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Tenacigenin B Derivatives Reverse P-Glycoprotein-Mediated Multidrug Resistance in HepG2/Dox Cells

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Tenacissimoside A (**1**) and 11α-*O*-benzoyl-12β-*O*-acetyltenacigenin B (**2**), two derivatives of tenacigenin B (**3**) from the plant *Marsdenia tenacissima,* reversed multidrug resistance in P-glycoprotein (Pgp)-overexpressing multidrug-resistant cancer cells. The sensitivity of HepG2/Dox cells to the antitumor drugs doxorubicin, vinblastine, puromycin, and paclitexel was increased by 18-, 10-, 11-, and 6-fold by 20 μg/mL (or 25 μM) of **1** and 16-, 53-, 16-, and 326-fold by 20 μg/mL (or 39 μM) of **2**, respectively. A preliminary mechanistic study has suggested that **1** might modulate Pgp-mediated multidrug resistance through directly interacting with the Pgp substrate site.

Multidrug resistance (MDR) is a major obstacle for the successful chemotherapy of cancer. One of the most clearly evident mechanisms is the overexpression of P-glycoprotein (Pgp), an ATP-dependent membrane transporter.[1] Many currently used anticancer drugs including vinblastine, puromycin, paclitaxel, and doxorubicin are Pgp substrates. Overexpressed Pgp actively transports the drugs out of cancer cells, leading to remarkable decreases in cellular drug accumulation.[2] Pgp modulators (or inhibitors) resensitize MDR cells to anticancer drugs mainly through inhibiting Pgp-mediated drug efflux. Verapamil was the first MDR modulator discovered.[3] Natural organic compounds belonging to different chemical families such as coumarins, terpenoids, and steroids have also been found with MDR-modulating activity.[4–7] *Marsdenia tenacissima* (Roxb.) Moon. is a plant of the Asclepiadaceae family distributed mainly in the southwest of mainland China, and its dried rhizomes and roots have been used for the treatment of cancer.[8] Some of the polyoxypregnane compounds isolated from this plant showed cytotoxic activity against cancer cell lines.[9] In an attempt to find anticancer constituents or MDR-reversal agents, we have isolated two compounds from *M. tenacissima* and investigated their activities on cancer cells. Here we report that tenacissimoside A (**1**) and 11α-*O*-benzoyl-12β-*O*-acetyltenacigenin B (**2**), two derivatives of tenacigenin B (**3**), increased drug sensitivity in Pgp-overexpressed human hepatoma cells.

As reported previously, human hepatoma HepG2/Dox cells are highly resistant to the Pgp substrate drugs vinblastine, doxorubicin, puromycin, and paclitaxel when compared with HepG2.[10] In this study, compounds **1** and **2** themselves showed no significant growth inhibitory effect in both sensitive and resistant cell lines, as their concentrations to achieve 50% inhibition of cell growth were both greater than 80 μM, but they remarkably increased drug sensitivity of HepG2/Dox cells when coadministered with a drug substrate (Table S1, Supporting Information). Compared with HepG2/Dox cells in the absence of any Pgp modulator, HepG2/Dox cells in the presence of 20 μg/mL (25.2 μM) of **1** were 18, 10, 11, and 6 times more sensitive to doxorubicin, vinblastine, puromycin, and paclitaxel, respectively. Corresponding increases in the presence of 20 μg/mL (39.2 μM) of **2** were 16, 53, 16, and 326 times, respectively. Compounds **1** and **2** at 10 μg/mL also exhibited activity in effectively reversing MDR (Table S1, Supporting Information). Increases in drug sensitivity by addition of **1** or **2** were not observed in drug-sensitive HepG2 cells, implying that the two compounds acted mainly on the overexpressed Pgp.

The cell-cycle analysis revealed that **1** at 20 μg/mL did not affect cell proliferation but markedly enhanced the action of doxorubicin in HepG2/Dox cells. Doxorubicin is a topoisomerase II inhibitor that induces G2/M arrest in the cell cycle. In drug-sensitive HepG2 cells, doxorubicin concentration to achieve a complete G2/M arrest was 0.2 μM. In HepG2/Dox cells, however, Pgp extrudes the substrate drug out of the cells, and the concentration to achieve a complete G2/M arrest was up to 50 μM. With the addition of 20 μg/mL of compound **1**, the doxorubicin concentration to achieve a complete G2/M arrest decreased from 50 μM to 1 μM (Table S2, Supporting Information).

