The Effects of Traditional Chinese Medicine on P-Glycoprotein Mediated Multidrug Resistance and Approaches for Studying the Herb–P-Glycoprotein Interactions

Yuhong Cao[1], Yiwei Shi[1], Ying Cai[1,2], Zhanying Hong[1]*, Yifeng Chai[1]

[1] School of Pharmacy, Second Military Medical University, Shanghai Key Laboratory for Pharmaceutical (Chinese Materia Medica) Metabolites Research, Shanghai 200433, China

[2] School of Pharmacy, Fujian University of Traditional Chinese Medicine, Fuzhou 350122, China

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* Corresponding author:

Zhanying Hong, Professor

Tel: 86-21-81871269 (Zhanying Hong)

E-mail address: hongzhy001@163.com

School of Pharmacy, Second Military Medical University, Shanghai Key Laboratory for Pharmaceutical (Chinese Materia Medica) Metabolites Research, Shanghai 200433, China

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ABBREVIATIONS:

A2780/T	trabectedin-resistant ovarian carcinoma cell
A549	human non-small cell lung cancer cell
ABC	ATP-binding cassette
ADR	adriamycin
BBB	Blood-Brain Barrier
B-CSF	Blood-Cerebrospinal Fluid
BTB	Blood-Testis Barrier
Caco-2	human colon cancer cell
CEM	human leukemia cell line
CR	Coptidis Rhizoma
CYP1A1	cytochrome P450, family 1, members A1
EGCG	Epigallocatechin-3-gallate
GQ	7-O-geranylquercetin
GSK 3β	Glycogen synthase kinase 3β
HeLaS3	human cervical cancer cell
HepG2	liver cancer cell
IC50	concentration which inhibits cell viability by 50%
K562	human erythroleukemia cell
KB	human mouth epidermal carcinoma
KB-C2	multidrug-resistant subclone human mouth epidermoid

LC-MS/MS	liquid chromatography tandem mass spectrometry
LS180	human colon carcinoma cell
MAPK/ERK	mitogen-activated protein kinase/ extracellular
MCF7	human breast adenocarcinoma cell line
MDCK-MDR1	MDR1-transfected Madin–Darby canine kidney cell
MDR	multidrug resistance
MDA-MB-231/MDR1	drug-induced resistant human breast cancer cell
MRP	Multidrug Resistance associate Protein
MSI	Mass spectrometry imaging
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NBD	nucleotide-binding domain
NCI/ADR-RES	adriamycin-resistant ovarian cancer cell
NF-κB	Nuclear factor-κB
P_{app} (AP-BL)	the apparent permeability coefficient of apical to
P_{app} (BL-AP)	the apparent permeability coefficient of basolateral to
P-gp	P-Glycoprotein
PI3K/AKT	phosphatidylinositol 3-kinase/ protein kinase B
PTX	paclitaxel
Rh123	rhodamine 123
RT-PCR	reverse transcription-polymerase chain reaction
TCM	traditional Chinese medicine
TMD	transmembrane domain
VCR	vincristine

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ABSTRACT

As a member of anATP-dependent membrane transport proteins, P-Glycoprotein (P-gp) is known to pump substrates out of cells in the ATP-dependent mechanism. The over-expression of P-gp in tumor cells reduces the intracellular drug concentrations, which decreases the efficacy of extensive antitumor drugs and leads to multidrug resistance (MDR) clinically. The combination of anti-cancer drugs with P-gp inhibitor has been an attractive and promising strategy to reverse MDR in cancer treatment. However, non-specific or non-selective distribution of P-gp inhibitors to non-target organs is one of the most fatal shortcomings in clinical application. Thus, there is an urgent need for effective and non-toxic MDR reversal agents, particularly in P-gp-mediated MDR. Traditional Chinese medicine (TCM) natural products may provide less toxic for use in P-gp inhibition to promote MDR reversal. P-gp modulatory effects have been previously demonstrated using selected TCM, including the flavonoids, alkaloids, terpenoids, coumarins, and quinonoids compounds, and some Chinese medicine extracts. Moreover, the approaches for screening active components from TCM are necessary and facing more challenges. At present, the approaches to study the interaction between TCM and P-gp are divided into *in vitro*, *in vivo* and *in silico* methods. This review will provide an overview and update on the role of TCM in overcoming P-gp mediated MDR and the approaches to study the interaction between TCM and P-gp.

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Significance statement: This review summarized some traditional Chinese medicine identified to have a modulatory effect towards P-gp, including flavonoids, alkaloids, terpenoids, coumarins, quinonoids compounds and some Chinese medicine extracts, and introduced the possible mechanisms. The approaches to study the interaction between TCM and P-gp are divided into *in vitro*, *in vivo*, and *in silico* methods.

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Introduction

Molecular Structure of Human P-glycoprotein (P-gp). P-gp is the 170-kD

protein product of the human gene *MDR1* (Gottesman MM and Pastan I, 1993;

Schinkel AH, 1997). P-gp is an ATP-binding cassette (ABC) transporter, using the

energy from ATP hydrolysis to pump substrates across the membrane. It consists of

two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding

domains (NBDs). P-gp is comprised of 1280 amino acids divided into two

symmetrical halves with 43% sequence homology between the two halves (Chen et al.,

1986). Each half contains six TMD that are separated by a sequence of 75 amino acids

that connects the C-terminal TMD with the N-terminal ATP binding domain (Shustik

C et al., 1995) (Fig.1).

Physiological Function of P-gp. P-gp is located in many physiological barriers,

such as Blood-Cerebrospinal Fluid (B-CSF), Blood-Brain Barrier (BBB), and

Blood-Testis Barrier (BTB). P-gp plays a vital role in regulating the absorption and

the efflux of various exogenous substances. P-gp is also present in multiple tissues

and organs, including gut, kidney, liver, and placenta. Therefore, its activity is highly

significant to the metabolism of numerous drugs (Hussaarts K et al., 2019), and the

behavior of drug absorption will be influenced significantly through co-administered

with P-gp inhibitor or inducer. Specifically, P-gp inhibitor may increase the

bioavailability of drugs, while the inducer may reduce the drug absorption.

