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Pharmacological Characterization of *N*-Substituted Phenoxazines Directed toward Reversing *Vinca* Alkaloid Resistance in Multidrug-Resistant Cancer Cells

JULIE K. HORTON,[1] KUNTEBOMMANAHALLI N. THIMMAIAH, FRANKLIN C. HARWOOD, JOHN F. KUTTESCH, and PETER J. HOUGHTON

Departments of Molecular Pharmacology (J.K.H., F.C.H., P.J.H.) and Hematology/Oncology (J.F.K.), St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Department of Graduate Studies in Chemistry, University of Mysore, Mysore, India (K.N.T.)

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SUMMARY

Previously we reported the synthesis and partial characterization of 21 N^{10}-substituted phenoxazines in reversing *Vinca* alkaloid resistance. Here we report on a subset of these compounds; we have compared their activities in increasing *Vinca* alkaloid accumulation and reversing drug resistance in KB-Ch^{R}8–5 and GC_3/c1 (human colon carcinoma) cell lines. Results demonstrated that 1) *N*-substituted phenoxazines increase accumulation of vinblastine; 2) within this series, there is little correlation or ranking of activity between the two cell lines when *Vinca* alkaloid accumulation is compared at equal concentrations of modulator; 3) *N*-substituted phenoxazines demonstrate both quantitative and qualitative differences, compared with verapamil, a standard modulator; and 4) the series includes at least two compounds, 10-[3′-[*N*-bis(hydroxyethyl)amino]propyl]phenoxazine and 10-(*N*-piperidinoacetyl)phenoxazine, which increase *Vinca* alkaloid accumulation but do not significantly inhibit efflux. Additionally, certain of these multidrug resistance modulators significantly enhance accumulation (8–50-fold) of *Vinca* alkaloids in cell lines with very low or undetectable P-glycoprotein levels, where verapamil has little activity. It is concluded that at least part of the activity of some of these *N*-substituted phenoxazine modulators may be mediated through a P-glycoprotein-independent mechanism.

Modulation of MDR mediated by overexpression of *mdr1*, which encodes P-glycoprotein, has become an area of intense research interest (reviewed in Refs. 1–4). Skovsgaard (5) initially made the observation that a noncytotoxic analogue, *N*-acetyldaunorubicin, competitively inhibited energy-dependent efflux of daunorubicin from MDR but not sensitive parental Ehrlich carcinoma cells. However, it was only after the report (6) that the slow calcium channel blocker verapamil reversed resistance that extensive searches for MDR-reversing agents were initiated. Over the last decade several hundred compounds capable of reversing *in vitro* P-glycoprotein-mediated resistance have been reported. These include quite diverse chemical and pharmacological groups, including calcium channel antagonists, calmodulin antagonists, steroids and hormonal analogues, noncytotoxic drug analogues, cyclosporins and other peptides (reviewed in Ref. 1), amino acids, and many other hydrophobic basic compounds (7).

However, despite extensive studies little information is available on structure-activity relationships for MDR modulators. Kessel and Wilberding (8) examined 14 structural analogues of verapamil and showed that tiapamil, which contains a dithiane tetroxide substituent, was 50-fold less potent than verapamil in causing partial sensitization of the 100-fold resistant P388/ADR murine leukemic cell line. Nogai *et al.* (9) examined the modulating activity of a series of dihydropyridine modulators and, although several potent modulators were identified, no structural correlates were apparent to guide further analogue development. Similarly, only vague structural requirements have been related to function for phenothiazine-type MDR modulators. Ford *et al.* (10) examined a series of phenothiazines and found that hydrophobicity of the tricyclic ring structure and the structural features of the N^{10} substituent were independently important for reversing resistance. Phenothiazines with at least four methylene groups between the N^{10}-position and the substituent amine possessed greatest activity, and activity was enhanced by substitution at the C_2 position. However, agents such as prochlorperazine, trifluperazine, and fluphenazine, which fulfilled these criteria, were relatively poor reversing agents. Based upon these data, Ford *et al.* (11) identified the thioxanthine class of antipsychotic agents as more effective modulators. This class differs from phenothiazines in

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[1] Present address: Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX 77555.

ABBREVIATIONS: MDR, multidrug resistant or resistance; VLB, vinblastine; VCR, vincristine; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; MoAB, monoclonal antibody; HEPES, 4-(2-hydroxyethyl)-1-piperazine-N′-(2-ethanesulfonic acid).

having carbon at the N^{10}-position of the tricyclic ring structure and an exocyclic double bond, conferring stereoisomerism to these agents. Of note was the finding that the *trans*-isomer of each agent in the series showed greater activity than the *cis*-isomer in reversing resistance in MCF7/Adr^r cells. The most effective agent, *trans*-fluphenthixol, was approximately 15-fold more effective than the *cis*-isomer at modulating doxorubicin resistance in the cell line examined. However, it is of note that no such differential effect was seen with NIH/3T3 fibroblasts transfected with the *mdr1* gene (11). Overall, even quite sophisticated modeling used to identify pharmacophores appears to have limited value in the design of new modulators (12), and two parameters, hydrophobicity and molar refractivity (and therefore surface area), appear to be directly related to the activity of compounds as modulators of MDR (13–16).

