Celastraceae Sesquiterpenes as a New Class of Modulators That Bind Specifically to Human P-Glycoprotein and Reverse Cellular Multidrug Resistance

Francisco Muñoz-Martínez,1 Pelhua Lu,2 Fernando Cortés-Selva,1 José María Pérez-Victoria,1 Ignacio A. Jiménez,3 Ángel G. Ravelo,1 Frances J. Sharom,1 Francisco Garmarrow,1 and Santiago Castanys1

1Instituto de Parasitología y Biomedicina “López-Neyra,” Consejo Superior de Investigaciones Científicas, Granada, Spain; 2Guelph-Waterloo Centre for Graduate Work in Chemistry and Biochemistry, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada; and 3Instituto Universitario de Bio-Orgánica “Antonio González,” Universidad de La Laguna –Instituto Canario de Investigación del Cáncer, Tenerife, Spain

ABSTRACT

Overexpression of ABCR1 (MDR1) P-glycoprotein, a multidrug efflux pump, is one mechanism by which tumor cells may develop multidrug resistance (MDR), preventing the successful chemotherapeutic treatment of cancer. Sesquiterpenes from Celastraceae family are natural compounds shown previously to reverse MDR in several human cancer cell lines and Leishmania strains. However, their molecular mechanism of action has not been characterized. In the present work, we have studied the ability of 28 dihydro-β-agarofuran sesquiterpenes to reverse the P-glycoprotein-dependent MDR phenotype and elucidated their molecular mechanism of action. Cytotoxicity assays using human MDR1-transfected NIH-3T3 cells allowed us to select the most potent sesquiterpenes reversing the in vitro resistance to daunomycin and vinblastine. Flow cytometry experiments showed that the above active compounds specifically inhibited drug transport activity of P-glycoprotein in a saturable, concentration-dependent manner (Ki down to 0.24 ± 0.01 μM/L) but not that of ABCC1 (multidrug resistance protein 1; MRP1), ABCC2 (MRP2), and ABCG2 (breast cancer resistance protein; BCRP) transporters. Moreover, sesquiterpenes inhibited at submicromolar concentrations the P-glycoprotein-mediated transport of [3H]elcichine and tetramethyl-rosamine in plasma membrane from CH1B30 cells and P-glycoprotein-enriched proteoliposomes, supporting that P-glycoprotein is their molecular target. Photoaffinity labeling in plasma membrane and fluorescence spectroscopy experiments with purified protein suggested that sesquiterpenes interact with transmembrane domains of P-glycoprotein. Finally, sesquiterpenes modulated P-glycoprotein ATPase activity in a biphasic, concentration-dependent manner: they stimulated at very low concentrations but inhibited ATPase activity as noncompetitive inhibitors at higher concentrations. Sesquiterpenes from Celastraceae are promising P-glycoprotein modulators with potential applications in cancer chemotherapy because of their MDR reversal potency and specificity for P-glycoprotein.

INTRODUCTION

The ability of cancer cells to develop resistance to multiple structurally and functionally nonrelated cytotoxic drugs, so-called multidrug resistance (MDR), is a major barrier to successful chemotherapy. Among the cellular mechanisms that contribute to MDR, overexpression of ABCB1 (MDR1) P-glycoprotein is the best studied by far (1). P-glycoprotein is a M, 170,000 multidrug transporter that belongs to the ATP-binding cassette (ABC) superfamily of proteins (2). Other ABC transporters, such as ABCB1 (multidrug resistance protein 1; MRP1; ref. 3), ABCC2 (MRP2; ref. 4), and ABCG2 (breast cancer resistance protein; BCRP) transporters have also been shown to confer MDR on cells in vitro. Moreover, expression of P-glycoprotein and MRP1 in many human cancers correlates with response to therapy and survival (6, 7).

These findings have prompted the interest of many researchers throughout the last two decades to develop P-glycoprotein inhibitors as a way to revert MDR in human cancers (8) or even to prevent the emergence of MDR in cancer patients (9). Many agents that modulate the function of P-glycoprotein are able to restore the cytotoxicity of chemotherapeutic drugs to MDR cells in vitro and in experimental drug-resistant tumors in vivo (10). Clinical trials with MDR modulators have shown some response in tumors that were otherwise nonresponsive to chemotherapy (11, 12). However, most P-glycoprotein modulators that were shown to be effective in in vitro assays have proved to be weak MDR reversers in patients and toxic at high doses (13). Moreover, most of them adversely and dramatically influence the pharmacokinetics and biodistribution of coadministered chemotherapeutic drugs (8, 14). Third-generation modulators that specifically and potently inhibit P-glycoprotein have been developed to overcome the limitations of the previous ones (8, 14). Although the preliminary results of ongoing clinical trials are hopeful, their efficacy in cancer patients has not yet been shown (15). Therefore, in anticipation of a possible clinical failure of the third-generation modulators currently under study or to complement them in case of success, it is still necessary to search for new, efficient P-glycoprotein modulators without undesirable side effects.

Plant extracts of the Celastraceae family have been used for centuries in traditional medicine. Among the active compounds identified in those extracts are sesquiterpenes, which constitute a wide family of natural compounds with a considerable range of bioactive properties and with potential clinical applications as anticancer drugs, and MDR reversal agents in cancer cells (16) and in the protozoan parasite Leishmania (17, 18). Because of these previous findings, we have initiated research to determine the cellular target(s) for sesquiterpenes and to characterize their molecular mechanism of action to rationally design new, more efficient modulators based on their chemical structure.