Models of P-gp-mediated Substrates Efflux. Drug resistance mediated by P-gp

depends on ATP hydrolysis, and the ATPase activity of P-gp is stimulated by the

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transported drugs (Mollazadeh S et al., 2018). P-gp inhibitors or substrates can enter

the cell from extracellular compartment to cytosol via filtration, simple diffusion, or

specialized transport. The first step of substrates efflux is the combination of P-gp and

the substrates promoted by ATP binding and hydrolysis. Vanadate trapping and

photo-cleavage experiments showed that P-gp contains two active ATPase sites, but

only one ATP is hydrolyzed at a time (Hrycyna CA et al., 1998). The hydrophobic

'vacuum cleaner' model and the flippase model have been proposed to describe

P-gp-mediated drug translocation (Binkhathlan Z and Lavasanifar A, 2013). In the

hydrophobic 'vacuum cleaner' model, P-gp pulls its substrates from the lipid bilayer

and pumps them out of the cell. The flippase model hypothesizes that P-gp 'scans' the

inner leaflet of the lipid bilayer and binds specific lipids and hydrophobic drugs

before their extrusion by 'flipping' the phospholipids from the inner to outer leaflets

of the lipid bilayer. The flippase model and the hydrophobic 'vacuum cleaner' model

are not mutually exclusive (O'Brien FE et al., 2012), and the combination of the two

models is illustrated (Fig. 2).

Multidrug Resistance (MDR) and the Mechanisms Mediated by P-gp. MDR

is common due to the overexpression of transporters on the tumor cell membranes.

Several transporters belonging to the ABC pump family, such as P-gp and BRCP, are

overexpressed in resistant tumor cells compared with normal cells (Robey RW et al.,

2018). This induced membrane state determines the efflux of chemotherapeutic agents

through the process where they are captured into the bilayer by the transporter and

then effluxed out of the cells. Consequently, the efficacy of antitumor therapy is

lowered due to the insufficient drug concentration inside the tumor cells.

Role of P-gp in Cancer MDR. P-gp was first identified in the plasma membrane of mammalian cells that had been selected for resistance to drugs (Gottesman MM and Ling V, 2006; Sharom FJ, 2011). P-gp can intercept drugs before they reach their specific target in the cell by facilitating drug efflux and promote MDR. Hence, it is always selected as the target for MDR reversal agents. The past three decades has witnessed the rapid development of strategies to reverse P-gp-mediated MDR. Many small molecular compounds were identified as P-gp inhibitors and can reverse P-gp-mediated MDR (Dong et al., 2020; Leopoldo M et al., 2019). Many anticancer agents are P-gp substrates, such as vinca alkaloids (Gherbovet O et al., 2016), anthracyclines, and taxanes (Nguyen PH et al., 2020; Li et al., 2019; Xie et al., 2020). The P-gp transport of anticancer agents can be inhibited with a competitive or non-competitive mechanism of some small-molecule reversal agents. These reversal agents are co-administered with an anticancer drug to reduce its efflux, thus increasing its efficiency. Some compounds have shown promising experimental results *in vitro*. However, at present, there is no compound being approved for clinical use as reversal agents (Kumar A and Jaitak V, 2019). Studies of certain reversal agents were terminated as a result of unacceptable toxicities in clinical trials or insufficient efficacy (Varma MV et al., 2003). Consequently, there remains a great challenge in searching for effective and less toxic agents to reverse tumor MDR.

Development of P-gp inhibitors in MDR modulation

To date, plenty of P-gp inhibitors have been found to modulate MDR activity in

clinical trials. Based on their affinity with P-gp and different side effects, these inhibitors could be classified into first, second, and third-generation modulators. The first-generation modulators had unacceptable toxicities in clinical trials (Varma MV et al., 2003). The second-generation modulators are inhibitors with higher affinity, efficacy, lower toxicity, and absence of intrinsic pharmacological activity, belonging to the analogs of the first generation. Most of them can be metabolized by cytochrome P450 (CYP450) enzymes family and interact with chemotherapeutic drugs. When used together with anticancer drugs, their metabolism or interaction with the drugs lead to their unacceptable toxicity and the treatment failure in turn (Kathawala RJ et al., 2015). The third generation is more specific and effective in reducing pharmacokinetic interactions compared to the second generation (Mistry P et al., 2001); some have been reported to increase the sensitivity of anticancer drugs in preclinical trials. However, most of these inhibitors are still underperformed in clinical trials because of diverse reasons such as high toxicity. Examples of first, second, and third-generation P-gp inhibitors are shown in Table 1.

Although progress has been made in the field of MDR, a suitable P-gp inhibitor with significant MDR reversal and acceptable toxicity has yet to be identified. The main reasons are summarized as follows: firstly, P-gp is a complex protein owing to its plastic conformational (Ma et al., 2018) and the existing inhibitors are non-specific for P-gp. Besides, some inhibitors may interact with chemotherapeutic drugs when co-administrated and may influence the integrity and functions of the brain. Furthermore, there lacks enough preclinical data and appropriate animal models to

evaluate the efficiency of P-gp inhibitors.

Traditional Chinese Medicine (TCM) Modulation of P-gp

At present, researchers have performed intensive efforts in the area of identifying MDR modulators from TCM. Various naturally originated compounds and plant extracts were reported for their modulation effect of MDR (Li et al., 2014; Abdallah et al., 2015). This review focuses on major classes of TCM including flavonoids, alkaloids, coumarins, terpenoids, and quinonoids, and other TCM extracts. In this section, TCM that have an MDR modulation effect would be discussed. These compounds could inhibit or decrease the activity of P-gp (Table 2).

Flavonoids. Flavonoids are ubiquitously existed in many vegetables and herbs and are closely related to a series of human life benefits. Current research has demonstrated that flavonoids have various pharmacological properties, including antioxidant, anti-inflammatory, anticancer, antifungal, and antiviral activity (Karabin M et al., 2015). Flavonoids have also been reported to inhibit ABC transporters that contribute to the development of MDR.

