Psorospermin structural requirements for P-glycoprotein resistance reversal

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Abstract

Resistance to chemotherapy reduces its effectiveness, resulting in increased mortality. Psorospermin, a natural product, is a topoisomerase II–directed DNA alkylating agent active against multidrug-resistant (MDR) cell lines, including multiple myeloma. In this study, the mechanism of the P-glycoprotein (P-gp) modulation activity of psorospermin and that of its associated pharmacophore were examined. Flow cytometry shows that doxorubicin-resistant multiple myeloma cells (8226/D40) pretreated with psorospermin enhance intracellular retention of doxorubicin compared with control (75% versus 38%). Because the overexpression of P-gp is the primary cause of drug resistance in the 8226/D40 cells, psorospermin-induced sensitization was likely due to mdr1/P-gp expressional or functional inhibition. As shown by PCR and Western blot, neither transcription of mdr1 nor translation of P-gp was down-regulated by psorospermin treatment. Therefore, the mechanism of psorospermin-induced resistance reversal is most likely through a direct interaction between psorospermin and P-gp. Furthermore, because only the (2′R,3′R) isomer of psorospermin showed any resistance reversal activity, the side chain of psorospermin is apparently a crucial moiety for resistance reversal. By understanding the mechanism of psorospermin-induced MDR modulation, psorospermin and similar compounds can be combined with other chemotherapies to treat resistant cancers. [Mol Cancer Ther 2008;7(11):3617–23]

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Introduction

Many cancer patients, especially those diagnosed in the late stages of disease, do not respond to standard treatments. This failure is also frequently observed in patients who relapse after periods of remission (1). Therefore, to treat drug-resistant cancers, new chemotherapeutics must be developed.

Cancers resistant to a variety of structurally unrelated chemotherapeutic agents display the multidrug-resistant (MDR) phenotype (1, 2). One major factor associated with MDR is altered drug transport, that is, overactive drug efflux (2, 3). Overactive drug efflux prevents drugs from reaching their intended target because transport proteins pump the drug out of the cell in an energy-dependent manner (2–4). The ATP-binding cassette protein superfamily has been identified to play an integral role in drug efflux and invariably drug resistance. Three ATP-binding cassette proteins frequently overexpressed in many human cancers are P-glycoprotein (P-gp), MDR-associated protein, and breast cancer resistance protein (5, 6).

P-gp, the most studied human ATP-binding cassette transporter, is a 170-kDa dimeric protein, which is the product of the mdr1 gene (7). Containing two homologous nucleotide-binding domains and two six-membered transmembrane domains, P-gp forms a pore in the cellular membrane, and the interface between the two transmembrane domains is the site for substrate binding (8). Although the mechanism of efflux is not fully understood, studies suggest that substrate binding results in ATP-driven dimerization of the nucleotide-binding domains. This reaction produces a conformation change of the transmembrane domains, leading to a decrease in the binding affinity of the substrate to the binding site, thus releasing the substrate into the extracellular space (8–10). Although P-gp functions in normal tissues to remove toxins from the body, it is frequently overexpressed in neoplastic tissues, resulting in the efflux of a variety of structurally unrelated compounds, including chemotherapeutics (5, 6, 9, 11).

Psorospermin was isolated from the stembark and roots of the African plant *Psorospermum febrifugum* (12, 13). Although studied for over 30 years, the four isomers of psorospermin were only recently synthesized in 2005 (Fig. 1A; ref. 14). Significantly, psorospermin has activity against drug-resistant cell lines, including breast cancer, leukemias, and AIDS-related lymphomas (15, 16). The cytotoxic activity of psorospermin stems from its ability to intercalate with DNA at the major groove by site-directed electrophilic addition, resulting in a covalent psorospermin-DNA adduct (Fig. 1B; ref. 17). We have shown previously that the alkylation activity of psorospermin is greatly increased in the presence of topoisomerase II, an

enzyme that assists in DNA breakage and religation. This increase is due to the ability of psorospermin to trap the topoisomerase II–DNA complex in the initial noncovalent binding step of the topoisomerase II catalytic cycle, resulting in topoisomerase II–mediated DNA cleavage (18). Due to its mechanism of topoisomerase II–directed alkylation, psorospermin has been characterized as a novel topoisomerase II poison (17, 18).