The present work focuses on the study of 28 dihydro-β-agarofuran sesquiterpenes from different Celastraceae plants as specific inhibitors of human P-glycoprotein. A previous screening of sesquiterpenes with in vitro tests with MDR1-overexpressing intact cells allowed us to identify the most potent sesquiterpenes reversing P-glycoprotein-dependent MDR and to assess the interaction of sesquiterpenes with P-glycoprotein. Similar experiments using MRP1-, MRP2-, and BCRP-expressing cells showed that sesquiterpenes active against P-glycoprotein do not substantially modify the activities of these ABC transporters. The direct molecular interactions of the active sesquiterpenes were assessed in experiments with plasma membrane from CH1B30 cells and purified protein. Binding of sesquiterpenes to P-glycoprotein was studied by competition of [3H]azidopine photoaffinity labeling of the protein and by a fluorescence quenching technique. Moreover, the modulating effect of these natural compounds on P-glycoprotein ATPase activity has also been characterized.
MATERIALS AND METHODS

Chemicals. ATP, vinblastine, verapamil, cyclosporin A, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT), sodium orthovanadate, and cholic acid from Sigma-Aldrich (Madrid, Spain). GF120918 was from GlaxoSmithKline (Madrid, Spain). The [3H]Azidopine (52 Ci/mmol) and NAMPI100 amplifying solution for fluorography were from Amersham Biosciences (Barcelona, Spain). The [3H]colchicine (15 to 25 Ci/mmol) was purchased from DuPont NEN (Boston, MA). The C219 monoclonal antibody, directed against P-glycoprotein, was from Calbiochem (Madrid, Spain). Daunomycin was from Pfizer (Madrid, Spain); calcein-acetoxymethyl ester (AM) was from Molecular Probes Europe BV (Leiden, The Netherlands). The 2,4-(5,6)-dihydrobenzofuran-3-carboxylic acid (DMEM, 2,4-6-trinitrophenyl-amino-5-triphosphate (TNP-ATP), Hoechst 33342, and tetramethylrhodamine were from Molecular Probes (Eugene, OR). The 1-palmitoyl-2-myristoyl-phosphatidylcholine (PMPc) was obtained from Avanti Polar Lipids (Alabaster, AL). Asolectin was obtained from Fluka (Ronkonkoma, NY).

Dihydro-β-agarofuran sesquiterpenes from Celastraceae plants Maytenus cucuinea, Maytenus canariensis, Maytenus magellanica, and Maytenus chubutensis were isolated, purified, and characterized as described previously (18–21). The structures of all of these compounds are depicted in Fig. 1.

Cell Cultures. Mammalian cell lines were cultured at 37°C in a humidified atmosphere with 5% CO2 with their respective media supplemented with 10% heat-inactivated fetal bovine serum. The cell lines were as follows: NIH-3T3 drug-sensitive parental and transfected with human mdr1-G185 (22); the parental drug-sensitive AuxB1 and the colchicine-selected line CHP/B30 (23); the drug-sensitive parental MDCKII and the transfected with the human mdr2 (24); the 2008 and the transfected with the human mrp2 (25); and the parental MDA-MB-231 and the transfected with the human bcrp (26).

Modulation of Sensitivity to Daunomycin and Vinblastine. The dose-response curves of NIH-3T3 and NIH-3T3 MDR1-G185 cells to daunomycin and vinblastine were determined by MTT colorimetric assay (27) in 96-well plates. After 72 hours incubation in the presence of different concentrations of drugs and sesquiterpenes, 100 µL of a 0.45 mg/ml MTT solution in complete DMEM + 10% heat-inactivated fetal bovine serum was added to each well. Dose-response curves were generated by nonlinear regression of the data points to a four parameters logistic curve using SigmaPlot 2000 for Windows (SPSS Inc., Chicago, IL). y = [min + (max – min)/1 + 10log (C0 – S)]/[1 + 10log (C0 – S)]; (Equation A), where y is the cell growth for each drug concentration x, max is the maximal cell growth, min is the minimal cell growth, IC50 is defined as the drug concentration that inhibited cell growth by 50%, and n is the Hill coefficient.

Inhibition of P-Glycoprotein-mediated Transport of Daunomycin in Intact Cells. A direct functional assay for P-glycoprotein in NIH-3T3 MDR1-G185 cells was done by flow cytometry. Twenty-four hours before the experiment, cells in logarithmic phase of growth were seeded in 24-well plates at a density of 105 cells per well. For assessment of the inhibition of P-glycoprotein-mediated daunomycin efflux, cells were first incubated for 30 minutes at 37°C in DMEM + 10% heat-inactivated fetal bovine serum in the presence or absence of sesquiterpenes with 2 µmol/L of daunomycin. After that, cells were washed twice with ice-cold PBS and incubated for an additional 30 minutes in DMEM + 10% heat-inactivated fetal bovine serum in the presence or absence of sesquiterpenes but without the fluorescent probe. After this second incubation period, cells were washed with ice-cold PBS, hypotonicized, and resuspended in 0.2 ml of ice-cold PBS for immediate analysis. Fluorescence measurements of individual cells were done with a Becton Dickinson FacScan (BD European HQ, Erembodegem-Aalst, Belgium).

For determination of the Ki for inhibition of P-glycoprotein-mediated daunomycin efflux, defined as the concentration of modulator that inhibited daunomycin efflux by 50%, the geometric mean of the fluorescence intensity of 105 cells incubated at different sesquiterpene concentrations was used for comparison. Vanadate was selected as a positive control that maximally inhibited the P-glycoprotein efflux of daunomycin. All of these values were converted to percentage inhibition of P-glycoprotein normalized for vanadate inactivation, plotted, and fitted to the Hill equation for allosteric interactions, using SigmaPlot 2000 software: i = (Imax × S^n)/(K_i + S^n); (Equation B), where i is the inhibition of daunomycin efflux at a given sesquiterpene concentration, Imax is the maximal inhibition (caused by 5 mM/L vanadate), S is the concentration of sesquiterpene, and n is the Hill coefficient.

Effect of Sesquiterpenes on Drug Transport Mediated by MRPI, MRP2, and BCRP in Intact Cells. To test whether selected sesquiterpenes have an inhibitory effect on the transport activity of MRPI, MRP2, and BCRP, functional assays similar to that described for P-glycoprotein were done, with intact cells expressing the appropriate ABC transporters, and testing by flow cytometry their ability to extrude drugs in the presence of sesquiterpenes. In the case of MRPI- and MRP2-expressing cells, calcein-AM (0.25 µmol/L) was used as a probe and cyclosporin A as positive control inhibitor. In the case of BCRP-expressing cells, the probe and the control inhibitor used were rhodamine 123 (1.5 µmol/L) and GF120918, respectively.

