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Structural Features Determining Activity of Phenothiazines and Related Drugs for Inhibition of Cell Growth and Reversal of Multidrug Resistance

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SUMMARY

Phenothiazines and structurally related compounds inhibit cellular proliferation and sensitize multidrug-resistant (MDR) cells to chemotherapeutic agents. To identify more potent pharmaceuticals, we studied the structure-activity relationships of 30 phenothiazines and related compounds on cellular proliferation and MDR in sensitive MCF-7 and resistant MCF-7/DOX human breast cancer cells. Substitutions on the phenothiazine ring that increased hydrophobicity increased antiproliferative and anti-MDR activities. For example, −Cl and $-CF_3$ groups increased whereas −OH groups decreased potency. Modifying the length of the alkyl bridge and the type of amino side chain also influenced potency. Compounds with increased activity against cellular proliferation and MDR possessed a four-carbon bridge rather than a three- or two-carbon bridge and a piperazinyl amine rather than a noncyclic amino group. Compounds with tertiary amines were better anti-MDR agents than those with secondary or primary amines but were equipotent antiproliferative agents. The effects of these substituents were unrelated to hydrophobicity. The structure-activity relationships suggest that an ideal phenothiazine structure for reversing MDR has a hydrophobic nucleus with a $-CF_3$ ring substitution at position 2, connected by a four-carbon alkyl bridge to a *para*-methyl-substituted piperazinyl amine. We subsequently studied related compounds having certain of these properties. Substitution of a carbon for a nitrogen at position 10 of the tricyclic ring, with a double bond to the side chain (thioxanthene), further increased activity against MDR. For example, (*trans*)-flupenthixol, the most potent of these compounds, increased the potency of doxorubicin against MDR cells by 15-fold, as compared with its stereoisomer (*cis*)-flupenthixol (5-fold) or its phenothiazine homolog fluphenazine (3-fold). (*cis*)- and (*trans*)-flupenthixol were equipotent antiproliferative agents. (*trans*)-flupenthixol was not accumulated more than (*cis*)-flupenthixol in MDR cells, implying that their stereospecific anti-MDR effects were not the result of selective differences in the access of the drugs to intracellular targets. Both drugs increased the accumulation of doxorubicin in MDR cells, but not in sensitive cells, suggesting that they modulate MDR by interacting with a uniquely overexpressed cellular target in these resistant cells. The apparent lack of clinical toxicity of (*trans*)-flupenthixol makes it an attractive drug for further investigation.

PTZs and structurally related antipsychotics inhibit the activity of several cellular enzymes (1–3) and block the function of critical cellular receptors, such as those for dopamine (4). Among these cellular targets is CaM, a multifunctional calcium-binding protein (5) that has been implicated in the regulation of numerous cellular events, including that of normal and abnormal cellular proliferation (6–8). Consistent with these observations was the demonstration that PTZs and other CaM antagonists possess antiproliferative and cytotoxic effects (9) that were proportional to their anti-CaM activity (10–13).

The recent demonstration and elucidation of the phenomenon of MDR has led to the search for drugs that could sensitize highly resistant cancer cells to chemotherapeutic agents. MDR is the process whereby malignant cells become resistant to structurally diverse chemotherapeutic agents following exposure to a single drug (14). Certain MDR cell lines have been associated with decreased drug accumulation due to enhanced efflux of chemotherapeutic drugs (15). This effect appears to be attributable to the overexpression of a 170,000-Da membrane glycoprotein (P-glycoprotein), which structurally resembles transport proteins in prokaryotic cells (16) and may function as an energy-dependent drug efflux pump in mammalian cells (17, 18).

PTZs have been shown to be among the group of drugs known to modify MDR (19). Although the mechanism by which PTZs and other drugs modulate MDR is not clear, it has been suggested that their pharmacological properties may be mediated by the calcium messenger system, because the active compounds are known to inhibit voltage-dependent calcium channels (20), CaM (5), and protein kinase C (21).

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ABBREVIATIONS: PTZ, phenothiazine; CaM, calmodulin; MDR, multidrug resistance; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

To elucidate the structural features required for the activity of PTZs and related compounds against cellular proliferation and MDR, we have studied the effects of a series of drugs with single molecular alterations for their ability to inhibit cell growth and to sensitize the human MDR breast cancer cell line MCF-7/DOX to doxorubicin. Furthermore, to clarify the role of CaM in these processes, we have compared the anti-CaM effects of these compounds with their effects on cellular proliferation and MDR.

Materials and Methods

Cell lines and culture. MCF-7 human breast cancer cells and the MDR subclone MCF-7/DOX (kindly supplied by Dr. Ken Cowan, National Cancer Institute, Bethesda, Maryland), selected by stepwise exposure of parental cells to increasing concentrations of doxorubicin, were maintained in exponential growth in Corning 75-cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 5% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air. MCF-7/DOX cells were approximately 200-fold more resistant to doxorubicin than the parental cell line and maintained a stable MDR phenotype while grown in drug-free medium for a period of at least 3 months, after which they were discarded. Cell lines were routinely tested and found to be free of contamination by mycoplasma or fungi.

Effect of drugs on cellular proliferation and MDR. Cells in exponential growth were digested with 0.5% trypsin (GIBCO, Grand Island, New York) in 10 mM PBS, disaggregated into single cell suspensions, counted electronically (Coulter, Hialeah, FL), and dispensed in 100-μl volumes into 96-well microtiter plates with a multichannel pipet (Titertek; Flow Laboratories, McLean, VA) at a concentration of 0.5–1.0 × 10^4 cells/well. Cells were allowed to attach to the plastic and to resume growth for 24 hr before the addition of 100 μl of drug-containing medium. Drugs were dissolved in small amounts of sterile water or 1% DMSO (final culture concentration, <0.05% DMSO) before dilution with medium. Controls were exposed to vehicle-containing medium. After a 48-hr incubation at 37°, the cellular supernatants of each well were gently aspirated, and cells were fixed and stained with 100 μl of 0.5% methylene blue (Sigma Chemical Co., St. Louis, MO) in 50% ethanol (w/v) for 30 min at room temperature, as previously described (22). Unbound stain was removed by decanting, followed by emersion in three 1-liter washes of distilled deionized water. The plates were dried for 12 hr and the stained protein was solubilized with 200 μl of sodium N-lauroyl sarcosine (Fluka, Buchs, Switzerland) solution (1%, v/v, in PBS). The optical density of each well was determined by absorbance spectrophotometry at a wavelength of 600 nm with a microculture plate reader (Titertek Multiscan MCC/340) interfaced to an Apple IIe computer. Inhibition of cell growth was expressed as a percentage of absorbance of vehicle-treated control cultures.