As a Pgp modulator, **1** was tested for activity on Pgp-mediated drug transport by flow cytometry. It was found in a drug accumulation assay that **1** increased significantly doxorubicin uptake in MDR cells. Compared with fluorescence in HepG2/Dox cells incubated with doxorubicin alone, cellular fluorescence in HepG2/Dox cells incubated with doxorubicin in combination with 10, 20, or 40 μg/mL of **1** was increased by 18%, 28%, or 41%, respectively (Figure 1). This substance also inhibited significantly the efflux of rhodamine-123 (Rh-123) and Hoechst 33342, which are two fluorescent Pgp substrates, in HepG2/Dox cells. Compared to cells in the absence of Pgp modulator, cellular Rh-123 retention was increased respectively by 50% and 90% by 40 and 80 μg/mL of **1**. Corresponding Hoechst 33343 retention was increased by 160% and 180%, respectively (Figure 2).

Some Pgp modulators may suppress Pgp expression. Western blot analysis revealed that treatment with 20 μg/mL of **1** for 72 h did not alter Pgp expression level in HepG2/Dox cells (Figure 3), implying **1** is not a gene suppressor but a direct Pgp inhibitor that reverses MDR through directly inhibiting Pgp transport function.

The mAbs UIC2 is a conformation-sensitive antibody of Pgp. Pgp reactivity to UIC2 can be increased by the binding of a transport substrate or competitive inhibitor such as cyclosporin A and can be decreased by the binding of an allosteric inhibitor such as sodium vanadate (Na_3VO_4).[11,12] In this experiment, **1** increased UIC2 labeling to HepG2/Dox cells like cyclosporin A, suggesting a substrate-like activity (Figure 4).

In summary, compounds **1** and **2** increased drug sensitivity of HepG2/Dox cells but not the sensitivity of the parental sensitive HepG2 cells, suggesting that they affected mainly the overexpressed Pgp. As a Pgp modulator, compound **1** reversed Pgp-MDR not through suppressing Pgp expression but through directly interacting

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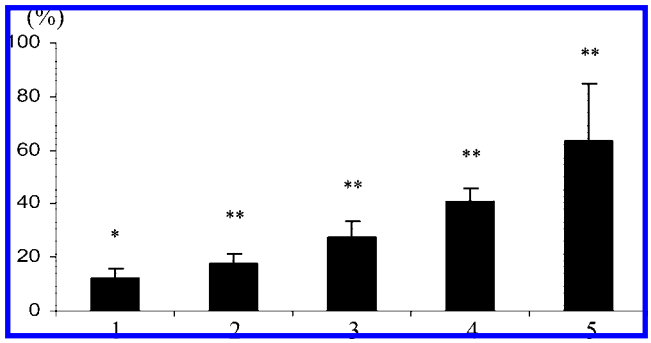


Figure 1. Effect of **1** on doxorubicin accumulation in HepG2/Dox cells. HepG2/Dox cells were treated with 10 μM doxorubicin in the absence (control) or presence of 5 μg/mL (column 1), 10 μg/mL (column 2), 15 μg/mL (column 3), or 20 μg/mL (column 4) of **1**, or 10 μM verapamil (column 5), at 37 °C for 40 min. Increase in cellular fluorescence (%) = 100 × (F_T − F_C)/F_C, where F_T is the fluorescent intensity in cells treated with **1** or verapamil (VRP) and F_C is the fluorescent intensity in control cells. Results are expressed as means ± SD of three experiments. Compared to control, data were significantly different at $*p < 0.05$ and $**p < 0.01$.

