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Reversal of multidrug resistance by *Marsdenia tenacissima* and its main active ingredients polyoxypregnanes

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Mitoxantrone (PubChem CID4212)
Verapamil (PubChem CID2520)
Sulforhodamine B (PubChem CID65191)
Rhodamine 123 (PubChem CID65217)
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ABSTRACT

Ethnopharmacological relevance: Multidrug resistance (MDR) of cancer is often associated with the over-expression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated protein-1 (MRP-1) and breast cancer resistance protein (BCRP or ABCG2), in cancer cells, which facilitates the active efflux of a wide variety of chemotherapeutic drugs out of the cells. *Marsdenia tenacissima* is a traditional Chinese medicinal herb that has long been clinically used for treatment of cancers, particularly in combinational use with anticancer drugs. Polyoxypregnanes (POPs) are identified as main constituents of this herb, and three of them have been reported to exhibit P-gp modulatory effect and thus reverse MDR. Therefore, it is of great necessity to investigate more POPs that have potential to reverse transporters-mediated MDR.
Aim of the study: We aimed to identify POPs as the chemical basis responsible for circumventing ABC transporters-mediated MDR by *M. tenacissima*.
Materials and methods: The MDR reversal effects of *M. tenacissima* crude extract together with a series of isolated POPs were evaluated on several MDR cancer cell lines that overexpress P-gp, MRP1 or ABCG2. The activities of P-gp, MRP1 and ABCG2 were determined by the flow cytometry-based substrate efflux assay. Molecular docking of POPs to a three-dimensional human P-gp homology structure was also performed.
Results: The crude extract of *M. tenacissima* was firstly found to circumvent P-gp-mediated MDR. Then, 11 polyoxypregnane compounds (POPs) isolated from this herb were found to overcome P-gp-, MRP1- and/or ABCG2-mediated MDR. Further mechanistic study delineated that the reversal of MDR by these POPs was due to significant increase in the intracellular concentrations of the substrate anticancer drugs via their inhibition of different ABC transporter-mediated efflux activities. Furthermore, molecular docking revealed that POPs with P-gp modulatory effect bound to P-gp and fitted well into the cavity between the alpha and beta subunit of P-gp via forming hydrogen bonds. In addition, several key structural determinants for inhibition of P-gp, MRP1 or ABCG2 by POPs were illustrated.
Conclusions: Our findings advocated the rational use of *M. tenacissima* to enhance efficacies of conventional anticancer drugs in tumors with ABC drug transporters-mediated MDR. Furthermore, 11 POPs were found to contribute to MDR reversal effect of *M. tenacissima* via inhibition of different ABC efflux transporters.

Abbreviations: ABC, ATP-binding cassette; BCRP/ABCG2, breast cancer resistance protein; Bz, benzoyl; FTC, fumitremorgin C; mBu, methyl isobutyl; LBE, lowest binding energies; MDR, multidrug resistance; MRP1, multidrug-resistance-associated protein 1; POP, polyoxypregnane; P-gp, P-glycoprotein; PhA, pheophorbide A; R123, rhodamine 123; SAR, structure-activity relationship; Tig, methyl isobutenyl