To determine the optimal conditions for this assay, plates were inoculated with various initial cell concentrations. One half-plate was assayed daily for 5 consecutive days by standard trypsin digestion and electronic cell counting, whereas the other half of each plate was stained as described above. There was a linear correlation between A_{600} from stained wells and actual cell number for both MCF-7 cell lines at final cell densities between 0 and 50,000 cells per well. Final assay conditions were chosen to ensure that optical density measurements fell on the linear portion of this curve. This system has proven extremely reproducible, with less than 5% variability between IC_{50} values from dose-response curves from different experiments run on different days.

The effect of PTZs on cellular proliferation was examined by exposing cells grown in the dark to 0–100 μM drug, as described above, with each condition repeated in quadruplicate. IC_{50} was the concentration of drug that reduced staining (A_{600}) to 50% of vehicle-treated controls. Final IC_{50} values represent the average of three to five separate experiments.

The effect of PTZs on MDR was studied by exposing cells to 0–100 μM doxorubicin in the absence or presence of a concentration of PTZ derivative that alone produced 10% inhibition of growth (IC_{10}), as determined in three to five experiments run in quadruplicate. Dose-response curves were corrected for the 10% inhibition of cell growth caused by the PTZs alone. The MDR Ratio was defined as the ratio of the IC_{50} for doxorubicin alone divided by the IC_{50} for doxorubicin in the presence of modifier. This ratio represents the increase in apparent potency of doxorubicin produced by each PTZ derivative.

$$\text{MDR Ratio} = IC_{50}\ \text{doxorubicin alone} \div IC_{50}\ \text{doxorubicin + drug}$$

Isobologram analysis. After determining the IC_{50} for doxorubicin and the IC_{50} for individual MDR modifiers against MCF-7/DOX cells, a series of dose-response curves to a single modifier in the presence of fixed concentrations of doxorubicin were obtained using the microtiter assay system. The concentrations of doxorubicin plus modifier that resulted in 50% inhibition of growth of MCF-7/DOX cells were plotted and the IC_{50} isobole was compared with the calculated line of additivity, using criteria previously described (23).

Cellular accumulation of thioxanthenes. Duplicate aliquots of 3 × 10^6 resistant cells in a total volume of 2 ml were incubated at 37° for 3 hr in the presence of 0–100 μM concentrations of each drug. Cells were washed three times in cold PBS and centrifuged at 100 × g for 10 min, resuspended in 2 ml of 0.3 N HCl in 50% ethanol, and sonicated for 10 pulses at 200 watt seconds with a Tekmar cell sonicator (Tekmar, Cincinatti, OH). After centrifugation at 1000 × g for 30 min the cell supernatant was removed and assayed for drug concentration with a Perkin-Elmer 512 spectrofluorometer (Norwalk, CT). Optimal excitation and emission wavelengths for both thioxanthene isomers were determined to be 320 and 400 nm, respectively. Cellular drug content (nmoles/10^6 cells) was computed from standard curves prepared with known amounts of drug in 0.3 N HCl in 50% ethanol.

Cellular accumulation of doxorubicin. Sensitive and MDR MCF-7 cells were each seeded in three 40-ml volumes of medium at a density of 1.67 × 10^6 cells/ml. Concentrated stock drug solutions (100 μl) were added to obtain a final doxorubicin concentration of 10 μM, in the absence or presence of either 3 μM (*cis*)-flupenthixol or 6 μM (*trans*)-flupenthixol (concentrations that alone produce 10% inhibition of cell growth). Cells were incubated at 37° for 3 hr. At various times after the addition of drugs, three 1.5-ml aliquots of cellular suspension were removed from each cell solution, immediately centrifuged for 60 sec at 11,000 × g, and washed three times with cold PBS using an Eppendorf 5415 microcentrifuge. Control samples were removed at time 0, immediately before drugs were added. Cell pellets were extracted with 0.3 N HCl in 50% ethanol, sonicated, and centrifuged as described above. Cell supernatants were removed and assayed fluorometrically for doxorubicin content using excitation and emission wavelengths of 470 and 585 nm, respectively, as previously described (19). Cellular content of doxorubicin was computed from standard curves prepared with known amounts of drug in 0.3 N HCl in 50% ethanol. The presence of thioxanthene isomers was shown not to effect the absorbance or emission spectra of doxorubicin.

Drugs. Doxorubicin was obtained from Sigma and was freshly prepared in distilled water for each experiment. PTZ derivatives and related drugs were generously donated as follows: chlorpromazine hydrochloride, trifluoperazine dihydrochloride, chlorpromazine sulfoxide hydrochloride, 2-chloro-10-[2-(dimethylamino)ethyl]phenothiazine hydrochloride, 2-chloro-10-[4-(dimethylamino)butyl]phenothiazine hydrochloride, promazine hydrochloride, trifluopromazine hydrochloride, 2-thiomethylpromazine hydrochloride, 1-chloropromazine hydrochloride, 3-chloropromazine hydrochloride, 4-chloropromazine hydrochloride, and prochlorperazine ethanedisulfonate by Dr. Charles Zirkle of Smith Kline and French Laboratories (Philadelphia, PA); 7-hydroxychlorpromazine, 3,8-dihydroxychlorpromazine, 7,8-dihydroxychlorpromazine, desmethylchlorpromazine hydrochloride, and didesmethylchlorpromazine hydrochloride by Dr. Albert Manian of the National Institute of Mental Health (Bethesda, MD); promethazine hydrochloride by Wyeth Laboratories (Radnor, PA); chlorproethazine

hydrochloride by Rhone-Poulenc (Paris, France); imipramine hydrochloride and 2-chloroimipramine hydrochloride by Geigy Pharmaceuticals (Summit, NJ); haloperidol, pimozide, penfluridol, and 4-(4-chloro-α,α,α-trifluoro-*m*-tolyl)-1-[4,4-bis(*p*-flourophenyl)butyl]-4-piperidinol (R-6033) by Dr. Pierre Laduron of Janssen Pharmaceutica (Beerse, Belgium); quinacrine dihydrochloride by Sterling-Winthrop Research Institute (Renssalear, NY); fluphenazine by Dr. S. J. Lucania of E. R. Squibb and Sons; and (*cis*)- and (*trans*)-flupenthixol by Dr. John Hyttel of H. Lundbeck (Copenhagen, Denmark). Perphenazine was obtained from Sigma. Other reagents were of analytical grade and were obtained from general commercial sources.

Statistical analysis. Statistical analysis of each dose-response curve was performed by the method of Finney (24). Accordingly, IC_{50} values ± standard errors for the inhibition of cellular proliferation by drugs alone or in combination were determined by linear regression analysis of the logit-transformed data. The significance of each MDR Ratio was then determined using Student's two-tailed *t* test, and was expressed in terms of *p* values 95% confidence intervals for each MDR Ratio were calculated by Fieller's ratio of means as modified by Bliss (25). Student's *t* test was also used to analyze the significance of differences between MDR Ratios or IC_{50} values for inhibition of cellular proliferation by various drugs.

