greens), whereas other 20 South Pacific countries intake less lutein in diets[139]. Thus, there was a clear inverse association with lutein intake and lung cancer incidence.

Regarding colorectal cancer, inverse associations with dietary lutein intake have been reported[124], and serum ZX concentration by Okuyama *et al*[140]. However, no association has been detected between the levels of plasma lutein and the risk of gastric cancer[141].

Regarding skin cancer risk, the specific effects of lutein are not fully known. The only reported data is that a combination of carotenoids may protect erythema development in human skin[142], and that may be correlated with the presence of skin cancer or precancerous lesions[124].

Regarding breast cancer, there is some possibility for protective effects of lutein[6,14,143]. Intake of lutein-rich foods significantly lowered the risk of premenopausal breast cancer. The Nurse's Health Study demonstrated a weak inverse association, but significant, between lutein and ZX intake and the breast cancer risk among premenopausal women[6]. Of note, the protective effect of lutein and ZX was strongest in patients have a family history of breast cancer. Also there is a report that increasing serum levels of lutein and ZX were associated with a reduced breast cancer risk, but the trend was only marginally significant in a case-control study[143]. There is a report comparing biopsy samples from breast cancer tissue and benign mammary tissue. In this report, increasing lutein and ZX concentrations tends to decrease the risk of breast cancer[144]. Meanwhile, Other studies have shown that there are no differences of lutein and ZX concentrations in mammary adipose tissue between benign breast tumors and breast cancer[145]. New York University Women's Health Study, a nested case-control prospective study, demonstrated an inverse relationship between plasma levels of lutein, but not ZX, and risk of breast cancer[146].

Regarding other cancers, significant inverse relations were observed for lutein and ZX in oral cavity and pharyngeal cancer[147].

Safety profile

No toxicities or adverse reactions for intake of lutein/ZX have been reported at doses up to 40 mg/d for 2 mo[131,148]. High doses of β-carotene supplements (> 30 mg/d) are well known to be associated with carotenodermia[149], and the same could happen when we consume high doses of lutein and ZX. Also it has been demonstrated that lutein has no mutagenic effect in the Ames test[150].

Preclinical study and anticancer mechanism

Lutein/ZX is thought to have a superior anti-oxidant ability to scavenge free radicals than other carotenoids. An *in vitro* study showed that lutein could quench peroxy radicals and play a guarding role against oxidative injury[151,152]. In this experiment, a synergistic antioxidant effect was obtained with a combination of lutein and lycopene[153]. Carotenoids also show a superb function for immune response[154].

Lutein could also function as an anti-carcinogenic reagent, such as a modulator of cell growth and apoptosis signaling. Lutein induces cell cycle arrest in human prostate and esophageal cancer cell[155,156]. Lutein induces apoptosis in transformed cancer cells but do not induce apoptosis in normal human mammary cells through modulating the ratio of Bcl-xL/Bax protein expression[157]. Meanwhile, ZX, structural isomer of lutein, induced cell cycle arrest in human beast cancer cells[158]. Lutein stimulates some genes involved in T-cell transformations activated by antigens, cytokines and mitogens[159]. Lutein interacts with carcinogens such as 1-nitropyrene and aflatoxin B1, and lowered its carcinogenetic activity[150,160]. In a recent report, female BALB/c mice were fed a diet containing lutein for 14 d, and then inoculated with 0 to 2.5 × 10^3 mammary tumor cells. The results demonstrated that 0.002% and 0.02% lutein lowered both mammary tumor incidence and tumor growth[161].

FUTURE ASPECTS

The versatile functions of xanthophylls have shown great potential for the prevention of metabolic syndrome and cancers, both *in vitro* and *in vivo*. Xanthophylls have been verified as safe with no side events, and several xanthophylls provide other health benefits, including improvements in inflammation, dyslipidemia, hypertension and liver function, as shown in this review. The accumulated evidence indicates the functionality of xanthophylls as anti-obesity and anti-insulin-resistance functional foods, implying that xanthophylls could be useful in preventing obesity-associated cancer.

The chemical synthesis of each xanthophyll is not impossible, but it may be very expensive. However, the

promising results obtained from *in vivo* studies encourage researchers to undertake more clinical studies in humans. We have some information about xanthophylls trials, and we should further promote human clinical studies to obtain information about the adequate dosage of xanthophylls needed to prevent cancers.

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P- Reviewer: Gagliardi G, Muscarella P, Schweiger U
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5497/wjp.v3.i4.153

World J Pharmacol 2014 December 9; 3(4): 153-161
ISSN 2220-3192 (online)
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MINIREVIEWS

Pharmacological role of efflux transporters: Clinical implications for medication use during breastfeeding

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Received: June 28, 2014 Revised: September 3, 2014
Accepted: September 23, 2014
Published online: December 9, 2014

Abstract

The World Health Organisation recommends exclusive breastfeeding for the first six months of an infant's life and in combination with solid food thereafter. This recommendation was introduced based on research showing numerous health benefits of breastfeeding for both the mother and the infant. However, there is always concern regarding the transfer of medications from mother to their breastfed baby *via* milk. Pharmacokinetic properties of a drug are usually used to predict its transferability into breast milk. Although most drugs are compatible with breastfeeding, cases of toxic drug exposure have been reported. This is thought to be due to active transport mechanisms whereby efflux transporter proteins expressed in the epithelial cells of the mammary gland actively secrete drugs into milk. An example of such efflux transporters including the breast cancer resistance protein which is strongly induced during lactation and this could result in contamination of milk with the substrates of this transporter which may place the suckling infant at risk of toxicity. Furthermore, there is little known about the substrate specificity of most efflux transporters as we have highlighted in this review. There also exists some degree of contradiction between *in vivo* and *in vitro* studies which makes it difficult to conclusively predict outcomes and drug-drug interactions.

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Key words: Active efflux transporters; Lactation; Breastfeeding; Mammary gland; Breast cancer resistance protein; P-glycoprotein; Breast milk; ABC transporters

Core tip: The aim of this review was to analyse the available literature on psychoactive drugs specifically selective serotonin reuptake inhibitors, antipsychotics and antiepileptic drugs that are commonly prescribed during lactation and pregnancy. This review investigated whether these drugs are substrates and/or inhibitors of efflux transporters especially of P-glycoprotein and breast cancer resistance protein and whether this has any effect on adverse outcomes in the breastfed infant of mothers who use these pharmacotherapeutic agents. Current evidence on acute adverse effects in breastfed infants due to the aforementioned drug groups either as sole treatment or their use in combination with other drugs was also explored.

Ahmadzai H, Tee LBG, Crowe A. Pharmacological role of efflux transporters: Clinical implications for medication use during breastfeeding. *World J Pharmacol* 2014; 3(4): 153-161 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/153.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.153

INTRODUCTION

It is widely accepted that breastfeeding is the best way of ensuring a good start in an infant's life as it not only has a favourable nutrient content but also provides passive immunity and various growth hormones to the breastfed infant[1-4]. However, there is always concern regarding the transfer of medications taken by the mother to the infant *via* breast milk. To understand the safety of medicines in a

nursing mother, it is important to elucidate the mechanism of such drug transfer.

The major determinant of the transferability of drugs from mother to baby *via* breast milk is usually calculated using parameters such as the physicochemical properties of the drug and the composition of the milk[4]. However, research has shown that active transport *via* efflux transporters may have a significant role in the transfer of drugs from the maternal plasma to breast fed infant. It has been shown that breast cancer resistance protein (BCRP) (also known as ABCG2), belonging to the ATP-binding cassette (ABC) superfamily of transporters is strongly induced during lactation[5]. There are also many other transporters belonging to the ABC family such as P-glycoprotein (P-gp) (also known as ABCB1 or MDR1) which like BCRP have some toxicological significance in lactation. Other transporters belonging to the solute carrier family such as the organic cation and anion transporters, peptide and nucleoside transporters also found in the mammary gland play an important role in the active transport of many nutrients, endogenous substances and xenobiotics[6].

The protective role of P-gp in the blood brain barrier is well established. It inhibits a wide variety of substrates from entering the central nervous system[7]. The main focus of research has so far been on the role of these transporters in the gastrointestinal tract and the blood brain barrier (BBB) affecting bioavailability of drugs, pharmacoresistance leading to ineffective drug treatment. Modulation of these efflux transporters can have an impact on drug absorption, disposition and consequently therapeutic outcome. However, we know that P-gp is also widely expressed in many other human tissues including the liver, kidneys, testes, placenta and the mammary epithelial cells[8]. It is also well known that these ABC transporters play a crucial role in the protective mechanism during embryogenesis in the placenta which is continued during lactation providing foetal protection against naturally occurring toxins[9]. Our area of interest is the role of these transporters in the lactating human mammary epithelial cells (HMEC) where they could potentially influence transfer of drugs from mother to their breastfed baby *via* breast milk. However, this area remains poorly researched and there is lack of controlled studies that can provide conclusive evidence. On the basis of other organ systems, co-administration of medications that are substrates or inhibitors of these transporters in a nursing mother could have significant drug-drug interactions which may lead to adverse effects in their breastfed infant. Several *in vitro* and animal studies have been conducted which address this area of concern. However, the applicability of these *in vitro* findings may not always be conclusive and are often contradictory due to differences in experimental design and use of species other than humans[10-12]. Most animal species such as rats and mice used in the laboratory have two genes (*Mdr1a* and *Mdr1b*) that code for P-gp, which further complicates issues regarding induction, expression and drug-drug interactions[11].

TRANSFER OF DRUGS FROM MATERNAL PLASMA TO THE BREASTFED INFANT

The transfer of drugs from maternal plasma to breast milk occurs *via* passive and active mechanisms[9,13]. The critical determinants of passive transfer include drug protein binding, drug ionisation and fat partitioning[4,13]. These factors can be used to predict milk to plasma (M:P) ratio where passive diffusion is thought to predominate. Other pharmacokinetic parameters such as half-life of the drug, protein binding, water and lipid solubility, route of drug administration, bioavailability, dissociation constant, volume of distribution, molecular size and ionisation potential can further help to determine the transfer of drugs from mother's plasma into the breast milk[14]. Drugs with the shortest plasma half-life, highest protein binding and lowest lipid solubility usually have the lowest ductal milk transport. The dose of a drug that an infant receives during breastfeeding depends on the amount excreted into the breast milk, the daily volume of the milk ingested and the average plasma concentration of the mother. Thus, M:P ratio has large inter-subject variability[15]. Although the transfer of most drugs into breast milk can be explained by passive diffusion theories, a review of the literature shows that there are several drugs where the actual measured M:P ratio is significantly greater than predicted[16-19]. Nitrofurantoin, acyclovir and cimetidine are some drugs which exhibited a significantly higher observed M:P ratio than predicted[5,7,16,18,20]. In one study nitrofurantoin had an observed M:P ratio of 6 as opposed to the predicted 0.28[17]. It has also been shown that several members of the ABC drug efflux transporters significantly affect the pharmacokinetic disposition of drugs such as the quinolones, thereby increasing their secretion into breast milk[17,19].

ROLE OF EFFLUX TRANSPORTERS IN THE TRANSFER OF DRUGS FROM MATERNAL PLASMA TO THE BREAST FED INFANT

The extent of the involvement of these ABC transporters in the transfer of many nutrients including essential vitamins and drugs into the breast milk has been recently considered[21]. There are several efflux transporters in human mammary epithelial cells that line the alveoli within the mammary gland[8,22,23]. This leads us to believe that there may be a more substantial role of these transporter proteins in the transfer of many compounds from maternal plasma to the breast milk than currently perceived. Alcorn *et al*[8] have shown that there is some of variability between the level of RNA expression of various transporters in the HMEC from lactating *vs* non lactating breast tissue, indicating a graded expression change during induction of the lactation process that could lead to significant changes in substrate transport

during lactation. Using immunocytochemical analysis and functional studies in primary human mammary epithelial cells culture, our group have demonstrated the presence of MDR1 (ABCB1), MDR3 (ABCB4) and MRP1 (ABCC1) in these cells[21].

Gilchrist and colleagues showed that there is a stage dependent change in the expression of transporters in rat mammary gland and isolated mammary epithelial organoids[24]. Using quantitative reverse transcription polymerase chain reaction, they demonstrated that the various solute carrier and ABC transporters showed a changing pattern in the different stages of lactation. Ling *et al*[25] studied the M:P ratio of cefepime, an actively transported drug at four and ten days post-partum in rats and found a significant reduction in the amount of cefepime excreted at these two time points. This leads us to believe that as lactation progresses from stages of mammogenesis to lactogenesis to galactopoiesis, changes in the expression of efflux transporters along with changing hormones may influence the transfer of endogenous and exogenous substances from mother to baby *via* breast milk. However, currently there are no studies in humans to confirm this. Our laboratory is presently investigating whether the expression of efflux transporters in humans follow a stage dependent pattern as seen in animal studies.

The expression of MRP1 (ABCC1) and MDR1 (ABCB1) are significantly lower in the lactating HMEC as compared to non-lactating HMEC[8] whereas that of BCRP is significantly higher[5]. It is important to note that there is a substantial overlap in the substrate specificities of these transporters[6]. These findings highlight the importance of possible drug-drug interactions between various transporter substrates and/or inhibitors when co-administered at different stages of lactation. In addition, it is important to take into account the localization of these transporters, such that their presence in the apical surface (MDR1 and BCRP) may pump drugs into milk and further place the suckling infant at risk of xenobiotic exposure[20]. Alternatively, if these transporters are located in the basolateral membrane of the cell (MRP1), then the substrate will be pumped out of the milk and into the mother's blood, thereby reducing infant exposure.

The active efflux transporters usually help in preventing accumulation of drugs into the tissues as they work against a concentration gradient and push drugs from the tissues back into the blood[7,8,24]. There have also been reports of transporter proteins being involved in the transfer of essential nutrients and vitamins to the breast fed infant which are mostly located in the basolateral side[7,22,26]. However, the extent to which they affect drug transfer in the mammary gland is not fully known[6]. Similar to P-gp, BCRP has a protective role at the blood side of many organ systems by facilitating the extrusion of toxins, xenobiotics and drugs out of the capillaries prior to interstitial accumulation with an important role being in the blood-placental barrier, where they protect the foetus from endogenous and exogenous toxins[27]. A current vexing question is how relevant is the role of efflux transporters at other blood-tissue barriers such as in HMECs of a lactating female who is breastfeeding her infant compared to the plethora of studies examining gastrointestinal or BBB transport. Can P-gp or BCRP modulation by an inhibitor drug that the mother consumes cause less drug to be transferred to the breastfed infant *via* milk? Also if P-gp is located on the apical membrane of the HMEC, the evidence that RNA expression of P-gp is lower in the lactating HMEC[8] could have a relatively protective effect on the breastfed infant, by virtue of less P-gp to excrete drug into the milk (Figure 1).

The expression of BCRP in pregnant mice was found to be strongly induced during late pregnancy and lactation, which is the opposite to that of P-gp[5]. Lindner and colleagues reported that the expression of BCRP in the mammary glands of several species of animals including sheep, goats and cows were significantly increased (up to 10 fold) during pregnancy and lactation[28]. Both P-gp and BCRP are located in the apical membrane of alveolar epithelial cells of the mammary gland and actively transport their substrates into breast milk as confirmed by animal studies[5,20]. BCRP has a significant role in accumulation of drugs and xenotoxins in breast milk which could be either beneficial or detrimental to the breastfed infant's health depending on the drug administered[29]. A BCRP substrate that is toxic can accumulate in milk and result in adverse effects in the infant whereas the accumulation of a drug such as aciclovir could be beneficial in reducing transmission of milk borne viruses from mother to baby. There is some evidence that the role of P-gp in the lactating HMEC is relatively insignificant in the transfer of medications which could possibly be due to its down regulation in lactation. Animal studies have shown that the transfer of nelfinavir, a known P-gp substrate is not affected by P-gp in the mammary gland[28]. However, many other studies have shown that the role of BCRP is much more significant, given that BCRP is strongly induced during pregnancy and lactation[5,16,18,30].

As there is a significant overlap between P-gp, BCRP, other efflux transporters and CYP3A4 substrates[6], it is crucial that each drug or the combination of drugs is considered with respect to these transporters and metabolism pathways in addition to the usual pharmacokinetic parameters to ensure minimum inadvertent exposure to a breastfed infant.

SPECIFIC DRUGS

Antidepressants - selective serotonin reuptake inhibitors

Post-natal depression is considered to be a significant problem in women of child bearing age with approximately 14% of all women affected by this condition at some stage[31]. Psychotherapy is considered quite useful in the management of post natal depression but due to a lack of adequate service provision in the community, it is often necessary to treat women with pharmacotherapeutic agents[31]. Selective serotonin reuptake inhibitors (SSRI) are considered the mainstay of postnatal depression due to their perceived low transferability into breast milk and safety profile unlike tricyclic antidepressants that may have a higher breast milk ex-

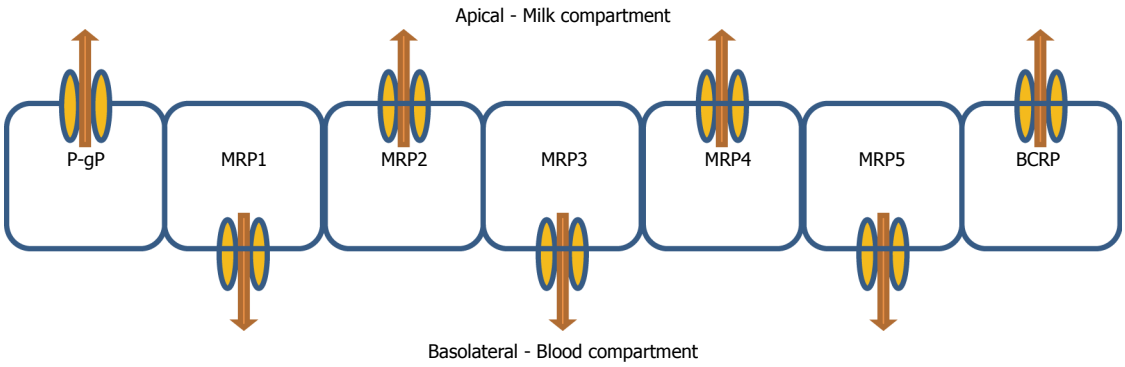

Figure 1 Schematic representation of expected localisation of drug efflux transporters in the human mammary epithelial cells. P-gp: P-glycoprotein; BCRP: Breast cancer resistance protein; MRP: Multidrug resistance protein.

posure leading to reduced usage[31]. The recommended SSRIs for post-natal depression are sertraline and paroxetine as they have been widely studied and are not associated with many adverse effects[32]. Fluoxetine and citalopram have measurable plasma levels in some infants[33,34]. However, these levels were usually low. Some women may choose to continue using one of the other SSRIs that they have been stabilised on before and during pregnancy. Nonetheless, most SSRIs are considered to be fairly safe in breastfeeding.

Paroxetine and fluoxetine are thought to be P-gp substrates[35]. Citalopram and its enantiomer, escitalopram have also been found to be substrates of P-gp in animal studies as demonstrated by Weiss *et al*[36]. The implications and relevance for this evidence in lactation remains elusive. However, *in vivo* it has been shown that the peak plasma concentration and area under the curve of paroxetine, is significantly increased by itraconazole (a P-gp and CYP3A4 inhibitor)[37]. Again, this could mean that a P-gp substrate which normally has low transferability into breast milk may potentially transfer in significantly higher amounts if given with a P-gp inhibitor.

Furthermore, it has been found that sertraline, a P-gp substrate and inhibitor, can modulate P-gp both *in vivo* and *in vitro* at the BBB and blood testes barrier sites[38]. This finding suggests that concurrent administration of other P-gp substrates with sertraline potentially increase CNS penetration of that substrate[39]. Again, the significance and implication of this finding in lactation needs to be investigated further as another study found modulation of P-gp by sertraline was site specific with different tissues reacting to sertraline in different ways[40]. It is important to investigate whether this finding has any potential for causing interactions in the lactating HMEC leading to adverse effects in the breastfed infant as this tissue was not directly investigated. A different study by Bhuiyan *et al*[41] found that a single dose of sertraline does not affect the pharmacokinetic profile of fexofenadine, another P-gp substrate but paroxetine and fluvoxamine do. There was no data with regards to BCRP substrate specificity in the selective serotonin reuptake inhibitors.

Of other new antidepressants, duloxetine, which is a serotonin noradrenaline reuptake inhibitor (SNRI), not used often in pregnancy and breastfeeding due to lack of safety data in this population, was found to cause no immediate adverse effects in an exclusively breastfed 32 d old infant[42]. The measured M:P ratio and the relative infant dose for this drug were also found to be very low[43]. Venlafaxine, another SNRI was found to induce BCRP in brain tissue and is thought to be a P-gp substrate whereas desvenlafaxine, the active metabolite of venlafaxine was found to have no effect on BCRP induction or P-gp modulation[44,45].

Another case study on sertraline reported signs of serotonergic overstimulation in a preterm baby whose mother had therapeutic levels of both the drug and its metabolite, desmethylsertraline. The symptoms disappeared on discontinuation of breastfeeding[46]. This adverse reaction was attributed to immaturity in the development of the infant's clearance mechanisms and lack of development of the BBB. Interestingly, the plasma sertraline and desmethylsertraline levels of the infant were significantly below the threshold levels considered to cause symptoms. There was no record of other medications that may have been used by the mother acutely or long term during the postpartum period. Hence, it raises the question of whether another drug(s) administered acutely, able to modulate P-gp activity, could have resulted in the adverse effects experienced by the breastfed baby. The lack of a full medication record makes it difficult to draw conclusions regarding the drug-drug interactions.

Current guidelines place paroxetine and sertraline amongst the recommended antidepressant drugs for use in lactation. It is imperative that infants of mothers taking these drugs are regularly monitored for adverse effects especially when the mothers are also treated acutely or chronically with another pharmacologic agent that could modulate active transporters as these drugs have the potential to do[35,47].

Antipsychotics

About one third of pregnant women with psychotic illness use antipsychotics at least once during pregnancy or whilst breastfeeding[48]. About 10% of women of child bearing age have a postpartum psychiatric disorder, with a significant number warranting the use of an antipsychotic medication[49]. Although the second generation (atypical)

antipsychotics are considered the best treatment option for schizophrenia, female patients who are pregnant or breastfeeding are often excluded from this treatment option due to safety concerns.

Several atypical antipsychotics are substrates for P-gp in therapeutic concentrations. These include amisulpride, aripiprazole, olanzapine, risperidone, quetiapine and paliperidone, with quetiapine and risperidone having high affinity for P-gp[50]. Friedman *et al*[50] found olanzapine and quetiapine to be P-gp substrates whereas another *in vitro* study by Müller *et al*[47] contradicted this finding and identified that quetiapine, haloperidol, olanzapine and clozapine are not P-gp substrates. As the first study assessed P-gp activity by measuring ATPase activity whereas the second study was an inhibition study carried out using Caco-2 cell monolayers, these different methods of P-gp substrate identification may be responsible for their different conclusions. Our studies using Caco-2 monolayers support the latter work, demonstrating the lack of P-gp mediated efflux associated with quetiapine and olanzapine. Hence, it is important to exercise caution when interpreting results from different cell lines and membrane systems[35]. *In vivo* studies using knockout and wild type mice may be more reliable and may provide a clearer picture of functional consequence[51]. However, it is worth noting that these rodents may have more than one gene coding for P-gp, hence may differ from human P-gp structurally.

Several antipsychotics including clozapine, quetiapine, paliperidone and chlorpromazine also exert some inhibitory effects on BCRP[52]. Risperidone has major inhibitory effects on BCRP, making it a potential contributor of adverse effects with co-administered with BCRP modulators such as commonly prescribed pantoprazole and omeprazole[52,53]. Aripiprazole, an atypical antipsychotic was found to have an inhibitory effect on BCRP[54]. Most antipsychotics are thought to act as inhibitors of P-gp/BCRP and therefore can influence plasma and brain concentrations of other substrates[55].

