Pervellite A, a Novel Tropane Alkaloid that Reverses the Multidrug-resistance Phenotype

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ABSTRACT

P-Glycoprotein-mediated drug efflux can yield a multidrug-resistance (MDR) phenotype that is associated with a poor response to cancer chemotherapy. Pervellite A, a novel tropane alkaloid obtained from a chloroform extract of Erythroxylum pervellite as the result of bioactivity-guided fractionation, was found to restore the vinblastine sensitivity of cultured multidrug-resistant KB-V1 and CEM/VLB100 cells, with IC_{50} values of 0.36 and 0.02 μM, respectively. Similarly, the chemosensitivity of KB-8-5 cells to colchicine was restored with an IC_{50} value of 0.61 μM. The mechanism of this response was evaluated with a number of model systems. First, incubation of multidrug-resistant KB-V1 and CEM/VLB100 cells with up to 45 μM pervellite A for 72 h did not significantly affect either the transcription of MDR1, as revealed by reverse transcriptional-PCR-based analysis of MDR1 mRNA, or levels of P-glycoprotein, as shown by Western blots. ATP-dependent binding of [3H]vinblastine observed with isolated multidrug-resistant KB-V1 cell membrane vesicles was inhibited by pervellite A in a dose-dependent manner, and kinetic analysis indicated competitive inhibition with respect to vinblastine binding with a K_i of 7.3 μM. Consistent with this effect, intracellular accumulation of [3H]vinblastine was increased from 0.18 pmol [3H]vinblastine/50 × 10^4 cells to approximately 5 pmol [3H]vinblastine/50 × 10^4 cells in the presence of 40 μM pervellite A. To explore the potential relevance of these responses, KB-V1 or KB-8-5 cells were placed in hollow filters and implanted into NCr nude mice. Cell growth was not significantly inhibited when vinblastine or pervellite A were administered as single agents, but when used in combination, inhibition of up to 75% was observed. Equimolar doses of verapamil were less effective. These data suggest that pervellite A is an effective inhibitor of P-glycoprotein and should be further evaluated for clinical utility.

INTRODUCTION

One serious problem associated with cancer chemotherapy is the development of multidrug-resistant tumor cells during the course of treatment. An important mechanism of acquiring the MDR phenotype in mammalian cells is the enhanced expression of a membrane glycoprotein, which has been termed Pgp (1). With a M_i of 170,000, the Pgp is coded by the MDR1 gene. Pgp functions as an energy-dependent multidrug membrane transporter that rapidly extrudes a variety of hydrophobic antitumor drugs from target cancer cells and thereby prevents the drugs from exerting cytotoxic effects. Initial physiological and pharmacological studies with multidrug-resistant mutant cell lines correlated resistance as being attributable to reduced accumulation of drugs within the cell as a result of increased efflux or decreased influx (1). Because the efflux pump is an ATP-dependent transport system (2), agents that are good inhibitors of ATP-dependent drug transport should inhibit the efflux of hydrophobic drugs from resistant cells and increase intracellular accumulation. Accordingly, a variety of agents have been reported to overcome or at least partially circumvent MDR (3).

First-generation modulators were originally developed for other therapeutic indications (4). Included in this category are calcium channel blockers such as verapamil (5), the immunosuppressive agent cyclosporin A (6), analogues of the antihypertensive reserpine and yohimbine (7), the antipsychotic trifluoperazine (4), and antiestrogens, such as tamoxifen (1). Second-generation modulators were developed that lack the original pharmacological activities of the first-generation compounds and usually possess higher affinity for Pgp. These agents include the R isomer of verapamil (8), a nonimmunosuppressive analogue of cyclosporin D, SDZ PSC-833 (6), and others such as MS-209 (9), S-9788 (10), GF120918 (11), and LY335979 (4). However, although many of these pharmacological agents have been found to completely overcome drug resistance with in vitro models, the number of reports showing such phenomena in vivo systems is more limited (12–15). The lack of in vivo activity of chemosensitizers results mainly from problems associated with maintaining active doses without causing serious side effects (16). Thus, clinical Phase I and Phase II studies have been disappointing, often because of limited tolerance to prototype MDR inhibitors by themselves, which precluded attainment of potentially active levels in patients (11, 17); e.g., full reversion of MDR by verapamil requires a concentration of approximately 10 μM in most cell culture models, whereas plasma levels above 1 μM result in atrioventricular blocks (5, 18). Immunosuppressive effects and nephrotoxicity limit the clinical usefulness of cyclosporin A (6), and toxicities such as cerebellar ataxia and hyperbilirubinemia are caused by SDZ PSC-833 (19, 20). Also, cyclosporin A, verapamil, and SDZ PSC-833 have profound effects on the pharmacokinetics of doxorubicin, etoposide, and other oncolytic drugs (4).

As described recently (21), the MDR modulator valspador is currently being evaluated in Phase III, randomized trials for the treatment of acute myeloid leukemia, multiple myeloma, and ovarian cancer. Also, Ontogen Corporation has announced completion of a Phase I study conducted with the MDR-reversing agent OC 144-093 (22), and a second Phase I study to examine oral administration is underway.

