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Non-glucocorticoid steroid analogues (21-aminosteroids) sensitize multidrug resistant cells to vinblastine

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Abstract. Several members of a group of compounds developed to treat stroke and trauma of the central nervous system are shown also to reverse multidrug resistance in human KB-V1 cells. The most potent reversal agents studied are 21-aminosteroid derivatives (lazaroids), tirilazad mesylate (tirilazad, U-74006F) and U-74389F. Tirilazad sensitizes resistant human cells (KB-V1) to killing by vinblastine by 66-fold, but does not change the sensitivity of the nonresistant parental line, KB-3-1, to vinblastine. KB-V1 cell membranes have high levels of P-glycoprotein, a protein that acts as an efflux pump and is thought to be the major cause of multidrug resistance in these cells. Tirilazad inhibits the photoaffinity labeling of P-glycoprotein with [^{3}H]azidopine in KB-V1 cells more effectively than does verapamil, a standard reversal agent. In addition, tirilazad causes the increased accumulation of [^{3}H]vinblastine in multidrug resistant KB-V1 cells. Studies of the resistance reversal potential of related compounds suggest that the complex amine portion of tirilazad is important for its reversal activity, while the steroid portion is less important.

Introduction

Multidrug resistance has been shown to be a significant impediment to cancer chemotherapeutic treatment in patients. Strategies to overcome this resistance would be of great advantage to patients that can benefit from effective chemotherapy. We report here the identification of a novel 21-aminosteroid compound, tirilazad, and related compounds that appear to be effective in reversing multidrug resistance in vitro.

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Multidrug resistance in vitro has been modeled by the study of resistance of cell lines that have been selected for growth in high levels of agents such as colchicine, colcemid, taxol, vinblastine or Adriamycin. Although these resistant cells have been selected for resistance to one agent, they are often cross-resistant to other hydrophobic natural product cytotoxic agents. Several molecular mechanisms may account for increases in drug resistance (for reviews see [7, 31]). It has been shown in in vitro studies that many instances of multidrug resistance (MDR) are correlated with amplified expression of a 170 kDa membrane protein, P-glycoprotein [24]. The P-glycoprotein is thought to act as an efflux pump, whose role is to excrete xenobiotics from cells. It has also been shown that transfection of the gene encoding for P-glycoprotein confers MDR [15]. Cells from many human tumors have elevated levels of P-glycoprotein [11, 14, 19]. These increased levels have been shown to correlate in some cases with drug resistance and negative response to chemotherapy [9, 21].

Recently, several laboratories have reported studies on diverse agents such as verapamil and cyclosporin that will sensitize multidrug resistant cells to the action of drugs such as vinblastine (for reviews see [12, 13, 34]). The range of modulating molecules appears to be quite wide. However, several features seem to be common to many compounds and may be of importance in predicting whether a compound can act as a sensitizer. Many sensitizing compounds are amphipathic and hydrophobic [29, 37]. There is a good correlation between relatively high lipophilicity [17, 33] and ability to cause MDR reversal. Zamora et al. have shown that heterocyclic amine compounds that are cationic, relatively lipophilic and have similar molar refractivities are fairly likely to have the ability to act as sensitizing compounds. They suggest that important characteristics of sensitizing compounds are two planar aromatic domains, a tertiary basic nitrogen atom and their relative disposition [23, 37]. Another important feature may be the ability of the compound to modulate binding on the P-glycoprotein molecule [27, 34, 36], as shown by altered binding of photoaffinity labels. Several of the

