L-4 Multidrug-Resistance Modulators from Medicinal Plants

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Introduction

The multidrug resistance(MDR) is characterized by the cross resistance to broad range of structurally and functionally unrelated drugs. MDR in tumors is one of the major obstacles to successful cancer chemotherapy. A primary mechanism of MDR is attributed to the overexpression of P-glycoprotein (P-gp) in plasma membrane of resistant cell where the P-gp acts as an energy dependent efflux pump, reducing intracellular accumulation of anticancer drugs(1). Thereby MDR phenomenon greatly limits the efficacy of many anticancer drugs originated from natural products such as vinca alkaloids, anthracyclines and many antitumor antibiotics and this problem may be extended to newly developed taxol and campthotecin. Many kinds of drugs such as calcium channel blockers, calmodulin inhibitors and indole alkaloids are known to reverse MDR by competing with anticancer drugs for binding to P-gp(2).

In an attempt to overcome MDR, significant efforts have been accumulated in identification of a number of compounds, i.e. taxoids, welwitindolinone, bisbenzylisoquinoline, torilin and dihydro-β-agarofuran, that are able to reverse P-gp-mediated MDR in *in vitro(3-7)*. Clinical trials of MDR reversing agents continue, however, problems with systematic toxicity of agents have thus far limited their utility(8). Thus, there remains a need to discover and characterize new classes of MDR reversing agents.

Results and Discussion

In our search for MDR reversing agents from natural product, methanol extracts from 450 plants were screened for multidrug-resistance reversing activity using drug sensitive KB-3-1 and multidrug-resistant KB-V1 cells(9). Among them, the extracts of *Cynanchum wilfordii*, *Torilis japonica*, *Celastrus orbiculatus*, *Melia toosendan,* and *Taxus cuspidata* strongly potentiated vinblastine cytotoxicity in KB-V1 cells. But their cytotoxicities to both sensitive KB-3-1 and resistant KB-V1 cells are in the same order of magnitude. We isolated 23 active principles by bioactivity-guided fractionation and separation of the MeOH extract of plants and identified their structures by spectroscopic analyses. Among them torilin isolated *Torilis japonica* and wilfosides isolated from *Cynanchum wilfordii* were extensively studied their mechanism of action, *in vitro* MDR reversing activity and antiangiogenic activity.

The effects of isolated compounds on the cell growth were determined in both drug sensitive KB-3-1 and MCF7 cells and resistant KB-V1 and MCF7/ADR cells. All the compounds did not show significant cytotoxicity to the cell lines tested. They did not show distinguishable difference in cytotoxicities between sensitive and MDR cells.

The relative resistances of MDR cell lines, KB-V1 and MCF7/ADR to various anticancer drugs were determined by exposing each cell to various concentrations of ADR, VLB, TX and COL for 48 h. The KB-V1 and MCF7/ADR cells displayed typical MDR phenotype, having significant cross-resistance to multiple anticancer or cytotoxic drugs unrelated to selecting agent in structures and mechanism of action.

To evaluate MDR reversing activities of torilin, wilfoside and orbiculin the sensitivity of each cell to various anticancer drugs was determined in the absence or presence of MDR modulators. All of them did not induce significant effects on the sensitivity to anticancer drugs of KB-3-1 and MCF7 cells. However, they strongly enhanced cytotoxicities of anticancer drugs against MDR cells and decreased RR of MDR cells in dose-dependent manners. For example, 10 mM of torilin reversed partially the resistances of KB-V1 cells to ADR from 762-fold to 92.08-fold, to VLB from 2198-fold to 37.82-fold, to COL from 673-fold to 112.43-fold and to TX from 8039-fold to 87.84-fold. The similar patterns of effect of other compounds on MDR were observed in KB-V1 and MCF7/ADR cells.

To study the mode of action of torilin and wilfoside, their effects on the accumulation and efflux of VLB in KB-V1 cells were investigated in relation to verapamil. Torilin and wilfoside not only greatly increased accumulation of drug resistant KB-V1 cells in dose-dependent manners but also inhibited VLB efflux in KB-V1 cells. To investigate any association of torilin and wilfoside to the expression of mdr1 gene which is overexpressed in MDR cells, levels of mdr1 mRNA and P-gp were measured in cells treated with torilin, wilfoside and verapamil. However, there was no significant change in the expression of mdr1 mRNA by compounds treated, nor did they influence on the contents of P-gp detected by immunoblot. On the other hand, when the ATPase activity of membrane fraction of KB-V1 cells was measured, torilin and wilfoside enhanced membrane ATPase activity of KB-V1 cells. These results indicated that MDR reversing effects of torilin and wilfoside might be directly associated with the increase of the intracellular accumulation of anticancer drugs by blocking the drug efflux exerted by P-gp.