A prospective controlled observational study of olanzapine use in 30 pregnant women who were taking olanzapine during pregnancy and whilst breastfeeding found that no adverse effects were imputable to the use of olanzapine by the mother. However, the rate of breastfeeding was significantly lower in the treated group. Also three out of thirty babies (10%) experienced withdrawal symptoms after birth and interestingly all three mums were on multiple medications including zuclopenthixol, lithium and paroxetine[56]. A case report of a lactating patient who was taking olanzapine after a psychotic episode reported low infant plasma levels of olanzapine and no adverse effects in the breastfed baby[57]. Another case study reported no adverse effects in the infant of a woman who was initiated on olanzapine during her third trimester and continued breastfeeding six months postpartum[49]. There is no mention of concurrent therapy if any. A case report of risperidone by Lutz *et al*[58] showed that risperidone, a P-gp substrate, and its metabolite 9-hydroxyrisperidone were moderately transferred into breast milk. A dose (concentration) of less than 10% of that received by the mother (on a mg/kg basis) has been suggested and is widely accepted as a "safe" dose (or concentration) in the infant[45]. Although, the amount transferred (4.3% of maternal dose) was below the notional 10% threshold, the mother was encouraged not to breastfeed due to concerns for the safety of her infant. Again, no information was provided on whether the mother was on any other concurrent medications. Certainly, we would prefer clinical studies to state that there were no other medications in the study rather than just omitting this crucial information. Often when the focus is on one particular drug and snapshot studies such as M:P ratio are being investigated, other medications do not make it into the clinical notes. Given our presumption that in psychiatry multiple medications are often used concurrently, this makes subsequent contextual analysis very difficult. Furthermore, a series of case reports by Illet's group[59] confirmed the findings from the first report that risperidone on its own is not transferred into breast milk in levels high enough to be considered a clinical issue for the safety of the breastfed infant.

Ziprasidone, an atypical antipsychotic was found to be excreted in very low concentrations in breast milk from a treated patient while no adverse effects were observed in the infant of a different patient with psychotic depression treated with citalopram and ziprasidone[60,61]. It is not known whether ziprasidone is a P-gp substrate or BCRP modulator at this time[55]. Further research and more long term studies are warranted to ensure the safety of the newer antipsychotics on infant growth and development.

Amisulpride, another atypical antipsychotic has been used in one woman who was breastfeeding her 13 mo old infant. An unusually high M:P ratio for this drug compared to predicted values based on pharmacokinetic parameters was found[62]. This high M:P ratio was attributed to amisulpride being a P-gp substrate[47,63]. Amisulpride used in conjunction with desvenlafaxine in a partially breastfed infant yielded no higher than expected relative infant dose. Given that Amisulpride is a P-gp substrate, the combination with desvenlafaxine did not appear to alter its pharmacokinetics reaffirming the *in vitro* evidence that desvenlafaxine does not modulate efflux transporters[44,45]. Olanzapine is considered a weak substrate whereas data for quetiapine are contradictory with one study identifying it as a substrate and the other not a substrate[47,63]. Another *in vitro* study found that olanzapine and risperidone may inhibit P-gp activity. Most other drugs in this therapeutic group though, such as clozapine, haloperidol chlorpromazine and quetiapine did not inhibit P-gp[64]. Much of the studies discussed above were cell based, and projecting this data into clinical studies has been sorely lacking. Nonetheless, it is appropriate to exercise caution when using these agents especially in combination with another agent which may modulate P-gp especially in a lactating mother who is breastfeeding or exclusively breastfeeding, given that some studies sup-

port the notion that blocking P-gp (or other efflux transporters) can elevate milk concentration of these drugs.

Antiepileptic drugs

Many women with epilepsy require treatment with antiepileptic drugs (AED) during pregnancy and post-partum when they may be breastfeeding their baby. Usually women with epilepsy do not have a choice to discontinue treatment while pregnant or breastfeeding. All antiepileptic drugs are transferred across the placenta and to a lesser extent into breast milk in varying amounts. The implications of AED exposure *via* breast milk is still not fully understood. A large prospective study of epileptic mothers on AED prenatally showed adverse development in their children regardless of breastfeeding status[65,66]. Some other large studies also showed no damaging effects on neurodevelopment of breastfed children of women who were prescribed AED during breastfeeding[67]. Nevertheless, most antiepileptic drugs are known to be teratogenic and increase the risk of foetal malformations[68].

The role of efflux transporters such as the MRP group and P-gp in the transport of AED showed variable results[69]. Luna-Tortós *et al*[10] initially found that several AED were substrates of the human P-gp. Lamotrigine and phenobarbital were also found to be MRP substrates. However, in a subsequent study they indicated that AED were not substrates of the human P-gp and that the different interpretations from the two studies were attributed to the different experimental designs used[11]. Several other studies also suggested that AED were not human P-glycoprotein substrates[12,68-70]. Studies performed in animals yielded conflicting results showing that AED were indeed substrates of P-gp[71-74]. The variability in these results may be attributed to the differences between human and rat P-gp. The cell lines and experimental designs used for conducting these studies can also have a significant impact on the results[71]. Another study in mice found that levetiracetam, topiramate and phenytoin demonstrate biphasic modulation of P-gp in BBB whereby at therapeutic doses they act as inducers of efflux. Sodium valproate and lamotrigine were found not to interact with P-gp[11,73,75]. Carbamazepine, itself was not a P-gp substrate but its metabolites were[76,77]. Dickens and co-workers also identified that lamotrigine and carbamazepine were not affected by P-gp[78,79]. Several case reports show that lamotrigine was transferred into breast milk in moderate amounts and in one case, where the mother was on 850 mg/d, it led to a severe apnoeic reaction in the exclusively breast fed infant[80]. It is not specified whether the mother was on any other medications at the time that could have contributed to this adverse reaction. Another study has shown that although lamotrigine's M:P ratio is highly variable, it is transferred into breast milk in moderate amounts[67].

In vitro studies carried out on MDCKII cells by Nakanishi *et al*[75] found that phenobarbital, clobazam, zonisamide, gabapentin and levetiracetam were BCRP substrates. Contrary to their findings, another group found that phenobarbital as well as other AED including phenytoin, ethosuximide, primidone, sodium valproate, carbamazepine, clonazepam, and lamotrigine did not interact with BCRP[79]. The differences in the experimental design and human and animal transporters may partly explain the variations in these results, with the animal studies appearing to favour anti-epileptic P-gp affinity while the human P-gp studies do not support this[68,77,79].

It is interesting that genetic polymorphism of P-gp is not associated with drug response in epileptic patients[80,81]. Again this concurs with the evidence that AED may not be transported by efflux transporters such as P-gp and BCRP[81]. Weiss *et al*[36] indicate that P-gp may not be of great significance in the transport of AED. Further studies are required to elucidate if there are any other active transport mechanisms which may have significant clinical implications in breastfeeding mothers.

DISCUSSION

It is well known that exposure to medications in utero is significantly higher than exposure *via* breast milk. However, exposure *via* breast milk is voluntary as opposed to in utero where they are inadvertently exposed to medications through placental transfer. Infant exposure to medications *via* breast milk, especially in the first six months when the infant is likely to be exclusively breastfed can have severe adverse effects possibly due to the underdeveloped metabolic and excretory mechanisms in the infant during this time. Hence, even small exposure to medication *via* breast milk may result in accumulation of drug at a level that may cause side effects in the infant, which calls into question the notional 10% of mother's plasma concentration in the milk as the threshold for concern. It is also important not to discontinue breastfeeding unnecessarily as there are many benefits associated with breastfeeding. Drug pharmacokinetic parameters usually give a good indication of the transferability of the drug from mother to baby *via* breast milk. However, there have been incidents when the actual M:P ratio of certain drugs have been much higher than predicted which is thought to be due to the involvement of active transport mechanisms. Yet, the significance of such active transport mechanisms and efflux transporters in the human mammary epithelial cells is still not fully understood. *In vivo* and *in vitro* studies have confirmed their presence but the clinical relevance of their role remains elusive. There is also contradiction and conflict with regards to the current findings as to whether particular drugs are substrates of these transporters and the degree of commonality between transporter substrates. Data can vary depending on the experimental design and the cell lines used. There is also significant inter species variability which can affect interpretation of results. Nonetheless, it is important to exercise caution when prescribing CNS acting drugs, such as psychotropics to breastfeeding mothers as even small unnecessary drug exposure may have disturbing side effects in the very young.

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P- Reviewer: Catania VA, Pan W **S- Editor**: Ji FF **L- Editor**: A
E- Editor: Lu YJ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i4.162

World J Pharmacol 2014 December 9; 3(4): 162-173
ISSN 2220-3192 (online)
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MINIREVIEWS

Pharmacophore approaches in protein kinase inhibitors design

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Received: June 27, 2014 Revised: October 15, 2014
Accepted: October 28, 2014
Published online: December 9, 2014

Abstract

Protein kinases constitute a superfamily of therapeutic targets for a number of human and animal diseases that include more than 500 members accordingly to sequencing data of the human genome. The well characterized nature of protein kinases makes them excellent targets for drug development. Pharmacophore approaches have become one of the major tools in the area of drug discovery. Application of pharmacophore modeling approaches allows reducing of expensive overall cost associated with drug development project. Pharmacophore models are important functional groups of atoms in the proper spatial position for interaction with target protein. Various ligand-based and structure-based methods have been developed for pharmacophore model generation. Despite the successes in pharmacophore models generation these approaches have not reached their full capacity in application for drug discovery. In the following review, we summarize the published data on pharmacophore models for inhibitors of tyrosine protein kinases (EGFR, HER2, VEGFR, JAK2, JAK3, Syk, ZAP-70, Tie2) and inhibitors of serine/threonine kinases (Clk, Dyrk, Chk1, IKK2, CDK1, CDK2, PLK, JNK3, GSK3, mTOR, p38 MAPK, PKB). Here, we have described the achievements of pharmacophore modeling for protein kinase inhibitors, which provide key points for further application of generated pharmacophore hypotheses in virtual screening, de novo design and lead optimization.

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Key words: Protein kinase; Inhibitor; Pharmacophore model; Receptor-based method; Ligand-based method

Core tip: In the following review, we summarize the published data on pharmacophore models for inhibitors of tyrosine protein kinases (EGFR, HER2, VEGFR, JAK2, JAK3, Syk, ZAP-70, Tie2) and inhibitors of serine/threonine kinases (Clk, Dyrk, Chk1, IKK2, CDK1, CDK2, PLK, JNK3, GSK3, mTOR, p38 MAPK, PKB). Here, we have described the achievements of pharmacophore modeling for protein kinase inhibitors, which provide key points for further application of generated pharmacophore hypotheses in virtual screening, de novo design and lead optimization.

Starosyla SA, Volynets GP, Bdzhola VG, Golub AG, Yarmoluk SM. Pharmacophore approaches in protein kinase inhibitors design. *World J Pharmacol* 2014; 3(4): 162-173 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/162.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.162

INTRODUCTION

Protein kinases are a group of enzymes which covalently modify proteins by adding phosphate groups from adenosine triphosphate (ATP) to serine, threonine or tyro-

sine residues and therefore, transduce a variety of signals in eukaryotic cells[1]. Kinases play a vital role in diverse cellular processes, functions, deregulations and now represent the second most important class of drug targets for pharmaceutical industry, after G-protein-coupled receptors[2]. Over the past decade about 20 drugs targeting kinases have been approved for clinical application, and much more are currently undergoing clinical studies[3].

Pharmacophore modeling is an important tool in drug development. A pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response[4]. There are two approaches for pharmacophore construction-receptor-based methods that allow building pharmacophore models based on the interactions of ligands with receptors, and ligand-based methods allowing generation of pharmacophore models based on the training sets of active compounds.

The pharmacophore models are applicable for screening large compound libraries in silico for the search of new small molecule inhibitors because they allow select compounds exhibiting binding features complementary oriented to an active binding pocket[5].

In this review we discuss the published data on pharmacophore models for inhibitors of several tyrosine protein kinases and serine/threonine protein kinases (Table 1).

PHARMACOPHORE MODELING FOR PROTEIN TYROSINE KINASE INHIBITORS

Protein tyrosine kinases (PTKs) are a family of enzymes that can transfer phosphate group from ATP to tyrosine amino acid residues of target proteins in cell. This covalent post-translational modification is a crucial event for regulation of various biological processes including growth, metabolism, differentiation and apoptosis. Recent advances have demonstrated that tyrosine kinases play significant role in development of different diseases suggesting PTKs as attractive targets in the search for therapeutic agents.

Tyrosine kinases are classified as receptor tyrosine kinases such as FGFR, epidermal growth factor receptor (EGFR), Vascular endothelial growth factor receptor (VEGFR), TEK tyrosine kinase, endothelial (Tie2) and non-receptor tyrosine kinases such as Spleen tyrosine kinase (Syk), Zeta-chain-associated protein kinase 70 (ZAP-70), ABL, SRC, FAK, Janus kinase (JAK)[6]. Each of receptor tyrosine kinases contains extracellular ligand-binding domain, transmembrane hydrophobic helix, and intracellular tyrosine protein kinase domain[7]. Non-receptor tyrosine kinases are cytosolic proteins, possessing considerable structural variability. The non-receptor tyrosine kinases have a kinase domain and often include several additional protein-protein interacting domains like SH2, SH3 and the PH domain[6].

EGFR inhibitors pharmacophore models

The EGFR family comprises four cell surface receptors: HER1 (EGFR/erbB1), HER2 (erbB2), HER3 (erbB3) and HER4 (erbB4)[7]. Binding of specific ligands to three of these receptors causes their dimerization and activation. HER2 is called an "orphan receptor" because it does not interact with any ligand, but it dimerizes with other ligand-bound members of EGFR family[8].

HER1 (EGFR/erbB1): HER1 overexpression and overactivity are often associated with a wide range of cancers, including prostate, gastric, breast, colorectal, pancreatic, ovarian, lung cancers, head and neck squamous cell carcinoma and glioma[9]. Aberrant EGFR signaling has been implicated in psoriasis, eczema and atherosclerosis[10,11]. Therefore, the EGFR inhibitors can be used for the amelioration of these diseases.

Furet *et al*[12] reported the first data concerning pharmacophore model for ATP-competitive inhibitors of EGFR. Accordingly to this Novartis pharmacophore hypothesis, the ATP-binding pocket in protein tyrosine kinases can be divided into five regions. In these five regions, three regions, namely, adenine region, sugar pocket, and hydrophobic region Ⅰ, are primarily important to the binding affinity. Two other regions, hydrophobic region Ⅱ and phosphate binding region are not of primary significance with respect to binding affinity, though they can be useful to enhance the inhibitor selectivity.

Also, pseudoreceptor model for EGFR was developed using a method FLARM. This model indicates the possible interactions between the receptor and ligand including two hydrogen bonds, one hydrophobic interaction and one sulfur-aromatic interaction, which are in accord with those in the Novartis pharmacophore model. Pharmacophore can be obtained according to the Novartis pharmacophore model and the pseudoreceptor model given by the FLARM method. 3D searching can then be done with the compound databases to find the lead compound of EGFR inhibitors[13].

HER2 (erbB2): HER2 has been demonstrated to play an essential role in the development and progression of about 25%-30% of human primary breast and ovarian cancers[14]. It was shown that the application of herceptin (a monoclonal antibody toward the HER2 receptor ectodomain) in combination with chemotherapy, leads to considerable regression of HER2-overexpressing metastatic breast tumors[15]. Therefore, the inhibition of HER2 has been considered a promising way of controlling malignant tumors.

Two groups of small molecules have been found to possess inhibitory activity toward HER2. A ligand-based approach was used for building HER2 pharmacophore model[16]. In the course of that work pharmacophore model generation was performed with Catalyst applying the Poling algorithm. From the calculated results, the best hypothesis bore good correlation with four features such as hydrogen bond donor, hydrogen bond acceptor, aliphatic and aromatic hydrophobic points. It seems that the formations of hydrogen bonds and the hydrophobic

Table 1 Protein kinases discussed in the review

Tyrosine protein kinases	
Epidermal growth factor receptor (EGFR; Erb-1; HER1 in humans)	Receptor
Human epidermal growth factor receptor 2 (HER2; erbB2; protooncogene Neu)	Receptor
Vascular endothelial growth factor receptor 2	Receptor
Janus kinase 2	Non-receptor
Janus kinase 3	Non-receptor
Spleen tyrosine kinase	Non-receptor
Zeta-chain-associated protein kinase 70	Non-receptor
TEK tyrosine kinase, endothelial	Receptor
Serine/threonine protein kinases	
Dual-specificity tyrosine-phosphorylation regulated kinase 1A	Non-receptor
Cdc2-like kinase	Non-receptor
Checkpoint kinase 1	Non-receptor
Human inhibitor nuclear-factor κB kinase 2	Non-receptor
Cyclin-dependent kinase 1	Non-receptor
Cyclin-dependent kinase 2	Non-receptor
Polo-like kinase	Non-receptor
c-Jun N-terminal kinase 3	Non-receptor
Glycogen synthase kinase 3	Non-receptor
Mammalian target of rapamycin	Non-receptor
p38 mitogen-activated protein kinase	Non-receptor
Protein kinase B	Non-receptor

interactions are crucial for ligand binding.

Pharmacophore modeling for VEGFR inhibitors

VEGFR signaling regulates vascular development, angiogenesis, lymphangiogenesis[17] and has been involved in a wide range of human pathologies including cancer, atherosclerosis, and inflammatory diseases[18]. Therefore, VEGFR has been emerged as an attractive therapeutic target. The pharmacophore models for VEGFR inhibitors were reported in[18,19].

The ligand-based pharmacophore models were generated with Catalyst using the Poling algorithm and the "best conformational analysis" method. The best obtained hypothesis comprised four pharmacophore features: hydrogen bond donor, hydrogen bond acceptor, hydrophobic, and ring aromatic. The three-dimensional structure of ligand extracted from the crystal structure of 1YWN has been taken for shape query generation. The combined shape and hypothesis model was further used as a search query to screen Maybridge database. The query has been effectively performed to find one novel promising inhibitor of VEGFR kinase which possesses activity in cell lines[18].

Two structure-based pharmacophore models of VEGFR-2 kinase inhibitors were built using the SBF software. The first pharmacophoric hypothesis was based on the crystal structure of 1Y6A, with the selection hydrogen bonding interaction for Glu915, Cys917, and Asn921. The second pharmacophoric model was based on the crystal structure of 1YWN; the backbone amide-NH of Cys917 and Asp1044 were used as hydrogen bond donors; and the backbone carbonyl oxygen of Glu883 was the hydrogen bond acceptor. The results suggest the importance of the five features for pharmacophores: the presence of two hydrogen bond donors, one hydrogen bond acceptor and two hydrophobic groups. The screening accuracy was assessed using a series of known inhibitors[19].

JAK 2 and JAK 3: JAK2 and JAK3 are non-receptor protein tyrosine kinases involved in B-cell- and T-cell-mediated diseases[20]. The inhibition of these kinases can be a potential strategy for the treatment of lymphoid-derived disorders.

Pharmacophore models for JAK2 and JAK3 were generated with PHASE, a high-performance program module of Schrödinger for ligand-based drug design. PHASE provides six pharmacophoric features: hydrogen bond donor (D), hydrogen bond acceptor (A), positively charged (P), negatively charged (N), hydrophobic (H), and aromatic ring (R) features. Two ligand-based pharmacophore hypotheses were constructed for the dataset of inhibitor molecules of JAK2 and JAK3 to dig out the essential structural features required for inhibition of both enzymes. These models can be helpful for screening of novel molecules having inhibitory activity toward both enzymes. The best hypothesis of JAK2 was ADRR, indicating that JAK2 inhibitors have one hydrogen bond acceptor (A), one hydrogen bond donor (D) and two ring aromatic (R) features. The best model of JAK3 was ADDRR. Pearson correlation coefficient calculated for test set molecules demonstrated excellent predictive power of these hypotheses[20].

Syk and ZAP-70: Syk and ZAP-70 are cytoplasmic non-receptor tyrosine kinases which play critical roles in the intracellular signal transduction of hematopoietic cells[21]. Syk is a key mediator of immunoreceptor signalling in B-lymphocytes, mast cells, macrophages and neutrophils[22-24], and ZAP-70 in T-lymphocytes, basophils and natural killer cells[24,25]. Syk was shown to be an attractive drug target for therapy of type I hypersensitivity reactions such as allergic rhinitis, asthma, urticaria, anaphylaxis and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus[23,26].

For a number of Syk and ZAP-70 small molecule inhibitors two reliable pharmacophore models were built with PHASE. The generated pharmacophore hypotheses combined with docking calculations were taken for further multi-step systematic virtual screening and finally 27 dual inhibitors of Syk and ZAP-70 were obtained as hits[24].

Also, 3D pharmacophore model of Syk inhibitors was developed by other authors applying HipHop and HypoRefine modules within Catalyst program package. Based on this model six compounds with good inhibitory potency against Syk were found[21].

Tie2: Tie2 is a receptor tyrosine protein kinase expressed almost exclusively in endothelial cells which plays an important role in blood vessel formation. This receptor negatively regulates the inflammatory response in endothelial cells, suppressing VEGF- and TNFα-induced expression of leukocyte adhesion molecules and procoagulant tissue factor[27]. Tie2 signaling also regulates pathologic angiogenesis, which includes tumor, psoriasis, choroidal neovascularization and rheumatoid arthritis angiogenesis[28]. The implication of Tie2 in pathologic angiogenesis makes this cellular receptor an attractive therapeutic target.

All the pharmacophore modeling calculations of type I and type Ⅱ kinase inhibitors of Tie2 were performed with HipHop and HypoRefine modules within Catalyst program package. In connection with the lack of highly active type I protein kinase inhibitors and restricted their structural diversity, only qualitative HipHop pharmacophore models were generated for this type inhibitors of Tie2. The best hypothesis comprised five pharmacophore features, namely, hydrogen bond donor, hydrogen bond acceptor, general hydrophobic, hydrophobic aromatic and ring aromatic. For type Ⅱ kinase inhibitors of Tie2, at the first step, a HipHop model was built with the aim to identify the common pharmacophore features which can be essential for potent inhibitors. Then, based on the information obtained from the HipHop hypothesis for type Ⅱ kinase inhibitors, the quantitative pharmacophore models were created with the aid of HypoRefine module. The best HypoRefine hypothesis included two hydrogen bond donors, one hydrophobic aromatic, two general hydrophobic features, and two excluded volumes. The validation of this HypoRefine model with the test set method demonstrated good correlation between the experimental and estimated IC_{50} values, suggesting a good predictive power[29].

PHARMACOPHORE MODELING FOR SERINE/THREONINE PROTEIN KINASE INHIBITORS

Serine/threonine protein kinases phosphorylate hydroxyl groups of serine or threonine residues of target proteins. Eukaryotic serine/threonine kinases can be classified into six groups: AGC, CaMK (for calcium-calmodulin dependent), CMGC (for CDK, MAP kinase, GSK and CDK-like), STE (homologs of STE11 and STE20), CK1 (for casein kinase-1), and TKL group (tyrosine kinase like). Accordingly to analysis of available structural data for members of each of the large groups it was revealed that the protein kinases possess similar architecture[30].

Dual-specificity tyrosine-phosphorylation regulated kinases and Cdc2-like kinases

Dual-specificity tyrosine-phosphorylation regulated kinases (Dyrk) proteins are defined as dual-specificity protein kinases because they can phosphorylate serine, threonine and tyrosine residues. Dyrk1A has increased expression in Down Syndrown individuals and is implicated in the development of other pathologies, such as neurodegeneration, cardiac hypertrophy and bone homeostasis[31,32]. Hence, inhibition of Dyrk1A may have possible application as a therapeutical strategy for treatment of these diseases. Cdc2-like kinases (Clk) is implicated in the regulation of alternative splicing of mRNA isoforms, indicating that small molecule compounds able to modulate Clk activity may represent an important mechanism for the control of mRNA splicing[33].

Pharmacophore models of Dyrk1A and Clk4 inhibitors were built based on the structure of the five most active compounds. Both hypotheses are represented with AAARR, indicating they comprise three hydrogen bond acceptors and two hydrophobic groups. The models associated with Dyrk1A and Clk4 have pharmacophore features located at the similar positions, considering both active sets have common structural cores. For both models, two hydrogen bond acceptors and one hydrophobic group are mapped to the quinazoline ring, which is shared among all studied compounds. The other two features, or one hydrogen bond acceptor and one hydrophobic group, are mapped to the R3 substituent 1,3-benzodioxol, which is common for tested inhibitors[34].

Checkpoint kinase 1

Checkpoint kinase 1 (Chk1) is a serine/threonine protein kinase which plays an integral role in the regulation of cell cycle progression, normal cell division and is critical component for DNA damage response. The inhibition of Chk1 kinase has been shown to result in interrupting of the G_2/M checkpoint, which would permit premature mitotic entry in the presence of DNA damage, leading to cell death. This suggests a potential therapeutic use of Chk1 inhibitors in cancer therapy[35].