ATPase Activity Measurements. The ATPase activity in the presence of sesquiterpenes of P-glycoprotein in CHP/B30 cell plasma membrane vesicles and purified P-glycoprotein in CHAPS solution was determined by measuring the release of Pi from ATP as reported previously (28). Samples incubated with 500 µmol/L vanadate (which inhibited 100% of P-glycoprotein ATPase activity) were obtained in parallel and subtracted from the measurements to account for the contribution to activity of any other ATPases or phosphatases. Results of experiments carried out with plasma membrane at increasing concentrations of sesquiterpenes were fitted to a bell-shaped curve, which is characterized of compounds that stimulate P-glycoprotein ATPase activity at low concentration but inhibit it at higher concentrations. The equation that defines such a curve is V(s) = [(K1 / K2 V0) + (K2 V1 S) + (V2 S1)] / (K1 / K2 + (K1 V2 S1) S1); (Equation C), where V(s) is the ATPase activity as a function of the sesquiterpene concentration S, V0 is the activity in the absence of sesquiterpene, V1 is the maximal enzyme activity (if only activation occurred), and K2 is the sesquiterpene concentration that gives half this maximal increment in the ATPase activity. V2 is the activity at infinite concentration of the sesquiterpene, and K1 is the sesquiterpene concentration that gives half-maximal reduction of ATPase activity from the value V1. For experiments with purified P-glycoprotein at increasing concentrations of sesquiterpenes, the resulting plots were fitted to an equation similar to Equation A, but in this case, y is the ATPase activity for each sesquiterpene concentration x, max is the maximal ATPase activity, min is the minimal ATPase activity at infinite concentration of sesquiterpene, K1 (instead of IC50 in Equation A) is defined as the drug concentration that inhibits ATPase activity by 50%, and n is the Hill coefficient.

In the case of ATPase measurements at increasing ATP concentrations and different fixed concentrations of sesquiterpenes, the results were plotted and fitted to the Hill equation for allosteric interactions to obtain the kinetic parameters for P-glycoprotein ATPase activity in the presence or absence of sesquiterpenes; this equation resembled Equation B but substituted i and imax with V1(i) and Vmax respectively, where V1(i) is the ATPase activity as a function of the ATP concentration, Vmax is the maximum ATPase activity at saturating concentrations of ATP, S is the concentration of ATP, n is the Hill coefficient, and K1 is the concentration of ATP that gives half Vmax. The constants Kmax and Kdiss (dissociation constant for free P-glycoprotein and for P-glycoprotein in complex with ATP, respectively) and the type of the inhibition of P-glycoprotein ATPase activity by sesquiterpenes were determined with the “Exploratory Enzyme Kinetics” application of SigmaPlot 2000 for Windows software, whose enzymological basis is the direct linear plot (29).

Plasma Membrane Preparation, P-Glycoprotein Purification, and Reconstitution. Plasma membrane vesicles from the colchicine-selected MDR Chinese hamster ovary cell line CHP/B30 were isolated as described previously (30). P-glycoprotein was purified to 90 to 95% by a procedure involving a differential two-step extraction of CHP/B30 plasma membrane with the zwitterionic detergent CHAPS followed by removal of contaminant glycoproteins on concanavalin-A Sepharose (31). Highly purified P-glycoprotein was reconstituted into proteoliposomes of PMPC by gel filtration chromatography on a Sephadex G-50 column (32). P-glycoprotein made up >85% of the reconstituted protein in the proteoliposomes as indicated by SDS-PAGE.

[3H]Azidopine Photoaffinity Labeling. Photoaffinity labeling of P-glycoprotein in CHP/B30 plasma membrane vesicles with [3H]azidopine (100 nM; 52 Ci/mmol) was carried out as described previously (33) in the presence of a 100-fold molar excess of sesquiterpenes (10 µmol/L). Membrane proteins were analyzed by SDS-PAGE on a 9% gel followed by fluorography.
Colchicine Transport in Vesicle Systems and Real-time Fluorescence Measurement of Drug Transport in Proteoliposomes. Steady-state uptake of $[^1H]$colchicine into CHB30 plasma membrane vesicles was determined by rapid filtration using protocols outlined previously (34). Fluorescence measurements of tetramethylrhodamine transport in proteoliposomes were carried out as described previously (32). Data from the measurements of drug transport activities at increasing concentrations of sesquiterpenes were plotted and fitted to an equation similar to Equation A but with a different meaning for each parameter: y is the transport activity at each sesquiterpene concentration; x_max is the maximal transport activity in the absence of sesquiterpene; min the minimal transport activity at infinite concentration of sesquiterpene; and IC$_{50}$ is replaced here by K, defined as the sesquiterpene concentration that inhibited P-glycoprotein-dependent transport of colchicine or tetramethylrhodamine by 50%.

Binding of Sesquiterpenes to P-Glycoprotein and Their Effects on Hoechst 33342 and TNP-ATP Binding to the Protein. The binding affinity of sesquiterpenes to purified P-glycoprotein without labeling, or P-glycoprotein labeled with MIANS at the nucleotide-binding domains, was determined using equation 1 as described in Materials and Methods. Results are of two to four independent experiments performed in triplicate; mean ± SD (P < 0.05).

RESULTS

Reversal of P-Glycoprotein-mediated Resistance to Daunomycin and Vinblastine in NIH-3T3 MDR1-G185 Cells by Sesquiterpenes. The in vitro multidrug resistance reversing activity of sesquiterpenes was studied by determining the cytotoxicity of daunomycin and vinblastine (two classical P-glycoprotein substrates) in NIH-3T3 cells expressing P-glycoprotein. The ratio between IC$_{50}$ in the absence and in presence of a P-glycoprotein modulator gives the resistance reversal index, a parameter that allows quantitative comparisons between the efficiencies of different P-glycoprotein modulators. Table 1 summarizes the reversal indexes for daunomycin and vinblastine with dihydro-β-aragofuran sesquiterpenes tested at increasing concentrations of each cytotoxic drug and three fixed concentrations (10, 3, and 1 μmol/L) of sesquiterpenes. Seven sesquiterpenes (Cuzco5, Cuzco7, Mama6, Mama12, Mama14, Machu1, and C-3) reversed resistance to daunomycin with efficiencies comparable with that of verapamil (a classical P-glycoprotein modulator), and two of them (Mama5 and Machu4) had higher potentials (Fig. 1). Testing a representative subset of sesquiterpenes with vinblastine, we found essentially the same profile of relative drug resistance reversal efficiencies as described above. Interestingly, almost all of the sesquiterpenes tested showed

Table 1 Drug resistance reversal ability of sesquiterpenes in mdr1-transfected NIH-3T3 cells

