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Physical-Chemical Properties Shared by Compounds that Modulate Multidrug Resistance in Human Leukemic Cells

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SUMMARY

Multidrug resistance (MDR), typified by resistance to *Vinca* alkaloids and anthracyclines, is a well characterized experimental phenomenon that may have some clinical correlates. Verapamil, chloroquine, and related drugs have been shown previously to be capable of enhancing anticancer drug cytotoxicity in multidrug-resistant cells, but the mechanism(s) by which these agents do this is(are) unclear. Since these agents did not seem to have common features, we studied these and other compounds for their ability to "modulate" *Vinca* alkaloid resistance in order to determine whether they possessed any common chemical or physical features. In addition to verapamil, 24 compounds, consisting of indole alkaloids, lysosomotropic agents, and amines, were tested for their ability to enhance the cytotoxicity of vinblastine and/or vincristine in our human leukemic multidrug-resistant cell line, CEM/VLB$_{100}$. Seventeen compounds that enhance the cytotoxicity of the *Vinca* alkaloids by more than 5-fold have been identified. These include quinolines (chloroquine, quinine, chinchonidine, and primaquine), acridines (acridine, acridine orange, and quinacrine), and indole alkaloids (yohimbine, corynanthine, reserpine, physostigmine, and the vindoline and catharanthine moieties of the *Vinca* alkaloids), as well as other alkaloids and amines (chlorpromazine, propranalol, atropine, and tryptamine). Vindoline, catharanthine, and quinacrine also enhanced the cytotoxicity of doxorubicin and teniposide in these cells, indicating that this "modulation" was not limited to *Vinca* alkaloids. We examined some well known lysosomotropic compounds (methylamine, epinephrine, suramin, and trypan blue) and found that they were not able to enhance the cytotoxicity of vincristine in the CEM/VLB$_{100}$ cells, indicating that lysosomotropic activity per se is not required for modulator activity. Three-dimensional computer modeling permitted molecular comparisons of conformationally related congeners of vinblastine, vindoline, and verapamil and revealed three regions of structural homology. We measured the hydrophobicity (by oil/water partitioning) and calculated the molar refractivity (by the additive substituent constant method) of active and inactive compounds. We found that those cationic agents—verapamil, quinacrine, indole alkaloids, and quinolines—that were lipid soluble at physiologic pH and had similar molar refractivities were best able to enhance the cytotoxicity of the *Vinca* alkaloids in our multidrug-resistant cells. Thus, we conclude that, although structural similarities may play a role in the ability of some compounds to modulate drug resistance, it appears that lipid solubility at physiological pH, cationic charge, and molar refractivity are important physical properties for modulators of MDR. Our studies provide direction for the development of new compounds that may be useful in understanding the mechanisms of MDR and may also suggest possible chemotherapeutic strategies for its circumvention.

Multidrug resistance (MDR) is now a well documented experimental phenomenon (1–5). Cells selected for MDR are characterized by cross-resistance to a wide variety of natural product drugs and their semisynthetic derivatives (1–6). The pharmacologic basis for MDR appears to be due to a decreased accumulation and retention of drugs by these cells (7, 8). It has been postulated that the altered pharmacology is mediated by a high molecular weight integral membrane protein, called Pgp or P170, that may be capable of binding drug and possibly even extruding it from the cell (9–12). Since recent studies have shown that this type of Pgp-associated MDR may occur clinically (13–15), strategies designed to either block expression or circumvent this form of drug resistance are warranted.

Several classes of membrane-active agents, including calcium channel blockers and calmodulin inhibitors, have been shown to be capable of enhancing the cytotoxic activity of (primarily) *Vinca* alkaloids and anthracyclines in multidrug-resistant cells (16–18). This "modulation" of cytotoxicity is much less pronounced or nonexistent in drug-sensitive cells. We and others have shown that the lysosomotropic agent, CLQ, is also capable

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ABBREVIATIONS: MDR, multidrug resistance; Pgp, P-glycoprotein (P170); VLB, vinblastine sulfate; VCR, vincristine sulfate; DOX, doxorubicin; DNR, daunorubicin; CLQ, chloroquine; VM-26, teniposide, [4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidine-β-D-glucopyranoside)].

of enhancing the cytotoxicity of *Vinca* alkaloids and anthracyclines in multidrug-resistant cells (19, 20). Recent findings with calcium channel-blocking drugs have suggested that they enhance anticancer drug retention through a competition for a common binding site on the resistance-associated protein, Pgp (21, 22), and not through any effect on voltage-gated calcium channels (23, 24). Structure-activity studies of such modulators may thus provide insights into the fundamental mechanisms of MDR.

Our observations with lysosomotropic agents [which include verapamil (25, 26)] prompted the present investigation to attempt to characterize the physical-chemical features of modulators of MDR. Specifically, we wished to determine whether there was some general class of drug or general drug properties that enhance *Vinca* alkaloid and anthracycline cytotoxicity. Since it was clear that basic compounds and some lysosomotropic agents were active in this regard, we asked if indole alkaloids and aromatic amines, as well as other agents that either accumulate in lysosomes or alter lysosomal pH could enhance drug cytotoxicity in our human multidrug-resistant cell line, CEM/VLB_{100}. Physical, chemical, and biological characteristics were compared in order to determine whether we could develop any general rules for a compound to modulate anticancer drug cytotoxicity. A preliminary account of some of this work has been presented (27).

Materials and Methods

Chemicals and supplies. VLB, VCR, vindoline, and catharanthine hydrochloride were obtained from Eli Lilly and Co. (Indianapolis, IN), and DOX and DNR were from Adria Laboratories (Columbus, OH). CLQ, quinine, primaquine, chinchonidine, quinacrine, acridine, acridine orange, yohimbine, corynanthine, physostigmine, reserpine, atropine, colchicine, D,L-propranolol, chlorpromazine, tryptamine, D,L-tryptophan, isoniazid, epinephrine, and methylamine were all purchased from Sigma Chemical Co. (St. Louis, MO). Teniposide (VM-26) was generously provided by Bristol-Myers (Wallingford, CT), suramin was obtained from Bayer (Leverkusen, FRG), and trypan blue was obtained from GIBCO (Grand Island, NY). VM-26 was dissolved in 100% dimethyl sulfoxide, the final concentration of which, <0.3%, had no independent effect on cell growth. Catharanthine and reserpine were solubilized in acidic ethanol (90% ethanol in 0.1 N HCl), the final concentration of which, <1%, had no independent effect on cell growth. Vindoline and acridine were dissolved in acidic saline (0.9% NaCl in 0.1 N HCl), the final concentration of which ($\leq 2 \times 10^{-3}$ N HCl) had no effect on cell growth.