All the pharmacophore modeling calculations for Chk1 were performed with Catalyst software package. The common pharmacophore features essential for promising Chk1 inhibitors were found with HipHop module. The best model involves four types of features, namely, hydrogen bond donor, hydrogen bond acceptor, ring aromatic and hydrophobic feature, indicating that the four types of features are important for potent Chk1 inhibitors[36].

Human inhibitor nuclear-factor κB kinase 2

The human inhibitor nuclear-factor κB kinase 2 (hIKK-2) is a serine/threonine protein kinase which belongs to the

IKK complex and implicated in the activation of nuclear-factor κB transcription factor under inflammatory conditions. The inhibitors of hIKK-2 could have strong therapeutic potential for treatment of chronic inflammatory diseases.

The structure-based pharmacophore model for hIKK-2 was built by using LigandScout software based on the protein-ligand complexes which were obtained by the docking process of ATP-competitive inhibitors into active site of hIKK-2.

The ligand poses which satisfied the common pharmacophore features of protein kinase inhibitors necessary for interaction with ATP-binding site [ability to form hydrogen bonds with the amino acid residues in the hinge region (segment 96-99 in hIKK-2 sequence) and hydrophobic interactions with the hydrophobic cavity in the active site of hIKK-2 (for example, Val29, Lys44, Ile65 and Val152)] were taken as knowledge-based coherent. As a result of this analysis, 43 poses of the 21 hIKK-2 inhibitors were considered as knowledge-based coherent, and their corresponding sites (functional groups which form intermolecular interactions with the kinase domain of hIKK-2) were selected to generate structure-based pharmacophore model. This hypothesis comprised two hydrogen bond donors, one hydrogen bond acceptor and one hydrophobic group common to most of the 43 poses[37].

Cyclin-dependent kinase 1

Cyclin-dependent kinase 1 (CDK1) is a serine/threonine protein kinase which plays a key role in promoting mitosis[38]. It was shown, that CDK1 inhibitors effectively blocked cell cycle progression in human tumor cell lines, indicating their potential clinical application as anticancer drugs[39].

A number of reliable binding hypotheses for CDK1 inhibitors were constructed with HypoGen module within Catalyst software package. HypoGen identifies a three-dimensional array of a maximum of five chemical features shared among the active ligands from training set providing relative alignment for each input molecule compatible with binding to target protein active site. The considered pharmacophore features can be hydrogen bond donors, hydrogen bond acceptors, aromatic planes, aliphatic, hydrophobic, positive and negative ionizable groups. The conformational flexibility of compounds from training set is modeled by generating multiple conformers covering a specified energy range for each input molecule. Successful pharmacophore models are complemented with exclusion spheres. Optimal sterically refined models obtained for CDK1 inhibitors were selected as search queries to screen the NCI, drugs and agrochemicals libraries. As a result, ten compounds demonstrated low micromolar activity toward CDK1, suggesting that generated pharmacophore hypothesis can be useful for search of potential anti-CDK1 agents[40].

Cyclin-dependent kinase 2

Cyclin-dependent kinase 2 (CDK2) is important protein kinase for initiation of DNA synthesis in higher eukaryotes and is required for promoting the cell division cycle and for successful progression through S and G_2 phases[41]. The importance of CDK2 for cell cycle progression has led to an active search of small molecule compounds inhibiting this enzyme as potential anticancer drugs.

Several ligand-based pharmacophore models for CDK2 small molecule inhibitors were generated independently with Catalyst by Hecker *et al*[42], Toba *et al*[43] and Vadivelan *et al*[44]. The multicomplex-based comprehensive pharmacophore map was built with LigandScout software by Zou *et al*[45]. It should be noted that during pharmacophore model construction Catalyst software takes into consideration only ligand information whereas LigandScout adds pharmacophore feature to the model when important interaction pattern between inhibitor and receptor is identified.

The authors of multicomplex-based comprehensive pharmacophore map compared their hypothesis with other reported CDK2 inhibitors models. It was revealed that each pharmacophore feature in the ligand-based models was mapped to the corresponding feature in comprehensive pharmacophore map suggesting that the last one includes more information over all three other models. Detailedly, during alignment of the final Hecker model and multicomplex-based comprehensive map it was shown that hydrogen bond acceptor feature in Hecker model was mapped to the feature of comprehensive map reflecting the interaction of small-molecule inhibitor with hinge region (A1); the hydrogen bond donor feature in Hecker model was matched to the feature representing the interaction with Gln131 (D5); the hydrophobic features were mapped to the features located at the solvent-accessible region (H1) and in the ribose-phosphate binding site (H3). The pharmacophore model generated by Toba *et al*[43] comprised two hydrogen bond donor features and three hydrophobic features. Each of these features was matched to the corresponding features D1, D5, H1, H2 and H3 of comprehensive pharmacophore map. The pharmacophore hypothesis constructed by Vadivelan *et al*[44] included two hydrogen bond acceptors, one hydrogen bond donor, and one hydrophobic feature. In comparison with comprehensive pharmacophore map, one of the hydrogen bond acceptor features was mapped to the feature that is located near Asp86 (A3), other one was matched to the feature representing the interaction with Lys33 (A4). The hydrogen bond donor feature and hydrophobic feature of Vadivelan model correspond to D1 and H3 features of multicomplex-based comprehensive pharmacophore map, respectively[45].

Polo-like kinases

Polo-like kinases (PLKs) belong to a family of serine/threonine protein kinases and exist in four isoforms, namely, PLK1, PLK2, PLK3, and PLK4. The only one of these isoforms, PLK1, is shown to be implicated in the regulation of chromosome segregation, centrosome maturation, bipolar spindle formation and execution of

cytokinesis[46]. The activity of PLK1 is increased in many tumor types, including lung, breast, colon, pancreatic, prostate and ovarian indicating its capability as a drug target[47].

The chemical feature-based pharmacophore models of PLK1 inhibitors were constructed by using HipHop and HypoGen modules within Catalyst program package. The best qualitative HipHop pharmacophore hypothesis contains seven features, namely, hydrophobic aromatic feature, two hydrophobic aliphatic moieties, three hydrogen bond acceptors and one hydrogen bond donor. The best quantitative HypoGen pharmacophore model, possessing the lowest rmsd value and the highest correlation coefficient, includes four features, namely, general hydrophobic, hydrophobic aliphatic, hydrogen bond acceptor and hydrogen bond donor. The results of validation for HypoGen pharmacophore model, which were obtained with the aid of the test set method, have demonstrated a really good correlation between the experimental and estimated IC_{50} values suggesting a good predictive ability[48].

c-Jun N-terminal kinase 3

The c-Jun N-terminal kinase 3 (JNK3) is a member of the mitogen-activated protein kinase (MAPK) family, which activates signaling pathways under environmental stress conditions[49]. JNK3 is expressed selectively in brain, heart, and testis[50]. It was shown, that JNK3 phosphorylates β-amyloid precursor protein, a conserved and ubiquitously expressed transmembrane glycoprotein involved in the development of Alzheimer's disease[51]. Therefore, JNK3 appears to be an attractive therapeutic target for this neurodegenerative disease.

Pharmacophore models for JNK3 small-molecule inhibitors were generated with Catalyst software package. X-ray crystal structure of JNK3 (PDB ID: 2R9S) was taken for structure-based pharmacophore modeling and molecular docking simulations. A structure-based pharmacophore hypothesis was built using interaction generation module implemented in Discovery Studio. The most important interaction patterns were transformed into pharmacophore features, such as hydrophobes, hydrogen bond donors and hydrogen bond acceptors along with their direction vectors. The final refined structure-based pharmacophore model included hydrogen bond donor features with Lys68, Gly71, Ser72, Gln155, Met149 and hydrogen bond acceptors with Lys68, Gly71, Met149, Gln155 and three hydrophobic features.

The features obtained in the ligand-based pharmacophore model are well compared to the features of structure-based pharmacophore model. But, structure-based pharmacophore model had three additional features, not present in ligand-based pharmacophore hypothesis, which would be helpful for development of novel JNK3 inhibitors[49].

Glycogen synthase kinase 3

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine protein kinase highly expressed in the nervous system, which regulates glycogen metabolism by insulin, and is involved in many different biological processes such as tumorigenesis, cell survival, and developmental patterning[52]. GSK-3 has recently emerged as a promising therapeutic target for the search of small molecule inhibitors which can be potential novel drug candidates for treatment of several human pathologies, including cancer, Alzheimer's disease, stroke, bipolar disorders, type II diabetes and chronic inflammatory processes[53].

Pharmacophore models for GSK-3 inhibitors were constructed with the HypoGen module within the Catalyst software package based on list of 152 GSK-3 inhibitors. HypoGen allows automatic pharmacophore generation based on a library of at least 16 molecules with inhibitory activity toward proposed molecular target ranging over 4 orders of magnitude[5].

3D pharmacophore mapping methodology based on distance comparison technique was designed for the three GSK-3 inhibitors using DISCOtech™ module implemented in SYBYL 8.0. DISCOtech™ is a well established module in constructing pharmacophoric map. Taking into consideration a set of molecules which are characterized by the ability to interact with the same protein receptor, DISCOtech™ identifies features that could be components in a pharmacophore hypothesis. DISCOtech™ operates in distance space and can perform clique detection to build pharmacophore models on up to 300 conformers per molecule. Therefore, DISCOtech™ can be efficiently applied with at least 3-5 compounds to design reliable pharmacophore hypotheses[54].

Mammalian target of rapamycin

Mammalian target of rapamycin (mTOR) is a ubiquitous serine/threonine protein kinase that regulates several important physiological functions like protein synthesis, metabolism, cell growth, proliferation, and autophagy. mTOR is also critical for a number of brain-specific mechanisms, such as synaptic plasticity, learning, and cortical development[55]. Recent studies have implicated mTOR to several human pathologies including cancer, diabetes, obesity, cardiovascular diseases and neurological disorders[56]. The pharmaceutical attractiveness of small molecule mTOR inhibitors coupled with the deficiency of crystallographic structural data for mTOR kinase domain, were starting point for development of ligand-based QSAR and pharmacophore models[57].

The Hip-Hop pharmacophore model was created with the Common Feature Pharmacophore Generation module implemented in Accelrys Discovery Studio 2.1. This pharmacophore hypothesis provides a geometrical representation of the features necessary for ligands to interact favorably with a receptor site and demonstrate biological activity. Hip-Hop identifies configurations or three-dimensional spatial arrangements of chemical features that are shared among all molecules in the set. Under the Common Feature Pharmacophore Generation protocol, were used four features such as hydrogen bond donor, hydrogen bond acceptor, ring aromatic, and hy-

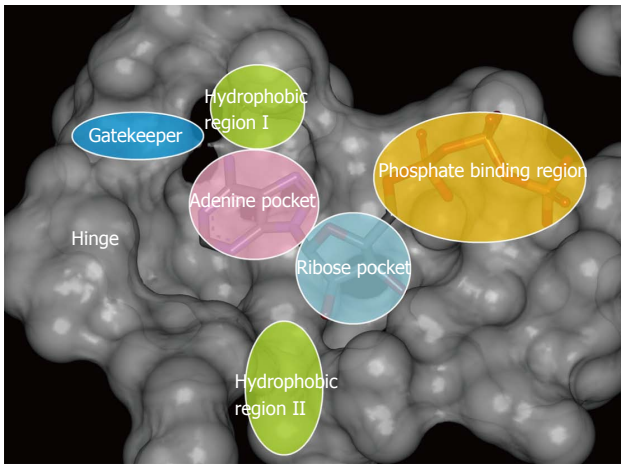


Figure 1 Pharmacophore model for type I protein kinase inhibitors.

drophobic to build the pharmacophore model.

The best pharmacophore hypothesis generated from 27 ATP-competitive inhibitors of mTOR comprised two hydrogen bond acceptors, one hydrophobic feature and one aromatic ring feature[58].

p38 mitogen-activated protein kinase

The p38 mitogen-activated protein (MAP) kinase (p38MAPK) is a serine/threonine protein kinase which plays a very important role in the pathophysiology of several inflammatory human diseases, such as, asthma, osteoarthritis and rheumatoid arthritis, a chronic obstructive pulmonary disease. Therefore, the inhibition of p38MAPK can be an effective strategy to prevent the development of these diseases[59].

Catalyst HypoGen pharmacophore approach was applied to obtain models for a collection of p38MAPK inhibitors[59,60]. Eight out of ten best hypotheses comprise the identical four features: one hydrogen bond acceptor, two hydrophobic aromatic and one hydrophobic feature, which indicates the stability of the models[59]. The obtained hypotheses are readily interpretable and can be applied for the rational discovery of new p38MAPK inhibitors[60].

Protein kinase B (PKB; Akt)

The protein kinase B (PKB; Akt) family of serine/threonine kinases consists of three members: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ[61]. Akt is a central component in cell signaling pathways regulated by growth factors, cytokines, and other cellular stimuli. The activation of Akt leads to cell cycle progression (inhibiting apoptosis)[62]. Ligand-based pharmacophore model of Akt inhibitors was built using DISCOtech and GASP (genetic algorithm similarity program) module[63].

A crystal structure of Akt2 complexed with a known inhibitor (PDB ID: 3E8D) was taken for construction of structure-based pharmacophore hypothesis. The Interaction Generation protocol within DS program was used to create pharmacophoric features corresponding to all important interaction points at the ATP-binding pocket of Akt2. The obtained pharmacophore model consisted of seven pharmacophoric features, namely, hydrogen bond donor (HD), two hydrogen bond acceptors (HA1-2), and four hydrophobic groups (HY1-4), besides, eighteen exclusion volume spheres were also taken into account. HD is at the neighborhood of the carboxyl group of Asp293. HA1 is positioned to interact with the amino group of Ala232. HA2 is located near amino group of Phe294 and Asp293. Groups in accordance with these pharmacophoric features potentially able form hydrogen bonds with adjacent amino acid residues. HY1 is located in a hydrophobic pocket formed by Ala178, Met282 and Phe439. HY2 is situated in another hydrophobic pocket composed by Gly159, Gly161, Gly164 and Val166. HY3 is close to Lys181 and Met229 and HY3 is near to Phe294. There are short distances between HY4 and hydrophobic amino acids Phe163 and Lys181. Groups in accordance with these hydrophobic features may be involved in hydrophobic interactions with enzyme. Therefore, small-molecule compounds matching with some of these features may be potential inhibitors of Akt2[64].

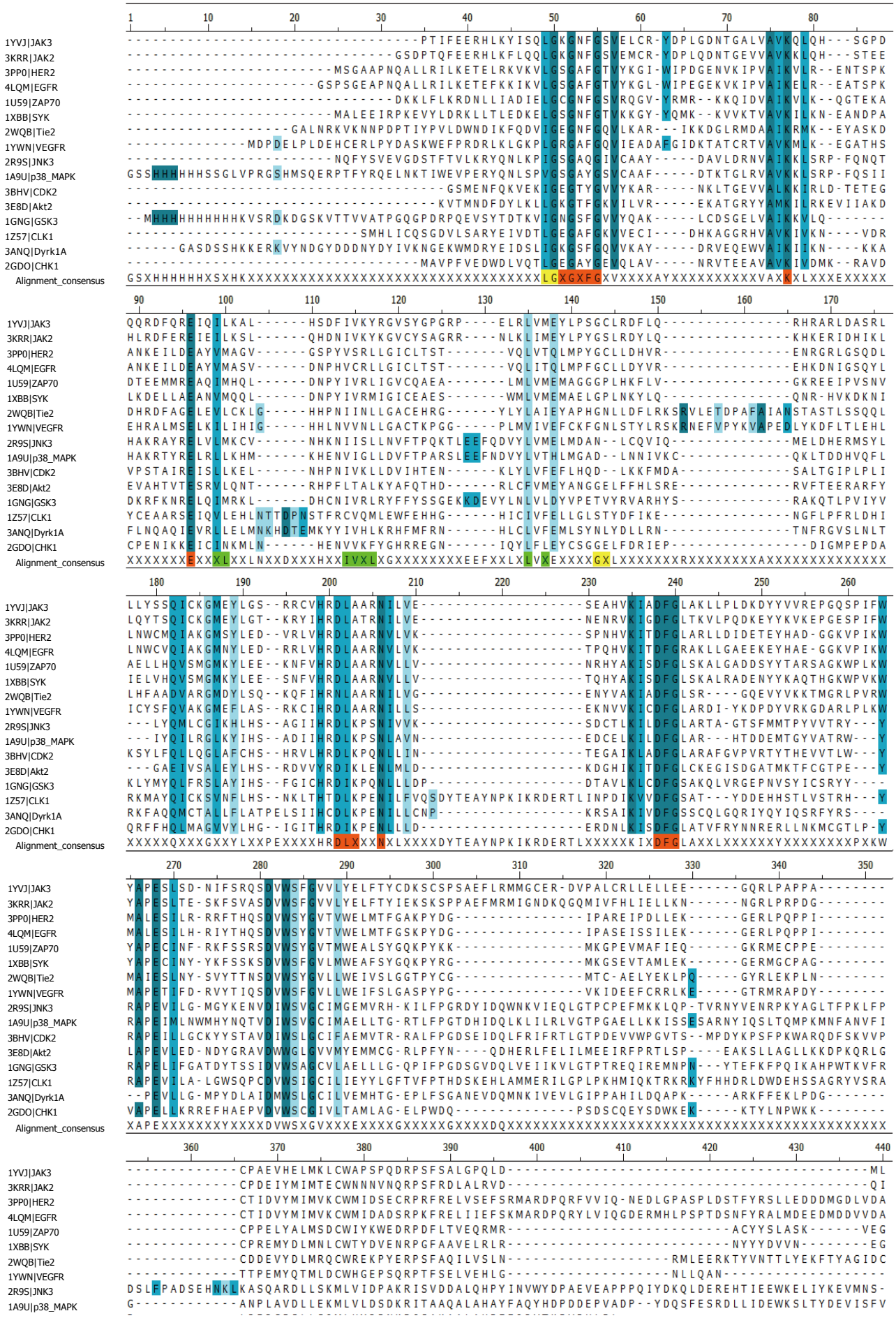
DISCUSSION

The methods for pharmacophore model generation are divided in two categories: receptor-based and ligand-based. Receptor-based approaches can be used when the structure of molecular target is determined. In other case, ligand-based approaches can be applied for pharmacophore hypothesis generation.

During analysis of the pharmacophore approaches in protein kinase inhibitors design, it was revealed that despite of large amount of the structural data for protein kinases, the ligand-based approaches are more widely used for protein kinase pharmacophore model generation than the receptor-based. Ligand-based methods for pharmacophore elucidation include ALADDIN, DISCO, GERM, COMPASS, GASP, Catalyst HipHop, SCAMPI, Catalyst HypoGen, Phase, CLEW GAMMA, PARM, DANTE, *etc.*

3D-QSAR methods Catalyst HypoGen and Phase which use the known activity values of the small molecule inhibitors in the training set to build the hypothesis, are the most applied for protein kinase inhibitors pharmacophore models generation. These models include features common only for highly active compounds and also can contain excluded volumes (obtained based on the structure of inactive compounds), which couldn't be occupied by inhibitors. The methods Catalyst HipHop, PharmaGist, DISCO can be used only for qualitative pharmacophore models generation which don't take into consideration the information concerning activity of compounds. Qualitative pharmacophore model can be taken as a basis for further 3D-QSAR hypothesis generation.

The receptor-based methods of pharmacophore model elucidation are more rarely used for protein kinase pharmacophore design. These approaches can be useful


1YVJ|JAK3
3KRR|JAK2
3PP0|HER2
4LQM|EGFR
1U59|ZAP70
1XBB|SYK
2WQB|Tie2
1YWN|VEGFR
2R9S|JNK3
1A9U|p38_MAPK
3BHV|CDK2
3E8D|Akt2
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3ANQ|Dyrk1A
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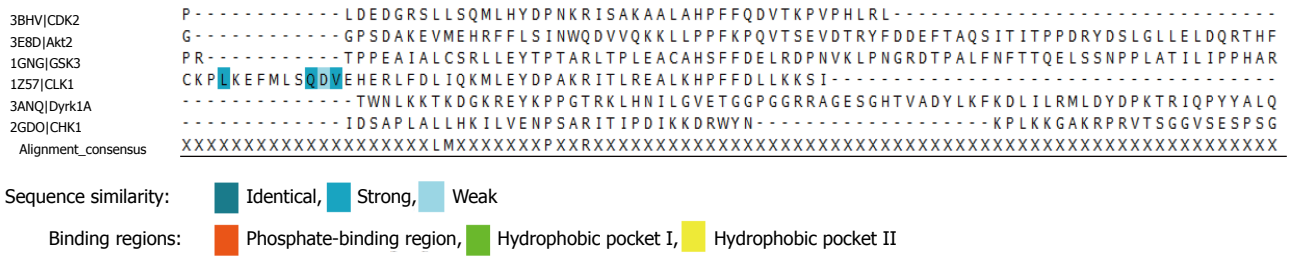

Figure 2 Alignment of amino acid sequences of tyrosine and serine/threonine protein kinases for which pharmacophore models were reported.

for study of ligand-receptor interactions. The easiest way to build receptor-based 3D-pharmacophore model is to manually select key amino acid residues in the active site of enzyme, to determine the most important interactions with ligand and then based on this information to set pharmacophore features with specific properties. Other way to obtain 3D-pharmacophore model is to apply receptor-oriented algorithms which automatically evaluate the interactions between ligand and receptor. This modeling is performed with software packages Pharmer, Accelrys Discovery Studio, Schrödinger, MOE, LigandScout, *etc*.

The combination of receptor-based and ligand-based methods allows improve efficiency of novel bioactive compounds development[49].

The pharmacophore models, developed for search of novel inhibitors for tyrosine and serine/threonine protein kinases don't have significant differences. For the best of our knowledge, the most protein kinase pharmacophore models are reported for inhibitors competing for the ATP-binding site and are in accord with the Novartis[12] and Traxler's[65] pharmacophore models. Traxler's pharmacophore model includes the same regions as Novartis pharmacophore model: adenine region, sugar pocket, hydrophobic region Ⅰ, hydrophobic region Ⅱ, phosphate binding region and also has one additional element - gatekeeper residue that plays an essential role in inhibitor binding and selectivity (Figure 1). In spite of the significant role of gatekeeper residue for inhibitor binding, in most cases the authors didn't pay attention to this residue during pharmacophore model generation for protein kinase inhibitors.

The average number of pharmacophore features in the analyzed tyrosine and serine/threonine protein kinase inhibitors pharmacophore models is 4-5. Several models have more pharmacophore features but it should be noted that increasing of their number leads to improvement of model specificity which decreases ability of the model to identify hit compounds from diverse chemical classes.

The most pharmacophore hypotheses have 1-2 features (hydrogen bond acceptor and/or hydrogen bond donor) in adenine region. As a rule, these features are indicated as vectors directed to main chain of amino acid residue in the hinge region. Several pharmacophore models have additional aromatic or hydrophobic feature in adenine region.

Almost all pharmacophore models have hydrophobic or aromatic pharmacophore feature in hydrophobic pocket Ⅰ. Several hypotheses also have hydrogen bond donor and/or hydrogen bond acceptor in this region which can additionally stabilize the inhibitor in ATP-binding pocket.

Only some models have hydrogen bond donor pharmacophore feature in sugar pocket. This region is not so important for binding affinity of inhibitors with protein kinase active site, as adenine region or hydrophobic pocket Ⅰ.

Phosphate-binding region has 1-2 pharmacophore features (hydrogen bond acceptor and/or hydrogen bond donor); hydrophobic pocket Ⅱ has one hydrophobic or aromatic pharmacophore feature. We have performed multiple alignment of amino acid sequences for tyrosine and serine/threonine protein kinases for which pharmacophore models were reported. It was revealed that phosphate-binding regions are highly conservative in contrast to hydrophobic pockets Ⅰ and Ⅱ (Figure 2). Therefore, pharmacophore features located in phosphate-binding region don't supply specificity for hit compounds, but correctly predicted hydrophobic or aromatic features in hydrophobic pockets Ⅰ and Ⅱ can be useful to improve the selectivity of inhibitors.

The most reported pharmacophore models were built for type Ⅰ protein kinase inhibitors. Several pharmacophore hypotheses were also generated for type Ⅱ protein kinase inhibitors. The models for both types are very similar, but in a case of type Ⅱ protein kinase inhibitors, the model has pharmacophore features in the deep pocket of ATP-binding site.

In this article, the existing data concerning protein kinase inhibitors pharmacophore models were reviewed. Ligand-based and receptor-based methods can be equally applied for protein kinase pharmacophore models generation. The combination of these approaches can be useful for improving of efficiency of bioactive compounds design. With the help of the discovered pharmacophore models, the identification of possible protein kinase inhibitors can be much more accelerated.

