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A Structure-Function Relationship Among Reserpine and Yohimbine Analogues in Their Ability to Increase Expression of *mdr1* and P-Glycoprotein in a Human Colon Carcinoma Cell Line

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SUMMARY

We previously showed that there is a structure-function relationship among reserpine and yohimbine analogues in their ability to inhibit the function of P-glycoprotein (P-gp) and reverse multidrug resistance (MDR). Because some P-gp inhibitors (e.g., verapamil and nifedipine) can increase *mdr1* and P-gp expression in human colon carcinoma cell lines, we used our reserpine/yohimbine analogues to determine whether there was a structural requirement for this induction. We found that 10 μM reserpine increased both *mdr1* and P-gp expression by 4–10-fold in 48 hr in a human colon carcinoma cell line that expresses moderate levels of *mdr1* (LS180-Ad50) but not in several other cell lines that expressed no *mdr1*. The reserpine/yohimbine analogues rescinnamine, trimethoxybenzoylyohimbine, and LY191401 (compound G), all of which contain the three structural elements used to describe the MDR pharmacophore, also increased both *mdr1* and P-gp expression significantly. Despite some exceptions, we found that there was a good association between the ability of these analogues to induce *mdr1* and P-gp expression and their ability to reverse vinblastine and doxorubicin resistance, revealing a structure-function relationship for this phenomenon. The increased P-gp expressed by these cells appeared to be functional, as determined by flow cytometric detection of rhodamine 123 retention. The increased expression was suppressed by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, an RNA synthesis inhibitor, whereas the protein synthesis inhibitor cycloheximide enhanced the expression several-fold, suggesting that induction of *mdr1* by these analogues is regulated at both the transcriptional and post-transcriptional levels. Given that the effect is seen only in cell lines that express some P-gp and is mediated only by active, P-gp-interacting compounds, we speculate that induction of *mdr1* and P-gp by these agents may be mediated through a feedback mechanism.

MDR is a well-documented experimental phenomenon (1, 2) that appears to have clinical importance (3). Although recent studies have revealed that MDR may have different mechanistic bases (4), the most widely studied form is that associated with overexpression of the *mdr1* gene and its product, P-gp. Over the past few years, effort has been focused on understanding the regulation of *mdr1* expression. The expression of *mdr1* in some cell lines was increased when treated with cytotoxic drugs (5), differentiating agents (6), heat shock, and arsenite (7). The transcription of reporter genes carrying the *mdr1* promoter was also reported to be activated by cytotoxic drugs (8), UV irradiation (9), heat shock (10), and transfection with oncogenes (11).

Many different compounds, such as verapamil, nifedipine, cyclosporin A, chloroquine, vindoline, and reserpine, have been shown to decrease P-gp-mediated drug efflux and thus increase the cytotoxicity of natural product anticancer drugs in tumor cells (12). Some of these modulators are undergoing clinical trial (13). Recently, Herzog *et al.* (14) reported that *mdr1*/P-gp expression was increased in two colon carcinoma cell lines (LS180-Ad50 and DLD-1) when treated with verapamil, nifedipine, diltiazem, or cyclosporin A but not with quinidine or chlorpromazine. Reserpine, the antihypertensive plant alkaloid, has been shown to be a potent modulator of anticancer drug cytotoxicity in MDR cells, probably by binding to P-gp and inhibiting its efflux function (15, 16).

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ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzinedazole; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide, TMBY, trimethoxybenzoylyohimbine; VLB, vinblastine; DOX, doxorubicin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Earlier work from our laboratories revealed that a series of reserpine and yohimbine analogues could reverse MDR and compete with a photoaffinity analogue of VLB for binding to P-gp in CEM/VLB_{100} cells in a structurally defined manner (16). Effective modulators had a pendent benzoyl function in a certain conformational arrangement. Accordingly, we used these and other reserpine and yohimbine analogues to determine whether they increase *mdr1* and P-gp expression and reverse MDR and, if so, whether there is a structure-function relationship in the ability of these agents to do so.

Materials and Methods

Cell lines. LS180-Ad50, a DOX-selected subline of LS180 colon carcinoma cells (14), was kindly provided by Dr. A. T. Fojo (Medicine Branch, National Cancer Institute, Bethesda, MD). The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine in a 37° incubator in 5% CO_2 atmosphere. The parental LS180 cell line was derived from a primary colon cancer resected from a patient who had never received chemotherapy (17). LS180-Ad50 expressed moderate levels of *mdr1* and P-gp. All experiments were carried out with exponentially growing cells.

Drugs. Most reserpine and yohimbine analogues (compounds A through G and I) were synthesized in the Lilly Research Laboratories (Indianapolis, IN) (Fig. 1). Reserpine, rescinnamine, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions (10 mM) of reserpine and analogues were prepared in DMSO and stored at −20°. Doxorubicin and VLB were dissolved in phosphate-buffered saline, aliquoted, and stored at −20° in the dark. Stock solutions of cycloheximide, actinomycin D, novobiocin, and DRB were all made in DMSO. The anti-P-gp monoclonal antibody C219 was obtained from Signet Laboratories (Dedham, MA). The secondary antibody, AP-rabbit antimouse IgG (H+L), was obtained from Zymed Laboratories (South San Francisco, CA).

Reserpine and yohimbine analogues. Fig. 1 provides the structures of the analogues. TMBY and compound B were prepared as previously reported (21). Compound A was prepared by selective hydrogenation of rescinnamine (5 atm H_2, 5% Pd/C, ethanol at 23° for 24 hr). Compound C was prepared by oxidation of yohimbine as previously reported (18). Compound G was prepared by treating yohimbine with sodium hydride and trimethoxybenzylbromide in *N,N*-dimethylformamide (23° for 3 hr). Compound F was prepared by treating yohimbine (compound C) with trimethoxyaniline and *N,N*-dimethylaminopyridine in toluene (reflux for 24 hr). Compound D was prepared as previously described (19). Compound E (calpurnine) was obtained from natural sources (20). All compounds exhibited physical chemical data (proton NMR, IR, mass spectrum, and elemental analysis) consistent with their assigned structures.