Because the cytotoxic activity of psorospermin is not affected by overexpression of P-gp or other ATP-binding cassette transport proteins (16), psorospermin may play a role in MDR modulation. In this study, we investigate the mechanism underlying the P-gp modulation activity of psorospermin in multiple myeloma cells. Our studies have shown that treatment with the natural isoform ($2'R,3'R$), and not the other isoforms, of psorospermin inhibits the doxorubicin export in these cells. Furthermore, we observed that neither mdr1 transcription nor P-gp translation is inhibited by psorospermin, leading to the hypothesis that psorospermin affects P-gp directly.

Materials and Methods

Cell Culture and Compounds

8226/S (parental) human multiple myeloma cells were purchased from the American Type Culture Collection. The doxorubicin-resistant 8226 cell line (8226/D40) was a gift from the Dalton laboratory (University of Arizona; ref. 19). Both 8226/S and 8226/D40 cell lines were cultured in RPMI 1640 (CellGro) supplemented with 8% fetal bovine serum (Gemini) and 1% penicillin/streptomycin (CellGro). Cells were maintained at 37°C in a humidified incubator at 5% CO_2. Cells were fed with medium every 2 to 3 days and passaged weekly. Doxorubicin was purchased from Fluka Biochemika and diluted in water to a stock concentration of 1.8 mmol/L. (±)Verapamil hydrochloride was purchased from Fluka Biochemika and diluted to 2.2 mmol/L in 100% methanol. All four isomers of psorospermin (PS-RR, PS-RS, PS-SR, and PS-SS) were obtained from Cylene Pharmaceuticals and diluted in DMSO to a stock concentration of 10 mmol/L. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, and phenazine methosulfate were purchased from Promega. P-gp antibody was purchased from Abcam. The goat anti-mouse IgG horseradish peroxidase-conjugated antibody was purchased from Bio-Rad.

