Multidrug-Resistance Modulators from *Stephania japonica*

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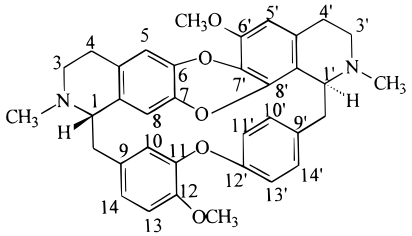
An alkaloidal extract of the vines of *Stephania japonica* showed multidrug-resistance-reversing activity as demonstrated by the bicinchoninic acid assay. Two known bisbenzylisoquinoline alkaloids, isotrilobine (**1**) and trilobine (**2**), were isolated by bioassay-directed fractionation and separation. Isotrilobine (**1**) was shown to be as active as verapamil (**3**) in reversing doxorubicin resistance in human breast cancer cells.

The ability to overcome multidrug resistance (MDR) is crucial to successful cancer chemotherapy. It has been demonstrated in preclinical trials that drug resistance could be partially reversed by the use of verapamil.[1,2] Previous research has shown that the alkaloids reserpine and quinidine reverse MDR.[3−8] Other natural products have been shown to reverse MDR, and You *et al.* demonstrated that three indole alkaloids inhibit the binding of [^{3}H]vinblastine to membrane vesicles derived from MDR cells.[9] Taxoids isolated from *Taxus cuspidata* and welwitindolinone alkaloids isolated from *Hapalosiphon welwitschii* were shown to increase intracellular accumulation of [^{3}H]vincristine.[10,11]

Preliminary screening of extracts of plant samples was carried out using the bicinchoninic acid (BCA) assay.[12] The BCA assay is a microtiter bioassay that measures cell density. It provides an easy, indirect method with which to screen large numbers of crude fractions or pure compounds for reversal of MDR. The assay allows the MDR-reversing effectiveness of compounds and extracts to be assessed independent of their cytotoxicity. This is accomplished by performing the assay with concentrations of the compound that are lower than the ED_{20} of the compound; this is estimated from the ED_{50} of the compound. In brief, the ED_{50} of doxorubicin on MCF-7/ADR (Adriamycin-resistant human breast adenocarcinoma) cells is divided by the ED_{50} of doxorubicin with the compound, giving the cytotoxicity enhancement factor; and the concentration of the compound tested is divided by the ED_{100} of the compound on MCF-7/ADR cells giving the cytotoxicity factor. The relative efficacy of the compounds tested is determined based on slopes by plotting the cytotoxicity enhancement factor against the cytotoxicity factor.

The alkaloid fraction of *Stephania japonica* (Thunb.) Miers (Menispermaceae) was shown to possess MDR-reversing activity. Alkaloids have been previously isolated from *S. japonica*,[13−18] with some having antitumor activity.[19] Our preliminary screening indicated that the alkaloid extract could reverse MDR activity of doxorubicin-resistant MCF-7 cells. The alkaloid fraction gave a slope of approximately 300, while another fraction gave a slope of 4, and verapamil gave a value of 160 at a concentration of 10 μg/mL (the estimated ED_{20} of verapamil).

An alkaloid extraction procedure was therefore carried out on 3 kg of the dried and ground vines of *S. japonica*. After bioactivity-directed fractionation using open-column chromatography, two bisbenzylisoquinoline alkaloids were isolated, isotrilobine (**1**) and trilobine (**2**). The compounds were not significantly toxic to resistant cells (MCF-7/ADR) in the absence of doxorubicin (ED_{50}: 2 μg/mL for isotrilobine and 1 μg/mL for trilobine).