Cells, culture conditions, and cytotoxicity assays. CCRF-CEM human leukemic lymphoblasts and their VLB-resistant derivatives, CEM/VLB_{100}, were grown in minimal essential medium (Earl's salts) containing 10% fetal bovine serum, as described previously (28). The CEM/VLB_{100} cells display the "classic," i.e., Pgp-associated MDR phenotype (4,29). Drug cytotoxicity was determined by the inhibition of cell growth in 48 hr, compared to controls. This assay has been shown to be appropriate for relative comparisons (30) and can reflect cell kill (18). Cells were grown in multiwell dishes (Costar no. 3424) at an initial density of ~2.5–3 $\times 10^5$ cells/ml. At 48 hr, the cell number was determined using a Coulter Counter (model ZBI), with a Channelizer to distinguish cells from debris. The IC_{50} is defined as the concentration of drug required to inhibit the 48-hr growth of treated cells by 50% compared to the controls. The fold-decrease in IC_{50} was determined by dividing the IC_{50} value of the controls by that of the treated cells.

Octanol/H_2O partitioning studies. We used a slight modification of the procedure of Owellen *et al.* (31) for these studies. Drug solutions were prepared as indicated above. These solutions were added to either 0.1 M sodium phosphate buffer, pH 7.4, or 0.1 M sodium acetate buffer, pH 4.5, to achieve a final concentration of 10^{-4} M. High performance liquid chromatography-grade octanol (Aldrich Chemical Co., Milwaukee, WI) was added to the drug solutions, vortexed vigorously, and allowed to separate. Using a Beckman model 25 spectrophotometer, the optical density of the aqueous phase was measured before and after octanol/H_2O partitioning and compared to stock solutions of known concentrations. The difference between the concentrations before and after partitioning was considered to be the concentration in the octanol phase. The partition coefficient, P, was determined by dividing the concentration of drug in the octanol phase by that in the aqueous phase. The $\log_{10} P$ was used as measure of lipid solubility.

Molar refractivity calculations. Molar refractivities were calculated by the additive substituent constant method using the computer program CLOGP-3 (32).

Computational studies. All calculations were performed using a Digital Equipment Corp. VAX 8800 series computer. Molecular graphics analyses were supported by the computer software SYBYL from TriPos Associates (St. Louis, MO). Three-dimensional starting geometries were obtained as follows. Coordinates for VLB and vindoline were taken from X-ray crystallographic coordinates of a related *Vinca* alkaloid, vinzolidine (33), and appropriately modified to account for differences in functionality. The final geometry of each was refined using the molecular mechanics program MM2 (34). The starting geometry of verapamil was derived from three-dimensional computer models (SYBYL) and refined using MM2 and the semi-empirical method, AM1 (35). Molecular comparisons of conformationally related congeners were generated using the MULTIFIT option in SYBYL wherein the conformations of VLB, vindoline, and verapamil were simultaneously varied in such a way as to minimize the differences in the coordinates of selected atoms. Common volume elements in this series were generated under the MVOL option in SYBYL and represent Boelean intersections of the molecular volumes (Van der Waals) of each final conformation.

Results

Screening for candidate "modulators" of MDR. Prior results with CLQ suggested that a survey of indole alkaloids, lysosomotropic agents, and amines might reveal other modulators of MDR. These results also suggested that, for a modulator to be active in our studies, it had to have some slight cytotoxicity of its own (19). Shown in Fig. 1 are structures of most of the compounds chosen for screening and are mainly indole alkaloids or related compounds that are derived from and contain tryptophan nuclei and have protonatable groups. Shown in Table 1 are IC_{50} values, the concentrations at which all the compounds were tested for enhancement of *Vinca* alkaloid cytotoxicity, and their independent effects on the growth of the CEM/VLB_{100} cells when used at the listed concentrations. For ease of comparison, the compounds are classified as quinolines, acridines, indole alkaloids, other alkaloids, and amines. It is clear from these data that these agents, in general, had very high IC_{50} values ($>10^{-4}$ M), indicating that they were relatively nontoxic, and at the concentrations used in combination with the anticancer drugs, most inhibited the 48-hr growth of cells by no more than ~25%.

Enhancement of *Vinca* alkaloid cytotoxicity in CEM/VLB_{100} cells. We examined the compounds in Table 1 for their ability to modulate *Vinca* alkaloid cytotoxicity in CEM/VLB_{100} cells. Since we showed previously that verapamil is equally effective in enhancing either VLB or VCR cytotoxicity (18), we tested the potential modulators against either or both of these drugs. As can be seen in Table 2, quinolines (including CLQ, quinine, primaquine, and chinchonidine), acridines (including quinacrine, acridine, and acridine orange), and indole

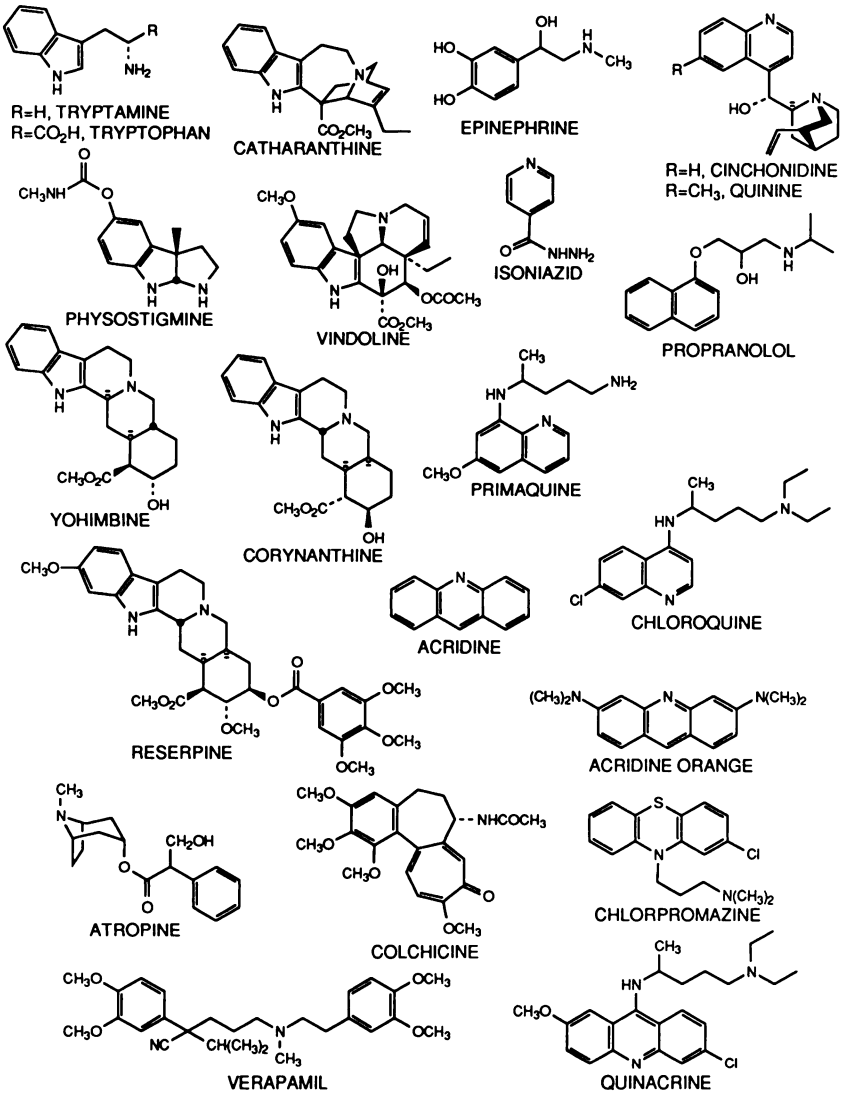

Fig. 1. Structures of most of the compounds tested for their ability to enhance the cytotoxicity of *Vinca* alkaloids in CEM/VLB$_{100}$ cells. These structures are of indole alkaloids, their precursors, and other "modulators."