A few flavonoids, including naringin(Zhu et al., 2018) and taxifolin (Chen et al., 2018) have been identified as substrates of P-gp. The cellular uptake of 40 flavonoids were measured in parental human mouth epidermal carcinoma (KB) cells and KB/MDR cells with or without elacridar. Molecular docking was also performed to investigate the structure affinity relationship between P-gp and the flavonoids. The results indicated that 3-OH, 5-OH, 3'-OCH3, and 4'-OCH3 are crucial for the interplay between flavonoids and P-gp (Fang et al., 2019). Epigallocatechin-3-gallate

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(EGCG) could downregulate the expression level of P-gp and BCRP but could not affect the expression of MDR associate Protein 1(MRP1) in adriamycin (ADR)-resistant human leukemia (CEM/ADR) cell (Li et al., 2018). 7-O-geranylquercetin (GQ), a derivative of quercetin, could reverse drug resistance of ADR-resistant human breast adenocarcinoma (MCF-7/ADR) cells. GQ inhibited the efflux of ADR by down-regulating the expression of P-gp protein and its encoding *MDR1* gene in MCF-7/ADR cells (Zhang et al., 2019). Silychristin A modulate MDR by the direct inhibition of P-gp, while anhydrosilychristin and isosilychristin modulate MDR by downregulating the expression of P-gp (Viktorova J et al., 2019). Chen et al. (2018) evaluated the effects of some natural flavonoids on P-gp activity, including taxifolin, luteolin, (-)-gallocatechin, and (-)-catechin. They found taxifolin could significantly resensitize MDR cancer cells in combination with chemotherapeutic agents and enhance the efficacy. This result suggested that taxifolin could be considered as a potential P-gp modulator for the synergistic treatment of MDR cancers. Saeed M et al. found apigenin could not only inhibit the activity of P-gp but also inhibit the activity of BCRP by increasing cellular uptake of ADR and synergistic inhibition of cell viability in combination with ADR in MDR cells (Saeed M et al. 2015). The molecular docking experiment indicated that apigenin could bind to the NDBs of P-gp, which suggested that apigenin may compete with ATP on NDBs and lead to energy depletion to fuel the transport of P-gp substrates. Curcumin has been described to inhibit both the function of P-gp and the expression of P-gp (Lopes-Rodrigues V et al., 2016). Glabridin can increase the accumulation of ADR in

drug-induced resistant human breast cancer (MDA-MB-231/MDR1) cells by suppressing the expression of P-gp and competitively inhibiting the P-gp efflux pump and enhance the apoptosis of MDA-MB-231/MDR1 cells induced by ADR, and thus realize reversal effects on MDR. Therefore, the combination therapy of anticancer drugs and glabridin is a promising strategy to overcome P-gp-mediated MDR (Qian et al., 2019).

Some flavonoids have been reported to possess significant P-gp inhibitory activity via diverse mechanisms. Mohana S et al. (2018) studied the interplay between flavonoids (theaflavin, quercetin, rutin, epicatechin 3 gallate, and tamarixetin) and Glycogen synthase kinase 3β (GSK 3β) by performing molecular docking. Curcumin could enhance anti-cancer efficacy through the ablation of Nuclear factor-κB (NF-κB), Wnt/b-catenin pathways, as well as mammalian target of rapamycin (mTOR) signaling (Mohana et al., 2018). Dihydromyricetin, a dihydroflavonol compound with anti-inflamatory, anti-oxidant, anti-bacterial and anti-tumor actions, could reverses MDR in MCF-7/ADR and K562/ADR cell lines. It enhanced the cytotoxicity of ADR by downregulating *MDR1* mRNA and P-gp expression through MAPK/ERK pathway and inhibiting the function of P-gp significantly (Sun et al., 2018).

Alkaloids. Alkaloids are a group of naturally originated chemicals containing one or more basic nitrogen atoms and are classified into different groups based on the amino acid they are derived from. Many reports have revealed the ability of alkaloids to inhibit P-gp. The structural analysis of alkaloids suggested the P-gp inhibitory function is due to the presence of (A) a basic nitrogen atom and (B) two planar

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aromatic rings. Alkaloids have been reported to inhibit the function of P-gp via diverse mechanisms.

Amongst various alkaloids investigated in *in vitro* experiments as P-gp inhibitors or MDR reversal agents, a natural alkaloid called CBT-01® (tetrandrine) has already been tested on clinical trials. Tetrandrine can antagonize MDR in both drug-induced and *MDR1* gene-transfected cancer cells by downregulating the expression of P-gp, followed by increasing the intracellular concentration of chemotherapeutic agents. The combinational therapy using tetrandrine and other anticancer drugs could promote the treatment efficiency of drugs that are substrates of P-gp (Liao et al., 2019). Tetrandrine and fangchinoline, isolated from Stephania tetrandra, could significantly reduce the expression level of P-gp expression in a concentration-dependent manner. Tetrandrine and fangchinoline showed a significant synergistic cytotoxic effect in MDR human colon cancer cells (Caco-2) and CEM/ADR cancer cells in combination with ADR (Sun and Wink M, 2014).

Besides, there exists large number of natural alkaloids possessing potent inhibition of the P-gp responsible for the development of resistance (Joshi P et al., 2017). Chelidonine, isolated from Chelidonium majus, could also inhibit the function of P-gp and consequently upregulate the xenobiotic metabolism genes *CYP1A1* and *MDR1* (Herrmann R et al., 2018). Matrine, isolated from Sophora flavescens, is another quinolizidine alkaloid that has inhibition effect of P-gp. Zhou et al. (2018) reported that matrine could inhibit MCF-7/ADR cell growth, induce cell apoptosis, and reverse MDR for breast cancer cells through the mediation of downstream

apoptosis factors of phosphatidylinositol 3-kinase/ protein kinase B (PI3K/AKT) signaling pathway by decreasing the cell phosphorylation level of AKT.

Terpenoids. Terpenoid is one of the most extensively studied and structurally diverse classes of TCM. Based on the number of isoprene units in the parent structure, terpenoids can be classified as monoterpenoids, sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids, tetraterpenes, and polyterpenes. Terpenoids show promising inhibitory effects on primary ABC transporters. The rosemary-originated terpenoid ursolic acid (Zong et al., 2019) were found to inhibit P-gp and increase the cellular accumulation of ADR and rhodamine 123 (Rh123). The chemosensitization effect of ursolic acid was tested, the result indicated that these compounds could significantly reduce the growth of cells in the presence of ADR.