Results

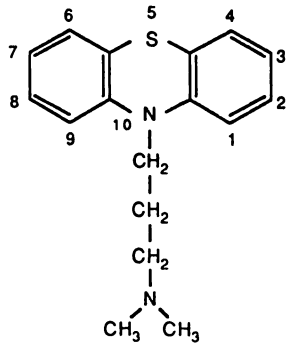
Effect of modifying the PTZ nucleus on cellular proliferation and MDR. Table 1 shows the structures, IC_{50} values for inhibition of cell growth, and MDR Ratios for a series of promazine derivatives having different substitutions on the PTZ nucleus. The unsubstituted PTZ, promazine, inhibited cell growth (IC_{50} = 26 μM) and sensitized MCF-7/DOX cells to doxorubicin by 20% (MDR Ratio = 1.2; $p < 0.05$ as compared with doxorubicin alone). Adding a chlorine at positions 1, 2, 3, or 4 increased potency against cell growth by up to 3-fold, with the most potent compound (chlorpromazine) having a chlorine at position 2 (IC_{50} = 8 μM). This latter compound also had the greatest effect against MDR, sensitizing resistant cells to doxorubicin by 60% (MDR Ratio = 1.6; $p < 0.001$ as compared with doxorubicin alone). Similarly, adding a $-CF_3$ group at position 2 increased potency against cell growth and MDR. Accordingly, trifluopromazine (IC_{50} = 16 μM) was a 1.6-fold more potent cytostatic agent than promazine ($p < 0.001$) and was 67% more potent ($p < 0.001$) against MDR (MDR Ratio = 2.0).

Conversely, adding a −OH to the PTZ nucleus decreased the potency against both processes. For example, 7-hydroxychlorpromazine was 2-fold less potent than promazine in inhibiting cell growth ($p < 0.001$), whereas the dihydroxylated analogs, 7,8- and 3,8-dihydroxychlorpromazine, were up to 15-fold less potent than promazine as inhibitors of cellular proliferation ($p < 0.001$), having IC_{50} values of 63 and 400 μM, respectively. In addition, the hydroxylated analogs had no significant activity against MDR and further increased resistance to doxorubicin (MDR Ratios ≤ 1.0). Oxidation of the bridge sulfur (chlorpromazine sulfoxide) markedly reduced antiproliferative activity (IC_{50} = 500 μM) but increased activity against MDR (MDR Ratio = 2.2).

TABLE 1

Effect of modifying the PTZ nucleus on activity against cell growth and MDR

Inhibition of cell growth was determined by exposing MCF-7/DOX cells to 0–100 μM of each PTZ. IC_{50} is the mean concentration ± standard error that produced 50% inhibition of cell growth compared with vehicle-treated controls, as described in Materials and Methods. To determine the MDR Ratio, MCF-7/DOX cells were exposed to 0–100 μM doxorubicin in the presence or absence of PTZ at a concentration that alone produced 10% inhibition of cellular growth. MDR Ratio is the IC_{50} for doxorubicin alone divided by the IC_{50} for doxorubicin in the presence of PTZ 95% confidence intervals and *p* values for comparison of IC_{50} values for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were derived as described in Materials and Methods. All values represent the mean of three to five separate experiments; each experiment was done in quadruplicate.



Substituent	Position	Name	Cell Growth Inhibition, IC_{50}	MDR Ratio
			μM	
		Promazine	26 ± 4	1.2 ± 0.2[a]
—Cl	1	1-Chloropromazine	21 ± 5	1.3 ± 0.2[b]
—Cl	2	Chlorpromazine	8 ± 1	1.6 ± 0.3[b]
—Cl	3	3-Chloropromazine	10 ± 2	1.3 ± 0.2[b]
—Cl	4	4-Chloropromazine	15 ± 3	1.4 ± 0.3[b]
—Cl,—OH	2, 7	7-Hydroxychlorpromazine	50 ± 7	1.0 ± 0.3[c]
—Cl,—OH,—OH	2, 3, 8	3,8-Dihydroxychlorpromazine	400 ± 45	0.9 ± 0.3[c]
—Cl,—OH,—OH	2, 7, 8	7,8-Dihydroxychlorpromazine	63 ± 22	0.8 ± 0.2[a]
—S—CH_3	2	Thiomethylpromazine	20 ± 3	1.5 ± 0.2[b]
—CF_3	2	Trifluopromazine	16 ± 3	2.0 ± 0.3[b]
=O	5	Chlorpromazine Sulfoxide	500 ± 95	2.2 ± 0.4[b]

[a] $p < 0.05$.
[b] $p < 0.001$.
[c] p, not significant.

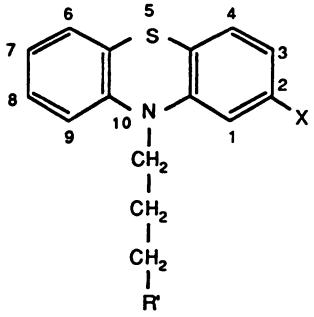
Influence of the side chain amino group on cellular proliferation and MDR. Table 2 shows that PTZs containing tertiary amines (chlorpromazine and chlorproethazine), secondary amines (desmethylchlorpromazine), and primary amines (didesmethylchlorpromazine) possess similar activity against cellular proliferation (IC_{50} values = 8–12 μM). However, PTZs having tertiary amines were more potent antagonists of MDR than were those with secondary or primary amines, producing a 1.6- to 2.2-fold increase in sensitivity to doxorubicin. For example, the IC_{50} values of chlorpromazine and desmethylchlorpromazine were equal (8 μM), whereas chlorpromazine was more potent than desmethylchlorpromazine against MDR (MDR Ratios = 1.6 versus 1.2; $p < 0.01$). Other changes in the type of amino group also affected anti-MDR activity. For example, piperazinyl derivatives increased potency against MDR. Accordingly, the MDR Ratios for trifluoperazine (3.4) and fluphenazine (2.7), compounds with piperazinyl amino side chains, were greater than that of trifluopromazine (MDR Ratio = 2.0; $p < 0.001$ and 0.001, respectively), a compound with an identical hydrophobic ring-substitution, but possessing an aliphatic side chain. Similarly, perphenazine and prochlorperazine (MDR Ratios = 2.0 and 2.6) were more potent antagonists of MDR than was chlorpromazine (MDR Ratio = 1.6; $p < 0.01$ and 0.001, respectively). This series also points out the importance of the $-CF_3$ substitution at position 2 for anti-MDR activity. For example, the MDR Ratio for trifluoperazine (3.4) was greater than that of prochlorperazine (MDR Ratio = 2.6; $p < 0.01$). These PTZs have identical structures except that the former has a $-CF_3$ instead of a $-Cl$ at position 2. A similar relationship is seen by comparing the MDR Ratio for fluphenazine (2.7) to perphenazine (MDR Ratio = 2.0; $p < 0.01$), also identical molecules except for the $-CF_3$ ring substitution. Finally, a *para*-methyl substitution on the piperazine appeared more potent than an ethanol group for anti-MDR activity of compounds, as seen by comparing the MDR Ratios for prochlorperazine (2.6) to perphenazine (2.0; $p < 0.05$), or trifluoperazine (3.4) to fluphenazine (2.7; $p < 0.001$).