Experimental Section

General Experimental Procedures. Mps were measured on a model of Electrothermal 9100 without correction. Optical rotations were determined on JASCO DIP-181 polarimeter. UV spectra were obtained on a Milton Roy 3000 spectronic array. 1H-NMR (300 MHz), 13C-NMR (75 MHz) spectra were obtained on a Varian Unity using CDCl3 as a solvent. HMBC spectrum was determined on a Bruker at 500 MHz. EIMS were measured on a Hewlett Packard 5989A, HRMS on JEOL HX 110 mass spectrometer. Kieselgel 60 (Merck No. 9385 and 7729) and LiChroprep RP-18 were used for CC. Prep. HPLC was carried out on a DELTA-PAK C18 (19mm300mm, Waters) with detection at 230 nm. Fetal calf serum, media and supplement materials for cell culture were purchased from GIBCO-BRL (Grand Island, NY). Anticancer drugs (ADR, VLB and TX) were obtained from Sigma Chemical Co. (St. Louis, MO)

Plant materials. The fruits of *Torilis japonica* and *Evodia officinalis* and the roots of *Cynanchum wilfordii* were purchased at herbal drug store in Taejon. The roots of *Celastrus orbiculatus* were collected at Cheongju, Chungbuk province in Korea in 1995 and identified by Kyong Soon Lee, a plant taxonomist at Chungbuk National University. The solvent extract of *Taxus cuspidata* needles were obtained from Hankook Sin Yak Pharm. Co..

Cytotoxicity. Human oral epidermal cancer KB-3-1 and its VLB selected multidrug-resistant KB-V1 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 2 mM l-glutamine and 10% heat inactivated fetal calf serum and splited twice a week at 1:16 and 1:8 ratio, respectively. KB-V1 cells were maintained in the presence of 1 M VLB. All cells were grown at 37 C in humidified atmosphere with 5 % CO2. Cell growth was measured using SRB method(10). Cells in exponential growth were trypsinized, disperserd in single cell suspension and dispensed in 100 L volumes into 96 well plates. 2.5-5.0 103 cells/well were inoculated in 100 L medium containing 5% fetal calf serum and allowed to attach and grow overnight. One hundred microliters of medium containing anticancer drug and/or reversing compound was added and further incubated for 48 h. Drugs were dissolved in small amounts of DMSO or MeOH before dilution with medium (final concentration of solvent < 0.5%). Controls were exposed to vehicle-containing medium. Cells were fixed by gently layering 50 L of cold 50 % trichloroacetic acid (final concentrations 10%) on the top of the growth medium in each well and incubated at 4C for 1 h and then washed five times with tap water. Plates were air-dried, stained with 0.4% (w/v) sulforhodamine B in 1% acetic acid for 15-30 min and rinsed four times with 1% acetic acid to remove unbound dye. Plates were air-dried and bound dye was solubilized with 10 mM unbuffered Tris base on a shaker for 5 min. Absorbance was read with microtiter plate reader at 570 nm.

MDR Reversing Activity. IC50 was the concentration of drug that reduced absorbance to 50% of vehicle-treated controls. The cytotoxicity of each compound was determined in KB-V1 cells in the absence or presence of 100 nM of VLB. VLB cytotoxicity enhancement by each compound was expressed as enhancement factor (EF).

$$EF = \frac{\text{IC50 of modulator on KB-V1 cells without VLB}}{\text{IC50 of modulator on KB-V1 cells with VLB}}$$

In another set of experiment, the effects of compounds on MDR were studied by exposing cells to a range of concentrations of anticancer drugs, ADR, VLB, COL and TX in the absence or presence of MDR reversing compounds. Drug resistance of MDR cells to various anticancer drugs and MDR reversing effect of each compound were expressed as relative resistance (RR).

$$RR = \frac{\text{IC50 of anticancer drug with MDR modulator}}{\text{IC50 of anticancer drug on sensitive parent cells}}$$

Drug Accumulation. KB-3-1 and KB-V1 cells were plated in 24 well tissue culture plates at a density of 2.5x104 cells and 4x104 cells /well, respectively, and allowed to grow to 90 % confluency. After 48 h, the medium was replaced by serum-free DMEM containing 0.25 Ci/ml of [3H] VLB (11.2 Ci/mmol) in the absence or presence of compounds. At indicated time, cells were washed with ice-cold PBS three times on ice, harvested in 200 l of 0.2 N NaOH and incubated overnight at 37C. One and a half ml of scintillation cocktail was added to it and mixed thoroughly. Then the radioactivity was counted and expressed as the concentration of cell-associated VLB from a standard curve.