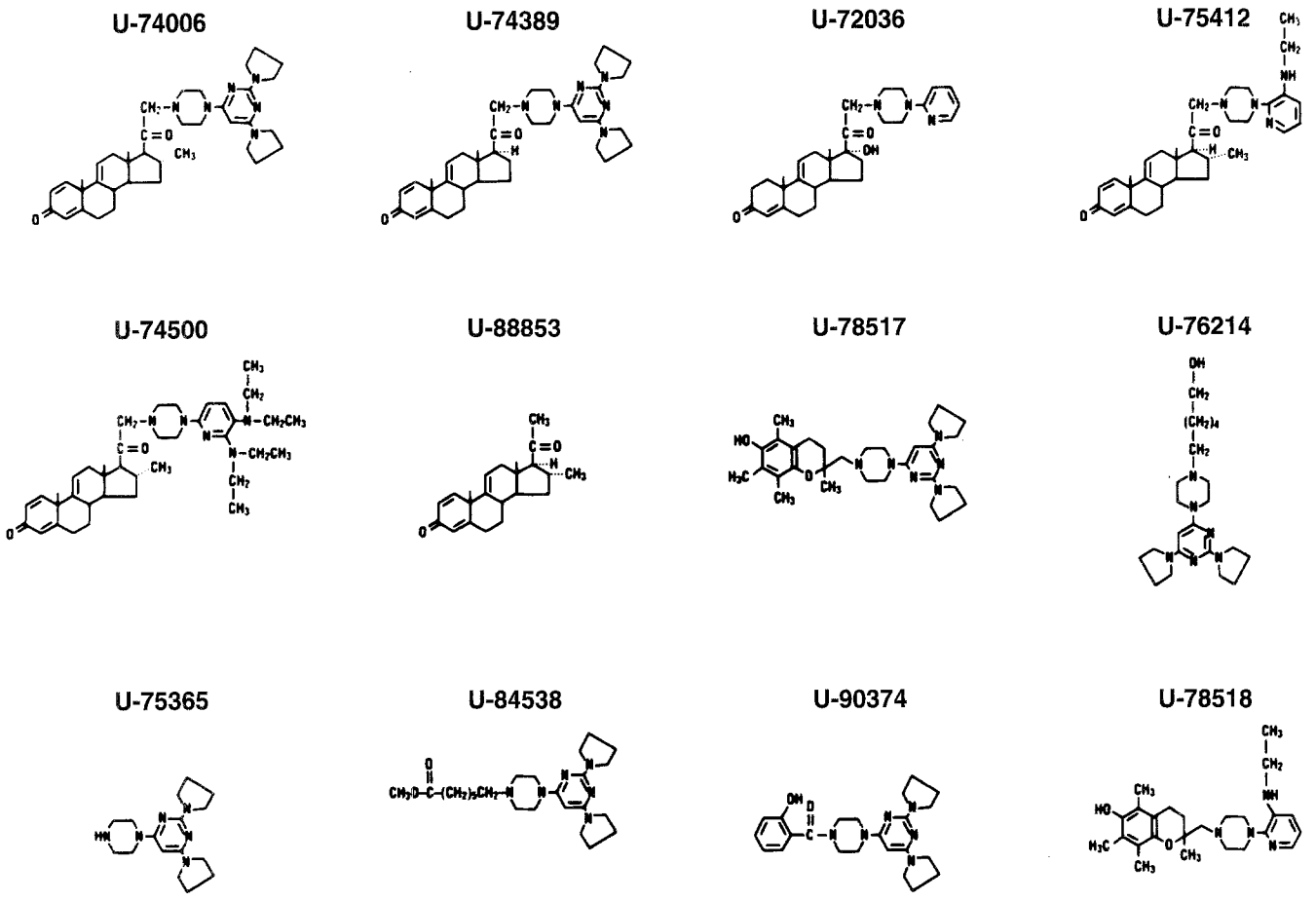

Fig. 1. Chemical structures of compounds described in text. U-74006, tirilazad

reversal compounds appear to act by competing for binding to the P-glycoprotein. However, not all reversal compounds have all these characteristics. For example, phenothiazines do not inhibit binding of [^{125}I]NASV, a vinblastine photoaffinity analogue [1]. This suggests that multiple functional classes of reversal agents may exist.

In this report we have studied the reversal properties of the non-glucocorticoid steroid (21-aminosteroid) tirilazad mesylate (tirilazad, U-74006F) and the closely related compound U-74389F. These novel compounds (lazaroids) have been developed primarily as agents to prevent free-radical damage in cells [18] and have not been previously shown to sensitize multidrug resistant cells. We show here that these compounds can sensitize multidrug resistant human KB-V1 cells to the toxic effects of vinblastine while having no effect on the parental sensitive KB-3-1 cells. KB-V1 cells have been shown to have high levels of P-glycoprotein [28].

The 21-aminosteroid compound tirilazad mesylate was the most effective compound of the group described here in its ability to sensitize the multidrug resistant cells to vinblastine and is at least as effective a sensitizer as verapamil, which has previously been shown to be a good sensitizer in both in vitro studies and in vivo mouse studies [30]. Verapamil has the disadvantage of causing cardiac toxicity to humans at doses that would be required for in vivo sensitization of cancer cells. While the toxicities of U-74006F in humans are under test, studies in other animals suggest relatively low toxicity (unpublished data). The results presented here suggest that some of the 21-aminosteroid analogues might have the potential to be used in anti-cancer therapy to sensitize cancer cells to traditional cytotoxic drugs that are P-glycoprotein substrates.