Influence of alkyl bridge length on cellular proliferation and MDR. Table 3 shows the effect on cell growth and on MDR of a series of 10-[alkyl-dimethylamino]phenothiazines in which the length of the alkyl bridge connecting the PTZ nucleus to the amino group was varied. Increasing the distance

TABLE 2

Effect of modifying the type of side chain amino group on activity against cell growth and MDR

MCF-7/DOX cells were treated as described in Table 1. IC_{50} values ± standard error and MDR Ratios with 95% confidence intervals and p values for comparison of IC_{50} values for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined as described in Table 1. Each value represents the mean of three to five separate experiments; each experiment was done in quadruplicate.



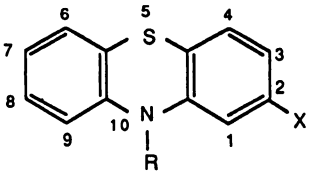
X	R'	Name	Cell Growth Inhibition, IC_{50}	MDR Ratio
			μM	
—Cl	—NH_2	Didesmethylchlorpromazine	11 ± 1	1.1 ± 0.1[a]
—Cl	—NH—CH_3	Desmethylchlorpromazine	8 ± 2	1.2 ± 0.2[b]
—Cl	—N(CH_3)(CH_3)	Chlorpromazine	8 ± 1	1.6 ± 0.3[a]
—Cl	—N(CH_2—CH_3)(CH_2—CH_3)	Chlorproethazine	12 ± 1	2.2 ± 0.4[a]
—Cl	—N(piperazine)—CH_2—CH_2—OH	Perphenazine	32 ± 4	2.0 ± 0.3[a]
—Cl	—N(piperazine)—CH_3	Prochlorperazine	22 ± 3	2.6 ± 0.4[a]
—CF_3	—N(CH_3)(CH_3)	Trifluopromazine	16 ± 3	2.0 ± 0.3[a]
—CF_3	—N(piperazine)—CH_2—CH_2—OH	Fluphenazine	23 ± 5	2.7 ± 0.3[a]
—CF_3	—N(piperazine)—CH_3	Trifluoperazine	19 ± 3	3.4 ± 0.4[a]

[a] $p < 0.001$.
[b] $p < 0.01$.

TABLE 3

Effect of modifying the length of the side chain amino group on activity against cell growth and MDR

MCF-7/DOX cells were treated as described in Table 1. IC_{50} values ± standard error and MDR ratios with 95% confidence intervals and p values for comparison of IC_{50} values for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined, as described in Table 1. Each value represents the mean of three to five separate experiments; each experiment was done in quadruplicate.



X	R	Name	Cell Growth Inhibition, IC_{50}	MDR Ratio
			μM	
—Cl	—CH_2—CH_2—N(CH_3)(CH_3)	2-Chloro-10-[2-(dimethylamino)ethyl] phenothiazine	27 ± 4	1.5 ± 0.3[a]
—Cl	—CH_2—CH_2—CH_2—N(CH_3)(CH_3)	Chlorpromazine	8 ± 1	1.6 ± 0.3[a]
—Cl	—CH_2—CH_2—CH_2—CH_2—N(CH_3)(CH_3)	2-Chloro-10-[4-(dimethyl-amino)butyl]phenothiazine	7 ± 1	2.0 ± 0.3[a]
—H	—CH_2—CH(CH_3)—N(CH_3)	Promethazine	29 ± 5	1.9 ± 0.5[a]
—H	—CH_2—CH_2—CH_2—N(CH_3)(CH_3)	Promazine	26 ± 4	1.2 ± 0.2[b]

[a] $p < 0.001$.
[b] $p < 0.05$.

between the ring nucleus and the amino group from two to four carbons increased the antiproliferative and the anti-MDR effects of these compounds. For example, 2-chloro-10-[4-(dimethylamino)butyl]phenothiazine, which has a four-carbon alkyl bridge, was a more potent antiproliferative agent (IC_{50} = 7 μM) and anti-MDR agent (MDR Ratio = 2.0) than any of the other four compounds with two- or three-carbon alkyl chains. Conversely, promethazine, which has an isopropyl side chain, was a less potent inhibitor of cell growth than promazine, which has a three-carbon chain.

Influence of hydrophobicity of PTZs on cellular proliferation and MDR. To determine the influence of hydrophobicity on the effect of PTZs on cellular proliferation and MDR, we compared the octanol/buffer partition coefficients for each of the 10 ring-substituted promazine derivatives, as previously determined (26), to the IC_{50} values for inhibition of cell growth and to the MDR Ratios. Fig. 1, A and B demonstrates the excellent correlation between hydrophobicity and both antiproliferative activity ($r = -0.73$; $p = 0.016$) and MDR antagonism ($r = 0.86$; $p = 0.0015$).

To determine whether the differences in potency of compounds with side chain alterations were also due to changes in overall hydrophobicity, we compared the octanol/buffer partition coefficients for each of the drugs, in Tables 2 and 3, that had —Cl substitutions at position 2 of the PTZ ring with their IC_{50} values for inhibition of cell growth and with their MDR Ratios (Fig. 1, C and D). In contrast to the results for ring-substituted PTZs, no statistically significant correlation was found between hydrophobicity and potency of compounds with side chain alterations for inhibition of cell growth ($r = 0.54$; $p = 0.27$) or antagonism of MDR ($r = 0.59$; $p = 0.21$).

Correlation between anti-calmodulin activity and inhibition of cellular proliferation and MDR. To examine the role of CaM as a possible target for the effect of PTZs on cellular proliferation and MDR, we compared the IC_{50} values for the inhibition of CaM by PTZs and structurally related compounds (26) with their IC_{50} values for the inhibition of cell growth and with their effect on MDR when used at equimolar (3 μM) concentrations. Fig. 2 shows a good correlation between anti-CaM activity and antiproliferative activity ($r = 0.58$; $p = 0.0009$), whereas no correlation was found between anti-CaM activity and effect on MDR ($r = 0.17$; $p = 0.44$).