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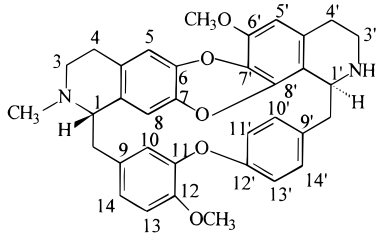

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A comparison of the MDR-reversing effects of isotrilobine (**1**) and trilobine (**2**) in relation to verapamil (**3**) is shown in Figure 1 and Table 1. It can be seen from the graph that isotrilobine has MDR-reversing activity comparable to verapamil at concentrations less than the ED_{20} of isotrilobine on MCF-7/ADR cells. The compounds were assayed three times and averaged results (not shown) indicated that trilobine has low activity with a slope of 28 as compared with slopes of 211 and 232 for isotrilobine and verapamil, respectively. The greater efficacy of isotrilobine (**1**) to trilobine (**2**) appears to be a result of the methyl group at N-2′; it is the only structural difference between the two compounds. This suggests that a tertiary amine is preferred at this position to a secondary amine. The slightly increased lipophilicity induced by the addition of another methyl group may also contribute to the increased activity. It is known that MDR inhibitors are lipophilic.[20]

Other requirements for effective MDR activity, outlined by Klopman *et al.*,[21] are also met by trilobine (**2**) and isotrilobine (**1**). This study used a methodology that generates structural fragments required for the activity

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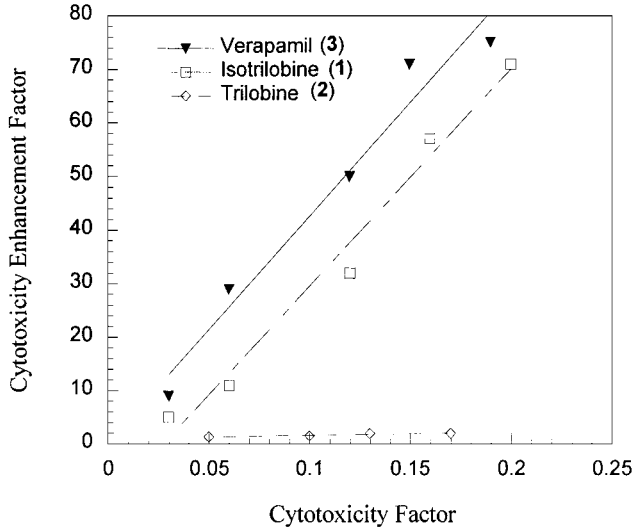


Figure 1. MDR-reversing activity of isotrilobine (**1**) (□), trilobine (**2**) (◇) and verapamil (**3**) (▼) on MCF-7/ADR cells.

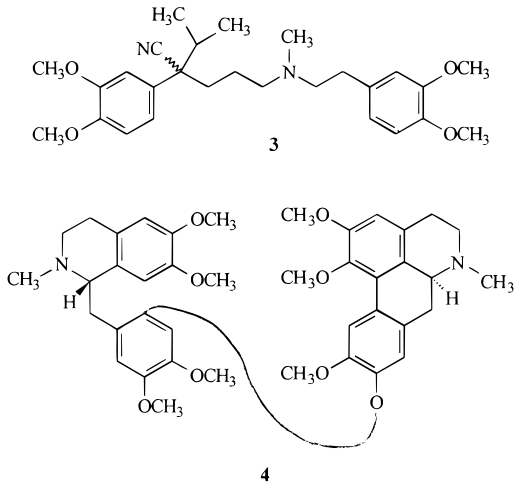
Table 1. Bicinchoninic Acid (BCA) Assay Data

compound	concentration (μg/mL)	ED_{50} of Adriamycin (μg/mL)	cytotoxicity factor	cytotoxicity enhancement factor
isotrilobine (**1**)	1.0	1.7×10^{-2}	0.20	70
	0.8	2.1×10^{-2}	0.16	57
	0.6	3.7×10^{-2}	0.12	32
	0.3	1.1×10^{-1}	0.06	10
	0.15	2.5×10^{-1}	0.03	5
trilobine (**2**)	1.0	6.0×10^{-1}	0.17	2.0
	0.8	6.1×10^{-1}	0.13	2.0
	0.6	8.3×10^{-1}	0.10	1.5
	0.3	1.2	0.05	1.0
verapamil (**3**)	10.0	1.6×10^{-2}	0.19	75
	8.0	1.7×10^{-2}	0.15	70
	6.0	2.4×10^{-2}	0.12	50
	3.0	4.1×10^{-2}	0.06	30
	1.5	1.3×10^{-1}	0.03	10
doxorubicin		1.2		