<table>
<thead>
<tr>
<th>Sesquiterpene</th>
<th>10 μM</th>
<th>3 μM</th>
<th>1 μM</th>
<th>10 μM</th>
<th>3 μM</th>
<th>1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil†</td>
<td>16.11±2.50</td>
<td>13.45±1.41</td>
<td>7.04±3.10</td>
<td>18.76±5.00</td>
<td>13.18±0.24</td>
<td>6.21±1.10</td>
</tr>
<tr>
<td>Cuzco1</td>
<td>2.82±0.70</td>
<td>2.43±0.55</td>
<td>2.21±0.60</td>
<td>20.00±2.00</td>
<td>5.10±1.50</td>
<td>2.10±0.50</td>
</tr>
<tr>
<td>Cuzco2</td>
<td>10.03±0.60</td>
<td>7.80±0.70</td>
<td>7.10±0.22</td>
<td>15.83±3.50</td>
<td>14.86±2.60</td>
<td>8.98±1.80</td>
</tr>
<tr>
<td>Cuzco2hydrolyzed</td>
<td>1.30±0.60</td>
<td>0.86±0.25</td>
<td>0.80±0.31</td>
<td>21.50±4.26</td>
<td>12.52±4.50</td>
<td>10.90±3.22</td>
</tr>
<tr>
<td>Cuzco2acetylated</td>
<td>2.90±1.20</td>
<td>1.60±0.21</td>
<td>0.90±0.12</td>
<td>29.30±8.00</td>
<td>25.00±3.00</td>
<td>10.90±3.22</td>
</tr>
<tr>
<td>Cuzco3</td>
<td>11.29±3.00</td>
<td>7.57±2.10</td>
<td>4.23±1.10</td>
<td>31.30±5.20</td>
<td>20.86±2.60</td>
<td>13.40±3.40</td>
</tr>
<tr>
<td>Cuzco4</td>
<td>2.90±0.80</td>
<td>2.10±0.40</td>
<td>1.50±0.40</td>
<td>10.70±1.90</td>
<td>7.60±1.10</td>
<td>3.20±1.10</td>
</tr>
<tr>
<td>Cuzco5</td>
<td>21.50±4.26</td>
<td>16.31±4.08</td>
<td>12.52±4.50</td>
<td>21.50±4.26</td>
<td>12.52±4.50</td>
<td>10.90±3.22</td>
</tr>
<tr>
<td>Cuzco5hydrolyzed</td>
<td>1.00±0.10</td>
<td>0.80±0.13</td>
<td>0.80±0.30</td>
<td>4.00±1.20</td>
<td>3.40±1.00</td>
<td>2.80±0.70</td>
</tr>
<tr>
<td>Cuzco5acetylated</td>
<td>1.60±0.20</td>
<td>1.00±0.40</td>
<td>0.80±0.21</td>
<td>19.91±0.90</td>
<td>17.96±0.50</td>
<td>7.51±0.24</td>
</tr>
<tr>
<td>Cuzco8</td>
<td>4.70±1.30</td>
<td>2.20±0.60</td>
<td>2.00±0.50</td>
<td>8.76±1.60</td>
<td>3.66±0.80</td>
<td>1.83±0.70</td>
</tr>
<tr>
<td>Mama1</td>
<td>5.00±1.10</td>
<td>3.30±1.10</td>
<td>1.80±0.50</td>
<td>10.50±1.30</td>
<td>4.60±1.00</td>
<td>2.30±0.40</td>
</tr>
<tr>
<td>Mama2</td>
<td>10.70±3.00</td>
<td>4.90±1.00</td>
<td>2.90±0.60</td>
<td>5.00±1.10</td>
<td>3.30±1.10</td>
<td>1.80±0.50</td>
</tr>
<tr>
<td>Mama3</td>
<td>6.00±1.20</td>
<td>5.50±1.10</td>
<td>3.20±1.10</td>
<td>26.67±3.00</td>
<td>24.80±4.00</td>
<td>8.70±3.70</td>
</tr>
<tr>
<td>Mama4</td>
<td>125.00±17.00</td>
<td>117.00±22.40</td>
<td>41.70±7.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama5</td>
<td>19.80±5.00</td>
<td>13.90±8.00</td>
<td>2.20±0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama6</td>
<td>7.10±4.80</td>
<td>2.70±0.90</td>
<td>1.60±1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama7</td>
<td>2.20±0.80</td>
<td>1.40±0.40</td>
<td>1.10±0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama10</td>
<td>4.10±1.80</td>
<td>2.00±0.10</td>
<td>1.50±0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama12</td>
<td>21.76±0.90</td>
<td>12.75±0.90</td>
<td>6.13±2.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama13</td>
<td>3.30±1.10</td>
<td>1.60±0.60</td>
<td>1.40±0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mama14</td>
<td>19.10±4.00</td>
<td>10.30±2.90</td>
<td>5.60±0.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Machu1</td>
<td>18.80±4.20</td>
<td>16.00±5.00</td>
<td>11.40±6.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Machu4</td>
<td>33.51±3.20</td>
<td>31.11±2.95</td>
<td>11.10±4.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>103.21±13.00</td>
<td>94.48±8.00</td>
<td>33.67±7.90</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Screening of sesquiterpenes reversing P-glycoprotein-dependent resistance to daunomycin and vinblastine in NIH-3T3 cells transfected with human MDR1 protein. The reversal index was defined as the ratio between the IC$_{50}$ of cells without sesquiterpene and the IC$_{50}$ with sesquiterpene. IC$_{50}$ values were determined using equation 1 as described in Materials and Methods. Results are of two to four independent experiments performed in triplicate; mean ± SD (P < 0.05).

† The maximum reversal index with daunomycin (ratio between IC$_{50}$ for wild-type and MDR cells) is 27.5.
‡ The maximum reversal index with vinblastine (ratio between IC$_{50}$ for wild-type and MDR cells) is 128.3.
§ Verapamil is a classical P-glycoprotein modulator used for comparison.

7132
substantially higher reversal indexes with vinblastine than with daunomycin, and the most potent of them (Machu4, Mama5, and Mama12) reverted vinblastine resistance at 1 μmol/L with potencies that were from 5- to 9-fold greater than that of verapamil. This means that at this low concentration, the above-mentioned sesquiterpenes were able to decrease the resistance to vinblastine of the MDR1-overexpressing cells from 128.3 to 2 to 4 times the intrinsic resistance of wild-type cells and from 27.5 to 2 to 3 times in the case of daunomycin.

