Pregnane Glycoside Multidrug-Resistance Modulators from *Cynanchum wilfordii*

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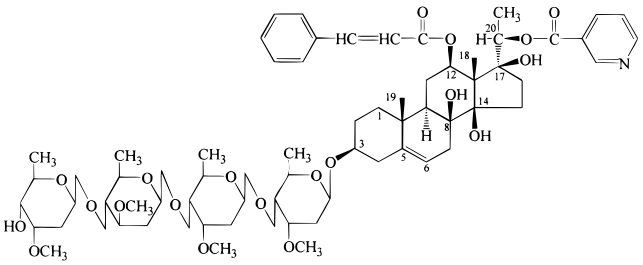
The methanol-soluble extracts of the roots of *Cynanchum wilfordii* showed a significant multidrug-resistance-reversing activity, and four known pregnane glycosides were isolated by bioassay-directed fractionation and separation. Their structures were identified as gagaminin 3-*O*-*β*-D-cymaropyranosyl-(1→4)-*β*-D-oleandropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside (**1**), wilfoside K1N (**2**), wilfoside C1N (**3**), and cynauricuoside A (**4**). In particular, compound **1**, at a concentration level of 1 *μ*M, was found to completely reverse the multidrug-resistance of KB-V1 and MCF7/ADR cells to adriamycin, vinblastine, and colchicine.

Multidrug resistance (MDR) by tumors is one of the major obstacles to successful cancer chemotherapy. A primary mechanism of MDR is often associated with the overexpression of P-glycoprotein (P-gp) in plasma membranes of resistant cell where the P-gp acts as an energy-dependent efflux pump, reducing intracellular accumulation of anticancer drugs.[1] Since P-gp plays a central role in MDR, efforts have been made to identify compounds that interact with P-gp. Extensive studies have been done with verapamil and cyclosporins to investigate their ability to overcome MDR.[2−5] Other classes of compounds such as progesterone and other steroids and antiestrogenic agents have been found to increase the sensitivity of MDR cells to anticancer drugs.[6−8]

Steroidal glycosides, which closely resemble the steroid hormone, are widely distributed in plants, and medicinal plants containing steroidal glycosides are commonly used for medicinal purposes in many Asian countries. In a search for MDR-reversing agents from natural sources, it was found that an extract of *Cynanchum wilfordii* Hemsley (Asclepidaceae) roots strongly potentiated the cytotoxicity of vinblastine in multidrug-resistant KB-V1 cells but not in drug-sensitive KB-3-1 cells.[9] Several steroidal glycosides including C/D-*cis*-polyoxypregnane esters have been isolated from the roots of *Cynanchum* species,[10−16] but their biological activity has not been reported. The MDR modulating activity of four pregnane glycosides (**1**−**4**), which were identified by their enhancing activity of vinblastine-cytotoxicity in multidrug-resistant KB-V1 cells, is described herein.

Bioactivity-guided fractionation of the MeOH extract of the roots followed by repeated column chromatography and HPLC separation led to the isolation of four active pregnane glycosides (**1**−**4**). All compounds showed positive Keller−Killiani and Liebermann−Burchard reactions, which indicated the presence of steroidal glycosides with a 2-deoxy sugar in the structure, and were identified as gagaminin 3-*O*-*β*-D-cymaropyranosyl-(1→4)-*β*-D-oleandropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside (**1**), kidjoranine 3-*O*-α-L-cymaropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-cymaropyranoside (wilfoside K1N) (**2**), caudatin 3-*O*-α-L-cymaropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-cymaropyranoside (wilfoside C1N) (**3**), and kidjoranine 3-*O*-*β*-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-cymaropyranoside (cynauricuoside A) (**4**), respectively, by comparison of their physical and spectral data with those of previous reports.[10,11,13,17]



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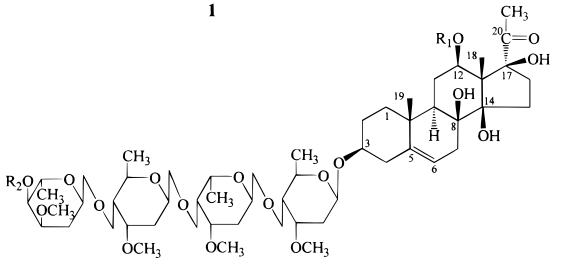

