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A myrsinol diterpene isolated from *Euphorbia prolifera* reverses multidrug resistance in breast cancer cells

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P-glycoprotein (P-gp), a member of ATP-Binding Cassette transporter superfamily, can expel a variety of anti-cancer drugs so that it impairs the effect of cancer chemotherapy and results in multidrug resistance (MDR). The P-gp inhibitors are important to circumvent MDR and improve efficacy of cancer chemotherapy. The dried root of *Euphorbia prolifera* Buch.-Ham. has been used to treat cancer and inflammation in Chinese folk medicine for several hundred years. A myrsinol diterpene derived from *Euphorbia prolifera* Buch.-Ham. (J196-9-4) could modulate multidrug resistance. Cytotoxicity assays were performed to measure the reversal efficiency of J196-9-4. Efflux assay and ATPase assay were used to elucidate the mechanism of the chemical. J196-9-4 potentiated cytotoxicity of anti-cancer drugs in the P-gp over-expressing resistant breast cancer cell line MCF-7/Adr as compared to MCF-7 cells. Concentrations of 5 and 10 μM J196-9-4 could reverse the resistance to daunorubicin, vincristine, and topotecan significantly. Since J196-9-4 inhibited P-gp mediated efflux and stimulated ATP hydrolysis, J196-9-4 was a substrate of P-gp. Thus J196-9-4 is a competitive inhibitor of P-gp and reverses multidrug resistance induced by the transporter.

1. Introduction

Euphorbia prolifera Buch.-Ham., belonging to the Euphorbiaceae family, is a perennial herbaceous plant. It is mainly found in southwest China (Xu et al. 2011; Zhao et al. 1999). The dried roots were called Lang-du and used in local folk medicine to treat cancer and inflammation.

Cancer cells frequently acquire cross-resistance to different anti-cancer drugs during the course of chemotherapy (Gillet et al. 2007). Highly expressed P-glycoprotein (P-gp) is one of the most important reasons leading to multidrug resistance (MDR). P-gp is encoded by the ABCB1 (MDR1) gene, which is a member of the ATP Binding Cassette (ABC) superfamily (Schinkel and Jonker 2003). P-gp is a transmembrane exporter, which can bind and transfer a variety of lipophilic drugs from the cell membrane and cytoplasm to body fluids (Seeger and van Veen 2009). This process decreases the intracellular concentration of the drugs. Thus highly expressed P-gp makes cancer cells resistant to anti-cancer drugs and results in failure of chemotherapy. P-gp inhibitors are necessary to block P-gp dependent efflux and reverse MDR (Wink et al. 2012). Although a number of MDR reversal agents were synthesized and clinically tested, they did not produce satisfying outcomes. Thus, natural products from traditional herbal medicine became promising sources of MDR reversal agents (Eichhorn and Efferth 2012).

It was reported that constituents isolated from *Euphorbia prolifera* inhibited proliferation of tumor cells (Li et al. 2011a). Different kinds of diterpenes isolated from *Euphorbia* species can inhibit P-gp and modulate MDR (Appendino et al. 2003; Corea et al. 2004a, b; Madureira et al. 2006). The components of *Euphorbia prolifera Buch.-Ham.* were analyzed to elucidate the effects of Lang-du against MDR cancer cells (human breast cancer cells). We found that the myrsinol diterpene J196-9-4 (Fig. 1) could effectively inhibit P-gp dependent efflux as a competitive inhibitor and reverse MDR of the p-gp over-expressing cell line MCF-7/Adr.

Abbreviations:
ABC, ATP Binding Cassette; DMSO, Dimethyl sulfoxide; FBS, fetal bovine serum; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; P-gp, P-glycoprotein.

2. Investigations and results

2.1. Cytotoxicity of J196-9-4

J196-9-4 did not exhibit cytotoxicity against MCF-7 and MCF-7/Adr when the concentrations were lower than 50 μM.