of a compound from information entered into the program MULTICASE.[21] The structural requirements were generated from compounds found to reverse MDR activity as determined by increased [^{3}H]vinblastine uptake. It was determined that −CXCC− where X = O, N is necessary for activity as well as a terminal aromatic group and an internal π-environment. Trilobine and isotrilobine contain these structural requirements for MDR activity.

Thalicarpine (thaliblastine) (**4**) is a bisbenzylisoquinoline−aporphine alkaloid isolated from the genus *Thalictrum* (Ranunculaceae), which has also been shown to reverse MDR.[22] The alkaloid completely blocked [^{3}H]-azidopine photoaffinity labeling of P-glycoprotein (Pgp) encoded by the *mdr*1 gene and increased intracellular doxorubicin concentration at a concentration of 8 μM after a 4-h incubation period. It was also determined that this alkaloid reverses MDR though its interaction with Pgp and not by altering glutathione *S*-transferase.[23] Thalicarpine and isotrilobine are structurally similar. Both contain a benzylisoquinoline moiety and two tertiary nitrogens. These data suggest that benzylisoquinolines may be good lead compounds for further investigation of their reversal activity.

Isotrilobine (**1**) has been previously isolated from *Cocculus pendulus*[24] and *Cocculus hirsutus*,[25] and trilobine (**2**) has been found previously as a constituent of



Cocculus hirsutus,[26] *Pachygone ovata*,[27,28] *Anisocycla cymosa*,[29] *Anisocyclea grandidieri*,[30] and *Cocculus macrocarpus*.[31] Both compounds were therefore identified by comparison of their ^{1}H-NMR, UV, MS, and mp data with previously reported values.[24−31]

Experimental Section

General Experimental Procedures. All 1D and 2D NMR spectra were obtained using a Varian VXR-500S (500 MHz) spectrometer. Chemical shifts of compounds in distilled $CDCl_3$ are recorded in parts per million relative to the $CDCl_3$ peak (7.24 ppm). EIMS were recorded on a Finnigan 4000 mass spectrometer. FABMS were obtained with a Kratos MS-50 mass spectrometer. UV profiles were obtained using a Beckman DU-7 spectrophotometer, and IR data were obtained with a Perkin-Elmer 600 series FTIR spectrometer using NaCl plates. All melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. A Perkin-Elmer 241 polarimeter was used to determine optical rotation. Dragendorff's spray reagent and UV light were used for detection of compounds.

Plant Material. The vines of *Stephania japonica* (Thunb.) Miers (Menispermaceae) were collected in 1991, by Mr. W.-L. Chu, Taiwan Forestry Research Institute, Heng-Chuen Branch Station, where a voucher sample has been deposited.

Extraction and Isolation. An alkaloid extraction procedure was carried out on 3 kg of the dried and ground vines of *S. japonica*. The plant material was first moistened with CH_2Cl_2−MeOH−5% NaOH (aqueous) (1:2:1). The mass was then extracted with CH_2Cl_2−MeOH (1:1). The resulting extract volume was then concentrated by rotoevaporation. Methylene chloride was added, and this solution was partitioned three times with 0.01 N HCl. The aqueous fractions were combined and back-extracted with CH_2Cl_2. The organic fractions were then combined and condensed to dryness using a rotoevaporator yielding 37.3 g of fraction C. The aqueous fractions were combined and titrated with 4% NaOH to pH ≃ 9. This basic solution was then extracted three times with CH_2Cl_2. The combined organic fractions were condensed to dryness under vacuum to give 23 g of fraction A. The aqueous solution was neutralized with 0.01 N HCl, condensed under vacuum, and lyophilized. This 80-g residue was extracted with 250-mL portions of CH_2Cl_2−MeOH (1:1)

three times. The organic solution was condensed to dryness under vacuum to yield 70 g of fraction B, and the insoluble salt was discarded. All crude fractions were tested for their ability to reverse MDR using the BCA assay, and results indicated that both fractions A and C were active.