2 R_1 = —C(=O)—CH=CH— (phenyl); R_2 = H

3 R_1 = —C(=O)—CH=C(CH$_3$)—CH(CH$_3$)$_2$; R_2 = H

4 R_1 = —C(=O)—CH=CH— (phenyl); R_2 = -β-D-glc

The cytotoxicity of **1**−**4** was measured in both drug-sensitive KB-3-1 and multidrug-resistant KB-V1 cells in the absence or presence of vinblastine (VLB), and the IC_{50}

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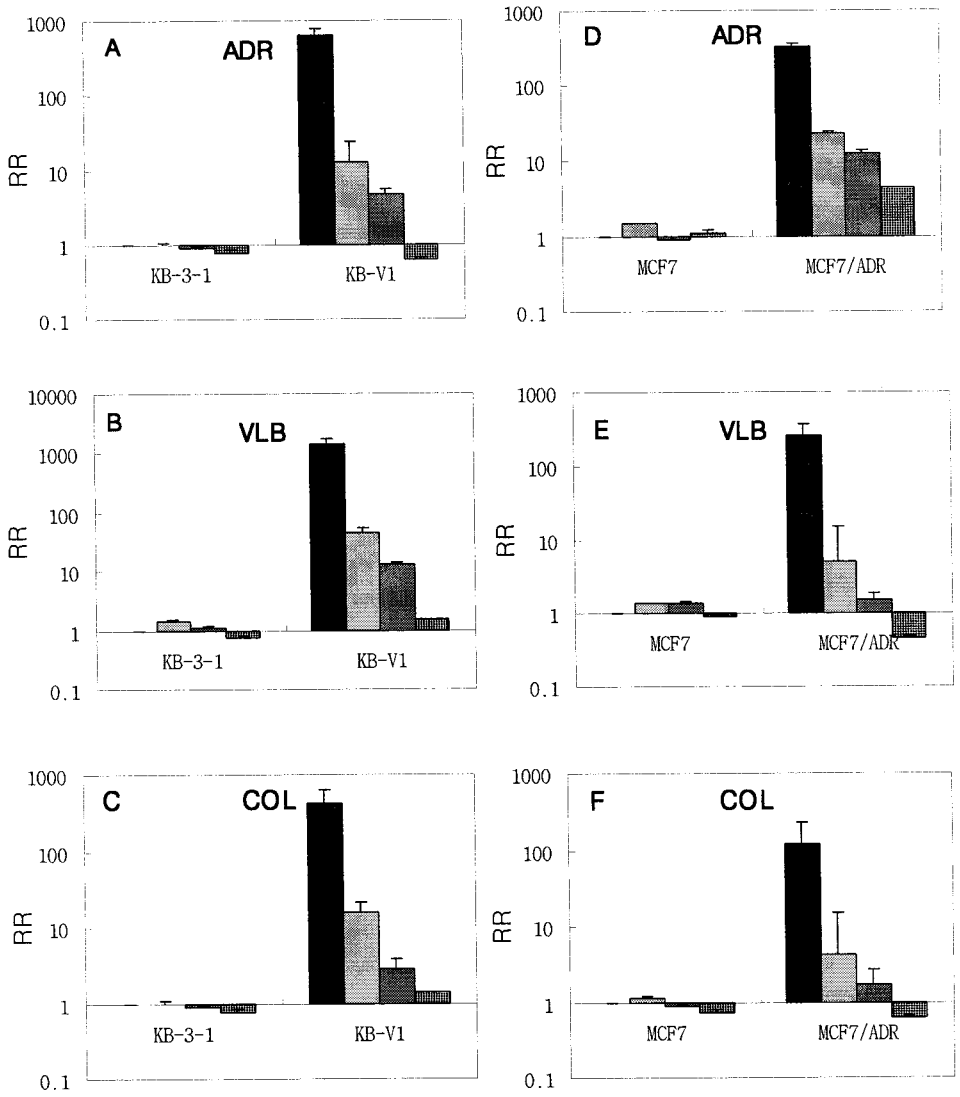