2.2. J196-9-4 reversed resistance of MCF-7/Adr to cytotoxic drugs

MCF-7/Adr was resistant to different kinds of cytotoxic agents. The IC_{50} values to daunorubicin, vincristine, and topotecan were higher than the IC_{50} values of the sensitive MCF-7 cells. The IC_{50} values decreased substantially when the cytotoxic drugs were applied to MCF-7/Adr combined with non-toxic concentration of J196-9-4 (Tables 1, 2, 3). Obviously, multidrug resistance was reversed by this diterpene.

2.3. J196-9-4 inhibited rhodamine 123 efflux

Since the MCF-7/Adr cells expressed higher levels of P-gp than the parental cell line MCF-7, rhodamine 123 (a fluorescent substrate of P-gp) was quickly exported from the cytoplasm. Fluorescence efflux assays were performed to investigate if the compound inhibited P-gp mediated efflux. Fluorescent intensity in MCF-7/Adr was much lower (and transporter activity higher) than that in MCF-7 cells (Fig. 2). After exposure to J196-9-4, the fluorescence

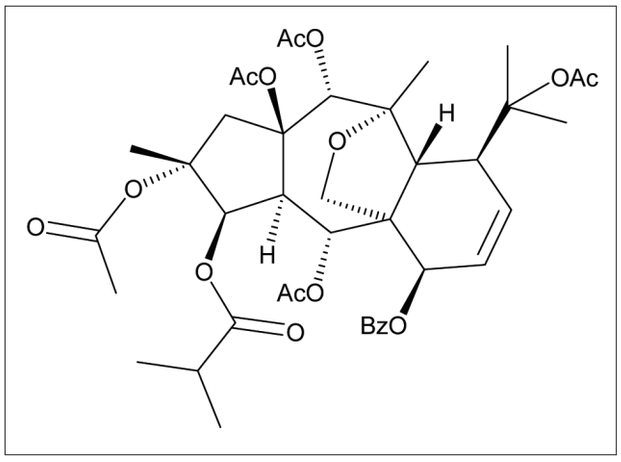

Fig. 1: Structure of J196-9-4

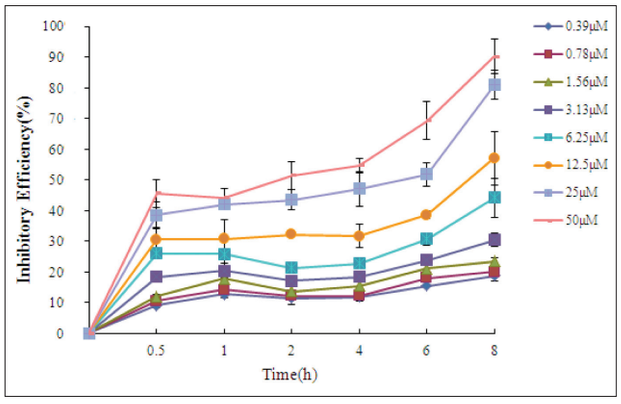

Fig. 2: Efflux inhibition efficiency of J196-9-4.
MDR cells were treated with different concentrations of J196-9-4 and the fluorescence intensity was measured at different time points. Inhibition efficiency was calculated by the formula (fluorescence intensity of treated resistant cells minus fluorescence intensity of untreated resistant cells) / (fluorescence intensity of sensitive cells minus fluorescence intensity of untreated resistant cells). Values are expressed as mean ± SD.

Table 1: IC_{50} of daunorubicin in MCF-7/Adr with or without reversal agents. Values are expressed as mean ± SD.

Cell	Drug	IC_{50}(μM)	Reversal ratio
MCF-7/ADR	Daunorubicin	29.65±1.62	
	Daunorubicin +10 μM verapamil	1.10 ±0.10	27.03
	Daunorubicin +5 μM J196-9-4	10.20±0.30	2.91
	Daunorubicin + 10 μM J196-9-4	2.70±0.082	10.98
MCF-7	Daunorubicin	0.41±0.12	

Table 2: IC_{50} of vincristine in MCF-7/Adr with or without reversal agents. Values are expressed as mean ± SD.