alkaloids (including yohimbine, corynanthine, physostigmine, reserpine, and the two halves of the *Vinca* alkaloid molecule, vindoline and catharanthine) all caused substantial enhancement of VLB and/or VCR cytotoxicity in the CEM/VLB$_{100}$ cells. Since many of these agents are lysosomotropic compounds, we also examined several well known lysosomotropic amines to determine whether they, too, could potentiate the cytotoxic activity of the *Vinca* alkaloids. To our surprise, methylamine, suramin, and trypan blue did not greatly potentiate the cytotoxicity of VCR (Table 2). We conclude from these experiments that the ability to impair lysosomal function is probably not sufficient to cause an enhancement of *Vinca* alkaloid cytotoxicity. It should be noted, however, that we did not measure lysosomal function after treatment with these various agents.

Enhancement of DOX and VP-16 cytotoxicity. Because of the structures of most of the modulators, we wished to determine whether their activity was specific for *Vinca* alkaloids or extended to other anticancer drugs. Accordingly, we examined the effects of several of these agents on the cytotoxicity of DOX and VM-26. It can be seen in Table 3 that, at the concentrations used, vindoline, CLQ, and quinacrine enhanced the cytotoxicity of DOX in our CEM/VLB$_{100}$ cells, and quinacrine also potentiated the cytotoxicity of VM-26. We showed earlier that verapamil was most effective in enhancing the cytotoxicity of the *Vinca* alkaloids, but it also had some effectiveness in increasing DOX and VM-26 cytotoxicity (18). We conclude from the results in Table 3 that the effectiveness of some of these other modulators is not limited to the *Vinca* alkaloids.

Lipid solubilities of compounds tested for their ability or inability to modulate *Vinca* alkaloid cytotoxicity. Since the ability of a compound to enhance the cytotoxicity of VLB and VCR seemed to depend more on some characteristic chemical feature rather than its structure or biochemical action, and since hydrophobicity was an important feature of modulators in earlier studies (36, 37), we examined the lipid solubilities of some of the agents listed in Table 2 both at physiological pH and at an approximate lysosomal pH of 4.5. Our results are shown in Table 4. The compounds are separated according to whether they did or did not enhance the cytotoxicity of the *Vinca* alkaloids in multidrug-resistant cells. Several facts are evident from the data in this table. First, almost all of the modulators are lipid-soluble compounds at physiologic pH whereas, with the sole exception of colchicine, the agents that do not enhance *Vinca* alkaloid cytotoxicity are all water soluble

TABLE 1
Cytotoxicity of candidate modulators of multidrug resistance*

Compound	IC_{50} in CEM/VLB_{100} cells	Concentration used in combination with *Vinca* alkaloids	Effect on CEM/VLB_{100} growth
	M	*μM*	*% of control*
Quinolines			
CLQ	1.4×10^{-4}	50	78
Quinine	2.3×10^{-4}	100	84
Primaquine	2.1×10^{-4}	100	84
Cinchonidine	$>4.8 \times 10^{-4}$	100	94
Acridines			
Quinacrine	1.4×10^{-5}	5	76
Acridine	1.6×10^{-4}	50	75
Acridine orange	3.4×10^{-6}	2.5	59
Indole Alkaloids			
Vindoline	1.8×10^{-4}	50	89
Catharanthine	$>10^{-5}$	10	89
Yohimbine	$>2.5 \times 10^{-4}$	100	88
Corynanthine	$>2.5 \times 10^{-4}$	100	88
Physostigmine	$>10^{-3}$	500	77
Reserpine	$>10^{-5}$	5	89
Other Alkaloids			
Atropine	$>8.6 \times 10^{-4}$	500	85
Colchicine	8.1×10^{-7}	0.5	72
Amines			
Propanolol	1.7×10^{-4}	100	76
Chlorpromazine	2.3×10^{-5}	10	89
Tryptamine	2.2×10^{-3}	1000	79
D-Tryptophan	$>3.6 \times 10^{-3}$	1000	83
L-Tryptophan	$>1.0 \times 10^{-3}$	1000	100
Isoniazid	$>7.4 \times 10^{-3}$	1000	79
Suramin	8.8×10^{-5}	50	76
Epinephrine	1.5×10^{-3}	500	78
Trypan blue	1.1×10^{-4}	50	67
Methylamine	$>3.5 \times 10^{-2}$	10,000	87

* Cytotoxicity was assessed in a 48-hr growth-inhibition assay.

at this pH. Second, the lipid solubility of the modulators decreased at pH 4.5. Primaquine proved to be the exception here in that its lipid solubility did not change with pH. In this regard, this drug was more like the "non-modulators" whose (relative) lipid solubility did not change with decreasing pH. Finally, it should be noted that verapamil is very similar to the other modulators in terms of its lipid solubility at physiologic pH and its increase in water solubility at the lower pH.

Relationship between lipid solubility and ability to modulate *Vinca* alkaloid resistance. Based on the results in Tables 2 and 4, it appeared that there was a relationship between these two parameters. This proved to be the case, as seen in Fig. 2, which is a plot of the $\log_{10}$ of the minimally effective modulator concentration versus the partition coefficient of the modulator (Fig. 2, *solid circles*), expressed as the $\log_{10} P$ value. The correlation coefficient for this relationship, 0.79, proved to be highly significant ($p < 0.05$), indicating that lipid solubility is an important property for a compound, regardless of its structure, to enhance the cytotoxicity of *Vinca* alkaloids, thus substantiating earlier studies (36, 37). Non-modulators such as suramin, epinephrine, trypan blue, and colchicine, are included in Fig. 2 (*open circles*) at the concentrations that produced ~20–30% inhibition of CEM/VLB_{100} cell growth without potentiating *Vinca* alkaloid cytotoxicity. These compounds were not used in determining the line in this figure.

Lipid solubilities of drugs involved in the MDR phenotype. Based on the results in Fig. 2, it became of interest to examine the octanol/water partitioning of the various drugs against which the multidrug-resistant cells express resistance and cross-resistance. It is seen in Table 5 that, with the exception of DOX, all of the "natural product" drugs against which the CEM/VLB_{100} cells are resistant or cross-resistant are lipid soluble at physiological pH. It can also be seen in this table that, whereas VCR, VLB, and DNR exhibit pH-dependent decreases in lipid solubility, as did the modulators of MDR, DOX, colchicine, and VM-26 did not. These results suggest that pH-dependent drug "trapping," originally postulated by Tritton and colleagues (38), may play less of a role in the diminished retention of these latter agents than in the former ones.

