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Reversal of Multidrug Resistance by Morning Glory Resin Glycosides in Human Breast Cancer Cells

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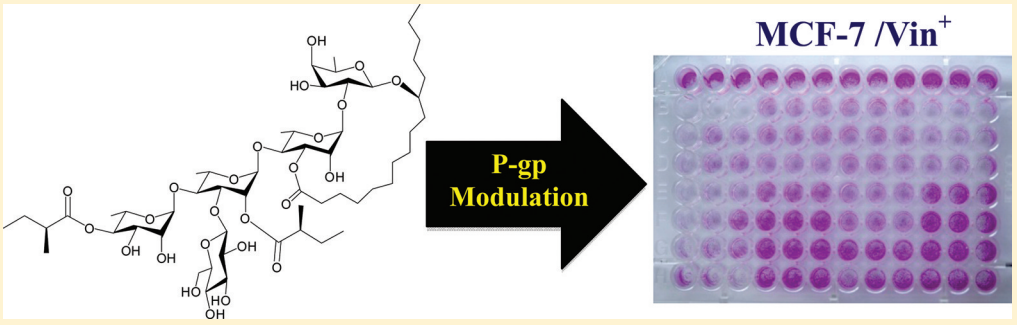
Supporting Information



ABSTRACT: Reversal of multidrug resistance (MDR) by thirty resin glycosides from the morning glory family (Convolvulaceae) was evaluated in vinblastine-resistant human breast carcinoma cells (MCF-7/Vin). The effects of these amphipathic compounds on the cytotoxicity and P-glycoprotein (P-gp)-mediated MDR were estimated with the sulforhodamine B colorimetric assay. Active noncytotoxic compounds exerted a potentiation effect of vinblastine susceptibility by 1- to over 1906-fold at tested concentrations of 5 and 25 μg/mL. Murucoidin V (**1**) enhanced vinblastine activity 255-fold when incorporated at 25 μg/mL and also, based on flow cytometry, significantly increased the intracellular accumulation of rhodamine 123 with the use of reserpine as a positive control for a MDR reversal agent. Incubation of MCF-7/Vin cells with **1** caused an increase in uptake and notably lowered the efflux rate of rhodamine 123. Decreased expression of P-glycoprotein by compound **1** was detected by immunofluorescence flow cytometry after incubation with an anti-P-gp monoclonal antibody. These results suggest that resin glycosides represent potential efflux pump inhibitors for overcoming MDR in cancer therapy.

Multidrug resistance (MDR) is one of the major obstacles and serious problems associated with cancer chemotherapy. Multiple resistance mechanisms in tumor cells complicate treatment and increase both human morbidity and financial cost in healthcare systems.[1] Chemoresistance in cancer cells presents multiple mechanisms, such as loss or inhibition of cell surface drug receptor or transporter, specific drug metabolism, or mutation of specific drug targets.[2] MDR phenotype is often the overexpression of a 170 kDa plasma membrane associated glycoprotein known as P-glycoprotein (P-gp), encoded by the human MDR1 gene.[3] This protein has been found in the intestine, liver, pancreas, kidney, ovary, testicle, and brain.[4] P-gp belongs to the superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporters and functions as an energy-dependent multidrug membrane translocase that extrudes a wide range of structurally and functionally diverse lipophilic and amphipathic anticancer drugs, carcinogens, toxins, and other xenobiotics from inside cells and thereby prevents drugs from exerting their cytotoxicity by means of decreasing intracellular accumulation.[5]