Effect of PTZs and structurally related compounds on doxorubicin-sensitive cells. The activity of all 30 compounds against cell growth and as modulators of sensitivity to doxorubicin was examined against the doxorubicin-sensitive MCF-7 cell line. Each of the drugs tested were equally potent antiproliferative agents against the doxorubicin-sensitive cell line, as compared with their activity against the resistant MCF-7/DOX cell line (data not shown). No compound sensitized the MCF-7 cells to doxorubicin.

Effect of compounds structurally related to the PTZs on inhibition of cellular proliferation and MDR. Table 4 shows the structures, IC_{50} values for inhibition of cell growth, and effect on MDR for several compounds that incorporate certain structural features identified as important for activity

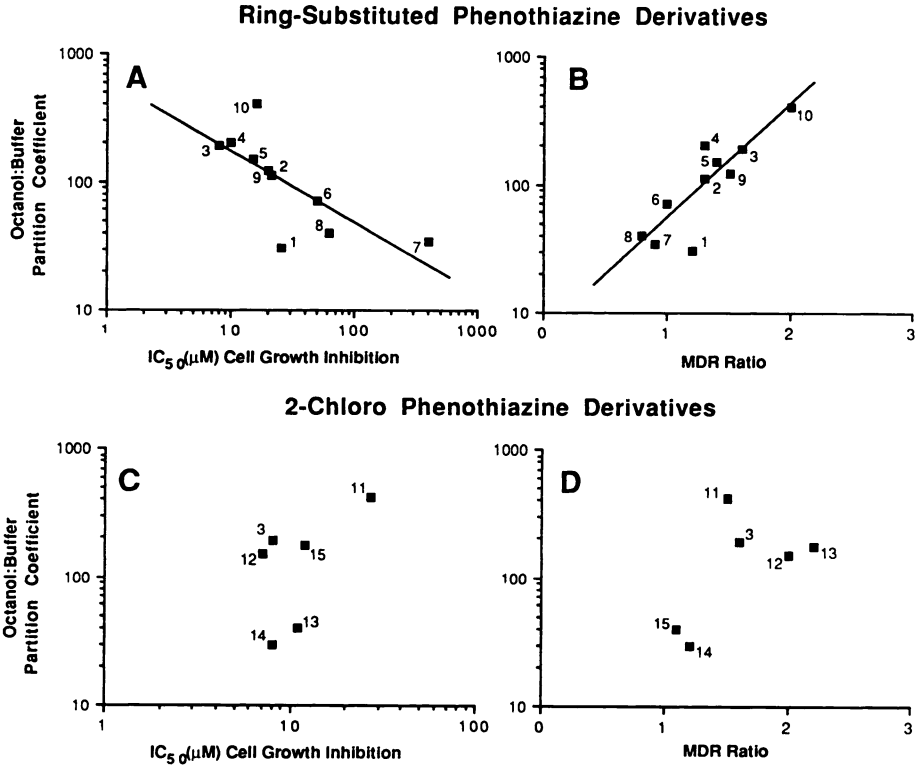


Fig. 1. Relationship between hydrophobicity and activity of PTZ derivatives as antiproliferative and anti-MDR agents. A and B, Correlation between octanol/buffer partition coefficients, as previously determined (26), and the IC_{50} values for inhibition of cell growth ($r = -0.73$; $p = 0.016$) and antagonism of MDR ($r = 0.86$; $p = 0.0015$)) for a series of phenothiazine derivatives with ring substitutions (Table 1). C and D, Lack of correlation between octanol/buffer partition coefficients and IC_{50} values for inhibition of cell growth ($r = 0.54$; $p = 0.27$) and antagonism of MDR ($r = 0.59$; $p = 0.21$) for a series of 2-Cl-substituted phenothiazine derivatives with side chain alterations (Tables 2 and 3). *Numbered points* represent **1**, promazine; **2**, 1-chloropromazine; **3**, chlorpromazine; **4**, 3-chloropromazine; **5**, 4-chloropromazine; **6**, 7-hydroxychlorpromazine; **7**, 3,8-dihydroxychlorpromazine; **8**, 7,8-dihydroxychlorpromazine; **9**, thiomethylpromazine; **10**, trifluopromazine; **11**, 2-chloro-10-[2-(dimethylamino)ethyl] phenothiazine; **12**, 2-chloro-10-[4-(dimethylamino)butyl] phenothiazine; **13**, didesmethylchlorpromazine; **14**, desmethylchlorpromazine; and **15**, chlorproethazine.

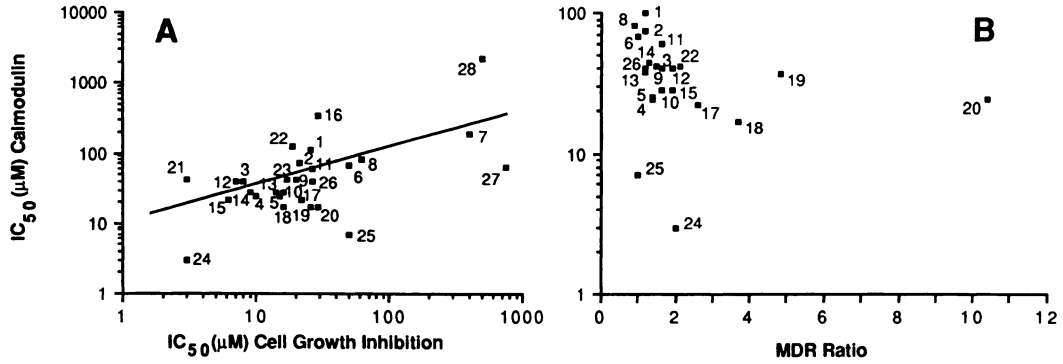
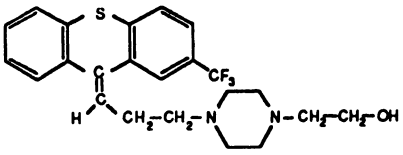
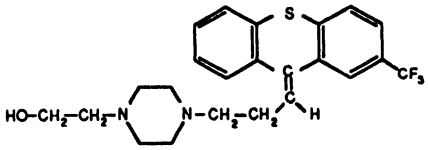
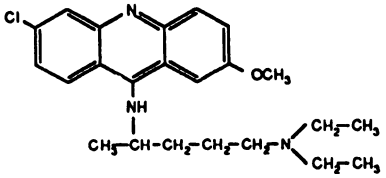
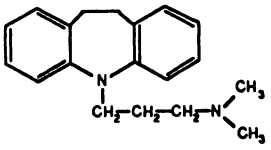
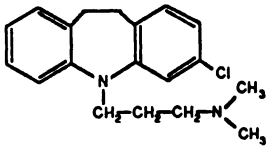
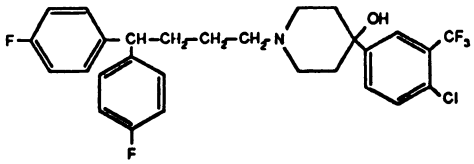
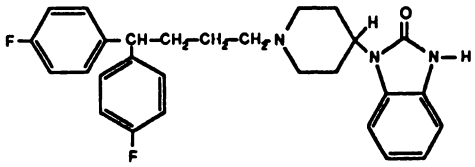
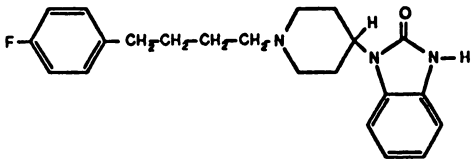
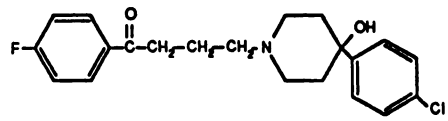