Are there structural requirements for a compound to reverse MDR? Our results in Table 2, showing that both halves of the VLB molecule, vindoline and catharanthine, are capable of enhancing *Vinca* alkaloid cytotoxicity, suggest that there may be a structural requirement for a compound to "reverse" MDR. Moreover, the recent results of Cornwell *et al.* (39), showing competition by several calcium channel blockers for the binding of the photoactivatable analog of VLB to the MDR-associated protein, Pgp, permitted a similar conclusion. Our results in Table 3, however, suggest that if there is a structural requirement for this effect, it is not readily apparent in two-dimensional representations.

The space-filling properties of these compounds may be similar. One approach to determine whether such similarity exists in a series of molecules is to compare their molar refractivities. The molar refractivity of a substance reflects the fundamental property of molecular volume. Since dispersion forces undoubtedly play a role in all ligand-receptor interactions and the molar refractivity of a molecule is related to these forces, this parameter should be a simple measure of structural similarity. The molar refractivities (in $Å^3$) of the modulators, related anticancer agents, and selected non-modulators are given in Table 6. Note that the modulators have molar refractivities that fall within the range 9.19–16.03 $Å^3$. The anticancer agents involved in MDR have generally greater molar refractivities, ranging between 13.7–22.07 $Å^3$. These higher values for the anticancer drugs (compared to the modulators) most likely reflect the presence of additional functionalities required by the specific targets of anticancer drug action. The two lysosomotropic non-modulators bracket these two groups of compounds with extreme values of 1.01 and 30.01 $Å^3$. However, the molar refractivity of colchicine, another non-modulator, is 10.86, suggesting that the space-filling properties of a compound are probably less important than others in determining whether or not it will modulate MDR.

Another approach to assess similarity within a series of unrelated structures involves computer graphics analysis (40). Within the limited series of verapamil, vindoline and VLB, there exist both planar, aromatic domains and a tertiary nitrogen atom. If the tertiary nitrogen in the three compounds is assumed to be the first important common feature of this group, then it is possible to project structural homology within the 2-phenethylamine moieties of each structure. This aromatic domain represents a second region of homology (the dihydroindole substituent in vindoline and VLB and the 3, 4-dimethoxyphenylethane substituent in verapamil). The remaining aromatic domain in verapamil and VLB comprises the third structural

TABLE 2

Effects of indole alkaloids on *Vinca* Alkaloid Cytotoxicity in CEM/VLB$_{100}$ cells

Compound	Concentration[a]	VLB IC_{50}[b]	-Fold decrease	p Value	VCR IC_{50}[b]	-Fold decrease	p Value
	μM	$M \times 10^{-8}$			$M \times 10^{-7}$		
None		42			32		
Verapamil	10	1.9	22.1				
Quinolines							
CLQ	50	4.1[c]	10	<0.001	1.9[c]	16	0.01
Quinine	100	<1.3	>32	<0.001	NA[d]		
Primaquine	100	<1.6	>26	<0.001	NA		
Cinchonidine	100	2.3	18	<0.001	NA		
Acridines							
Quinacrine	5	3.6	12	<0.001	2.2	15	<0.001
Acridine	50	7.2	5.8	<0.01	NA	NA	
Acridine orange	2.5	NA	NA		1.9	16	<0.01
Indole Alkaloids							
Vindoline	50	<1.0	>42	<0.001	0.3	107	<0.001
Catharanthine	10	3.5	12	<0.001	3.2	10	<0.001
Yohimbine	100	4.1	10	<0.001	NA	NA	
Corynanthine	100	4.5	9.3	<0.001	NA	NA	
Physostigmine	500	2.4	17	<0.001	NA	NA	
Reserpine	5	<1.0	>42	<0.001	<0.24	>133	0.02
Other Alkaloids							
Atropine	500	6.2	6.8	<0.001	NA	NA	
Colchicine	0.05	NA	NA		12	2.6	0.02
Amines							
Propanolol	50	3.5	12	<0.001	NA	NA	
Chlorpromazine	10	4.0	11	<0.001	NA	NA	
Tryptamine	1,000	NA	NA		5	6.4	0.02
D-Tryptophan	1,000	NA	NA		18	1.8	0.22
L-Tryptophan[e]	1,000	42	1.0	NA	NA	NA	
Isoniazid	1,000	NA	NA		18	1.8	0.18
Suramin	50	NA	NA		15	2.1	0.06
Epinephrine	500	NA	NA		29	1.1	0.30
Trypan blue	50	NA	NA		14	2.2	0.04
Methylamine	10,000	NA	NA		29	1.1	0.06

[a] Modulator concentrations were slightly toxic to nontoxic.
[b] IC_{50} concentration that inhibits 48-hr cell growth by 50%.
[c] Mean of three to five experiments.
[d] NA, not assayed.
[e] One experiment.

TABLE 3

Effects of "modulators" on multidrug resistance in CEM/VLB$_{100}$ cells

Compound	Concentration	DOX IC_{50}[a]	-Fold decrease	VM-26 IC_{50}[a]	-Fold decrease
	μM	$M \times 10^{-8}$		$M \times 10^{-8}$	
None		290		200	
Vindoline	50	24	12.1	110	1.8
Catharanthine	10	210	1.4	NT[b]	
Quinacrine	5	150	1.9	33	6.1
CLQ	50	74	3.9	NT	

[a] Concentration of drug that inhibits cell growth by 50% in 48 hr. Values are the means of four to five experiments.
[b] NT, not tested.

domain (which is absent in vindoline). Using these three sites as regions of structural homology, the structures of each were refined using a molecular mechanics algorithm in such a way as to not allow high energy interactions (1,2 and 1,3 eclipsing interactions). The results of these calculations are presented in Fig. 3. It is important to note that vindoline, lacking the third homologous domain, is a less effective modulator than verapamil, which expresses all three domains. Indeed, the planar aromatic rings and nitrogen atoms are common features shared by all modulators as well as the *Vinca* alkaloids and anthracyclines. While this result suggests a basis for the verapamil effect, a parallel structural analysis has yet to be applied to other modulators of MDR, including other calcium channel blockers, indole alkaloids, and CLQ.

These computational studies were not meant to deduce an accurate "bound" conformation of each molecule examined but, rather, to test for non-obvious elements of structural commonality in an energetically reasonable way. Since the starting geometries of each molecule were refined with energy minimization routines using a single molecular mechanics force field, all structural parameters (bond lengths and angles) must be internally consistent. The final step in this analysis—suggesting structural commonality within the series—varies all torsional angles that are considered rotatable (i.e., not in a ring). The final subset of these conformations was then further refined using molecular mechanics. Because of the high degree of conformational flexibility presented by an acyclic molecule such as verapamil, this structure was subjected to the more rigorous quantum mechanical method, AM1. This additional step confirmed the presence of the final verapamil conformation on an energy surface. The structure did not change, indicating that it may reasonably be considered as an allowed conformational minimum.