In order to reverse MDR in cancer cells, some studies have focused on inhibiting P-gp function and thus increasing the intracellular drug accumulation.[6] Many natural and synthetic pharmaceutical agents including calcium channel blockers (e.g., verapamil),[7] calmodulin antagonists (e.g., trifluoperazine and chlorpromazine),[8,9] and antimalarial (e.g., chloroquine),[7] antiarrhythmic (e.g., quinidine),[7] and immune suppressive drugs (e.g., cyclosporine A)[10] inhibit P-gp function. These findings indicate the need to select natural or synthetic leads for developing new potent efflux pump inhibitors (EPIs). P-gp has also been inhibited by herbal medicines.[10] Some plant flavonols (e.g., kaempferol, quercetin, and galangin),[11] ginsenosides,[10] polyphenols,[12] omega-6 polyunsaturated fatty acids,[13] macrocyclic diterpenoids,[14] and alkaloids[7,15] (e.g., reserpine and yohimbine) have been reported to modulate P-gp activity.[16]

The modulatory effect of microbiologically inactive resin glycosides,[17] amphipathic glycolipids, evaluated on multidrug-

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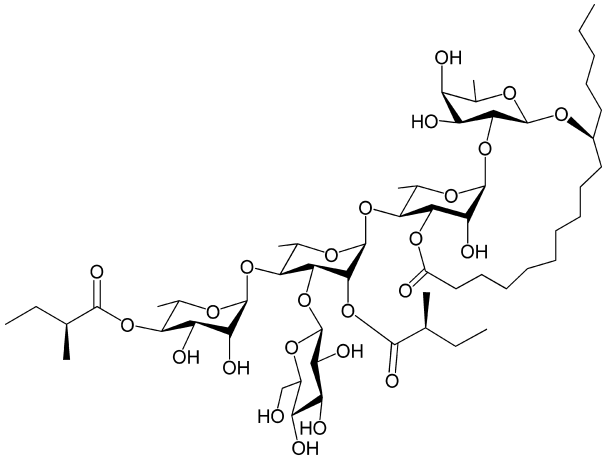
resistant Gram-positive (*Staphylococcus aureus*)[18−21] and -negative bacteria (*Escherichia coli* Rosetta-gami, *Salmonella typhi*, and *Shigella flexneri*)[22] led to the characterization of these compounds as substrates for efflux pumps. Using SA-1199B, an effluxing strain overexpressing the Nor A multidrug transporter, some members of the orizabin and murucoidin series were as inhibitory of ethidium bromide efflux as reserpine (an EPI used as a positive control). It has been suggested that MDR is a variation of a regular translocase that simply broadened its substrate spectrum, providing a cell with a simple defense against toxins.[23] Thus, this similarity in design, and the high incidence of apparently independent evolution of bacterial and eukaryotic MDR pumps,[23] was the rationale for an evaluation of the modulatory activity of resin glycosides from the morning glory family (Convolvulaceae) in human breast cancer cells



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resistant to vinblastine (MCF-7/Vin) as prototypes for effective EPIs.

RESULTS AND DISCUSSION

Thirty convolvulaceous resin glycosides (**1**−**30**; Figures S4−S16, Supporting Information) were tested for cytotoxicity and modulatory activity against human breast cancer, using a vinblastine-resistant line (MCF-7/Vin) and its parental cells (MCF-7). Proliferation curves were established for each cell line by the sulforhodamine B (SRB) colorimetric assay[24] (Figure S1, Supporting Information). As expected, the developed MDR cell lines MCF-7/Vin$^-$ (cells growing in vinblastine-free medium) and MCF-7/Vin$^+$ (cells growing in vinblastine-supplemented medium) displayed lower vinblastine susceptibility up to 22- and 26-fold, respectively, than that of MCF-7 drug-sensitive cells. Cross-resistance to adriamycin, colchicine, and ellipticine was also observed (Table 1). Screening for cytotoxicity of all tested glycolipids (**1**−**30**) was performed using both vinblastine-sensitive and -resistant cell lines to determine IC_{50} values (Table S1, Supporting Information). These results identified noncytotoxic compounds (IC_{50} > 25 μg/mL) for further evaluation as EPIs. Most of the tested compounds were weakly cytotoxic (IC_{50} = 10−25 μg/mL) or inactive as previously reported for other resin glycosides on various drug-sensitive cell lines.[17] Only members of the orizabin (**19**−**25**) series displayed moderate to marginal cytotoxicity against the evaluated MDR cell lines (IC_{50} = 1.5−10 μg/mL). Lack of cytotoxicity is an important requirement for performing modulation assays with effluxing cell lines to be able to clearly distinguish any potentiation effect (through inhibition of efflux pumps) from possible synergism of an active compound with vinblastine.[18,22]