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P- Reviewer: Choi CY, Huang Y, Moens U **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5497/wjp.v3.i4.174

World J Pharmacol 2014 December 9; 3(4): 174-185
ISSN 2220-3192 (online)
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MINIREVIEWS

Role of antipsychotics for treating behavioral and psychological symptoms of dementia

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Received: July 29, 2014 Revised: October 2, 2014
Accepted: October 23, 2014
Published online: December 9, 2014

Abstract

Over the past three decades, concerns about the high prevalence of antipsychotic use in the nursing homes (NHs) for the management of behavioral and psychological symptoms of dementia continue to be emphasized and intervened by many. However, despite the numerous side effects and the recent blackbox warning by the United States Food and Drug Administration about the increased risks for stroke and sudden death associated with the use of antipsychotics in dementia, the prevalence of antipsychotic use in NHs remains high. While the use of antipsychotics appeared to have modest efficacy in reducing symptoms of aggression and psychosis in dementia, there is insufficient evidence to routinely recommend the use of alternative psychopharmacological treatments for these symptoms. Hence, clinicians have to balance the safety warnings against the need to treat these symptoms in order to prevent harm to the resident that may result from his/her dangerous behaviors. Although the use of antipsychotics may be warranted in some cases, organizational, resource and training support should be provided to encourage and equip NH staff to participate in interventions so as to minimize inappropriate use of these medicines in NHs. This review will discuss the place in therapy, the trend and appropriateness of antipsychotic use in NHs, as well as the effectiveness of current and future strategies for reducing antipsychotic use in the NHs.

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Key words: Antipsychotic agents; Psychotropic drugs; Prescribing appropriateness; Dementia; Behavioral and psychological symptoms of dementia; Nursing homes

Core tip: While antipsychotics may be used to manage symptoms of severe aggression and psychosis when the safety of the resident is threatened, there should be routine reviews of the appropriateness of antipsychotic use as well as training and support of the care staff in providing psychosocial intervention to treat the symptoms so as to reduce antipsychotic use in nursing homes. Reported studies evaluating interventions to improve antipsychotic use appropriateness in nursing homes are limited by the small sample sizes and absence of control groups. Future research should address these methodological issues while exploring safer therapeutic alternatives to manage these symptoms.

Yap KZ, Chan SY. Role of antipsychotics for treating behavioral and psychological symptoms of dementia. *World J Pharmacol* 2014; 3(4): 174-185 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/174.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.174

OVERVIEW OF BEHAVIORAL AND PSYCHOLOGICAL SYMPTOMS OF DEMENTIA

With the rapidly aging population worldwide, the number of persons with dementia is projected to double every 20 years, from 35.6 million in year 2010, to 65.7 million, and 115.4 million by years 2030 and 2050 respectively[1]. Dementia is marked by features of progressively worsening

memory impairment and cognitive disturbances[2]. As the illness advances, the resulting decline in functional capacity naturally exerts its toll on the patient's family and/or the society, demanding significant expenditure in time, energy and resources in caregiving for extended periods. In year 2010, the cost of informal care (unpaid care provided by family and others) and direct cost of social care (provided by care professionals in the community and in residential long-term care institutions) for dementia each contributed to about 42% of the estimated USD 604 billion total cost[3].

In addition to delaying cognitive and functional decline, dementia-related research has become increasingly focused on defining, measuring and managing behavioral and psychological symptoms of dementia (BPSD)[4]. BPSD is a term that encompasses a heterogeneous range of non-cognitive symptoms, such as disturbed perception, thought content, mood, and behavior[4]; and are broadly classified as "behavioral" or "psychological"[5]. These symptoms may be present in up to 97% of persons with dementia over a five-year period[6], and were reported to be a significant source of patient distress, caregiver stress[7,8], increased costs of care and nursing home (NH) admissions[9]. Hence, it was not surprising that higher point prevalence of BPSD were reported in the NHs compared to that in the community care setting[10].

EFFICACY AND SAFETY OF ANTIPSYCHOTICS FOR TREATING BPSD

Over many decades, antipsychotics have been prescribed for managing BPSD, particularly symptoms of severe agitation, aggression and psychosis. Although the precise mechanism of antipsychotics' influence on agitation is not known, their antagonistic effect on postsynaptic dopamine receptors was postulated to play a role in the amelioration of psychotic symptoms in dementia[11]. In a meta-analysis, haloperidol appeared to be clinically effective in reducing symptoms of aggression in dementia compared to controls[12]. The lack of a significant difference in the overall drop-out rates between the haloperidol treatment and control groups despite more drop-outs from the haloperidol group due to the presence of side effects further suggests the possible effectiveness of the drug[12]. However, the authors concluded that the routine use of haloperidol for the treatment of agitation in dementia should not be recommended, due to insufficient evidence of benefits from this treatment[12].

Among the atypical antipsychotics, risperidone and olanzapine were deemed by Sink and his team to have the best evidence for efficacy in the treatment of aggression and psychosis[13]. Specifically, a meta-analysis reported that risperidone (1 to 2 mg/d) was effective in the treatment of aggression and psychosis related to dementia, while the use of olanzapine (5 to 10 mg/d) showed significant benefits in reducing aggression when compared to placebo[14]. These findings were consistent with that reported in another meta-analysis[15] and the CATIE-AD trial[16,17]. In another randomized, placebo-controlled study, the use of aripiprazole resulted in a reduction of psychotic symptoms[18]. Although quetiapine was observed by Tariot and his co-investigators to improve symptoms of agitation[19], this finding was not replicated in other studies[16,17,20].

While typical antipsychotics are primarily D_2 receptor antagonists and inhibit dopaminergic neurotransmission in a dose-dependent manner, atypical antipsychotics vary in their binding affinities to other receptors[21]. As a result, each atypical antipsychotic has a different side effect profile. Unlike haloperidol, atypical antipsychotics generally have lesser propensities to cause neuroleptic-induced movement disorders or extrapyramidal symptoms (EPS)[22] due to a lower D_2 receptor blocking effect or a partial D_2 agonistic effect[23]. However, EPS may become more apparent with risperidone at doses higher than 2 mg/d due to its dose-dependent dopamine receptor blocking effect[14]. Other side effects and adverse events of risperidone also included somnolence, peripheral edema, cerebrovascular adverse events, urinary incontinence, urinary tract infection and falls[14]. For olanzapine and quetiapine, somnolence side effect was more prominent compared to other antipsychotics due to their higher affinity to block H_1 receptors[24]. In addition, significant weight gains, increased waist circumferences and decreased high-density lipoprotein cholesterol levels were also reported as characteristic side effects of olanzapine and quetiapine[14,25]. A decline in cognitive function could be a side effect of antipsychotic use, but this was found to be more significant among individuals who were treated with olanzapine and risperidone[26], possibly due to the more pronounced anticholinergic effects of these antipsychotics[21].

Besides the above-mentioned side effects, several studies and reviews have also highlighted the safety concerns regarding the use of antipsychotics in dementia. In a systematic review by Trifiró *et al*[27], an increase in risk for mortality was reported to be associated with the use of both typical and atypical antipsychotics in a dose-dependent fashion, where the highest risk was estimated to be shortly after exposure[27]. Although the related causes were postulated to include cerebrovascular event, pneumonia, peripheral vascular effects and/or metabolic effects, the differential risks of the individual antipsychotics and predisposing patient factors have yet to be established[27].

Another safety concern was the increased risk for cerebrovascular events (CVEs) associated with antipsychotics, particularly olanzapine and risperidone, which were linked to a threefold increase in risk[14,28]. Yet, based on currently reported studies, a fair conclusion on the difference in risks between individual antipsychotic agents cannot be drawn. The plausible mechanisms of antipsychotic-related CVEs were deemed to be linked to side effects of antipsychotics including EPS[29], orthostatic hypotension, hyperprolactinemia[30], thromboem-

bolic events and excessive sedation[29,31]. However, while the elevated risk was thought to be temporal with the potential to decrease over time[32], the contribution of predisposing patient factors to the development of CVEs (such as presence of vascular dementia) independent of antipsychotic use was not ascertained[27].

Antipsychotic use was also associated with an increased risk for pneumonia. Specifically, threefold and 1.6-fold increases in risk were observed when atypical and typical antipsychotics were used respectively[33]. These could possibly be due to aspiration pneumonia linked to underlying mechanisms of antipsychotic-induced side effects including dysphagia, sedation and EPS[34]. Due to the poor prognosis of pneumonia in older persons with dementia, this adverse event may in turn contribute to the increased risk of their mortality associated with the use of these medicines.

ROLE OF ALTERNATIVE PSYCHOPHARMACOLOGICAL AGENTS

Benzodiazepines

Compared to antipsychotics, there was no or little evidence for recommending the use of other psychopharmacologicals such as benzodiazepines and anticonvulsant mood stabilizers to treat symptoms of severe agitation, aggression and psychosis. Specifically, the use of benzodiazepines among older persons for the treatment of agitation, especially in the presence of dementia, should be avoided as these individuals are more sensitive to side effects including sedation, ataxia and withdrawal symptoms, which may potentiate confusion, falls and fractures leading to adverse clinical outcomes[35]. To the authors' knowledge, there are also no published systematic review, meta-analysis or randomized controlled study to provide any evidence to support the treatment of severe agitation, aggression and psychosis in dementia with benzodiazepines.

Anticonvulsant mood stabilizers

With regards to anticonvulsant mood stabilizers, valproate was ineffective in reducing BPSD or agitation symptoms according to two recent reviews[13,36] and a meta-analysis[37]. Although carbamazepine appeared to have some effect in reducing symptoms of aggression[38,39], these reports were countered by negative findings of another study[40]. Furthermore, carbamazepine has clinically significant drug-drug interactions with medicines commonly used by older persons such as verapamil[41,42] and warfarin[43]. Carbamazepine also carries black box warnings for potentially fatal severe adverse drug reactions, specifically hematologic toxicity and serious dermatologic reactions especially for individuals with HLA-B*1502 allele[44,45]. As such, the clinical use of carbamazepine, especially in the NHs, would be highly inconvenient due to the need for pre-treatment genotype screening and regular hematological monitoring to minimize the occurrence of these serious adverse drug reactions. Therefore, anticonvulsant mood stabilizers should not be used to treat aggression and psychosis related to dementia.

Antidepressants

While serotonin has been postulated to be involved in the underlying pathophysiological mechanisms for psychosis and aggression[46,47], the evidence for the clinical use of antidepressants is primarily for the treatment of depression in dementia[48]. Although Lyketsos and his colleagues observed a beneficial reduction in non-mood behavioral symptom scores of the Neuropsychiatric Inventory (NPI-NM)[49,50] among individuals with Alzheimer's disease who had responded fully to the treatment of depression with sertraline, the difference in the reported NPI-NM scores between the treatment and control groups of the study was not statistically significant[51]. In a recent meta-analysis[52], the use of serotonin reuptake inhibitors (SSRIs) sertraline and citalopram were associated with a larger mean change in the Cohen Mansfield Agitation Inventory[53] score (compared to placebo: -0.89, 95%CI: -1.22 to -0.57) and appeared to be better tolerated than typical and atypical antipsychotics. However, these findings were limited by the small sample sizes[52]. Furthermore, an evaluation of the effectiveness of citalopram for the treatment of BPSD noted improvements that were limited to symptoms of agitation and lability. The results were also potentially biased with a high dropout rate of more than 50% due to possible side effects and lack of efficacy[54]. Hence, more large-scale studies would be required to ascertain the safety and efficacy of SSRIs in the treatment of aggression and psychotic symptoms in dementia.

Acetylcholinesterase inhibitors and memantine

Besides improving cognitive symptoms, the effects of acetylcholinesterase inhibitors (donepezil, rivastigmine and galantamine) and an N-methyl D-aspartate antagonist (memantine) in reducing BPSD were described in many case reports[55-57], clinical studies[58-64], randomized controlled trials[65-74] and systematic reviews[63,75-77]. A review on the randomized controlled trials concluded that the findings for donepezil and memantine appeared to be conflicting[13]. In addition, there is no landmark head-to-head study to offer a fair comparison for the differences in efficacies of these pharmacological agents. Meta-analyses of these drugs were also limited by different methodologies and measures of BPSD used in the clinical trials of each drug[78]. Furthermore, there were also reports of paradoxical worsening of both behavioral symptoms related to the use of donepezil in frontotemporal dementia[79] and parkinsonism associated with the use of donepezil in dementia with Lewy bodies[57]. Significant side effects of rivastigmine were also observed, which included nausea, vomiting, tremor and dizziness[66]. In all, it appears however, that rivastigmine[64,66,77] and memantine[71] may be safer alternatives for the management of aggression and psychotic symptoms, particularly for individuals with Parkinson's disease dementia and dementia with Lewy bodies, as they are likely to be susceptible to

the severe adverse effects of antipsychotics such as worsening of Parkinsonian symptoms and life-threatening severe neuroleptic malignant syndrome[80,81].

GUIDELINES AND TRENDS OF ANTIPSYCHOTIC USE IN DEMENTIA

Although there is limited evidence supporting the efficacy of non-pharmacological interventions for reducing aggression and psychosis related to dementia, these are recommended as the first-line strategy over the use of antipsychotics in all practice guidelines[82-84]. The obvious reasons are the numerous side effects[12,25,26,85] and higher risks for stroke and death associated with antipsychotics, which out-weigh their modest efficacies[86-89], and their limited benefits with long-term use[90]. Despite the introduction of the black box warning for antipsychotics by the United States Food and Drug Administration in 2005 against its use in view of the increased risks for stroke and death, the reported prevalence of antipsychotic use in most NHs in the United States remained unchanged[91]. A recent report by the Centers for Medicare and Medicaid Services estimated that about 40% of NH residents with dementia were prescribed with antipsychotics in 2010[92]. Interestingly, this corresponded with the prevalence of delusion (54%) and hallucination (39%) found among these residents with dementia[93]. In a cross-national comparison, while about a quarter of NH residents in the United States were prescribed with antipsychotics, this prevalence varied between 11%-40% in Hong Kong, Canada, Switzerland and Finland and other countries[94].

Within NHs, comparisons between the older persons with dementia residing in special dementia units *vs* traditional care wards found that antipsychotics were used more often in the former, as these residents were more likely to exhibit behavioral problems[95,96]. However, no statistical difference in the prevalence of antipsychotic use between these two cohorts of elderly NH residents with dementia was reported in another study, where the researchers attributed it to the effect of increased number of activities and psychosocial interventions which reduced the need for antipsychotics[97].

Nevertheless, the use of antipsychotics in the NHs will continue due to the lack of alternative evidence-based pharmacological treatments for dementia-related severe agitation, aggression and psychosis for the residents with risk of physical harm as a result of uncontrolled behavior[98,99]. In addition, although many guidelines advocate prescribing antipsychotics for a minimal duration with attempts to taper off and discontinue at least once every 6 mo, a recent study suggested that the use of antipsychotics for up to 9 mo in individuals with severe baseline symptoms may confer benefits of having a reduction in symptom relapses compared to those who were taken off antipsychotics after 4 mo[100]. Similarly, another review concluded that though antipsychotics can be withdrawn within 6 mo without detrimental effects on behavior for most individuals, the use of antipsychotics could be extended for those with more severe symptoms at baseline to prevent relapses[90]. Yet, concerns regarding the inappropriate use of antipsychotics in NHs were raised, which included the lack of proper documentation (especially pertaining to indications for use)[92,101,102], prescribing of inappropriately high doses[103] and inadequate monitoring[104] for managing adverse effects and evaluating the need for continued use.

APPROPRIATENESS OF ANTIPSYCHOTIC PRESCRIBING

While there appears to be a lack of specific clinical guidance for antipsychotic prescribing in dementia[105], the literature is replete with criteria for defining what are considered as "inappropriate". Firstly, clinicians have to balance the safety warnings associated with antipsychotic use in dementia against the need to alleviate the caregiving stress of providing the basic needs of the aggressive patient and to protect the resident from his/her own dangerous behaviors[99,106,107]. A failure to address these needs is considered "inappropriate"[99,108]. On the other hand, the patient's and/or family's wishes to refrain from antipsychotic use have to be considered and respected[108].

Secondly, antipsychotic prescribing decisions without documented reasons are considered as inappropriate. As suggested in the algorithm by Oborne *et al*[102], proper documentation of prescribing rationale when initiating antipsychotics would include the specific description of the target behavior and/or symptoms and its impact on the patient that the prescribed medicine was intended to treat and resolve. During subsequent medical follow-ups and reviews, documentation of the patients' responses to the prescribed treatment in terms of the changes in the details and impact of the target indications would be required to make informed decision for attempting a dose reduction or continuing with the antipsychotic treatment at the minimum effective dose, according to the guiding principle of "start low and go slow"[109]. Since the recommended antipsychotic doses for managing aggression and psychosis in dementia is generally much lower than that for treatment of psychiatric conditions, the prescribing of high doses and/or quick upward titration of doses may inappropriately expose the older person with dementia to unnecessary side effects such as drug-induced movement disorders, gait disturbances, and somnolence, potentially contributing to falls and adverse events such as fractures[12,85,110].

Lastly, prescribing of antipsychotics for use in older persons with dementia would be inappropriate without proper monitoring for clinical responses and side effects. As prescribing decisions made during the short consultation time often depend on feedback from the caregivers, detailed and specific accounts of the changes in behavior, symptoms and complaints of patients may prompt timely interventions such as titrating the antipsychotic dose downwards for abating side effects. Specifically in the NH setting, the lack of proper monitoring and feedback

processes may be attributed to the low staff-to-resident ratio[111], nurse-resident miscommunication[112], inadequate training and the lack of a structured monitoring framework for antipsychotic use[113], resulting in a lack of proper documentation of the rationale for antipsychotic use for each resident throughout his stay at the NH[104].

INTERVENTIONS TO REDUCE INAPPROPRIATE PRESCRIBING OF ANTIPSYCHOTICS IN NURSING HOMES

In order to reduce inappropriate prescribing of antipsychotics in the NHs, many interventions have been detailed. The first widespread changes in antipsychotic use trends were reported across most NHs in the US during the early 1990s. This was in response to the implementation of the OBRA'87 legislation which restricted the unjustified use of antipsychotics as a chemical restraint in the NHs for the management of difficult behaviors such as wandering, restlessness, anxiety and uncooperativeness[114]. In tandem with this legislation was the mandatory conduct of routine drug regimen reviews by pharmacists[115]. Although these brought about remarkable reductions in antipsychotic use, evidence of its positive impact on other clinical outcomes such as reduction in adverse events among NH residents was elusive. In contrast, a retrospective cross-sectional study noted that the NH residents in the United States were more likely to sustain falls, despite lower prevalence of psychotropic use, compared to those in Denmark, Iceland, Italy, Japan and Sweden[116]. Furthermore, adequate level of staffing could be more crucial towards the successful reduction of antipsychotic use and improved outcomes in the NHs[117,118].

A literature search using a combination of terms "antipsychotics", "neuroleptics", "prescribing", "nursing homes" and "intervention" was conducted to identify original studies that reported interventions targeting to reduce inappropriate antipsychotic use in NHs. A total of 12 interventions involving strategies such as audit-feedback processes, education and training for prescribers and/or nurses, medication review, multi-disciplinary case conferencing, early screening and intervention, structured monitoring program, as well as patient-centered psychosocial interventions are discussed in this review (Table 1).

Interventions involving audits and feedback

Among the interventions using the audits and feedback approach, Westbury's and Castle's reports showed statistically significant reduction in the use of antipsychotics[119,120]. However, Westbury's group postulated that the positive outcome was likely to be attributed to the impact of the academic detailing with physicians, nursing staff training and follow-up medication review component[119], but the outcome was not sustainable after 18 mo and the antipsychotic use prevalence returned to baseline levels[121]. This suggests for the management of BPSD to be a long-term process, requiring constant reviews to ensure the appropriateness of antipsychotic prescribing. Similarly, another study showed that the audit-feedback process resulted in a reduction in antipsychotic use when it was carried out in combination with providing education and practical tools for nurses on how to document behaviors as well as the use of non-pharmacological interventions by nurses to manage agitation and challenging behaviors[122]. However, the intervention employed by Castle[120] did not include the component of education. Instead, it focused on communicating "important legislative efforts to reduce the prevalence of antipsychotics" as well as each NH's "performance" compared to other NHs across the board, in a manner which facilitated the use of these information by the NHs to change their care processes[120]. However, as inadequate information was provided on the selection or randomization of the NHs included in Castle's study, there could be a potential bias of more NHs that are motivated in change in the intervention group.

Interventions involving education of healthcare professionals

Unlike educational interventions previously reported in the early 1990s[123,124], two studies published more recently in 2005 and 2010 did not report significant reduction of antipsychotic use in the intervention NHs[125,126]. Reasons for these could be related to the small sample size of residents using antipsychotics at baseline as well as the use of non comparable control NHs with regards to the BPSD severity and use of antipsychotics of the NH residents. However, Monette *et al*[127] reported a large number of antipsychotic discontinuation and dose reduction following an inter-disciplinary educational intervention, which was coupled with active monthly clinical follow-up by pharmacists to remind physicians to review antipsychotic prescriptions, monthly charting of residents' BPSD severity by nurses, as well as regular inter-disciplinary team meetings. A re-evaluation of this complex intervention involving education and inter-disciplinary efforts five years' later continued to demonstrate a reduction in the prevalence of antipsychotic use from 30.5% in year 2004 to 17.2% in year 2009[128].

Interventions involving medication review

In the United States, pharmacist-conducted medication reviews yielded a positive impact on improving the appropriateness of antipsychotic use in nursing homes[116]. This effect was also observed in Northern Ireland where a significant reduction in antipsychotic use was observed among NH residents receiving a structured pharmacist-led medication review program compared to those receiving usual care with no pharmacist intervention[129]. Despite the involvement of resident interviews and multidisciplinary meetings with nurses and physicians, this intervention was estimated to be more cost-effective than usual care[130]. Positive reduction in antipsychotic use was also reported in a medication review intervention led by

Table 1 Selected original intervention studies aimed to improve antipsychotic use in the nursing home

Intervention type	Study design	Study duration	Healthcare disciplines providing intervention	Changes in antipsychotic use
Audit-feedback				
Castle[120]	CT	1 yr	NA	4.8% reduction in intervention group; SS
Westbury *et al*[119]	CT	26 wk	P, Ph, N	1.7% reduction in intervention group; SS
Watson-Wolfe *et al*[122]	SSBAS	2 mo	N	4.9% reduction
Education				
Hagen *et al*[126]	CT	1 yr	Ph	Increases in antipsychotic use; no SS in intervention group but SS in control group
Testad *et al*[125]	CRCT	1 yr	P	Increases in antipsychotic use; no SS
Monette *et al*[127]	SSBAS	7 mo	P, Psy, Ph, N	49% discontinued antipsychotics, 13.6% had dose reduction
Medication review				
Patterson *et al*[129]	CRCT	1 yr	P, Ph, N	9.4% reduction in intervention group; odds ratio of antipsychotic use for intervention group *vs* control = 0.26 (95%CI: 0.14–0.49); SS
Chakraborty *et al*[131]	MSBAS	2 yr	Psy, N	13.4% reduction
Case conferencing				
Dahl *et al*[133]	SSBAS	1 yr	P, Ph, N	1.3% reduction
Structured monitoring				
Yap *et al*[104]	SSBAS	24 wk	P, Ph, N	4 times increase in antipsychotic prescribing decisions due to side-effects reported; SS
Psychosocial intervention				
Fossey *et al*[134]	CRCT	10 mo	Psychologist, occupational therapist, N	19.1% reduction in intervention group; lower prevalence in intervention group (19.1% *vs* 42.1%); SS
Bird *et al*[135]	CT	9 mo	P, Psy, N, Psychologist	15.7% reduction in intervention group; SS

CRCT: Cluster-randomized controlled trial; CT: Controlled trial (non-randomized); MSBAS: Multi-site, before-and-after study; N: Nurse; NA: Not assessed; P: Physician; Ph: Pharmacist; Psy: Psychiatrist; SSBAS: Single site, before-and-after study; SS: Statistically significant.

psychiatrists and nurses[131]. However, the study did not include a control group or cost-effective analysis. The frequency and duration of visits by the psychiatrist-nurse team was also not known. Interestingly, the NHs in this study had an overall higher prevalence of antipsychotic use compared to non-nursing residential homes. This could be attributed to NH residents having more severe BPSD, which supports the continuous need to identify safer and more effective approaches to manage BPSD in NHs.