Doxorubicin Accumulation Assay

8226/S and 8226/D40 cells were plated at a concentration of 1×10^6/mL in 250 mL conical tubes. The cells were pretreated with 5 μmol/L psorospermin or 13 μmol/L verapamil or were not pretreated. After 15 min, all cells were treated with 10 μmol/L doxorubicin and incubated at 37°C for 1 h. The reaction was quenched with ice-cold PBS, and the cells were pelleted by centrifugation (2,000 rpm, 2 min). The cells were resuspended at a concentration of 2.5×10^5/mL in medium with or without psorospermin or verapamil. The cells were incubated at 37°C. At time 0 and after 60 min, aliquots of 1×10^6 cells were removed from each sample in triplicate. The reaction was quenched with ice-cold PBS, and the cells were pelleted by centrifugation. The cells were resuspended in ice-cold PBS, and intracellular doxorubicin content was quantitated by a FACScan flow cytometer (BD Biosciences) at 488ex/585em nm.
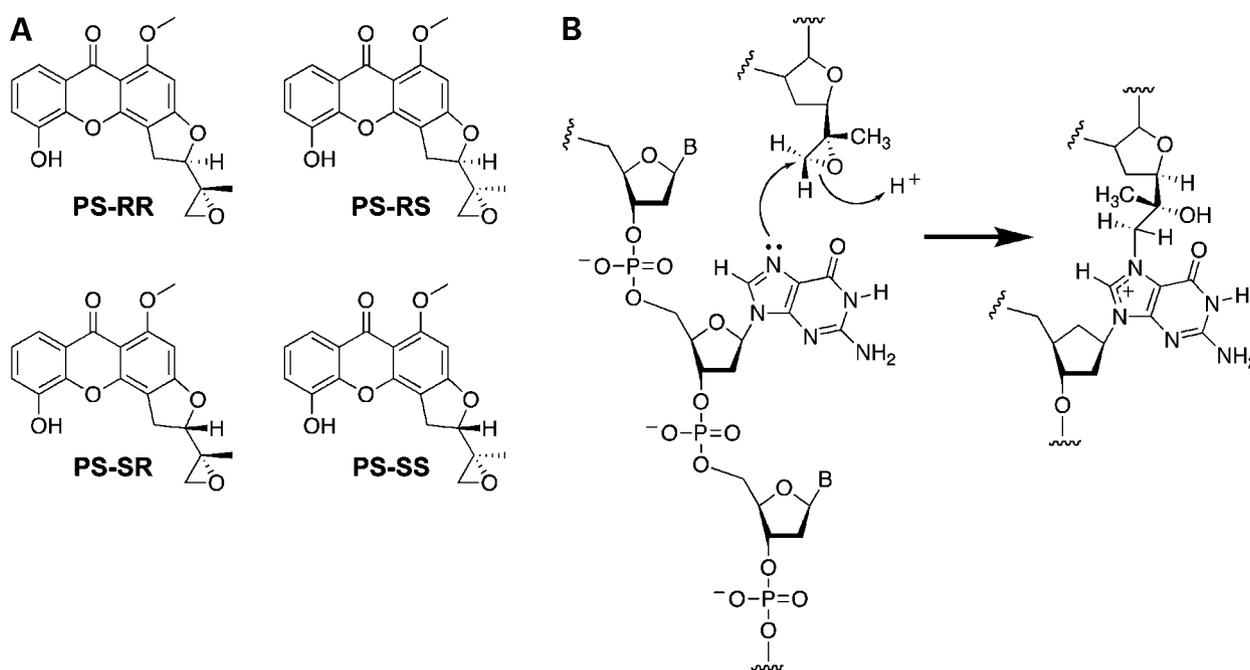
Figure 1. **A,** structures of the four isomers of psorospermin. **B,** covalent alkylation reaction between psorospermin and N^7 of guanine of DNA (reproduced from ref. 17).
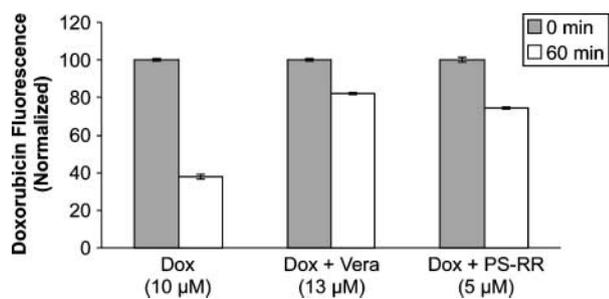
Figure 2. Intracellular doxorubicin fluorescence in 8226/D40 cells treated with psorospermin (PS-RR) or verapamil. Doxorubicin (10 μmol/L) fluorescence was measured after 60 min of uptake (0 min) and following efflux (60 min) in the absence or presence of verapamil (13 μmol/L) or PS-RR (5 μmol/L) using flow cytometric analysis. Doxorubicin fluorescence was normalized to the measurement at time 0.

Cell Viability Assay

Cells were plated at a concentration of 5×10^3 per well in a 96-well plate and treated with drug at indicated concentrations. The cells were incubated at 37°C for 3 days. Cell viability was measured using a CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Kit (Promega). Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, and phenazine methosulfate were combined at a 20:1 ratio, and 20 μL were added to each well. The cells were incubated for 3.5 h, and absorbance was read at 490 nm using a plate reader.

Transcription and Translation of mdr1/P-gp

In the mdr1 transcription experiments, cells were plated at a concentration of 2×10^6 in T75 flasks. The cells were treated with (2′R,3′R) psorospermin (PS-RR) at concentrations of 2.5 and 5 μmol/L. DMSO was used as a vehicle control. After incubating the cells at 37°C for 2 h, the cells were pelleted and washed with ice-cold PBS. Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel) and quantitated by spectroscopy (260 nm). cDNA was made with 1 μg RNA using the iScript cDNA Synthesis Kit (Bio-Rad). For use in real-time PCR, primers for mdr1 were designed using MacVector. The primer sequences used were 5′-CATTGGTGTGGTGAGTCAGG-3′ (forward) and 5′-CATAGGCATTGGCTTCCTTG-3′ (reverse). β-Actin was used as a housekeeping gene. Real-time PCR was done using the Bio-Rad MyiQ Single-Color Real-time PCR Detection System with SYBR Green 1 fluorophore (Bio-Rad). The Bio-Rad protocol was followed with an optimized annealing temperature of 59°C.