TABLE 4

Partition coefficients of modulators and non-modulators of *Vinca* alkaloid cytotoxicity in CEM/VLB$_{100}$ cells

Drug	$Log_{10} P$[a]		$\Delta Log_{10} P$[b]
	pH 7.4	pH 4.5	
Modulator			
Verapamil	4.8[c]	0.7	4.1
Vindoline	≥2.5	0.7	≥1.8
Catharanthine	3.1[c]	NA[d]	
Chlorpromazine	≥3.8	1.6	≥2.2
Quinine	2.1	0.6	1.5
Quinacrine	1.9	−1.7	3.6
Propranolol	1.2	0.3	0.9
CLQ	0.6	−2.0	2.6
Primaquine	0.2	0.2	0
Atropine	1.83[c]	NA	
Tryptamine	−0.92	NA	
Physostigmine	0.17[c]	NA	
Non-modulator			
Colchicine	1.1	1.1	0
Methylamine	−0.57[c]	NA	
Isoniazid	−0.50[c]	NA	
Tryptophan	−1.04[c]	NA	
Epinephrine	−2.8	−2.7	0.1
Suramin	−3.5	−3.4	0.1
Trypan blue	−2.2	NA	

[a] The log_{10} of the octanol/water partition coefficient (P) was determined as described under Materials and Methods.

[b] The change in the $log_{10} P$ was calculated by subtracting the $log_{10} P$ at pH 4.5 from that at pH 7.4.

[c] Data from Ref. 27.

[d] NA, not assayed.

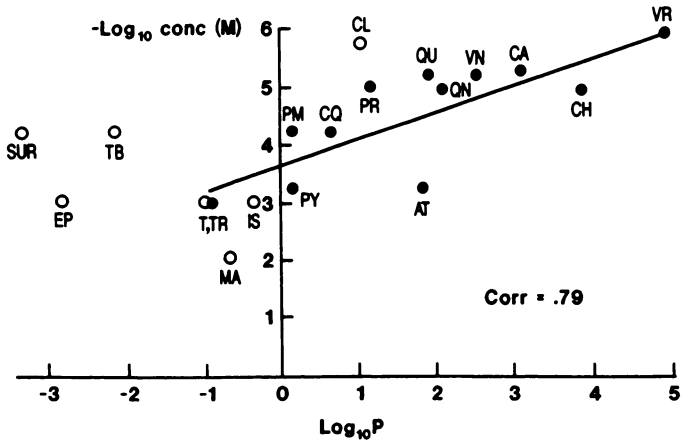

Fig. 2. Relationship of the effective or toxic concentrations of "modulators" and their lipid solubilities. The minimum effective concentrations of compounds that enhanced *Vinca* alkaloid cytotoxicity in CEM/VLB$_{100}$ cells (●) are plotted against their partition coefficients. The compounds are identified by the letter code next to the symbols. Agents that did not modulate *Vinca* alkaloid cytotoxicity (○) are also shown. The concentrations of the "non-modulators" inhibited cell growth by ~20–30% without potentiating the cytotoxicity of the *Vinca* alkaloids. The correlation coefficient (*Corr.*) and the *line* in the figure are for the modulators only. Only one modulator, tryptamine, was water soluble, and only one non-modulator, colchicine, was lipid soluble. The former compound was only moderately effective at mM concentrations. The latter compound caused more than 50% inhibition of cell growth at 1 μM, and lower concentrations did not enhance *Vinca* alkaloid cytotoxicity. *VR*, verapamil; *CH*, chlorpromazine; *CA*, catharanthine; *VN*, vindoline; *QN*, quinine; *QU*, quinacrine; *AT*, atropine; *PM*, primaquine; *CL*, colchicine; *CQ*, chloroquine; *PR*, propanaolol; *PY*, physostigmine; *IS*, isoniazid; *MA*, methylamine; *TR*, tryptamine; *T*, tryptophan; *TB*, trypan blue; *EP*, epinephrine; *SUR*, suramin.

TABLE 5

Octanol/water partition coefficients of anticancer agents

Drug	$Log_{10} P$[a]		$\Delta Log_{10} P$
	pH 7.4	pH 4.5	
VCR	2.2	−0.7	2.9
VLB	>4.2	0.2	>4.0
DNR	0.3	−0.2	0.5
DOX	−0.6	−0.8	0.2
Teniposide	2.0	1.8	0.2
Colchicine	1.1	1.1	0

[a] The log_{10} of the octanol/water partition coefficient (P) was determined as described under Materials and Methods.

[b] The change in the $Log_{10} P$ was obtained by subtracting the $Log_{10} P$ at pH 4.5 from that at pH 7.4.

TABLE 6

Calculated molar refractivities of some anticancer drugs and modulators and non-modulators of *Vinca* alkaloid cytotoxicity[a]

Drug	Molar refractivity
	Å^3
Anticancer Agents	
VCR	22.07
VLB	22.04
Teniposide	15.76
DOX	13.17
DNR	13.17
Modulators	
Reserpine	16.03
Verapamil	13.15
Vindoline	12.02
Catharanthine	9.75
CLQ	9.57
Quinine	9.48
Nifedipine	9.19
Non-modulators	
Suramin	30.41
Colchicine	10.86
Methylamine	1.01

[a] Molar refractivities were calculated as described under Materials and Methods.

Discussion

The results of the present study indicate that a variety of compounds have the ability to enhance the cytotoxic activity of *Vinca* alkaloids in the multidrug-resistant cell line, CEM/VLB$_{100}$. These compounds have in common certain chemical and physical features, including hydrophobicity and cationic charge. Earlier studies have shown that detergents (37) and hydrophobic compounds (36) were able to enhance drug cytotoxicity in multidrug-resistant rodent cells. More recent investigations revealed that noncytotoxic analogs of *Vinca* alkaloids (41) and anthracyclines (42) could potentiate the cytotoxicity of VCR and DOX, respectively, in murine multidrug-resistant cells. The present studies evolved from our initial observations with verapamil (18) and CLQ (19), and suggested that a study of similar agents or of similar classes of drugs might yield other modulators of MDR, or at least of *Vinca* alkaloid cytotoxicity.