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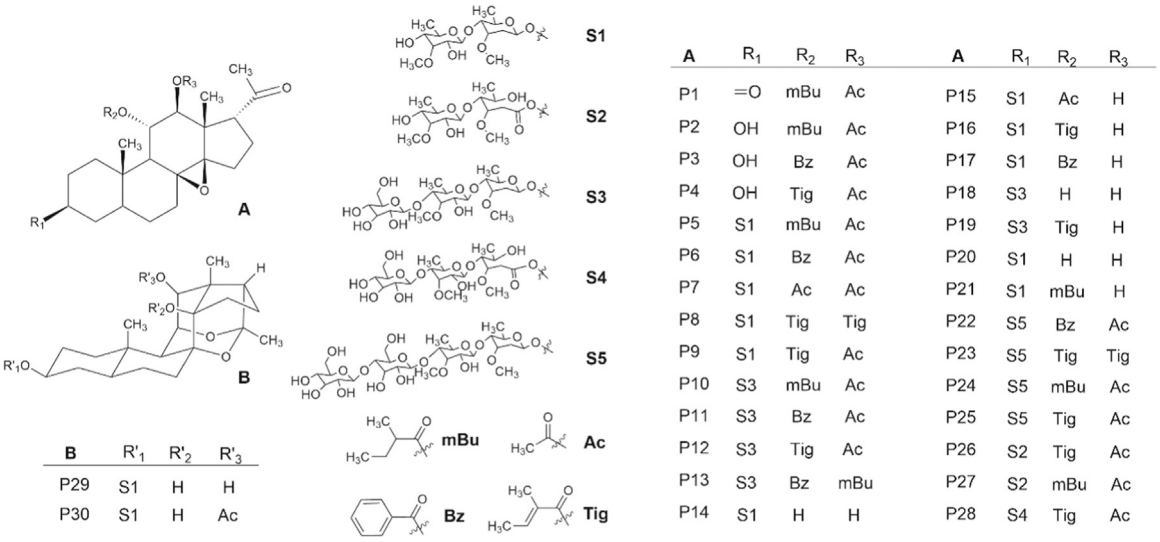

A	R_1	R_2	R_3	A	R_1	R_2	R_3
P1	=O	mBu	Ac	P15	S1	Ac	H
P2	OH	mBu	Ac	P16	S1	Tig	H
P3	OH	Bz	Ac	P17	S1	Bz	H
P4	OH	Tig	Ac	P18	S3	H	H
P5	S1	mBu	Ac	P19	S3	Tig	H
P6	S1	Bz	Ac	P20	S1	H	H
P7	S1	Ac	Ac	P21	S1	mBu	H
P8	S1	Tig	Tig	P22	S5	Bz	Ac
P9	S1	Tig	Ac	P23	S5	Tig	Tig
P10	S3	mBu	Ac	P24	S5	mBu	Ac
P11	S3	Bz	Ac	P25	S5	Tig	Ac
P12	S3	Tig	Ac	P26	S2	Tig	Ac
P13	S3	Bz	mBu	P27	S2	mBu	Ac
P14	S1	H	H	P28	S4	Tig	Ac

B	R'_1	R'_2	R'_3
P29	S1	H	H
P30	S1	H	Ac

Fig. 1. Structures of 30 POPs isolated from *M. tenacissima*, which are the derivatives of tenacigenin B (A) and tenacigenin A (B).

1. Introduction

The ability of cancer cells to acquire resistance to multiple drugs, termed multidrug resistance (MDR), is often mediated by ATP-binding cassette (ABC) transporters that remove substrates out of the cell against a concentration gradient (Gottesman et al., 2002). To date, forty-nine ABC transporters have been identified in the human genome and are divided into seven subfamilies (A-G) based on sequence similarities (de Vries et al., 2007). Overexpression of the three major ABC transporters, namely P-glycoprotein (P-gp) encoded by the *ABCB1* (or *MDR-1*) gene, multidrug-resistance-associated protein 1 (MRP1) encoded by the *ABCC1* (or *MRP1*) gene and breast cancer resistance protein (BCRP/ABCG2) encoded by the *ABCG2* gene, is frequently observed in MDR cancer cells (Szakács et al., 2006) and critical to clinical drug resistance (Leonard et al., 2003). These transporters exhibit broad substrate specificities for a variety of drugs, toxins, metabolites and endogenous compounds (Glavinas et al., 2004).