Materials and methods

Chemicals. [^{3}H]Azidopine (52 Ci/mmol) and [^{3}H]vinblastine (16 Ci/mmol) were purchased from the Amersham Corp. Verapamil and vinblastine were obtained from Sigma Chemicals. Tirilazad mesylate (tirilazad; U-74006), U-78517, U-72036, U-75412, U-78518, U-74500, U-74389, U-88853, U-76214, U-75365, U-84538F, U-90374, and their respective salts (U-74006F, U-78517F, U-72036E, U-75412E, U-78518E, U-74500A, U-74389F) were synthesized at the Upjohn Company. Compounds were dissolved in DMSO. Final DMSO concentrations did not affect the viability of cells.

Cell lines and growth. The wild-type KB-3-1 cell line was derived from a single clone of human KB epidermoid carcinoma cells after two subclonings [1]. The multidrug-resistant KB-V1 line was derived from the

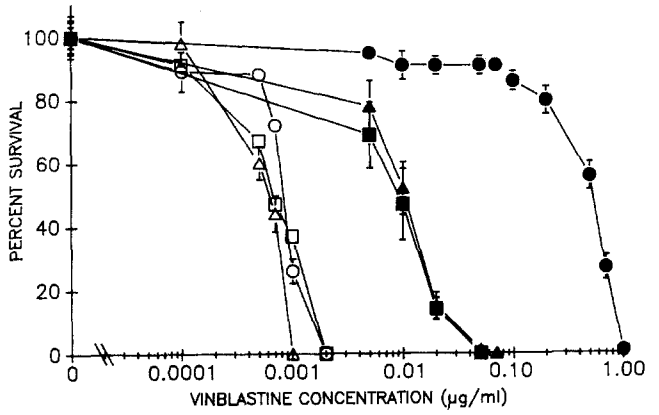

Fig. 2. Effect of verapamil or tirilazad on resistance of KB cells to vinblastine. Survival of KB-3-1 cells (○, □, △) and KB-V-1 cells (●, ■, ▲) in the presence of varying concentrations of vinblastine alone (○, ●) or vinblastine and 5 μM verapamil (△, ▲) or vinblastine and 5 μM tirilazad (□, ■) was examined by colony formation assay (see "Materials and methods"). Percent survival was obtained by normalizing number of colonies in the vinblastine-treated wells to those in the absence of vinblastine. Values are the mean of 1-6 trials, performed in duplicate. Bars indicate standard essor od the mean (SEM)

KB-3-1 line through selection in increasing concentrations of vinblastine [28]. Cells were maintained in monolayer culture as described. Periodically, the cell lines were tested for the presence of mycoplasma infections and were always found to be free of infection.

Viability Tests. Cell viability was measured by colony formation after 9 days growth. KB-3-1 or KB-V1 cells were plated in duplicate at 300 cells/well in 24-well plates. Drugs were added 24 h after plating of the cells. Percent viability was determined by dividing the number of colonies in the drug-treated wells by the untreated control. IC_{50} values were determined from these values through the use of a computer program for estimating the four-parameter logistic function based on the method of Rodbard [25].

[^{3}H]Azidopine photoaffinity labeling of P-glycoprotein in membrane fraction. KB-V1 cells were sonicated in buffer A (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 0.1 mM PMSF) and centrifuged at 3000 *g* for 10 min, and supernatants were centrifuged at 100 000 g for 1 h at 4° C. The membrane fraction pellet was resuspended in buffer B (50 mM Tris-HCl, pH 7.5, with 10 μg/ml each of leupeptin, aprotinin, and pepstatin A). Protein concentration were determined using the Bio-Rad Protein Assay. Each reaction contained 100 μg of membrane protein at 1 μg/μl in buffer B in the presence of 50 nM [^{3}H]azidopine [32]. Competing drugs were used at doses ranging from 10- to 1000-fold molar excess, or 0.5–50 μM final concentration per reaction. Following the labelling reaction, samples were immediately subjected to SDS-PAGE on a 6% gel, without heat denaturing prior to loading. The gel was treated with EnLightning fluorography enhancer (NEN-DuPont), dried in vacuo, and exposed to Kodak X-Omat AR film. The radiolabelled bands were excised from the gel, solubilized in Solvable (NEN-DuPont), and radioactivity was determined by liquid scintillation counting.