Fig. 2. Relationship between anti-CaM activity and antiproliferative or anti-MDR activity for PTZ derivatives. A, Correlation between the IC_{50} values for inhibition of CaM-induced activation of phosphodiesterase and the IC_{50} values for inhibition of cell growth ($r = 0.58$; $p = 0.0009$) for PTZ derivatives. B, Lack of correlation between the IC_{50} values for inhibition of CaM-induced activation of phosphodiesterase and antagonism of MDR by modifiers used at a standard concentration of 3 μM ($r = 0.17$; $p = 0.44$) for PTZ derivatives. All IC_{50} values for the inhibition of CaM are from Prozialeck and Weiss (26) and represent the concentration of drug necessary to inhibit by 50% the calmodulin-induced activation of the Ca^{2+}-dependent form of cyclic nucleotide phosphodiesterase. IC_{50} values for the inhibition of cellular proliferation are from Tables 1–4. MDR Ratios are the mean of two or more experiments, each run in quadruplicate, and were determined as described in Materials and Methods, except that an equimolar concentration (3 μM) of each modifier was used instead of an equitoxic concentration. *Numbered points* represent **1**, promazine; **2**, 1-chloropromazine; **3**, chlorpromazine; **4**, 3-chloropromazine; **5**, 4-chloropromazine; **6**, 7-hydroxychlorpromazine; **7**, 3,8-dihydroxychlorpromazine; **8**, 7,8-dihydroxychlorpromazine; **9**, thiomethylpromazine; **10**, trifluopromazine; **11**, 2-chloro-10-[2-(dimethylamino)ethyl]phenothiazine; **12**, 2-chloro-10-[4-(dimethylamino)butyl]phenothiazine; **13**, didesmethylchlorpromazine; **14**, desmethylchlorpromazine; **15**, chlorproethazine; **16**, promethazine; **17**, prochlorperazine; **18**, trifluoperazine; **19**, (*cis*)-flupenthixol; **20**, (*trans*)-flupenthixol; **21**, quinacrine; **22**, imipramine; **23**, 2-chloroimipramine; **24**, penfluridol; **25**, pimozide; **26**, R-6033; **27**, haloperidol; and **28**, chlorpromazine sulfoxide.

TABLE 4

Effect of compounds structurally related to the PTZs on cell growth and MDR

MCF-7/DOX cells were treated as described in Table 1. IC_{50} values ± standard error and MDR Ratios with 95% confidence intervals and *p* values for comparison of IC_{50} values for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined, as described in Table 1. Each value represents the mean of three to five experiments; each experiment was done in quadruplicate.

Compound	Structure	Cell Growth Inhibition, IC_{50}	MDR Ratio
		μM	
(*cis*)-Flupenthixol		24 ± 4	4.8 ± 0.6[a]
(*trans*)-Flupenthixol		25 ± 4	15.2 ± 1.9[a]
Quinacrine		3 ± .1	1.3 ± 0.1[b]
Imipramine		19 ± 5	2.5 ± 0.9[a]
2-Chloroimipramine		20 ± 2	2.0 ± 0.6[a]
Penfluridol		3 ± .2	2.0 ± 0.3[a]
Pimozide		50 ± 20	1.3 ± 0.5[c]
R-6033		27 ± 4	1.1 ± 0.2[d]
Haloperidol		750 ± 300	3.3 ± .7[a]

[a] $p < 0.001$.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] *p*, not significant.

from the previous studies. Penfluridol and quinacrine were potent antiproliferative agents (IC_{50} = 3 μM) but were relatively poor antagonists of MDR (MDR Ratios = 2.0 and 1.3, respectively).

The thioxanthene isomers (*cis*)- and (*trans*)-flupenthixol were more effective agents for sensitizing MDR cells to doxorubicin. These compounds differ from the PTZs by a carbon substitution at position 10 of the PTZ nucleus and an exocyclic double bond to a piperazinyl side chain. Both compounds possessed greater activity against MDR than the PTZs (MDR Ratios for *cis* and *trans*-isomers when used at IC_{10} doses were 4.8 and 15.2, respectively). At slightly higher concentrations (IC_{25}), (*trans*)-flupenthixol sensitized MDR cells to doxorubicin by more than 37-fold, a 10-fold greater anti-MDR activity than equitoxic doses of trifluoperazine, the most potent PTZ antagonist of MDR (data not shown).

Table 4 also demonstrates that the thioxanthene isomers displayed stereospecificity in their effect on MDR. Accordingly, the *trans*-isomer was 3-fold more potent than the *cis*-isomer ($p < 0.001$). (*trans*)-Flupenthixol was 6-fold more potent than the PTZ homolog fluphenazine (MDR Ratio = 2.7; $p < 0.001$), when tested at equitoxic concentrations (Fig. 3) or equimolar concentrations (data not shown).

In contrast to the anti-MDR effects of these compounds, the thioxanthene isomers displayed a relative lack of potency and stereospecificity as antiproliferative agents. Accordingly, (*cis*)- and (*trans*)-flupenthixol possessed only modest activity against cell growth (IC_{50} values of 24 and 25 μM), similar in potency to the PTZ homolog fluphenazine (IC_{50} = 23 μM) and less potent than many of the other PTZs tested.

Drug accumulation studies. We next determined whether the difference in the anti-MDR activity of the thioxanthenes could be attributed to differences in their cellular accumulation. After a 3-hr incubation in 3–100 μM concentrations of each drug, cell-associated (*cis*)- and (*trans*)-flupenthixol concentrations (nmol/10^6 cells) were 645 ± 55 versus 205 ± 14 at 3 μM ($p < 0.001$), 2170 ± 325 versus 1110 ± 81 at 10 μM ($p < 0.001$), and 7260 ± 330 versus 6100 ± 330 at 100 μM ($p > 0.1$).