Preliminary assays using MCF-7/Vin$^-$ cells showed that all tested compounds displayed modulation of vinblastine susceptibility (Table S2, Supporting Information) and allowed for the selection of five compounds (**1**, **2**, **3**, **16**, and **17**) with a positive response (>20-fold) as MDR reversal agents (Tables 2 and S3, Supporting Information). In terms of the relationship between chemical structure and the observed nonspecific modulatory activity, neither the size of the lactone ring nor the length of the oligosaccharide chain was crucial for activity potentiation. The degree, type, or position of saccharide core acylation also had no clear influence on the activity of these compounds.

Although all selected compounds enhanced the cytotoxicity of vinblastine to MCF-7/Vin cells (Table 2), murucoidin V (**1**) was less toxic and the only compound in adequate supply for further evaluation of its inhibitory effect on both P-gp function and expression by flow cytometry. Intracellular rhodamine 123 (Rh123)-associated mean fluorescence intensity in MCF-7/Vin and MCF-7 cells was employed to study the effects of resin glycosides on the inhibition of P-gp function. As expected from their MDR phenotype,[10] the accumulation of Rh123 by MCF-7/Vin cells preincubated with compound **1** (25 μg/mL) was less than a third of that registered for the parental cells (Figure 1). This accumulation was comparable to that observed for reserpine, which was used as the EPI control. In the experiment of intracellular Rh123 efflux (Figure 2), a rapid decrease of intracellular Rh123 level was observed in MCF-7/Vin$^+$ cells after incubation in Rh123-free medium. However, in the presence of compound **1**, Rh123 efflux was suppressed in

Table 1. Cross-Resistance and Cytotoxicity of Vinblastine against Drug-Sensitive MCF-7 and Multidrug-Resistant MCF-7/Vin Cells

	cell line				
	MCF-7sens	MCF-7/Vin$^-$		MCF-7/Vin$^+$	
	IC_{50}[a] (μg/mL)	IC_{50}[a] (μg/mL)	resistance value	IC_{50}[a] (μg/mL)	resistance value
vinblastine	0.047 ± 0.01	1.02 ± 0.18	22-fold	1.22 ± 0.14	26-fold
adriamycin	0.644 ± 0.05	4.52 ± 1.3	7-fold	2.42 ± 0.48	4-fold
colchicine	0.016 ± 0.003	1.05 ± 0.06	66-fold	0.90 ± 0.19	56-fold
camptothecin	<0.00064	<0.00064		<0.00064	
ellipticine	0.359 ± 0.04	0.38 ± 0.02	1-fold	0.43 ± 0.07	1-fold

[a]Each value represents the mean ± SD from three independent experiments.

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Table 2. Modulation of Vinblastine Cytotoxicity in Drug-Sensitive MCF-7 and Multidrug-Resistant MCF-7/Vin Cells by Selected Resin Glycosides

	IC_{50} (μg/mL)			reversal fold[c]		
compound[a]	MCF-7/Vin$^-$	MCF-7/Vin$^+$	MCF-7 sens	$RF_{MCF\text{-}7/Vin^-}$	$RF_{MCF\text{-}7/Vin^+}$	$RF_{MCF\text{-}7\ sens}$
vinblastine	1.02 ± 0.18	1.22 ± 0.14	0.047 ± 0.01			
1	0.004 ± 0.002	0.156 ± 0.13	0.001 ± 0.002	255	7.8	47
2	<0.00064	<0.00064	<0.00064	>1593.8	>1906.3	>73.4
3	<0.00064	<0.00064	<0.00064	>1593.8	>1906.3	>73.4
16	<0.00064	<0.00064	<0.00064	>1593.8	>1906.3	>73.4
17	<0.00064	<0.00064	0.001 ± 0.0003	>1593.8	>1906.3	47
reserpine[b]	0.037 ± 0.01	0.31 ± 0.19	0.003 ± 0.001	27.6	3.9	15.7

