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(−)-ROEMERINE, AN APORPHINE ALKALOID FROM *ANNONA SENEGALENSIS* THAT REVERSES THE MULTIDRUG-RESISTANCE PHENOTYPE WITH CULTURED CELLS

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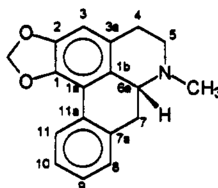
ABSTRACT.—A known aporphine alkaloid, (−)-roemerine [**1**], isolated from the leaves of *Annona senegalensis*, was found to enhance the cytotoxic response mediated by vinblastine with multidrug-resistant KB-V1 cells. In the absence of vinblastine, no significant cytotoxicity was observed with KB-3 or KB-V1 cells (ED_{50} >20 μg/ml), and several other human tumor cell lines were also relatively insensitive. As indicated by its ability to inhibit ATP-dependent [^{3}H]vinblastine binding to multidrug-resistant KB-V1 cell membrane vesicles, (−)-roemerine appears to function by interacting with P-glycoprotein. In addition to alkaloid **1**, three inactive compounds [the aporphine alkaloid (−)-isocorydine (reported in the levo-configuration for the first time), and the lignans (±)-8,8′-bisdihydrosiringenin [**2**] (a new natural product), and (+)-syringaresinol] were also isolated.

Several plant parts of *Annona senegalensis* Pers. (Annonaceae) are used in traditional medicine in various countries of tropical Africa for the treatment of many diseases and symptoms, including cancer (1–3), convulsions (3), diarrhea (4,5), dysentery (1), fever (1), filariasis (6), male impotency (5), pain of the chest and intestines (4), swelling (1), trypanosomiasis (7), venereal disease (1), and snake-bite (1). Root extracts of *A. senegalensis* have been found to exhibit antineoplastic activity in mice bearing sarcoma 180 ascites tumor cells (1,2), and antiprotozoal activity in mice infected with *Trypanosoma brucei brucei* (7), but the active principles were not determined in either case. Reported constituents of *A. senegalensis* include an aliphatic ketone, alkanes, alkanols, fatty acids, flavonoids, and sterols from the leaves (8,9); monoterpenoids and sesquiterpenoids from the essential oil of the leaves and fruit (10); amino acids from the stem bark (11); and *ent*-kaurenoids from the root bark (3,12).

In our continuing search for naturally occurring antineoplastic agents from higher plants, we have investigated an EtOAc-soluble extract of *A. senegalensis* leaves, which showed initial activity against a multidrug-resistant KB-V1 cell line (ED_{50} 17.4 μg/ml) in the presence of vinblastine, while exhibiting no significant cytotoxicity with KB-V1 cells in the absence of vinblastine (ED_{50} >50 μg/ml), nor with parental KB cells (ED_{50} >50 μg/ml) (Figure 1). Activity-guided fractionation using KB-V1 cells (co-incubated with vinblastine) led to the isolation of the aporphine alkaloid (−)-roemerine [**1**, (−)-*N*-methylanonaine] as the sole active principle. Also obtained as inactive compounds from the active fraction containing **1** were the alkaloid (−)-isocorydine (which has not been previously reported to occur in the levo-enantiomeric form), the known lignan (+)-syringaresinol, and (±)-8,8′-bisdi-



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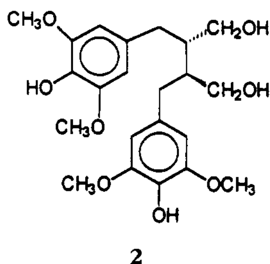
hydrosiringenin [**2**]. This is the first report of **2** as a natural product, although it has been derived previously by hydrolysis of a glycoside derivative (13,14). In the structure provided for **2**, only the (+)-enantiomeric form is shown, for simplicity of presentation.