The effect of the thioxanthenes on the accumulation of doxorubicin in both sensitive and MDR cell lines was also studied. Fig. 4 demonstrates that after a 3-hr incubation in 10 μM doxorubicin, by which time steady state concentrations were reached, MCF-7/DOX cells accumulated approximately 10-fold less doxorubicin than did the sensitive cell line. The addition of 3 μM (*cis*)- or 6 μM (*trans*)-flupenthixol had no significant effect on the accumulation of doxorubicin in the sensitive MCF-7 line. However, they increased by 2.4- and 4.6-fold, respectively, the accumulation of doxorubicin in the resistant MCF-7/DOX cells.

Isobologram analysis. To rigorously study the magnitude of potentiation of doxorubicin by (*trans*)-flupenthixol, their multiple drug effects were studied by isobologram analysis. Fig. 5 demonstrates the synergistic action of doxorubicin and (*trans*)-flupenthixol, evident by comparing the actual concentrations necessary for 50% inhibition of cell growth with those predicted for drugs that are simply additive.

Discussion

The present study identifies certain structural features of the PTZ molecule that affect its activity against cellular proliferation and MDR. The results suggest that these two actions of the PTZs are mediated by different mechanisms.

Specifically, increasing the hydrophobicity of the PTZ nucleus increased potency against cellular proliferation and against MDR, whereas decreasing the hydrophobicity decreased potency (Table 1). Thus, the $-CF_3$-substituted compounds were the most potent drugs, whereas −OH-substituted compounds were the least potent drugs. Chlorpromazine sulfoxide, the oxidative metabolite of chlorpromazine, lost most of its antiproliferative effect. However, it retained its effect against MDR, suggesting that first-pass hepatic metabolism of these drugs may not present a major impediment to their clinical use.

The type of amino group also significantly affected potency against MDR but not against cellular proliferation. For example, tertiary amines were more potent than primary or second-

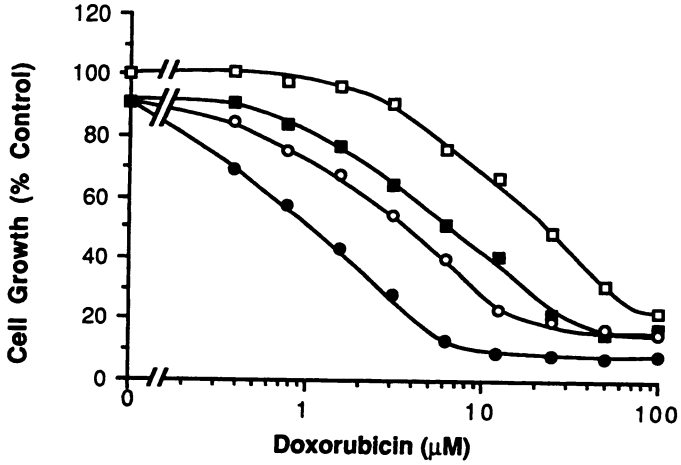


Fig. 3. Effect on the sensitivity of MDR cells to doxorubicin by PTZs and structurally related modifiers. MCF-7/DOX cells were exposed to 0–100 μM doxorubicin for 48 hr in the absence (□) or presence of fluphenazine (■), (*cis*)-flupenthixol (○), or (*trans*)-flupenthixol (●) at concentrations that alone produced 10% inhibition of cell growth. Cell growth was determined by a microtiter assay as described in Materials and Methods. Each *point* represents the mean of quadruplicate determinations, which differed by less than 5%.

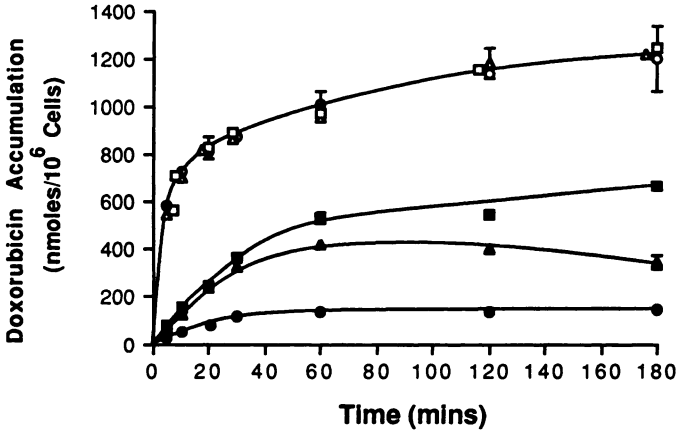


Fig. 4. Effect of thioxanthene isomers on the accumulation of doxorubicin in sensitive MCF-7 (*open symbols*) and MDR MCF-7/DOX cells (*closed symbols*). Cells were incubated with 10 μM doxorubicin in the absence (*circles*) or presence of 3 μM (*cis*)-flupenthixol (*triangles*) or 6 μM (*trans*)-flupenthixol (*squares*). Cell-associated doxorubicin at various times after the addition of drug was determined spectrofluorometrically as described in Materials and Methods. Values are means from triplicate determinations, with standard error bars when greater than 5% of the mean.

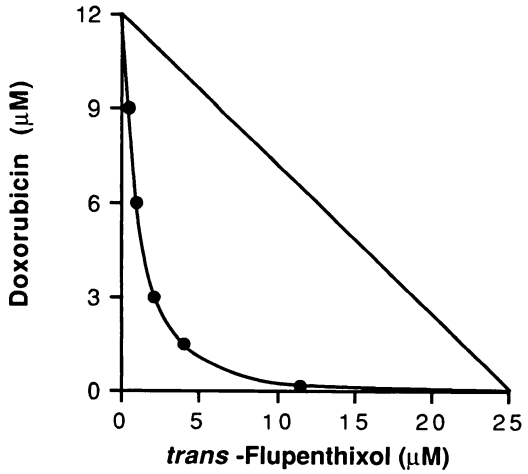


Fig. 5. Isobologram analysis of the interaction between doxorubicin and (*trans*)-flupenthixol. IC_{50} isobole for inhibition of MCF-7/DOX cell growth by various combinations of doxorubicin and (*trans*)-flupenthixol (●) was determined by exposing cells to drug combinations for 48 hr, as described in Materials and Methods. The *straight line* represents the predicted IC_{50} isobole for drugs that have additive antiproliferative effects. Doxorubicin (12 μM) and 25 μM (*trans*)-flupenthixol alone caused 50% inhibition of cell growth. Each *point* represents the mean value of quadruplicate determinations, which differed by less than 5%.

ary amines, and piperazinyl amines were more potent than noncyclic groups. Moreover, piperazinyl structures that possessed a *para*-methyl group had consistently greater activity than others (Table 2).

The distance between the amino group and the PTZ nucleus was important for both inhibition of cell growth and antagonism of MDR. A four-carbon chain was superior to alkyl bridges of shorter lengths (Table 3). Whether an alkyl bridge of greater than four carbons would further increase activity could not be determined in the present study because these derivatives were not available.