The intrinsic toxicity of the most potent sesquiterpenes, used at concentrations up to 10 μmol/L in wild-type drug-sensitive NIH-3T3 cells, was generally lower than that of verapamil (43.70 ± 2.80, 14.20 ± 2.20, 26.40 ± 1.30, 18.70 ± 1.50, and 17.95 ± 0.05% of growth inhibition at 10 μmol/L for verapamil, Cuzco5, Mama5, Mama12, and Machu4, respectively; P < 0.05).

Inhibition of P-Glycoprotein-mediated Daunomycin Efflux by Sesquiterpenes in Intact Cells. When determining the inhibition of daunomycin efflux at increasing concentration of sesquiterpenes, we

\[
\begin{array}{|c|c|c|c|c|c|c|c|c|}
\hline
\text{Source and Compound} & R_1 & R_2 & R_3 & R_4 & R_5 & R_6 & R_7 & R_8 \\
\hline
\text{Maytenus cuzcoina} & \text{Cuzco1} & \text{OAc} & \text{αOBz} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco2} & \text{OAc} & \text{αOFu} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco2 hydrolyzed in H} & \text{OAc} & \text{αOFu} & \text{H} & \text{OH} & \text{OH} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco2 acetylated in 6} & \text{OAc} & \text{αOFu} & \text{H} & \text{OH} & \text{OAc} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco3} & \text{OAc} & \text{H} & \text{OH} & \text{OAc} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco4} & \text{OAc} & \text{αOMeBut} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco5} & \text{OAc} & \text{αOAc} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco6 hydrolyzed in 6} & \text{OAc} & \text{αOAc} & \text{H} & \text{OH} & \text{OH} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco6 acetylated in 6} & \text{OAc} & \text{αOAc} & \text{H} & \text{OH} & \text{OAc} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco6} & \text{OAc} & \text{αOPr} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{H} \\
\text{Cuzco7} & \text{OAc} & \text{αOAc} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{OAc} \\
\text{Cuzco8} & \text{OAc} & \text{αOAc} & \text{H} & \text{OH} & \text{OAc} & \text{H} & \text{βOFu} & \text{OAc} \\
\text{Cuzco9} & \text{OAc} & \text{αOH} & \text{H} & \text{OH} & \text{OFu} & \text{H} & \text{βOFu} & \text{H} \\
\text{Maytenus megellancia} & \text{Mama1} & \text{OBz} & \text{βOAc} & \text{ONic} & \text{OH} & \text{OAc} & \text{H} & \text{βOCin} & \text{H} \\
\text{Mama2} & \text{OBz} & \text{βOAc} & \text{ONic} & \text{OH} & \text{OAc} & \text{H} & \text{βOBz} & \text{H} \\
\text{Mama3} & \text{ONic} & \text{βOAc} & \text{OAc} & \text{OH} & \text{H} & \text{βOBz} & \text{OBz} & \text{H} \\
\text{Mama4} & \text{ONic} & \text{βOAc} & \text{OBz} & \text{OH} & \text{H} & \text{βOAc} & \text{OBz} & \text{H} \\
\text{Mama5} & \text{OBz} & \text{βOAc} & \text{OAc} & \text{OH} & \text{H} & \text{βOBz} & \text{OBz} & \text{H} \\
\text{Mama6} & \text{OBz} & \text{βOAc} & \text{OAc} & \text{OH} & \text{H} & \text{βOAc} & \text{OBz} & \text{H} \\
\text{Mama7} & \text{OCin} & \text{βOAc} & \text{OAc} & \text{OH} & \text{H} & \text{βOBz} & \text{H} \\
\text{Mama10} & \text{OBz} & \text{βOAc} & \text{OH} & \text{OH} & \text{H} & \text{βCin} & \text{H} \\
\text{Mama11} & \text{OBz} & \text{βOAc} & \text{OH} & \text{H} & \text{H} & \text{βOBz} & \text{H} \\
\text{Mama12} & \text{OAc} & \text{ONic} & \text{H} & \text{OH} & \text{OBz} & \text{H} & \text{βOBz} & \text{OAc} \\
\text{Mama13} & \text{OBz} & \text{OAc} & \text{H} & \text{H} & \text{OBz} & \text{H} & \text{βOBz} & \text{H} \\
\text{Mama14} & \text{OBz} & \text{OBz} & \text{H} & \text{OH} & \text{OAc} & \text{βOBz} & \text{H} \\
\text{Maytenus chubutensis} & \text{Machu1} & \text{OBz} & \text{βOAc} & \text{OH} & \text{H} & \text{OAc} & \text{βOBz} & \text{H} \\
\text{Machu4} & \text{OAc} & \text{ONic} & \text{H} & \text{H} & \text{OBz} & \text{H} & \text{βOBz} & \text{OAc} \\
\text{Maytenus canariensis} & \text{C-3} & \text{OAc} & \text{H} & \text{OH} & \text{OAc} & \text{αOMeBut} & \text{αOBz} & \text{OAc} \\
\end{array}
\]
obtained saturable, concentration-dependent curves that were monophasic with no signs of substantially cooperativity, except for sesquiterpenes C-3 (data not shown). From these curves, we obtained the $K_i$ values for inhibition of daunomycin efflux. The most potent sesquiterpenes inhibiting daunomycin efflux had $K_i$ values in the submicromolar range (as low as 0.24 ± 0.01 μmol/L and 0.33 ± 0.01 μmol/L for Machu4 and Mama12, respectively; $P < 0.001$).

Ability of Sesquiterpenes to Inhibit the BCRP, MRP1, and MRP2 Transporters in Mammalian Cells. Five sesquiterpenes shown previously to reverse P-glycoprotein-mediated MDR in vitro (Cuzz05, Cuzz07, Mama5, Mama12, and Machu4) were assayed. In calcine accumulation experiments, 10 μmol/L cyclosporin A was able to restore the intracellular calcine accumulation levels in MRP1- and MRP2-expressing cells to 95% and 81%, respectively, of that reached in the wild-type cells. Sesquiterpenes at the same concentration were able to increase intracellular calcine accumulation to levels no higher than 30% and 45%, respectively. In rhodamine 123 efflux experiments, GF120918 at 1 μmol/L increased rhodamine 123 intracellular accumulation of rhodamine 123 in BCRP-expressing MDA-MB-231 cells to levels up to 83% of those observed in drug-sensitive cells. However, sesquiterpenes at 10 μmol/L increased rhodamine 123 intracellular accumulation to levels no higher than 26% of the levels in wild-type cells. In contrast, sesquiterpene Machu4 at 2 μmol/L was able to block 97% of P-glycoprotein-mediated daunomycin efflux from MDR1-expressing cells.