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Evaluation of the cytotoxic potential of (−)-roemerine [**1**] was conducted with cultured P-388 cells and a battery of human tumor cell lines. Also, to investigate the effect of (−)-roemerine [**1**] on reversing multidrug resistance, KB-V1 cells were treated with different concentrations of the test substance in the presence or absence of 1 μg/ml vinblastine. This concentration is lethal with drug-sensitive KB-3 cells, but does not affect the growth of drug-resistant KB-V1 cells. As summarized in Table 1, alkaloid **1** showed general albeit weak cytotoxic activities against many of the tumor cell lines that were tested. However, with KB-V1 cells, alkaloid **1** did not mediate a cytotoxic response in the absence of vinblastine, but on addition of vinblastine to the culture medium, an ED_{50} value of 0.6 μg/ml was obtained. Further, alkaloid **1** did not demonstrate appreciable activity with the drug-sensitive cell line, KB-3.

These data clearly reveal a reversion of multidrug resistant KB-V1 cells to drug sensitivity that is related to the dose of alkaloid **1** added to the incubation medium (Figure 2). To further explore the MDR reversing activity of alkaloid **1**, KB-3 and KB-V1 cells were treated with various concentrations of vinblastine in

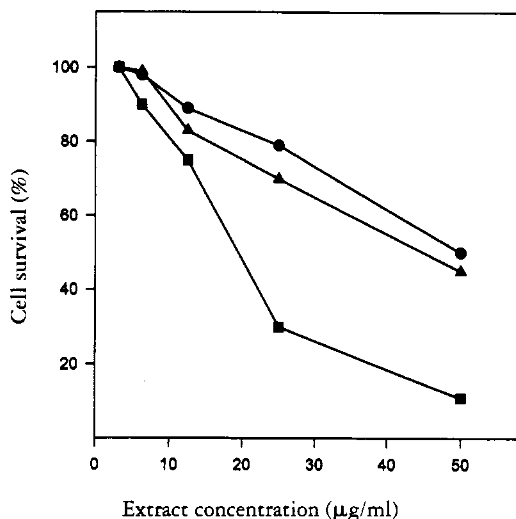

FIGURE 1. Percent survival of KB-V1 and KB-3 cells treated with various concentrations of the EtOAc extract of *A. senegalensis* leaves, measured as described in the Experimental. Results were obtained in the presence of the indicated amount of extract with KB-3 cells (●), KB-V1 cells with 1 μg/ml vinblastine in the medium (■), and KB-V1 cells without vinblastine in the medium (▲).

TABLE 1. Evaluation of the Cytotoxic Potential of (−)-Roemerine [**1**] Obtained from *Annona senegalensis*.

Cell line[a]												
A431	BC1	Col2	HT	KB-3	KBV-V1^{+}	KBV-V1^{-}	LNCaP	Lu1	Mel2	P-388	U373	ZR-75-1
4.3	8.3	5.8	6.4	>20	0.6	>20	10.7	6.6	>20	>5	5.0	>20

[a]Key: A431=human epidermoid carcinoma; BC1=human breast cancer; Col2=human colon cancer; HT=human fibrosarcoma; KB-3=human oral epidermoid carcinoma; KB-V1^{+}=drug-resistant KB-3 assessed in the presence of vinblastine (1 μg/ml); KB-V1^{-}=drug-resistant KB-3 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. Results are expressed as ED_{50} values (μg/ml).

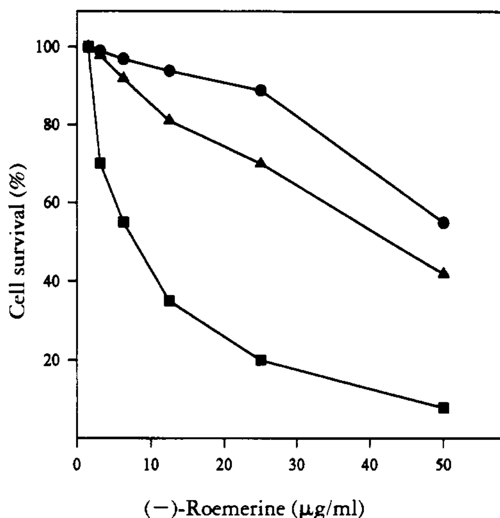

FIGURE 2. Percent survival of KB-V1 and KB-3 cells treated with various concentrations of (−)-roemerine [**1**], measured as described in the Experimental. Results were obtained in the presence of the indicated amount of (−)-roemerine [**1**] with KB-3 cells (●), KB-V1 cells with 1 μg/ml vinblastine in the medium (■), and KB-V1 cells without vinblastine in the medium (▲).