Figure 1. Effects of **1** on the resistance of MDR cells to standard anticancer drugs. Drug-sensitive and drug-resistant cells were treated with adriamycin (ADR), vinblastine (VLB), and colchicine (COL) in the presence of 0 (filled), 0.1 (light dot), 0.3 (dark dot), and 1 (hatched) μM of compound **1**. Cell growth was measured, and relative resistance (RR) was calculated as described in the Experimental Section. A−C: KB cells. D−F: MCF cells. Bars: mean ± SD of triplicate.

Table 1. Effects of Compounds **1**−**4** and Verapamil on the Growth of Drug-Sensitive and Drug-Resistant Cells

	IC_{50}[a] (μg/mL)			
compd	KB-3-1	KB-V1	KB-V1 (+VLB)	EF[b]
1	15.3 ± 1.9	17.3 ± 0.1	0.02 ± 0.01	865.0
2	23.3 ± 4.4	23.9 ± 1.8	0.82 ± 0.11	29.1
3	34.9 ± 3.3	37.2 ± 4.2	1.32 ± 0.08	28.2
4	225.0 ± 22.5	>300	99.1 ± 18.6	3.0
verapamil	20.5 ± 2.6	15.3 ± 2.7	0.69 ± 0.12	22.2

[a] Data are mean ± SD of three separate experiments. [b] EF (enhancement factor) of VLB (100 nM) cytotoxicity by **1**-**4** was calculated as follows: IC_{50} of KB-V1 cells without VLB/IC_{50} of KB-V1 cells with VLB.

of each compound for KB-V1 cells was divided by the IC_{50} of compound with 100 nM of VLB to give a cytotoxicity enhancement factor (EF). As shown in Table 1, all of the compounds were not significantly toxic at the dose levels used and showed no discernible difference in cytotoxic activity between drug-sensitive and drug-resistant cells. However, in the presence of 100 nM VLB, the IC_{50} values of **1**−**3** for KB-V1 cells were remarkably reduced by between 28- and 865-fold. The concentration of VLB added was lethal to drug-sensitive KB-3-1 cells but had no effect on the growth of drug-resistant KB-V1 cells. These results clearly demonstrate that **1**−**3** reverse MDR in KB-V1 cells. As a pentaglycoside, **4** differs structurally from **2** by having one more glucose moiety and showed the weakest cytotoxicity as well as the least potent MDR-reversing activity. Tetraglycosides **2** and **3** showed similar levels of cytotoxicity and MDR-reversing activity, comparable to those of verapamil. The only structural difference between **2** and **3** is the substituent at C-12; **2** has a cinnamoyl group instead of an ikemaoyl group as in **3**. Compound **1** was shown to be the most active with an EF value of 865, which was 30-fold higher than those of **2** and **3**. The major structural difference of **1** from **2** and **3** is the presence of a pyridine-3-carboxyl group at C-20 in addition to the stereochemistry of the three deoxy sugars. These results point out that the MDR-reversing activity of pregnane glycosides may be attributed to the polarity of the glycosidic linkage and to the nature of the hydrophobic ester linkage at C-12 and C-20. Previous research has shown that certain steroids, progesterone and corticosterone, inhibited [^{3}H]vinblastine binding to P-gp, which resulted in an increased cellular accumulation of anticancer drugs.[6,7] Interestingly, the effect of these steroids on drug accumulation was proportional to the hydrophobicity of steroid.[6] Most MDR inhibitors are known to interact with P-gp, thereby inhibiting the efflux

of anticancer drugs. They share some physicochemical characteristics such as hydrophobicity, a conjugated planar ring, and a substituted tertiary amino group.[18] Compounds **1**−**3** do not have common structural elements but share hydrophobic features and a terminal aromatic group. However, considering the potent effect of **1** on reversing MDR, it is likely that the pyridine-3-carboxyl group may play an important role. It has been demonstrated that nicardipine and dexniguldipine with the dihydropyridine-3-carboxyl group are potent MDR modulators.[18,19]