In part, this confusing picture of what contributes to an effective modulator may be due to an assumption that all modulators work through a common mechanism, i.e., they directly inhibit the function of P-glycoprotein by competitive or noncompetitive mechanisms. For example, it is unusual for detailed analysis of mechanisms of modulation to be reported for a series of modulators. Rather, it is assumed that within a series there are quantitative and not qualitative differences that account for different modulator activities. However, there are now several reports to indicate that modulation of MDR may occur without a corresponding increase in drug accumulation (17–20) and that several classes of compounds shown to modulate MDR, including propranolol, phenothiazines (chlorpromazine, promethazine, and trifluoperazine), calcium antagonists, and cyclosporin, have multiple effects on membrane structure (21–28). Also, the failure to reverse MDR *in vitro*, often the end-point for assessing a modulator, may be associated with degradation of the modulator under culture conditions. Stability of modulators is rarely determined (29). The predominant focus upon P-glycoprotein as the sole target for modulation, rather than consideration of the action of modulators within the context of the metabolic characteristics of tumor cells upon which P-glycoprotein-mediated resistance is superimposed, predicts that modulators should exert qualitatively uniform activities. However, data reported suggest both quantitative and qualitative differences in the action of modulators, depending upon the drug and cell line used (10). Thus, the results discussed above (11), in which MCF7/Adr^r cells demonstrated differential modulation by isomers of fluphenthixol, whereas NIH/3T3 cells did not, could indicate that cell type may significantly influence the activity of putative modulators.

To more fully understand the mechanisms for modulating MDR, we have synthesized a series of phenoxazine derivatives (30), based upon our initial observation that phenoxazine, where the 5-position sulfur of phenothiazine is replaced by an oxygen atom (see Table 1), was a more effective modulator of *Vinca* alkaloid accumulation than was phenothiazine. The studies presented here suggest that within the series there are compounds that inhibit efflux (verapamil-like activity), whereas others markedly increase *Vinca* alkaloid accumulation without having significant detectable inhibitory activity on the efflux component. Several of the phenoxazine modulators characterized enhance *Vinca* alkaloid accumulation in cell lines where verapamil has no significant activity. Data support the concept that influx of *Vinca* alkaloids may be enhanced in a P-glycoprotein-independent manner by several *N*-substituted phenoxazines.

Materials and Methods

Cell lines and cell cultures. A cloned line of human colon adenocarcinoma, GC_3/c1 (31), that is intrinsically resistant to VCR (≈4-fold, relative to KB3–1) was routinely grown at 37° in antibiotic-free RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), in a humidified atmosphere of 5% CO_2/95% air. Human epidermoid carcinoma KB3–1 cells and a colchicine-selected MDR variant, KB8–5, were obtained from Dr. M. Gottesman (National Cancer Institute) (32). KB8–5 cells were cross-resistant to VCR and VLB and were grown in monolayer culture at 37° in antibiotic-free Dulbecco's modified Eagle's medium with 10% fetal bovine serum and L-glutamine, in a humidified atmosphere of 10% CO_2/90% air. The resistance of the KB8–5 cells was maintained by culturing them with colchicine (10 ng/ml). Cell lines derived from childhood rhabdomyosarcoma (Rh18, Rh28, and Rh30) have been described previously (33–35) and were maintained in culture as described for GC_3/c1 cells.

Accumulation studies. Two milliliters of cell suspensions (2×10^6 cells) were plated in 35- × 10-mm "easy grip" culture dishes (Becton Dickinson Co., Lincoln Park, NJ). Cells were allowed to attach to plastic overnight at 37°. Medium was aspirated and cells were washed with 2 × 2 ml of physiological Tris buffer (20 mM Tris containing 120 mM NaCl, 3 mM K_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose, pH 7.4). Monolayers were incubated at room temperature in physiological Tris buffer for 10 min before aspiration of buffer and addition of 1 ml of serum-free RPMI 1640-HEPES buffer (10.4 g of RPMI 1640 medium in 1 liter of 25 mM HEPES, pH 7.4) containing [^{3}H]VLB (specific activity, 10.1 Ci/mmol), with or without phenoxazine modulators or verapamil dissolved in H_2O or DMSO (final culture concentration, <0.1% DMSO). After the appropriate period of incubation at room temperature, medium was rapidly aspirated to terminate drug accumulation and was replaced with medium with or without modulator for efflux studies. To determine cell-associated radiolabel, monolayers were washed four times with ice-cold PBS (in g/liter: NaCl, 8.0; $Na_2HPO_4 \cdot 12H_20$, 2.9; KCl, 0.2; KH_2PO_4, 0.2) and drained. To each dish, 1 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) was added. After 1 min, monolayers were triturated to give a uniform suspension of cells, and radioactivity in 0.75 ml was determined by scintillation counting. Cell number per dish was determined with 200 μl of suspension, as described previously (31).

Effect of *N*-substituted phenoxazines on cell survival. KB8–5 cells were plated in triplicate at a density of 1000 cells/well and GC_3/c1 cells were plated at 3000 cells/well in Falcon six-well flat-bottomed tissue culture plates (Becton Dickinson). After 24 hr, incubation medium was replaced with 3 ml of fresh medium containing modulators at concentrations ranging from 1 to 100 μM (final culture concentration, 0.1% DMSO), and cells were incubated at 37° for an additional 7 days. The medium was aspirated, and cells were washed once with 2 ml of 0.9% NaCl and dried overnight. Colonies were stained with 1 ml of 0.1% crystal violet, followed by two washes with distilled water, and were counted using an automated ARTEK model 880 colony counter. The IC_{50} values were determined from concentration-percentage of cell survival curves and were defined as the concentrations of phenoxazines required for 50% reduction in colonies, compared with controls.

Effect of *N*-substituted phenoxazines on *in vitro* cytotoxicity of VLB and VCR. Cells were treated with graded concentrations of VCR and VLB (either for 2 hr or continuously for 7 days) in the absence or presence of nontoxic concentrations of modulators (continuous exposure for 7 days). The plates were then transferred to a CO_2 incubator and, after incubation for an additional 7 days at 37°, colonies were enumerated as described.