the presence or absence of alkaloid **1**. As shown in Figure 3A, with KB-V1 cells the cytotoxic response mediated by vinblastine was augmented in the presence of a fixed concentration of alkaloid **1** (10 μg/ml), which was nontoxic in the

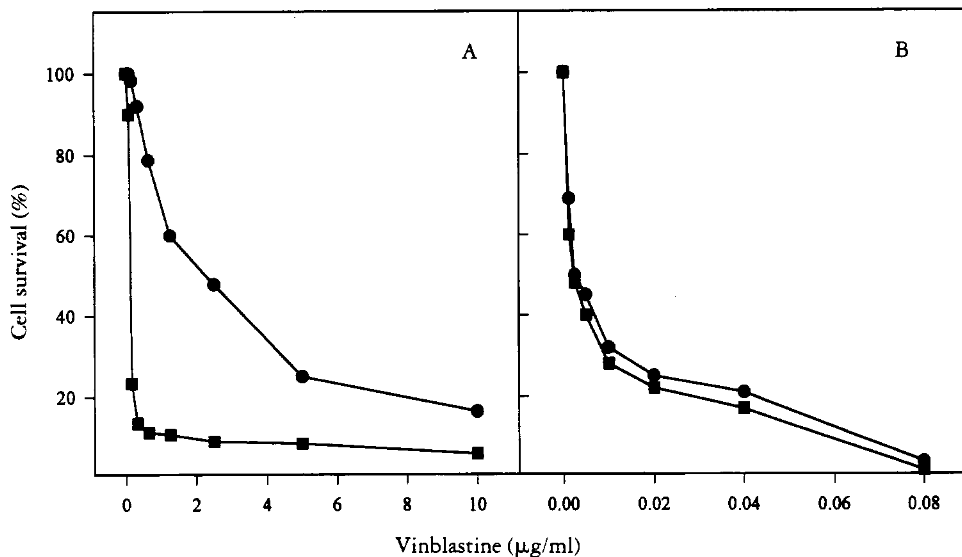

FIGURE 3. (A) Percent survival of KB-V1 cells treated with various concentrations of vinblastine in the presence (10 μg/ml) (■) and absence (●) of (−)-roemerine [**1**]. (B) Percent survival of KB-3 cells treated with various concentrations of vinblastine in the presence (10 μg/ml) (■) and absence (●) of (−)-roemerine [**1**].

absence of vinblastine. Augmentation of dose-dependent vinblastine-mediated toxicity by alkaloid **1** was not observed with parental KB-3 cells (Figure 3B). Finally, 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 alkaloid **1**. As shown in Figure 4, inhibition of ATP-dependent vinblastine binding to vesicles isolated from multidrug-resistant KB-V1 cells was facilitated by alkaloid **1** in a dose-dependent manner.

It has been reported that the most potent MDR reversing agents are hydrophobic molecules with two planar aromatic rings and a tertiary basic nitrogen atom (15). These agents may modulate the MDR phenotype by competitive or noncompetitive inhibition of the binding of drugs to hydrophobic domains within the P-glycoprotein molecule, and this leads to intracellular drug accumulation. We currently demonstrate that (−)-roemerine [**1**] reversed the MDR phenotype, as might be expected on the basis of its structural features. Due to the selectivity of this response, we are currently evaluating compound **1** for its potential to mediate analogous responses with MDR animal tumor models. However, to avoid toxic side-effects, it may eventually be necessary to consider an analogue of (−)-roemerine [**1**], because mutagenic activity has been demonstrated with *Salmonella typhimurium* strain TA98 in the presence of a metabolic activating system derived from the post-mitochondrial supernatant fraction of rat liver (16).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. Uv spectra were obtained on a Beckman DU-7 spectrometer. Ir spectra were taken with a Midac Collegian FT-IR spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were measured with TMS as internal standard, using a Varian XL-300 instrument operating at 300 MHz and 75.6 MHz, respectively, with ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR nmr experiments performed using standard Varian pulse sequences. Eims were obtained using a Finnigan MAT 90 instrument. Prep. tlc was performed on Merck Si gel G plates (0.5-mm layer thickness).