[^{3}H]Vinblastine accumulation. KB-V1 cells were plated in DME plus 10% FBS in 6-well dishes 1 day before assay without selective agent (vinblastine) for a concentration of 1×10^6 cells per well at time of testing. Medium was replaced by DME with 1% FBS with or without sensitizing agents 15 min prior to the addition of [^{3}H]vinblastine to 1 μM, at 50 mCi/mmol specific activity. Plates were incubated at 37° C during the pretreatment as well as for 1 h after the vinblastine addition. Wells were then rinsed twice with 5 ml cold phosphate-buffered saline (PBS). Cells were lysed in 0.1 *N* NaOH at room temperature and transferred to scintillation vials. Total radioactivity was determined by scintillation counting. Extra wells of untreated cells were washed in PBS, trypsinized, and lysed by sonication; the amount of protein per well was then determined by Bio-Rad Protein Assay.

Table 1. Effect of 21-aminosteroids and related agents on toxicity of vinblastine to KB-V1 cells

Compound	Concentration (μM)	Relative viability	IC_{50} (μM)	Fold increase
Control	–	1.00	0.5573±0.0272	1.0
U-74389F	5.0	0.99	0.0082±0.0002	68.0*
Tirilazad	5.0	0.97	0.0085±0.0009	65.6*
Verapamil	5.0	0.93	0.0099±0.0006	56.3*
U-78517F	5.9	1.13	0.0131±0.0019	42.5*
U-72036E	5.0	0.99	0.0138±0.0014	40.4*
U-76214	5.0	0.74	0.0181±0.0034	30.8*
U-75412E	3.0	0.67	0.0197±0.0001	28.3*
U-75365	5.0	0.91	0.0369±0.0039	15.1*
U-78518E	5.0	0.90	0.0630±0.0069	8.8*
U-84538F	5.0	0.87	0.1018±0.0145	5.5*
U-90374	1.5	0.74	0.1104±0.0126	5.0*
U-88853	5.0	0.67	0.1495±0.0170	3.7*
U-74500A	2.5	0.83	0.2064±0.0195	2.7*
U-79206	2.5	0.97	0.4919±0.0134	1.1

IC_{50} is the amount of vinblastine (μM) ± SEM required to cause 50% decrease in colony number. Each compound was tested at several doses of vinblastine, in duplicate, as described in "Materials and methods." The relative increase in sensitivity to vinblastine toxicity as compared to control is indicated as fold increase

* $P < 0.0001$ as compared with control, determined by Student's *t*-test

Results

Reversal of multidrug resistance was tested by growing resistant cell lines in the presence of a potential sensitizer and in varying concentrations of vinblastine. Structures of some of the compounds used in this study are shown in Fig. 1. The toxicity of vinblastine to KB-V1 cells is increased by the presence of verapamil (Fig. 2). Tirilazad appears to be as effective as verapamil in increasing toxicity to vinblastine. Neither tirilazad nor verapamil caused any increase in toxicity in the parental cell line, KB-3-1.

Table 1 summarizes our results with tirilazad and several related compounds in the colony formation assay. Compounds were tested at concentrations that avoided significant toxicity to the cells. The compounds are listed in order of their effectiveness in increasing toxicity of vinblastine to KB-V1 cells. The compounds tested can be divided into three groups. Group 1 consists of those compounds that sensitized KB-V1 cells at least 50-fold to the effects of vinblastine. This includes tirilazad and U-74389F, a close analogue of tirilazad, and verapamil. As can be seen, tirilazad sensitized the cells 66-fold to the effects of vinblastine, while verapamil sensitized about 56-fold. Results with tirilazad were further confirmed with dose-response studies with KB-V1 cells and with Adriamycin-resistant murine P388 cells (data not shown). Group 2 consists of compounds U-78517F, U-72036E, U-76214 and U-75412E. These are also somewhat effective, within 50% of the effectiveness of verapamil, and can be considered as