Competition for [^{3}H]azidopine labeling of P-glycoprotein. Competition for photolabeling of P-glycoprotein used membranes from KB-V1 cells, which have higher P-glycoprotein levels than do KB8–5 cells. The method was essentially similar to that used previously for

CEM/VLB$_{6K}$ cells (29). Briefly, >10^9 KB-V1 cells were harvested, washed several times in 25 mM HEPES, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 5 mM NaH_2PO_4, pH 7.4, finally resuspended at 10^7 cells/ml in 20 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4, and lysed by nitrogen cavitation as described (36); membrane pellets were resuspended in 10 mM Tris, pH 7.4, and frozen at −70° until use. For photoaffinity labeling, membrane protein (200 μg) in buffer containing 250 mM sucrose and 10 mM Tris·HCl, pH 7.4 at 25°, was mixed with 100 nM [^{3}H]azidopine (50 Ci/mmol; Amersham) in the absence or presence of modulators (25–100 μM), in a total of 150 μl. After 20 min of incubation in the dark, mixtures were exposed to UV light (20 min, 10-cm distance). Proteins were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by autoradiography. Bands were quantitated by scanning densitometry of the autoradiograms.

Immunohistochemistry. Tumor cell lines were grown on plastic chamber well slides (four-well) at an initial cell density of 30,000/well. When the cells were in midlogarithmic growth phase (approximately 48 hr), slides were washed twice with PBS and air dried overnight. Slides were then fixed in cold acetone (on dry ice) for 10 min. MoABs C219 (Centocore, Malvern, IL) and HYB241 (a gift from Dr. L. Grauer, Hybritech, San Diego, CA) were used for the detection of P-glycoprotein, and pooled mouse immunoglobulins were used as a negative control. The slides were initially incubated in the presence of blocking serum (PBS/5% rabbit serum/0.05% Tween) for 15 min at room temperature. The slides were then incubated overnight at 4° with the MoABs or negative control at final concentrations of 10 μg/ml blocking serum. MoAB binding was demonstrated using the peroxidase-antiperoxidase immunoenzymatic method described by Chan *et al.* (37). P-glycoprotein expression was quantitated by image analysis of 50 cells (Bioquant image analysis system).

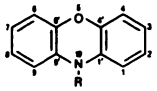
Results

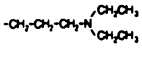
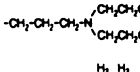
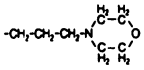
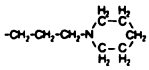
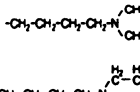
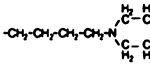
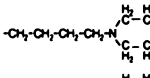
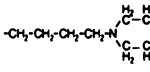
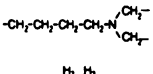
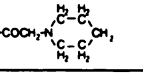
***Vinca* alkaloid accumulation.** Our initial evaluation (30) showed that at 100 μM verapamil increased accumulation of VLB by 1124 and 238% in KB8–5 and GC_3/c1 cells, respectively. At the same concentration (100 μM) 20 of 21 phenoxazine compounds significantly increased accumulation of VLB in KB8–5 cells, with compounds **2**, **3**, **4**, **5**, **11**, and **18** being more effective than verapamil (Table 1). Compounds **2**, **3**, and **10-14** increased accumulation of VLB >10-fold in GC_3/c1 colon adenocarcinoma cells, whereas the prototype modulator verapamil had little effect in these cells. Of note, however, was the poor correlation between the degree of enhancement of VLB accumulation by *N*-substituted phenoxazines in these two cell lines ($r^2 = 0.05$).

In contrast, for the two cell lines there were similar cytotoxic potencies after 7 days of continuous exposure ($r^2 = 0.74$) (Table 1). As a consequence of their relative lack of toxicity, compounds **1**, **3**, **4**, and **18** were studied further. Concentrations of modulator that reduced cloning efficiency of KB8–5 and GC_3/c1 cells by 10% after 7 days of exposure were determined, and modulation of VLB accumulation at the IC_{10} concentration is given in Table 2. Modulators were used at their IC_{10} concentration, to allow comparison of MDR-reversing properties at equitoxic concentrations. Cells were exposed continuously for 7 days to modulator and for 2 hr or 7 days to VCR or VLB (Table 2). Data for compounds **1**, **3**, **4**, and **18** and verapamil are presented in Figs. 1 and 2. In KB8–5 cells (Fig. 1), compound **4** [10-(3′-*N*-morpholinopropyl)phenoxazine] was more effective than verapamil in potentiating VLB toxicity after 2-hr or continuous (168-hr) exposure to the *Vinca* alkaloid (Table 2) but was less effective than verapamil in sensitizing cells to VCR. Compound **3** (10-[3′-[*N*-bis(hydroxyethyl)amino]propyl]

TABLE 1

Biological Properties of *N*-(alkylamino) or *N*-acylamino derivatives of phenoxazine



Compd. No.	R	VLB Accumulation (% increase) KB-8-5	VLB Accumulation (% increase) GC_3/c1	IC_{50} (μM) KB-8-5	IC_{50} (μM) GC_3/c1
1		342	570	57	83
2		2123	1025	15	10
3		1666	1070	36	37
4		1717	960	73	40
5		1227	633	ND	ND
10		403	1165	<10	16
11		2684	1175	17	27
12		1071	1121	<10	7
13		188	1340	<10	7
14		477	1315	<10	8
16		2023	448	73	66

* ND, not determined.

phenoxazine) and compound **4** were equally effective (2-hr exposure) or more effective (168-hr exposure) than verapamil in sensitizing GC_3/c1 cells to VLB but were significantly less effective in modulating VCR (Fig. 2). Compound **18** [10-(*N*-piperidinoacetyl)phenoxazine] also sensitized KB8–5 cells, although it was less effective than verapamil (Table 2).