PLANT MATERIAL.—The leaves of *Annona senegalensis* were collected in February 1991, at the Mutorashanga District, Zimbabwe. Voucher specimens have been deposited in the herbarium of the

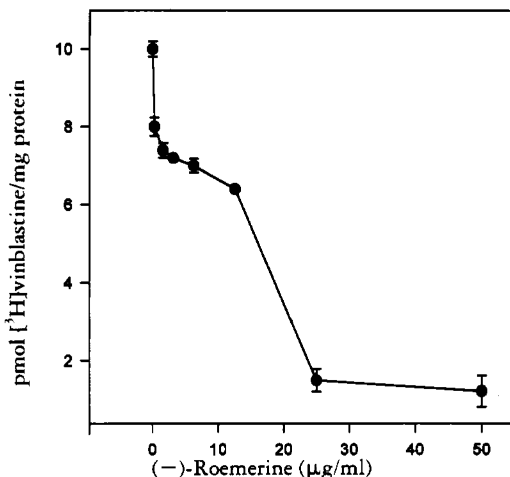

FIGURE 4. Dose-dependent inhibition of [^{3}H]vinblastine binding to KB-V1 cell membrane vesicles. Incubations were performed as described in the Experimental in the presence of the indicated concentrations of (−)-roemerine [**1**].

Field Museum of Natural History, Chicago, Illinois (V0034) and at the Herbarium Zimbabwense, Harare, Zimbabwe (V341).

EXTRACTION AND ISOLATION.—The air-dried, milled leaves (3.4 kg) were extracted three times overnight with MeOH (3×15 liters). The resultant extracts were combined, concentrated, and then diluted with H_2O to afford an aqueous MeOH (1:9 v/v, 1.5 liter) solution, which was defatted with petroleum ether (3×1 liter). The methanolic layer was concentrated to dryness and partitioned three times between EtOAc (3×1 liter) and H_2O (3×1 liter). The combined EtOAc layers were dried ($MgSO_4$), filtered, and concentrated to give an extract (16.5 g).

A portion (16 g) of the EtOAc extract was adsorbed onto Si gel (65 g), and separated over Si gel (130 g) by vlc, using petroleum ether/$CHCl_3$ and $CHCl_3$/MeOH mixtures, before being finally eluted with MeOH. A total of 12 combined fractions was collected. Fraction 5 (500 mg), which was eluted with $CHCl_3$-MeOH (99:1), showed the most pronounced activity against the KB-V1 (+VLB) cell line (ED_{50} 7 μg/ml). Further purification of this active fraction by flash chromatography over Si gel using $CHCl_3$/MeOH mixtures gave five combined fractions. Sub-fraction 4 (100 mg) was further separated by flash chromatography [hexane-$(CH_3)_2CO$, 2:3] yielding two compounds that were positive to Dragendorff's reagent. They were purified by prep. tlc, using as solvent petroleum ether-$CHCl_3$-MeOH (10:10:1), and identified as the aporphine alkaloids (−)-roemerine (**1**, N-methylanonaine; 8 mg, 0.0002%) and (−)-isocorydine (10 mg, 0.0003%), respectively. Sub-fraction 1 (70 mg) was further separated on Sephadex LH-20 ($CHCl_3$-MeOH, 1:1), followed by purification on prep. tlc (solvent, $CHCl_3$-MeOH, 9:1) to afford (±)-8,8′-bisdihydrosiringenin (**2**, 10 mg, 0.0003%). Sub-fraction 3 on purification by repeated prep. tlc (solvent, $CHCl_3$-MeOH, 9:1) afforded a second lignan, (+)-syringaresinol (6 mg, 0.0002%).