Drug Efflux. KB-3-1 and KB-V1 cells were incubated for 48 h and then the medium was replaced by serum-free DMEM. KB-3-1 cells and KB-V1 cells were incubated with 0.25 Ci/ml and 2.5 Ci/ml [3H] VLB, respectively, for 4 h to obtain same accumulation of [3H]VLB in both cell lines. Each well was washed with PBS and added to fresh serum-free DMEM in the presence or absence of compounds. At indicated time, cells were washed, harvested and the radioactivity was counted on a liquid scintillation counter. Cell-associated radioactivity was expressed as % of radioactivity at zero time.

Northern Blot. KB-3-1 and KB-V1 cells were inoculated a density of 3.5x 104 cells /cm2 in 56 cm2 tissue culture dishes, and incubated overnight. Cells were further incubated in the absence or presence of compounds. At indicated time, cells were harvested with ice-cold PBS and centrifuged at 1,000 g at 4℃ for 10 min. Total cellular RNA was isolated by TRIzolTM reagent. The cDNA 5A (*EcoR1-EcoR1*, 1.38 kb), which was provided by Dr. M. Gottesman, was labeled by random primed DNA labeling method using Megaprime DNA labeling system. The specific activity of the probe was approximately 1x109 cpm/g. Twenty microgram of total RNA was electrophoresed in 1% agarose-2.2 M formaldehyde gel using MOPS buffer (0.2 M MOPS pH 7.0, 80 mM sodium acetate, 10 mM EDTA), transferred onto nylon membrane and fixed by UV irradiation for 2-3 min. The membrane was prehybridized at 42℃ for 2h, added to 32P-labeled probe (1x107 cpm) and hybridized overnight at 42℃ in 50% (w/v) formamide, 6xSSC, 5x Denhardts solution, 0.5% SDS and 100 g/ml salmon sperm DNA. The membrane was washed twice with 2x SSC, 1% SDS at room temperature for 10 min and then twice with 0.1 SSC, 0.1% SDS at 65℃ for 10 min, dried and exposed to X-ray film at -70℃ for 2-3 days. To verify the amount of RNA on each lane, membrane was simultaneously hybridized with 32P-labeled β-actin cDNA probe.

Membrane ATPase Activity. KB-3-1 and KB-V1 cells were grown in the 500 cm2 tissue culture plates and harvested at confluency by scraping in ice-cold PBS. All steps were performed at 4C using ice-cold buffer. Cells were washed twice in PBS and collected by centrifugation at 1,000g for 5 min. The pellets were resuspended to be 5x 107 cells/ml in a hypotonic lysis buffer containing 10 mM Tris-HCl pH 7.4, 0.25 M sucrose, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptine, 1 g/ml pepstatin A and 1 mM EGTA, and incubated on ice for 20 min. Swollen cells were homogenated in a glass homogenizer with 30-40 strokes. Homogenates were centrifuged at 1,000g for 10 min. The supernatant was then layered on a 35% sucrose cushion in the lysis buffer and centrifuged at 16,000 x g for 1h. Membrane was collected at the interface and diluted two-fold with lysis buffer and ultracentrifuged at 100,000g for 1h. The pellet was washed and resuspended at 1 mg/ml in a lysis buffer supplemented protease inhibitors using 25-gauge needle and stored at -70 C. ATPase activity was determined by measuring of Pi released from ATP using the Fieske and Subbrow method(12). Ten microgram of membrane protein was incubated at 37 C for 30 mim in a 100 l of assay medium, which included 10 mM Tris-HCl pH 7.4, 0.25M sucrose, 1 mM MgCl2, 10 mM NaN3, 1 mM ouabain and 5 mM phosphoenol pyruvate and 3.6 units/ml pyruvate

kinase as an ATP regenerating system. Compounds were dissolved in DMSO (final conc. 1%). Reaction was started by the addition of 3 mM of MgATP. At indicated time, 40 l of 1 % NH4MoO4 in 2 N H2SO4 and 10 l of Fieske and Subbrow reducer was added and absorbance was measured at 650 nm after 10 min. Each reaction was also assayed in the presence of 1mM of sodium vanadate. The activity of P-gp ATPase was defined as vanadate sensitive portion of total ATPase activity.

