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INDOLE ALKALOIDS FROM *PESCHIERA LAETA* THAT ENHANCE VINBLASTINE-MEDIATED CYTOTOXICITY WITH MULTIDRUG-RESISTANT CELLS

MIN YOU, XIANJIAN MA, RABINDRANATH MUKHERJEE,[1] NORMAN R. FARNSWORTH, GEOFFREY A. CORDELL, A. DOUGLAS KINGHORN, and JOHN M. PEZZUTO*

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612

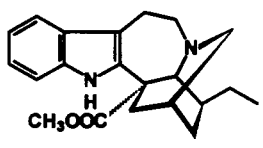
ABSTRACT.—Coronaridine [**1**], conoduramine [**2**], and voacamine [**3**], three indole alkaloids isolated from *Peschiera laeta*, have been found to enhance the cytotoxic response mediated by vinblastine [**4**] with multidrug-resistant KB cells. Inhibition of vinblastine binding with membrane vesicles isolated from this cell line was also assessed, and the bisindole alkaloids conoduramine [**2**] and voacamine [**3**] were found to be more potent inhibitory agents than the monomeric alkaloid, coronaridine [**1**]. Thus, these compounds appear to function by binding with P-glycoprotein.

Peschiera laeta (Mart. ex A. DC.) Miers (syn. *Tabernaemontana laeta* Mart.) (Apocynaceae) is a small tree indigenous to Brazil (1). Although there have not been any prior investigations on the biological activities of extracts of *P. laeta*, a number of indole alkaloids have been reported as constituents of the combined twigs and leaves of this species (2). Other species in the genus *Peschiera* have also afforded indole alkaloids (3–10), as well as triterpenes and a sterol (3).

In the present investigation, an EtOAc extract derived form the stem bark of *P. laeta* was shown to inhibit the growth of drug-resistant KB-V1 cells in the presence of vinblastine [**4**], but not parental KB cells themselves. Activity-guided fractionation resulted in the isolation and characterization of three active indole alkaloids of known structure, namely, coronaridine [**1**], conoduramine [**2**], and voacamine [**3**]. The individual activities of these three compounds in inhibiting ATP-dependent vinblastine binding to P-glycoprotein-associated cell membrane vesicles isolated from multidrug-resistant KB-V1 cells have been evaluated.

RESULTS AND DISCUSSION

An EtOAc extract of *P. laeta* stem bark exhibited significant cytotoxicity with multidrug-resistant (MDR) KB-V1 cells in the presence of vinblastine (ED_{50} = 1.7 μg/ml), while exhibiting no cytotoxicity with KB-V1 cells in the absence of vinblastine [**4**]



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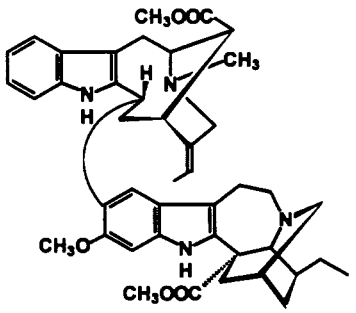

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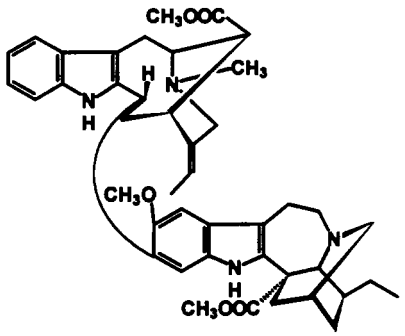

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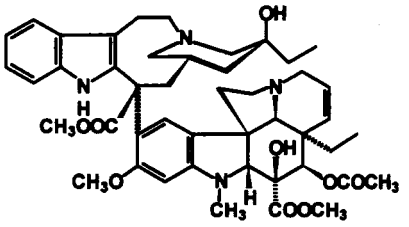

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(ED_{50} >20 μg/ml), nor with parental KB cells (ED_{50} >20 μg/ml) (Figure 1). Activity-guided fractionation using KB-V1 cells (treated with vinblastine) led to the purification of three indole alkaloids, coronaridine [**1**], conoduramine [**2**], and voacamine [**3**], which were identified by comparison of their physical and spectroscopic data with literature values, and in the case of **3**, by direct comparison with an authentic sample. Although compounds **1** and **3** have been found previously as constituents of *Peschiera* (2, 7–9), conoduramine [**2**] has not been previously isolated from any plant in this genus.