Terpenoids have been reported to possess significant P-gp inhibitory activity via several mechanisms. Parthenolide inhibited P-gp up-regulation and promoted the intracellular accumulation of ADR in ADR-resistant human non-small cell lung cancer (A549/ADR) cells. Parthenolide inhibited the development of the resistance toward ADR, which exhibited an inhibitory effect on the overexpression of Nrf2 and P-gp (Carlisi et al.,2017). Costunolide dramatically enhanced ADR-induced antiproliferative activity against ADR-resistant human erythroleukemia cell (K562/ADR) cells through inhibition of the PI3K/Akt pathway and downregulation of P-gp expression (Cai et al., 2019). Hu et al. (2014) found that cryptotanshinone and dihydrotanshinone could increase the intracellular accumulation of anticancer drugs by down-regulating P-gp mRNA and protein levels and inhibiting P-gp ATPase

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activity. Tenulin and isotenulin significantly inhibited P-gp efflux by stimulating P-gp ATPase activity. The combinations of tenulin and isotenulin with chemotherapeutic drugs significantly resensitized MDR cancer cells (Chang et al., 2019).

Coumarins. Coumarins belonging to the benzopyrone family of TCM are mostly found in plants rich in oils. They can be classified as simple coumarins, furanocoumarins, pyranocoumarins, and pyrone-substituted coumarins based on the position of the substituents. The P-gp inhibitory activity of galbanic acid was evaluated using Rh123 efflux assay in Multidrug-Resistant Leukaemia HL60 Cells. It was found to be more potent than verapamil in terms of P-gp inhibition (Maruszewska and Tarasiuk, 2019). Decursinol inhibited ADR-resistant ovarian cancer cell proliferation and induced apoptosis via P-gp expression inhibition (Choi HS et al., 2016).

Coumarins have been reported to inhibit P-gp through multiple mechanisms. Osthole decreased the expression of P-gp at both mRNA and protein levels. Further experiments demonstrated that osthole could suppress P-gp expression by inhibiting the PI3K/Akt signaling pathway, which is possibly the main mechanism accounting for the reversal potential of osthole in K562/ADR cell (Wang et al., 2016).

Quinonoids. Quinone compounds are a class of chemical components with the quinoid structure in TCM, mainly divided into four types: benzoquinone, naphthoquinone, phenanthrenequinone, and anthraquinone. Miltirone, an abietane-type diterpene quinone isolated from Salvia miltiorrhiza, demonstrate anticancer activities in P-gp-overexpressing human cancer cells. Current studies have

suggested that the miltirone can inhibit the activity of P-gp and apoptotic induction in human hepatoma (HepG2) cell line and its P-gp-overexpressing HepG2/ADR cell line (Zhou et al., 2015). The inhibition activities of six anthraquinones (alizarin, purpurin, chrysophanol, emodin, aloe-emodin, and 1,3,8-trihydroxyanthraquinone) toward P-gp were estimated using docking analysis. The results indicated that all the investigated anthraquinones were potential inhibitors of P-gp under physiological conditions, indicating their roles as potential protectors against patient resistance toward various anticancer drugs (Jeremić et al., 2018). Emodin reversed ADR resistance in K562/ADR cells by decreasing the expression of P-gp. It can increase the accumulation of Rh123 in both K562/ADR and Caco-2 cells and hence inhibit P-gp efflux (Min et al., 2017). Shikonin/paclitaxe (PTX) co-treatment led to synergistically enhanced cytotoxicity and apoptosis in PTX-resistant ovarian cancer cells, reflecting the reverse of MDR. Further studies indicated that the MDR reversal effect of shikonin was independent of inhibiting the activity of P-gp (Wang et al., 2019). Moreover, the shikonin derivatives (acetylshikonin and acetoxyisovalerylshikonin) showed inhibition of P-gp and increased uptake and reduced efflux of anticancer drugs in the malignant cancer cells, suggesting that chemotherapy in combination with shikonin compounds may be beneficial to cancer cells (Wang et al., 2019).

Traditional Chinese Medicine extracts. B. garizans (Asiatic toad), a popular Chinese herb, showed a reversal effect on P-gp-mediated MDR in colorectal cancer cells (CRC). More studies showed the representative ingredient, cinobufagin, significantly enhanced the sensitivity of P-gp-overexpressing cells to ADR without

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affecting the corresponding parental cells. Studies further revealed that the mechanism of action involved noncompetitive inhibition of P-gp(Yuan et al., 2017). Salvia miltiorrhiza is a Chinese herb with significant antifungal, antioxidant, anti-inflammatory, and anticancer activities. The diterpenoid tanshinone co-existed in the Salvia extract could significantly enhance the absorption of cryptotanshinone. Danxiongfang is a useful preparation composed of the Salvia extract, which mainly includes lipophilic diterpenoid tanshinones, water-soluble salvianolic acids, and ferulic acid. It could be used to treat coronary heart diseases and cerebrovascular disease. Danxiongfang could influence the absorption of cryptotanshinone. It has been proposed that the oral bioutilization of cryptotanshinone can be enhanced by reducing the efflux and transport of P-gp in combination with diterpenoid tanshinones and danxiongfang (Dai et al., 2012). S. chinensis, known as Wu Wei Zi in Chinese, could inhibit the efflux of P-gp. Its active components include schizandrol A, γ-schisandrin, schizandrin B, and schizandrin C. Some experiments showed that schizandrin B increased the intracellular accumulation of ADR through inhibiting expression and activity of P-gp (Wang et al., 2017). The MDR reverse effect of Schizandrol A was demonstrated with P-gp overexpressed drug-resistant K562 cells (Arken, 2019). Coptidis Rhizoma (CR), the rhizome of Coptis chinensis, is a well-known Chinese herb. CR contains abundant isoquinoline alkaloids such as berberine, coptisine, and palmatine. Cell studies have shown that CR decoction, berberine, coptisine, and palmatine can activate the efflux of P-gp (Yu et al., 2018). Wutou-Gancao herb-pair is extensively used to attenuate the toxicity and enhance the efficacy of aconite. The

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active components of liquorice (Gancao) could inhibit the efflux of P-gp through

Caco-2 cell. Since aconitine from Aconitum (Wutou) has been shown to be a P-gp

substrate, the synergic effect is attributed to the inhibition of P-gp by liquorice. The

results showed the potential synergic mechanism of Wutou-Gancao herb-pair, which

could help to elucidate the compatibility principle of the two herbs by inhibiting P-gp

function and enhancing the systemic circulation exposure of aconitine and further

anti-inflammatory effects (He et al., 2020).