To measure the protein expression of P-gp, 2×10^8 cells treated with PS-RR for 2 h (as above) were lysed and fractionated as follows. Cells were resuspended in Hypotonic Lysis Buffer [10 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 10 mmol/L Tris-HCl (pH 7.4), Protease Inhibitor Tablet; Roche] and ruptured with a 15 mL homogenizer (25 strokes). Lysate was centrifuged at 5,700 rpm, 4°C, for 10 min. The supernatant was centrifuged at 25,000 rpm, 4°C, for 1.5 h and the pellet was frozen at -80°C overnight. Pellet was resuspended in a 1:1 mixture of 5 mmol/L

Tris-HCl (pH 7.4) and 16% sucrose and homogenized using a 7 mL homogenizer. The lysate/sucrose mixture was added to the top of a 16% to 60% noncontinuous sucrose gradient and centrifuged at 27,000 rpm, 4°C, for 4 h. The membrane vesicles were collected from the 16% to 31% and 31% to 45% interfaces, diluted with 5 mmol/L Tris-HCl (pH 7.4), and centrifuged at 27,000 rpm, 4°C, for 1 h. The membrane pellet was resuspended in 5 mmol/L Tris-HCl (pH 7.4; 25 μL) and the protein concentration was measured by BCA assay (Bio-Rad). Total protein (30 μg) were separated by SDS-PAGE using a 4% to 20% Tris-HCl gel. The protein was transferred to nitrocellulose using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell (6 V for 20 min followed by 20 V for 3 h). After blocking the filter with 5% nonfat dry milk/TBST [20 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl, 0.1% Tween 20], the membrane was agitated in blocking buffer with P-gp primary antibody (1:1,000) for 2 h at room temperature. After washing the membrane with TBST, it was agitated for 1 h in blocking buffer with the goat anti-mouse secondary antibody (1:1,000). The membrane was again washed with TBST and stained with chemiluminescent solution (Pierce). The membrane was exposed to film and developed. Using the ImageJ software (NIH), absorbance analysis was used to quantitate changes in P-gp expression.

Psorospermin Superposition Molecular Modeling

Psorospermin, fluphenazine, and GF-120918 were constructed using the Insight II Builder program (Insight II 2005L, Molecular Modeling Software; Accelrys). Molecules were then minimized using the Discover 3.0 minimization program within the Insight II software, and the minimized energy structures were used for superimposition. The psorospermin molecule was superimposed with fluphenazine and GF-120918 using structural similarity and manual superimposition.
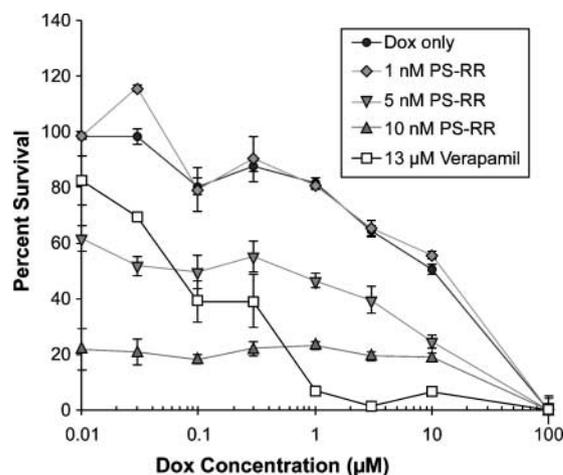
Figure 3. Doxorubicin dose-response curve of 8226/D40 cells treated with PS-RR or verapamil. Cells were pretreated with vehicle, PS-RR, or verapamil and increasing concentrations of doxorubicin. After 3 d, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay.

Results

Psorospermin Can Induce Drug Resistance Reversal in Multiple Myeloma

Previous data showed equivalent psorospermin cytotoxicity in the 8226/S and 8226/D40 cell lines. In comparison, in the same pair of cell lines, doxorubicin showed a 500-fold level of resistance (16). These data indicate that psorospermin is either not a substrate for P-gp or perhaps a modulator of it.