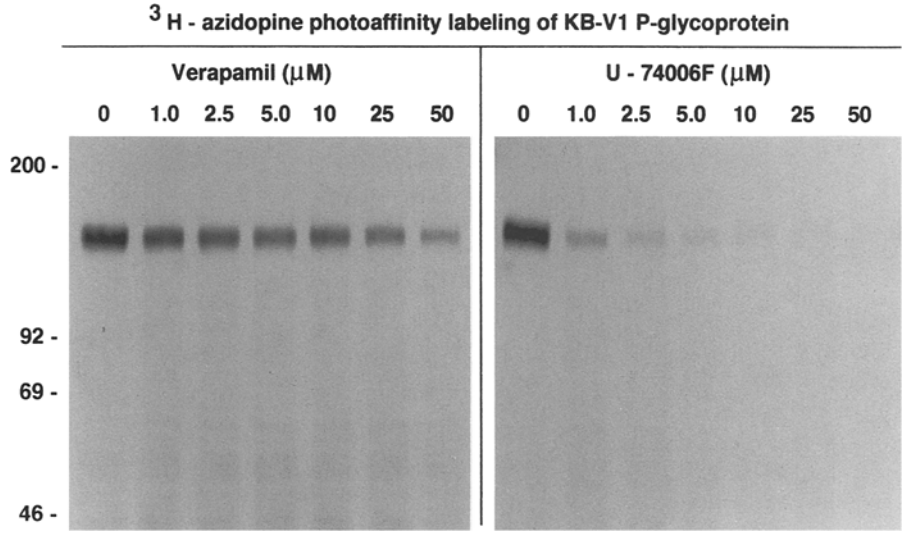

Fig. 3. [^{3}H]Azidopine photoaffinity-labeled membrane proteins from drug-resistant KB-V1 cells in the presence of verapamil or U-74006F (tirilazad). Membranes were prepared and photoaffinity labeled as described in "Materials and methods." Membranes were incubated with the amounts of verapamil or tirilazad indicated, in the presence of 50 nM [^{3}H]azidopine

Table 2. Effect of compounds on [^{3}H]vinblastine accumulation in KB-V1 cells

	% Control,[a] $x \pm$ SE (n)	
Compound	2.5 μM	5 μM
Verapamil	–	434 ± 33 (9)
Tirilazad	387 ± 28 (4)	465 ± 32 (9)
U-74389F	542 ± 39 (5)	663 ± 54 (5)
U-78517F	173 ± 5 (5)	249 ± 12 (5)
U-72036EF	489 ± 58 (5)	618 ± 70 (5)
U-75412E	512 ± 56 (5)	756 ± 86 (5)
U-74500A	103 ± 15 (5)	153 ± 15 (5)
U-75365	145 ± 9 (3)	186 ± 5 (3)
U-76214	202 ± 6 (3)	253 ± 4 (3)
U-88853	174 ± 4 (3)	190 ± 4 (3)

The effect of 2.5 μM or 5 μM of test compounds on the accumulation of [^{3}H]vinblastine was measured as described in "Materials and methods." Increase in accumulation ± SEM was determined relative to control values of accumulation with no added test compound. Numbers in parentheses indicate numbers of replicate experiments

[a] Control = 9.45 ± 0.76 dpm/mg protein $\times 10^{-2}$, $n = 9$

partial modulators. All of these compounds have a complex amine. In this group, U-72036E and U-75412E both have a steroid attached to the amine, while neither U-78517F nor U-76214 have a steroid group. Group 3 consists of those compounds which sensitized cells 15-fold or less.

Variations in the amine group (compare U-78517F and U-78518E or U-75365 and U-79206, Fig. 1) greatly affected ability to reverse resistance. The isolated steroid moiety of tirilazad (U-88853) had very little activity. The amine function alone of tirilizad (U-75365) had a somewhat higher amount of sensitization ability (15X) than the steroid group. The same complex amine group with the addition of a long hydrophobic carbon chain (U-76214) was associated with an increase in sensitization ability.

