Reversal of Multidrug Resistance by Two Novel Indole Derivatives

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ABSTRACT

Two new fused indoles were found to overcome multidrug resistance in P388/Adr cells in vitro. These agents potentiated the cytotoxicity of the antitumor drugs Adriamycin, vinblastine, and vincristine in multidrug-resistant cells with no effect on drug-sensitive parent P388 cells. They significantly increased the ATP-dependent accumulation of [3H]-vinblastine and inhibited efflux of the labeled drug from resistant cells. These compounds also inhibited photoaffinity labeling of P-glycoprotein by [3H]azidopine in P388/Adr cells and membranes isolated from these cells. In addition, the calcium antagonist activity of these compounds was very weak compared with that of verapamil. These data suggest that the compounds reported here may specifically overcome multidrug resistance without the serious hypotensive effects associated with calcium antagonists and that this activity may be independent of their ability to block calcium transport.

INTRODUCTION

MDR is characterized by the development of resistance to several structurally unrelated classes of carcinostatic compounds. It is a major problem in cancer chemotherapy. Over the past several years, evidence has accumulated to show the association between the development of MDR and the appearance of a specific P-glycoprotein, P-170, in resistant cells (1, 2). The human gene coding for the P-glycoprotein MDR1 has been cloned and sequenced (3). MDR1 was shown to be sufficient with the presence of P-170 to previously sensitive cells (4, 5). P-170 is a membrane-associated protein thought to actively efflux cytotoxic agents such as anthracyclines and Vinca alkaloids. The function of P-glycoprotein is ATP dependent (6). It contains ATPase activity and binds photoactive analogues of certain drugs (7). Based on these findings, it was proposed that P-glycoprotein functions as an energy-dependent drug transporter. Several compounds are known to reverse MDR in vitro and to enhance the cytotoxic effects of anticancer drugs. These include calcium channel antagonists (phenylalkylamines such as verapamil) (8), calmodulin inhibitors (phenothiazines) (9), indole alkaloids (reserpine) (10), quinolines (quinine, chloroquine), and cyclosporins (A, C, G, and others) (11, 12). Verapamil has attracted much attention as a chemosensitizing agent; however, several combination therapy trials using verapamil were discontinued after the onset of toxic effects ascribed to the high levels of verapamil (13). In this study, we describe two compounds that modulate the MDR phenotype in vitro. These compounds have been analyzed for their ability to sensitize MDR cells, their effect on accumulation and efflux of labeled vinblastine, their affinity for P-170 in whole cells and isolated membrane vesicles, and their effect on calcium transport in isolated rat aorta.

MATERIALS AND METHODS

Compounds and Reagents. MDR modulators A-30312 and A-39355 were synthesized according to the method of Spialter and Pappalardo (14). The structures of these compounds are shown in Fig. 1. Other chemicals used in this study were ADR, VBL, VCR, verapamil, MTT, and Nonidet P-40 (Sigma Chemical Co.); cyclosporine A (Sandoz); and [3H]AZD, [3H]VBL, [14C] molecular weight standards, and Amplify (Amersham).

Cell Lines and Cell Culture. P388 and P388/Adr cells were both obtained from the American Type Culture Collection and maintained on RPMI 1640 with 10% fetal bovine serum. These P388/Adr cells are known to overexpress the P-170 glycoprotein constitutively and do not require maintenance in ADR for expression of the MDR phenotype. Cells were routinely cultured in 25-cm


disks containing 8 ml of medium. To prepare cell membranes, 50 ml of growth medium from each dish was dispensed into a microfuge tube. Modulators were dissolved in 100 ml of dimethyl sulfoxide. In many cases, the solubility of the formazan was improved by the addition of acid isopropanol (250:1:5, isopropl alcohol:HCl:Triton X-100). This was later used as the method of choice over dimethyl sulfoxide. The absorbance of the dissolved formazan was read at 570 nm in a microtiter plate reader (Bio-Tek Instruments). IC50 was measured as the concentration required to inhibit cell growth by 50%.

Depletion of Cellular ATP. Cells were adjusted to 2 x 106/ml of medium without fetal bovine serum and incubated at room temperature for 1 h for recovery. Following recovery, the cells were centrifuged and resuspended in complete PBS. Cellular ATP was depleted by sequential treatment with rotenone (2 nmol/l, 37°C for 20 min) and 2-deoxyglucose (2 mm, 37°C for 20 min). Non-ATP-depleted cells used for comparative studies remained in glucose and glutamine (1 mM each for 40 min).