Part of our natural product drug discovery program involves monitoring the potential of plant extracts to reverse MDR. Standard cell survival assays are used to determine the dose of plant extracts or compounds required to inhibit cell growth by 50% with drug-sensitive human epidermoid carcinoma parental KB-3 cells and Pgp-associated multidrug-resistant KB-V1 cells. To investigate the potential of plant extracts or compounds to reverse multidrug-resistance, KB-V1 cells are treated with different concentrations of plant extracts or compounds in the presence (1 μg/ml) or absence of vinblastine. This concentration of
vinblastine is lethal to KB-3 cells but does not affect the growth of KB-V1 cells. Therefore, KB-3 cells serve as a control to differentiate between nonspecific cytotoxicity and selective MDR antagonism. This assay uses 96-well microtiter plate technology, and over 3000 different plant extracts have been tested. Using the model for bioassay-guided isolation of active principles, we have identified previously (23, 24) four moderate inhibitors of MDR: coronaridine, conoduramine, voacamine, and (-)-roemerine. More recently, an extremely potent novel tropane alkaloid, pervilleine A (3a, 4, 5-trimethoxybenzoyloxy)-6β-(3, 4, 5-trimethoxy-cinnamoyloxy)-7β-hydroxytropane (Fig. 1), was obtained from a chloroform-soluble extract derived from the roots of *Erythroxylum pervillei* Baillon (Erythroxylaceae). 7 Appreciable activity was only demonstrated when vinblastine was added to KB-V1 cells in the presence of pervilleine A. The compound did not inhibit the growth of BC1 (human breast cancer), L1210 (human lung cancer), Co12 (human colon cancer), LNCaP (hormone-dependent human prostate cancer), SKNSH (human neuroblastoma cancer), M109 (mouse lung cancer), or SW626 (human ovarian cancer) cells in culture (IC50 values, >34 μM).

As described herein, we have partially characterized the mechanism by which pervilleine A reverses the MDR phenotype. Obviously, however, cancer treatments that appear promising with *in vitro* models are often less effective against solid tumors. One method for providing a preliminary indicator of therapeutic efficacy has been described recently by Hollingshead et al. (25). The majority of the human tumor cell lines currently used in cell culture can be grown inside hollow fibers (26) to form a heterogeneous solid tumor model. Therefore, semipermeable hollow fibers containing human tumor cells are implanted at the i.p. or s.c. compartments of host mice, and the mice are treated with the test substances of interest. Through determination of the potential to inhibit cell growth versus the potential to mediate a toxic response toward the host, a preliminary estimate of therapeutic efficacy is provided in a cost- and time-effective manner (27). We currently report the use of this model for assessing MDR-reversing agents in combination with conventional chemotherapeutic agents and the results obtained with pervilleine A.

**MATERIALS AND METHODS**

**Chemicals and Cell Cultures.** [3H]Vinblastine (4.8 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). All of the other chemicals were purchased from Sigma Chemical Co. Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY). Human oral epidermoid carcinoma KB-3 was purchased from the American Type Culture Collection (Rockville, MD), and KB-V1 and KB-8-5 cells were supplied by Dr. I. B. Roninson (Department of Molecular Genetics, University of Illinois at Chicago, Chicago, IL). KB-3 cells were maintained in DMEM supplemented with 10% heat-inactivated calf serum and 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B. KB-V1 cells were grown in the same medium, which was further supplemented with vinblastine (1 μg/ml). Similarly, KB-8-5 cells were cultured in the same medium supplemented with colchicine (10 ng/ml). Human leukemic lymphoblast CEM cells and their multidrug-resistant counterpart CEM/VLB100 cells (provided by Dr. W. T. Beck, Department of Pharmacuetics and Pharmacodynamics, University of Illinois at Chicago, Chicago, IL) were cultured as described previously (28).

**Cytotoxic Potential.** The cytotoxic potential of test substances with KB-3, KB-V1, and KB-8-5 cells was determined as described previously (24). Briefly, various concentrations of test compounds (dissolved in 10 μl of 10% DMSO) were transferred to 96-well plates, and 190-μl aliquots of cell suspensions (5 × 104 cells/ml) were added to each well. The plates were then incubated for 72 h at 37°C (100% humidity with a 5% CO2 atmosphere in air), and 100 μl of cold 20% aqueous trichloroacetic acid were added to the growth medium in each well to fix the cells. The cultures were incubated at 4°C for 30 min, washed, air-dried, stained with sulforhodamine B solution, and washed with 1% acetic acid. Finally, 200 μl of 10 mM Tris base were added to each well, and the optical densities were determined at 515 nm using an ELISA plate reader. In each case, a zero-day control was performed by adding an equivalent number of cells to several wells, incubating at 37°C for 30 min, and processing as described above. Absorbance values obtained with the zero-day control were subtracted, and cell survival, relative to control (solvent-treated) cultures, was calculated. The cytotoxic potential of the compounds with CEM and CEM/VLB100 cells was determined as described by Beck et al. (29). Cells were grown in 24-well plates at a density of 5–6 × 105 cells/ml. At 48 h, the cell number was determined using a Coulter counter, with a channelizer to distinguish cells from debris. The IC50 was defined as the concentration of drug required to inhibit the 48-h growth of treated cells by 50% compared with untreated controls.