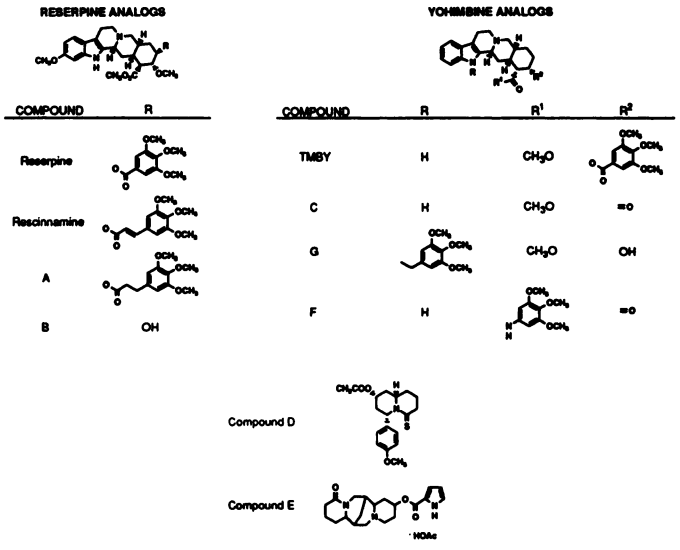

Fig. 1. Structures of reserpine/yohimbine analogues and two related compounds studied for their ability to increase *mdr1*/P-gp expression and reverse MDR in LS180-Ad50 cells.

RNA isolation and Northern blot analysis. Total cellular RNA was extracted by a modification of the acid guanidinium thiocyanate/phenol/chloroform extraction method (21). Equal amounts (30 μg) of RNA were electrophoresed on 1% agarose-formaldehyde gels, transferred to nitrocellulose membrane (Nitroplus, Micron Separations, Westboro, MA), and UV cross-linked in a Stratalinker (Stratagene, La Jolla, CA). The blots were hybridized (Quick-Hyb solution from Stratagene) at 65° to a 3′ human *mdr1* cDNA probe. The ^{32}P-labeled probe was prepared by random priming (Quick Prime Kit, Pharmacia, Piscataway, NJ) with a 3.04-kilobase *Eco*RI/*Pvu*I restriction fragment of *mdr1* cDNA as template and purified by passing through Nick Spin columns (Pharmacia). The plasmid ($PHDRV_1$) containing the *mdr1* cDNA insert was kindly provided by Dr. Igor B. Roninson (Department of Genetics, University of Illinois, Chicago, IL). The blots were washed, autoradiographed, and quantified by an image analyzer (Bioimage, Millipore Corporation, Bedford, MA). To correct for slight variations in the amount of RNA loaded, the blots were stripped and reblotted with either human G3PDH- or β-actin-specific cDNA probes (Clontech Laboratories, Palo Alto, CA).

Immunoblot analysis. Cells (5×10^6) were harvested by being scraped in phosphate-buffered saline. After centrifugation, the cell pellets were lysed by sonication in hypotonic buffer (10 mM Tris, pH 7.8, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$) containing 1 mM phenylmethylsulfonyl fluoride and 20 μg aprotinin/ml. Protein concentrations were determined by the Bio-Rad (Bio-Rad, Richmond, CA) assay with bovine serum albumin as the standard. Equal amounts of protein (100 μg) were loaded without boiling and separated on 8% SDS-PAGE gels (22), along with molecular weight standards (Bio-Rad). Proteins were transferred to nitrocellulose membranes (SCS, NC, Schleicher and Schuell, Keene, NH) by a semidry method using a Milli-Blot-SDS system (Millipore Corporation, Bedford, MA) according to the manufacturer's instructions. The membranes were preincubated for 2 hr at 37° with Blotto (23) (0.9% NaCl, 10 mM Tris·HCl, pH 7.5, 0.02% sodium azide, 5% dry milk, 3% IgG-free bovine serum albumin, and 0.2% Tween 20) followed by overnight incubation with C219 monoclonal antibody in Blotto (1:400 dilution) at 4°. After several washes with washing buffer (0.9% NaCl, 10 mM Tris·HCl, pH 7.5), the blot was incubated with the secondary antibody, AP-rabbit antimouse IgG, in Blotto (1:400 dilution) at room temperature for 90 min. The washings were repeated and the blots were developed according to the 2,2′-di-*p*-nitropheynl-5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4α-diphenylene]ditetrazolium chloride/-bromo-4-chloro-3-inodolyl phosphate system (NBT/BCIP).

Cytotoxicity and resistance-reversal studies. Cytotoxicity was determined by the MTT assay (24). Exponentially growing LS180-Ad50 cells were plated (~3000–4000/well in 200 μl of media) into 96-well microtiter plates (Costar, Cambridge, MA) in quadruplicate. After 24 hr of incubation at 37° in a 5% CO_2 atmosphere, the reserpine and yohimbine analogues (diluted in RPMI 1640 medium without fetal bovine serum) were added to final concentrations of 1 and 5 μM, and the plates were incubated at 37° for 2 hr. Serial dilutions of VLB or DOX were then added to the wells, and the incubation was continued for an additional 68 hr. The medium from each well was carefully aspirated, and 200 μl of a solution of MTT (2 mg/ml) in RPMI 1640 (without serum) was added to each well and incubated for an additional 4 hr. After the MTT solution was aspirated, the resulting formazan crystals were dissolved in DMSO (200 μl/well) by vigorous shaking for 15 min. The absorbance of the solution was read at 540 nm in a microplate reader (Bio-Tek Instruments, Winnoski, VT) and was proportional to the fraction of the living cells in each well (24). IC_{50} values in the presence or absence of the reserpine/yohimbine analogues were calculated as the mean ±

standard deviation of three separate experiments. Fold-reversal of resistance was determined by dividing mean IC_{50} with VLB or DOX alone with the mean IC_{50} in the presence of the modulator. Cytotoxicity of reserpine/yohimbine analogues was also determined by the MTT assay.