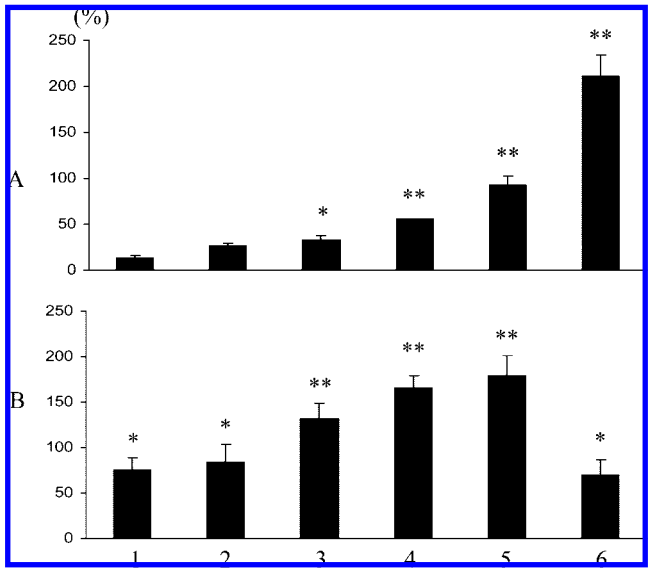


Figure 2. Inhibitory effect of **1** on drug efflux in HepG2/Dox cells. Cells were incubated with 5 μg/mL Rh-123 (graph A) or 20 μg/mL Hoechst 33342 (graph B) for 1 h followed by an additional hour of incubation in Rh-123- or Hoechst 33343-free medium containing 0 μg/mL (control), 5 μg/mL (column 1), 10 μg/mL (column 2), 20 μg/mL (column 3), 40 μg/mL (column 4), or 80 μg/mL (column 5) of **1**, or 15 μM verapamil (column 6). Increase of cellular fluorescence is shown in percentage (= 100 × (F_T − F_C)/F_C) as described in legend for Figure 1. Results are expressed as means ± SD of three experiments. Compared with control, data were significantly different at $*p < 0.05$ and $**p < 0.01$ level by the Student's *t* test.

with the substrate site(s) on Pgp, therefore hindering the binding and transportation of drug substrate.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a PE343 polarimeter. NMR spectra were recorded on a Bruker AM-400 MHz spectrometer using the tetramethylsilane signal as an internal reference. ESIMS were recorded on a Bio TOF-Q mass spectrometer.

Plant Material. A sample of *Marsdenia tenacissima* (Roxb.) Moon. was collected from Xishuangbanna, Yunnan, People's Republic of China, in December 2005. The plant was identified by Dr. Li-Gong Lei, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (2005-12-03m) has been deposited at Kunming Institute of Botany, Kunming, People's Republic of China.

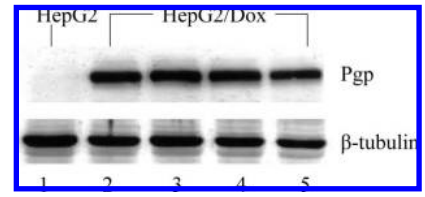


Figure 3. Compound **1** did not alter Pgp expression level in HepG2/Dox cells. Cells were incubated without (lane 2) or with 5 μg/mL (lane 3), 10 μg/mL (lane 4), or 20 μg/mL (lane 5) of **1** for 72 h. Lysed cell extracts containing 50 μg of total cellular protein were separated on 10% SDS-PAGE. Pgp was detected by antibody labeling. Samples in lane 1 were from untreated HepG2 cells.