Cell	Drug	IC_{50}(μM)	Reversal ratio
MCF-7/ADR	Vincristine	13.84±0.47	
	Vincristine +10μM verapamil	0.054±0.0055	256.3
	Vincristine +5μM J196-9-4	1.60±0.06	8.65
	Vincristine + 10μM J16-9-4	1.41±0.054	9.82
MCF-7	Vincristine	0.25±0.026	

Table 3: IC_{50} of topotecan in MCF-7/Adr with or without reversal agents. Values are expressed as mean ± SD.

Cell	Drug	IC_{50}(μM)	Reversal ratio
MCF-7/ADR	Topotecan	4.61±0.13	
	Topotecan +10μM verapamil	0.80±0.073	5.76
	Topotecan +5μM J196-9-4	1.21±0.23	3.81
	Topotecan + 10μM J16-9-4	0.65±0.13	7.09
MCF-7	Topotecan	1.6±0.24	

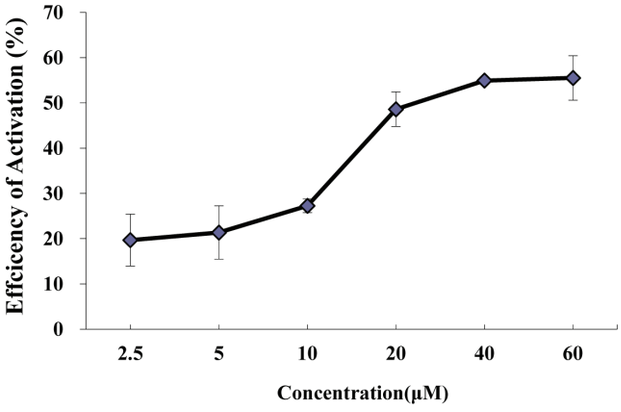

Fig. 3: Effects of J196-9-4 on ATPase activity of P-gp
ATPase activity of P-gp increased as concentration of J196-9-4 rose.

intensity rose in a time and concentration depending manner. Thus compound J196-9-4 inhibited P-gp mediated fluorescence export.

2.4. J196-9-4 stimulated the ATPase activity of P-gp

Since P-gp hydrolyzed ATP to drive substrate extrusion, the ATPase activity of P-gp was activated when substrates were present (Sauna et al. 2004). As shown in Fig. 3, J196-9-4 treatment enhanced ATP consumption of Human P-gp membrane (Gentest™). Therefore, J196-9-4 stimulated ATPase activity of P-gp and was apparently a substrate of P-gp.

3. Discussion

MDR is an important obstacle in cancer chemotherapy, as it makes cancer cells resistant to higher doses of anti-cancer drugs. Over-expressed P-gp is one of the most important factors leading to MDR. The multidrug resistant MCF-7/Adr cells can tolerate higher concentrations of daunorubicin, vincristine, and topotecan than the sensitive MCF-7 cells. Since MCF-7/Adr expresses higher level of ABCB1 than MCF-7 and verapamil (a P-gp inhibitor) can re-sensitize MDR of MCF-7/Adr, P-gp contributes to MDR of MCF-7/Adr evidently.

A number of reversal agents were synthesized to antagonize P-gp and overcome MDR. The inhibitors made promising results in preclinical research (Cripe et al. 2010; Hyafil et al. 1993; Baer et al. 2002). However, because of the unacceptable side-effects or interference of pharmacokinetics of co-administrated anticancer drugs, no reversal agents were used in clinical cancer therapy until now. Therefore, the development of new inhibitors is needed. A variety of traditional Chinese herbal medicines were used in cancer therapy. Some of them could reverse MDR (Chang et al. 2004; Wang et al. 2010; Wu et al. 2003; Yang et al. 2011). Lang-du has been used in cancer therapy by traditional Chinese healers for several hundred years. J196-9-4 is a myrsinol diterpene isolated from *Euphorbia prolifera* Buch.-Ham and did not exhibit cytotoxicity to the cancer cells below 50 μM.