Photoaffinity Labeling of P-Glycoprotein with [3H]Azidopine in the Presence of Sesquiterpenes. Plasma membrane vesicles from CHB30 cells (which express P-glycoprotein up to 15% of the total membrane protein) were coincubated with the radioligand and a 100-fold molar excess of different sesquiterpenes (10 μmol/L: a concentration in which they clearly display their MDR reversal effect). For comparison, parallel samples were also coincubated with the same concentration of the classical P-glycoprotein substrates verapamil and vinblastine, which are known to compete with photoaffinity labeling by direct binding and displacement of [3H]azidopine from the drug-binding sites of P-glycoprotein. Fig. 2 shows that verapamil and vinblastine compete for azidopine photoaffinity labeling of the protein with efficiencies in accordance with their respective binding affinities to P-glycoprotein, reported elsewhere (35, 39). All but one of the tested sesquiterpenes inhibited labeling of P-glycoprotein with [3H]azidopine, with relative efficiencies that correlate roughly with their efficiencies as blockers of P-glycoprotein-mediated drug transport activity. Surprisingly, the only sesquiterpene that did not seem to compete for photoaffinity labeling was Machu4, which is the most potent sesquiterpene-reversing P-glycoprotein-mediated MDR found in the present work. In this regard, Mama12, which is almost as efficient as Machu4 at blocking P-glycoprotein drug transport activity and does compete for photoaffinity labeling of P-glycoprotein by [3H]azidopine, differs from Machu4 only by the presence of a hydroxyl group at position 4 (see Fig. 1). It is possible that although sesquiterpenes may share a common mechanism of action on P-glycoprotein, the specific binding site(s) that they are interacting with within the transporter could be different, depending on subtle changes in the molecular structure of these compounds.

Effect of Sesquiterpenes on P-Glycoprotein ATPase Activity. Multiple P-glycoprotein substrates and modulators are known to modulate P-glycoprotein ATPase activity. When we examined the effect of a set of sesquiterpenes on the ATPase activity of CHB30 plasma membrane, we found the typical bell-shaped activation curves reported previously for many P-glycoprotein substrates and inhibitors (Fig. 3A). These curves are suggested to arise from the presence of two binding sites for the modulator, one that activates ($K_a$) and one (of lower affinity, $K_a$) that inhibits (39). When the data points were fitted to a modified form of the classical Michaelis-Menten equation (Equation C; see Material and Methods), half-maximal stimulation ($K_a$) and inhibition ($K_a$) constants could be extracted, which are a measure of the effectiveness of the modulator as an activator or inhibitor. For the tested sesquiterpenes, the $K_a$ values were in the low submicromolar range (18 to 53 nM), and the $K_a$ values were in the submicromolar to micromolar range (250 nM to 4.6 μmol/L). The concentration range in which sesquiterpenes sensitize MDR P-glycoprotein-overexpressing cells matched with the $K_a$ for inhibition of P-glycoprotein ATPase activity.
activity, with no substantially toxic effects in intact cells at the same concentrations. Surprisingly, when we studied the effect of the same sesquiterpenes on the ATPase activity of purified P-glycoprotein in CHAPS solution, we found that sesquiterpenes had lost their ability to stimulate P-glycoprotein ATPase activity and only retained their inhibitory effect (Fig. 3B), with $K_2$ values almost unchanged with respect to those measured in plasma membrane. For comparison, verapamil was still able to stimulate ATPase activity of purified protein as it did in plasma membrane (data not shown), which suggests that P-glycoprotein, despite not being localized within a lipid membrane, conserved its native conformation. Therefore, it seems likely that these sesquiterpenes lost their stimulatory effect not because of modifications in P-glycoprotein conformation but because of changes in their mode of interaction to the transporter, which seems to require the presence of membrane lipids. Such interesting phenomenon deserves additional investigation.

![Fig. 4. Modulation of P-glycoprotein drug transport activity by sesquiterpenes, and kinetic analysis of inhibition of P-glycoprotein-dependent tetramethylrosamine transport in proteoliposomes.](image)
Kinetic analysis of the inhibition of ATPase activity in plasma membrane by the sesquiterpenes Mama5 and Cuzco9 revealed that they functioned as noncompetitive or mixed-type inhibitors of ATPase activity (data not shown). This means that these compounds inhibited ATPase activity by negative allosteric effects, presumably resulting from direct interactions at the transmembrane domains, rather than direct competition with ATP at the nucleotide-binding domains.

**Inhibition of P-Glycoprotein-mediated Drug Transport by Sesquiterpenes in Plasma Membrane Vesicles and P-Glycoprotein-enriched Proteoliposomes.** Equilibrium uptake of [3H]colchicine into CHB30 plasma membrane vesicles (Fig. 4A) and real-time tetramethylrosamine transport into P-glycoprotein-containing PMPC proteoliposomes (Fig. 4B) was determined in the presence of increasing concentrations of various sesquiterpenes. In both systems, sesquiterpenes showed similar behavior; colchicine transport was 90% inhibited at 1.4, 2, and 3.5 μmol/L of Mama5, Cuzco5, and Mama12, respectively, whereas 5 μmol/L verapamil was needed to reach the same inhibition (data not shown). In the proteoliposome system, Mama5 inhibited tetramethylrosamine transport by 90% at around 4.2 μmol/L, which was an efficiency similar to that observed for cyclosporin A (4 μmol/L). This means that these compounds inhibited ATPase function as noncompetitive or mixed-type inhibitors of ATPase activity (data not shown). This means that negative allosteric effects, presumably resulting from direct interactions at the transmembrane domains, rather than direct competition with ATP at the nucleotide-binding domains.