[a]Serial dilutions from 0.000 64 to 10 μg/mL of vinblastine in the presence or absence of glycolipid (25 μg/mL). [b]Reserpine = 5 μg/mL as positive control. [c]RF = IC_{50} vinblastine/IC_{50} vinblastine in the presence of glycolipid. Each value represents the mean ± SD from three independent experiments.

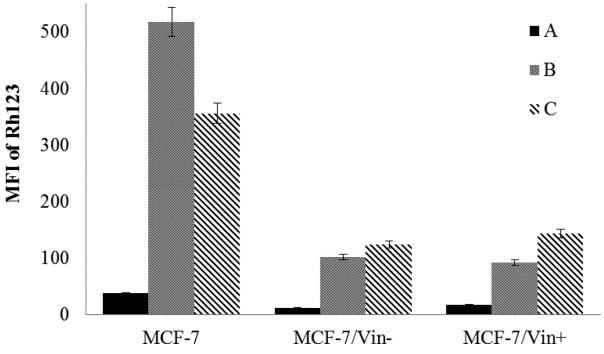

Figure 1. Effects of murucoidin V (**1**) on Rh123 accumulation in drug-sensitive MCF-7 cells and multidrug-resistant MCF-7/Vin cells. Cells were respectively (A) untreated, (B) pretreated with 25 μg/mL **1**, (C) pretreated with 5 μg/mL reserpine. Each bar represents the mean ± SD from three independent experiments.

MCF-7/Vin$^+$, which result was almost equipotent to the effect observed for the positive control. This inhibition continued even after the tested compound was removed from the medium for over 90 min (Figure S2, Supporting Information). It would appear that this cellular uptake of resin glycosides is facilitated by their amphipathic properties, resulting in an easy interaction with P-gp.[2,5]

Decreased expression of P-gp by compound **1** was detected by immunofluorescence flow cytometry after incubation with an anti-P-gp monoclonal antibody. The fluorescent intensity of labeled antibody in parental MCF-7 cells was used as a blank control. MCF-7/Vin$^+$ exhibited a strong fluorescent area that corresponded to the expression of P-gp. After 30 min incubation with compound **1** (25 μg/mL), the expression level of P-gp was decreased by 30% when compared with untreated MCF-7/Vin$^+$ (Figure 3). This effect was similar to the one exerted by reserpine (5 μg/mL) with a 38% reduction in the P-gp expression (Figure S3, Supporting Information). Therefore, these data suggest that resin glycosides are substrates of the P-gp transporter and hold promise as leads in the search for more potent EPIs. Their inhibitory effects could be used to overcome the acquired resistance to common antineoplasic drugs by lowering current effective therapeutic doses, thereby decreasing toxic side-effects in refractory malignancies. These results also indicate that combining an anticancer agent with a MDR inhibitor is an approach that promises positive possibilities for future cancer treatments.