Rhodamine 123 accumulation and retention. Rhodamine 123 has been shown to be a good substrate for P-gp and a marker for P-gp function (25). LS180-Ad50 cells treated with 10 μM reserpine for 4 and 12 days and untreated control cells were rinsed with fresh RPMI 1640 media (calcium free), trypsinized, disaggregated by pipetting several times, and washed twice more with medium. The cells were resuspended in fresh calcium-free medium, rhodamine 123 (10 μg/ml final concentration) was added, and the cells were incubated at 37° for 30 min. The dye-containing medium was removed by centrifugation, the cells were washed twice and resuspended in fresh medium, and the dye was allowed to efflux for 30 min at 37°. The cells were then collected by centrifugation, and rhodamine 123 fluorescence was analyzed with a Becton-Dickinson Vantage flow cytometer, with an excitation wavelength of 488 nm and an emission wavelength of 530 ± 30 nm. Approximately 5000 cells were counted for each measurement. Mean fluorescence was used as a measure of fluorochrome concentration. Rhodamine 123 retention in untreated CEM, CEM/VLB_{10}, CEM/VLB_{55}, and CEM/VLB_{100} cells was examined in parallel experiments.

Calculated three-dimensional structures of selected analogues. Conformational analyses were carried out with the molecular modeling program MacroModel V4.5 (26) operating on a Silicon Graphics Iris workstation. These calculations produced minimum energy conformations of reserpine, compound G, and compound F. Energy minimizations were made with the MM2* molecular mechanics force field under the Polak-Ribiere conjugate gradient algorithm. Graphic displays and overlay calculations were developed with Chem3D Plus™ V3.0 operating on an Apple MacIntosh IIci computer.

Results

Effect of reserpine on *mdr1* induction. LS180-Ad50 cells express a low but detectable level of *mdr1* transcripts, as detected by Northern blotting. Because initial experiments showed a clear increase in *mdr1* mRNA levels 48 hr after reserpine treatment, we examined the effects of different concentrations of reserpine at this time point. It is seen in Fig. 2 that reserpine treatment (2–10 μM) produced an increase in *mdr1* mRNA expression in LS180-Ad50 cells by 4–10-fold; concentrations of >10 μM caused no greater stimulation. As seen in Fig. 3, cells treated with 10 μM reserpine showed a clear 2–3-fold increase in *mdr1* mRNA within 24 hr, which was further increased to 6–8-fold in 48 hr. The mRNA levels continued to increase, although at a slower rate, on longer incubation with reserpine (120 hr). Finally, the reversibility of *mdr1* induction by reserpine was examined by removing the drug from cells that had been treated for 48 hr. This resulted in a rapid decrease in *mdr1* mRNA in 3–4 days, reaching levels slightly higher than those of untreated cells (data not shown).

Effects of analogues of reserpine and yohimbine on *mdr1* induction. We then sought to determine whether there was a structural basis for the induction of *mdr1* mRNA by using different reserpine/yohimbine analogues in LS180-Ad50 cells. The concentration of the analogues was 10 μM, at which *mdr1* induction was maximal while growth inhibition was <30% (data not shown). The duration of treatment with the analogues was 48 hr because a significant large portion of the effect of the drug was observed at this time, as was seen with reserpine. Furthermore, treatment for a longer duration was avoided to exclude the possibility of drug selection. As shown in Fig. 4, compounds A through F had a modest effect in inducing *mdr1* mRNA (~1.5–2.5-fold), whereas reserpine, rescinnamine, TMBY, and compound G increased *mdr1* mRNA levels 4–8-fold in 48 hr. Reserpine was the most active compound in inducing *mdr1* mRNA. Verapamil (10 μM), which increased *mdr1* mRNA 4–6-fold in this cell line, is shown in the figure as a control.

Effect of reserpine on P-gp expression. Our next aim was to determine whether reserpine-induced *mdr1* mRNA results in increased P-gp levels in the LS180-Ad50 cells. It is seen in Fig. 5 that, as assessed by immunoblotting, there was

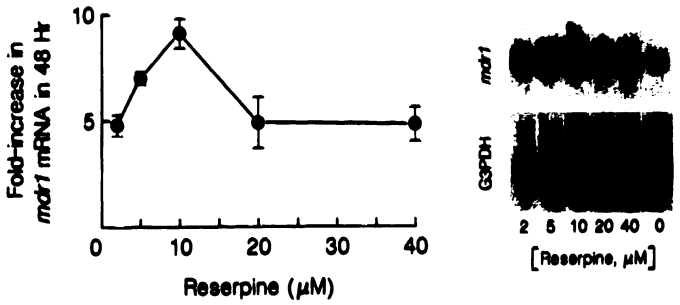

Fig. 2. Effect of reserpine on *mdr1* expression in LS180-Ad50 cells. Cells were treated with 2, 5, 10, 20, or 40 μM reserpine for 48 hr, after which total RNA was extracted and analyzed for *mdr1* mRNA expression by Northern as detailed in Materials and Methods. Each point represents mean ± standard error (4–10 experiments) fold-increase in *mdr1* mRNA in treated cells compared with its level in untreated control cells. *Right top*, representative Northern blot probed with *mdr1* cDNA probe. *Right bottom*, same blot reprobed with human G3PDH-specific cDNA, indicating that equal amounts of total RNA were loaded.