Interventions involving multi-disciplinary case conferencing

A regular multidisciplinary team intervention study reported a significant decrease in the prevalence (-19%) of antipsychotic use[132]. However, at the end of the study, the prevalence of use remained at 19% after the intervention study period, while only 5% of the study population had psychotic disorders. Additional tools for facilitating the assessment, documentation of symptoms and reporting during multi-disciplinary meetings were described in other published studies[104,133]. In order to circumvent the challenge of coordinating the schedules of visiting physicians, psychiatrists and pharmacists for face-to-face case conferencing at the NHs, the intervention reported by Yap's group[104] emphasized on the process of monitoring, documentation and feedback of changes in residents' behavior, clinical responses and side effects to the prescribed antipsychotics by the nursing staff, including nursing aides and healthcare attendants. These caregivers were motivated in providing the intervention as the monitoring-feedback processes were readily incorporated into their usual duties and did not require them to perform additional interviews or physical assessments on the residents. This resulted in a significant increase in prescribing decisions, specifically dose reduction and switching of agents to one with less propensity for drug-induced movement disorders, in response to side effects of antipsychotics reported by the nursing staff. Hence, this intervention would be useful in settings with low staff-to-resident ratios and where potential language and/or cultural barriers between the care staff and residents are present.

Interventions involving psychosocial intervention

The use of psychosocial care was found to be an effective alternative to the use of antipsychotics in managing BPSD according to two studies. Specifically, the use of person-centred care approach for managing BPSD demonstrated significant reduction in antipsychotic use in NHs[134,135]. However, it may be challenging for NHs with staffing caps to implement full psychosocial care in managing BPSD as it is labor-intensive. The provision of continuous support from prescribers and NH administrators as well as training of staff[134] and adequate staff-to-resident ratio is needed[135].

Overall, the majority of the published interventions with positive outcomes of reducing inappropriate antipsychotic use involved education for clinicians and care staff. While nurses were involved in all the interventions

with positive changes in antipsychotic use, they were not part of those interventions which reported no significant reduction in antipsychotic use. This observation suggests that interventions should involve healthcare providers from more than one discipline, especially the nursing staff, as their input as direct caregivers would be a significant influence on antipsychotic prescribing in the NHs[136]. Furthermore, it was noted that many interventions focused on reducing the use of antipsychotics, which is synonymous with preventing the "overuse" and "mis-use" of antipsychotics, while only the intervention reported by Yap's group[104] expressedly addressed the potential "underuse" of antipsychotics due to under- or mis-identification of symptoms such as psychosis, which could respond to short-term antipsychotic treatment[83].

Most of the intervention studies cited in this review employed a variety of intervention types and methodological designs, and some are limited by small sample sizes and the lack of a suitable control. As only one study evaluated the cost-effectiveness of the pharmacist-led medication review intervention[130], the comparisons of effectiveness in the other studies were descriptive at best. However, a study making direct comparison of the 4 interventions, namely medication review, recreational therapy, exercise and patient-centered care is ongoing[137]. Its results would provide deeper insights on the effectiveness of these interventions. Although some of the intervention studies[125,127,134] included in this review measured the change in BPSD using various instruments, positive results for this outcome measure can not be entirely attributed to the appropriateness of antipsychotic use as BPSD, specifically agitation, is intermittent in nature[138]. Hence, future studies should address the highlighted methodological concerns and measure the long-term effects of reducing antipsychotic use on BPSD and adverse outcomes among NH residents[139].

CONCLUSION

It appears that despite the modest efficacy and concerns for adverse outcomes of antipsychotic use in the management of BPSD, the use of these medications in the NHs is inevitable. However, future research should continue to explore the use of safer alternatives for the treatment of these symptoms. Although current guidelines recommend the use of psychosocial care over antipsychotics for the management of BPSD, organizational, resource and training support are essential to encourage and equip the NH staff to participate and provide these interventions. At present, there is no alternative solution to antipsychotic treatment and no gold standard in clinical practice to reduce inappropriate antipsychotic use. While the use of antipsychotics to manage BPSD symptoms may be warranted in cases when the safety of the NH resident and others around him/her is threatened, multidisciplinary interventions such as routine medication reviews to promote the appropriate use of antipsychotics may contribute as a long-term sustainable solution.

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P- Reviewer: Myers CS, Mauri MC **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i4.186

World J Pharmacol 2014 December 9; 3(4): 186-192
ISSN 2220-3192 (online)
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MINIREVIEWS

Use of eltrombopag in thrombocytopenia of liver disease

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Received: May 16, 2014 Revised: July 4, 2014
Accepted: September 17, 2014
Published online: December 9, 2014

Abstract

Second generation thrombopoietin agonists including eltrombopag and romiplostim act on the thrombopoietin receptor to increase the megakaryocyte production. These agents were needed as use of first generation recombinant products was associated with formation of autoantibodies. Eltrombopag is an oral thrombopoietin agonist found effective in raising platelet counts in patients with immune thrombocytopenia. The drug has now been found to be useful in raising platelet counts in thrombocytopenia related to liver disease including cirrhosis and chronic viral hepatitis. Although the drug may help enable adequate interferon therapy in patients with HCV infection and help carry out invasive procedures in patients with cirrhosis, concerns have been raised of possible thrombotic complications including portal vein thrombosis. Randomized trials have shown that use of eltrombopag concomitant with pegylated interferon and ribavirin increased the chances of sustained virologic response while decreasing the dose reductions of interferon. The data on use of romiplostim in these clinical indications is also emerging. However, in the future, availability of interferon free regimens is likely to decrease the use of eltrombopag for enabling antiviral therapy. The review discusses the role of eltrombopag in management of liver disease related thrombocytopenia in wake of recent data as also the dosage, precautions and adverse effects associated with its use.

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Key words: Cirrhosis; Thrombopoietin; Eltrombopag; Romiplostim; Hepatitis; Hepatitis C virus; Splenectomy; Thrombocytopenia

Core tip: Thrombocytopenia associated with liver disease is multifactorial. Eltrombopag, a thrombopoietin agonist, has been found useful in increasing platelet counts in these patients. It has been clinically used to increase platelet counts in cirrhotic patients prior to invasive procedures and in patients with chronic hepatitis C to enable administration of interferon based antiviral therapy. However, there are concerns regarding its safety and possible increased risk of portal vein thrombosis.

Sharma V. Use of eltrombopag in thrombocytopenia of liver disease. *World J Pharmacol* 2014; 3(4): 186-192 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/186.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.186

INTRODUCTION

The recent advances in management of immune thrombocytopenia seem to have rubbed off on the management of thrombocytopenia in liver disease. The availability of thrombopoietin agonists in recent times has added to the armamentarium to manage liver disease related thrombocytopenia. The present review focuses on the evidence regarding clinical use of eltrombopag (marketed as Revolade and Promacta) in liver disease related thrombocytopenia.

THROMBOCYTOPENIA IN LIVER DISEASE

Thrombocytopenia is an important complication of chronic liver disease but may accompany non-cirrhotic liver disease. Although various authors have used different definitions, any level of platelets below 150000/mm^3

Table 1 Mechanisms of thrombocytopenia in liver disease

In chronic liver disease
Decreased Thrombopoietin production
Splenic sequestration
Autoantibodies against platelets
Expansion of plasma volume
Bone marrow suppression (Alcohol)
In specific liver diseases
Viral or alcohol related marrow suppression
Autoimmune thrombocytopenia
Cryoglobulins
Drugs: Interferon mediated marrow suppression

would qualify as thrombocytopenia[1]. The incidence of thrombocytopenia has been reported from 15% to 75%[2,3]. Multiple mechanisms may contribute to the genesis of thrombocytopenia in association with liver disease[2,4]. These may include sequestration in the enlarged spleen and reduced thrombopoietin production by the diseased liver (Table 1). The role of antiplatelet antibodies has also been alluded to in the genesis of liver disease related thrombocytopenia[5]. Antibodies may also account for thrombocytopenia associated with viral hepatitis[6,7]. The improvement in thrombocytopenia after liver transplantation has been ascribed to the normalization of thrombopoietin production[8].

The incidence of thrombocytopenia in chronic hepatitis C is higher than the general population and variable incidence (0.16%-45%) has been reported from multiple reports[1,7,9,10]. The reason is that different definitions have been used to define thrombocytopenia and different disease stage of liver disease of included patients. A report indicated that the likelihood of having a platelet count of less than 100000/mm^3 was 12 in the cirrhotic population vis-à-vis the general population[10]. Indeed thrombocytopenia is considered an indirect marker of severity of chronic liver disease and may predict the presence of cirrhotic complication especially esophageal varices[11,12]. In fact a ratio of platelet to spleen size may help predict the presence of esophageal varices in patients with liver disease[12]. Thrombocytopenia of liver disease is usually not life-threatening. The importance of liver disease related thrombocytopenia relates to the difficulties in management of such patients including in administration of antiviral therapy in hepatitis C virus (HCV), or the difficulty in doing invasive procedures in chronic liver disease[2,13]. Most importantly low platelet counts are a contraindication to start pegylated interferon related therapy in patients with HCV infection. Interferon itself causes thrombocytopenia in around 30%-35% of patients[14,15]. In spite of recent advances in management of HCV and number of interferon free regimens becoming available, interferon remains a therapy of first choice in many countries due to the lack of availability and high costs of the newer regimens[16].

Traditionally many treatment options were available for management of liver disease related thrombocytopenia but these were either invasive or had significant risks associated with them. Platelet transfusion remained the standard especially in emergent settings but with the caveat that there were attendant risks of transfusion transmitted infections, febrile reactions, lung injury and alloimunisation[17]. Other options included splenectomy or splenic artery embolization with an intent to reduce the effects of hypersplenism to raise thrombocyte counts[2]. Danazol, in a dosage of 300-600 mg daily has also been found effective for management of thrombocytopenia in chronic hepatitis C thereby enabling administration of antiviral therapy with pegylated interferon and ribavirin[18]. However the availability of thrombopoietin agonists has remarkably altered the management of liver disease related thrombocytopenia.

THROMBOPOIETIN AGONISTS

Formation of platelets is a complex process wherein pleuripotent hematopoietic stem cells undergo maturation to form the megakaryocytes. The production of megakaryocytes is controlled and can increase ten folds in times of need[19]. This increase involves regulation by many factors including interleukin (IL)-3, IL-6, IL-11 and most importantly thrombopoietin. Although its existence was postulated in 1958, thrombopoietin (TPO) was discovered in 1994 by five different laboratories independently[20]. TPO is synthesized in the liver and mediates its actions through interaction with Human anti-thrombopoietin receptor receptor resulting in downstream activation of various signaling pathways like janus kinase/signal transducer and activator of transcription, Shc/Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt which result in activation and proliferation of erythroid, myeloid and megakaryocytic progenitors. Therefore, TPO has been also termed as a pan-hematopoietic cytokine[21].

Discovery of TPO and its effects on megakaryocyte proliferation resulted in efforts to use TPO and its congeners in clinical situations. Two recombinant thrombopoietins entered clinical development: recombinant full length thrombopoietin (rhTPO) and pegylated recombinant human megakaryocyte growth and development factor (PEG-MGDF). The essential difference between the two is whilst rhTPO is a full length glycosylated form of TPO, PEG-MGDF is a truncated and non-glycosylated form of TPO[22]. Both these agents were effective in elevating platelet counts and were used in various clinical indications. rhTPO resulted in elevation in platelet counts from day 4 to day 21 of administration with peak levels on day 12[23]. Pharmacokinetics with PEG-MDGF were also similar. However further clinical development of these otherwise excellent drugs was halted because of development of neutralizing antibodies especially with PEG-MDGF[24,25]. However the second generation of thrombopoietin agonists soon entered clinical realm and have since added to the armamentarium available for management of immune thrombocytopenia and other thrombocytopenic disorders. Romplostim is a peptibody which was synthesized by combining a pair of 14 amino

acid TPO peptide into IgG type 1 heavy chain resulting in a drug with significant with potent action on megakaryocyte production[26]. Romiplostim is used as once in a week subcutaneous injection[20,27]. Since romiplostim has no molecular homology with the human TPO, no problem of neutralizing antibodies has been noted with its use[26]. Eltrombopag is the other second generation molecule which is orally active and is a biaryl hydrazone[27,28].

ELTROMBOPAG

Eltrombopag is a non-peptide TPO agonist which acts on the thrombopoietin receptor to increase the production of platelets. It was first approved in 2008 for treatment of immune thrombocytopenia. Since then its role has been recognized in management of thrombocytopenia of diverse etiologies[19,27,29]. Being structurally different from the endogenous TPO, the interaction with TPO receptor is non-competitive and additive[30]. In relapsing or refractory immune thrombocytopenia, eltrombopag was seen to exhibit a dose dependent increase in platelet counts over doses of 30, 50 and 75 mg/d[31]. The dosages available are 12.5, 25, 50, 75 and 100 mg. In immune thrombocytopenia the lowest dosage which achieves a platelet count of 50000/μL is used. Use of higher doses in chronic liver disease may predispose to more chances of portal vein thrombosis. In such a situation an initial dosage of 25 mg/d for 2 wk has been recommended[32]. Certain precautions need to be observed whilst prescribing eltrombopag. The drug must be taken empty stomach with a 1-2 h interval between the drug intake and the meals. Concomitant calcium supplements or other polyvalent cations must be avoided and the patient must not take the drug more than once in a 24 h period. In a pharmacokinetic study in healthy volunteers it was noted that intake of calcium or magnesium and aluminum containing antacids reduced the systemic availability of the drug[33].

Eltrombopag is absorbed to the extent of around 50% after oral ingestion and peak plasma levels are achieved in 2-6 h[27]. Twenty percent of the drug is excreted unchanged in faeces[34]. With increasing hepatic impairment the area under curve increased suggesting that liver plays an important role in elimination of eltrombopag[35]. Eltrombopag also has low-distribution performance and liver is the primary site for its distribution and elimination both[34]. Apart from immune thrombocytopenia eltrombopag has also been used in myelodysplastic syndrome, chemotherapy related thrombocytopenia, and aplastic anemia[19]. Eltrombopag is a fairly safe drug. The most common side effects noted include headache, malaise, fever, deranged liver function tests including transaminase elevations and indirect hyper-bilirubinemia[36,37]. Other reported adverse events include cutaneous hyperpigmentation, erythroderma, pruritic exanthema and episodes of venous thrombosis at various sites[37-39]. Although increased cataracts had also been reported but patients had received steroids for immune thrombocytopenia[37]. Possible reasons for stopping eltrombopag treatment include severe adverse events like thrombosis, lack of response to maximal dose for 4 wk, or elevation in transaminases more than three folds of the baseline[40].

ELTROMBOPAG IN HEPATITIS C THERAPY

Therapy for HCV has been an area of much contemporary interest and has seen many new drugs emerge which are likely to become available globally and will result in increased rates of sustained virologic response and reduced side-effects[41]. Interferon free regimens are now a clinical reality for all HCV genotypes[42]. The combinations of ledipasvir and sofosbuvir given for a 12 wk duration provide standard variable rate (SVR) of more than 90% in HCV genotype 1[43,44]. Even for genotype 2 and 3 a combination of sofosbuvir and ribavirin for 12 and 24 wk respectively provides good SVR rates[45,46]. However pegylated interferon and ribavirin combination remains the therapy of choice for many patients due to issues of affordability and availability of the newer agents.

As previously discussed HCV is known to cause thrombocytopenia. It may also increase the risk of developing immune thrombocytopenia[47,48]. The matter is further complicated in the patients receiving pegylated interferon and ribavirin. Interferon is known to cause thrombocytopenia by causing bone marrow suppression[49]. In a large report on interferon therapy in HCV patients, baseline thrombocytopenia was present in 44% of patients. In patients with severe thrombocytopenia (< 75000/μL), the need to stop interferon or reduce its dose was much higher. Severe bleeding events were uncommon but a platelet count of < 50000/μL predicted an increased risk of bleeding[50].

ENABLE-1 and 2 trials provided data regarding use of eltrombopag to ensure initiation and completion of interferon and ribavirin therapy (Table 2). In ENABLE-1 trial patients with HCV infection and platelet count of < 75000/μL received progressively increasing doses of eltrombopag (25, 50, 75 and 100 mg) to achieve a platelet count of > 90000/μL. With this strategy initiation of interferon treatment was possible in 95% cases and only in 2% cases did the count not increase to the desired level. Also 88% patients benefited with a dose of 50 mg/d or less. The group was now randomized to either receive pegylated interferon-2a and ribavirin with placebo *vs* with eltrombopag. Although the rates of RVR were similar in the two groups the rates of EVR and SVR were increased in those receiving eltrombopag. Dose reductions of interferon were higher in the placebo arm. ENABLE-2 had a similar study design except for the use of pegylated interferon 2b instead of 2a used in ENABLE-1. The results were similar with 96% patients achieving the target platelet counts. Median time to achieve the target was 2 wk. Discontinuations were higher with the placebo arm but thromboembolic events including portal vein thrombosis

Table 2 Studies of Eltrombopag in patients with liver disease

Ref.	Population	Type	Results
McHutchison *et al*[55]	Compensated HCV cirrhosis with thrombocytopenia	Phase Ⅱ RCT, placebo controlled	Dose dependent increase noted with eltrombopag
Kawaguchi *et al*[32]	Cirrhosis	Phase Ⅱ Randomised Open label study	Risk of thrombotic phenomenon, recommends lower dose in Japanese
Afdhal *et al*[56] ELEVATE trial	Cirrhosis patients, periprocedural use	Phase Ⅲ, RCT, placebo controlled	Decreased platelet transfusion with eltrombopag with increased risk of portal vein thrombosis
Afdhal *et al*[51] ENABLE 1 and 2 trial	HCV related thrombocytopenia, to enable SVR	Phase Ⅲ, RCT, placebo controlled	Decreased dose reduction in eltrombopag group, Higher SVR

HCV: Hepatitis C virus; SVR: Standard variable rate.

were higher in the eltrombopag arm as were the rates of hepatic decompensation[51-53]. The occurrence of portal vein thrombosis may compromise the feasibility and outcomes of liver transplantation which may be needed in these patients. Interestingly neither the dosage of eltrombopag nor the platelet counts predicted the risk of thromboembolic events in the ENABLE trials[49]. With advent of interferon free therapies, the use of eltrombopag or other thrombopoietin agonists for enabling interferon based therapies is likely to decrease in the near future. However, there is still time before the majority of world population especially in the low income countries has access to direct acting antivirals and till that time role of eltrombopag to support difficult to treat groups like those with cirrhosis will remain[54].

ELTROMBOPAG IN CIRRHOSIS

Compensated cirrhosis with HCV is also an indication for treatment with interferon but the presence of thrombocytopenia complicates the management. In a trial evaluating multiple dose regimens of eltrombopag for management of thrombocytopenia in HCV related cirrhosis so as to initiate interferon and ribavirin therapy, 74 patients were assigned to receive placebo, 30, 50 or 75 mg of eltrombopag daily for 4 wk. Twelve weeks therapy with the antivirals was possible only in 6% patients receiving placebo whilst a progressively larger number of patients (36%, 53%, and 65%) were able to receive therapy with increasing doses of eltrombopag (30, 50, and 75 mg respectively)[55]. In this trial three patients required withdrawal of eltrombopag for various reasons including new onset ascites, retinal exudates and neutropenia; effects not entirely related with use of eltrombopag. Further information on use of eltrombopag in cirrhosis came from the ELEVATE trial in which patients with cirrhosis and a platelet count of < 50000/μL who were planned for an invasive procedure received either placebo or eltrombopag in a dosage of 75 mg daily for 2 wk before the planned procedure (Table 2). In a significantly higher number of patients receiving the drug (72%) vis-à-vis the placebo (19%), the transfusion of platelets could be avoided. However, there were no differences in significant bleeding episodes[56]. This, however, came at an increased risk of portal vein thrombosis in the treatment arm raising concerns about the safety. Interestingly the dosage used in this trial was a higher one at 75 mg and the risk increased with higher platelet count levels[56,57]. There are reports suggesting a higher drug exposure in East Asian population and lower initial doses have been recommended[58,59]. In a report from Japan on 38 patients with chronic liver disease even a dosage of 12.5 mg daily resulted in a mean platelet elevation of 24000/μL suggesting that lower doses may be effective in this population. More side effects as also serious events like portal vein thrombosis were noted in the 37.5 mg group[32]. Other case reports have also described similar events with the usage of eltrombopag or romiplostim in chronic liver disease[60,61]. Romiplostim has also been effective for management of HCV and cirrhosis related thrombocytopenia[62,63]. The use of romiplostim was reported to be effective in raising the platelet count in majority of patients (33 out of 35) with chronic hepatitis C related cirrhosis to a level of > 70000/μL thereby enabling surgical procedures. No major bleeding or thrombotic episodes were reported in this Phase Ⅱ study[64,65].

CONCLUSION

Eltrombopag is effective in treatment of thrombocytopenia of liver disease and may help in certain clinical situations. The drug may be of use to initiate and complete interferon based anti-HCV therapy and may have a role prior to invasive procedures in patients with cirrhosis. However the use must be tempered by the possible risk of thrombotic complications including portal vein thrombosis. Importantly the minimum possible dose which can achieve the requisite platelet count should be used.

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P- Reviewer: Ali AEM, Chuang WL, Hekmatdoost A, Kurtoglu E
S- Editor: Song XX **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Pharmacology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5497/wjp.v3.i4.193

World J Pharmacol 2014 December 9; 3(4): 193-198
ISSN 2220-3192 (online)
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Perspective of antiviral therapeutics for hepatitis C after liver transplantation

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Received: June 24, 2014 Revised: September 22, 2014
Accepted: October 1, 2014
Published online: December 9, 2014

Key words: Hepatitis C; Recurrence; Liver transplantation; Therapeutics; Fibrosis

Core tip: Management of hepatitis C virus (HCV) recurrence after liver transplantation used to be a bothering issue due mostly to the interferon-based therapy. Current available data from treatment of chronic HCV infection shows promising results of interferon-free, or even ribavirin-free, pan-genotypic, all oral medications will soon reform the treatment of HCV recurrence after liver transplantation.

Ho CM, Hu RH, Lee PH. Perspective of antiviral therapeutics for hepatitis C after liver transplantation. *World J Pharmacol* 2014; 3(4): 193-198 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/193.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.193

Abstract

Hepatitis C virus (HCV) almost recurs after liver transplantation for HCV-related liver cirrhosis or hepatocellular carcinoma. Management of HCV recurrence after liver transplantation is challenging because the traditional interferon-based therapy is often patient-intolerable and inducing cytopenia, and dose reduction is needed. The response rate in liver recipients is inferior to those of chronic HCV infection. About 5 percent of liver recipients receiving interferon-based therapy would develop immune-mediated graft injury and may need retransplantation. Recent advances of anti-HCV therapy for chronic HCV infection has evolutionary changing the schema from interferon-based, to interferon-free, and even to ribavirin -free, all oral combinations for pan-genotypes. Management of HCV recurrence after liver transplantation is currently evolving too and promising results will soon come to the stage. This "fast-track" concise review focuses on the issues relevant to HCV recurrence after liver transplantation and provides up-to-date information of the trend of the management. A real-world case demonstration of management was presented here to illustrate the potential complications of anti-HCV therapy after liver transplantation.

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INTRODUCTION

Hepatitis C-related liver cirrhosis or hepatocellular carcinoma is the main indication of liver transplantation worldwide[1-4]. Almost all recipients experiences post-transplant recurrence of hepatitis C virus (HCV) and some degrees of long-term graft injury[5,6]. Although hepatitis C is a potentially curable disease because it is caused by an RNA flavivirus with 6 major genotypes[4], and, unlike hepatitis B virus which integrates its DNA into host DNA genome and causes viral clearance difficult, therapeutic outcomes were inferior in liver recipients compared to patients of chronic HCV infection[7].