(−)-Roemerine [(−)-N-*Methylanonaine*] [**1**].—Amorphous solid, mp 89–91°, $[\alpha]^{25}D$ −54.0° (c=0.10, EtOH) [lit. (17), mp 85–86°, $[\alpha]^{25}D$ −79.7° (c=0.1, EtOH)]; ^{13}C nmr ($CDCl_3$) δ 142.6 (C-1), 116.4 (C-1a), 126.4 (C-1b), 146.7 (C-2), 107.5 (C-3), 126.9 (C-3a), 29.0 (C-4), 53.5 (C-5), 62.0 (C-6a), 34.6 (C-7), 153.3 (C-7a), 127.5^a (C-8), 126.9^a (C-9), 127.0^a (C-10), 128.2^a (C-11), and 131.1 (C-11a). [Values denoted by the superscript are interchangeable with the assignments made by comparison with analogue alkaloids in the literature (18)]. The identity was confirmed by comparison with published spectroscopic data for (−)-roemerine (uv, ir, ^1H-nmr, ms) (18).

(−)-Isocorydine.—Amorphous powder, mp 78–80°, $[\alpha]^{25}D$ −190° (c=0.02, $CHCl_3$) [lit. (18), $[\alpha]^{25}D$ +210° (c=0.10, $CHCl_3$) and lit. (19), $[\alpha]^{25}D$ +196° (c=0.2–1.5, $CHCl_3$)]. The identity of this isolate was confirmed by comparison with published spectroscopic (uv, ir, ^1H-nmr, ^{13}C-nmr, ms) data for (+)-isocorydine (18,20).

(±)-8,8′-Bisdihydrosiringenin [*(±)-1,4-dihydroxy-2,4-di-(4-hydroxy-3,5-dimethoxybenzyl)propane*] [**2**].—Amorphous powder, mp 180–181°, $[\alpha]^{25}D$ +3.6° (c=0.14, $CHCl_3$) [lit. (13) for 7*S*,7′*S* [i.e., (+)-form], $[\alpha]^{25}D$ +30.6° (c=0.82, $CHCl_3$)]. The identity was confirmed by comparison with published spectroscopic (uv, ir, ^1H-nmr, ^{13}C-nmr, ms) data for the 7*S*,7′*S*-(+)-form of 8,8′-bisdihydrosiringenin [**2**] (13,14).

(+)-Syringaresinol.—Colorless needles, mp 170–173°, $[\alpha]^{25}D$ +10.4° (c=0.07, $CHCl_3$) [lit. (21), mp 173–175°; $[\alpha]^{25}D$ +12.8° (c=0.05, $CHCl_3$)]. The identity was confirmed by comparison with published spectroscopic (uv, ir, ^1H-nmr, ^{13}C-nmr, ms) data for (+)-syringaresinol (21–23).

EVALUATION OF THE CYTOTOXIC POTENTIAL OF (−)-ROEMERINE [**1**].—The cytotoxic potential of (−)-roemerine [**1**] was determined using procedures described previously (24). Briefly, various concentrations of the test compound (dissolved in 10 μl of 10% DMSO) were transferred to 96-well plates and 190 μl of a cell suspension were added to each well. The plates were then incubated for 72 h at 37° (100% humidity with a 5% CO_2 atmosphere in air). 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 an ELISA 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.

(−)-ROEMERINE [**1**]-MEDIATED INHIBITION OF VINBLASTINE BINDING TO KB-V1 VESICLES.—Binding assays were performed in 96-well microtiter plates. In brief, plasma membranes were prepared from cultured KB-V1 cells (25,26) and used as a source of P-glycoprotein. An aliquot of plasma membrane (60 μg protein) was added to 0.01 M Tris-HCl buffer, pH 7.5, containing 0.125 M sucrose, 5 mM $MgCl_2$, 0.5 mM ATP, as well as 0.16 μM [^3H]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 pre-treated with 0.2% bovine serum albumin dissolved in the buffer described above. Radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by performing similar incubations that contained a thousand-fold excess of unlabeled vinblastine in addition to the components listed above. Nonspecific 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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