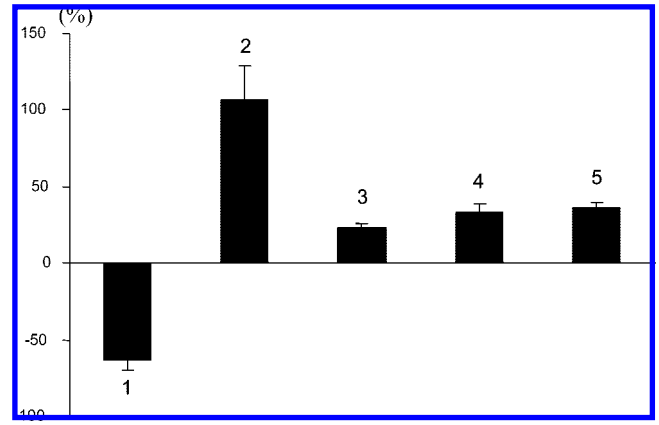


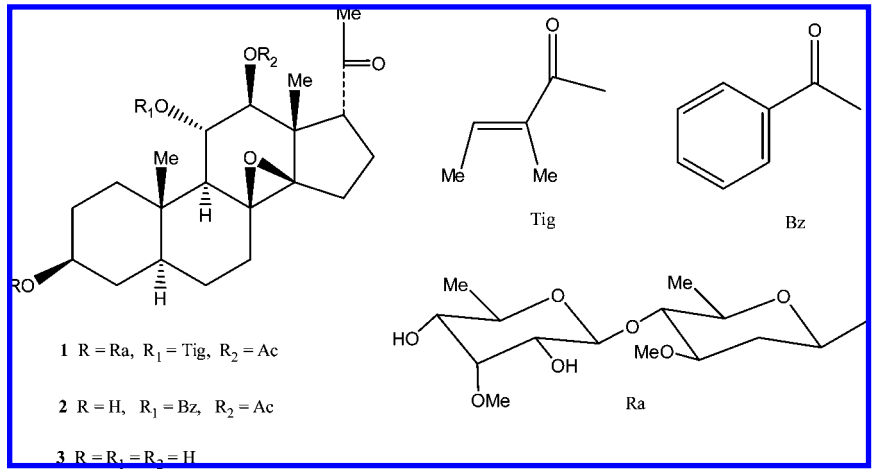
Figure 4. Compound **1** increased Pgp reactivity to mAb UIC2. HepG2/Dox cells were reacted with mAb UIC2 at 37 °C, in the presence or absence (control) of 1 mM Na_3VO_4 (column 1), 5 μM cyclosporin A (column 2), 10 μg/mL (column 3), 20 μg/mL (column 4), or 40 μg/mL (column 5) of **1**. UIC2-bound Pgp was detected with a fluorescent secondary antibody, and cells were analyzed by flow cytometry. Results are expressed as percentage increase of cellular fluorescence intensity (= 100 × (F_T − F_C)/F_C) as described in the legend for Figure 1.

Extraction and Isolation. Air-dried rhizomes and roots of *M. tenacissima* were pulverized (1.7 kg) and extracted with EtOH. The ethanol extract was partitioned in H_2O, defatted with petroleum ether, and extracted with EtOAc. The EtOAc extract (50.0 g) was passed over silica gel columns by elution with mixed solvents of $CHCl_3$−MeOH (100:0 to 80:20) and further purified by repeated ODS silica gel column chromatograph, eluting with MeOH−H_2O (7:3 to 9:1), to afford **1** (900 mg, 0.053%) and **2** (29 mg, 0.0017%). Both **1** and **2** appeared as white, amorphous solids with $[\alpha]^{25}_D$ −26.3 (*c* 0.076, CH_3OH) and +16.2 (*c* 0.037, CH_3OH), respectively. They were identified as tenacissimoside A (**1**) and 11α-*O*-benzoyl-12*β*-*O*-acetyltenacigenin B (**2**) by comparing their ^1H and ^{13}C NMR spectroscopic data, and their ESIMS data with those reported in the literature.[9,13]

Cell Line and Cell Culture. The HepG2 human hepatoma cell line and a doxorubicin selected Pgp-MDR subline HepG2/Dox were grown in RPMI 1640 medium containing 10% FBS and 100 U antibiotics, at 37 °C in a humidified 5% CO_2 incubator. To maintain the drug resistance phenotype, 1.2 μM doxorubicin was added into the medium of HepG2/Dox cells. The MDR cells were grown in drug-free medium for at least 7 days before use.

Growth Inhibitory Assay. Inhibitory effects of various drugs on cell growth were determined by a SRB assay and evaluated with the respective IC_{50} values, as described previously.[10] The growth inhibition experiment was repeated at least three times, and the results were expressed as means ± standard deviation (SD). Solvents and media were included as blank control.