Modulation of VLB accumulation and retention in KB8–5 cells. Compounds **3** and **4** were studied further, because they significantly enhanced accumulation of VLB in both cell lines. In addition, modulator **4** was less toxic to cells than the butyl analogue **11**. Compound **18** was studied further because initial experiments suggested quantitative differences between modulation of VLB accumulation in KB8–5 and GC_3/c1 cells. Accumulation of VLB (49.5 nM) was examined in KB8–5 cells after 2-hr exposure to varying concentrations of modulator. As shown in Fig. 3, verapamil and compound **3** had similar activities, whereas both phenoxazines **4** and **18** enhanced accumulation of VLB to a significantly greater extent (approximately 2-fold), compared with the maximum effect of verapamil. At a concentration of 200 μM compound **3** caused monolayers to detach from the plastic culture dish in this experiment.

To elucidate the basis for the qualitative differences, compared with verapamil, efflux studies were conducted. KB8–5 cells were incubated for 2 hr with VLB (49.5 nM), and mono-

TABLE 2
Biological and biochemical activities of *N*-substituted phenoxazines

Cell line	Compound	IC_{10}[a]	Accumulation of VLB[b]	VLB cytotoxicity			
				2-hr exposure		168-hr exposure	
				IC_{50}	Fold reversal	IC_{50}	Fold reversal
		μM	% of control	nM		nM	
KB8-5	**3**	25	361 ± 36	500	12.0	0.65	12.9
	4	40	1233 ± 140	110	54.5	0.21	40.0
	18	40	324 ± 71	560	10.7	1.0	8.4
	Verapamil	30	667 ± 67	220	27.3	0.39	21.5
GC_3/c1	**3**	25	655 ± 55	560	8.4	0.21	14.8
	4	25	706 ± 17	510	9.2	0.19	16.3
	Verapamil	40	231 ± 8	510	9.2	0.30	10.3

[a] 7-day exposure.
[b] At IC_{10} concentration of modulator.

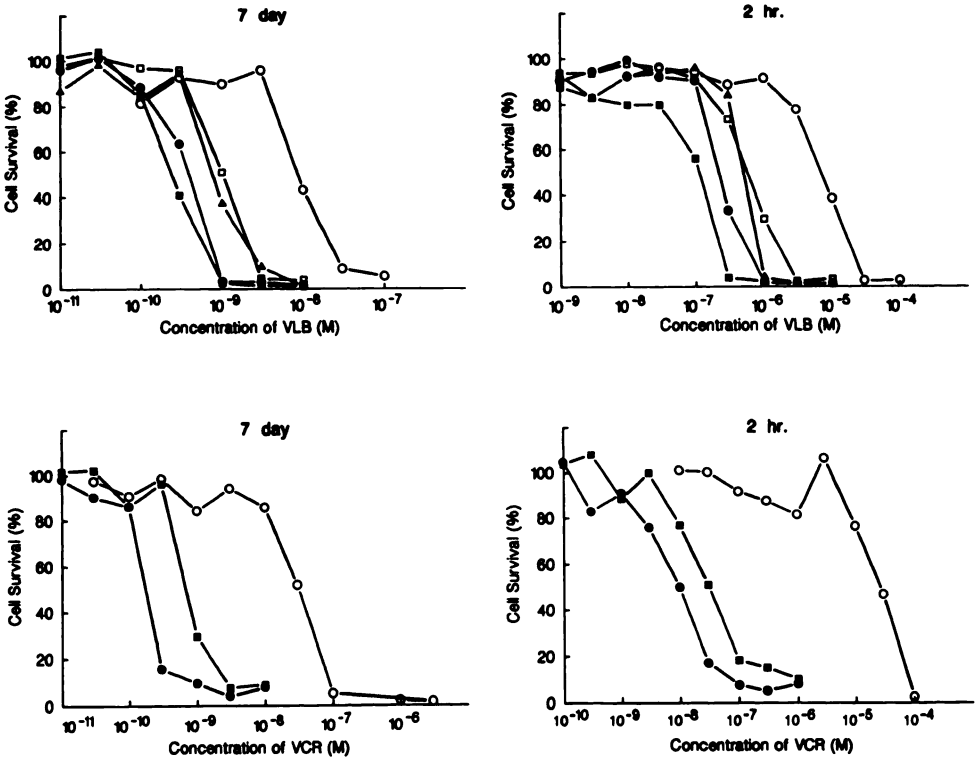


Fig. 1. Potentiation of *Vinca* alkaloid cytotoxicity by *N*-substituted phenoxazines and verapamil in KB8–5 cells. Cells were exposed to VLB or VCR for 7 days (*left*) or 2 hr (*right*) in the absence or presence of modulator (IC_{10} concentration; see Table 1), which was maintained for 168 hr. ○, No modulator; ▲, compound **3**; ■, compound **4**; □, compound **18**; ●, verapamil.

layers were washed in ice-cold buffer and incubated for up to 2 hr in buffer containing 100 μM modulator, during which time cell-associated VLB was determined (Fig. 4A). Results showed that verapamil and compound **4** significantly inhibited the efflux of VLB, whereas compounds **3** and **18** did not. To determine whether compound **3** inhibited efflux at higher VLB concentrations, cells were incubated with VLB (49.6, 248, or 10,000 nM) for 2 hr, and efflux was determined in the presence of **3** (25 or 100 μM) or in the absence of modulator. As shown in Fig. 4B, there was no inhibition of VLB efflux.