Another way of measuring the reversal of drug resistance is to compare accumulation of [^{3}H]vinblastine intracellularly with and without treatment of putative sensitizers. Drug resistant cell lines accumulate less intracellular drugs than their sensitive parent cell lines, due to the action of the P-glycoprotein as a drug efflux pump [10]. The drug uptake can be increased in resistant cell lines by treatment with known sensitizers such as verapamil. Our results indicate that tirilazad and U-74389F (group 1) were as effective as verapamil in causing an increased accumulation of [^{3}H]vinblastine in cells (Table 2). The compounds in group 2 were variable in their ability to allow increased accumulation of [^{3}H]vinblastine. Two of the compounds, U-72036E and U-75412E, were effective in increasing vinblastine accumulation, but U-78517F and U-76214 were not particularly effective. This was unexpected, since both U-78517F and U-72036E were about equivalent in their sensitizing ability. It is possible that U-78517F and U-76214 sensitized cells to vinblastine toxicity through additional mechanisms, other than simply increased accumulation. Alternatively, U-78517 and U-76214 may have been metabolized by cells more slowly than U-72036E because of structural dissimilarities. Persistence of U-78517F and U-76214 in the cell over the 9-day course of the cytotoxicity measurements may explain their effectiveness despite the relatively modest increase in [^{3}H]vinblastine uptake over 1 h. In group 3, U-74500A, U-75365, and U-88853 were unable to cause increased accumulation of vinblastine.

[^{3}H]Azidopine has been shown to specifically photolabel the P-glycoprotein, and its binding is inhibited by agents known to reverse multidrug resistance [27]. Figure 3 shows that tirilazad effectively inhibited the photoaffinity labeling of P-glycoprotein by [^{3}H]azidopine. It inhibited labeling at a lower concentration than did verapamil. Quantitation of further results with tirilazad and other compounds are shown in Table 3. All the compounds tested, except ouabain, showed some inhibition of [^{3}H]azidopine photoaffinity labeling of P-glycoprotein. Tirilazad was more efficient in inhibiting the labeling of P-glycoprotein than was verapamil. Ouabain was used as an example of a compound that is known not to interact

Table 3. Inhibition of [^{3}H]azidopine labeling of P-glycoprotein

Compound	% Inhibition of azidopine binding		
	1 μM (20×)[a]	5 μM (100×)	20 μM (400×)
Verapamil	41.5	63.08±3.9	74.0±7.8
Tirilazad	68.2±6.2	87.2 ±1.5	96.0± .7
U-78517F		81.3	93.9
U-72036E		69.7	80.0
U-75412E		87.2	92.3
U-74500A		77.7	86.3
			50 μM (1000×)
Ouabain			−7.0

Isolated membranes were incubated with 50 nM [^{3}H]azidopine and the indicated compounds at concentrations shown. Percent inhibition of azidopine binding was derived in comparison with control values of membranes treated with [^{3}H]azidopine alone

[a] Values in parentheses indicate the fold-molar-excess of compound compared with [^{3}H]azidopine used

with P-glycoprotein and did not inhibit binding even at a 1000-fold molar excess. One of the compounds in group 3, U-74500A, was a very poor sensitizer and did not cause an increase in [^{3}H]vinblastine accumulation, yet showed a substantial inhibition of [^{3}H]azidopine binding. The reason for this unexpected result is not clear, but it suggests that the inhibition of [^{3}H]azidopine binding to P-glycoprotein is not necessarily due to competitive inhibition and that it is not always an indication of potential sensitizing activity of a compound.

Discussion

These results show that two non-glucocorticoid steroid analogues (21-aminosteroids) are effective in sensitizing multidrug resistant cells to the toxic effects of vinblastine. Tirilazad and the closely related compound U-74389F sensitized KB-V1 cells to vinblastine. A second class of compounds, group 2, are partial modulators. Analysis of results with these compounds suggest that the critical components of sensitizing activity for this set of compounds is a complex amine and a steroid or other hydrophobic group. However, these components are not sufficient for effective modulatory activity. Group 3 compounds, U-74500A and U-78518E, were ineffective as modulators, despite structures similar to those of group 2.

We compared the effect of these compounds with that of verapamil, a well-characterized sensitizer. Tirilazad and U-74389F appear to be at least as efficacious as verapamil in sensitizing cells to vinblastine. Tirilazad also caused an inhibition of labeling of P-glycoprotein by [^{3}H]azidopine that was greater than that caused by verapamil. In addition, tirilazad caused an increased intracellular accumulation of [^{3}H]vinblastine that was equivalent to that of verapamil. The results suggest that these drugs might be effective in reversing MDR in vivo.