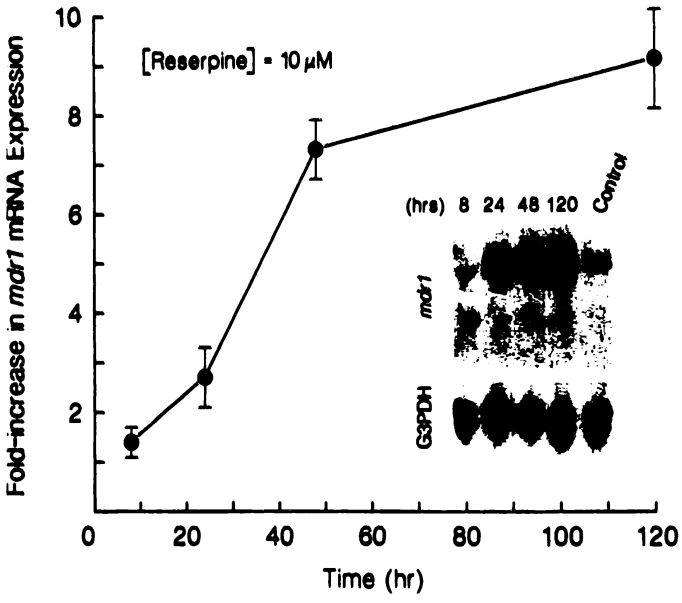

Fig. 3. Time course of *mdr1* induction by reserpine in LS180-Ad50 cells. Cells were incubated with 10 μM reserpine for 8, 24, 48, or 120 hr, after which total RNA was extracted and subjected to Northern blot analysis with *mdr1*-specific cDNA probe. Values represent the mean ± standard error (4–10 experiments) fold-increase in *mdr1* mRNA by reserpine treatment compared with untreated controls. *Inset top*, representative Northern blot probed successively with *mdr1*-specific cDNA probes. *Inset bottom*, representative Northern blot probed successively with human G3PDH-specific cDNA probes.

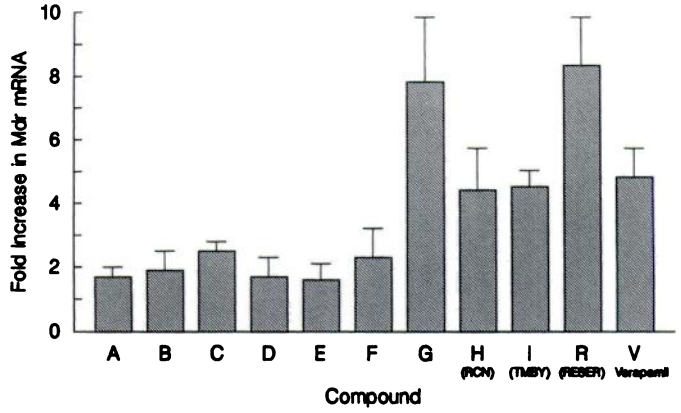

Fig. 4. Effect of reserpine/yohimbine analogues on *mdr1* expression in LS180-Ad50 cells. Cells were treated with various analogues at 10 μM for 48 hr, after which total RNA was extracted and subjected to Northern blot analysis to measure *mdr1* mRNA levels. *Bars*, mean ± standard error (4–14 experiments) fold-increase in *mdr1* mRNA when treated with the analogues compared with its level in untreated cells. Fold-increase in *mdr1* mRNA when the cells were treated with 10 μM verapamil for 48 hr is shown as a control.

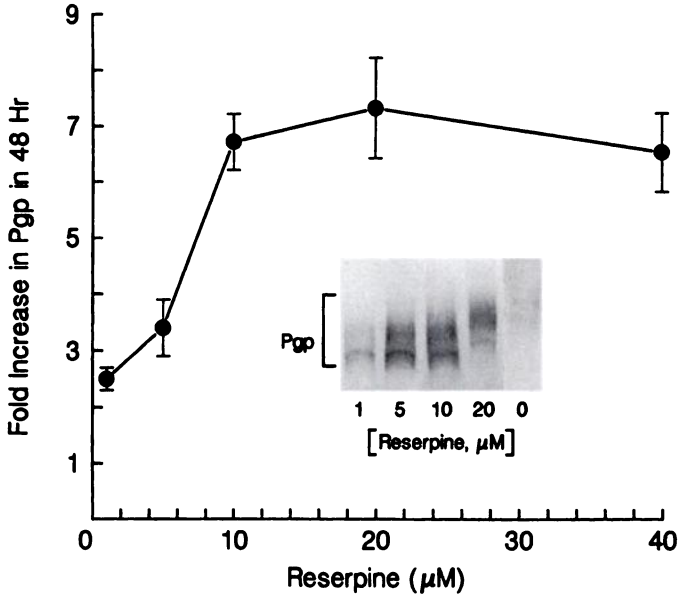

Fig. 5. Effect of reserpine on P-gp expression in LS180-Ad50 cells. Cells were incubated with 1, 5, 10, 20, or 40 μM reserpine for 48 hr, and total cell lysates were prepared. Lysates, each containing 100 μg of total protein, were separated on 8% SDS-PAGE and immunoblotted with monoclonal antibody C219 as described in Materials and Methods. Each point represents mean ± standard error fold-increase in P-gp level compared with that in untreated cells for four or more independent experiments. *Inset*, representative Western blot.

a progressive increase in P-gp expression at 1–10 μM reserpine; a maximum (7–8-fold) increase was seen when cells were treated with 10–20 μM reserpine for 48 hr. In contrast to the mRNA results shown in Fig. 2, it is seen in Fig. 5 that concentrations of reserpine of >10 μM still supported P-gp expression. Regarding the time course of P-gp induction, most of the increase was seen within 48 hr of reserpine treatment, but further increases were seen beyond this time (Fig. 6).