Although these results suggested qualitative differences between compound **3** and verapamil, the data could have indicated that compound **3** modulation of VLB retention or uptake was time dependent (i.e., slow onset). Therefore, the effect of compound **3** on accumulation of VLB was examined over 2 hr in KB8–5 cells after simultaneous exposure to VLB and modulator (Fig. 5). Compound **3** significantly increased cell-associated VLB within 5 min of incubation, and the effect was concentration dependent. To examine whether the lack of effect on VLB efflux was a consequence of slow onset of the modulator effect, KB8–5 cells were incubated with VLB (24.8 nM) plus 100 μM modulator **3** or with 300 nM VLB (with no modulator) for 2 hr, to give approximately equivalent cell-associated VLB. Efflux of VLB was examined over 10 hr in the presence or absence of modulator. Results from a representative experiment are shown in Fig. 6. Modulator **3** had very little effect on efflux, which was similar to that from cells loaded with 300 nM VLB alone (no modulator) and incubated without modulator during the efflux phase. In contrast, when cells were loaded in the presence of phenoxazine **3** and then verapamil (85 μM) was substituted during the period of efflux, there was a very marked inhibition of VLB efflux (Fig. 6).

Competition for [^{3}H]azidopine labeling of P-glycoprotein. These data suggested that the effect of compound **3** on increasing the rate of VLB accumulation could not be explained by an effect on drug efflux and was possibly independent of a P-glycoprotein-dependent mechanism. To examine this further, competition between [^{3}H]azidopine and each phenoxazine modulator was examined. Data for compounds **3** and **4** for labeling of membrane vesicles from KB-V1 cells are shown in

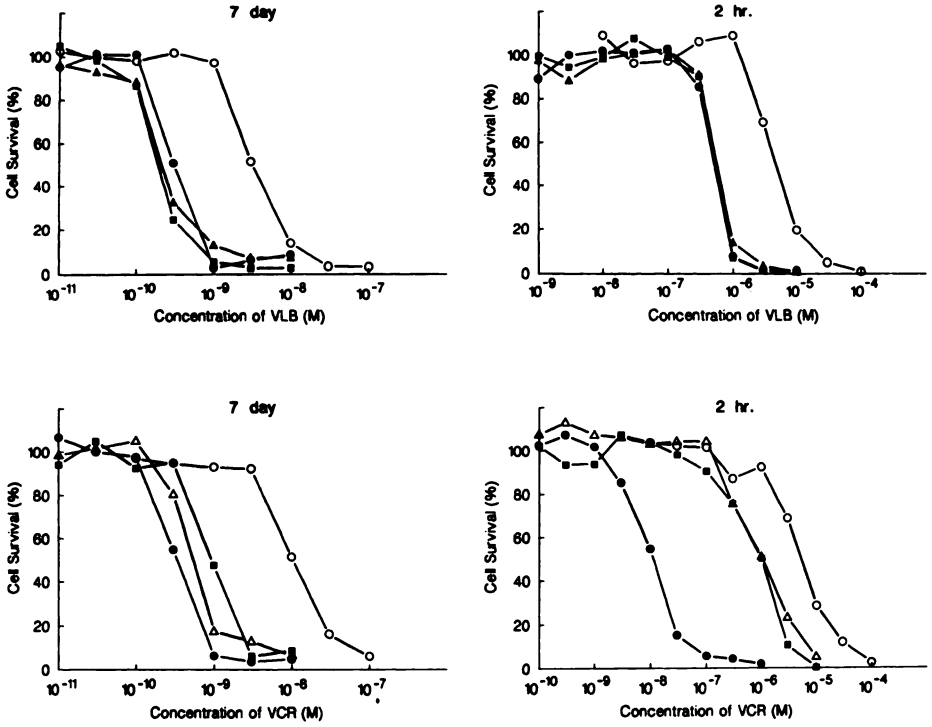


Fig. 2. Potentiation of *Vinca* alkaloid cytotoxicity by *N*-substituted phenoxazines and verapamil in $GC_3/c1$ colon adenocarcinoma cells. Cells were exposed to VLB or VCR for 7 days (*left*) or 2 hr (*right*) in the absence or presence of modulator (IC_{10} concentration), which was maintained for 168 hr. ○, No modulator; △, compound **1**; ▲, compound **3**; ■, compound **4**; ●, verapamil.

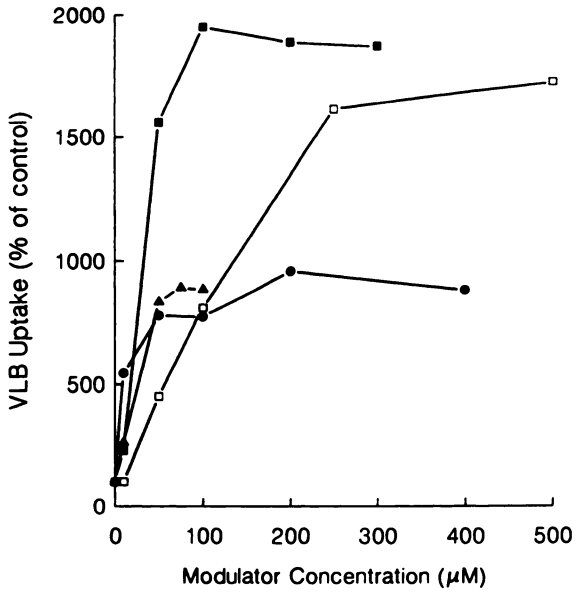


Fig. 3. Accumulation of [^{3}H]VLB (49.5 nM) in KB8–5 cells with increasing concentrations of *N*-substituted phenoxazines or verapamil. Monolayer cell lines were exposed simultaneously to modulator and VLB for 2 hr. Each *point* represents the mean of three determinations. ▲, Compound **3**; ■, compound **4**; □, compound **18**; ●, verapamil.

Fig. 7, and quantitative data for all modulators are presented in Table 3. At 100 μM (1000-fold excess) compound **4** reduced [^{3}H]azidopine labeling of P-glycoprotein by 50% and verapamil inhibited labeling by 38%. In contrast, modulators **3** and **18** had no effect (band intensity was 98% and 96%, respectively, of that obtained with no modulator; Table 3).