Drug Accumulation and Efflux. For each reaction condition, a 600-ul aliquot was dispensed into a microtube. Modulators were added at 6.6 


ul followed by the addition of 10 


ul [3H]VBL. At various times, 100-ul samples were removed, and the cells were separated by centrifugation through a cushion of silicone oil. The tubes were frozen quickly in ethanol/dry ice, and the tips containing the cell pellets were clipped off into scintillation vials containing 500 ul of 0.5 M NaOH (for cell lysis), neutralized, and counted with liquid scintillant. For drug efflux studies, cells were loaded with 10 ul [3H]VBL in the presence or absence of 10 ul verapamil for a 1-h incubation at 37°C. The cells were washed quickly in cold PBS (pH 7.2) and resuspended in prewarmed PBS with or without 20 ul modulators. At the indicated times, cells were harvested and counted as described above.

Preparation of Membrane Vesicles. Membrane vesicles from P388 and P388/Adr cells were prepared as described by Cornwell (6), except that the cells were lysed by nitrogen cavitation at 500 psi for 20 min.

Protein concentration of membrane preparations was determined using BioRad protein assay reagent with bovine serum albumin as the standard.

4735
Photoaffinity Labeling of P-Glycoprotein in Whole Cells and Membrane Vesicles. For whole cell studies, P388 and P388/Adr cells were adjusted to 5 x 10^6 in PBS and incubated with 1 µg/ml [3H]AZD for 20 min at room temperature in the presence or absence of varying concentrations of modulator. After exposure to UV (366 nm) light for 20 min, the cells were simultaneously washed and lysed in PBS containing 1% Nonidet P-40 and 1 mM EDTA. The lysate was centrifuged at 12,000 x g for 10 min, and the supernatant was analyzed with urea containing SDS-polyacrylamide gel. Membrane samples (25 µg of protein) were labeled with 0.5 µM [3H]AZD (40 Ci/mmol) in the presence or absence of various modulators for 20 min at room temperature (7). After continuous irradiation at 366 nm for 20 min, the samples were solubilized in an SDS sample buffer.

SDS-Polyacrylamide Gel Electrophoresis. Samples labeled with [3H]AZD were analyzed by electrophoresis on a 5.6% polyacrylamide gel containing 4.5 M urea without a stacking gel. The gels were dried, autoradiographed, and developed after 48–72 h. The molecular weight of the P-170 glycoprotein was estimated from 14C-labeled protein molecular weight standards. The developed autoradiographs were analyzed using a laser densitometer (LKB) to determine the intensity of binding of label to P-170 in each lane, and results were represented in absorbance units.

Isolation of Rat Aorta. Male Sprague-Dawley rats (300–350 g) were anesthetized i.p. with 50 mg/kg sodium pentobarbital and exsanguinated. The thoracic aorta was quickly removed and placed in a Krebs-Henseleit buffer of the following composition (mM): NaCl (18.0); KCl (4.7); KH2PO4 (1.2); MgSO4·7H2O (1.2); EDTA·Na2 (0.03); CaCl2 (2.5); D-glucose (11.1); and NaHCO3 (25.0); the aorta was gassed with 95%/5% O2/CO2 to maintain pH at 7.4. The vessels were cleared of extraneous tissue and segmented into 4–5-mm-wide rings which were suspended in 2-ml jacketed tissue baths maintained at 37°C. Each aortic ring was then attached via gold chain to an isometric force transducer linked with a physiograph for monitoring tension changes. Baseline tension was set at 2.0 g, and the tissues were allowed to equilibrate for 2 h. During this period, the tissues were washed every 15 min with fresh buffer, and the tension was twice readjusted to baseline.

Determination of Calcium Channel Activity. Aortic tissues were contracted by depolarization with 55 mM potassium. Cumulative concentration-response curves to verapamil (1 x 10^-9 to 3 x 10^-6 M), MDR modulators (1 x 10^-9 to 1 x 10^-4 M), or their vehicle were then performed. Upon completion of these concentration-response curves, maximal relaxation was induced by papaverine (10 µM). Data were compiled from individual experiments, and means ± SE were calculated.