Chart 1



MDR Reversing Potential Evaluation. The activities of **1** and **2** in increasing drug sensitivity of cancer cells was evaluated by comparing IC_{50} values of an anticancer drug in the absence or presence of **1** or **2** and expressed as fold decrease in IC_{50} values of an anticancer drug achieved with **1** or **2**. Cells treated with **1** or **2** alone at its working concentration are used as a drug negative control. Verapamil, a known Pgp inhibitor, was used as a positive control.

Cell Cycle Analysis. Approximately 2×10^5 cells in 1 mL of medium were seeded in a 35 mm culture dish and incubated overnight. Next 1 mL of medium with or without drug was added and incubated for 48 h. Cells were collected, washed with PBS twice, and then fixed with 70% EtOH at −20 °C overnight. After this, the cells were resuspended in 1 mL of PBS containing 100 μg/mL RNase A and incubated at 37 °C for 30 min. Propidium iodide (PI) staining solution was added at a final concentration of 40 μg/mL and incubated at room temperature for 5−10 min. Samples were then placed in 12 × 75 Faclon tubes, and analysis was performed by a FACSCalibur flow cytometer.

Doxorubicin Accumulation Assay. Approximately 1×10^6 HepG2/Dox cells were suspended in 1 mL of medium containing 10 μM doxorubicin with or without **1** and incubated at 37 °C for 40 min. Cells were washed with ice-cold PBS twice and resuspended in 1 mL of ice-cold PBS. Cellular doxorubicin fluorescence was monitored by a FACSCAN flow cytometer. Verapamil was again used as a positive control.

Rhodamine-123 Efflux Assay. Rhodamine-123 (Rh-123) (5 μg/mL) was added to 1×10^6 cells in a complete growth medium (1 mL), and the cells were incubated at 37 °C for 1 h to allow Rh-123 uptake. Rh-123 loaded cells were washed with ice-cold PBS twice and resuspended in fresh medium with or without **1**. After 1 h of incubation at 37 °C, cells were washed with ice-cold PBS twice and resuspended in 1 mL of ice-cold PBS and analyzed by a FACSCAN flow cytometer.

Hoechst 33342 Efflux Assay. The HepG2/Dox cells (5×10^4 cells in 100 μL of medium per well) were seeded in 96-well plates and incubated overnight. The medium was replaced with fresh medium containing 20 μg/mL of Hoechst 33342, and the cells were incubated at 37 °C for 1 h. The cells were washed with 100 μL of ice-cold PBS twice, added with a new medium containing a Pgp modulator of various concentrations, and were further incubated at 37 °C for 1 h. The cells were washed with ice-cold PBS twice. The cellular fluorescence intensity was measured at $\lambda_{ex} = 365$ nm ($\lambda_{em} = 460$ nm) by a BMG FLUOstar OPTIMA microplate reader. The inhibitory effects of **1** on Rh-123 or Hoechst 33342 efflux were expressed as the percentage increases of retained Rh-123 or Hoechst 33342 in MDR cells. Verapamil was used as a positive control.

Pgp-UIC2 Reactivity Assay. Reactivity of Pgp to monoclonal antibody UIC2 was performed as described previously.[10] Briefly, HepG2/Dox cells were washed with PBS and resuspended in UIC2 binding buffer (PBS plus 1% BSA). Approximately 1×10^6 cells in 800 μL of binding buffer were prewarmed at 37 °C for 10 min and incubated with drugs at 37 °C for another 10 min, and 1 μg of the monoclonal antibody UIC2 was added. After 15 min at 37 °C, 700 μL of ice-cold UIC2 buffer was added to stop the reaction. The cells were washed with ice-cold UIC2 binding buffer twice and resuspended in 500 μL of ice-cold UIC2 binding buffer, and 2 μL of goat anti-mouse IgG_{2a}-PE was added. After 15 min at 4 °C in the dark, the cells were washed and resuspended in 1 mL of ice-cold UIC2 binding buffer. The cellular fluorescence intensity was analyzed using a FACSCalibur flow cytometer. Normal mouse IgG_{2a} served as a negative control.

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Supporting Information Available: Tables showing the sensitizing effects by compounds **1** and **2** on Pgp-overexpressing HepG2/Dox cells to anticancer drugs. This information is available free of charge via the Internet at http://pubs.acs.org.

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