Effects of reserpine and yohimbine analogues on P-gp expression. The pattern of increase in P-gp levels when treated with different reserpine/yohimbine analogues

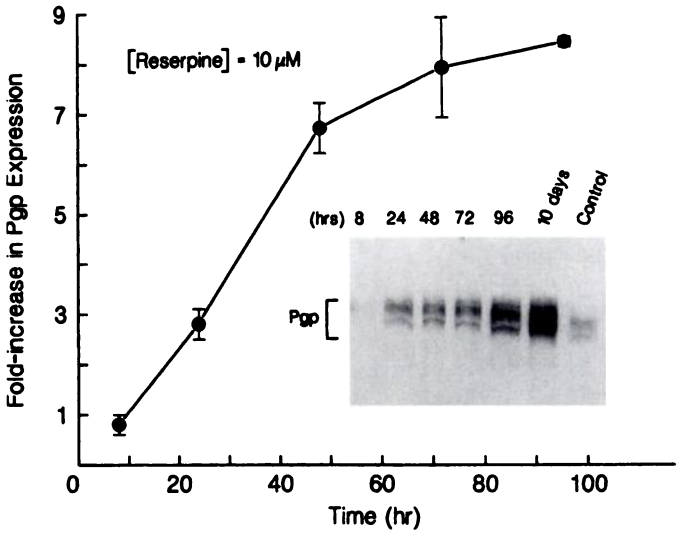

Fig. 6. Time course of P-gp induction by reserpine in LS180-Ad50 cells. Cells treated with 10 μM reserpine for various lengths of time (8 hr to 10 days) were lysed, separated on 8% SDS-PAGE, and immunoblotted with monoclonal antibody C219. The fold-increase in P-gp at each time point is shown as mean ± standard error of four or more separate experiments. One blot is displayed.

at 10 μM for 48 hr is similar to that observed with *mdr1* induction. As seen in Fig. 7, compounds A through F had little stimulatory effect, whereas compounds G, H, I, and R (reserpine) increased P-gp levels 3–7-fold.

Molecular modeling studies: comparison of reserpine with compounds F and G. It was interesting to note that reserpine and compound F, in which the pendent aromatic ring is attached to the E ring, and compound G, in which the pendent aromatic ring is attached to the B ring, produced different effects in LS180-Ad50 cells. A detailed conformational analysis of these compounds revealed structural differences in the deposition of the pendent aromatic ring relative to the basic nitrogen atom and indole ring system. In reserpine, we previously reported a twist-boat conformation in the E ring with an equatorial configuration of the 18-trimethyoxybenzoyl group (16). In compound F, the

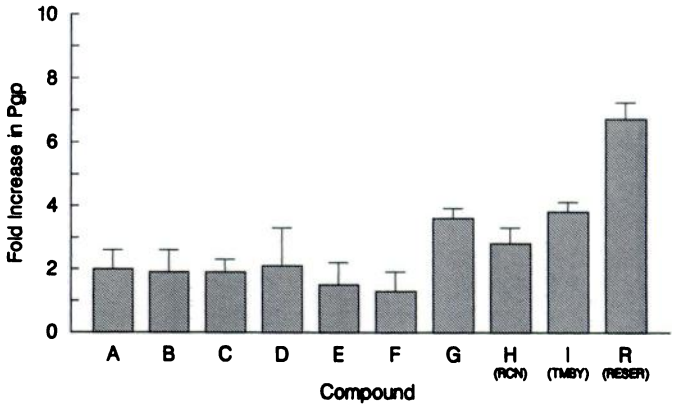

Fig. 7. Effect of reserpine/yohimbine analogues on P-gp expression in LS180-Ad50 cells. After incubation of the cells with different analogues (all at 10 μM) for 48 hr, lysates were prepared, separated on 8% SDS-PAGE, and analyzed by immunoblotting with C219 antibody. *Bars*, fold-increase in P-gp in treated cells compared with level in untreated controls and given as mean ± standard error for four or more experiments.

17-carbonyl slightly flattens the chair conformation of the E ring, placing the 16-trimethoxyanilide in an equatorial configuration (data not shown). The preferred *trans*-amide geometry (>10 kcal/mol) further defines the configuration. In contrast to reserpine and compound F, compound G has a trimethoxybenzyl group attached at the indole nitrogen of the B ring. The conformation is such that the benzyl group is β relative to the yohimbine nucleus. The preference of this conformation may be due to the α configuration of the 3-hydrogen because the conformation that places the benzyl group α, near the 3-hydrogen, is disfavored by ~5 kcal/mol. Our molecular modeling studies confirm what can be discerned from examining the two-dimensional structures in Fig. 1: there is little apparent structural overlap among compound G and reserpine or compound F to account for some of the similarities of their actions.

Reversal of VLB and DOX resistance by reserpine and yohimbine analogues. We used the MTT assay to examine the ability of these compounds to reverse VLB and DOX resistance in LS180-Ad50 cells. In these experiments, analogues were used at 5 μM for 3 days, which are optimal concentration and time for enhancing DOX and VLB cytotoxicity, whereas analogue cytotoxicity was <10%. As shown in Table 1, reserpine and yohimbine analogues have a range in their ability to reverse VLB or DOX resistance in LS180-Ad50 cells. For example, compound C was inactive, whereas the VLB and DOX resistances were decreased by reserpine 8- and 3-fold, respectively. Of interest, the ability of these analogues to reverse VLB resistance was greater than their ability to reverse DOX resistance and thereby enhance its cytotoxicity.

Correlation of fold-increase in *mdr1* and P-gp expression with fold-reversal of VLB and DOX resistance. Fig. 8 shows the relationship of fold-increase (mean values) in *mdr1* mRNA in LS180-Ad50 cells when treated with reserpine/yohimbine analogues for 48 hr versus fold-reversal of VLB (left panel) and DOX (right panel) resistance. There is a good correlation between the ability to increase *mdr1* expression and their ability to reverse both VLB (r = 0.67) and DOX (r = 0.76) resistance. Similarly, as shown in Fig. 9, the ability of reserpine/yohimbine analogues to increase P-gp expression correlates even better with their ability to reverse both VLB or DOX resistance (r = 0.91 and 0.79, respectively).