Approaches for studying the interaction of P-gp and TCM

In vitro **Approaches for TCM Interactions with P-gp.** Several *in vitro*

screening assays can be utilized to characterize the interplay between test compounds

and P-gp to identify drugs as P-gp substrates or inhibitors (Polli JW et al., 2001). The

basic principle of the classification method is determining the effect on P-gp function

and expression. Functional assays used to screen P-gp inhibitors include the detection

of the changes in P-gp content and the expression level of the *MDR1* gene, the

evaluation of the P-gp efflux by colorimetric chemosensitivity assay, and bidirectional

transcellular transport assays. Colorimetric chemosensitivity assay was used to

determine the effect of reversal agent on P-gp via the calculation of IC50 and the

degree of drug resistance. Concentration changes in P-gp content and the levels of

MDR1 genes were typically measured in cells by reverse transcription-polymerase

chain reaction (RT-PCR), immunoblotting, and/or flow cytometry (Xia et al., 2017).

Li et al. (2018) used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) assay to determine the cytotoxicity of doxorubicin, polyphenols, and digitonin,

and the cytotoxicity of their combinations. Rhodamine 123 and Calcein-AM were further used to detect the effects of polyphenols on the activity of P-gp. It has been validated that the combination of non-toxic concentrations of each polyphenol with ADR could synergistically improve the drug efficacy towards Caco-2 and CEM/ADR cells. Polyphenols could modulate the activity of P-gp and may be used as chemosensitisers.

Bidirectional transcellular transport assay is the gold standard for identifying P-gp substrates *in vitro* and represents the most accurate predictive model for the identification of P-gp inhibitors *in vivo* (Feng et al., 2008). Drug-resistant cell lines with overexpression of P-gp are widely used to investigate the effect of test compounds as potential inhibitors of P-gp in this method. There are many cell lines suitable for use in bidirectional transcellular transport assays, including the naturally P-gp-expressing Caco-2 cells and polarized *MDR1*-transfected MDCK-MDR1, with distinct apical and basolateral membrane domains. P_{app}BL-AP/P_{app}AP-BL was used to judge whether the intestinal absorption of a drug would be affected by P-gp. It was considered that the transport of a drug was directional when P_{app}BL-AP/P_{app}AP-BL was more than 1.5 (Liang et al., 2012). When the efflux rate of a probe substrate decreased, it was considered that the agent may inhibit the activity of P-gp; the schematic diagram of the method is shown in Fig.3A. The effect of different tanshinones on the P-gp efflux function was first investigated by using a digoxin bi-directional transport assay. According to the results, among the five tanshinones tested, only cryptotanshinone and dihydrotanshinone could decrease the efflux ratio of

digoxin bidirectional transport across Caco-2 cell monolayer, indicating their inhibition on P-gp function (Hu et al., 2014). Meanwhile, these *in vitro* methods have their limitations and should be fully considered in practice. Different studies have used the same assay for the same compound but reported contrasting results. In addition to the discordance in the results obtained from different *in vitro* studies, there have also been some disagreements between *in vitro* and *in vivo* findings regarding the P-gp substrates or inhibitors (Lund M et al., 2017).

***In vivo* Approaches for TCM Interactions with P-gp.** The generation of *MDR1*-knockout mice has boosted the study of the pharmacological influence on the function of P-gp (Li et al., 2020). By comparing brain/plasma ratios between these P-gp-deficient mice to those of the wild-type controls, it has been possible to screen drugs as potential P-gp substrates *in vivo* (Mittapalli RK et al., 2012). Alternatively, P-gp inhibitors can be evaluated by investigating the influence of P-gp inhibition on drug pharmacokinetics in wild-type animals. Co-administration of digoxin with verapamil or emodin can increase the AUC (area under the curve) of digoxin by 55% and 51%, respectively. Emodin demonstrated inhibition of P-gp to specific extents *in vivo*(Li et al., 2014).

Tumor-bearing mice were also conducted for *in vivo* studies. The chemosensitization effects of TCM have been investigated through changes in tumor growth or survival rate of tumor-bearing mice (Fig.3B) (Tiwari AK et al., 2013). If a TCM has MDR reversing activity, tumor growth should be inhibited and the survival period of tumor-bearing rats should be prolonged. *In vivo* antitumor study showed that

co-encapsulated PTX and baicalein in nanoemulsions demonstrated higher antitumor efficacy than other PTX formulations. The antitumor effect of various PTX formulations *in vivo* was evaluated in mice bearing MCF-7/Tax xenograft tumors models (Meng et al., 2016). Combining PTX with vitamin D3 and curcumin could potentially synergize their ability to decrease drug resistance by decreasing P-gp. *In vivo*, the triple therapy group (PTX + curcumin +D3) resulted in the smallest tumor size, indicating the addition of curcumin and D3 could enhance the therapeutic effect on the tumor (Attia et al., 2020).

In Silico Prediction method. With the rapid development of machine learning and artificial intelligence, *in silico* methods have acquired an increasing interest in P-gp inhibitor discovery. Compared with the classical methods, these *in silico* methods have an enormous potential to speed up the preclinical development processes at minimal costs. Using *in silico* virtual screening to detect novel P-gp inhibitors is an important application of molecular simulations.

At present, no human crystallized P-gp has been published. Since the sequence identity between the human protein and the murine protein is 87%, the available structures of the murine protein are important for molecular docking from different perspectives (Pajeva IK et al., 2009; Jabeen I et al., 2011). Shityakov *et al.* ran molecular docking in the crystallized murine structure with polynomial empirical scores using a P-gp inhibitor library of 1,300 molecules (Shityakov S and Forster C, 2014). To understand the interaction of ADR and miltirone with P-gp, molecular docking analysis was conducted. The results indicated the most energetically optimal

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binding modes of ADR and miltirone at the drug binding cavities of mouse P-gp

(PDB ID: 3G61), which is shown in Fig. 3C (Zhou et al., 2015). However, the human

P-gp homology model showed more limitations to predict the experimental data. In

addition, no human crystallized P-gp has been made available and extrapolation of the

results from animals to humans is not always satisfactory (Montanari F and Ecker GF,

2015).