J196-9-4 treatment reversed the resistance to daunorubicin, vincristine, and topotecan to different extents, which indicated that this compound reversed MDR of breast cancer cells. P-gp mediated efflux expelled the P-gp substrate rhodamine 123 and reduced cellular fluorescence intensity. The fluorescence intensity increased after J196-9-4 treatment indicating that it was an inhibitor of P-gp. The P-gp dependent efflux was coupled with ATP hydrolysis (Al-Shawi et al. 2003). After treatment with J196-9-4, ATP consumption increased in a dose-dependent manner. J196-9-4 is likely a substrate of P-gp and can be expelled by the transporter. Therefore, this compound might act as a competitive inhibitor. It competes with cytotoxic agents for the substrate binding sites of P-gp and prevents them from extrusion. In consequence, this process enhances cytotoxicity of the drugs.

J196-9-4 can re-sensitize MDR of P-gp overexpressing cancer cells to daunorubicin, vincristine, and topotecan effectively. This compound blocks rhodamine 123 efflux mediated by P-gp and stimulated ATPase activity of P-gp. Therefore J196-9-4 is a competitive inhibiter of P-gp and can reverse MDR.

4. Experimental

4.1. Chemicals and reagents

Daunorubicin, vincristine, topotecan, doxorubicin, MTT, DMSO, verapamil, and rhodamine 123 were purchased from Sigma-Aldrich. DMEM medium, fetal bovine serum (FBS) was purchased from Gibco.

4.2. Cell culture

Human breast adenocarcinoma MCF-7 and its multidrug resistant subline MCF-7/Adr were maintained in 90% RPMI 1640 supplemented with 10% FBS and 10 μg/ml human insulin at 37 °C and 5% CO_2. Doxorubicin was applied to MCF-7/Adr every week at 1 μg/mL to maintain resistance. Both cell lines were kindly provided by Prof. Yuanfu Xu (Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin, China).

4.3. Cytotoxicity assay

Aliquots containing 1×10^4 cells per well were seeded into 96-well plates. Different concentrations of cytotoxic agents with or without the compound were added into wells after the cells were incubated for 24 h at 37 °C and 5% CO_2. Then cells were incubated for another 48 h. MTT at a final concentration of 0.5 μg/ml were added and incubated for 4 h. The medium was discarded and formazan crystals dissolved in 150 μL DMSO. The plates were read at 570 nm with a spectrophotometric microplate reader (Model 680, Bio-Rad). IC_{50} was defined as the concentration of compounds required to inhibit growth of treated cells by 50% compared with control. Reversal ratio was defined as (IC_{50} of MDR cells / IC_{50} of reversal agents treated MDR cells).

4.4. Efflux assay

Efflux assays were performed as described before with some modification (Li et al. 2011b). Aliquots containing 1×10^4 cells per well were inoculated into 96-well plates, which were incubated at 37 °C and 5% CO_2 for 4 days. The compound was applied at different concentrations into wells 15 min after 1 μg/mL rhodamine 123 had been added. After incubation, the plates were washed twice with ice cold PBS. The plates were then scanned at excitation wavelength of 480 and emission wavelength of 550 with the fluorometer (Twinkle LB 970, Berthold). Sensitive cells and MDR cells treated with fluorescent dyes alone represented 100% inhibition and 0% inhibition of transporters, respectively. Inhibition efficiency was defined as (fluorescence intensities of treated MDR cells - fluorescence intensities of untreated MDR cells) / (fluorescence intensities of sensitive cells - fluorescence intensities of untreated MDR cells) × 100%.

4.5. ATPase assay

Effects of the compound on the ATPase activity of P-gp were measured with Human P-gp membrane (Gentest™) and ATPase Assay Kit (Gentest™). Since orthovanadate blocks ATP hydrolysis of ABC transporters, sodium orthovanadate treated membrane was considered as 0% ATPase activity, whereas untreated membrane represented the basal level of ATPase. Experiments were conducted following the manufacturer's instructions. ATPase activation efficiency was defined as [(treated groups – sodium orthovanadate treated groups) / (untreated groups – sodium orthovanadate treated groups)-1] × 100% (Lei et al. 2013).

4.6. Data analysis

All assays were performed in triplicate at least three times. Data are presented as mean±SD. Differences between groups were determined using a two-sided Student's t-test.

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Conflicts of interest: None declared.

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