Ongoing evolutionary positive results in "general" (non-recipient) population shed promising lights on HCV liver recipients who are still struggling to suffer from the recurrence. The aim of this concise review is to illustrate the current and, more importantly, future pictures of the anti-HCV therapeutics after liver transplantation for non-hepatologists by summarizing a great varieties of reviews

Table 1 Category, mode of action and major side-effects of anti-hepatitis C virus therapy

Category	Specific target	Mechanism of action	Major side-effect
Interferon α	Host hepatocytes and immune cells	Enhance host immune response	Flu-like symptoms
Ribavirin (nucleoside inhibitor)	Nucleoside guanosine analogue	Stop viral RNA synthesis and viral mRNA capping Enhance interferon response	Cytopenia
DAA			
Protease inhibitor (NS3 inhibitor or NS3/4A inhibitor)	NS 3 serine protease ± NS4A cofactor	Inhibit cleavage of the HCV proteins from the polyprecursor	Transfusion-dependent anemia; drug interaction with calcineurin inhibitors
HCV polymerase inhibitor (NS 5B inhibitor)	NS 5B protein (RNA-dependent RNA polymerase)	Inhibit HCV replication	Minimal
NS 5A replication complex inhibitor	NS 5A protein (protein for viral RNA replication and inteferon-resistance)	Stop viral RNA replication	Minimal

DAA: Direct acting antiviral agent; NS: Non-structural; HCV: Hepatitis C virus.

and articles. Detailed or extensive dissections of single agents are beyond the scope of this fast-track review and will be suggested to references.

COURSES OF HEPATITIS C AFTER LIVER TRANSPLANTATION-CONSIDER MORE THAN JUST VIRAL LOAD IN LIVER RECIPIENTS

Early experiences showed that after liver transplantation for HCV-related cirrhosis, persistent HCV infection can cause severe graft damage, and such damage is more frequent in patients infected with HCV genotype 1b than with other genotypes[8].

As more experiences accumulated around the world, the natural history of HCV is accelerated after liver transplantation with 20%-40% progressing to cirrhosis within 5 years[9-12]. Evidence of markers and risk factors, including clinical, serum, histopathological, or donor-related, to early predict this group of patients is summarized well in Howell *et al*[5] and Mariño *et al*[13] review and is still being identified. Crespo *et al*[6] proposed simplified algorithm, combining risk factors (old donors, female recipients, diabetes mellitus, cytomegalovirus infection and corticosteroid boluses) and non-invasive liver stiffness measurement, for management of HCV recurrence after liver transplantation and further consolidated the timing for antiviral intervention.

The primary goal in this scenario is prevention of graft loss from fibrosis progression[12]. Viral load does not correspond to graft injury[14]. Clinicians should note that progression of fibrosis is occasionally observed even in patients who responded to treatment; in these cases, progression may be related to other factors, such as smoldering rejection, nonalcoholic steatohepatitis, other graft-related issues, or older donor or patient age[14]. About 5% of liver recipients receiving interferon-based regimens would develop rejection or graft fibrosis, in the absence of HCV[14]. They could be related to interferon-induced innate alloimmune response or fluctuation of immunosuppressant levels *via* drug interactions[15-18].

Once graft injury progresses irreversibly, either through fibrosis due to HCV recurrence or through therapeutic-induced, immune-mediated injury, retransplantation is often the only option of treatment[19,20]. Patient and graft survival rates after retransplantation in these circumstances, however, are inferior to those after primary liver transplantation[19] although it seems no different compared to those of retransplantation due to other reasons[20].

TRENDS OF ANTI-HCV THERAPY RELEVANT TO LIVER TRANSPLANTATION- NEW INSIGHTS TOWARD THE NEAR FUTURE

HCV was first isolated and identified in 1989[21]. Lai *et al*[22] pioneer study found that interferon-based therapy increased the sustained virologic response and thereafter was considered as the standard of care for HCV treatment. Interferon-based therapy augments the innate immune response to cure the virus[23]. Ribavirin synergistically increase the interferon effect through NK cell activation[24]. As the 3D crystalized structure of HCV was identified, more and more targets disclosed and large upcoming amounts of new drugs designed to achieve better effect[25,26]. Non-structural (NS)3/4A protease inhibitors were added to the interferon-based regimen and increased the sustained virological response (SVR) further[25].

The effective therapy against chronic HCV infection has improved dramatically recently, with expected SVR rates of near 75% in all previously untreated patients and the current treatment guidelines was summarized by Gane *et al*[12].

Recently, NS 5B polymerase inhibitor further raised the response rate substantially and open the era of interferon-free, all oral, regimens[25]. NS 5B and NS 5A fixed, once daily combination further suggest the possibility of ribavirin-free regimens in the near future[27,28]. Shorter treatment course and pan-genotypic response, in previous treatment failure or cirrhotic patients make anti-HCV treatment revolutionary[27-29]. The evolutionary trend of

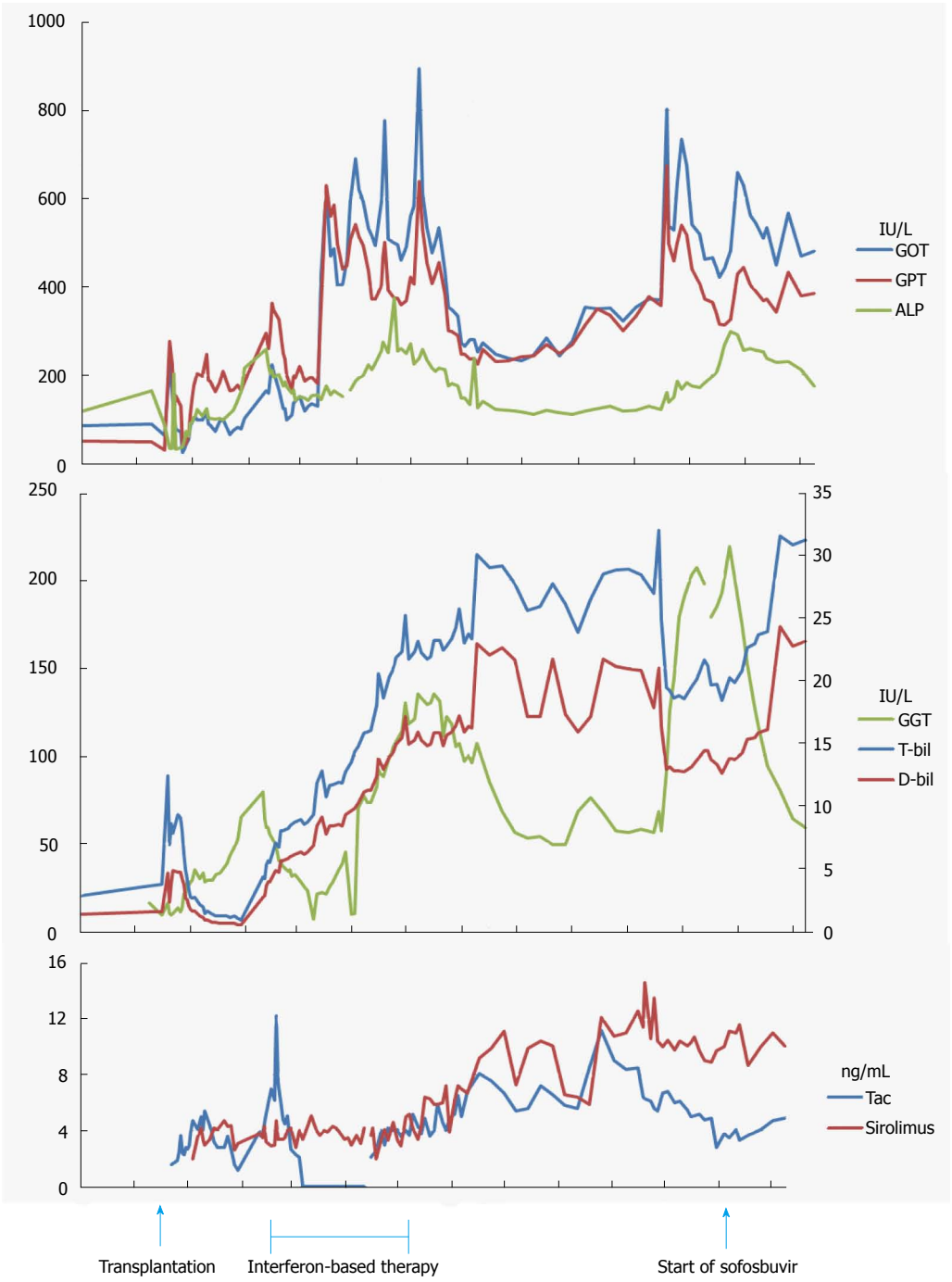

Figure 1 Post-transplant course of a liver recipient with hepatitis C recurrence.

anti-HCV therapy was nicely presented in Heim's work[25].

Table 1 summarized the major anti-HCV therapeutic agents, mode of actions, and major side effects. High cost will be the major issue of anti-HCV therapy in the world instead[30]. In the future, the indication for ribavirin would be limited only to non-nucleotide-based combinations or failure of other oral combination[7,31].

ANTI-HCV THERAPY AFTER LIVER TRANSPLANTATION-THE REAL WORLD

Interferon-based regimens after liver transplantation achieved 30% SVR in liver recipients, with higher rates achievable in patients with non-1 genotypes[32,33]. A lot of HCV liver recipients, however, are rejected for the regimens from the start or early in the course of treatment because of the side effect intolerance. In fact, the most negative predictor for viral response is lack of tolerability from pegylated interferon/ribavirin-more than 80% of patients dose reduce and almost 30% cease therapy because of adverse effects[12]. These results highlight the critical need for better tolerated and more efficacious HCV therapies for HCV transplant recipients[12].

Interferon-based, NS 3A/4A added regimens after

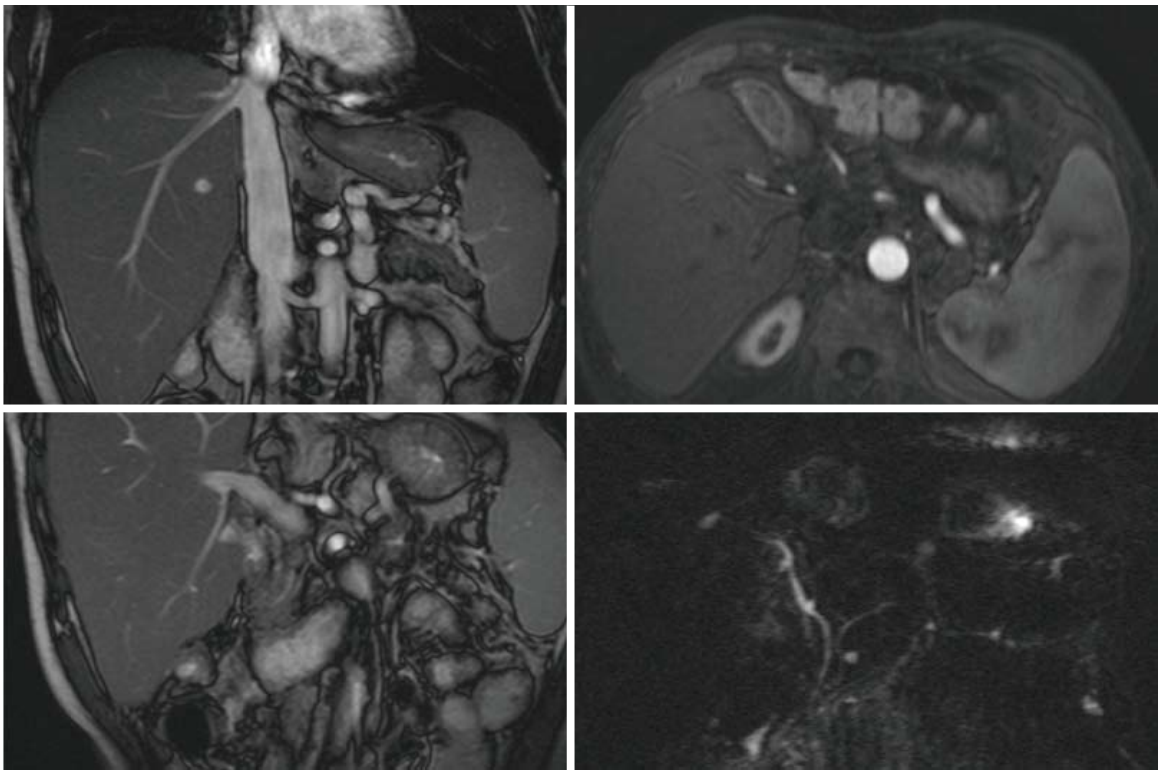

Figure 2 Magnetic resonance imaging screening for liver graft vascular or biliary complications.

liver transplantation could be further increased the SVR to 50%[34]. Discontinuation rates were still observed high and over one third of patients need blood transfusion[12]. In addition, antiviral therapy utilizing boceprevir in liver transplant recipients requires close monitoring of cyclosporine (5-fold) or tacrolimus (70-fold increase) due to the enzyme inhibition of the cytochrome P450 3A[12].

A case report of the HCV liver recipient with good virological response using NS 5B inhibitor show promise of translating the success in "general" HCV population to HCV liver recipients[35]. Sarkar *et al*[31] reported preliminary, multi-center, promising results of sofosbuvir and ribavirin for post-transplant HCV recurrence (more than 80% had HCV genotype 1) in the Liver Meeting 2013. They found a rapidly decline of HCV after starting therapy, and over 70% of 40 recipients had SVR 4 wk after completing treatment[36,37]. Only 2 (5%) had side effects that led to treatment discontinuation[36,37]. These studies had actively formulating the future all-oral treatment regimens in HCV liver recipients.

REAL-WORLD CASE DEMONSTRATION

A 59 year-old man, received liver transplantation for HCV-related liver cirrhosis, was referred for prolonged and progressive jaundice since 2 mo following liver transplantation. Serial liver biopsies showed chronic hepatitis C and serum viral load was 5.8×10^6 IU/mL with the genotype 1B. Interferon-based therapy was initiated soon but lasted for 3 mo because of patient intolerance. The viral load was 1.3×10^4 IU/mL at this time. Jaundice, however, was progressive. Laboratory data of liver profiles were shown in Figure 1. Image survey showed no evidence of vascular or biliary complications (Figure 2). Immunosuppressant regimens were tacrolimus-based initially, withdrawal transiently for the threat of drug-related cholestasis, and followed by re-initiation. The serum levels of immunosuppressant (tacrolimus and sirolimus) were shown in Figure 1. Interferon-based therapy with add-in sofosbuvir were restarted 9 mo after liver transplantation. Ribavirin and sofosbuvir were used 3 wk later without interferon because of the patient intolerance. Serum viral load dropped dramatically to undetectable 3 mo after the use of sofosbuvir, 11 mo after transplantation and remained thereafter. The latter serial liver biopsies (from 5 mo to 11 mo after liver transplantation), however, showed first acute rejection, and chronic rejection later. Steroid bolus therapy was not responsive. Liver retransplantation was suggested.

CONCLUSION

In summary, with the rapid advances of anti-viral therapy in HCV hepatitis, the prognosis of HCV liver recipients is expected to improve greatly once the all oral, interferon-free, ribavirin-free regimens come to the stage of the standard of care with reasonable cost.

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DOI: 10.5497/wjp.v3.i4.199

World J Pharmacol 2014 December 9; 3(4): 199-208
ISSN 2220-3192 (online)
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EVIDENCE-BASED MEDICINE

Vitamin D and bone fracture healing

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Received: July 28, 2014 Revised: September 22, 2014
Accepted: October 14, 2014
Published online: December 9, 2014

Abstract

AIM: To examine whether vitamin D is of potential relevance in the healing process of fractures.

METHODS: The present narrative review examined the bulk of the evidence based literature on the topic of vitamin D and bone healing in key electronic data bases from 1980 onwards using the terms vitamin D and bone healing, callus, fracture healing. All data were examined carefully and categorized according to type of study. A summary of the diverse terms and approaches employed in the research, as well as the rationale for hypothesizing vitamin D has a role in fracture healing was detailed.

RESULTS: The results show very few human studies have been conducted to examine if vitamin D is effective at promoting post fracture healing, and the different animal models that have been studied provide no consensus on this topic. The terms used in the related literature, as well as the methods used to arrive at conclusions on this clinical issue are highly diverse, there is no standardization of either of these important terms and methodologies, hence no conclusive statements or clinical guidelines can be forthcoming. There is a strong rational for continuing to examine if vitamin D supplements should be administered post-fracture, and ample evidence vitamin D is an essential hormone for functioning in general, as well as bone health and muscle as this relates to bone density.

CONCLUSION: Whether those with low vitamin D levels can benefit from supplements if their nutritional practices do not cover recommended daily amounts, remains in question.

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Key words: Bone healing; Callus formation; Fractures; Fracture healing; Vitamin D

Core tip: This work describes the status of research on the role of vitamin D in bone healing, and offers suggestions for future research and current clinical practice.

Ray M. Vitamin D and bone fracture healing. *World J Pharmacol* 2014; 3(4): 199-208 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/199.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.199

INTRODUCTION

Bone fractures are an important cause of morbidity and often, premature mortality among the older population. Among athletes and others, bone fractures due to trauma or excessive stress can seriously impair function and future activities and aspirations. In both older persons as well as younger persons minimizing the bone healing time, while maximizing bone strength of the fracture site during healing are important outcomes of the therapeutic process. Because inactivity as a result of a fracture is detrimental both to bone healing and health, and may exacerbate or foster vitamin D insufficiency or deficiency, it appears early or accelerated fracture healing would be highly desirable for returning fracture patents to function as soon as possible with minimal side effects.

The term vitamin D or cholecalciferol, which refers to a group of structurally related metabolites obtained either from dietary sources, supplementation, or sunlight and, bound by vitamin D binding protein is transported to the liver where hydroxylating enzymes initially catalyze it to form 25(OH)D (25-hydroxycholecalciferol). This product is then transported to the kidney where a second hydroxyl group is added to form 1,25-dihydroxycholecalciferol, the biologically active form of vitamin D[1]. Vitamin D is critically important for the development, growth, and maintenance of a healthy skeleton. Calcitriol or $1,25(OH)_2D_3$, the dominant D(3)-hormone and active form produces a wide array of biological responses by interacting with vitamin D nuclear receptors [VDR(nuc)] that regulate gene transcription in over 30 target organs and with a putative cell membrane receptor [VDR(mem1,25)] that mediates rapid biological responses[2]. A second type of receptor is a cell surface vitamin D receptor[1].

Not surprisingly, even though the nomenclature is highly varied in the related literature[1], a substantive body of research implies low vitamin D levels can significantly increase fracture risk, as well as increase the risk of fragility fractures[3]. By contrast, vitamin D supplements can reportedly reduce bone loss, especially at common fracture sites due to its effect on bone mineralization and maintenance[4]. As well, physical activities alone, and especially those that improve muscular loading of bone may enhance bone health and reduce fracture risk, whilst inactivity or muscle weakness may increase the risk of falls and subsequent fractures, and here again vitamin D can play a positive role as suggested by research conducted by Beaudart *et al*[5] and Shuler *et al*[6] and Tieland *et al*[7].

As outlined by Schindeler *et al*[8], fracture healing is a complex event involving a variety of differing processes. To better understand if fracture healing itself can be accelerated by the use of vitamin D supplements, either as a result of its impact on bone, or muscle or both, as suggested by Schunak[2] and Smith *et al*[3] this present review was designed to examine more closely, if vitamin D levels consistently predict the extent or rate of post-fracture bone healing, either directly through their osteogenic effects or indirectly through their effects on muscle function.

Since the literature remains equivocal about whether supplementation may be desirable for promoting bone healing in fracture cases, despite considerable prior discussions on this topic, it was felt a broad examination of the available literature would be helpful in this regard. The term fracture healing in this paper refers to the different stages during one of the four stages of fracture repair, but these are not strictly delineated as there is overlap in these stages, namely inflammation, soft callus formation, hard callus formation, and bone remodeling[8]. The terminology adopted to describe vitamin D in this paper is that most commonly used in the related literature, rather than any generic term as there is considerable diversity in this respect and it is highly challenging to interpret or standardize successfully (Table 1).

That is, employing the terminology of the authors whose work is reviewed, this review sought to examine whether deficiencies or insufficiencies in serum levels of 25 hydroxyvitamin D, the metabolite recommended for determining vitamin D status in humans[1], and 1,25-dihydroxyvitamin D, the hormone related to bone and muscle health, are specifically related to the fracture healing process.

At the same time it was hoped the review would provide recommendations for future research and practice in this area, given that the paper by Esche *et al*[9] published in 2011 concluded there were too few human based studies to arrive at conclusive recommendations.

MATERIALS AND METHODS

Using the same search strategy as Esche *et al*[9], the search term Vitamin D and Fracture Healing: produced 130 citations (of which 43 were relevant); Vitamin D and Bone Healing: produced 318 cited studies; Vitamin D and Callus Formation: produced 51 cited studies. Compared to Vitamin D alone: that had 59559 cited studies, it can be seen that although the topic is increasing in terms of citations, it is still understudied relative to other topics in the field. Accepted as valid sources of information were literature reviews, case studies, cross-sectional studies, prospective studies, and topics related to healing both direct and indirect that involved the topic of vitamin D and fracture healing or fracture non-union situations, and that appeared to address the topic of interest in this review.

RESULTS

Animal studies

Briggs *et al*[10] mention that dihydroxylated vitamin D metabolites may play a key role on fracture healing as shown by enhanced serum levels of 24R, 25-dihydroxyvitamin D levels in the long bone post fracture period. This idea has been examined for almost three decades and was supported early on by a number of studies using various animal models, such as the chick[11,12], mice[13], rat[14], and rabbit[15].

Melhus *et al*[16] who examined if osteoporosis and the healing of fractured osteoporotic bone were related, studied this issue in vitamin-D depleted ovariectomized rats known to induce weakening of the femoral neck. After initial ovariectomy, the rats were allocated to vitamin D deficient diets and sham operated rates received normal diets. At 12 wk, a fracture was induced in the tibia and fixed with a nail. Bone and callus formation were monitored with bone scans and vitamin D serum levels were measured. The results showed the experimental group had reduced bone mass, but no differences were found in the mechanical properties of the callus between the groups. The authors concluded that vitamin D is not crucial for fracture healing or for enhancing the mechanical properties of callus. This was a similar overall finding to that of Mao *et al*[17] who examined the influence of

Table 1 Diverse vitamin D terminology and modes of assessment in the related literature and related source

Serum 25(OH)D, 24R,25(OH)$_2$D, 1,25(OH)$_2$D[10]
Vitamin D$_2$[25]
24R,25-dihydroxyvitamin D$_3$ [24R,25(OH)$_2$D$_3$], and 1 α,25-dihydroxyvitamin D$_3$[1 α, 25(OH)$_2$D$_3$] hormonally active vitamin D metabolites[24]
Plasma 1,25-dihydroxyvitamin D$_3$ 25(OH)$_2$D$_3$[28]
24R,25-dihydroxyvitamin D$_3$[29]
25OHD concentration[36]
Serum 25-hydroxyvitamin D[37]
Serum 25(OH)D$_3$[38]
Serum 25-hydroxyvitamin D (25-OH-D$_3$, 24,25 dihydroxyvitamin D$_3$ [24,25(OH)$_2$D$_3$], 1,25 dihydroxyvitamin D3 [1,25(OH)$_2$D$_3$][44]
Serum 25-hydroxyvitamin D, 1,25 dihydroxyvitamin D$_3$, 24,25 dihydroxyvitamin D$_3$ metabolites[46]
25-hydroxyvitamin D [25(OH)$_2$D$_3$], 1,25 dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$], and 24,25 dihydroxycholecaciferol; 24,25(OH)$_2$D$_3$-active metabolites of vitamin D$_3$[30]
1,25 dihydroxyvitamin D [1,25(OH)$_2$D]-biologically active metabolite of vitamin D; 24,25(OH)$_2$D$_3$-a metabolite of vitamin D[54]
1,25(OH)D[58]
Vitamin D 25(OH)D[63]

Vitamin D refers to an inactive compound ingested from the diet or produced after exposure of skin to sunlight. 25-hydroxyvitamin D [25-(OH)D] is an inactive metabolite produced in the liver that is hydroxylated in the kidney to form 1-alpha,25-dihydroxyvitamin D [1,25(OH)$_2$D] is the active form of vitamin D that binds to vitamin D receptor or VDR on target tissues[11]. 24R,25-dihydroxyvitamin D3 [24R,25(OH)$_2$D$_3$] is an essential vitamin D metabolite[24,29].

both diabetes and vitamin D deficiency on bone repair in female mice. Although vitamin D deficiency aggravated the decrease in bone mineral density according to the diabetic state of the mice, it did not affect bone repair delayed by the diabetic state.