RESULTS

Potentiation of Cell Cytotoxicity by MDR Modulators. Cells were first tested to determine their level of resistance to ADR, VBL, and VCR. Relative resistance for each drug was calculated as the ratio of the IC50s of the anticancer agent for P388/Adr and normal P388 cells. The two active compounds A-30312 and A-39355 (Fig. 1) were identified by the MTT reduction assay for cell cytotoxicity using P388/Adr cells. Initially several compounds were tested at a concentration of 10 µM and selected for further analysis if VBL potentiation was greater than 50% (data not shown). After determination of cytotoxicity (data not shown), modulators were tested for chemosensitization at concentrations below their IC50s. Verapamil and A-39355 were tested at 2–6.6 µM, while A-30312 was tested at 0.1–1 µM. P388/Adr cells were significantly resistant to all of the antitumor compounds tested with relative resistance ranging from 114-fold for ADR to 443-fold for VBL (Table 1) as compared with normal P388 cells. The addition of verapamil (2–6.6 µM) reduced VBL resistance in a dose-dependent manner from 40-fold to 2-fold, while VCR and ADR reduced sensitivity to the same level as in normal cells. A-30312, although tested at lower concentrations to avoid toxic effects, compared favorably with verapamil for sensitization to VBL but was not as effective for VCR or ADR. A-39355, on the other hand, enhanced the cytotoxicity of all three drugs tested at 6.6 µM and significantly reversed resistance to VBL and VCR at 3.3 µM. A-39355 was also much more effective than A-30312 or verapamil at 2 µM (Table 1).

Effect of Modulators on Drug Accumulation and Efflux. To determine how A-30312 and A-39355 might act as chemosensitizers, we tested their effect on the accumulation of [3H]VBL, a cytotoxic drug that is normally rapidly effluxed by MDR cells. As shown in Fig. 2, the attainable intracellular level of label in P388/Adr cells was less than 25% of that in normal P388 cells. Furthermore, the accumulation of [3H]VBL in P388/Adr cells was clearly dependent on the presence of ATP. This was not the case with nonresistant P388 cells. ATP-depleted P388/Adr cells accumulated as much label as normal P388 cells did, whereas non-ATP-depleted P388/Adr cells retain label in the presence of A-30312 and A-39355 (Fig. 3). In the presence of A-30312, maximal retention was close to 60% of levels retained by normal P388 cells, whereas in the presence of A-39355, resistant cells retained as much drug as normal cells. Based on this assay, A-39355 and verapamil appeared to be equally effective in causing drug retention.

To determine the effect of A-30312 and A-39355 on active drug efflux, P388/Adr cells that had been previously loaded with [3H]VBL in the presence of verapamil were washed and resuspended in complete PBS with or without modulators. In the absence of modulator, 80% of the label was lost within the...
Table 1: Potentiation of the cytotoxicity of anthracycline and Vinca alkaloids in P388/Adr cells by modulators of MDR

<table>
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<tr>
<th>Modulator (μM)</th>
<th>Cell type</th>
<th>VBL IC₅₀ (nM)</th>
<th>Relative resistance</th>
<th>VCR IC₅₀ (nM)</th>
<th>Relative resistance</th>
<th>ADR IC₅₀ (μM)</th>
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<td></td>
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<td>443</td>
<td>858.0</td>
<td>408</td>
<td>11.4</td>
<td>114</td>
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Fig. 2. Accumulation of [³H]vinblastine sulfate in P388 and P388/Adr cells. The intracellular level of [³H]vinblastine in P388 (○), ATP-depleted P388 (□), P388/Adr (●), and ATP-depleted P388/Adr (□) cells was determined as described in “Materials and Methods.”

Fig. 3. Retention of [³H]vinblastine sulfate by P388/Adr cells. The intracellular level of [³H]vinblastine was determined in the absence (●) and presence of various modulators, verapamil (○), A-30312 (△), and A-39355 (□), as described in “Materials and Methods.”

The intracellular level of [³H]vinblastine sulfate in P388 and P388/Adr cells. The intracellular level of [³H]vinblastine in P388 and P388/Adr cells was determined as described in “Materials and Methods.”