The effect of modulators on accumulation of VLB in cells demonstrating low or undetectable levels of P-glycoprotein, determined by image analysis of stained cells, was next determined. Both phenoxazines **3** and **4** demonstrated concentration-dependent enhancement of VLB accumulation in each cell line, whereas verapamil had only slight effects. Enhancement of VLB accumulation in cells exposed to phenoxazine modulators or verapamil is presented in Fig. 8. Of note was the finding that verapamil did enhance VLB accumulation (2.4-fold) in this subline of KB3–1 cells, consistent with the low levels of P-glycoprotein expression detected by immunostaining. As shown in Table 4, at 100 μM compound **3** increased VLB accumulation 8–37-fold in rhabdomyosarcoma cells (Rh18, Rh28, and Rh30), whereas verapamil enhanced accumulation by only 2.3–3.4-fold. Similarly, modulator **4** enhanced VLB accumulation by nearly 50-fold in Rh18 cells.

Discussion

In this study, we have extended our initial observations on *N*-substituted phenoxazines as modulators of MDR. These earlier studies (29, 30) showed that phenoxazine and *N*-substituted analogues were in general more effective in modulating accumulation of VLB than the structurally similar VCR. However, although 20 of 21 compounds significantly enhanced accumulation of VLB, of greater interest was the finding that certain modulators [e.g., **13** [10-[4′-[(β-hydroxyethyl)-piperidino]butyl]phenoxazine] and **18**] had significantly different activities in the two cell lines, whereas other compounds (**3** and **4**) had significant activity in increasing uptake of VLB in both KB8–5 and $GC_3/c1$ cells. These data suggested that potential qualitative as well as quantitative differences may exist between modulators in this series.

Four of the modulators (compounds **10** and **12-14**) were cytotoxic at low concentrations. This is not surprising, because antitumor activity has been reported among the phenoxazine dyes (reviewed in Ref. 38). Thus, although **13** demonstrated differential activity against $GC_3/c1$ cells, its toxicity precluded further study. Compound **18** demonstrated relatively low toxicity and differential effects between the cell lines, whereas compounds **3** and **4** were among the most active in increasing accumulation of VLB in both cell lines. Each compound signif-

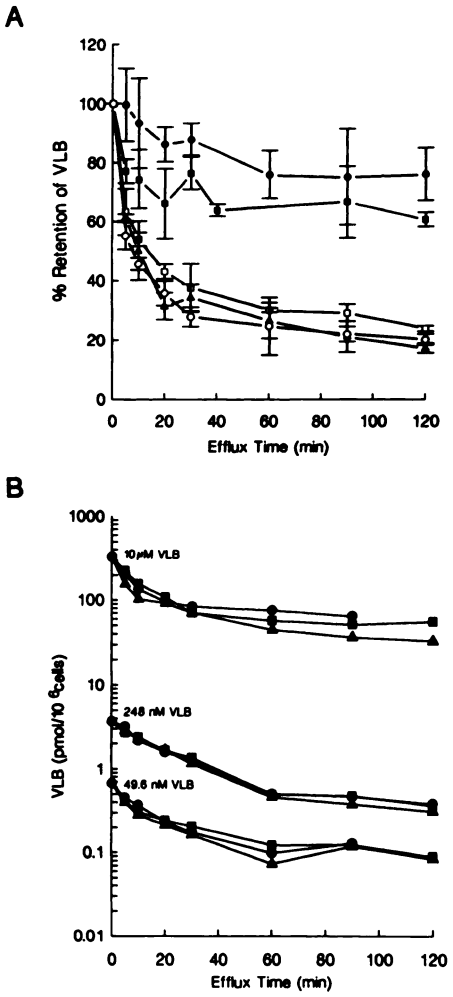


Fig. 4. Effect of modulators on VLB efflux. A, KB8–5 monolayers were exposed to VLB (49.5 nM) for 2 hr and washed (2°), and efflux of radiolabel was determined over an additional 2 hr in the absence of modulator (○) or in the presence of 100 μM compound **3** (▲), compound **4** (■), compound **18** (□), or verapamil (●). B, KB8–5 cells were incubated for 2 hr with 49.6 nM, 248 nM, or 10,000 nM VLB and washed, and cell-associated radiolabel was determined over an additional 2 hr in the absence (●) or presence of 25 μM (▲) or 100 μM (■) modulator **3** (three determinations; standard deviation, <5% of mean).

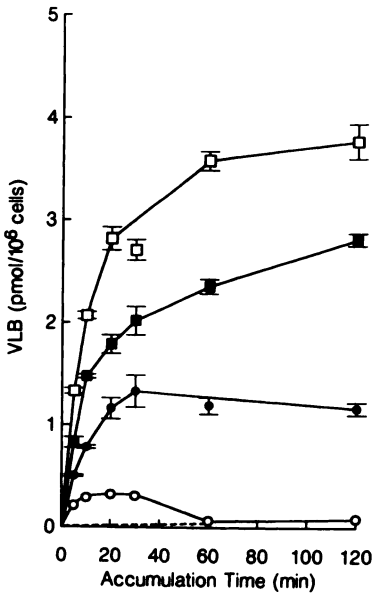


Fig. 5. Concentration-dependent effect of compound **3** on initial rates of VLB accumulation in KB8–5 cells. ○, No modulator; – – –, 2°, no modulator; ●, 25 μM; ■, 50 μM; □, 100 μM compound **3**. Each *point* represents the mean ± 1 SD (three determinations).