Conclusions and Outlooks

Despite the promising preclinical results of P-gp inhibitors, no compound has yet

been approved for clinical use as MDR reversal agents. Studies were terminated due

to unexpected toxicities in clinical trials or insufficient efficacy *in vivo*. Consequently,

how to identify MDR reversal agents with high efficacy and limited toxicity remains a

big challenge. Due to the characteristic of low toxicity, an increasing number of TCM

including flavonoids, alkaloids, terpenoids, coumarins, quinonoids, and other TCM

extracts have been investigated for their potential as MDR reversal agents and have

shown a good efficacy of reversing MDR in experimental model systems. TCM has

been reported to reverse P-gp mediated MDR with diverse mechanisms, such as

inhibiting P-gp mediated drug efflux (Abdallah HM et al., 2015), hindering the

activity of P-gp ATPase, and reducing P-gp levels by downregulating the expression

of the *MDR1* gene.

Currently, studies on identifying TCM as P-gp reversal agents have mainly been

conducted *in vitro,* using cell models, rat models, and *in silico* method. However, the

in vitro approaches for screening are labor-intensive and time-consuming at present.

The molecular docking results from the human P-gp homology model is not always satisfactory. Technologies like proteoliposome, which can predict the interaction of the active compound with the target protein, should be explored in order to establish a new high-throughput screening method.

On the other hand, the influence of P-gp inhibition on tissue pharmacokinetics and intra-tumoral distribution of anticancer drugs are very important when co-administrated with P-gp inhibitor. More efforts should be made to explore the effects of TCM on the pharmacokinetic processes of anticancer drugs *in vivo* to choose the optimal therapeutic protocol for cancer treatment. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is commonly used to determine the drug concentration in homogenized tissues, while it's not accurate for tumors due to heterogeneous characteristics. Since the heterogeneous tissue and the tumor microenvironment markedly affect drug penetration, it is necessary to detect not only the level of exposure but also the spatial distribution of the drug within the tumor tissue. Thus, the use of LC-MS/MS analysis only is insufficient for clarifying the behavior of anti-tumor drugs. Mass spectrometry imaging (MSI), which could offer spatial information for the drug in the tumor tissue, has been recently utilized as an innovative tool for detecting the molecular distribution of pharmacological agents in heterogeneous targets. Compared with LC-MS/MS, MSI does not require homogenization of tumor tissue, so it can provide information on the spatial distribution of the detected drugs in the tissue. This technique would certainly help understand the heterogeneous and irregular tumor drug distribution.

In summary, TCMs play a crucial role in the development of effective MDR modulators in the future. The *in vitro* techniques such as bidirectional transcellular transport assays and *in silico* prediction method will greatly influence the drug development. These approaches can help predict the effect of TCM on P-gp and provide an impetus to the discovery of more MDR reversal agent candidates for clinical trials. However, precise techniques such as MSI discussed in this review were needed for *in vivo* validation. In brief, the challenge is on the shoulders to identify suitable P-gp inhibitors with significant MDR reversal and acceptable toxicity from TCM and thus improve the treatment effect of chemotherapy drugs in clinical cancer therapy.

Authorship Contributions

Participated in research design: Cao, Hong, Chai

Conducted experiments: Cao, Hong, Shi, Cai

Contributed new reagents or analytic tools: Cao, Shi, Cai

Performed data analysis: Cao, Hong, Chai

Wrote or contributed to the writing of the manuscript: Cao, Shi, Hong

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Footnotes.

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Legends for Figures

Fig.1. Secondary structure models of P-gp.

Fig.2. Model of P-gp substrate transport. (A) substrate-binding conformation, the molecule travels through one of two portals, to enter the substrate-binding pocket (SNP) of P-gp. Substrate–P-gp interactions lead to the binding of two ATP molecules to the NBD. The binding of ATP to the NBDs causes dimerization of the NBDs. (B) This leads to a conformational change, resulting in an outward facing configuration presenting the substrate and drug-binding site(s) to the outer leaflet/extracellular space. Above broken line = outer leaflet of phospholipid bilayer. Below broken line = inner (cytoplasmic) leaflet of phospholipid bilayer.

Fig.3. Approaches to study TCM Interactions with P-gp. (A) *in vitro* using bidirectional transcellular transport assay; (B) *in vivo* conducted on tumor bearing rats and (C) *in silico* prediction by molecular docking (Zhou et al., 2015)

TABLES:

TABLE 1

Examples of P-gp multidrug resistance reversal agents.

Generation	Representative agent	Mechanisms of action	Clinical response	Reference
1st	Verapamil; Reserpine Quinine; Tamoxifen Cyclosporine A; Toremifene	Altering ATP hydrolysis pathway (Tariquidar; Verapamil; Cyclosporine A; Toremifene; Quinine; Tamoxifen);	Toxicities; nephrotoxicity, myelosuppression, and neurotoxicity	Varma MV et al., 2003
2nd	Dexverapamil; Valspodar; Biricodar	Competition for Binding Sites (Verapamil; Reserpine; Quinine; Elacridar; Cyclosporine A);	More affinity, efficacy; low toxicity and interaction with cytochrome P450 enzymes	Kathawala RJ et al., 2015
3rd	Elacridar; Laniquidar; Zosuquidar; Dofequidar; Mitotane; Annamycin; Tariquidar	Alteration in P-gp expression (Verapamil; Cyclosporine A; Valspodar)	More specific and potent with diminutive pharmacokinetic interactions	Pusztai L et al., 2005

TABLE 2

Inhibition of P-gp function and/or expression by TCM.