P-gp is known to transport a wide range of chemotherapeutic drugs (Szakács et al., 2006), and widely expressed in different tissues in the body (Cordon-Cardo et al., 1990; Thiebaut et al., 1987). High expression of P-gp is most often detected in renal carcinoma, colon carcinoma, adrenal carcinoma and teratocarcinoma (Cordon-Cardo et al., 1990). Verapamil was the first known P-gp inhibitor reported to increase the intracellular concentration of anticancer agents in MDR cells by binding P-gp and inhibiting the P-gp-mediated drug efflux (Tsuruo et al., 1981). This observation fueled hopes that anticancer drug resistance could be reversed by inhibiting drug efflux. To improve the efficacy of chemotherapy in MDR tumors, a range of P-gp inhibitors have been developed (Leonard et al., 2003) and numerous clinical trials have been carried out to test this MDR reversal hypothesis. However, therapeutic benefit by inhibiting P-gp was not successfully observed in clinics thus far (Robey et al., 2007) due to either adverse pharmacological effect or unwanted pharmacokinetic interaction via inhibition of cytochrome P450s (Leonard et al., 2002). Although some of the third generation inhibitors such as tariquidar are under clinical development with higher specificity and lower toxicity (Abraham et al., 2009; Pusztai et al., 2005), the discovery of potent MDR inhibitors devoid of other biological activities is still a primary goal to test the MDR reversal hypothesis in clinics.

Moreover, the recognition that not all MDR cells with reduced drug uptake express P-gp prompted a search for additional drug transporters responsible for MDR and for their inhibitors. The emergence of several other members of the ABC transporter family (e.g., MRP1 and ABCG2) has necessitated the development of antagonists that can inhibit more than one transporter. The number of different inhibitors that have been described to date is remarkable. However, to date no clear structure-activity relationship (SAR) has been identified to explain the definitive requirements for an inhibitor of P-gp, MRP1 and/or ABCG2.

Apart from the importance in anticancer drug resistance, inhibition of P-gp and ABCG2 has also been applied to increase oral bioavailability and brain penetration of their substrate drugs. Previous studies have shown that co-administration of the ABCG2 inhibitor elacridar with topotecan enhanced oral bioavailability of the latter in mice (Kruijtzer et al., 2002). Another ABCG2 inhibitor, gefitinib, was also reported to enhance the oral bioavailability and antitumor activity of irinotecan in mice (Stewart et al., 2004). Moreover, the inhibition of the two transporters has also been shown to increase brain penetration of topotecan (Zhuang et al., 2006), again highlighting the need for potent inhibitors of P-gp and/or ABCG2.

Natural products and derivatives are rich sources of novel therapeutics (Newman and Cragg, 2007). Naturally occurring compounds belonging to different chemical families such as coumarins, terpenoids and steroids have been found with MDR-modulating activity (Chearwae et al., 2004; Jain et al., 2007; Wang et al., 2004). *Marsdenia tenacissima* (Roxb.) Wight et Arn is a plant of *Asclepiadaceae* family distributed mainly in the southwest of Mainland China. Its dried stems have been widely used either alone or in combination with other chemotherapeutics for the treatment of cancer (Huang et al., 2013; Wang et al., 2009; Xueyuan, 1976). Polyoxypregnanes (POPs) as main ingredients present in this plant (Supplementary Fig. S1) have been studied. Except for a few studies on cytotoxic activities of some POPs (Si-Qi et al., 1993; Ye et al., 2014), it have been reported by our research team and others that several POPs possessed remarkable MDR reversal effects. Interestingly, three POPs (**P1**, **P6** and **P9**, Fig. 1) were found to be P-gp modulators and showed the ability of restoring the sensitivity of anticancer drugs in P-gp-overexpressing human MDR cancer cells (Hu et al., 2008; Yao et al., 2014).

To further identify the chemical basis responsible for MDR-reversal effect of herb *M. tenacissima*, in the present study, we systematically evaluated the crude extract of *M. tenacissima* and 30 POPs (Fig. 1), which were previously isolated from this herb by our group (Yao et al., 2016, 2014) for their potential MDR-reversal activity in cancer cell lines with overexpression of the three major ABC transporters. To investigate the underlying mechanisms, we further examined the inhibition of ABC transporter mediated efflux activity by flow cytometry in P-gp, MRP1 and ABCG2 stably transfected cell lines. In addition,