**Effect of Sesquiterpenes on the Binding of Hoechst 33342 and TNP-ATP to P-Glycoprotein.** Sesquiterpenes modulate both the drug transport and the ATPase activity of P-glycoprotein. To address the possibility that these compounds may affect the binding of nucleotides and substrates to P-glycoprotein, we took advantage of the intrinsic fluorescent properties of the transport substrate, Hoechst 33342, and the nucleotide analog TNP-ATP. Both compounds alone are weakly fluorescent in aqueous solution, but their quantum yields are greatly enhanced when they are transferred to a hydrophobic environment, such as the binding pocket within a protein active site. Moreover, both compounds cause the quenching of intrinsic Trp fluorescence on binding to P-glycoprotein. Therefore, there are two ways in which to monitor the process of binding of these compounds to P-glycoprotein and to study if this process is affected in some manner by sesquiterpenes. Both approaches gave similar results, but we have shown the curves obtained after the fluorescence enhancement of the two probes (Figs. 5, A and B), because they were more reproducible from one experiment to another. Sesquiterpene Mama5 did not affect TNP-ATP binding to P-glycoprotein at concentrations up to 50 μmol/L, which is 50-fold higher than the \( K_d \) for inhibition of ATPase activity in CHB30 plasma membrane vesicles. On the other hand, addition of Mama5 steadily decreased the binding of Hoechst 33342 to P-glycoprotein at the same time that it decreased the extent of binding, and both were reduced in the same proportion at all of the tested concentrations of Mama5. This result suggests that Mama5 behaves as an uncompetitive inhibitor of Hoechst 33342 binding, affecting the binding of Hoechst 33342 to its binding site from another site that is only accessible in the P-glycoprotein-Hoechst 33342 complex. The only \( K_i \) that could be extracted from the kinetic analysis was that for Mama5 binding to P-glycoprotein in complex with Hoechst 33342 (K<sub>i</sub> of Mama5, 0.45 ± 0.10 μmol/L) and 0.74 ± 0.15 μmol/L for inhibition of colchicine and tetramethylrosamine transport, respectively). However, because these processes were measured in different systems (plasma membrane versus artificial proteoliposomes composited only of PMPC), it cannot be determined if the observed differences are because of the system itself or because the P-glycoprotein substrate used in each case binds to different regions within the transporter. In addition, the tetramethylrosamine experiments monitor an initial rate of transport, whereas the colchicine experiments measure equilibrium drug uptake.

**Quenching of the Fluorescence Trp Residues and MIANSLabeled P-Glycoprotein by Sesquiterpenes.** Binding of MDR spectrum drugs and modulators to native P-glycoprotein and MIANSLabeled P-glycoprotein has been reported to lead to substantial saturable quenching of intrinsic Trp fluorescence (35) and the bound MIANS probe (31). Similarly, addition of sesquiterpenes caused a saturable, concentration-dependent quenching of both native P-glycoprotein and MIANSP-glycoprotein (data not shown). The quench curves were monophasic for the two tested sesquiterpenes (Mama5 and Mama12), which suggests that they bind to a single site within P-glycoprotein. Whatever this binding site was, the binding affinity of Mama12 obtained by quenching of Trp fluorescence was very similar to that obtained from quenching of the fluorescence of MIANS-P-glycoprotein, with a low degree of quenching (<10%) in both cases. To check that the P-glycoprotein used for these experiments was functional and correctly folded, ATPase activity measurements and quenching curves with rhodamine 123 and TNP-ATP were done on the same preparation in parallel (data not shown). The latter two compounds were shown to give a high degree of quenching of Trp fluorescence as described previously (35, 37). The P-glycoprotein samples retained ATPase activity, which was stimulated by verapamil. In addition, rhodamine 123 and TNP-ATP generated saturable, concentration-dependent quenching curves as expected. The maximal Trp quenching values for rhodamine 123 and TNP-ATP were 96% and 85%, respectively, with \( K_q \) values of 82 μmol/L and 76 μmol/L, respectively. It is important to note that although the values of Trp quenching obtained for both P-glycoprotein substrates were very similar to those reported previously (35), the \( K_q \) values were 20 to 25% higher. A possible reason for this phenomenon could be the nature of the lipids used in each case (PMPC in the previous study and asolectin in the present one). The differences in lipid composition (asolectin versus PMPC) and different drug-lipid ratios could also explain why the calculated binding affinity for Mama12 and Mama5 in this experiment was almost 10 times higher than the \( K_i \) for inhibition of tetramethylrosamine transport in PMPC proteoliposomes.
whereas the enhancement of TNP-ATP fluorescence was monitored at 535 nm after excitation with unlabeled P-glycoprotein was monitored at 460 nm after excitation at 350 nm. P-glycoprotein. Enhancement of the intrinsic fluorescence of Hoechst 33342 on interaction with increasing concentrations of Hoechst 33342 or TNP-ATP at the fixed concentrations of Mama5 or cyclosporin was used for both experiments represented in Fig. 5. The present study has focused on the identification of P-glycoprotein as the cellular target of sesquiterpenes and on the study of their molecular mechanism of action.

Although none of the 28 sesquiterpenes tested showed greater potency in modulating P-glycoprotein than previously described third-generation modulators, such as LY335979 (40), many of them had a potency greater than the classical first-generation modulator verapamil, and three of them had comparable potency to cyclosporin A. Although none of the 28 sesquiterpenes tested showed greater potency in modulating P-glycoprotein than previously described third-generation modulators, such as LY335979 (40), many of them had a potency greater than the classical first-generation modulator verapamil, and three of them had comparable potency to cyclosporin A. One of the advantages of sesquiterpenes with respect to first-generation modulators is that Celastraceae plants containing high doses of sesquiterpenes have been used worldwide for centuries in traditional medicine with no deleterious effects on human health. Moreover, the most effective sesquiterpenes tested against P-glycoprotein had almost no effect on MRP1, MRP2, and BCRP drug transport activities in vitro in the same concentration range, which shows that the tested sesquiterpenes were specific modulators of P-glycoprotein. It is also worth noting that the most efficient sesquiterpenes were less toxic than verapamil toward cultured drug-sensitive cells but were specifically more toxic toward P-glycoprotein-expressing cells (data not shown). This finding is very interesting, considering that P-glycoprotein is presumed to be involved in malignancy of cancer cells as well as drug resistance (41) and that inhibition of P-glycoprotein by PSC-833 led to a selective direct elimination of MDR cells (42).