To measure the P-gp reversal activity of psorospermin, doxorubicin efflux assays were performed, and relative intracellular doxorubicin concentrations were measured by flow cytometry (Fig. 2). The 8226/D40 cells that were only treated with doxorubicin (no psorospermin or verapamil) showed a significant decrease in intracellular doxorubicin after 60 min of efflux, retaining only 38.2% of the initial doxorubicin. However, 8226/D40 cells that were treated with PS-RR retained the majority (74.7%) of the doxorubicin after 60 min. As a comparison, doxorubicin efflux in the presence of a known P-gp modulator, verapamil, was greatly inhibited: 82.2% of doxorubicin was retained in the same time interval. The 8226/S cells did not show any significant change in doxorubicin levels over 60 min of efflux with or without psorospermin or verapamil (data not shown).

Dose Dependency and Mechanism of Psorospermin-Induced Resistance Reversal Are Different from Those Producing the Cytotoxicity

To identify a psorospermin concentration at which only chemosensitization, and minimal cytotoxicity (<10%), is observed, PS-RR dose-response curves were generated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assays (data not shown). PS-RR (3 nmol/L) produced a decrease in cell viability of 10.2% in the 8226/D40 cells. Therefore, using three psorospermin doses (1, 5, and 10 nmol/L), the enhancement of doxorubicin cytotoxicity in the presence of psorospermin was examined. Because the 8226/D40 cell line had a 500-fold level of resistance to doxorubicin, a 4-log concentration differential was used, with a typical exposure period of 3 days. When psorospermin is combined with doxorubicin, even at very low concentrations (5 and 10 nmol/L), the cytotoxic effect overwhelmed the reversal effect (Fig. 3). Thus, the data indicate that the percent survival is reduced to ~50% and 20% for those two psorospermin concentrations, respectively. Then, as the doxorubicin concentration increases, the cytotoxic effect of doxorubicin is observed, but this appears to be an additive effect with psorospermin. At the lowest concentration of PS-RR (1 nmol/L), no effect is seen. The reversal effect (Fig. 2) requires a higher concentration (5 μmol/L) and shorter exposure time (1 h) than those of the PS alkylation-mediated cytotoxic effect (5-10 nmol/L and 3 days). This is a reflection of the short time but large dose required to induce P-gp versus the long time but small dose of psorospermin required to produce the cytotoxic effects.

Psorospermin Does Not Significantly Alter mdr1/P-gp Transcription or Translation

To determine if the P-gp reversal by psorospermin can be attributed to transcription or translation of the mdr1 gene
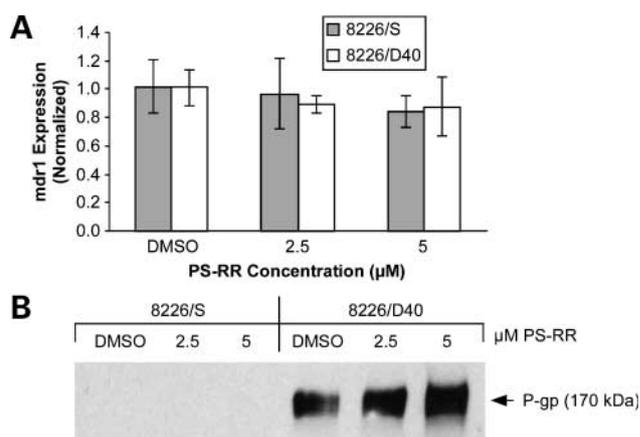
Figure 4. **A,** mdr1 gene expression in 8226/S and 8226/D40 cells treated with PS-RR. Cells were treated with DMSO or 2.5 and 5 μmol/L PS-RR for 2 h. mdr1 mRNA levels were quantitated by real-time PCR. The measurements were normalized to the untreated control. **B,** P-gp protein levels of membrane isolates from 8226/S and 8226/D40 cells treated with PS-RR for 2 h. Cells were treated as above. Membranes were isolated and P-gp levels were measured by Western blot.