[3H]Vinblastine Accumulation with KB-V1 Vesicles. Cell membrane vesicles were prepared from KB-V1 cells following literature procedures (30, 31) with some modifications. Medium was removed from KB-V1 cells in log growth phase (about 80% confluence), and the cells were rinsed with ice-cold PBS followed by ice-cold PBS containing 2 mM EDTA. Aprotinin (1 mg/ml) was then added, and, after a 10-min incubation at room temperature, the cells were harvested by gentle aspiration with a serological pipette and collected by centrifugation (100 × g for 5 min). The cells were suspended in 0.25 μM sucrose buffer [0.01 M Tris-HCl (pH 7.5) containing 0.25 μM sucrose, 0.2 mM CaCl2, and 1 mM EDTA] and homogenized with a Polytron at 2500 rpm for 30 s. After this procedure, <5% of the cells remained intact. The homogenate was then diluted with four volumes of 0.025 μM sucrose solution [0.01 M Tris-HCl (pH 7.5) containing 0.025 mM sucrose] and centrifuged (1000 × g for 10 min). The supernatant was layered onto a 35% sucrose cushion [35% w/v sucrose, 1 mM EDTA, and 0.01 M Tris-HCl (pH 7.5)] and centrifuged at 16,000 × g for 30 min. The interface (about 5 ml) was collected, diluted with four volumes of 0.25 μM sucrose, 0.01 M Tris-HCl (pH 7.5), and centrifuged at 100,000 × g for 1 h. The resulting vesicle pellet was suspended in PBS containing 1 mM phenylmethylsulfonyl fluoride, using a 25-gauge needle, and stored at −80°C. Protein content was determined using a bichinonic acid protein assay kit with BSA as a standard (32).

Vinblastine accumulation with membrane vesicles assays was performed in 96-well plates as described previously (23, 24). Plasma membrane vesicles (40 μg of protein) were incubated in 0.01 M Tris-HCl buffer (pH 7.5) containing 0.125 μM sucrose, 0.05 mM MgCl2, 0.5 mM ATP, and 0.16 μM [3H]vinblastine (4.8 Ci/mmol). Various concentrations of test samples, dissolved in 5 μl of DMSO, were then added (final volume, 100 μl), and incubations were conducted at ambient temperature for 20 min. For kinetic studies, various concentrations of [3H]vinblastine were used. Reactions were terminated by aspirating the contents of each well onto a glass filter (printed type A filtermat; Wallac) using a 96-well harvester (Harvester 96; Tomtec). Radioactivity was determined by liquid scintillation counting (1450 Microbeta; Wallac). Nonspecific binding was determined by performing similar incubations with reaction mixtures.

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containing a 1000-fold excess of unlabeled vinblastine. Nonspecific binding was subtracted from all of the total-binding data to yield specific binding.

**[3H]Vinblastine Accumulation with Intact Cells.** Measurement of the accumulation of [3H]vinblastine in cell monolayers was performed by the method of Fojo et al. (33) with some modifications. Studies were performed with 24-well plates. In preparing the cell monolayers, 2 ml of a cell suspension containing 2.5 × 10⁶ cells/ml of incubation medium (DMEM with 10% calf serum) were added to each well. Control wells contained 2 ml of assay medium. After the addition of [3H]vinblastine (16 nM, 4.8 Ci/mmol) and various concentrations of test samples (dissolved in 10 µl of DMSO), the plates were incubated at 37°C in a 5% CO₂ incubator for 1 h. Monolayers were washed three times with cold PBS and dried by inversion, and the cells were treated with trypsin. Cell suspensions were then transferred to vials containing 3 ml of fresh assay medium (CytoScint, ICN), mixed vigorously and counted. The quantity of [3H]vinblastine associated with incubations containing cells was corrected by subtracting the counts associated with incubations not containing cells.

**RT-PCR Analysis of MDR1 mRNA Expression.** Culture flasks (60 × 15 mm) containing 100 × 10⁶ KB-3, KB-V1, CEM, or CEM/VLB₁₀₀ cells were treated with various concentrations of pervinelle A (0–34 µM) or verapamil (0–44 µM) for 72 h at 37°C in a 5% CO₂ incubator. Total RNA was isolated from cultured KB-3, KB-V1, CEM, and CEM/VLB₁₀₀ cells with TRIZol reagent (Life Technologies, Inc.; Ref. 32) and quantified by UV absorbance. The RT of the RNA was performed by using the Superscript Pre amplification System (Life Technologies, Inc.) in a final volume of 20 µl containing 5 X first strain synthesis buffer, 2.5 µM MgCl₂, 0.5 mM each deoxynucleotide triphosphate, 10 µM DTT, 2 units of Rnase inhibitor, 200 units of Superscript II reverse transcriptase, 0.5 µg of oligo(dT)₂₀–₃₀, 2 µg of total RNA (3 µg for CEM and CEM/VLB₁₀₀ cells), and diethylpyrocarbonate-treated water. After incubation at 42°C for 50 min, the RT reaction was terminated by heating to 70°C for 15 min. To the newly synthesized cDNA (2 µl), a PCR mixture containing 1.4 mM MgCl₂ (1.25 mM for CEM and CEM/VLB₁₀₀ cells), 2.5 units of Taq polymerase, and 0.2 µM (0.6 µM for CEM and CEM/VLB₁₀₀ cells) of primers based on the MDR1 gene (34) and custom synthesized by Ana-Gen Technology, Inc. (Palo Alto, CA; 5'-ATATCTAGGCGGCGCACTATAC-3'; 5'-GAAGACATTGGATGTCGCG-3'; Ref. 34). As an internal control, 0.125 µM of oligo(dT)₁₂–₁₅ for CEM and CEM/VLB₁₀₀ cells of primers for glyceraldehyde-3-phosphate dehydrogenase (5'-CGGGAACGCTTGTGATCAATGG-3'; 5'-GGGACTGGTGATCATCGTCC-3'; Ref. 35) were added (final volume to 50 µl).