Our interest in determining the chemical moieties of tirilazad that were critical to the drug reversal properties led us to look at related structures. U-74389F, which was very similar to tirilazad except for the loss of the methyl group on the steroid portion, behaved as well or better than tirilazad as a sensitizer. Compounds that had retained the exact or a closely related steroid moiety of tirilazad but had an altered amine group, such as U-72036E or U-75412E (group 2), retained partial modulating activity. U-74500A, which is comprised of the steroid moiety of tirilazad and a different complex amine portion, on the other hand, had virtually no activity. Clearly the amine group is of critical importance in the sensitizing activity. Compounds that retained the complex heterocyclic amine group of tirilazad, such as U-74389F, U-78517F, U-76214 and U-75365, had varying activity. U-79206, with an amine lacking the piperazine ring and no steroid moiety, had virtually no activity. U-78517F, which has the amine of tirilazad connected to a portion of the vitamin E molecule, and U-76214, which has the amine connected to an aliphatic alcohol, were both in group 2 with partial modulatory activity. The vitamin E portion attached to another amine caused a further decrease in activity (U-78518E). Removing the aliphatic alcohol from the tirilazad amine moiety of U-76214 reduced the sensitizing activity by half (U-75365), as did other alterations to the attached groups (U-84538F, U-90374).

Several of the compounds tested here, for example tirilazad, U-78517F and U-74389F, inhibit lipid peroxidation [4, 18]. These compounds have been shown to prevent neuronal damage after various oxidative insults [20, 22]. This activity has been associated with the ability of these compounds to inhibit lipid peroxidation. However U-78517F, which is more effective at inhibiting lipid peroxidation than tirilazad or U-74389F, is less effective as a sensitizing agent. We conclude that the antioxidant properties of these compounds are not of great importance in determining sensitizing activity.

The mode of action of the compounds presented in this study and other compounds in sensitizing drug resistant cells is not fully understood. Several possibilities present themselves, however. One is that these compounds bind directly to the P-glycoprotein and interfere with efflux of vinblastine [3, 5, 8, 26]. Another possibility is that lipophilic compounds may enter the membrane and perturb the function of the P-glycoprotein pump by a more indirect mechanism, by altering or interacting with sites other than the drug binding sites on P-glycoprotein.

Our results show that inhibition of [^{3}H]azidopine binding on P-glycoprotein may not always be predictive of chemosensitizing activity. We have found that U-74500A is an effective inhibitor of [^{3}H]azidopine binding but does not cause the cell to be able to increase accumulation of vinblastine or be sensitized to vinblastine toxicity. In addition U-74500A did not increase accumulation of the fluorescent dye rhodamine (not shown). Increased rhodamine uptake has previously been used as a measure of reversion of MDR [35]. Yang et al. [33] found a similar result with hydrocortisone, which caused appreciable inhibition of [^{3}H]azidopine labeling of J7.V1-1 cells (67% inhibition at 1000-fold excess) but caused no increase in [^{3}H]vinblastine accumulation. It is not likely that U-74500A forms a covalent complex itself with [^{3}H]azidopine, since the steroid progesterone, which is

very similar to the steroid portion of U-74500, does not form a covalent complex with [^{3}H]azidopine [33]. It is possible that U-74500 interferes with the binding of azidopine by causing membrane pertubations without disabling the P-glycoprotein function. A further possibility is that this compound is binding to the P-glycoprotein and inhibiting azidopine binding, but in such a way as not to interfere with functioning of the P-glycoprotein. All these considerations suggest the conclusion that simple inhibition of azidopine binding is not sufficient to indicate that the test compound is a modulator of multidrug resistance.

The results presented here show a good correlation with increase in vinblastine accumulation to increased toxicity by vinblastine. Tirilazad and the closely related U-74389F showed large increases in vinblastine accumulation, as did U-78517F, U-72036F and U-75412E. On the other hand, U-74500A, U-75365 and U-88853 showed very little increase in accumulation and also showed small amounts of sensitization. U-76214 showed an intermediate degree of sensitization but a low increase in accumulation.

Our results support the view that effective modulators are lipophilic, amphipathic compounds. We do not see a direct correlation with lipophilicity and modulation, however. Instead our analysis of the relation of the structure and function of these compounds suggest that the disposition of charge and the shape and size of the molecule is of great importance, as has been previously suggested for other classes of sensitizers [12, 37]. This suggests that a further study of this series of compounds considered here may lead to more information about the determinants that make good modulating compounds and may lead to the discovery of more effective compounds. Future work will concentrate on the effectiveness of tirilazad, U-74389F, and analogues in other MDR cells and in in vivo models of drug resistance.

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