or P-gp, respectively, these endpoints were measured following treatment with psorospermin. Because resistance reversal has been observed within 2 h after psorospermin treatment (Fig. 2), it was important to determine how the mdr1 mRNA and P-gp expression levels changed within that period. Therefore, using the 8226/S and 8226/D40 cell lines, the cells were treated with PS-RR at concentrations of 0 (vehicle), 2.5, and 5 μmol/L for 2 h. The cells were then collected and processed for real-time PCR and Western blot analysis. The transcriptional analysis showed that the mdr1 mRNA levels did not change significantly in either cell line when treated with PS-RR (Fig. 4A). When P-gp from PS-RR-treated membrane isolates was measured, an increase of 23.1% and 51.4% (as determined by absorbance analysis) was observed in 8226/D40 cells treated with 2.5 and 5 μmol/L PS-RR, respectively, compared with vehicle control (Fig. 4B). As expected, no P-gp was detected in the 8226/S cells, which do not overexpress P-gp (19).

### Resistance Reversal Activity of Psorospermin Is Stereospecific

The cytotoxicity of psorospermin varies based on its stereochemistry. Previous studies have determined that, in the 8226/D40 cell line, the IC_{50} of psorospermin ranges from 0.037 μmol/L (PS-RR) to 0.335 μmol/L (PS-SS; ref. 16). Due to this variability of cytotoxic potency, we determined if stereochemistry was a factor in its resistance reversal activity. By doxorubicin accumulation assay, 8226/D40 cells that were treated with the PS-RR isomer showed a significant retention (80.4%) of doxorubicin after 60 min compared with cells treated with doxorubicin only (36.2%), similar to that seen in Fig. 2. As reported previously, verapamil retained doxorubicin at a comparable level (Fig. 5). The other three isomers of psorospermin (PS-RS, PS-SR, and PS-SS) did not show any significant retention of doxorubicin compared with the control.

Discussion

In this study, we have achieved a better understanding of the mechanism of psorospermin-induced P-gp resistance reversal. The resistance reversal activity of psorospermin appears to be due to its stereospecific interaction with P-gp directly and is not based on transcriptional or translational down-regulation.

Previous studies have shown that psorospermin has cytotoxic activity in drug-resistant cancers, including doxorubicin-resistant breast cancer and multiple myeloma (16). This cytotoxicity has been attributed to its interaction with topoisomerase II and DNA (18). However, currently, treatment of doxorubicin-resistant 8226 cells with PS-RR has resulted in a significantly enhanced intracellular retention of doxorubicin. Because the doxorubicin resistance of the 8226/D40 cell line is due to an overexpression of P-gp (16, 19), psorospermin-induced resistance reversal may be attributed to an inhibition of P-gp function. By reducing P-gp activity, doxorubicin efflux is decreased, allowing the drug to accumulate intracellularly.

Although it is apparent that psorospermin plays a critical role in the observed doxorubicin retention, the mechanism of P-gp inhibition of psorospermin is still unclear. Because inhibition of transcriptional and/or translational activity of mdr1/P-gp and inhibition by a direct interaction with the protein could both result in a decrease of drug efflux, each possible mechanism had to be examined. Treatment with PS-RR did not result in a decrease in either mdr1 transcription or P-gp translation within the time interval in which resistance reversal was observed. Therefore, it is unlikely that psorospermin-induced resistance reversal is due to transcriptional/translational regulation. In particular, psorospermin (5 μmol/L) increased P-gp protein expression in the 8226/D40 cells, yet P-gp function was decreased, further highlighting the likely physical interaction of PS-RR with P-gp. The observed increase in P-gp expression in the presence of PS-RR may be indicative of an attempt by the cells to counteract the resistance reversal
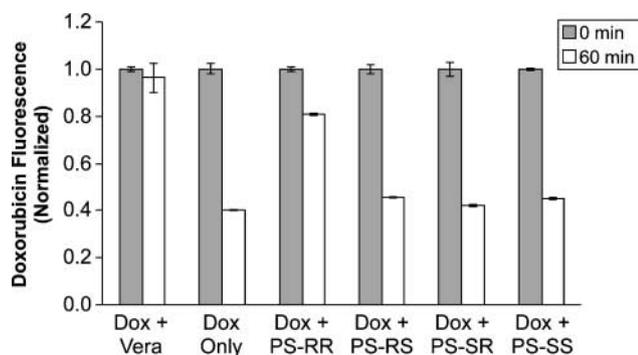
effect of PS-RR. However, the hypothesis that transcriptional/translational regulation is not involved in resistance reversal is still strengthened by the fact that psorospermin-induced resistance reversal is observed within 2 h of treatment, whereas the half-lives of the mdr1 mRNA and P-gp are 4 to 10 and 14 to 72 h in P-gp-overexpressing cell lines, respectively (20–23), indicating that psorospermin would most likely have to interact directly with the P-gp to inhibit drug efflux, as observed.