CLQ, a quinoline, is a synthetic indole alkaloid that is related to acridine compounds by an anthranilic acid intermediate. We showed here that several quinoline and acridine compounds can potentiate the cytotoxicity of *Vinca* alkaloids as well as DOX and VM-26 in the CEM/VLB$_{100}$ cell line. The quinoline, primaquine, is of interest in that it is an 8-amino compound whereas the others are 4-amino derivatives. The ability of primaquine to enhance VLB cytotoxicity suggests that the position of the side chain of the quinoline ring is not critical

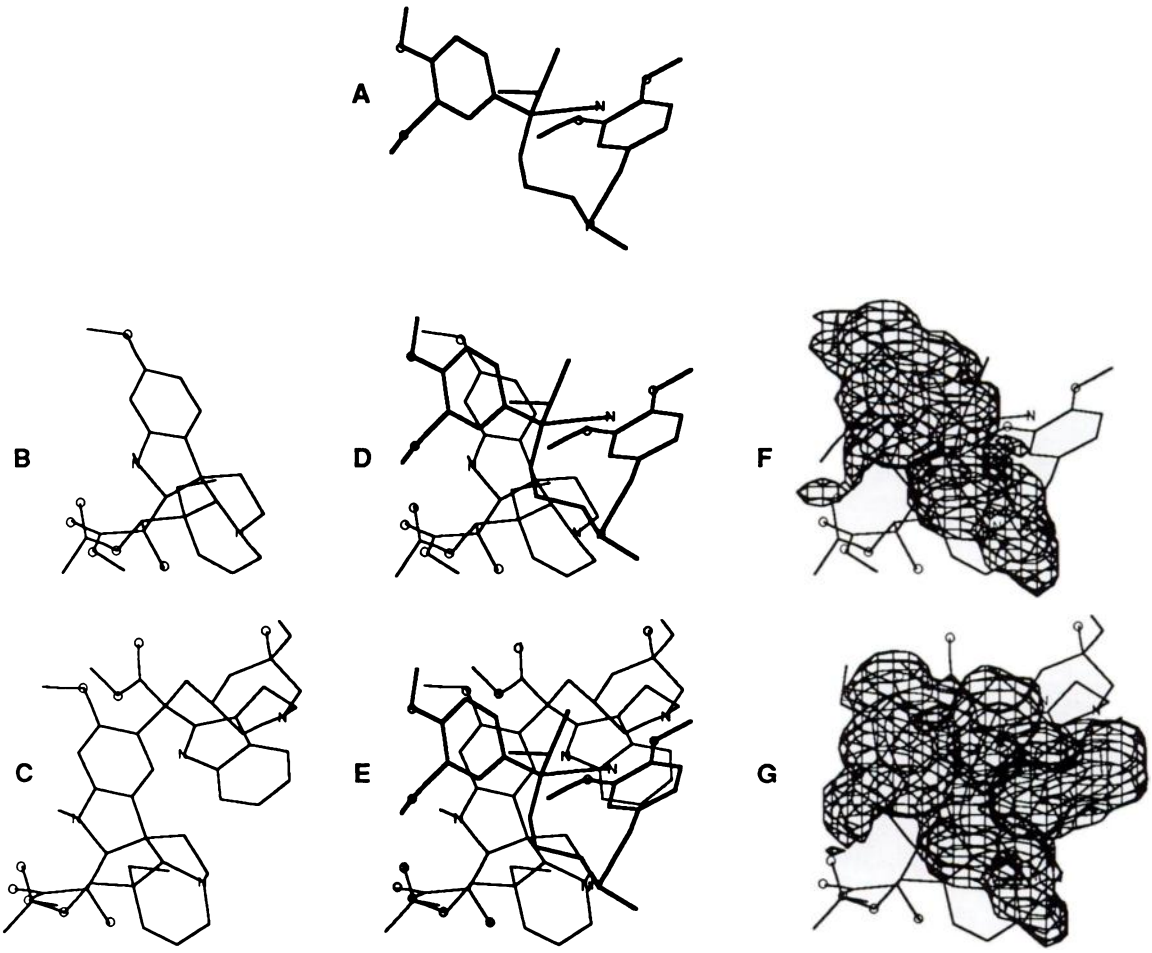

Fig. 3. Computer-generated three-dimensional structures of verapamil (A), vindoline (B), and VLB (C). Structures D and E are superimpositions of the verapamil/vindoline and verapamil/VLB structures, respectively. Structures F and G represent the common Van der Waals volume elements of D and E, respectively.

for this effect. That quinacrine, acridine orange, and acridine all potentiate *Vinca* alkaloid cytotoxicity suggests that the planar, aromatic part of the acridine structure may be more important than the side groups with respect to its ability to modulate drug cytotoxicity.

The apparent lack of importance of the side groups on quinolines and acridines indicates that some more general feature of the indole alkaloids is responsible for their ability to enhance *Vinca* alkaloid cytotoxicity. We thus tested other cationic compounds containing aromatic rings in our system. We found that atropine, chlorpromazine, and propranolol were weak modulators, suggesting that indole alkaloids represent but a subclass of compounds that possess this activity. Although chlorpromazine and propranolol are structurally similar to the quinolines, atropine, which is a pyrrolidine alkaloid derived from ornithine (43), is structurally unrelated to these agents but is nevertheless a weak modulator in our system, but only at very high concentrations. Thus, we found that most of the modulators of *Vinca* alkaloid cytotoxicity could be classified as alkaloids, a heterogeneous group of nitrogen heterocycles. Most alkaloids except colchicine are weakly basic and amphipathic. It was these properties that appeared to separate modulators from non-modulators in our system.

Most of the hydrophilic amines that we tested did not significantly enhance *Vinca* alkaloid cytotoxicity. Although these compounds had some effect on VCR cytotoxicity (Table 2), the enhancement was not the same order of magnitude as that of the alkaloids. We believe this is due to an additive toxicity of the amine and VCR. For this reason, a 5-fold increase in *Vinca* alkaloid cytotoxicity was chosen as significant. D-Tryptophan, isoniazid, and epinephrine were inactive, whereas tryptamine, the precursor of the indole alkaloids, was active, but only at mM concentrations; this latter activity most likely represents some nonspecific effect of the high concentration of drug, rather than any specific, pharmacologically relevant action. These compounds all have aromatic rings and protonatable nitrogens, but their inactivity indicates that these structures alone are not sufficient to modulate *Vinca* alkaloid cytotoxicity.

Methylamine, epinephrine, suramin and trypan blue are also hydrophilic compounds that do not enhance *Vinca* alkaloid cytotoxicity. These compounds are lysosomotropic agents, as are CLQ, quinine, quinacrine, acridine, and acridine orange (44–46), compounds that do enhance *Vinca* alkaloid cytotoxicity, suggesting that lysosomotropic activity of a compound per se is not sufficient to modulate MDR. However, suramin and trypan blue, which accumulate in lysosomes by "piggyback" endocytosis (45), are not basic compounds and therefore do not alter lysosomal pH. Moreover, these two agents are highly water

soluble and do not readily diffuse across membranes or interact with lipophilic proteins. Conversely, methylamine is a water-soluble lysosomotropic agent that does alter lysosomal pH (47). Its inactivity may be due to its relatively slow passage across cell membranes and slow entry into lysosomes (45, 47). In addition, compounds like methylamine might not be as easily "trapped" by a proton pump as would lipophilic amphipathic compounds (45, 47).