TABLE 1

Reversal of VBL and DOX resistance in LS180-AD50 cells by reserpine and yohimbine analogues[a]

Reserpine/yohimbine analogue	VBL		DOX	
	IC_{50}	Fold-decrease	IC_{50}	Fold-decrease
	nM		nM	
None	600 ± 75	1.0	1050 ± 50	1.0
A	260 ± 70	2.3	660 ± 60	1.6
B	460 ± 50	1.3	700 ± 75	1.5
C	450 ± 75	0.8	960 ± 90	1.1
D	250 ± 65	2.4	875 ± 30	1.2
E	500 ± 55	1.2	1170 ± 150	0.9
F	285 ± 35	2.1	500 ± 45	2.1
G (LY191401)	285 ± 50	2.1	525 ± 90	2.0
H (rescinnamine)	360 ± 80	1.7	650 ± 30	1.6
I (TMBY)	160 ± 35	3.8	590 ± 55	1.8
R (reserpine)	75 ± 15	8.0	360 ± 40	2.9

[a] Cytotoxicity of VBL and DOX in LS180-Ad50 cells was determined by the MTT assay (24) in both the presence and absence of 5 μM of the reserpine/yohimbine analogues, as described in Materials and Methods. The IC_{50} values for VBL and DOX were calculated in the presence or absence of each analogue in three separate experiments. Fold-increase in cytotoxicity was calculated by dividing the mean IC_{50} with VBL/DOX alone with mean IC_{50} in the presence of the analogue.

Reserpine-induced P-gp is functional. The results in Table 1 and Fig. 9 suggested that the P-gp induced by these agents is functional, based on the reduced IC_{50} values of VLB and DOX. We also assessed the accumulation and retention of rhodamine 123 by the LS180-Ad50 cells at different times after reserpine treatment. Rhodamine 123 has been shown to be a substrate for P-gp, and its retention, as measured by flow cytometric detection of fluorescence, is taken as an indication of P-gp activity, with cells with lower fluorescence (i.e., rhodamine "dark" cells) having more P-gp (25). We controlled for our experiments with our well-characterized VLB-

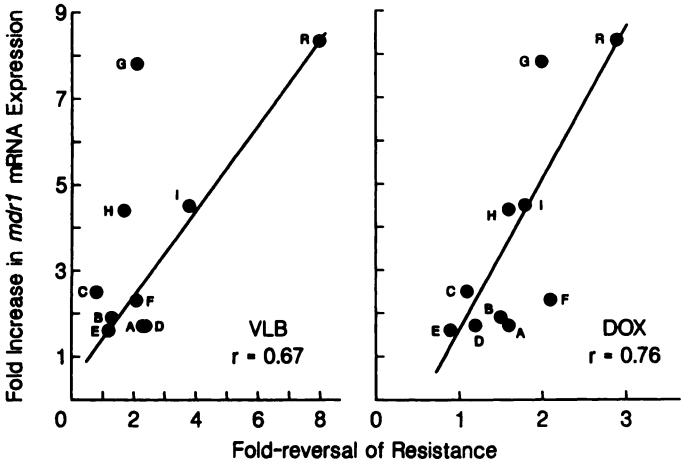

Fig. 8. Relationship of induction of *mdr1* mRNA and increased cytotoxicity of VLB or DOX in LS180-Ad50 cells by reserpine/yohimbine analogues. *Left*, mean fold-increase in *mdr1* mRNA (from Fig. 3) versus mean fold-increase in VLB cytotoxicity by different analogues at 5 μM (from Table 1). *Right*, a similar scatterplot correlates mean fold increase in *mdr1* mRNA (from Fig. 3) with fold-increase in DOX cytotoxicity (from Table 1) by different analogues.

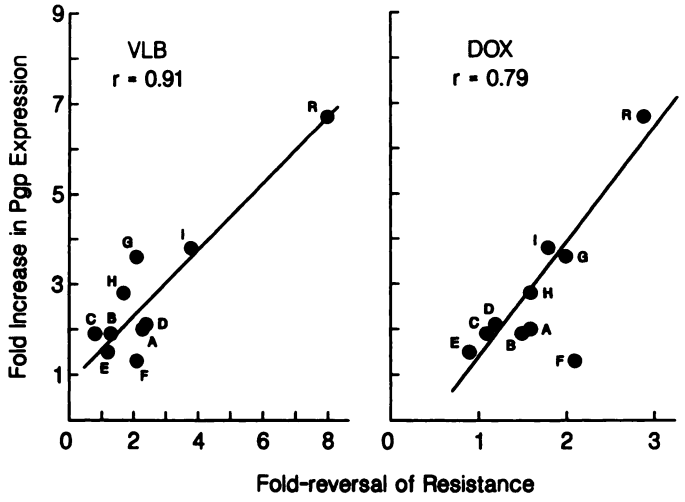

Fig. 9. Relationship of induction of expression of P-gp and increased cytotoxicity of VLB or DOX in LS180-Ad50 cells by reserpine/yohimbine analogues. *Scatterplots*, relationship between fold-increase in P-gp induced by reserpine/yohimbine analogues (see Fig. 7) and their ability to increase VLB (*left*) or DOX (*right*) cytotoxicity (from Table 1).

resistant CEM cells of increasing P-gp expression (27) (data not shown). Results of a typical experiment are shown in Fig. 10. On the left side of the figure, it is seen that there is little difference in rhodamine 123 retention between 4-day reserpine-treated and control cells (mean fluorescence, 1989 and 1860, respectively). However, after 12 days of treatment with reserpine, a rhodamine "dull" population is seen (Fig. 10, *bottom right*) (mean fluorescence, 965 versus 2166 for controls, respectively). Based on rhodamine 123 retention by the CEM/VLB cells, this population is probably somewhat less resistant than the CEM/VLB_{10} cells. These results, along with those in Fig. 9, indicate that the increased P-gp produced by reserpine is functional, at least in a subpopulation of cells.