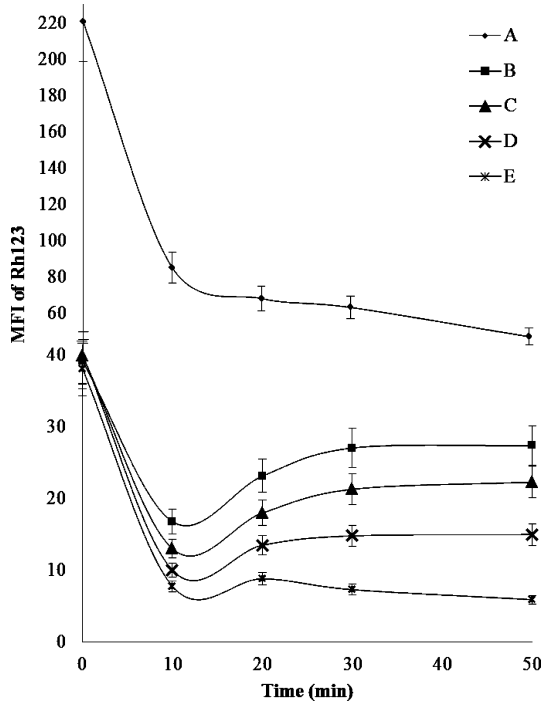

Figure 2. Effect of murucoidin V (**1**) on the Rh123 efflux assay in the drug-sensitive MCF-7 cells and multidrug-resistant MCF-7/Vin cells: (A) untreated drug-sensitive MCF-7 cells, (B) 5 μg/mL reserpine in MCF-7/Vin$^+$, (C) 25 μg/mL **1** in MCF-7/Vin$^+$, (D) 25 μg/mL **1** in MCF-7/Vin$^-$, and (E) untreated as a negative control in MCF-7/Vin$^+$. Each point represents the mean ± SD from three independent experiments.

EXPERIMENTAL SECTION

Chemicals. RPMI 1640 medium and fetal bovine serum were obtained from Gibco. Fluorescein conjugated mouse anti-human monoclonal antibody against P-gp Mdr-1 (UIC2) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sulforhodamine B, reserpine, vinblastine, ellipticine, colchicine, camptothecin, and rhodamine 123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Test Compounds. Glycolipids **1**–**30** were isolated as previously described from *Ipomoea batatas*,[25] *I. intrapilosa*,[26] *I. murucoides*,[19,20,27] *I. orizabensis*,[28] *I. pes-caprae*,[29,30] *I. purga*,[31] and *I. tricolor*.[32,33] The structures for all tested compounds are included in Figures S4–S16 (Supporting Information).

Cell Lines and Cell Cultures. Drug-sensitive human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection (ATCC HTB-22). The resistant counterpart MCF-

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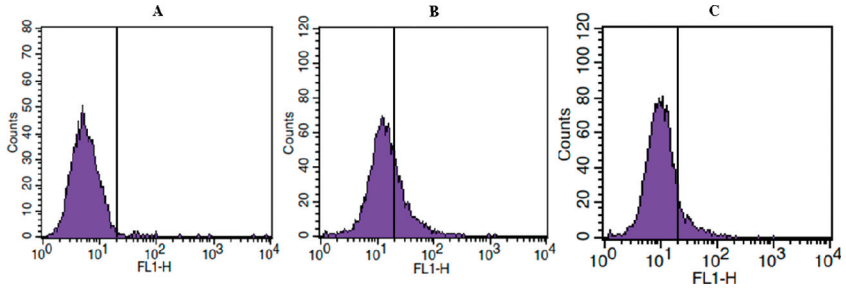

Figure 3. Mediated P-gp expression by murucoidin V (**1**) in drug-sensitive MCF-7 cells and multidrug-resistant MCF-7/Vin$^+$ cells: (A) untreated MCF-7 cells; (B) untreated MCF-7/Vin$^+$; (C) MCF-7/Vin$^+$ treated with 25 μg/mL **1**. The percentage area to the right indicates the relative quantity of P-gp expression.

7/Vin was developed through continuous exposition to vinblastine during three consecutive years. These cells are considered to express the MDR phenotype due to their cross-resistance to adriamycin, colchicine, and ellipticine as displayed in pre-experiments (Table 1). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and were cultured at 37 °C in an atmosphere of 5% CO_2 in air (100% humidity). To maintain drug resistance, MCF-7/Vin$^+$ cells were cultured in medium containing 0.192 μg/mL vinblastine. At the same time, a stock of MCF-7/Vin$^-$ cells was maintained in vinblastine-free medium.