Compounds **1–3** were evaluated for cytotoxic activity with 11 cultured tumor cell lines. In addition, with multidrug-resistant KB-V1 cells, the study was performed with various concentrations of compounds **1–3**, respectively, in the presence and absence of vinblastine [**4**] (1 μg/ml). The sensitivities of KB and KB-V1 cells to vinblastine, and the resulting cytotoxicity of compounds **1–3** against various tumor cell lines, are illustrated in Table 1. Compounds **1–3** showed general cytotoxic activities against many

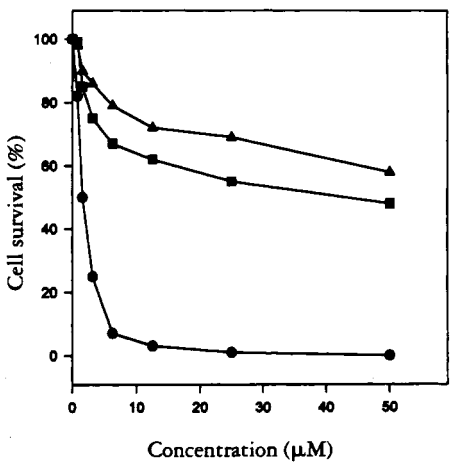

FIGURE 1. Percent survival of KB-V1 and KB-3 cells treated with various concentrations of the EtOAc extract of the stem bark of *P. laeta* was measured as described in the Experimental. Results were obtained with KB-3 cells (▲), KB-V1 cells with 1 μg/ml vinblastine in the medium (●), and KB-V1 cells without vinblastine in the medium (■).

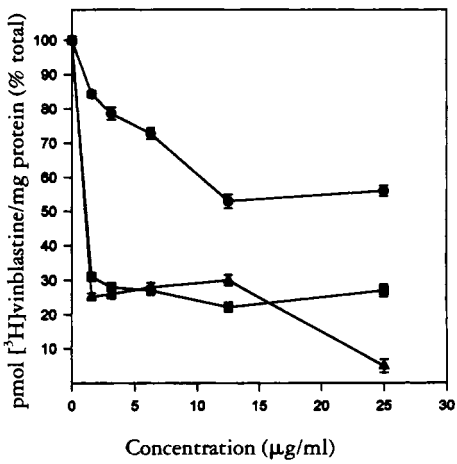

FIGURE 2. Dose-dependent inhibition of [^{3}H]vinblastine binding to KB-V1 vesicles. Incubations were performed as described in the Experimental in the presence of the indicated concentrations of coronaridine [**1**] (●), conoduramine [**2**](■), and voacamine [**3**](▲).

TABLE 1. Evaluation of the Cytotoxic Potential of Isolates Obtained from *P. laeta* [**1–3**] and Vinblastine [**4**].

Compound	Cell line[a]												
	A431[b]	BC1[b]	Col2[b]	HT[b]	KB[b]	KBV^{+}[b]	KBV^{-}[b]	LNCaP	Lu1[b]	Mel2[b]	P-388[b]	U373[b]	ZR-75-1
1	19.1	7.5	>20	>20	13.6	1.9	11.2	10.7	10.9	>20	3.8	12.0	>20
2	3.6	0.8	1.1	2.0	8.8	0.6	11.7	11.1	5.3	1.7	2.6	2.6	1.3
3	5.0	2.9	1.5	6.6	9.6	2.0	15.3	12.6	11.2	3.8	3.0	1.3	2.8
4	0.05	0.06	0.01	0.02	0.002	0.5	2.6	0.1	0.02	0.01	0.02	1.1	0.3

[a]Key: A431=human epidermoid carcinoma; BC1=human breast cancer; Col2=human colon cancer; HT=human fibrosarcoma; KB=human oral epidermoid carcinoma; KBV^{+}=drug-resistant KB assessed in the presence of vinblastine (1 μg/ml); KBV^{-}=drug-resistant KB assessed in the absence of vinblastine; LNCaP=human prostate cancer; Lu1=human lung cancer; Mel2=human melanoma; P-388=murine lymphocytic leukemia; U373=human glioma; ZR-75-1=hormone-dependent human breast cancer.

[b]Results are expressed as ED_{50} values (μg/ml).

of the tumor cell lines tested. Of particular note, however, with KB-V1 cells, the cytotoxicity mediated by compounds **1–3** was augmented by the addition of vinblastine to the culture medium. The ED_{50} values of vinblastine for KB and KB-V1 cells were 0.002 μg/ml and 2.6 μg/ml, respectively, indicating a greater than 1000-fold resistance. Therefore, KB-V1 cells were not affected by the presence of vinblastine [**4**] (1 μg/ml), unlike KB-3 cells that were not able to survive if treated with this concentration of vinblastine. Therefore, these data clearly reveal a reversion of multidrug-resistant KB-V1 cells to drug sensitivity by each of the three test compounds. This effect was similar to that mediated by the calcium channel blocker verapamil (data not shown).