To further test the MDR-reversing activity of compound **1**, both drug-resistant cells and the parent cells were treated with various concentrations of adriamycin (ADR), VLB, and colchicine (COL) in the presence of 0, 0.1, 0.3, and 1 μM of **1**. As shown in Figure 1, **1** did not greatly influence the sensitivities of KB-3-1 and MCF7 cells to ADR, VLB, and COL even at a concentration of 1 μM. On the other hand, KB-V1 and MCF7/ADR cells were sensitive to ADR, VLB, and COL in the presence of **1** in a dose-dependent manner. Compared to KB-3-1 cells, KB-V1 cells were 644-fold more resistant to ADR, 1466-fold more resistant to VLB, and 428-fold more resistant to COL. When KB-V1 cells were treated with various concentrations of ADR, VLB, or COL in the presence of 1 μM of **1**, the sensitivities of KB-V1 cells to each drug were completely restored to the level of drug-sensitive KB-3-1 cells. The relative resistance (RR) of KB-V1 cells to KB-3-1 was only 0.64 for ADR, 1.49 for VLB, and 1.37 for COL in the presence of 1 μM of **1**. Similar results were also obtained in experiments with MCF7 and MCF7/ADR cells. On the other hand, verapamil only partially restored the resistance of MDR cells to each anticancer drug at the concentration of 10 μM. These results demonstrated that compound **1** exhibited much more potent MDR-reversing activities than verapamil, under the conditions of the present investigation.

Experimental Section

General Experimental Procedures. Melting points were measured without correction on a Electrothermal model 9100. Optical rotations were determined on a JASCO DIP-181 polarimeter. UV spectra were obtained on a Milton Roy 3000 spectrometer and IR spectra on a JASCO FT/IR 300E spectrophotometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained on a Varian Unity NMR spectrometer using $CDCl_3$ as a solvent. HMBC spectra were determined on a Bruker AMX 500 NMR spectrometer. EIMS were measured on a Hewlett-Packard 5989A mass spectrometer and HRMS on JEOL HX 110 mass spectrometer. Kieselgel 60 (Merck No. 9385 and 7729) and LiChroprep RP-18 were used for column chromatography. Preparative HPLC was carried out on a Delta Pak C_{18} column (19 × 300 mm, Waters) with UV detection at 230 nm. Fetal calf serum, media, and supplement materials for cell culture were purchased from GIBCO-BRL (Grand Island, NY). Standard anticancer drugs adriamycin, vinblastine, and colchicine and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO).

Plant Material. The dried roots of *C. wilfordii* were purchased at an herbal drug store in Taejon, Korea, in October 1996 and identified by Dr. Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen (No. 961024) is deposited at our institute.

Extraction and Isolation. Dried roots of *C. wilfordii* (3 kg) were extracted with MeOH three times at room temperature. A MeOH extract (480 g) was obtained by evaporation of the solvent and partitioned with CH_2Cl_2 and H_2O. The CH_2Cl_2 layer (87 g) was subjected to silica gel column chromatography using a CH_2Cl_2−MeOH step gradient systems (CH_2Cl_2−MeOH, 20:1, 10:1, 5:1, 2:1, MeOH, each 2 L) as eluants to give five fractions (A1−A5). Fraction A2 (37.4 g) was repeatedly chromatographed on silica gel columns with hexane−acetone step gradient systems (hexane−acetone, 3:1, 2:1, acetone, each 2 L) to give six fractions (B1−B6). Fraction B2 (1.8 g) was chromatographed on a reversed-phase MPLC column (C_{18} column, 26 × 460 mm, 20−45 μm; mobile phase 80% MeOH; flow rate 10 mL/min) to yield the active fraction C (0.9 g). Rechromatography of fraction C on preparative HPLC (Delta Pak C_{18}, 19 × 300 mm, mobile phase 75% MeOH) gave compounds **1** (40 mg), **2** (57 mg), and **3** (45 mg). Another active fraction B4 (3.7 g) was purified on a RP MPLC column (C_{18} column, 26 × 460 mm, 20−45 μm; mobile phase 85% MeOH; flow rate 10 mL/min), and further separation by preparative HPLC (Delta Pak C_{18}, 19 × 300 mm, mobile phase 75% MeOH) afforded compound **4** (47 mg).