The PCR was heated to 94°C for 3 min and immediately cycled 23 times (35 times for CEM and CEM/VLB₁₀₀ cells) through a 1-min denaturing step at 94°C, a 1-min annealing step at 58°C, and a 1-min elongation step at 72°C with a Perkin-Elmer 2400 thermocycler. After the final cycle, a 7-min PCR product was electrophoresed on 2% agarose gels (Bio-Rad), and PCR fragments were visualized using ethidium bromide staining.

**Western Blot Analysis of MDR1 Expression.** To investigate MDR1 gene expression, Western blots were performed with KB-3, KB-V1, CEM, and CEM/VLB₁₀₀ cells treated with pervinelle A (0–45 µM) or verapamil (0–44 µM) for 72 h at 37°C in a 5% CO₂ incubator. Cells (7.5 × 10⁶ cells as a starting cell amount) were harvested with SDS lysis buffer [20 mM Tris-HCl (pH 6.8) containing 0.4% SDS (w/v), 5% glycerol (v/v), 0.006% bromphenol blue, and 2% β-mercaptoethanol], and lysates were boiled for 10 min and stored at −20°C. Aliquots of lysates were used for protein determinations (bicinchoninic acid protein assay kit with BSA as standard). Aliquots containing 20 µg of protein were electrophoresed on SDS-PAGE with prestained 7.5% Tris-glycine acrylamide gels (Novex, San Diego, CA). After transfer to polyvinylidene difluoride membranes, nonspecific binding sites were blocked with 5% nonfat dry milk. Blotting was performed with rabbit polyclonal antibody mdR (Ab-1; Oncogene Research Products, Inc.), analyzed by streptavidin horseradish peroxidase-conjugated secondary antibody (Amersham Life Science), and visualized using an enhanced chemiluminescence Western blotting detection system (Amersham Life Science).

**In Vivo Hollow Fiber Test.** In vivo hollow fiber test was performed using a literature procedure with some modifications (27). Confluent monolayers of KB-3, KB-V1, or KB-8-5 cells were harvested, collected by centrifugation, and resuspended in medium at a concentration of 7.5 × 10⁶ cells/ml. Fibers filled with cells were incubated in 6-well plates overnight at 37°C in a 5% CO₂ atmosphere. Female athymic NCr nu/nu mice at 5–6 weeks of age were obtained from Frederick Cancer Research Facility. Each mouse was inoculated with six fibers, which were cultured in two physiological compartments. For i.p. implants, a small incision was made through the skin and musculature of the dorsal abdominal wall, the fiber samples were inserted into the peritoneal cavity in a craniocaudal direction, and the incision was closed with skin staples. For s.c. implants, a small skin incision was made at the nape of the neck to allow insertion of an 11-gauge tumor implant trocar. The trocar, containing the hollow fiber samples, was inserted caudally through the s.c. tissues, and fibers were deposited during withdrawal of the trocar. The incision was closed with a skin staple.

In preliminary studies, cell growth was assessed with fibers containing various cell densities. As a result, a cell density of 7.5 × 10⁶ cells/ml was found to be suitable for drug studies for KB-3, KB-V1, and KB-8-5 cells. For treatment protocols, vinblastine and verapamil were dissolved in PBS. Pervinelle A was coprecipitated with polyvinyl pyrrolidone (36) to increase solubility and then dissolved in PBS. Mice were randomized into six groups (three mice/group): PBS vehicle control group; vinblastine treatment group; verapamil treatment group; pervinelle A treatment group; verapamil plus vinblastine group; and pervinelle A plus vinblastine group. Test compounds were administered once daily by i.p. injection from day 3–6 after implantation. Body weights were measured daily.

On day 7, mice were sacrificed, and fibers were retrieved. The fibers were placed into 6-well plates, with each well containing 2 ml of fresh, prewarmed culture medium, and allowed to equilibrate for 30 min at 37°C. To define the viable cell mass contained within the intact hollow fibers, a MTT dye conversion assay was used. Briefly, 1 ml of prewarmed culture medium containing 1 mg of MTT/ml was added to each dish. After incubating at 37°C for 4 h, the culture medium was aspirated, and the samples were washed twice with normal saline containing 2.5% protamine sulfate solution by overnight incubation at 4°C. To assess the absorbance of the samples, the fibers were transferred to 24-well plates, cut in half, and allowed to dry overnight. The formazan was extracted from each sample with DMSO (250 µl/well) for 4 h at room temperature on a rotation platform. Aliquots (150 µl) of extracted MTT formazan were transferred to individual wells of 96-well plates and assessed for absorbance at a wavelength of 540 nm. The effect of the treatment regimen was determined by the net growth percentage of the cells relative to change in body weight.

**RESULTS**

**Growth Inhibitory Potential.** An in vitro cell survival assay was used as an initial method for monitoring the potential of test compounds to reverse MDR. As summarized in Table 1, neither pervinelle A nor verapamil demonstrated appreciable growth inhibitory potential with KB-3 cells in culture. This cell line is highly susceptible to the therapeutic efficacy of compounds and extracts employing in vivo hollow fiber tests, submitted for publication.