Since a major mechanism of MDR in mammalian cells is increased expression of P-glycoprotein (11–13), the reversal of vinblastine resistance demonstrated by compounds **1–3** with multidrug-resistant KB-V1 cells suggests affinity for P-glycoprotein. In order to verify this hypothesis, ATP-dependent [^{3}H]vinblastine binding to multidrug-resistant KB-V1 cell membrane vesicles (a source of P-glycoprotein) was assessed in the presence and absence of compounds **1–3**. As shown in Figure 2, inhibition of ATP-dependent vinblastine binding to vesicles isolated from multidrug-resistant KB-V1 cells was mediated by each of the compounds in a dose-dependent manner. However, the bisindole alkaloids, conoduramine [**2**] and voacamine [**3**], were more potent inhibitory agents of [^{3}H] vinblastine binding with membrane vesicles isolated from multidrug-resistant KB-V1 cells, relative to the monomeric alkaloid, coronaridine [**1**].

Vinblastine [**4**] is a bisindole alkaloid consisting of catharanthine- and vindoline-derived moieties. It has been previously reported that both catharanthine and vindoline suppress the active outward transport of antitumor drugs from drug-resistant cells, thereby reversing the resistance phenotype (14). We currently demonstrate that coronaridine [**1**] reversed MDR, as would be expected on the basis of its structural relationship with catharanthine. On the other hand, the bisindole alkaloids conoduramine [**2**] and voacamine [**3**] somewhat resemble the structure of vinblastine and more effectively reverse MDR. Antitumor drugs such as vinblastine have been proposed to bind directly to P-glycoprotein, and this leads to active efflux from MDR cells through a pore or channel formed by the multiple transmembrane domains of P-glycoprotein using energy derived from P-glycoprotein-mediated ATP hydrolysis (13). Our findings suggest that coronaridine [**1**], conoduramine [**2**], and voacamine [**3**] are antitumor drug analogues that may modulate the MDR phenotype by competitive or noncompetitive inhibition of the binding of drugs to hydrophobic domains within the P-glycoprotein molecule. By similar mechanisms, it has been reported that calcium channel blockers such as verapamil (15) and many other agents can reverse multidrug-resistance with in vitro and in vivo biological systems (16). However, more effective agents with fewer toxic activities and side-effects than the present reversing agents are needed. As presently described, coronaridine [**1**], conoduramine [**2**], and voacamine [**3**] reverse the MDR phenotype, but, relative to vinblastine, cytotoxicity is greatly reduced. Thus, this study describes another new family of compounds, other than calcium antagonists, capable of inhibiting drug-binding and thereby circumventing multi-drug resistance, which should be of interest for more advanced biological testing.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were obtained on a Fisher-Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Uv spectra were recorded in MeOH on a Beckman DU-7 spectrometer, and ir spectra on a Midac Collegian Ft-ir spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were taken on a Varian XL-300 instrument, at 300 MHz and 75.6 MHz, respectively, with TMS as internal standard. Eims were obtained using a Finnigan MAT 90 instrument.

PLANT MATERIAL.—The stem bark of *Peschiera laeta* was collected in Brazil in December 1991. A voucher specimen (A00819) has been deposited in the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL.

EXTRACTION AND FRACTIONATION.—The dried, powdered bark of *P. laeta* (600 g) was percolated with MeOH (2×2 liters) at room temperature. After filtration and evaporation of the solvent, and defatting with *n*-hexane (2×250 ml), the MeOH residue was then partitioned between EtOAc and H_2O, and the EtOAc-soluble residue (8.5 g) was obtained as a brown powder on removal of solvent. This residue was subjected to cc over Si gel, using KB-V1 cells (+vinblastine) to monitor the fractionation. Elution with mixtures of $CHCl_3$ and Me_2CO of increasing polarity afforded 11 major fractions. Compound **1** (0.53 g, 0.18% w/w) was isolated from fraction 4 by elution with $CHCl_3$, and was purified by further Si gel cc by elution with *n*-hexane/$CHCl_3$. The bisindole alkaloids **2** and **3** occurred in fraction 8 of the initial cc (eluent $CHCl_3$-Me_2CO, 1:19), and were purified by elution over Si gel with *n*-hexane-EtOAc-dimethylamine (4:1:0.12), with **2** (84 mg, 0.028% w/w) obtained from fraction 14, and **3** (122 mg, 0.04% w/w) obtained from fraction 19 from this column.