We found that there was a strong relationship between the lipid solubility of a compound and its ability to modulate *Vinca* alkaloid cytotoxicity in our multidrug-resistant cells (Fig. 2). Although similar findings regarding hydrophobic compounds (36, 37) and noncytotoxic analogs of *Vinca* alkaloids and anthracyclines (41, 42) were made earlier using rodent cells, we are not aware of another systematic survey of the type and scope presented here. In the present study, the *Vinca* alkaloids and the most effective modulators (verapamil, quinine, and vindoline) have similar octanol/water partition coefficients at the two pH values examined, suggesting that the anticancer drugs themselves might be capable of modulating their own cytotoxicity. In this regard, early work from Ling's laboratory (48) showed that colchicine uptake and cytotoxicity in Chinese hamster ovary cells could be enhanced by 10 μM VCR, a concentration that is close to that predicted by Fig. 2. Moreover, colchicine is highly toxic to the cells at 100 μM, which is less than its predicted concentration necessary to modulate *Vinca* alkaloid cytotoxicity. Finally, although DOX is relatively water soluble compared to the other anticancer drugs, a considerable amount is still able to partition into the lipid phase, suggesting that it, too, might be capable of modulating the cytotoxicity of other agents.

We also found that, in addition to lipid solubility, effective modulators have other features in common: they are cationic compounds with similar molar refractivities. The three non-modulators examined covered a wider range of molar refractivities than did the modulators. Although we have not examined anionic or neutral hydrophobic compounds, the cationic modulators, with the exception of primaquine, all became more water soluble with a decrease in pH. The spectral properties of primaquine at pH 4.5 differed from those at pH 7.4,[2] suggesting a change in its protonation state. The importance of the cationic properties is also reflected in the behavior of the anticancer drugs themselves. We have shown that verapamil (18), CLQ (19), vindoline, catharanthine, and quinacrine (Table 3) are most effective in enhancing the cytotoxicity of anticancer drugs in multidrug-resistant cells in the following order: *Vinca* alkaloids > DOX ≥ VM-26. This suggests that the protonatable nitrogen of the anticancer drug itself may also be an important determinant in either its action or modulation.

It is possible that some modulators may share a common receptor with some of the anticancer agents whose activity is enhanced (12, 21, 39, 41). Computer graphics analysis suggests that a planar aromatic domain and a nitrogen atom are common features of verapamil, vindoline, and VLB. It is possible for two aromatic domains and a nitrogen atom to share a common volume element in verapamil and VLB, suggesting that these are important structural components of the MDR-modifying effect. Recent studies demonstrating that verapamil and other "calcium channel blockers" are capable of inhibiting the binding of the photoactivatible analog of VLB to Pgp (21, 39) support this notion. Studies are currently in progress in our laboratories to determine whether other modulators share structural features with the drugs involved in the MDR phenotype and whether they can block the binding of VLB to Pgp.

Based on our studies, we conclude that lipid solubility, cationic charge, molar refractivity, and perhaps structural similarity (41, 42) are important properties for compounds to be effective modulators of MDR. The compounds tested in the present study were structurally quite different, but the best modulators shared these properties. Finally, our studies provide direction for the development of new compounds that may yield insights into the nature of the receptor for the modulator. Moreover, while many of the compounds found to be modulators of MDR in this study are clinically available, they have other actions. A significant aspect of our studies is that we may be able to identify the pharmacophore(s) necessary to circumvent MDR and thus design agents whose major and perhaps only pharmacologic activity is the reversal of MDR.

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References

1. Beck, W. T. The cell biology of multiple drug resistance. *Biochem. Pharmacol.* **36**:2879–2887 (1987).
2. Pastan, I., and M. Gottesman. Multiple-drug resistance in human cancer. *N. Engl. J. Med.* **316**:1388–1393 (1987).
3. Riordan, J. R., and V. Ling. Genetic and biochemical characterization of multidrug resistance. *Pharmacol. Ther.* **28**:51–75 (1985).
4. Beck, W. T. Cellular pharmacology of Vinca alkaloid resistance and its circumvention. *Adv. Enzyme. Regul.* **22**:207–227 (1984).
5. Kaye, S., and S. Merry. Tumour cell resistance to anthracyclines—a review. *Cancer. Chemother. Pharmacol.* **14**:96–103 (1985).
6. Akiyama, S.-i., A. Fojo, J. A. Hanover, I. Pastan, and M. M. Gottesman. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somat. Cell. Mol. Genet.* **11**:117–126 (1985).
7. Beck, W. T., M. C. Cirtain, and J. L. Lefko. Energy-dependent reduced drug binding as a mechanism of *Vinca* alkaloid resistance in human leukemic lymphoblasts. *Mol. Pharmacol.* **24**:485–492 (1983).
8. Fojo, A., S.-i. Akiyama, M. M. Gottesman, and I. Pastan. Reduced drug accumulation in multiply drug resistant human KB carcinoma cell lines. *Cancer Res.* **45**:3002–3007 (1985).
9. Gros, P., J. Croop, and D. Housman. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell* **47**:371–380 (1986).
10. Chen, C. J., J. E. Chin, K. Ueda, D. B. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson. Internal duplication and homology with bacterial transport proteins in the *mdr-1* gene from multidrug-resistant human cells. *Cell* **47**:381–389 (1986).
11. Gerlach, J. H., J. A. Endicott, P. F. Juranka, G. Henderson, F. Sarangi, K. L. Deuchars, and V. Ling. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature (Lond.)* **324**:485–489 (1986).
12. Safa, A. R., C. J. Glover, M. B. Meyers, J. L. Biedler, and R. L. Felsted. Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug resistant cells. *J. Biol. Chem.* **261**:6137–6140 (1986).
13. Bell, D. R., J. H. Gerlach, N. Kartner, R. N. Buick, and V. Ling. Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. *J. Clin. Oncol.* **3**:311–315 (1985).
14. Fojo, A. T., K. Ueda, D. J. Slamon, D. G. Poplack, M. M. Gottesman, and I. Pastan. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. USA* **84**:265–269 (1987).
15. Gerlach, J. H., D. R. Bell, C. Karakousis, H. K. Slocum, N. Kartner, Y. M. Rustum, V. Ling, and R. M. Baker. P-Glycoprotein in human sarcoma: evidence for multidrug resistance. *J. Clin. Oncol.* **5**:1452–1460 (1987).
16. Tsuruo, T., H. Iida, S. Tsukagoshi, and Y. Sakurai. Increased accumulation of vincristine and adriamycin in drug resistant P-388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. *Cancer Res.* **42**:4730–4733 (1982).
17. Ganapathi, R., and D. Grabowski. Enhancement of sensitivity to adriamycin

[2] J. M. Zamora, unpublished observation.