Effects of RNA and protein synthesis inhibitors on reserpine-induced *mdr1* expression. Attempts to assess *mdr1* stability by inhibition of RNA synthesis with novobiocin (200 μM) were unsuccessful because the compound increased *mdr1* expression in LS180-Ad50 cells by ~2-fold in 48 hr (data not shown). Although actinomycin D at 5 μg/ml (it is cytotoxic above this concentration) was ineffective in suppressing *mdr1* mRNA synthesis, DRB, another RNA synthesis inhibitor (28), at 50 μg/ml almost completely inhibited the enhanced *mdr1* induction by reserpine in 24 hr, while having little effect on β-actin mRNA levels. These results suggest that the reserpine-increased expression of *mdr1* may be mediated in part at the transcriptional level.

We also sought to determine whether protein synthesis was involved in the reserpine-mediated induction or turnover of *mdr1* mRNA in LS180-Ad50 cells. Cycloheximide (10–40 μM) was added to cells after 48 hr of incubation with reserpine (10 μM). The effects on *mdr1* induction were similar in this concentration range, whereas higher concentrations of cycloheximide had a considerable cytotoxic effect on the cells. As shown in Table 2, treatment with cycloheximide alone increased *mdr1* expression in LS180-Ad50 cells by ~6-fold, but combined treatment with 10 μM cycloheximide and 10 μM reserpine increased the enhanced *mdr1* induction by reserpine to ~26-fold, which is more than the additive effect of the two compounds. These results indicate that inhibition of protein synthesis has marked effects on the reserpine-induced *mdr1* induction, and suggest that post-transcriptional events are also involved in regulating *mdr1* expression.

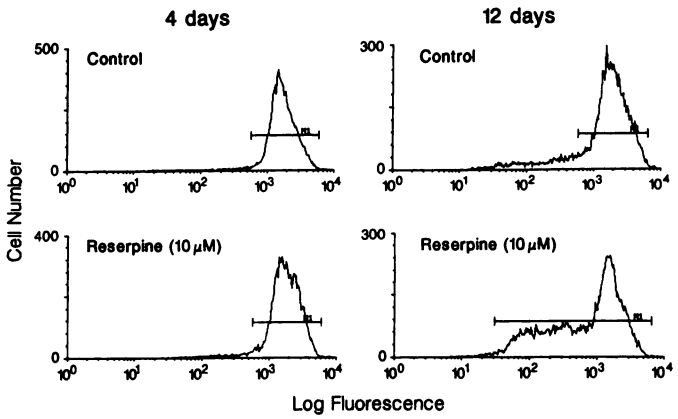

Fig. 10. Rhodamine 123 accumulation and retention in reserpine-treated and untreated control LS180-Ad50 cells. Cells treated with 10 μM reserpine for 4 days (*bottom left*) or 12 days (*bottom right*) and the corresponding untreated control cells (*top left and right*) were incubated with rhodamine 123 for 30 min and allowed to efflux for 30 min (both at 37°), and the fluorescence retained by the cells was analyzed by flow cytometry as detailed in Materials and Methods.

TABLE 2

***mdr1* mRNA in LS180-Ad50 cells treated with reserpine with and without cycloheximide[a]**

Treatment	Fold-increase in *mdr1* mRNA			
	0	6	12	24
	hr after cycloheximide addition			
Reserpine (10 μM)	7.3	6.6	8.2	8.8
Cycloheximide (10 μM)	1.0	1.4	1.9	5.8
Reserpine + cycloheximide	···	9.1	17.1	26.7

[a] Cycloheximide was added after incubation of the cells with reserpine for 48 hr. Total RNAs were extracted at indicated time points (after cycloheximide addition) and analyzed by Northern blotting.

Discussion

With a series of reserpine and yohimbine analogues, we have shown for the first time an apparent structure-function relationship in the ability of some MDR modulators to induce *mdr1* and P-gp expression. We observed this effect only in the colon carcinoma cell line, LS180-Ad50, but not in other human and rodent cell lines, including CEM, CEM/VLB_{100}, HL-60, H-446, H-460, H-661, EHR-2, EHR-2/DNR, and EHR-2/Didox, suggesting that the phenomenon may be cell line specific. In previous work, we showed that reserpine is a potent modulator of MDR (15, 16), and several reserpine/yohimbine analogues revealed a structural basis for this reversal of MDR in CEM/VLB_{100} cells (16). We also found that both *mdr1* and P-gp induction by reserpine is dose and time dependent. Furthermore, although expressed at low levels, probably because of the heterogeneity of the population, it appears that the reserpine-induced P-gp expressed by these cells is functional, as assessed by rhodamine 123 retention and resistance of the cells to VLB and DOX.

In studying the structural requirements for this effect, we found, despite some exceptions, a good relationship between the ability of these reserpine and yohimbine analogues to induce *mdr1* and P-gp and their ability to reverse MDR, although the latter effect was modest. Moreover, reserpine, rescinnamine, and TMBY, which increased *mdr1* and P-gp expression 4–10-fold in LS180-Ad50 cells, have been used previously to describe the elements of the MDR pharmacophore: a basic nitrogen and two planar aromatic rings in a defined spatial orientation (16). Compound G is also very active in increasing both *mdr1* and P-gp but, interestingly, was much less potent than other agents in reversing VLB and DOX resistance. Examination of the structure of compound G reveals that the pendent aromatic ring is in a different configuration than that of reserpine or TMBY, being attached to the indole nitrogen as opposed to the E ring of the nucleus. Molecular modeling studies also revealed these differences to be profound (data not shown). These results suggest a possible divergence in the structure-function relationship between compounds that induce *mdr1* and P-gp and compounds that reverse MDR. In contrast to these compounds, reserpine/yohimbine structures that lacked the pendent aromatic ring, such as compounds B and C, were poor at both inducing *mdr1* and P-gp and reversing MDR. The lack of activity of compounds A and F may be due to an unfavorable

conformation of their pendent aromatic rings. Unrelated structures, compounds D and E, also showed little activity. The predictions about the structures of compounds G and F were confirmed by molecular modeling and suggest that certain parts of the reserpine/yohimbine molecule (e.g., the core structure containing the indole ring) are necessary to mediate this effect. The overall conclusion, however, is that a series of yohimbine/reserpine analogues share certain structural features that appear to be important not only for induction of *mdr1* and P-gp but also for reversal of MDR.