Compound 1: white amorphous powder; mp 185−187 °C; $[\alpha]^{25}_D$ +20.0° (*c* 0.5, MeOH); HRFABMS *m/z* 1216.6073 ([M + Na]$^+$, calcd for $C_{64}H_{91}NO_{20}Na$, 1216.6032), and possessed physical and spectral data comparable to previously reported values.[13]

Compound 2: white amorphous powder; mp 183−187 °C; $[\alpha]^{25}_D$ −33.0° (*c* 1.0, MeOH); HRFABMS *m/z* 1109.5674 ([M + Na]$^+$, calcd for $C_{58}H_{86}O_{19}Na$, 1109.5661), and possessed comparable physical and spectral data to previously reported values.[10]

Compound 3: white amorphous powder; mp 141−145 °C; $[\alpha]^{25}_D$ −52.0° (*c* 1.0, MeOH); HRFABMS *m/z* 1089.6008 ([M + Na]$^+$, calcd for $C_{56}H_{90}O_{19}Na$, 1089.5974); and possessed comparable physical and spectral data to previously reported values.[11]

Compound 4: white amorphous powder; mp 167−173 °C; $[\alpha]^{25}_D$ −41.0° (*c* 1.0, MeOH); HRFABMS *m/z* 1271.6124 ([M + Na]$^+$, calcd for $C_{64}H_{96}O_{24}Na$, 1271.6189), and possessed comparable physical and spectral data to previously reported values.[17]

Cell Lines and Cell Culture. The human oral epidermoid cancer cell lines, KB-3-1 and KB-V1, which were derived by stepwise exposure to VLB of KB-3-1 cells, were obtained from Dr. M. M. Gottesman (NCI, Bethesda, MD). They were grown in Dulbecco's modified Eagles medium (DMEM) containing 2 mM L-glutamine and 10% fetal calf serum. KB-V1 cells were maintained in the continuous presence of 1 μM vinblastine. Human breast cancer MCF7 and MCF7/ADR cell lines were supplied by Dr. D. J. Newman (NCI, Frederick, MD) and were cultured in RPMI1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine and transferred twice a week by diluting at 1:8 or 1:16 split in fresh media. MCF7/ADR cells maintain resistance to ADR for 20 passages in the absence of ADR. All cells were grown at 37 °C in humidified atmosphere with 5% CO_2.

***In Vitro* Drug Sensitivity.** Cell growth was measured using the standard sulforhodamine B method.[20] Briefly, cells in exponential growth were trypsinized, dispersed in single cell suspension, and dispensed in 100 μL volumes into 96 well plates. For *in vitro* assays, 2.5 × 10^3 KB-3-1, 5 × 10^3 KB-V1, 5 × 10^3 MCF7, and 1 × 10^4 MCF7/ADR cells/well were inoculated in 100 μL medium containing 5% fetal calf serum and allowed to attach and grow overnight. One hundred microliters of medium containing anticancer drug and/or MDR-reversing compound were 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 μL of cold 50% trichloroacetic acid (final concentrations 10%) on the top of the growth medium in each well and incubated at 4 °C 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 (pH 10.5) on a shaker for 5 min. Absorbance was read with microtiter plate reader at 570 nm. The IC_{50} value was the concentration of drug that reduced absorbance to the 50% of vehicle-treated controls.

MDR-Reversing Activity. The effect of compound **1** on MDR was studied by exposing cells to a range of concentrations of standard anticancer drugs adriamycin (ADR), vinblastine (VLB), and colchicine (COL) in the absence or presence of 0.1, 0.3, and 1 μM of **1**. The IC_{50} value of each anticancer drug was obtained, and then the relative resistance (RR) of MDR cells was calculated as follows: IC_{50} of drug-resistant KB-V1 cells/IC_{50} of drug-sensitive KB-3-1 cells.

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