The results concerning the modulation of photoaffinity labeling of P-glycoprotein with [3H]azidopine with P-glycoprotein ATPase activity and the P-glycoprotein-mediated transport of [3H]colchicine support the proposal of a direct interaction between sesquiterpenes and P-glycoprotein. Modulation of tetramethylrhodamine transport in P-glycoprotein-enriched proteoliposomes, the quenching of both Trp and MIANS probes on binding of sesquiterpenes to purified P-glycoprotein and MIANS-P-glycoprotein, and the inhibition of Hoechst 33342 binding to purified P-glycoprotein by sesquiterpene Mama5 are definite evidence of the direct interaction of sesquiterpenes from Celastraceae with P-glycoprotein. Regarding the mechanism of action of sesquiterpenes as P-glycoprotein inhibitors, the results of the present study suggest that these compounds block drug transport activity of P-glycoprotein by binding to the transmembrane domains rather than the nucleotide-binding domains. Moreover, the kinetic analysis of ATPase activity inhibition by sesquiterpenes Mama5 and Cuzco9 revealed that these compounds act as noncompetitive/mixed-type inhibitors, affecting ATPase activity by negative allosteric effects as a consequence of direct interactions at transmembrane domains and not because of direct competition with ATP at the nucleotide-binding domains. This mechanism of action gains support from the fact that Mama5 did affect binding of Hoechst 33342 to transmembrane domains but not that of TNP-ATP to nucleotide-binding domains. Finally, sesquiterpenes inhibited drug transport more efficiently than ATPase activity of P-glycoprotein. At the concentration range that sesquiterpenes sensitized MDR P-glycoprotein-overexpressing cells, they efficiently inhibited drug transport, whereas a substantially ATPase activity still remained. Therefore, sesquiterpenes do not block drug transport by inhibiting the “ATP-fuelled engines” of the transporter (the nucleotide-binding domains). On the contrary, they should block drug transport itself at the transmembrane domains and, as a consequence of the coupling between the domains, ATPase activity may be consequently inhibited. Sesquiterpenes Mama5 and Cuzco9 have different efficiencies as P-glycoprotein modulators, yet inhibited P-glycoprotein ATPase activity in the same manner (as noncompetitive/mixed inhibitors). Because all of the tested sesquiterpenes share a common chemical structure with few modifications on the basic skeleton, it may be assumed that the general mechanism of action of sesquiterpenes is essentially common (by interaction with the transmembrane domains), independent of their respective reversal potencies.

Other questions that remain to be answered include whether all of the sesquiterpenes bind to the same binding site(s) within the transmembrane domains, how many binding sites they interact with (if more than one exists), and the location of such binding site(s). The modulation of [3H]azidopine photoaffinity labeling by sesquiterpenes was markedly affected by subtle changes in the molecular structure, which suggests that although sesquiterpenes should bind to transmembrane domains, they may not be doing so exactly at the same site. According to the model of Loo and Clarke (43), which considers P-glycoprotein-drug interactions at the level of only one poly specific binding site, it is not surprising that even closely related compounds may bind to different but overlapping sites within the transmembrane domains. In fact, the stereoisomers cis- and trans-flupentixol each bind to different sites within P-glycoprotein (44). This may also explain why the sequences in efficiencies obtained for the tested sesquiterpenes are slightly different from one kind of experiment to another, because different drug substrates were used in each experimental approach, and each of them may bind to different, overlapping drug binding sites. The bell-shaped profile of P-glycoprotein ATPase activity modulation suggests the existence of two different binding sites of high and low affinity for sesquiterpenes. However, only the low-affinity binding seems to be responsible for the
pharmacological effects associated with P-glycoprotein-sesquiterpene interactions, given that these compounds reverse in vitro MDR in the same concentration range as the \( K_d \) for ATPase activity inhibition. Moreover, no substantial cooperativity (except for sesquiterpene C-3) was observed in the curves of daunomycin efflux inhibition from intact cells obtained for 11 sesquiterpenes (data not shown), suggesting that only one functional binding site for sesquiterpenes exists in P-glycoprotein. In addition, quenching of P-glycoprotein and P-glycoprotein-MIANS by sesquiterpenes Mamã5 and Mamã12 was best fitted to monophasic curves describing binding to a single site. Therefore, although more than one site may be implicated in sesquiterpene binding, each one seems to bind to a single functional site within the transmembrane domains of P-glycoprotein.

In summary, all of the evidence shown in the present work supports P-glycoprotein as the molecular target for Celastraceae sesquiterpenes and shows that these natural compounds are efficient and specific P-glycoprotein modulators with promise for clinical application in the treatment of MDR malignancies. Additional improvement of their potency as blockers of P-glycoprotein-mediated drug transport activity would make them suitable for entry into clinical studies. Moreover, the identification of P-glycoprotein as their cellular target and improved knowledge of their molecular mechanism of action has prompted us to start the studies conducing to the development of a computer-assisted quantitative structural-activity relationship model that will allow the rational design of new molecules with higher potency and specificity based on the common molecular structure of sesquiterpenes.

ACKNOWLEDGMENTS

The authors thank Pilar Navarro for her excellent technical assistance with the cell cultures and Miguel Lugo-Álvarez for his valuable theoretical discussions regarding the ATPase and fluorescence quenching experiments. We also thank Dr. Ira Pastan (National Cancer Institute, NIH, Bethesda, MD) for providing the NIH-3T3 and NIH-3T3 MDR-G185 cell lines; Dr. Piet Borst (Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands) for providing the 2008, 2008-MRP1 clone 8, MDCKII, and MDCKII-MRP2 clone 17 cell lines; Dr. Douglas D. Ross (University of Maryland Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD) for providing the MDA-MB-231-BCRP cell lines; and Francisco Javier Pérez-Victoria for helpful discussion and contributions as this work progressed. Finally, we acknowledge Pfizer for providing the daunomycin used in this work.

REFERENCES

Celastraceae Sesquiterpenes as a New Class of Modulators That Bind Specifically to Human P-Glycoprotein and Reverse Cellular Multidrug Resistance

Francisco Muñoz-Martínez, Peihua Lu, Fernando Cortés-Selva, et al.

*Cancer Res* 2004;64:7130-7138.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/19/7130

Cited articles
This article cites 42 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/19/7130.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/64/19/7130.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.