Effect of A-30312 and A-39355 on the Photoaffinity Labeling of P-Glycoprotein by [³H]AZD. The effect of modulators on the binding of [³H]AZD to P-glycoprotein was examined in whole cells and isolated membrane vesicles from P388/Adr cells. In whole cells, verapamil, A-39355, and cyclosporine A all showed complete inhibition of [³H]AZD binding at 100 μM (Fig. 5, Lanes 5, 11, and 14), whereas A-30312 at the same concentration inhibited binding by less than 25%. At 10 μM, A-39355 reduced binding by 75% (Fig. 5, compare Lanes 1 and 10), while cyclosporine A caused 50% inhibition (Fig. 5, Lane 13). Interestingly, verapamil and A-39355 caused a slight increase in [³H]AZD binding at 10 μM, as did all of the compounds tested at 2 μM (Fig. 5, Lanes 3, 6, 9, and 12). In cell membrane preparations, 100 μM A-39355 caused complete inhibition of binding, while A-30312 and verapamil had 20% and 6% residual [³H]AZD bound to P-170 (Fig. 6). At 2 μM, A-39355 was still effective, while A-30312 and verapamil showed no significant difference in AZD binding compared with membranes without modulator. The whole cell studies demonstrated A-39355 and cyclosporine A to be equally effective in competing with AZD binding and much more effective than verapamil or A-30312. Similarly, A-39355 inhibited [³H]-AZD labeling of P-170 from isolated cell membranes, while A-30312 and verapamil were not as effective.

Effect of MDR Compounds on Calcium Channel Activity. In cumulative concentration-response curves for the relaxation of potassium-depolarized and contracted rat aortic rings, verapamil effectively reversed the contraction with an EC₅₀ (—log M) of 7.22 ± 0.02 and an efficacy index of 0.98 ± 0.00 (100 μM papaverine = 1.00) (Fig. 7; Table 2). A-30312 and A-39355 were tested in cumulative concentration-response curves ranging from 1 x 10⁻⁹ to 1 x 10⁻⁴ M. MDR modulator vehicle curves were performed simultaneously, and severe toxic effects of the ethanol vehicle were evident at a volume-concentration...
equivalent of $3 \times 10^{-4}$ M. This concentration of MDR modulator was omitted from concentration-response curves so as not to introduce aspects of vehicle toxicity or interfere with maximal relaxation by papaverine. The effects of verapamil and of the MDR modulators and their vehicle on the reversal of potassium-induced contractions are presented in Fig. 7. From data normalized against the maximal relaxant effect of papaverine, verapamil was found to be most potent and efficacious in reversing the contraction induced by depolarization. Compared with verapamil, the MDR modulators were less effective in reversing the contraction, with an EC$_{50}$ ($-\log \text{M}$) for relaxation for A-30312 of 5.07 ± 0.08 for the concentration range tested; A-39355 did not sufficiently relax the constricted tissues to determine an accurate EC$_{50}$ value.

**DISCUSSION**

While a major obstacle to successful cancer chemotherapy, particularly in solid tumors, is the emergence of MDR, several lipophilic agents have been identified that can chemosensitize MDR cell lines in vitro. The structures of these agents are as diverse as those of the substrates for P-glycoprotein efflux (16). Also, many of the compounds capable of MDR reversal do so at...
concentrations too high to be effective in vivo without significant toxic side effects. Verapamil is among the best characterized modulators of MDR. In vitro reversal of MDR by verapamil in many cell lines expressing different levels of P-glycoprotein has been previously demonstrated (17). However, in vivo results have been disappointing because of associated severe cardiovascular toxicities including conduction blockade, myocardial dysfunction, and systemic hypotension associated even with subtherapeutic doses (13, 18, 19). Recent reports on the immunosuppressant cyclosporine and its analogues also demonstrate in vitro MDR reversal (20, 21). In either case, it would be advantageous to dissociate calcium transport and immunosuppression from MDR reversal. We sought to discover potent, nontoxic compounds that caused MDR reversal through specific interaction with the P-170 protein known to be expressed in resistant cell lines. Several compounds were selected based on physiochemical commonalities such as the presence of a basic nitrogen and two aromatic rings, lipid solubility, and cationic charge, which fit the chemical profile for an MDR reversal agent as described by Zamora et al. (22) and extended by Pearce et al. (23). A-30312 and A-39355 were identified initially by their ability to reverse VBL resistance. Further comparative studies demonstrated that A-39355, like verapamil, enhanced the cytotoxicity of all three anticancer drugs tested, while A-30312 only enhanced the cytotoxicity of VBL and had a less pronounced effect on the cytotoxicity of VCR or ADR. A structural comparison of A-39355 and other potent MDR reversal agents such as the phenothiazines reported by Ford et al. (16) suggests the following similarities. Both compounds contain a tricyclic ring system with a nitrogen in the center ring. The distance between the tertiary amino group and the phenothiazine nucleus is three carbons long; compounds with shorter chain lengths were significantly less potent. Finally, the phenothiazine with the best MDR ratio had a piperazine substitution on the three-carbon chain similar to A-39355. The reason for increased sensitization by A-30312 to VBL, compared with VCR or ADR, is not clear. Ikeda et al. (15) have reported a similar result with naptho-7-pyrones in KB cell lines. They suggest that Vinea alkaloids and anthracyclics may have overlapping P-170 binding sites with different affinities. However, we cannot rule out reduced accumulation of VCR and ADR.