Cytotoxicity and Modulation of Multidrug-Resistance Assays. Cytotoxicity and reversal fold of the resin glycosides (**1**–**30**) were determined by using the SRB assay.[24] The cells were harvested at log phase of their growth cycle and were treated in triplicate with various concentrations of the test samples (0.2–25 μg/mL) and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Results are expressed as the concentration that inhibits 50% control growth after the incubation period (IC_{50}). The values were estimated from a semilog plot of the drug concentration (μg/mL) against the percentage of growth inhibition. Vinblastine was included as a positive control drug.

The reversal effects as modulators were further investigated with the same method. MCF-7 and MDR MCF-7/Vin cells were seeded into 96-well plates and treated with various concentrations of vinblastine (0.000 64–10 μg/mL) in the presence or absence of glycolipids at 25 and 5 μg/mL for 72 h. The ability of glycolipids to potentiate vinblastine cytotoxicity was measured by calculating the IC_{50} as described above. In these experiments, reserpine (5 μg/mL) was used as a positive control drug. The reversal fold (RF) value, as a parameter of potency, was calculated from dividing IC_{50} of vinblastine alone by IC_{50} of vinblastine in the presence of test compounds.

Intracellular Rh123 Accumulation Assay. MCF-7 and MDR MCF-7/Vin cells were seeded at 4.5 × 10^5 cells per well in six-well plates and cultured for 24 h at 37 °C in an atmosphere containing 5% CO_2. At 60% confluence, fresh media containing 2.5 μg/mL Rh123 and compound **1** (25 or 5 μg/mL) or reserpine (5 μg/mL) was added and incubated at the same conditions for 30 min. At the end of the incubation time, the accumulation of Rh123 was stopped by washing the cells three times with ice-cold PBS; then the intracellular mean fluorescence intensity (MFI) associated with Rh123 was measured with a FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA). Excitation was performed at 485 nm, and the emitted fluorescence was collected through a 530 nm pass filter. Data analysis was performed using Cell Quest software.

Rh123 Efflux Assay and Persistence of Activity. Cells were seeded and cultured in the same manner as described for the accumulation assay during 24 h. At the end of this time, cells were first incubated with fresh medium containing 2.5 μg/mL of Rh123 at 37 °C for 30 min; then they were washed three times with Rh123-free medium and incubated at 37 °C in the presence or absence of **1** (25 or 5 μg/mL) for 0, 10, 20, 30, and 50 min, respectively. For the persistence assay, fresh media was added and cells were incubated again containing 2.5 μg/mL Rh123 in the presence or absence of **1** (25 or 5 μg/mL) for 30 min and washed three times with Rh123-free medium. The ability of cells to accumulate Rh123 and the remaining inhibitory effect were measured after incubating for 30, 60, and 90 min. T_0 represents the end of the modulator incubation phase.[34] Reserpine (5 μg/mL) was used as a positive control for both efflux and persistence of activity assays. The MFI was measured as described for the accumulation assay.

P-gp Expression. To measure the cell surface P-glycoprotein levels by immunofluorescence flow cytometry,[35] cell lines were seeded into six-well plates at a density of 4.5 × 10^5 cells per well and then cultured for 24 h. After this period, cells were harvested, washed twice with ice-cold PBS, and then labeled with fluorescein conjugated mouse anti-human monoclonal antibody against P-gp Mdr-1 (UIC2) according to the manufacturer's instructions. The cells were exposed at the same time to compound **1** (25 μg/mL) or reserpine (5 μg/mL) as well as to the antibody during 30 min of ice-incubation. The MFI was measured as described above.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The structures of tested compounds, their cytotoxicity against drug-sensitive and MDR MCF-7 cell lines, primary screening on modulatory activity in MCF-7/Vin$^-$, modulatory activity for selected compounds (**1**, **2**, **3**, **16**, and **17**) at a concentration of 5 μg/mL against the drug-sensitive and MDR MCF-7/Vin cell lines, P-gp expression with 5 μg/mL reserpine, proliferative curves, persistence assays, and pictures of modulation assay plates are available free of charge via the Internet at http://pubs.acs.org.

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