Hong *et al*[18] examined the potential effects of vitamin D on bone regeneration in dogs. Their results indicated that when combined with calcium, vitamin D supplementation may have positive systemic effects that influence bone regeneration more speedily. Similarly, Fu *et al*[19] found the effect of 1,25-dihydroxy vitamin D on fracture healing and bone remodeling in ovariectomized rat femora to favor fracture healing by improving the histological parameters of the bone, its mechanical strength, and tendency to increase transformation of woven bone into lamellar bone. Blahos *et al*[14] who investigated the impact of 1,25-dihydroxycholecalciferol on local healing of artificially induced tibial fracture in the rat, found the contributory effect to increase the weight of the fractured tibias. This was explained by its stimulatory effect on callus formation. Omeroğlu *et al*[15] found a single high-dose of vitamin D$_3$ did show positive effects in the healthy rabbit as far as fracture healing goes. This was supported by observations of increases in the sites mechanical strength after the administration of the high-dose vitamin D3.

Likewise, Liu *et al*[20] who examined the effect of vitamin D supplementation on the fixation of titanium implants in mice with chronic kidney disease-a problem that negatively affects bone regeneration and fracture healing, showed the bone-implant contact ratio and bone volume around the implant were significantly increased in the vitamin D supplementation group. It was concluded that these results implied vitamin D supplementation is an effective approach for improving titanium implants fixation in cases of chronic kidney disease. This is consistent with the finding by Gigante *et al*[21] that vitamin D is able to stimulate osteoblast differentiation of fracture site derived mesenchymal stem cells, and that administration of 25-OH-vitamin D after a fracture can improve the fractured bone's mechanical strength[22] and accelerate the initial mineralization process in the healing fracture region[23]. It was also consistent with the observation by Kato *et al*[24] that there is a biological role for 24R, and 25 (OH)2D$_3$ forms of vitamin D in the fracture healing process. This group actually found the presence of its receptor/binding protein in a callus membrane fraction of a chick tibial fracture.

Contrary results however, were those of Sun *et al*[25] who found vitamin D binding protein had no effect on enhancing healing in rat bone defects. Melhus *et al*[16] too found vitamin D deficiency was not crucial for fracture healing or the mechanical properties of the callus, in rats with osteoporosis induced by ovariectomy. Lindgren *et al*[26] produced evidence that 1,25(OH)$_2$D$_3$ actually impairs fracture healing/in the rabbit, as did Andreen and Larsson in the rat[27]. Yet Jingushi *et al*[28] found serum 1 alpha, 25 dihydroxy vitamin D3 does accumulate into the fracture callous during rat femoral fracture healing. The authors suggested that plasma 1,25(OH)$_2$D$_3$ becomes localized in the callous, possibly regulating processes of fracture healing, a finding similar to that of Seo *et al*[29] Dekel *et al*[30] who examined fractures of the right tibia of chicks depleted of vitamin D, or given vitamin D$_3$ that were subsequently tested mechanically with respect to torsional stress, showed benefits of vitamin D. In this respect, they found repletion with 24,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ produced the most marked effects.

In sum, it is difficult to arrive at any consensus among the many approaches taken to examine the role of vitamin D on bone healing in the context of animal models. Results vary across models, as well as in the same models, and research approach, compounds, metabolites, and vitamin D derivatives are highly heterogeneous and unstandardized (Table 2).

Human studies

A good account of early clinical studies examining the

Table 2 Sample of studies using animal models to examine vitamin D influence on bone healing

Researchers	Model	Finding
Andreen *et al*[27]	Rat	Low doses $1,25(OH)_2D_3$ increased early callus mineralization
Blahos *et al*[14]	Rat tibia	$1,25(OH)_2D_3$ may produce a general response
Brumbaugh *et al*[45]	Chick	Chicks without 1 α, 25 dihydroxy D_3 supplementation showed prolonged fracture healing; 1 α, 25 dihydroxyvitamin D_3 promotes bone repair in the absence vitamin D_3, 25 hydroxyvitamin D_3, and 24, 25 dihydroxyvitamin D_3
Dekel *et al*[30]	Chick	$24,25(OH)_2D_3$, as well as $1,25(OH)_2D_3$ are essential for bone formation after fracture
Fu *et al*[19]	Rat	Vitamin D affected fracture healing positively for up to 12 wk compared to controls both biomechanically and histologically
Lindgren *et al*[49]	Adult rat	Rats given $1,25(OH)_2D_3$ had stronger fracture callus
Lindgren *et al*[26]	Rabbit	$1,25(OH)_2D_3$ impairs fracture healing
Lidor *et al*[12]	Chick	Active metabolites of vitamin D_3 are involved directly in fracture repair
Melhus *et al*[16]	Rat	Vitamin D deficiency does not impact fracture healing
Omeroğlu *et al*[15]	Rabbit	A single high dose of vitamin D_3 had Positive mechanical effects on fractured bone
Seo *et al*[29]	Chicken	$24,25(OH)D_2$ levels increased during fracture repair
Steier *et al*[23]	Rat	Vitamin D_2 accelerated initial mineralization in the fracture healing region

role of vitamin D in fracture healing has been provided by Gorter *et al*[31] Among these studies, research by Doetsch *et al*[32] tried to quantify the healing process of an osteoporotic fracture and to quantify the impact of vitamin D supplementation on the healing process among 30 women randomly assigned to a 800 IU vitamin D plus 1 g calcium or placebo in a double blinded prospective study. The researchers examined the mechanical properties of bone, as well as radiographs to evaluate healing. Bone mineral density was comparable among groups at baseline, and both increased over the 2 wk period. The authors found positive benefits of vitamin D_3 and calcium over the first 6 wk of the fracture for the active group.

Briggs *et al*[10] conducted a prospective study to examine the extent of bioavailable levels of vitamin D metabolites among 28 patients after a cross-shaft fracture of the long bone. They measured serum concentrations of 3 vitamin D metabolites within 48 h of a fracture, and at 1 wk and 6 wk post fracture. They found no change in serum concentrations of 25(OH)D or $24R,25(OH)_2D$ at any time. Mean serum $1,25(OH)_2D$ declined 21% over the course of the study, but no changes in bioavailable concentrations of any vitamin D metabolite were seen over the course of the study.

In a case study reported by Parchi *et al*[33] who examined the impact of vitamin D on the fracture healing process in a child, the authors found deficient vitamin D was a possible cause of the observed inadequate fracture healing process. More specifically, this research showed a significant effect on callus formation with the addition of vitamin D supplementation. Similarly, as reported by Pourfeizi *et al*[34] who conducted a case control study of 30 patients with tibial non union compared with 32 patients with normal bone healing, a high percentage of vitamin D deficiency was observed in tibial unexplained nonunion compared to normal union. Accordingly, the authors suggested vitamin D deficiency was a possible explanation for nonunion of traumatic fractures. This finding, which generally supported the observation of Van Denmark *et al*[35] of a relationship between non union of a distal tibial stress fracture associated with vitamin D deficiency, was contrary to that reported by Boszczyk *et al*[36] who compared vitamin D concentrations in patients with normal and impaired bone union. These authors found a vitamin D deficiency in 86% of examined patients. They found no difference though in 35 patients either with normal or with impaired bone healing. This was a retrospective case-control study, not a prospective randomized controlled study.

Ettehad *et al*[37] who recently examined changes in the vitamin D levels in the serum during healing with respect to fractures of the tibial and femoral shafts found levels of vitamin D declined by the end of the third week after the fracture. They felt this was demonstrative of the fact vitamin D is important in the formation and mineralization of the callus, and consequently supplements of vitamin D administered during the healing process might be helpful in those patients with tibial or femoral shaft fractures. Again, Wölfl *et al*[38] who examined the time course of 25(OH)D during fracture healing in persons with fractures of healthy bone *vs* osteoporotic bone over an eight week period found no inter group differences, making it difficult to establish a definite role for vitamin D in fracture healing in this case controlled study.

A more positive finding in favor of supplementation with vitamin D was reported by Gomberg *et al*[39]. This group described the outcome of efforts to heal subtrochanteric stress fractures caused by excessive long term treatment with alendronate. They found treatment with large doses of oral vitamin D increased serum 25-hydroxyvitamin D_3 to normal levels in 2 mo, after which it remained in the normal range using a maintenance dosage. Although fractures appeared worse on magnetic resonance imaging at 2 mo, 6 mo later, in conjunction with teriparatide treatment and calcium, there was faint bridging of cortical bone, and complete fracture healing occurred over the next year. The combined treatment seemed beneficial to the patient. Inklebarger *et al*[40] have also argued recently for the need to consider the presence

of low vitamin D levels when investigating the causes and possible interventions of femoral and tibial stress fractures in soldiers which may delay healing of these fractures, which is consistent with the finding that serum vitamin D levels are generally low in trauma cases in the United States[41]. In accord with the favorable results of Kato *et al*[42] in an *in vitro* experiment, and findings of low vitamin D levels among fallers[43], the most common cause of hip fractures in older people. Alkalay *et al*[44] found serum 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] was significantly reduced in the fracture patient, even though serum 25 hydroxyvitamin D_3(25-OH-D_3) and 24,25 dihydroxyvitamin D_3 [24, 25$(OH)_2D_3$] did not differ significantly between fracture patients and elective patients.

The data is confusing though because while Brumbaugh *et al*[45] indicated 1 alpha, 25-hydroxyvitamin D_3 promotes bone repair, Haining *et al*[46] found vitamin D metabolites had no influence in explaining fracture nonunion, even though vitamin D supplementation after traction avulsion fracture was recommended by Inkelbarger *et al*[47] and may have indirect beneficial bone effects[48] and appeared to have favorable healing effects in adult rats[49]. Tauber *et al*[50] found blood levels of several active vitamin D metabolites were decreased in some fracture patients, but not others, and attributed the decrease to their consumption during fracture healing. Meller *et al*[51] found a significant rise in plasma 24,25$(OH)_2$-D_3 on the day of the fracture compared to the level measured six weeks later, but no significant changes in plasma 25$(OH)D_3$ levels, in young patients with fractures, and suggested a physiological role for 24,25$(OH)_2$-D_3 in human fracture healing. In an animal model, Seo *et al*[29] implied that 24,25$(OH)D_3$ seems to be involved in the early stage of fracture repair and there is some form of physiological communication between the fractured bone and kidney that results in an increase of the renal derived 24-hydroxylase and circulating concentration of this metabolite. However contrary to research by Hoikka *et al*[52], Omeroğlu *et al*[53], and Lidor *et al*[54], Osório *et al*[55] found no changes in serum levels of 24R,25$(OH)_2D$, although levels of 1,25-dihydroxyvitamin D decreased after fracture over a 6 wk period.

In sum, as outlined in Table 1 and Table 3, the limited data in this area is highly variable and there is consequently little definitive data on whether vitamin D is helpful or not to the healing human fracture, although ample rationale for its post-fracture application exists (Table 4).

DISCUSSION

Fractures, especially those that occur among the elderly are considered to place an enormous burden on the individual, as well as on societies and their social and economic wellbeing. Considerable research shows that high rates of vitamin D insufficiency, referring to serum 25(OH)D concentrations less than 20 ng/mL[1], currently prevail in a high proportion of cases who sustain traumatic fractures[41], especially among the elderly. Consequently, improving vitamin D levels for these fracture patients has been advocated[41] to concentrations greater than 30 ng/mL[1] is advocated. But is there sufficient evidence for this idea? Esche *et al*[9] who conducted a short literature review that examined the question of whether vitamin D supplementation is beneficial for fracture healing found only two studies that were clinically oriented, and that most were studies using a wide variety of animal models. As they observed, both in the non human, as well as the human studies, there are negative, as well as positive results supporting vitamin D supplementation for enhancing fracture healing. As indicated by these authors, at a minimum, more research on larger samples, with more robust research designs, and a careful differentiation of baseline vitamin D status and agreed upon methods of determining vitamin D status is strongly recommended. In particular, more follow up studies, including a focus on events that take place at the four distinct phases of healing could be highly revealing, as opposed to those that simply measure short term fluctuations in vitamin D levels post fracture only, often with opposing results. Gorter *et al*[31] who conducted an updated literature review of 75 *in vitro* and 30 *in vivo* studies found inconsistent results concerning the mechanism of action of vitamin D on fracture healing. They found only four studies that examined the effect of vitamin D deficiency on human fracture healing and that indicated no effect. No studies examined the specific benefits of supplementation alone and studies discussing the cellular effect of vitamin D in fracture healing were nonconclusive.

Because one fracture is often followed by another, and preliminary evidence strongly supports a role for 24,25$(OH)D_2$, a vitamin D metabolite, in mammalian fracture repair[48], it would seem advantageous to strongly consider the use of nutritious sources, sunlight, and if not available, supplementary resources for those at greatest risk of second fractures, even if healing is not promoted. Given that a sizeable proportion of the population appears to suffer from vitamin D insufficiency[48], and that optimal muscle function is contingent on appropriate vitamin D levels[10-12], this alone might be helpful both in preventing future falls, and in enabling muscle forces around the fracture site to promote healing, while offering better protection of the bone while it is healing, even if the fracture site is not impacted directly. As well, the more generic benefits of vitamin D on physical wellbeing could serve to enhance activity levels that are key to building or maintaining bone mineral density, as well as preventing falls and future fractures, and fostering opportunities to be exposed to sunlight.

Corter *et al*[31] who specifically discussed the influence of vitamin D on bone mineralization and subsequent bone quality did not refer to the importance of vitamin D in fostering muscle function, as well as general wellbeing. Even though this group retrieved over 100 studies on this topic, the fact that they only found five *in vitro* studies performed on material from a fracture site, and only one *in vivo* study in the fracture patient, renders the role of vitamin D in this respect is very hard to discern.

Table 3 Sample of human studies designed to examine vitamin D influence on bone healing

Ref.	Type of Study	Finding
Briggs *et al*[10]	Examined vitamin D levels in 28 patents with diaphyseal long bone fractures at 48 h, 1 wk and 6 wk	Serum 1,25-dihydroxyvitamin D decreased from baseline, but serum 24R,25(OH)$_2$D levels did not change
Delgado-Martínez *et al*[22]	Investigated 25-OH-vitamin D effect in elderly with fractures	The addition of the vitamin D supplement improved strength of the fractured bone
Sun *et al*[25]	Examined effect of vitamin D$_3$ on the differentiation of mesenchymal stem cells from a human fracture site	Vitamin D$_3$ was able to modulate the the differentiation towards osteoblastic phenotype of the cells derived from fracture sites
Doetsch *et al*[32]	Quantified impact of vitamin D$_3$ + calcium on healing of osteoporotic fracture	Bone mineral density at 6 wk was higher in actively treated group suggesting vitamin D$_3$ had a positive effect 6 wk post fracture, but this was not maintained at 12 wk
Parchi *et al*[33]	Case report of child post-fracture	Hypovitaminosis D is a possible cause of inadequate fracture healing and refracture in children Vitamin D has a clear effect on callus formation
Boszczyk *et al*[36]	35 patients with inexplicable fracture healing impairments and controls were studied with regard to vitamin D	No impact of vitamin D deficiency noted
Ettehad *et al*[37]	Determined serum levels of vitamin D during fracture healing of 73 patients	Serum levels of vitamin D were reduced in curative period, suggesting vitamin D plays a role in the formation and mineralization of callus
Alkalay *et al*[44]	Assessed vitamin D metabolite levels in 28 patients after fracture, and 27 undergoing surgery	Serum 1,25-dihydroxyvitamin D$_3$ was significantly reduced in the fracture cases
Tauber *et al*[50]	Determined active metabolites of vitamin D$_3$ in 7 fracture patients	24,25(OH)$_2$D$_3$ levels showed a relative decrease, and a decrease in 1,25(OH)$_2$D$_3$ in 2 cases, suggesting these metabolites are consumed at fracture site during healing
Meller *et al*[51]	Levels of 25(OH)D$_3$ + 24,25(OH)$_2$D$_3$ were determined in 13 young patients with long bone fractures on admission and after 6-8 wk	Plasma 24,25(OH)$_2$,-D$_3$ levels rose over the 6 wk period, but no changes in 25(OH)D$_3$ levels occurred
Hoikka *et al*[52]	Treated 37 osteoporotic fracture cases with 1 α, OHD$_3$ – dosage 1 ug per day, plus 2.5 gm calcium	1 α-OHD$_3$ impacts fracture healing although 5/19 cases developed hypercalcemia

Table 4 Rationale for hypothesizing vitamin D as beneficial in fracture healing

Plays an essential role in bone formation and maintenance[1,58]
Has positive benefits on muscle strength[5,58]
Is involved in calcium and bone metabolism[1,29,37,54,57,58,64]
Deficiency is associated with fractures[58]
Can modulate cell growth and neuromuscular function[57,65]
May influence the inflammation stage of bone healing positively, as well as the callus formation stage[31]
Can help regulate inflammation and bone marrow and intramuscular fat deposits[58]
Protects older people from osteoporosis[58]
Enhance fixation of implants[58]
Deficiency may be associated with refrature[33]
Deficiency is associated with non union[34,35,67,69]

Although very few studies were evident in the data bases reviewed, this group noted vitamin D deficiency does not seem to hinder fracture healing, while supplementation with calcium increases the extent of the fracture callus at the fracture site and promotes healing.

In other research, Briggs *et al*[10] found decreased serum levels of 1,25-dihydroxyvitamin D in cases with diaphyseal long bone fractures but no changes in serum levels of vitamin D metabolites post fracture. However, Tauber *et al*[50] found a relative decrease in 24,25(OH)$_2$D$_3$ levels as well as a partial decrease in 1,25(OH)$_2$D$_3$ in cases suffering from delayed non unión and/or multiple fractures. Ettehad *et al*[37] too found these metabolites were reduced during the curative period in cases with either tibial or femoral fractures. They related this finding to the possible role of vitamin D in the formation and mineralization of the callus. Suzuki *et al*[43] found excessively low levels of 25(OH)D to be independently and significantly associated with an increased risk of falling in the elderly. Since adequate or high levels of supplementary vitamin D are protective of bone, and many elderly with fractures are already vitamin D deficient at the time of a fall, the most common reason for fracturing a bone, it seems taking supplements as a precaution against future fractures, as well as attempting to enhance fracture healing is potentially of great importance as supported by findings of Hoikka *et al*[52] who observed the addition of 1 alpha-OHD$_3$ to patients with osteoporotic hip fractures seemed to have a beneficial effect on fracture healing. Although this was also found to frequently cause hypercalcemia, and Boszczyk *et al*[36] found no difference in vitamin D concentrations in normal and impaired bone union, and disturbances in vitamin D metabolism are unlikely to play a major role in maintenance of non-union fractures[46],

vitamin D deficiency was present in 86% of examined patients. Inadequate levels of vitamin D were also found to prevail among patients undergoing orthopedic surgery who presented with bone healing complications[47].

Given that hypovitaminosis D could affect bone formation adversely[1], and that muscle strength capacity alone is found to benefit from vitamin D if taken orally[55], and in combination with calcium may decrease the incidence of non-vertebral fractures in older persons with low vitamin D levels[56] the sustained usage of these compounds may be more favorable than not for influencing fracture healing[57-60], despite the negative findings of the RECORD trial[61]. In addition, for those requiring internal fixation surgery post-fracture, the supplementation of vitamin D where this is found deficient may increase the bone-implant contact ratio and bone volume around the implant as reported by Liu *et al*[20] As well as fostering callus mineralization[62], resistance of the implant is also expected to increase favorably with appropriate supplementation[20].

However, the lack of definitive evidence precludes any conclusion or any set of useful guidelines concerning vitamin D supplementation post-fracture, where indicated, despite the magnitude of the societal burden incurred by the high prevalence of adults who experience delayed bone union, non-union, or future fractures due to suboptimal bone and muscle recovery post-fracture. Clearly, while *in vitro* models are helpful, a much greater effort in the clinical research arena appears warranted. In particular, more prospective long term follow-up studies of different vulnerable groups, and exposure to different levels and combinations of supplements appear desirable. For example, in the study by Omeroğlu *et al*[53] 116 guinea pigs who had received 50000 i.u./kg of vitamin D_3 intramuscularly benefited by this administration, suggesting this method of vitamin D delivery might be highly beneficial for accelerating the synthesis and organization of collagen fibers, the proliferation and differentiation of osteoprogenitor cells, and mineralization of the matrix. Alternately, Lidor *et al*[11,54] found the implantation of D_3 compounds directly into the fractures accelerated healing and prevented non-union. Another mode of delivery, namely subcutaneous delivery after an experimental fracture improved fracture strength in a dose dependent manner[22] as did vitamin D injections[20]. Thus different modes of delivering vitamin D post fracture may produce positive, albeit differential impacts on the healing bone that might be worth investigating. Another area for research may extend to testing different vitamin D metabolites and the affinity of callus membrane receptor/binding proteins for these as observed by Kato *et al*[42] in chick tibial fracture healing callus. Another form of study might be focused on assessing the viability of vitamin D receptors, and whether their functional status is linked to the outcomes of vitamin D analyses in the context of fracture healing, bearing in mind that vitamin D measures may not be useful for judging vitamin D in clinical studies. The consistent use of assays to examine plasma concentrations of 25-hydroxyvitamin D [25(OH)D] may provide the best method for assessing the presence of any prevailing vitamin D deficiency[63].

In sum, since the elderly in particular, who are highly prone to fractures, are at risk of vitamin D deficiency and insufficiency, as well as reduced exposure to sunlight, accelerated bone[57] loss, skeletal fragility and reduced muscle power[58], the application of post-fracture vitamin D supplements would appear beneficial[64,65]. In particular, as outlined in Table 4, vitamin D in its different physiological forms is implicated in bone metabolism[59], and muscle-bone interactions[58] and potentially promotes fracture healing and mineralization[62,66]. Consequently, identifying the optimal vitamin D level that is desirable in the post-fracture state, as well as the best mode of delivery appears highly warranted. Stressing the importance of compliance with recommendations regarding supplements, if indicated, acknowledging the importance of calcium supplementation when vitamin D levels are deficient, and applying doses of vitamin D known to have clinical efficacy is more likely than not to foster optimal post fracture bone remodeling processes and functional benefits especially for those at risk for osteoporotic fractures[57], falls that lead to fractures[58], fractures requiring fixation[20] or atrophic fracture nonunion in the presence of vitamin D deficiency[35]. As outlined by Maier *et al*[60] about 20% of seniors receive vitamin D at the time of their fracture and after the event despite the documented 81% prevalence of vitamin D deficiency. In this regard, it appears reasonable to suggest efforts to improve vitamin D supplementation in seniors both before and after a fracture event are warranted, especially if it is confirmed that serum levels of 1,25-dihydroxyvitamin D are deficient[61], non-union appears to prevail or is imminent[67], or the diagnosis of a stress fracture is forthcoming[68]. Based on findings of vitamin D deficiencies among patients with non-unions[69], studies that show calcium and vitamin D_3 supplementation may enhance callus formation in the osteopenic or osteoporosis patient[32], and animal models that show a combined effect of 1,25 $(OH)_2D_3$ on serum calcium and phosphate and bone matrix formation[62], fracture healing rates as well as bone quality or both may be forthcoming[70]. Alternately, it is possible, that by inadvertently delaying fracture healing, failure to provide adequate vitamin D supplementation in those suffering from vitamin D insufficiency may result in longer curative periods of inactivity and pain, thus potentially fostering further vitamin D insufficiency or depletion.

COMMENTS

Background

Fractures of the bone and their mechanisms of repair are topics that have been the subject of investigation for more than three decades. In both cases, both preventing and treating fractures and the role of vitamin D in both processes have received increasing attention in the literature due to the importance of minimizing post-fracture complications, especially among the older population.

Research frontiers

While vitamin D is of potential relevance in the healing process of fractures, it is unclear whether supplements should routinely follow fracture injuries, and if so

what is the evidence base for this.

Innovations and breakthroughs

The present narrative review examined the bulk of the literature present in key electronic data bases from 1980 onwards. The results show very few human studies have been conducted to examine if vitamin D is effective at promoting post fracture healing, and the different animal models that have been studied provide no consensus on this topic. While not new, this gap in the literature indicates much more attention is required in this realm than is currently evident.

Applications

Given that vitamin D is an essential hormone for functioning in general, those who have low levels of the hormone in general, can probably benefit from supplements in the post-fracture period if their nutritional practices do not cover recommended daily amounts, and they are at high risk for non union and/or subsequent fractures. Since those who experience non union or delayed union may inadvertently suffer from inadequate vitamin D exposure, and vitamin D insufficiency, or deficiency this approach appears worthwhile to contemplate.

Terminology

The term vitamin D in this paper refers to all forms of this hormone and/or its metabolites. The terms bone healing and fracture healing are used interchangeably.

Peer review

The review by Marks is well written.