Further separation was carried on the alkaloid-containing fraction A. Fraction A (10 g) was loaded onto a Si gel column (1 kg). The alkaloids were eluted with increasing MeOH in $CHCl_3$ ($CHCl_3$−MeOH, 99:1 to 92:8) giving 7 fractions. Fraction 4 (0.5 g) was loaded onto a 2-mm Chromatotron plate prepared with Kieselgel 60 PF_{254} (EM Reagents, Darmstadt, Germany), eluting pure compound **1** (isotrilobine, 120 mg) with the solvent system, $CHCl_3$−hexane−diethylamine (4%) (1:8, 2:7, 3:6, 4:5, 5:4). Fractions 5 and 6 (3 g) were combined and subjected to Si gel open-column chromatography, using $CHCl_3$−hexane−diethylamine (4%) 2:8 to 5:5 as solvents, resulting in six fractions. Pure compound **2** (trilobine, 150 mg) was obtained by crystallization of fraction 6.

Isotrilobine (1): white crystals; mp 218−220 °C (MeOH), mp 281 °C ($CHCl_3$-hexane) [lit.[25] mp 210 °C]; $[\alpha]^{30}_D$ +323° (*c* 0.5, $CHCl_3$); and possessed comparable spectroscopic data (UV, IR, MS and ^{1}H NMR) to previously published values for isotrilobine.[24,25]

Trilobine (2): white crystals; mp 245−246 °C (MeOH) [lit.[26] mp 232−233 °C (C_6H_6−MeOH)], mp 240 °C ($CHCl_3$−hexane); $[\alpha]^{30}_D$ +311° (*c* 0.5, $CHCl_3$) [lit.[30] $[\alpha]^{30}_D$ +305° (*c* 1.26, $CHCl_3$)]; and possessed comparable spectroscopic data (UV, IR, MS and ^{1}H NMR) to previously published values for trilobine.[28]

Bioassay. The BCA assay was carried out as previously described.[12] Briefly, MCF-7/ADR cells were seeded into 96-well microtiter dishes and were then incubated at 37 °C for 24 h prior to the addition of the compound. MCF-7/ADR and MCF-7/WT cells were maintained in RPMI medium with 10% FCSHI and penicillin/streptomycin. The compounds (or extracts) were solubilized first with a volume of DMSO that gave a concentration of less than 0.1% DMSO in the well and then were further diluted with media. On one 96-well plate, up to five concentrations of a compound were added to resistant cells. Cells were incubated at 37 °C with the compound for 30 min and then serial dilutions of doxorubicin were added to each concentration of compound. This allowed the dose at which doxorubicin is 50% effective (ED_{50}) to be determined for each concentration of the compound. The compound alone was also added to the resistant cells to determine its cytotoxicity. After a 6-day period the amount of protein present is assayed using the BCA reagents and a microtiter plate reader. ED_{50} values were calculated using linear regression. The ED_{50} of doxorubicin without the compound on MCF-7/ADR cells was divided by the ED_{50} of doxorubicin with the compound giving the cytotoxicity enhancement factor (*y*-axis). The concentration of the compound tested was divided by the ED_{100} of the compound on MCF-7/ADR cells giving the cytotoxicity factor (*x*-axis). The data was then graphed to depict the varying degrees of reversal of resistance by the compounds or crude extracts tested.

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