Coronaridine [**1**].—Obtained as white powder, mp 50–51°, $[\alpha]^{20}D$ −42° (c=4.1, $CHCl_3$) [lit. (11) mp 50–51°, $[\alpha]^{26}D$ −41° (c=2.0, $CHCl_3$)], and exhibited comparable spectroscopic (uv, ir, ^{1}H-nmr, ^{13}C-nmr, ms) data to published values for coronaridine (17).

Conoduramine [**2**].—Crystallized as needles from MeOH/Me_2O, mp 215–217°, $[\alpha]^{20}D$ −7.4° (c=6.6, $CHCl_3$) [lit. (12) mp 215–217°, $[\alpha]^{22}D$ −77° (c=1.0, $CHCl_3$)], and exhibited comparable spectroscopic (uv, ir, ^{1}H-nmr, ^{13}C-nmr, ms) data to published values for conoduramine (18–20).

Voacamine [**3**].—Crystallized from MeOH as rhombic crystals, mp 225–227°, $[\alpha]^{20}D$ −51° (c=0.18, $CHCl_3$) [lit. (3) mp 222–224°, $[\alpha]^{20}D$ −50° (c=0.94, $CHCl_3$)], and exhibited comparable spectroscopic (uv, ir, ^{1}H nmr, ^{13}C nmr, ms) to published values for voacamine (2,6,21), and was directly compared to an authentic sample. Voacamine [**3**] has been isolated previously as a constituent of the leaves and twigs of *P. laeta* (2).

EVALUATION OF THE CYTOTOXIC POTENTIAL OF CORONARIDINE [**1**], CONODURAMINE [**2**], AND VOACAMINE [**3**].—Cytotoxicity tests were performed as described previously (22). In brief, various concentrations of compounds were transferred to 96-well plates (10 μl/well in 10% DMSO), and 190 μl of a cell suspension were added to each well. The plates were incubated for 72 h at 37° in a humidified 5% CO_2 atmosphere. At the end of the incubation, 50 μl of cold 50% aqueous trichloroacetic acid were added to the growth medium in each well to fix the cells. The cultures were incubated at 4° for 1 h and then washed with tap H_2O. Plates were air-dried and stained with sulforhodamine B solution for 30 min. Stained cultures were washed with 1% HOAc. Finally, 200 μl of 10 mM Tris base was added to each well to solubilize the sulforhodamine B and the optical densities were determined at 515 nm utilizing a plate reader. In each case, a zero-day control was performed by adding an equivalent number of cells to several wells and incubating at 37° for 30 min. Cells were then processed as described above. Finally, the optical density values obtained with the zero-day control were subtracted, and cell survival, relative to control (solvent-treated) cultures, was evaluated.

Similar to the procedures described above, P-388 cells were enumerated and diluted to a concentration of 10×10^4 cells/ml, 190 μl of a cell suspension were added to each well containing the test compounds and the incubation was performed at 37° in a humidified incubation with a 5% CO_2 atmosphere for 48 h. After incubation, plates were centrifuged (10 min, 2000 rpm) and the supernatant fractions were removed. Cells were then treated with 100 μl of 20% trichloroacetic acid, incubated at 4° for 1 h, and rinsed with H_2O, dried, and treated with sulphorhodamine B solution as described above.

CORONARIDINE [**1**]-, CONODURAMINE [**2**]-, AND VOACAMINE [**3**]-MEDIATED INHIBITION OF VINBLASTINE BINDING TO KB-V1 VESICLES.—Binding assays were performed in 96-well microtiter plates. Briefly, plasma membranes were prepared from cultured KB-V1 cells (23) and used as a source of P-glycoprotein. An aliquot of plasma membrane (60 μg protein) was incubated in buffer consisting of 0.01 M Tris-HCl, pH 7.5, 0.125 M sucrose, 5 mM $MgCl_2$, and 0.5 mM ATP, as well as 0.16 mM [^{3}H]vinblastine (9 Ci/mmol), and various concentrations of test compounds that were initially dissolved in 5 μl DMSO (final volume, 100 μl). Incubations were conducted at room temperature for 20 min. Reactions were terminated by aspirating the contents of each well onto a glass fiber filter (Filtermats-Receptor Binding, Skatron Instruments, Inc., Sterling, VA), which had been pretreated with 0.2% bovine serum albumin dissolved in the buffer described above. Non-specific binding was determined by performing similar incubations that contained a 1000-fold excess of unlabeled vinblastine in addition to the components listed above. Non-specific binding was subtracted from all total-binding data to yield specific binding. All experimental points were obtained at least in triplicate, and standard deviations were calculated.

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