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P- Reviewer: Makishima M, Peng JB **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Lu YJ

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DOI: 10.5497/wjp.v3.i4.209

World J Pharmacol 2014 December 9; 3(4): 209-216
ISSN 2220-3192 (online)
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SYSTEMATIC REVIEWS

Association of serum bilirubin and non-alcoholic fatty liver disease: A feasible therapeutic avenue?

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Received: May 21, 2014 Revised: October 15, 2014
Accepted: October 23, 2014
Published online: December 9, 2014

Abstract

AIM: To the look at the current strength of evidence and the potential application of anti-oxidants in this setting.

METHODS: Two electronic databases (PubMed and Web of Knowledge) were searched to January 2013 to find studies addressing serum bilirubin levels in non-alcoholic fatty liver disease (NAFLD). The search used key word combinations in relation to NAFLD and serum bilirubin specific to human adults only. After screening selected studies were reviewed in depth by two independent reviewers. Data synthesis with further meta-analysis was planned but not possible due to the heterogeneity of the outcome measures in these studies.

RESULTS: Out of 416 studies screened only seven studies were considered suitable for inclusion. All seven studies consistently reported an inverse association of bilirubin with NAFLD despite the heterogeneous sample of studies. Only two studies were prospective. No negative studies were found.

CONCLUSION: Most studies suggest a correlation between high bilirubin levels of any type are inversely correlated with NAFLD. But to date most of these studies have been poorly designed to allow meaningful conclusions, except one cohort study. There is a need for a large prospective cohort study in multiple populations to test this hypothesis fully before mechanistic associations can be established and therapeutic options of the apparent anti-oxidant effect of bilirubin be explored in NAFLD. Furthermore these studies should include analysis of UGT1A1 gene to expound upon underlying cause of unconjugated hyperbilirubinaemia.

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Key words: Review; Systematic; Bilirubin; Hyperbilirubinemia; Anti-oxidants; Adult; Non-alcoholic fatty liver disease

Core tip: This systematic review summarises and highlights the deficiencies in the current studies on the association of serum bilirubin with non-alcoholic fatty liver disease (NAFLD). It explores the potential underpinning of the mechanistic association of NAFLD with bilirubin. Potential novel therapeutic avenues of bilirubin are explored in NAFLD, a common condition with oxidative damage as a core pathogenetic factor. Although this area of study is still in its infancy, this review is a timely summary of current key studies in this subject area and provides an up to date thought perspective with focus on future direction and potential therapeutic application.

Anwar MS, Dillon JF, Miller MH. Association of serum bilirubin and non-alcoholic fatty liver disease: A feasible therapeutic avenue? *World J Pharmacol* 2014; 3(4): 209-216 Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i4/209.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i4.209

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been

recognised as the most prevalent liver disease, with current estimations that it affects around 20%-30% of the general population in the western world[1,2]. NAFLD is considered to be the hepatic manifestation of the metabolic syndrome as it is closely related to insulin resistance, obesity, hypercholesterolemia, type 2 diabetes, and coronary artery disease[3-5]. NAFLD is composed of a histological spectrum of hepatic dysfunctions ranging from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma[6]. The pathogenic processes underpinning NAFLD remain unclear, although oxidative stress, fat transportation and inflammation are implicated. Furthermore oxidative stress has been suggested as an aetiopathogenic mechanism in NAFLD[7,8]. Additionally, mounting evidence suggests a link between serum ferritin, insulin resistance, and NAFLD[9,10]. Excessive hepatic iron accumulation in NAFLD is likely one of the potential cofactors involved in the enhanced oxidative stress, which triggers liver cell necrosis and activation of hepatic stellate cells, both leading to fibrosis[11]. The significance of the association serum ferritin in NAFLD may relate to heme catabolism and the anti-oxidant state of play, which will be discussed later. Studies involving administration of a free radical-generating azo compound to mice or rats induced fat accumulation in the liver by increasing triacylglycerol and decreasing phospholipids. Likewise fat accumulation in the liver was suppressed through the simultaneous administration of free radical-scavenging antioxidants such as Vitamin E, Therefore antioxidant agents have been proposed as an effective treatment[12-15].

Bilirubin, the end product of heme catabolism, is known to be a potent physiological antioxidant cytoprotectant due to its inhibitory effect on the activity of NAD(P)H oxidase. In addition it scavenges peroxyl radicals, hydroxyl radicals, and reactive nitrogen species preventing oxidation of intracellular lipids[16-20]. Bilirubin has also been proposed as having an anti-inflammatory role and has major anti-fibrogenic properties *via* heme oxygenase-1 (HO-1)[21-23]. Previous studies have shown that unconjugated hyperbilirubinemia is inversely associated with ischaemic heart disease, carotid stenosis, insulin resistance, diabetes, vascular complication of diabetes, peripheral vascular disease and even cancer[24-29]. Furthermore there is strong clinical evidence for the beneficial cytoprotective effects of unconjugated bilirubin as observed in Gilbert's syndrome[28-29].

Consequently, it can be hypothesised that elevated serum bilirubin levels reduce oxidative stress, decrease fibrosis and inflammation, and decrease the risk of NAFLD development and progression. If this hypothesis is confirmed then therapeutic options of inducing "iatrogenic" Gilbert's syndrome would be a key area of research. This systematic review evaluates the studies carried out to date to assess the reported association between bilirubin and NAFLD.

MATERIALS AND METHODS

Data sources

This systematic review included studies published in electronic databases over the time period ranging from their inception to January 2013. We searched two main stream public-domain data bases, PubMed and Web of Knowledge. Three categories were devised (1) conditions (SS, NASH, NAFLD, FLD); (2) bilirubin (unconjugated hyperbilirubinemia, bilirubin, anti-oxidant, protective marker); and (3) subjects (human). Each possible combination was searched in the above two databases, also the bibliographies of relevant systematic reviews were manually searched.

Study selection

We included all types of studies, which investigated the relationship between bilirubin and NAFLD. Paediatric studies were excluded due to the potential alternate pathophysiology in this category. Outcome measure for each study was bilirubin, but it should be noted there are three methods of reporting bilirubin. Total bilirubin which consists of direct (conjugated) and indirect (unconjugated) bilirubin. Each study focused on one of these during statistical analysis.

Statistical analysis

Each selected study was assessed independently by two reviewers for methodology, outcome measures, results, limitations, risks of bias. Data synthesis with further meta-analysis was not possible due to the heterogeneity of outcome measures, study designs and statistical analysis in each study. Therefore instead we have provided a summary of this for each study.

RESULTS

Study selection, characteristics and analysis

We considered 416 potentially relevant articles, after screening the abstracts and titles, 407 studies were excluded, (Figure 1). Nine articles were fully evaluated, with a further two excluded. First of these articles was an editorial rather than an original study[30] and the second article was not relevant, it discussed measures of oxidation stress in NASH[31]. Of the included studies, three were cross sectional, two retrospective and two prospective. Two of these studies had a large sample size and were conducted in South Korea[32,33]. The remaining five small sized studies used liver biopsy[34-38] to diagnose NAFLD but notably this does not include every patient in the study. Also only two studies[37,38] specifically states blinding of the pathologist to biochemical results and intention of study. All studies excluded patients using alcohol > 20 g/d, screened for viral hepatitis, alternative liver pathology and haemolysis, except the Tarantino[38] study which did not assess patient for possible haemolysis. The characteristics of each study are shown in Table 1. Due to the heterogeneity of these studies, further pooled analysis could not be carried out and therefore a summary of key results is provided instead in Table 2. Notably no study reported an insignificant association of bilirubin with NAFLD.

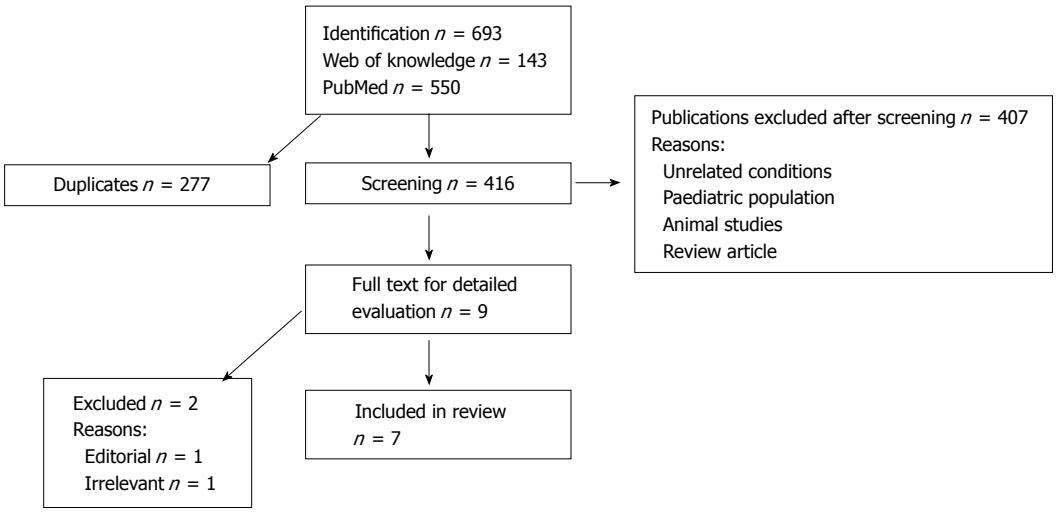

Figure 1 Flow chart describing the selection process for publication to be included in this review.

Of these studies, two were prospective[32,33] and one[32] of these was a large cohort study of young Korean men (5900) and showed all types of raised bilirubin were inversely associated with developing NAFLD but focused on conjugated hyperbilirubinaemia. In this study multivariate model analysis showed only conjugated hyperbilirubinaemia as independently association with risk of developing NAFLD. The study adjusted for confounding factors such as age, body mass index, current smoking, alcohol intake, exercise, diabetes mellitus, history of cardiovascular disease and history of malignancy, high density lipoprotein cholesterol, triglycerides, glucose, insulin, and uric acid.

The second[33] large sample study was also carried out in South Korea and showed an inverse association between total bilirubin and NAFLD. But due to the cross-sectional design of this study a causal relationship cannot be confirmed. These large sample studies confirmed NAFLD on the basis of typical ultrasound findings instead of a liver biopsy. This limitation is essentially unavoidable in such a large sample size given the morbidity and mortality associated with this procedure.

The smaller studies which were based at tertiary centres did carry out liver biopsies albeit not for every patient. Duseja[34] carried out the first study looking into association of hyperbilirubinaemia and NASH. This was a prospective study consisting of only 67 subjects and therefore did not show a statistically significant association between hyperbilirubinaemia and NASH. Given the small sample size of this study, it cannot be considered sufficient to have shown negative or positive correlation between hyperbilirubinemia and NASH. Furthermore the setting and criterion for patient selection is not adequately defined.

Hjelkrem *et al*[35] and Kumar *et al*[36] suggested that degree of fibrosis also appears to be related to bilirubin levels alongside NAFLD development and progression, although neither study blinded the pathologist to study intent. Interestingly although 508 patients had liver biopsy in the Hjelkrem *et al*[35] study including only thirty five who had elevated unconjugated bilirubin, their statistical analysis did not suggest any association between NAFLD severity and unconjugated bilirubin but this is probably due to small sample of elevated unconjugated bilirubin patients in this group. Kumar *et al*[36] reported a negative correlation between serum unconjugated levels and histopathological NAS score, stages of fibrosis on the basis of biopsy results from 42 patients in total. Given the sample size and study design of these two studies, these results can only be regarded as speculative at this stage.

Chisholm *et al*[37] carried out a study in bariatric patients prior to surgery for evaluation of markers predictive of steatohepatitis, they devised a ROC curve for prediction curve of NASH which included 3 variables: total bilirubin, ALT and HOMA-IR (Homeostasis model of assessment-insulin resistance). Of 370 patients in this study 275 had a liver biopsy, with the pathologist blinded to the intent of the study but a cohort of patients were also given very low calorie diet to study the influence of this on histology findings. Unfortunately insufficient data is given to infer any association between degree of fibrosis and bilirubin level.

Tarantino *et al*[38] study analysed hepatocytes after exposing them to free fatty acids with expected mitochondrial damage for the presence of anti-oxidant substances such as cytochrome c, gamma-glutamyl transferase, triglycerides and unconjugated bilirubin in patients with NAFLD of differing severity (n = 186) and controls (n = 27). The study showed unconjugated bilirubin to be elevated in all spectra of NAFLD, except in healthy controls (P = 0.008). The study authors felt the elevated unconjugated bilirubin represented anti-oxidant effect in this setting and unconjugated bilirubin could be used to monitor response to disease modifying treatment. Interestingly the level of unconjugated bilirubin was the highest in NASH subgroup (n = 44) with the most severe histological findings, contradicting all previous studies. All previous studies had suggested unconjugated bilirubin was protective

Table 1 Summary of characteristics of studies on the association of bilirubin with non-alcoholic fatty liver disease

Ref.	Study design Total number of patients	Study population	Main outcome measure
Chang *et al*[32]	Cohort-longitudinal *n* = 5900	Korean men from a single large semiconductor company (South Korea)	Conjugated bilirubin
Kwak *et al*[33]	Cross-section *n* = 17348	Health check-general Population (South Korea)	Total bilirubin
Duseja *et al*[34]	Prospective *n* = 67	Hospital setting unclear, probably Tertiary (India)	Unconjugated bilirubin
Hjelkrem *et al*[35]	Retrospective *n* = 641	Tertiary hospital patients undergoing liver biopsy (United States)	Unconjugated bilirubin
Kumar *et al*[36]	Cross-sectional *n* = 204	Tertiary hospital, outpatient NAFLD clinic (India)	Unconjugated bilirubin
Chisholm *et al*[37]	Cross-sectional *n* = 370	Liver biopsy prior to bariatric surgery (United States)	Total bilirubin
Tarantino *et al*[38]	Cross-sectional *n* = 186	Tertiary hospital, outpatient likely liver clinic (Italy)	Unconjugated bilirubin

NAFLD: Non-alcoholic fatty liver disease.

Table 2 Results and analysis summary of the studies on the association of bilirubin with non-alcoholic fatty liver disease

Ref.	Study analysis	Results
Chang *et al*[32]	(+) Large sample (+) Prospective (-) NAFLD on US	Hazard ratio for NAFLD comparing the highest to the lower quartile of serum conjugated bilirubin = 0.61 (95%CI: 0.54-0.68), after adjusting metabolic parameters 0.86[a] (95%CI : 0.76-0.98)
Kwak *et al*[33]	(+) Large sample (-) NAFLD on US	Total bilirubin inversely associated with NAFLD OR = 0.88, 95%CI: 0.80-0.97. An inverse, dose-dependent association between NAFLD and serum total bilirubin levels OR = 0.80[b], 95%CI: 0.71-0.90 in the fourth quartile *vs* lowest quartile
Duseja *et al*[34]	(-) Small sample (+) Prospective (+) NASH on biopsy (selected)	Patient sample too small to make any meaningful statistical inference
Hjelkrem *et al*[35]	(-)Retrospective design (-) Small sample (+) NAFLD on biopsy	Unconjugated hyperbilirubinaemia inversely associated with NASH OR = 16.1[b], 95%CI: 3.7-70.8
Kumar *et al*[36]	(-) Small sample (+/-) NAFLD on biopsy but not all patients	Unconjugated hyperbilirubinaemia (UCHB) and NAS score-negative correlation: *r*: -0.48[b]. UCHB and stage of fibrosis: *r*: -0.28[d]
Chisholm *et al*[37]	(-) Small sample (+) NAFLD on biopsy	Binary logistic regression showed independent association of total bilirubin with NASH [c]*P* = 0.016
Tarantino *et al*[38]	(-) Retrospective (-) Small sample (+) NASH on biopsy (selected)	Unconjugated bilirubin elevated in all spectra of NAFLD, except healthy control [f]*P* = 0.008 High UCHB in advanced NASH group

[a]*P* < 0.039; [b]*P* < 0.001; [d]*P* = 0.007; [c]*P* = 0.016; [f]*P* = 0.008. (+) favourable design; (-) unfavourable design; OR: odds ration; US: ultrasound; r: relative coefficient; P: Probability; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

for NASH and less commonly elevated in the advanced cohort of NASH. It is worth noting that the study did not demonstrate cytochrome c to be elevated as expected which contradicts multiple previous studies and notably did not check for underlying haemolysis in patient cohort either therefore a cautious approach should be taken with all results of this study.

DISCUSSION

Despite the heterogeneous nature of studies addressing the association of bilirubin with NAFLD including large variation in sample size and specific bilirubin type (s) measured there was consistent inverse association of raised total bilirubin, conjugated and uncojugated hyperbilirubinemia with NAFLD, across populations in Asia and America. Although Tarantino *et al*[38] data contradicted these findings, the poor study design and failure to demonstrate other expected findings make result interpretation from this study highly speculative. But given the sample size and study design variation of the remaining studies, these results can only be regarded as showing a tentative association with disease reduction but combined with a plausible biological mechanism, they raise the intriguing possibility of a causative relationship.

This review is limited due to the small number of studies on this topic and the heterogeneity of these studies. Given this pooled analysis could not be carried out.

Despite the shortcoming of the studies in this review, there is a consistent reported inverse association between high bilirubin and NAFLD, except in one study, which had very small sample size, and therefore no statistical inference can be attained from this study. Notably to date most studies have suggested undiagnosed Gilbert's syndrome as the cause of unconjugated hyperbilirubinemia although none of the studies have validated this with analysis of UGT1A1 genetic mutation. Although Gilbert's syndrome may account for some of these patients, another possibility is that unconjugated hyperbilirubinemia may be an initial acquired response to oxidative stress and thus represents the liver's intrinsic anti-oxidative capacity, but is not sustainable due to repeated insults. Notably Gilbert's syndrome is more commonly diagnosed in men and sex steroids may influence bilirubin metabolism with higher production in men[39]. Two studies included in this review[32,36] showed higher preponderance of men with unconjugated hyperbilirubinemia. Therefore future studies are required to verify the inverse correlation of bilirubin to NAFLD in large, prospective cohort study in multiple populations along with the analysis of *UGT1A1* gene to expound upon underlying cause of unconjugated hyperbilirubinaemia in this cohort.

Although a direct effect of bilirubin maybe likely, other parts of the heme catabolism such as the effect on iron homeostasis may also be relevant. Heme catabolism represents a key function in mobilising macrophage iron derived from ingested erythrocytes. Importantly, the storage and processing of iron from erythrophagocytosis by macrophages within plaque appear to play a vital role in plaque progression[40]. Accordingly, it has been demonstrated that erythrocytes induce plaque vulnerability in a dose-dependent manner in a rabbit model of intra-plaque haemorrhage[41]. Furthermore, it has been found that the effect of HO-1 on iron homeostasis within macrophages may represent a new tool to prevent foam cell formation and atherosclerotic lesion progression. A protective effect of iron depletion that may have multiple beneficial consequences is decreased availability of redox-active iron *in vivo*. The amount of free iron available at sites of oxidative or inflammatory injury appears to be a function of the stored iron level. Indeed, a recent study found that the cytoprotective effect of HO-1 induction or forced expression (which usually leads to concomitant elevated serum bilirubin level) may derive from temporary elevated expression of ferritin, and consequent reduction of redox-active iron[42]. Alongside this, there is accumulating evidence to suggest that excessive hepatic iron accumulation in NAFLD is one of the potential cofactors involved in the enhanced oxidative stress, which triggers liver cell necrosis and eventually fibrosis[9-11]. There are potentially multiple mechanistic underpinnings for the beneficial association of bilirubin with NAFLD.

The development of antioxidant therapeutics is gaining prominence as the pathophysiological foundation of oxidative stress in cardiovascular disease, neoplasia and NAFLD is better understood[43]. Significant numbers of studies have focused on using vitamin based antioxidant therapy to assess prevention of cardiovascular or neoplastic disease. Despite replicable *in vitro* evidence to support antioxidant vitamin use, this has poor correlation with *in vivo* subjects showing conflicting results at best to date. Further ongoing large randomised controlled trials results are awaited[44,45]. This disparity is likely due to any exogenous vitamin antioxidants' inability to meaningfully influence intracellular antioxidant levels[46].

Bilirubin is recognised to have antioxidant activity so inducing "iatrogenic Gilbert's syndrome" is another strategy for advancing antioxidant therapeutics which involves using drugs that promote the unconjugated hyperbilirubinaemic state. This strategy may overcome the difficulties of achieving sufficient antioxidants at intracellular level as bilirubin's main antioxidant action appears to act not as a direct radical scavenger given its low concentration in tissue. But instead as a potent and specific inhibitor of the membrane bound NADPH oxidase, a key source oxidants in both phagocytic and non-phagocytic cells[47]. Probenecid, a uricosuric agent is known to decrease hepatic glucuronidation activity, leading to hyperbilirubinaemia which has been observed only in multiple case reports but not formally evaluated in studies in dose-dependent manner[47,48]. It is known to be well tolerated but decreasing glucuronidation activity will increase half life of commonly used medication such as paracetamol and lorazepam and thus may hinder the application of this drug and strategy[49,50]. Rifampicin could be another possible candidate, it causes hyperbilirubinaemia by inhibiting the transporter which accelerates hepatocyte uptake of bilirubin[51-53]. But rifampicin can cause significant side effects which include liver failure although in a small proportion of patients, this would entail closer monitoring. Sodium valproate has a similar mechanism of action to rifampicin but due to many side effects and poor tolerability would be unsuitable for this application.

Oral administration of bilirubin or its precursor biliverdin which is more soluble is another possible avenue but this strategy is limited by the lack of evidence showing increased bilirubin levels after oral administration other than in mice and rat models[41,54]. Even if clear evidence was obtained the mass production of bilirubin is complex and costly thus would be a major obstacle to overcome[55]. Phycobilins that are structural analogues of biliverdin are produced readily by plants, algae and cynobacteia which may provide feasible alternatives to bilirubin[56]. Both in animal models and *in vitro* studies phycobilins have been shown to have comparable effect to bilirubin[57,58]. Further research in this area with human subjects is awaited.

To conclude once the protective association between uncojugated hyperbilirubinaemia and NAFLD is verified. The focus of preventing NAFLD progression and development should consider bilirubin induction therapy randomised double blind controlled trials to assess the clinical value of this treatment. Thereafter it remains to be shown how popular this treatment would be as patient

will be icteric. Although equally with weight reduction as the current mainstay of NAFLD prevention, this strategy has its own limitations and therefore optimal treatment options which are acceptable to patients continues to be a key challenge in this area.

COMMENTS

Background

Obesity is a growing epidemic in the western world and if it is not controlled and reversed it is associated with type 2 diabetes, hypercholesterolaemia and coronary artery disease. The hepatic manifestation of this multi-organ disease is non-alcoholic fatty liver disease (NAFLD). This hepatic dysfunction can eventually lead to cirrhosis and hepatocellular carcinoma. At present there is no effective treatment available and the key current management is prevention by weight reduction. High levels of bilirubin (hyperbilirubinaemia), which is naturally occurring anti-oxidant in the human body has been shown to prevent progression of NAFLD in some studies. This paper evaluates all studies in relation to this, to the look at the current strength of evidence and the potential application of anti-oxidants in this setting.

Research frontiers

Preventing NAFLD progression is a key area of research, inducing hyperbilirubinaemia represents a potential solution. Laboratory based research as well as on animal models has shown high levels of bilirubin to have protective and preventative effect on NAFLD and other diseases processes related to obesity. But this relationship needs to be substantiated in human subjects.

Innovations and breakthroughs

Although most studies evaluated in this review suggest there is likely a protective association between high bilirubin levels and NAFLD progression and prevention. The poor design of these studies is prohibitive to allow any meaningful conclusion. There is a need for good quality large prospective cohort study in multiple populations to test this hypothesis fully, which should also included analysis of UGT1A1 gene to expound upon underlying cause of unconjugated hyperbilirubinaemia.

Applications

This review allows readers to appreciate and evaluate current progress in this area of research. The case for protective association of hyperbilirubinaemia with NAFLD needs to be corroborated with good quality studies. They are multiple drug options available to induce hyperbilirubinaemia but these drugs need to be tested in randomised controlled trial setting.

Terminology

Bilirubin is a naturally occuring substance in the blood which comes from break down of red blood cells. It is an anti-oxidant (protects cells again harmful substances) and appears to also have an anti-inflammatory effect. High level of bilirubin gives the jaundiced appearance.

Peer review

It is a good review article, the authors provide updates of the fatty liver diseases with a Q and A format for easy reads.

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P- Reviewer: Ahmed M, Mascitelli L, Oeda S, Shen WJ, Tamano M **S- Editor**: Song XX **L- Editor**: A **E- Editor**: Lu YJ

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