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**Table 1** Reversal of MDR in KB-V1 and KB-8-5 cells by pervinelle A and verapamil

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pervinelle A</th>
<th>Verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-3</td>
<td>24 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KB-V1</td>
<td>&gt;34 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;44 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KB-8-5</td>
<td>0.36 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.79 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>KB-8-5</td>
<td>14 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;44 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>KB-3/KB-V1</td>
<td>0.61 &lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.9 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>[KB-V1–][KB-V1 (+)]</td>
<td>66.66 &lt;sup&gt;f&lt;/sup&gt;</td>
<td>47 &lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>[KB-V1–][KB-V1 (+)]</td>
<td>&gt;95 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;55 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>[KB-8-5–][KB-8-5 (+)]</td>
<td>40.3 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.1 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>[KB-8-5–][KB-8-5 (+)]</td>
<td>23.3 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;15.4 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as IC₅₀ values in µM. Data are the means of the two independent experiments, with each concentration tested in triplicate. For additional experiment details, see “Materials and Methods.”

<sup>b</sup> Incubations were performed in the presence (1 µg/ml) or absence (−) of vinblastine.

<sup>c</sup> Incubations were performed in the presence (10 ng/ml) or absence (−) of colchicine.

<sup>d</sup> Ratios of IC₅₀ values.
Table 2  MDR reversal ability in drug-resistant human leukemia CEM/VLB100 cells

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Pervilleine A</th>
<th>Verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modulator only</td>
<td>&gt;15 µM</td>
<td>&gt;15 µM</td>
</tr>
<tr>
<td>(Modulator + vinblastine)</td>
<td>0.02 ³, 0.025 ³</td>
<td>250 ³ 200 ³</td>
</tr>
<tr>
<td>Modulator (modulator + vinblastine)</td>
<td>&gt;750 µM</td>
<td>&gt;600 µM</td>
</tr>
<tr>
<td>(Modulator + daunorubicin)</td>
<td>0.065 µM 0.5 µM</td>
<td>15 µM 2 µM</td>
</tr>
<tr>
<td>Daunorubicin (modulator + daunorubicin)</td>
<td>&gt;231 &gt; 30</td>
<td></td>
</tr>
<tr>
<td>Modulator (modulator + daunorubicin)</td>
<td>&gt;231 &gt; 30</td>
<td></td>
</tr>
</tbody>
</table>

³ Results are expressed as IC₅₀ values in µM. Data are the means of the two independent experiments, with each concentration being tested in duplicate. For additional experiment details, see “Materials and Methods.”

² Pervilleine A concentration, 5 µM; verapamil concentration, 10 µM; vinblastine concentration, varied from 1 nM–10 µM. In the absence of modulator, the IC₅₀ value of vinblastine was 5 µM.

³ Ratios of IC₅₀ values.

Species A concentration, 5 µM; daunorubicin concentration, 10 µM; daunorubicin concentration, varied from 10 nM–100 µM. In the absence of modulator, the IC₅₀ value of daunorubicin was 1 µM.

Experimental conditions results in the accumulation of 0.18 ± 0.008 pmol vinblastine/50 × 10⁶ cells. When KB-V1 cells were treated with verapamil or pervilleine A in the 0–40 µM range (Fig. 4), accumulation of [³H]vinblastine increased from basal levels of 0.18 pmol [³H]vinblastine/50 × 10⁶ cells to approximately 5 pmol [³H]vinblastine/50 × 10⁶ cells. The enhanced accumulation was dose-dependent, and pervilleine A appeared more effective than verapamil.

RT-PCR Analysis of MDR1 mRNA Expression. RT-PCR studies confirmed that MDR1 mRNA gene was overexpressed in the MDR cell lines KB-V1 and CEM/VLB100 compared with the parental cell lines KB-3 and CEM. However, treatment of KB-V1 and CEM/VLB100 cells for 72 h with pervilleine A at various concentrations ranging up to 34 µM showed no significant effect at the level of MDR1 mRNA (data not shown).

Western Blot Analysis of MDR1 Expression. MDR1 expression with KB-3, KB-V1, CEM, and CEM/VLB100 cells was assessed using Western blot analysis. As expected, MDR1 was overexpressed in to vinblastine (IC₅₀ 0.04 µM) and colchicine (IC₅₀ 0.05 µM). In the absence of vinblastine, neither test substrate inhibited the growth of KB-V1 cells (IC₅₀ values, >34 or 44 µM for pervilleine A or verapamil, respectively). However, when vinblastine was added to the media in the presence of pervilleine A or verapamil, chemosensitivity was restored (IC₅₀ 0.36 or 0.79 µM, respectively). For pervilleine A, these data yielded IC₅₀ ratios of 66.6 and >95 for KB-3/KB-V1 (+ vinblastine) and KB-V1 (– vinblastine)/KB-V1 (+ vinblastine), respectively, which compare favorably with the corresponding values obtained with verapamil (47 and >55, respectively) and indicated a lack of nonspecific cytotoxicity. For KB-8-5 cells (Table 1), although pervilleine A showed greater growth inhibitory activity (IC₅₀ 14 µM) than that of verapamil (IC₅₀ >44 µM) in the absence of colchicine, the IC₅₀ ratios of KB-3/KB-8-5 (+ colchicine) and KB-8-5 (– colchicine)/KB-8-5 (+ colchicine) were still favorable (40.3 and 23.3, respectively, for pervilleine A, and 13.1 and >15.4, respectively, for verapamil).