The decreased ability of all analogues to reverse VLB and DOX resistance in LS180-Ad50 cells compared with their ability to do the same in CEM/VLB$_{100}$ cells (16) may be due to their unique ability to increase *mdr1* and P-gp expression in the colon carcinoma line, whereas no increase was seen in the CEM/VLB$_{100}$ cells (data not shown). Thus, the effect of these agents in reversing MDR may be self-limited by their ability to induce *mdr1* and P-gp in LS180-Ad50 cells.

The increased expression of *mdr1* and P-gp in LS180-Ad50 cells by reserpine and analogues is not likely to be due to selection of cells with higher levels of gene expression, a conclusion also reached by Herzog *et al.* (14). At the concentrations used (10 μM), the cytotoxicity of the analogues was <30%. Furthermore, the increased expression of the gene is seen even at 1 μM reserpine, and the effect is seen within 24 hr after the addition of drug, which is too soon to select a new population of cells. Moreover, the increased expression of *mdr1* is attenuated rapidly after removal of the drug (data not shown).

Our experiments with DRB and cycloheximide suggest that the enhanced expression of *mdr1* is regulated at both the transcriptional and post-transcriptional levels, and studies designed to clarify the mechanistic basis for this increased *mdr1* expression are under way. Herzog *et al.* (14) suggested that verapamil and nifedipine increased *mdr1* induction in LS180-Ad50 post-transcriptionally, but Muller *et al.* reported recently that verapamil down-regulated *mdr1* gene transcription in CEM/VLB$_{100}$ cells (29) and suggested that this may be due to aberrant gene regulation in this *mdr1* overexpressing cell line. Kohno *et al.* have shown that treatment with cytotoxic drugs (8, 30) activated a *mdr1* promoter-chloramphenicol acetyltransferase plasmid transfected into human cells. Choudhary and Roninson (5) and Hu *et al.* (31) reported that cytotoxic drugs induced *mdr1* expression in several human cell lines. Heat shock, arsenite, and cadmium chloride were also reported to increase *mdr1* mRNA levels in human cells (7). Studies of *mdr1* mRNA stability, turnover, and synthesis in our system are presently ongoing.

The effect of cycloheximide on *mdr1* expression may be mediated either transcriptionally or post-transcriptionally (32). The large, more-than-additive increase in *mdr1* mRNA by reserpine when treated with cycloheximide (Table 2) could be due to the inhibition of synthesis of proteins involved in the degradation of this mRNA, although evidence has been presented to suggest that inhibitors of protein synthesis may activate transcription by forming complexes with proteins that participate in signal transduction (33). However, if there is a transcriptional component to the activation of *mdr1* by reserpine, the effect of cycloheximide may be mediated through a transacting transcriptional repressor, as seen in hepatocytes exposed to cytotoxic drugs (32).

Recent studies suggest that the state of phosphorylation of P-gp is dependent on the individual agent used to modulate it (34). The MDR modulators verapamil and trifluoperazine have been shown to increase P-gp phosphorylation (35). It will be of interest to investigate whether different reserpine analogues modulate phosphorylation of P-gp and to determine whether there is a structural correlation between the ability to induce or modulate MDR and the ability to phosphorylate P-gp.

So, although there is no clear understanding of the mechanism by which reserpine and analogues increase *mdr1* and P-gp expression in the LS180-Ad50 cell line, it is unlikely that these agents bind directly to DNA and activate the *mdr1* promotor. Also unlikely is the possibility that these agents bind to or interact with *cis*- or *trans*-acting elements that interact with the *mdr1* promotor, although we have not performed gel-shift assays to exclude this possibility. Because we observed correlations between reversal of drug resistance and induction of *mdr1* and P-gp and because that we observed this only in cells that express some level of *mdr1* and P-gp, it is likely that the effect is mediated in some manner through the interaction of these agents with P-gp; we know from earlier studies that the active compounds bind to P-gp and inhibit it (15, 16). Accordingly, we offer the speculation that this inhibited P-gp could be "sensed" by the cell as a reduced P-gp level, and this might trigger the cell to produce more of the protein through a feedback mechanism. A sensory feedback might be operative if P-gp mediates other functions, such as ion transport (36) or ATPase activity (37). Inhibition of P-gp by reserpine and other analogues might interfere with these functions and induce a signaling pathway that might involve the *ras* cascade (11), NF-IL6 (48), or even p53 (11, 39), any or all of which could be part of a feedback mechanism. Although Herzog *et al.* (14) discounted a feedback mechanism, we suggest that it is a viable hypothesis, for the reasons stated. The lack of effect of quinidine and chlorpromazine in the study of Herzog *et al.* may reflect an inability or reduced ability of these agents to interact with P-gp and reverse MDR in the LS180-Ad50 cells. Furthermore, although some cytotoxic drugs that are not P-gp substrates have been reported to enhance *mdr1* and P-gp induction (5), it is possible that their action involves different mechanisms.

In conclusion, we have shown that there is a structure-activity basis behind the actions of some reserpine and yohimbine analogues to induce *mdr1* and P-gp in a human colon carcinoma cell line. Current efforts involve attempts to understand the mechanistic basis behind this phenomenon. That expression of P-gp can be induced by agents that inhibit its function has implications for MDR modulator studies and suggests that other strategies to circumvent MDR merit consideration (40).

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