in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine. *Cancer Res.* **43**:3696–3699 (1983).
18. Beck, W. T., M. C. Cirtain, A. T. Look, and R. A. Ashmun. Reversal of Vinca alkaloid resistance but not multiple drug resistance in human leukemic cells by verapamil. *Cancer Res.* **46**:778–784 (1986).
19. Zamora, J. M., and W. T. Beck. Chloroquine enhancement of anticancer drug cytotoxicity in drug resistant human leukemia cells. *Biochem. Pharmacol.* **35**:4303–4310 (1986).
20. Shiraishi, N., S.-i Akiyama, M. Kobayashi, and M. Kuwano. Lysosomotropic agents reverse multiple drug resistance in human cancer cells. *Cancer Lett.* **30**:251–259 (1986).
21. Cornwell, M. M., A. R. Safa, R. L. Felsted, M. M. Gottesman, and I. Pastan. Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150-170 kDa protein detected by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA* **83**:3847–3850 (1986).
22. Cornwell, M. M., M. M. Gottesman and I. Pastan. Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J. Biol. Chem.* **261**:7921–7928 (1986).
23. Yamashita, N., H. Hamada, T. Tsuruo, and E. Ogata. Enhancement of voltage-gated Na^+ channel current associated with multidrug resistance in human leukemia cells. *Cancer Res.* **47**:3736–3741 (1987).
24. Lee, S. C., C. Deutsch, and W. T. Beck. Comparison of ion channels in multidrug-resistant and -sensitive human leukemic cells. *Proc. Natl. Acad. Sci. USA*, **85**:2019–2023 (1988).
25. Akiyama, S., K. Tomita, and M. Kuwano. The effect of calcium antagonists on the proteolytic degradation of low density lipoprotein in HeLa cells. *Exp. Cell Res.* **158**:192–204 (1985).
26. Akiyama, S.-i., M. M. Gottesman, J. A. Hanover, D. J. P. Fitzgerald, M. C. Willingham, and I. Pastan. Verapamil enhances the toxicity of conjugates of epidermal growth factor with *Pseudomonas* exotoxin and antitransferrin receptor with *Pseudomonas* exotoxin. *J. Cell. Physiol.* **120**:271–279 (1984).
27. Zamora, J. M., M. C. Cirtain, and W. T. Beck. Physical-chemical requirements for compounds that modulate multiple drug resistance (MDR). *Proc. Am. Assoc. Cancer Res.* **28**:1319 (1987).
28. Beck, W. T., T. J. Mueller, and L. R. Tanzer. Altered surface membrane glycoproteins in Vinca alkaloid-resistant human leukemic lymphoblasts. *Cancer Res.* **39**:2070–2076 (1979).
29. Beck, W. T., M. C. Cirtain, M. K. Danks, R. L. Felsted, A. R. Safa, J. S. Wolverton, D. P. Suttle, and J. M. Trent. Pharmacological, molecular, and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. *Cancer Res.* **47**:5455–5460 (1987).
30. Conter, V., and W. T. Beck. Acquisition of multiple drug resistance by CCRF-CEM cells selected for different degrees of resistance to vincristine. *Cancer Treat. Rep.* **68**:831–839 (1984).
31. Owellen, R. J., D. W. Donigian, C. A. Harke, and F. O. Hains. Correlation of biologic data with physico-chemical properties among the Vinca alkaloids and their congeners. *Biochem. Pharmacol.* **26**:1213–1219 (1977).
32. Leo, A. Parameter and structure-activity data bases: management for maximum utility. *Environ. Health Perspect.* **61**:279–283 (1985).
33. Jones, N. D., S. Rolski, and J. K. Swartzendruber. The crystal structure of vinzolidine. *Acta Crystallogr. Sect. A Cryst. Phys. Diffr. Theor. Gen. Crystallogr.* **40**(Suppl. 3):3–14 (1984).
34. Allinger, N. L. Conformational analysis. 130. MM2. A hydrocarbon force field utilizing V_1 and V_2 torsional terms. *J. Am. Chem. Soc.* **99**:8127–8134 (1977).
35. Dewar, M. J. S., E. G. Zoebisch, E. F. Healy, and J. J. P. Stewart. AM1: a new general purpose quantum mechanical model. *J. Am. Chem. Soc.* **107**:3902–3909 (1985).
36. Biedler, J. L., and H. Riehm. Potentiation of drug effect by Tween 80 in Chinese hamster cells resistant to actinomycin D and daunomycin. *Cancer Res.* **32**:1195–1200 (1972).
37. Bech-Hansen, N. T., J. E. Till, and V. Ling. Pleiotropic phenotype of colchicine-resistant CHO cells: cross-resistance and collateral sensitivity. *J. Cell. Physiol.* **88**:23–32 (1976).
38. Siegfried, J. M., T. Burke, and T. R. Tritton. Cellular transport of anthracyclines by passive diffusion. *Biochem. Pharmacol.* **34**:593–598 (1985).
39. Cornwell, M. M., I. Pastan, and M. M. Gottesman. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J. Biol. Chem.* **262**:2166–2170 (1987).
40. Wender, P. A., K. F. Koehler, N. A. Sharkey, M. L. Del'Aquila, and P. Blumberg. Analysis of the phorbol ester pharmacophore on protein kinase C as a guide to the rational design of new classes of analogs. *Proc. Natl. Acad. Sci. USA* **83**:4214–4218 (1986).
41. Inaba, M., and K. Nagashima. Non-antitumor Vinca alkaloids reverse multidrug resistance in P388 leukemia cells *in vitro*. *Jpn. J. Cancer Res. (Gann)* **77**:197–204 (1986).
42. Inaba, M., K. Nagashima, Y. Sakurai, M. Fukui, and Y. Yanagi. Reversal of multidrug resistance by non-antitumor anthracycline analogs. *Gann* **75**:1049–1052 (1984).
43. Robinson, T. *Alkaloids. The Organic Constituents of Higher Plants. Their Chemistry and Interrelationships.* Cordus Press, North Amherst MA, 278–292 (1975).
44. Dean, R. T., W. Jessup, and C. R. Roberts. Effects of exogenous amines on mammalian cells with particular reference to membrane flow. *Biochem. J.* **217**:27–40 (1984).
45. DeDuve, C., T. DeBarsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof. Lysosomotropic agents. *Biochem. Pharmacol.* **23**:2495–2531 (1974).
46. Allison, A. C., and M. R. Young. Vital staining and fluorescence microscopy of lysosomes, in *Lysosomes in Biology and Pathology 2* (J. T. Dingle and H. B. Fell, eds.) Elsevier-North Holland Publishing, Amsterdam, 600–628 (1969).
47. Ohkuma, S., and B. Poole. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA* **75**:3327–3331 (1978).
48. Carlsen, S. A., J. E. Till, and V. Ling. Modulation of membrane drug permeability in Chinese hamster ovary cells. *Biochim. Biophys. Acta* **455**:900–912 (1976).
49. Safa, A. R., C. J. Glover, J. L. Sewell, M. B. Meyers, J. L. Biedler, and R. L. Felsted. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J. Biol. Chem.* **262**:7884–7888 (1987).

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