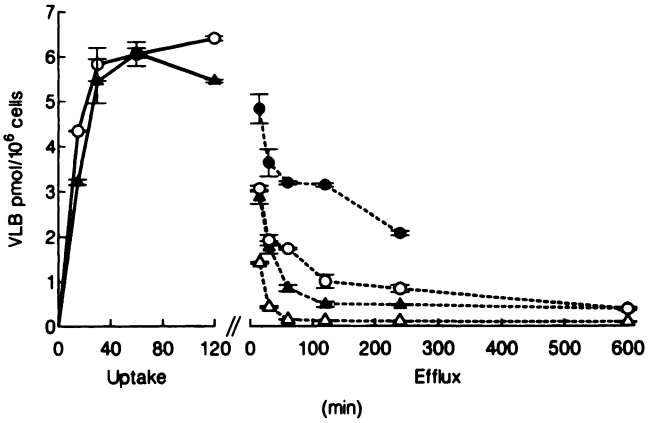


Fig. 6. Comparison of the effects of compound **3** upon accumulation and retention of VLB in KB8–5 cells. *Left*, cells were exposed to 24.8 nM VLB in the presence of 100 μM modulator **3** (▲) or 300 nM VLB without modulator (○). *Right*, monolayers were washed and retention of VLB was measured over 600 min without modulator (△), with 100 μM compound **3** (▲), with 85 μM verapamil (●), or in the absence of modulator after loading of cells for 2 hr in the presence of 300 nM VLB alone (○). Each *point* is the mean ± 1 SD (three determinations) and shows a representative experiment.

icantly enhanced accumulation of VLB when cells were incubated with equitoxic concentrations of modulator that reduced cloning efficiency by ≤10% (7-day exposure) (Table 2). In KB8–5 cells the increase in VLB accumulation at the IC_{10} concentration of modulator correlated well with the degree of chemosensitization for 2-hr (r^2 = 1.00) or 7-day (r^2 = 0.99) exposure to VLB. In KB8–5 cells, compound **4** was more effective than verapamil in reversing VLB resistance, whereas compounds **3** and **18** were less effective. In GC_3/c1 cells exposed to VLB for 2 hr or 7 days, compounds **3** and **4** were as effective as verapamil in reversing resistance. However, both phenoxazines were less effective than verapamil in modulating VCR cytotoxicity.

We next examined the concentration-effect relationship for three phenoxazine modulators (**3**, **4**, and **18**) and verapamil. Interestingly, in the KB8–5 cell line compounds **4** and **18** enhanced VLB accumulation to a significantly greater extent than the maximum activity of verapamil. This suggested qualitative differences between verapamil and these two modulators. Data presented in Fig. 3 show results of studies in which each modulator was examined in a single experiment. For modulator **3** it was more usual to see linearity between VLB accumulation and concentration of modulator (three of four independent experiments). This variation (see Fig. 5) is not understood but may relate to the culture conditions or to the toxicity of modulator **3** in KB8–5 cells. However, the major distinction between verapamil and certain of the phenoxazine modulators was that compounds **3** and **18** did not inhibit the efflux of VLB. Compound **3** was examined most extensively. Simultaneous exposure of KB8–5 cells to VLB and compound **3** showed that the enhanced accumulation of VLB occurred rapidly and was concentration dependent. In contrast, this modulator did not decrease the rate of VLB efflux at concentrations up to 100 μM. As shown in Fig. 6, modulator **3** enhanced the accumulation of VLB such that approximately equal concentrations were achieved in KB8–5 cells exposed to 25 nM

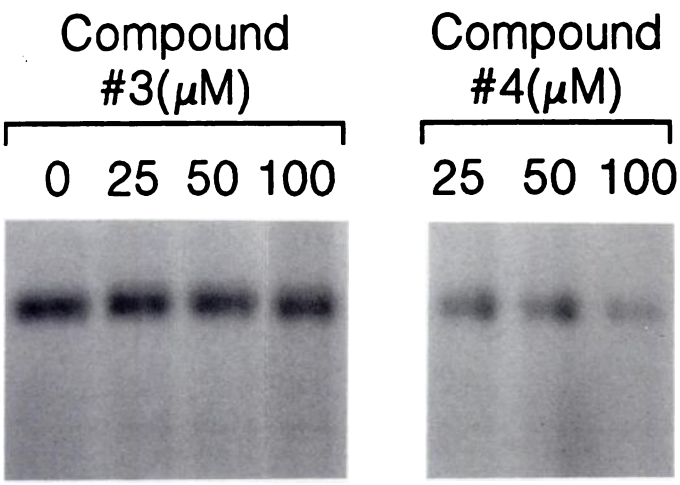


Fig. 7. Competition between [^{3}H]azidopine photoaffinity labeling of KB-V1 cell membranes and phenoxazines **3** and **4**. Membranes were incubated with 100 nM [^{3}H]azidopine in the presence of 0, 25, 50, or 100 μM modulator, as described in Materials and Methods. Reduction in photoaffinity labeling of P-glycoprotein was determined by quantitative densitometry of the autoradiogram.

TABLE 3
Competition by *N*-substituted phenoxazines and verapamil for [^{3}H] azidopine binding to KB-V1 membranes

Compound	[^{3}H]Azidopine binding[a]		
	25 μM	50 μM	100 μM
	% of control		
1	54	60	55
3	103	104	98
4	74	68	50
18	125	110	96
Verapamil	80	79	62

[a] Binding of [^{3}H]azidopine to P-glycoprotein is expressed as percentage of control (no competitor).