Several studies have shown discrepancies between the cellular uptake and the concentration of anticancer drug required to kill cells (11, 17). Our studies on cellular pharmacokinetics of VBL show a significant increase in the intracellular retention of VBL by A-39355 and verapamil. Although these experiments were done with 200-fold more cells than used for the cytotoxicity assay and uptake was only measured over a 60-min period, we found the ATP-dependent increased uptake to correlate with increased reversal of MDR in the cytotoxicity assay. As reported by others (24), increased accumulation was related to inhibition of drug efflux. This further supports the hypothesis that resistance is associated with the efflux function of P-glycoprotein. However, many compounds that inhibit efflux and reverse MDR also affect other normal functions. Calcium antagonists affect calcium transport, cyclosporins are immunosuppressive, calmodulin inhibitors affect a number of cellular events adversely (25), and lysomotropic agents are nonspecific (26). Recently, a nonimmunosuppressive analogue of cyclosporine, SDZ PSC 833, was shown to be orders of magnitude more effective in vivo than known chemosensitizers that have entered clinical trials for MDR reversal (27). Compared to verapamil, the compounds reported here had a significantly less pronounced effect on the relaxation of isolated rat aorta at levels which completely reversed MDR in the P388 murine leukemia cell line. This compares favorably with the dihydropyridines with reduced calcium antagonist activity, reported by Yoshinari et al. (24), although neither has been tested in vivo.

Previous studies have shown that photoactive analogues of ATP, VBL, and the calcium channel blocker AZD were able to bind P-glycoprotein in isolated membrane vesicles (7). Cyclosporine A and nonimmunosuppressive cyclosporine H also showed binding to P-170 in intact cells (20). We have obtained similar results with A-39355, which appears to be at least as potent as cyclosporine A in competing for binding with azidopine in intact cells and significantly better than verapamil or A-30312. This, in addition to the positive effect on drug accumulation and efflux, may explain its superior chemosensitization. Although most MDR modulators affect AZD binding (28), the mechanism by which binding results in inhibited transport is not clear. Some compounds such as bleomycin, an antitumor drug not affected by MDR, can actually stimulate AZD binding (29). We have also seen a similar stimulation of AZD binding in intact cells at low concentrations of modulator (Fig. 7). This might be explained if AZD was itself a substrate for P-glycoprotein, albeit a poor one. Then at lower concentrations of modulator, more AZD would be retained by intact cells and thus bind to P-170 upon photoactivation. At higher concentrations, however, the modulator would effectively compete with AZD for binding to P-170. Similarly, Goldberg et al. (30) have shown that the level of cyclosporine in resistant cells is increased by verapamil. It should be noted that stimulation of AZD binding to P-170 was not seen in experiments using isolated membrane vesicles.

In conclusion, the MDR reversal agents reported here enhance the cytotoxicity of several anticancer compounds in vitro through enhanced accumulation of the cytotoxics due to inhibition of P-glycoprotein-mediated efflux. They do so by interacting specifically with P-170 and have a markedly less pronounced effect than verapamil on normal calcium transport.

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REFERENCES

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