A related group of studies conducted with drug-resistant CEM/ VLB100 cells is summarized in Table 2. In the absence of vinblastine or daunorubicin, neither pervilleine A nor verapamil inhibited the growth of CEM/VLB100 cells (IC₅₀ >15 µM). In the presence of pervilleine A, the IC₅₀ values of vinblastine and daunorubicin were reduced to 0.02 and 0.085 µM, respectively, and the corresponding values in the presence of verapamil were 0.025 and 0.5 µM, respectively. These effects were specific, based on the high IC₅₀ ratios of modulator (modulator + vinblastine) and vinblastine (modulator + vinblastine) and on the corresponding ratios obtained with daunorubicin (Table 2).

[³H]Vinblastine Accumulation with KB-V1 Vesicles. Inhibition of ATP-dependent vinblastine accumulation with vesicles isolated from multidrug-resistant KB-V1 cells was mediated by pervilleine A or verapamil in the 0–100 µM concentration range (Fig. 2). Accumulation was reduced from about 5.93 pmol [³H]vinblastine/mg protein in control incubations to about 0.09 pmol [³H]vinblastine/mg protein in the presence of pervilleine A or verapamil. Reduction was clearly dose-dependent, with inhibition of 50% being obtained at concentrations of 3.84 and 3.59 µM for pervilleine A and verapamil, respectively (Fig. 2). Using similar methodology, data were obtained for the construction of double-reciprocal plots, and competitive inhibition was observed (Fig. 3). The Kᵢ values were approximately 7.3 and 8.8 µM for pervilleine A and verapamil, respectively.

[³H]Vinblastine Accumulation with Intact Cells. Pgp-MDR cells accumulate and retain fewer anticancer drugs than do their drug-sensitive counterparts. Treatment of KB-3 cells with [³H]vinblastine results in the accumulation of 7.1 ± 0.17 pmol vinblastine/50 × 10⁶ cells, whereas treatment of KB-V1 cells under the same
Materials and Methods.

Expressions were transferred to scintillation vials for counting. The quantity of \[^{3}H\]vinblastine monolayers were washed and treated with trypsin. Cells were enumerated, and suspensions were then added to each well. After a 1-h incubation period, verapamil or pervilleine A (0–40 \(\mu M\)) were then added to each well. In addition to colchicine resistance, KB-8-5 cells are known to be cross-resistant with vinblastine but to a lesser degree than KB-V1 cells (37, 38). With the in vivo hollow fiber models, treatment with 100 \(\mu g/kg\) of vinblastine inhibited the growth of KB-3 cells (Fig. 6A) but did not significantly influence the growth of KB-V1 cells (less than 1% growth inhibition; Fig. 6B). In addition to colchicine resistance, KB-8-5 cells are known to be cross-resistant with vinblastine but to a lesser degree than KB-V1 cells (37, 38). With the in vivo hollow fiber models, treatment with 100 \(\mu g/kg\) of vinblastine inhibited the growth of KB-3 cells (Fig. 6A) but did not significantly influence the growth of KB-V1 cells (less than 1% growth inhibition; Fig. 6B). None of these cell types was sensitive to pervilleine A (79.2 \(mg/kg\)) or verapamil (61.4 \(mg/kg\)) at a dose of 0.136 mmol/kg (less than 5.2% growth inhibition; Fig. 6B and C).

However, when vinblastine was coadministered with pervilleine A or verapamil, a significant growth inhibitory effect (P < 0.0001) was observed with KB-V1 (Fig. 6B) or KB-8-5 cells (Fig. 6C) implanted at the i.p. site.

The response observed with cells implanted at the s.c. site was less intense. As summarized in Table 3, when pervilleine A or vinblastine were administered as single agents, growth inhibition effects of 3.3 or 1.2%, respectively, were observed, but when given together, an inhibitory effect of 74.7% resulted. In each case, relative to the percentage inhibition that was calculated as a summation of the inhibition noted when the agents were administered singly, enhancements were observed when the agents were coadministered. Thus, because observed inhibitions were greater than those calculated, both agents were effective, and pervilleine A showed a stronger effect than verapamil. In all of the cases, no significant loss in mouse body weight was observed (Fig. 6D–F), based on established criteria (27).

DISCUSSION

Cell culture has proven to be an invaluable tool for the discovery and characterization of agents capable of altering the MDR pheno-

![Fig. 4. Enhanced accumulation of \[^{3}H\]vinblastine in KB-V1 cells as a function of added verapamil or pervilleine A (0–40 \(\mu M\)). Suspensions (2 ml) containing 5.0 \(x\) 10^5 KB-V1 cells in DMEM were placed in 24-well plates and incubated overnight in a 5% CO\(_2\) incubator. \[^{3}H\]Vinblastine (16 nM) and the indicated concentrations of verapamil (\(\bullet\)) or pervilleine A (\(\odot\)) were then added to each well. After a 1-h incubation period, monolayers were washed and treated with trypsin. Cells were enumerated, and suspensions were transferred to scintillation vials for counting. The quantity of \[^{3}H\]vinblastine observed with blank plates containing no cells was subtracted from total binding. Additional experiment details are provided in “Materials and Methods.”](image)

![Fig. 5. Western blot analysis of MDR1 expression. A, KB-3 (Lane 1) and KB-V1 cells (Lanes 2–11) were treated with DMSO (Lanes 1 and 2) or 1, 5, 10, 15, 20, 25, 30, 35, and 40 \(\mu M\) (Lanes 3–11) pervilleine A for 72 h. B, CEM (Lane 1) and CEM/VLB\(_{100}\) cells (Lanes 2–13) were treated with DMSO (Lanes 1 and 2) or 0.5, 1, 2, 5, 10, 15, 20, 25, 30, 35, and 45 \(\mu M\) (Lanes 3–17) pervilleine A for 72 h. For additional experiment details, see “Materials and Methods.”](image)