Compound	Test Model	Pharmacological results	References
Flavonoids			
EGCG	CEM/ADR	Down-regulation of P-gp	Li et al., 2018
GQ	MCF-7/ADR	Down-regulation of P-gp and *MDR1* gene	Zhang et al., 2019
Silymarin A	A2780/ADR	Inhibition of P-gp efflux; Down-regulation of P-gp	Viktorova J et al., 2019
Dihydromyricetin	MCF7/ADR; K562/ADR	Inhibition of P-gp efflux; Down-regulation of P-gp	Sun et al., 2018
Curcumin	KB-C2	Inhibition of P-gp	Lopes-Rodrigues V et al., 2016
Apigenin	CEM/ADR	Inhibition of P-gp	Saeed M et al., 2015
Taxifolin	HeLaS3; KB/VCR	Inhibition of P-gp	Chen et al., 2018
Alkaloids			
Chelidonine	Caco-2; CEM/ADR	Inhibition of P-gp	Herrmann R et al., 2018
Tetrandrine	MCF-7/ADR	Down-regulation of P-gp	Liao et al., 2019
Matrine	MCF-7/ADR	Inhibition of P-gp	Zhou et al., 2018
Terpenoids			
Ursoli acid	MCF-7/ADR;	Inhibition of P-gp	Zong et al., 2019

Parthenolide	A549/ADR	Down-regulation of P-gp	Carlisi et al., 2017
Costunolide	K562/ADR	Down-regulation of P-gp	Cai et al., 2019
Cryptotanshinone Dihydrotanshinone	CEM/ADR	Down-regulation of P-gp and *MDR1* gene	Hu et al. 2014
Coumarins			
Galbanic acid	HL60	Inhibition of P-gp	Maruszewska and Tarasiuk, 2019
Decursinol	NCI/ADR-RES	Inhibition of P-gp	Choi HS et al., 2016
Osthole	K562/ADR	Down-regulation of *MDR1* gene	Wang et al., 2016
Qinonoids			
Miltirone	HepG2	Inhibition of P-gp	Zhou et al., 2015
Emodin	K562/ADR; Caco-2 cells	Down-regulation of P-gp	Min et al., 2017
Shikonin	A2780/PTX	Inhibition of P-gp	Wang et al., 2019
TCM extracts			
Asiatic toad	CRC	Inhibition of P-gp	Yuan et al., 2017
Salvia miltiorrhiza	Everted rat gut sacs	Inhibition of P-gp	Dai et al., 2012
S.chinensis	K562/A02	Down-regulation of P-gp; Inhibition of P-gp	Wang et al., 2017
CR	LS180	Activation of P-gp transportion	Yu et al., 2018

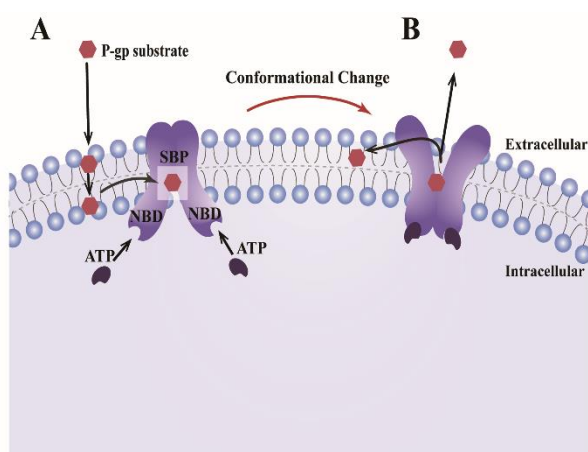| Liquorice | Caco-2 cell | Inhibition of P-gp | He et al., 2020 |

Abbreviations: EGCG, Epigallocatechin-3-gallate; CEM/ADR, adriamycin-resistant human leukemia cell line; MCF-7/ADR, adriamycin-resistant human breast adenocarcinoma cell line; GQ,7-O-geranylquercetin; A2780/ADR, adriamycin-resistant ovarian carcinoma cell; KB-C2, multidrug-resistant subclone human mouth epidermoid; HeLaS3, human cervical cancer cell; KB/VCR, vincristine-resistant human mouth epidermal carcinoma; Caco-2, human colon cancer cell; LS180/ADR, adriamycin-resistant human colon carcinoma cell; A549/ADR, adriamycin-resistant human non-small cell lung cancer cell; K562/ADR, adriamycin-resistant human erythroleukemia cell; CRC, colorectal cancer cell; HepG2, human hepatocellular carcinomas cell; LS180, human colon carcinoma cell ; NCI/ADR-RES, adriamycin-resistant ovarian cancer cells.
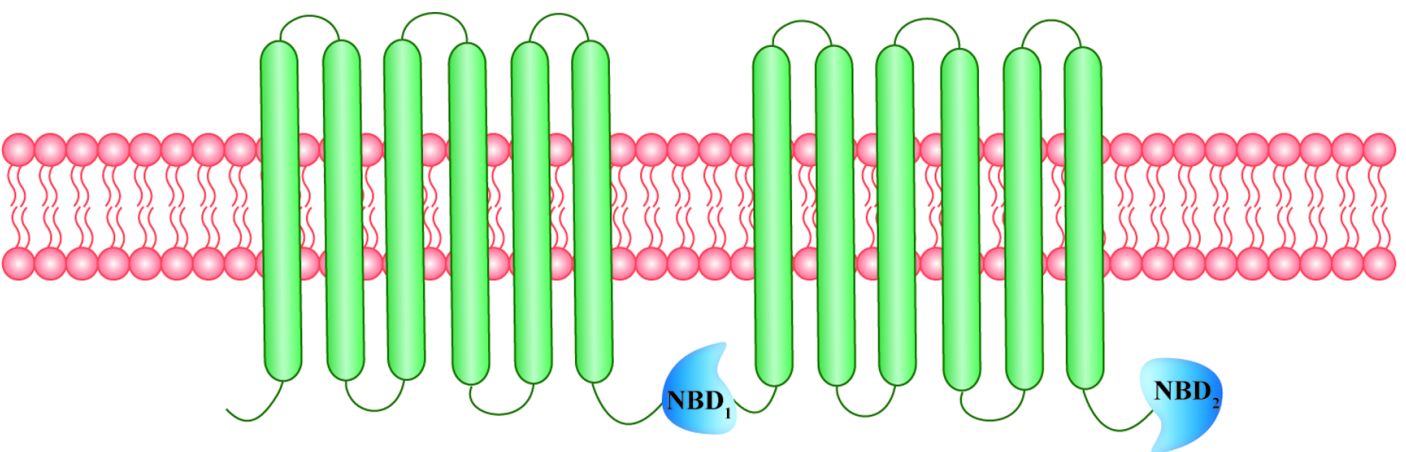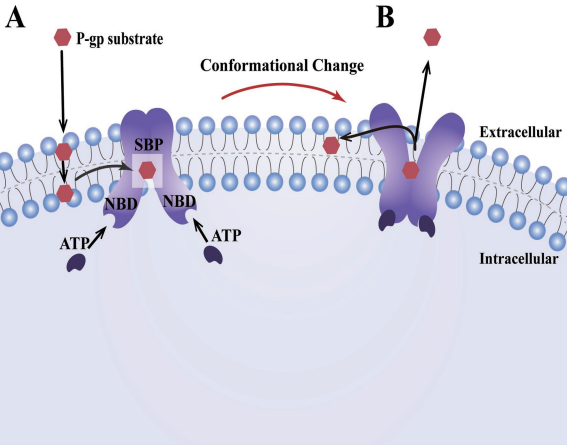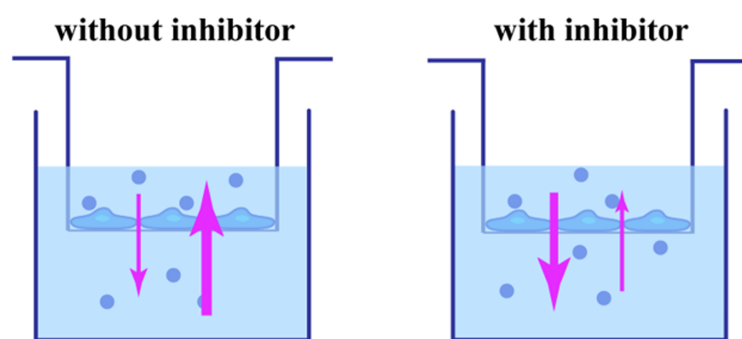
FIGURES:

Fig.1

Fig.2

Fig.3.

NBD₁

NBD₂

A

P-gp substrate

B

Conformational Change

SBP

NBD   NBD

ATP   ATP

Extracellular

Intracellular
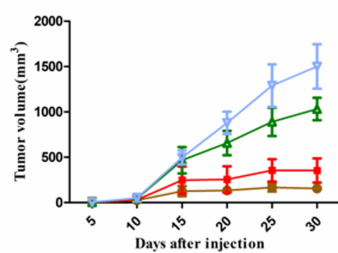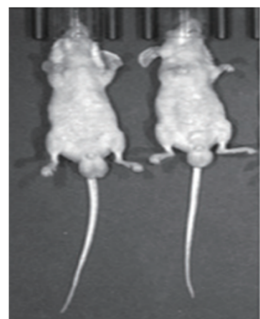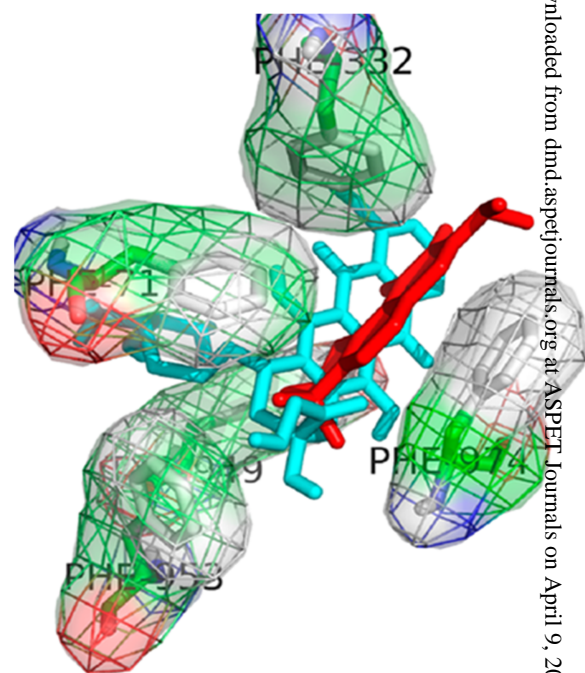
A **without inhibitor** **with inhibitor**
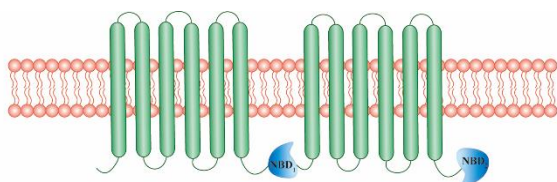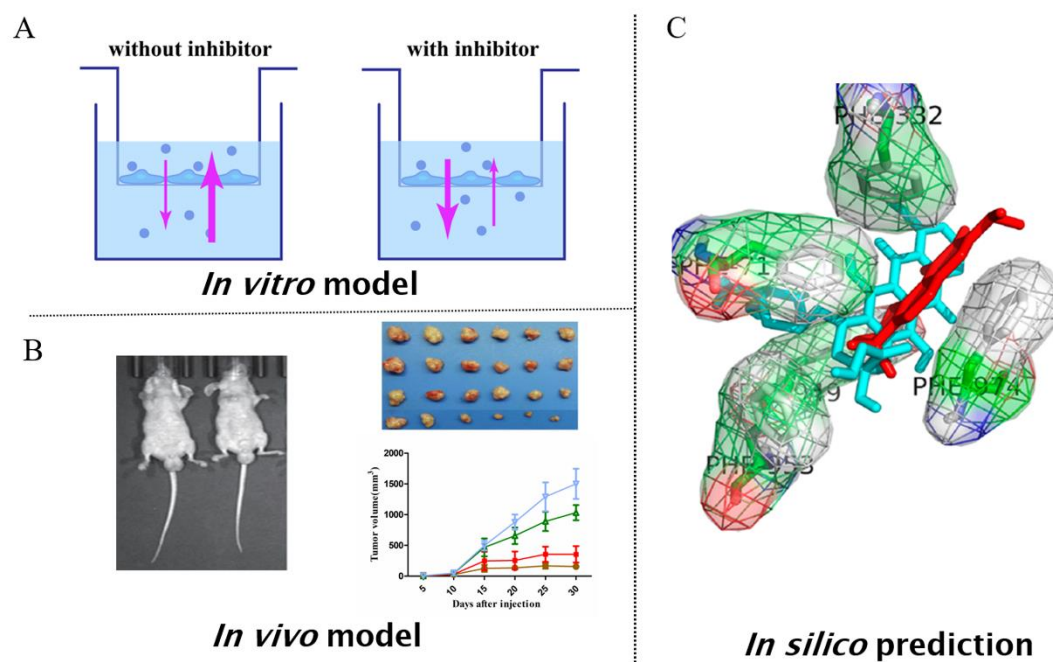
In vitro model

B *In vivo* model

C *In silico* prediction