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Fig. 1. Representative MDR reversing agents

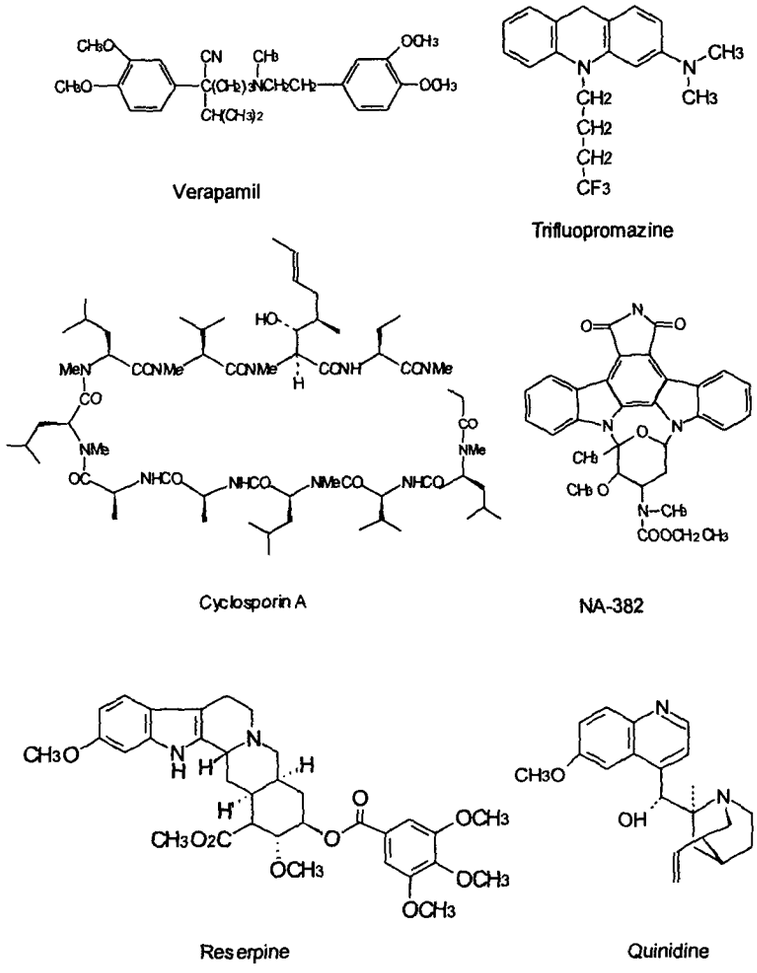
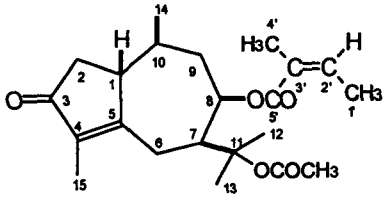
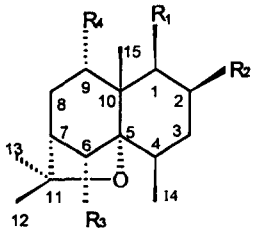
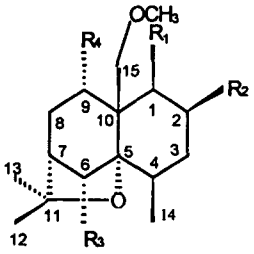


Fig. 2. Strucuures of compounds isolated from plant extracts reversing multidrug-resistance



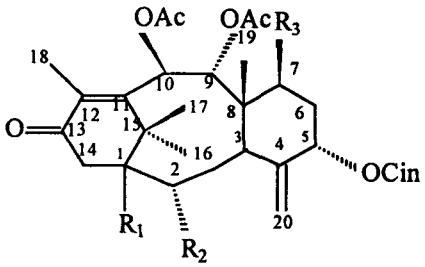
1

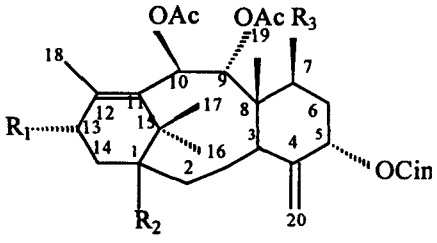




2	R_1=OAc	R_2=H	R_3=OFu	R_4=OBz
3	R_1=OAc	R_2=H	R_3=OBz	R_4=OFu
4	R_1=OAc	R_2=H	R_3=OFu	R_4=OFu
11	R_1=OAc	R_2=H	R_3=OBz	R_4=OBz
12	R_1=OAc	R_2=H	R_3=OCin	R_4=OBz
5	R_1=OAc	R_2=OAc	R_3=OFu	R_4=OBz
6	R_1=OAc	R_2=OFu	R_3=OFu	R_4=OBz
7	R_1=OAc	R_2=OBz	R_3=OBz	R_4=OBz
8	R_1=OAc	R_2=OAc	R_3=OBz	R_4=OBz
13	R_1=OAc	R_2=OAc	R_3=OAc	R_4=OBz

9	R_1=OAc	R_2=H	R_3=OAc	R_4=OBz
10	R_1=OAc	R_2=OBz	R_3=OAc	R_4=OBz





14	R_1=H	R_2=OH	R_3=OAc	17	R_1=OAc	R_2=OH	R_3=OAc
15	R_1=H	R_2=OAc	R_3=OAc	18	R_1=OAc	R_2=H	R_3=OAc
16	R_1=H	R_2=OAc	R_3=H				