![Fig. 6. In vivo hollow fiber studies conducted with KB-3 (A and D), KB-V1 (B and E), and KB-8-5 (C and F) cells implanted at the i.p. compartment of NCr nu/nu mice. Confluent monolayers of KB-3, KB-V1, and KB-8-5 cells were harvested and pelleted by centrifugation, and resuspended in conditioned medium at a concentration of 7.5 \(x\) 10^5 cells/ml. Fibers filled with the cells were incubated in 6-well plates overnight at 37°C in a 5% CO\(_2\) atmosphere and then inserted into the peritoneal cavity of NCr nu/nu mice in a cranio-caudal direction. The incisions were closed with skin staples. The animals were treated with PBS (control), vinblastine (VLB; 250 \(\mu g/kg\), A, D, B, and E; 100 \(\mu g/kg\), C and F), verapamil (VP; 0.136 mmol/kg), pervilleine A (PA; 0.136 mmol/kg), a combination of vinblastine and verapamil (VLB/VP), or a combination of vinblastine and pervilleine A (VLBP/PA). Doses of individual agents in combination regimens are the same as given above. Drugs were administered once daily by i.p. injection from day 3–6 after implantation. On day 7, mice were sacrificed, and fibers were retrieved. The effectiveness of the drugs was evaluated on the basis of net growth percentage of the cells determined by MTT assays (A–C). Body weight was determined on day 1 and day 7 of the study and expressed as the difference (D–F). Additional experiment details are provided in “Materials and Methods.” The calculated percentage inhibitions were significantly different from the observed percentage inhibitions (P < 0.0001) using Student’s t-test (n = 6).](image)
type. Relative to parental KB-3 cells, KB-V1 cells are >200-fold more resistant to the growth inhibitory effect of vinblastine (6), and we have used this as a model for the discovery of various agents capable of reversing MDR (23, 24). As a result of this process, the most interesting natural product MDR-reversing agent we have discovered is pervilleine A. Because pervilleine A bears structural resemblance to verapamil (Fig. 1), a well-known prototype-reversing agent, comparative studies were performed with the two compounds. In each case, the response mediated by pervilleine A was found to be equal to or greater than the response mediated by verapamil. In addition to KB-V1 cells, pervilleine A effectively reversed the MDR phenotype with KB-8-5 (Table 1) and CEM/VLB100 cells (Table 2).

Previous studies (39, 40) have demonstrated that the overexpression of Pgp is a major cause for developing MDR, and decreasing expression of Pgp at either the transcriptional or protein level is one mechanism for reversing MDR. To explore the mechanism by which pervilleine A enhanced the vinblastine sensitivity of MDR cells, we investigated the potential of pervilleine A to alter MDRI mRNA or protein expression with KB-V1 and CEM/VLB100 cells in culture. Treatment with various concentrations of pervilleine A for 72 h did not significantly alter protein (Fig. 5) or mRNA expression (data not shown), and similar negative results were obtained in parallel studies conducted with verapamil (concentrations ranging up to 44 μM). With verapamil, these results are consistent with those of Hu et al. (41), who demonstrated a limited effect on MDRI mRNA levels in KB-V1 cells after a 24-h incubation. With CEM/VLB100 cells, however, we did not observe the inhibition of Pgp expression noted previously (39) with verapamil. This is probably attributable to some differences in experimental conditions, because it is known that the effect of modulators on Pgp expression might be cell line- and modulator-dependent (41). In any event, it is clear that Pgp transcription and protein expression with cultured KB-V1 and CEM/VLB100 cells were not affected by pervilleine A.

On the other hand, pervilleine A clearly altered functional aspects of Pgp. When KB-V1 cells were treated with verapamil or pervilleine A in the concentration range of 0–40 μM (Fig. 4), [3H]vinblastine accumulation increased by about 27-fold (from basal levels of 0.18 pmol [3H]vinblastine/50 × 10^6 cells to approach 5 pmol [3H]vinblastine/50 × 10^6 cells). This level of accumulation approached that of sensitive cells (7.1 ± 0.17 pmol vinblastine/50 × 10^6 cells), indicating that restoration of the vinblastine sensitivity of resistant cells results from enhanced intracellular accumulation of vinblastine.

Plasma membranes obtained from MDR cells bind greater quantities of anticancer drugs than their counterparts derived from drug-sensitive cells (42), and [3H]vinblastine associated with plasma membrane vesicles isolated from MDR cells (23, 24, 31, 42, 43) can be used as a model to investigate the mechanism of Pgp-mediated drug transport/efflux. Treatment of membrane vesicles isolated from multidrug-resistant KB-V1 cells with pervilleine A or verapamil in the concentration range of 0–100 μM (Fig. 2) in the presence of 0.16 μM [3H]vinblastine reduced [3H]vinblastine accumulation by about 65-fold (from 5.93 pmol [3H]vinblastine/mg protein in control incubations to about 0.09 pmol [3H]vinblastine/mg protein in the presence of pervilleine A or verapamil). Thus, as is the case with verapamil, pervilleine A increased intracellular drug accumulation by inhibiting the efflux mechanism of Pgp.