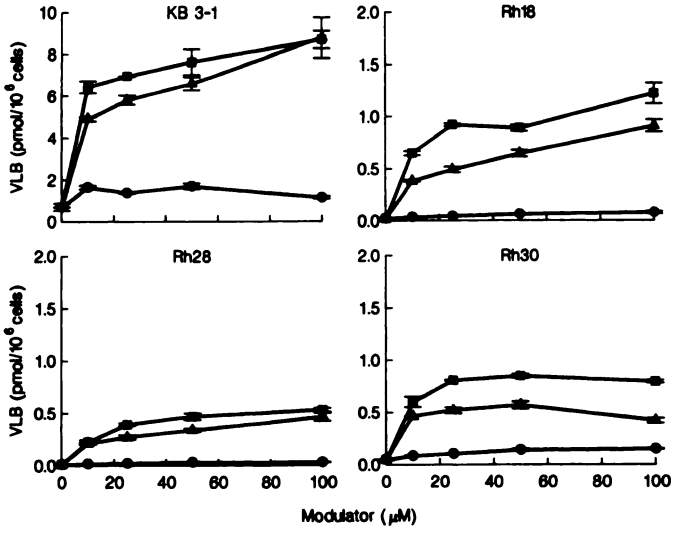


Fig. 8. Effect of phenoxazine modulators on accumulation of VLB in cell lines demonstrating low or undetectable levels of P-glycoprotein by immunostaining. Two-hour accumulation of VLB is shown as a function of modulator concentration. ■, Phenoxazine **4**; ▲, phenoxazine **3**; ●, verapamil. KB3–1 cells were exposed to 25 nM VLB; other cell lines were exposed to 2.5 nM VLB. Each *point* is the mean ± 1 SD (three determinations).

TABLE 4
Increase in VLB accumulation

	P-glycoprotein staining[a]	VLB accumulation		
		Verapamil	3	4
		%		
KB8-5	628 ± 68	1120	1670	1720
GC_3/c1	217 ± 20	240	1070	960
KB3-1	142 ± 12	240	1220	1210
Rh18	60 ± 8	340	3700	4940
Rh28	26 ± 3	230	3160	3630
Rh30	0[b]	290	830	1560

[a] Immunostaining was quantitated by image analysis of 50 cells (mean ± standard deviation).
[b] No staining was detected in any cell.

VLB plus modulator or 300 nM VLB alone. However, retention of VLB during the 10 hr after removal of VLB was not enhanced by modulator **3** (100 μM); rather, VLB efflux was more rapid, compared with retention of VLB in cells loaded to the same level when exposed to the higher concentration of VLB alone. The results presented could indicate that, whereas modulator **3** enhances cell association, the intracellular distribution and association (e.g., binding to tubulin) may differ from those obtained at similar cellular levels of VLB in the absence of modulator. However, in contrast to verapamil, phenoxazine **3** was relatively ineffective in causing prolonged retention of VLB in KB8–5 cells. Consequently, the major effect of compounds **3** and **18** appeared to be enhanced uptake of VLB, rather than decreased efflux.

The fact that **3** and **18** (data not shown) act at least partially independently of P-glycoprotein is supported by their failure to compete with radiolabeled azidopine for binding to P-glycoprotein in membrane vesicles. Further examination of phenoxazine **3** showed that this modulator enhanced VLB accumulation in cell lines with low or undetectable levels of P-glycoprotein (Rh18, Rh28, and Rh30), where verapamil had little activity. Verapamil did enhance accumulation of VLB in KB3–1 cells, in contrast to reported studies; however, the effect was modest, compared with phenoxazine modulators. Immunohistochemical staining of KB3–1 cells did reveal low levels of P-glycoprotein staining (Table 4), consistent with the slight activity of verapamil.

Our data suggest that, within a limited series of phenoxazine modulators, there are potentially at least two mechanisms of action. Compounds such as **3** and **18** appear to modulate VLB accumulation and resistance without significantly inhibiting drug efflux. Compound **4** inhibits efflux less effectively than does verapamil and competes for azidopine binding but also increases VLB accumulation to a greater extent than the maximal effect of verapamil. Both phenoxazines **3** and **4** enhanced accumulation of VLB in cell lines expressing low or undetectable levels of P-glycoprotein, suggesting at least partial P-glycoprotein-independent activity of these modulators. Thus, within this series of *N*-substituted phenoxazines, two distinct effects appear to be separated. Compound **4** had dual effects, i.e., inhibition of VLB efflux and competition for azidopine photoaffinity labeling (verapamil-like activity), but also increased VLB accumulation to a greater extent than did verapamil in KB8–5 cells and in several cell lines where verapamil had little activity. Compounds **3** and **18** had minimal "verapamil-like" activity but instead exerted effects upon VLB influx. The fact that this effect on the influx component is biologically significant is shown by reversal of VLB resistance

in both KB8–5 and GC_3/c1 cells. Whether this component is responsible for the greater activity of compound **4**, compared with verapamil, is unclear. However, these data would argue against phenoxazines causing increased accumulation of VLB in relatively unimportant sites such as lysozomes. The data presented most strongly support an hypothesis that the "influx-modulating" component is mediated through decrease of a permeability barrier, allowing greater interaction with some intracellular target (e.g., tubulin) or exposing cryptic binding sites. Preliminary studies[2] indicated that the unsubstituted parent compound, phenoxazine, caused a 2-fold increase in the plasma membrane potential of both KB8–5 and KB3–1 cells. This would be consistent with greater partitioning of VLB into cells.

The objective of our study was to define mechanisms by which this limited series of modulators caused VLB accumulation, rather than providing a detailed structure-activity analysis. Thus, we have not investigated the effect of extension of the methylene bridge between N^{10} and the nitrogen substituent or substitutions at C_2 of the phenoxazine ring, as reported by Ford *et al.* (10). However, our results indicate that within a series there may be multiple mechanisms by which modulators exert their effects and this may, in part, contribute to the difficulty of establishing structure-activity relationships for MDR modulators.

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Send reprint requests to: Peter J. Houghton, Department of Molecular Pharmacology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105.