Because a direct interaction between psorospermin and P-gp appears to be necessary for resistance reversal activity, it was not surprising that the stereochemistry of psorospermin significantly affects potency. Because only PS-RR was shown to have any resistance reversal activity, this activity is stereospecific. The two chiral centers of psorospermin, which define the four diastereomers, are on the side chain of the molecule and not the xanthone ring. Thus, the side chain is apparently necessary for P-gp functional inhibition. Whereas the side chain is necessary for any inhibitory activity, the xanthone ring is also likely to be functionally relevant. Established P-gp modulators, such as fluphenazine (a first-generation MDR modulator) and GF-120918 (a third-generation MDR modulator), contain planar ring structures that are similar to the xanthone ring of psorospermin. An overlay of these molecules and psorospermin showed that the xanthone ring of psorospermin fits well with the phenothiazine ring of fluphenazine (RMS value = 0.221; Fig. 6A) and the 9-acridinol ring of GF-120918 (RMS value = 0.09; Fig. 6B). Because fluphenazine and GF-120918 have been shown to have *in vitro* reversal activity in drug-resistant solid cancers, psorospermin, a compound with a similar structure, could be expected to display resistance reversal activity as well. It is possible that the planar xanthone ring is necessary to allow for psorospermin to access and interact with the binding pocket of P-gp.

Previous studies by Seelig and Landwojtowicz (24, 25) that identified general structural elements necessary for P-gp substrate recognition correlate with our psorospermin findings. Seelig proposed that substrates of P-gp must contain at least one type I (two electron donor groups separated by 2.5 ± 0.3 Å) or one type II (two or three electron donor groups separated by 4.6 ± 0.6 Å; outer groups) electron donor group to interact with P-gp. Using these criteria, it is not surprising that psorospermin may interact with P-gp because its structure contains two type I electron donor groups (a carbonyl and ether group separated by 2.71 Å and an alcohol and an ether separated by 2.77 Å; Fig. 6C), characteristic of a P-gp substrate. These structural elements are comparable with that of verapamil, an established resistance reversal agent (26, 27) that has two type I electron donor units but also one type II unit (25). Although the mechanism of resistance reversal of psorospermin is not yet known, but because both psorospermin and verapamil contain multiple type I electron donor groups and also display similar levels of resistance reversal activity, psorospermin is likely to share a similar mechanism with verapamil, which competitively inhibits P-gp
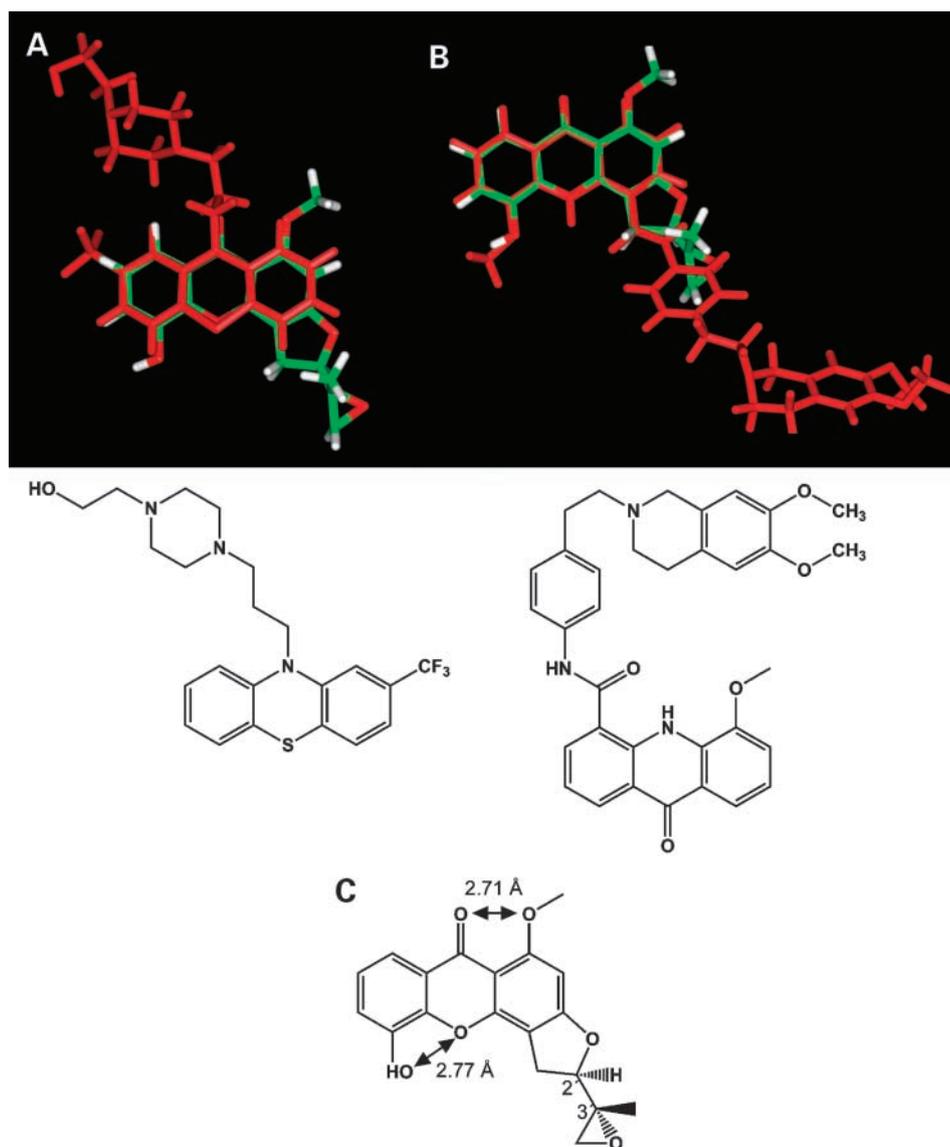