To characterize the mode of interaction, kinetic studies were performed with membrane vesicles derived from KB-V1 cells in which the effect of verapamil or pervilleine A on [3H]vinblastine accumulation was determined. With both test agents, competitive inhibition was observed, suggesting interaction with the binding site normally occupied by vinblastine. In a similar manner, Cornwell et al. (43) suggested verapamil and vinblastine bind to the same site in membrane vesicles from KB-V1 cells, based on the results of vinblastine binding and vinblastine photoaffinity labeling studies. However, Pascaud et al. (44) have proposed Pgp has distinct but interacting binding sites for cytotoxic drugs (e.g., vinblastine) and reversing agents (e.g., verapamil) through analysis of the ATPase activity of Pgp. It is presently uncertain whether pervilleine A action involves a unique common binding site or two mutually exclusive but distinct binding sites; this potential mechanism will be investigated in future studies.

Many studies (8, 45–49) have been performed to define the structural or physicochemical features of reversors that might account for their effectiveness. It has been suggested (47) that planar aromatic rings and nitrogen atoms are common features shared by all of the modulators. A CH₂–CH₂–N–CH₂–CH₂ sequence was observed (49) in most active compounds, and a methoxyphenyl moiety was found to enhance the reversing activity. The relative disposition of aromatic rings and the basic nitrogen atom are important for modulators of Pgp-associated MDR (8), suggesting a ligand-receptor relationship. Verapamil and pervilleine A (Fig. 1) bear features shared by other MDR reversors in having a tertiary nitrogen, two aromatic rings, and methoxyphenyl groups. Verapamil has three domains in common with vinblastine that appear to be important for its ability to interact with Pgp and modulate MDR (8): two aromatic rings and a basic nitrogen atom. In addition to these structural features, lipid solubility and molar refractivity might play dominant roles in reversing MDR (47). Additional studies are currently underway to elucidate the physical-chemical properties of pervilleine A.

Because pervilleine A demonstrated promising activities with in vitro studies, we used the hollow fiber model to further investigate in vivo potential. This is the first reported use of the hollow

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**Table 3 Calculated and observed growth inhibition of KB-V1 and KB-8-5 cells implanted at i.p. and s.c. sites**

<table>
<thead>
<tr>
<th>Cell line tested</th>
<th>Reversor</th>
<th>i.p. Calculated inhibition</th>
<th>Observed inhibition</th>
<th>s.c. Calculated inhibition</th>
<th>Observed inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-V1</td>
<td>Verapamil</td>
<td>56.6 (P &lt; 0.0001)</td>
<td>0 + 10.2 = 10.8</td>
<td>13.6 (P = 0.098)</td>
<td></td>
</tr>
<tr>
<td>KB-8-5</td>
<td>Pervilleine A</td>
<td>68.6 (P &lt; 0.0001)</td>
<td>0 + 3.3 = 3.3</td>
<td>14.6 (P = 0.188)</td>
<td></td>
</tr>
</tbody>
</table>

**Note:**
- Fibers filled with cells were implanted into the i.p. and s.c. compartments of host mice. The test compounds were administered once daily by i.p. injection from day 3–6 after implantation. Fibers were retrieved on day 7.
- The efficacy of drugs (expressed as percentage growth inhibition) was determined by quantifying cells using the MTT assay (n = 6).
- Significance (P) was evaluated using Student’s t-test (calculated percentage inhibition versus observed percentage inhibition; n = 6). For additional experiment details, see “Materials and Methods.”
- Calculated percentage inhibition is the summation of inhibition noted when vinblastine (first value) and the reversing agent (second value) were used as single agents.
- Observed percentage inhibition resulting from coadministration of agents as described in “Materials and Methods.”

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*Fibers filled with cells were implanted into the i.p. and s.c. compartments of host mice. The test compounds were administered once daily by i.p. injection from day 3–6 after implantation. Fibers were retrieved on day 7. The efficacy of drugs (expressed as percentage growth inhibition) was determined by quantifying cells using the MTT assay (n = 6). Significance (P) was evaluated using Student’s t-test (calculated percentage inhibition versus observed percentage inhibition; n = 6). For additional experiment details, see “Materials and Methods.”

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**Table 3 Calculated and observed growth inhibition of KB-V1 and KB-8-5 cells implanted at i.p. and s.c. sites**
fiber model to assess MDR-reversing agents in combination with cancer chemotherapeutic agents. As currently described, in combination with vinblastine, the hollow fiber test was used to assess the capacity of verapamil or pervililene A to effect the growth of multidrug-resistant tumor cells growing in the i.p. and s.c. compartments of mice. At pharmacologically relevant doses, the data in Table 3 indicate that both verapamil and pervililene A were capable of reversing vinblastine sensitivity with KB-V1 and KB-8-5 cells implanted at the i.p. site (P < 0.0001), which was consistent with in vitro data (Table 1). However, responses at the s.c. site were less clear (Table 3), and only pervililene A showed a significant reversing effect (P = 0.008) with KB-8-5 cells. Relative to verapamil, it is possible that pervililene A is distributed more effectively in the rodent body. Alternatively, with KB-8-5 cells, pervililene A may be more effective than verapamil in terms of reversing resistance to vinblasteine. Overall, the relatively poor responses observed at the s.c. site might be attributable to ineffective drug delivery or the lack of blood vessel development, because the present hollow fiber assay protocol would not enable angiogenesis (50). However, these results indicate the utility of the present hollow fiber model to assess MDR-reversing agents in combination with conventional in vivo animal models. Because pervililene A is an effective inhibitor of Pgp, with comparable or greater activities than verapamil, the results of more advanced in vivo tests will be pivotal in contemplating further development as a clinically useful pharmaceutical agent.

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