Figure 5. Intracellular doxorubicin fluorescence in 8226/D40 cells treated with psorospermin isomers or verapamil. Cells were pretreated with 5 μmol/L PS-RR, PS-RS, PS-SR, or PS-SS or 13 μmol/L verapamil, and doxorubicin uptake and efflux were examined. Doxorubicin fluorescence was normalized to the measurement at efflux time 0.

Figure 6. Superimposition of psorospermin and established P-gp modulators. Using the Insight II Builder program and its Discover 3.0 minimization program, the minimized energy structures of molecules were used to construct an overlay of psorospermin (colored by atoms) with (**A**) fluphenazine (*red*) and (**B**) GF-120918 (*red*). **C,** identification of the type I electron donor groups of PS-RR. Measurements were made between oxygen atoms.

(28). If this is the case, psorospermin would act as a competitive inhibitor of P-gp, thus reversing the cell's resistance mechanism and preventing efflux of doxorubicin and allowing doxorubicin to interact with topoisomerase II. This illustrates the unique dual mechanism of psorospermin. Psorospermin can act both by a topoisomerase II–mediated alkylation mechanism as well as by a specific mechanism that inhibits P-gp and causes resistance reversal. The stereochemistry required for both mechanisms is the same (2′R,3′R) and corresponds to the natural isomer of psorospermin. Taken together, these data show how important stereochemistry is for psorospermin activity.

Psorospermin is able to produce two distinct effects: topoisomerase II–directed DNA alkylation and P-gp-mediated resistance, which are both relevant to the ability of psorospermin to kill cells that overexpress P-gp; the dose required to reverse doxorubicin resistance is ~1,000 times higher than that required for the cytotoxic effect of psorospermin. This is really a reflection of the comparative cytotoxicities of the two drugs. The only way psorospermin could be used to reverse doxorubicin cytotoxicity would be to eliminate the alkylation ability of psorospermin. Nevertheless, the structural insight into the P-gp resistance reversal by psorospermin is an important step toward this goal.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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