It has been postulated that the effects of the PTZs may be due solely to nonspecific membrane interactions resulting from their high degree of lipophilicity (27). A careful analysis of the relationship between hydrophobicity and inhibition of cellular proliferation or antagonism of MDR showed a correlation for ring-substituted PTZ derivatives (Fig. 1, A and B) but not for compounds with specific side chain alterations (Fig. 1, C and D). Thus, the degree of lipophilicity of each drug, although important, was not the sole determinant of potency for the antiproliferative or anti-MDR activity of PTZs.

This profile is reminiscent of that displayed by the interaction of PTZs with CaM. Studies of the structural features of PTZs that influence CaM antagonism revealed that ring-substitutions that increased hydrophobicity increased potency, whereas modifications of the type or length of the amino side chain affected potency in a manner unrelated to hydrophobicity (26). Similarly, studies with *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (28), and a series of 15 derivatives of W-7 (29), demonstrated that both halogenation of the naphthalene ring with chlorine, iodine, or cyano groups and increasing the length of the alkyl side chain from 4 to 12 carbons increased their potency against CaM.

The site(s) of action of PTZs and structurally related compounds for inhibition of cellular proliferation and antagonism of MDR have not been identified. The current data demonstrate that the antiproliferative activity of these drugs used individually against the malignant breast cancer cell lines MCF-7 and MCF-7/DOX correlates with their potency as CaM antagonists (Fig. 2A), supporting previous observations with a limited number of PTZs in MDA-MB-231 human breast cancer cells (10), C_6 astrocytoma cells (12), HL-60 human leukemia cells, L1210 murine leukemia cells, and HCT-8 human colonic carcinoma cells (13). These data are consistent with the known role of CaM in cellular proliferation (6, 8). However, the lack of correlation between anti-CaM activity and antagonism of MDR by equimolar (Fig. 2B), as well as equitoxic (data not shown), concentrations of PTZs points toward an alternative mechanism of inhibition for this effect.

The information gained from these studies allowed us to identify drugs with certain of the important features for anti-MDR activity. For example, (*cis*)- and (*trans*)-flupenthixol have a $-CF_3$ substitution at position 2 of the hydrophobic thioxanthene ring, possess a piperazinyl amino side chain, and have a three-carbon alkyl bridge. Although the thioxanthene isomers are more hydrophobic than PTZs due to the substitution of a carbon for a nitrogen in the cyclic ring [octanol/buffer partition coefficients (log *P*) for both flupenthixol isomers = 4.25 versus 4.04 for chlorpromazine (30)], this alone cannot explain their cellular effects. For example, they are less potent antiproliferative agents than chlorpromazine and other less hydrophobic PTZs. In addition, although the isomers are equally hydrophobic, (*trans*)-flupenthixol is a 3-fold more potent anti-MDR agent (Fig. 3), and both isomers are more potent than agents with greater hydrophobicity, such as pimozide (log $P = 4.88$). The orientation of the side chain amine in relation to the tricyclic nucleus was an important determinant of anti-MDR activity but not of antiproliferative activity. For example, (*trans*)-flupenthixol displayed greater activity than (*cis*)-flupenthixol against MDR (Fig. 3) but was equal to that of the *cis*-isomer against cellular proliferation (Table 4).

One explanation for the differences in anti-MDR activity observed for the thioxanthene stereoisomers would be differences in their cellular accumulation and, therefore, differences in access to intracellular targets. However, MDR cells actually accumulate significantly more (*cis*)- than (*trans*)-flupenthixol at effective anti-MDR doses (3–10 μM), the opposite of what would be expected if their potency as antagonists of MDR Reflected differences in their intracellular accumulation. This implies that the difference in anti-MDR activity between these stereoisomeric thioxanthenes might be due to differences in their ability to interact with a unique cellular target(s).

Although the antiproliferative effects of the PTZs and related compounds were approximately equal in both the MCF-7 and the MCF-7/DOX malignant cell lines, the ability of these drugs to potentiate the effect of doxorubicin (Table 4), as well as their ability to increase the cellular accumulation of doxorubicin (Fig. 4), occurred only in the MDR cell line. This suggests that the latter effects were mediated through a target(s) overexpressed in MDR cells. One logical site would be the putative drug efflux pump, P-glycoprotein, the gene product encoded by the recently cloned *mdr*1 gene (16, 31). A current hypothesis suggests that the mechanism by which MDR cells reduce cellular accumulation of anthracyclines is through the increased expression of this plasma membrane glycoprotein in MDR cells (14) and that compounds that antagonize MDR compete with

cytotoxic drugs for specific drug-binding sites on the protein (32). Although calcium channel blockers can inhibit binding of a photoaffinity-labeled vinblastine analog to P-glycoprotein, PTZs were far less effective (18). However, the failure of PTZs to block vinblastine binding to P-glycoprotein does not rule out the interaction with other sites on the protein. For example, Hamada and Tsuruo (17) have recently demonstrated ATPase activity of the molecule and shown that agents that inhibit active drug efflux, such as trifluoperazine and verapamil, cause an increase in P-glycoprotein ATPase activity, whereas doxorubicin and vincristine, agents that may interact with the putative drug-binding region, do not affect this ATPase activity (33). Alternatively, Center (34) demonstrated that trifluoperazine increased phosphorylation of this protein in MDR Chinese hamster lung cells and enhanced doxorubicin accumulation and cytotoxicity, suggesting the PTZs may indirectly affect P-glycoprotein. Inasmuch as the most potent anti-MDR agent studied, (*trans*)-flupenthixol, reduced the 10-fold difference in doxorubicin accumulation between sensitive and MDR cells to a 2-fold difference (Fig. 4), it is likely that this modifier acts through either direct or indirect inhibition of P-glycoprotein-mediated chemotherapeutic drug efflux. However, because of the many cellular effects of the PTZs and thioxanthenes, it is possible these agents exert their effect through more than one mechanism.

Another cellular enzyme affected by the PTZs that may play an important role in MDR is protein kinase C (21, 35). MCF-7/DOX cells have been shown to have increased levels of protein kinase C compared with parental MCF-7 cells (36, 37), and phorbol esters, which stimulate protein kinase C, produce increased levels of anthracycline resistance in several cell lines (37, 38). However, the concentration of trifluoperazine, chlorpromazine, and fluphenazine required to inhibit protein kinase XC in isolated systems (21) were 10- to 50-fold greater than those necessary to antagonize MDR in the present study. Thus, although the activation and inhibition of protein kinase C offers an attractive hypothesis for the modulation of MDR, it appears that the anti-MDR effects of the PTZs are not likely to be mediated solely through this enzyme.

Taking into account all of the principles derived from this analysis of PTZ-like compounds, structural features important for a drug to alter MDR include a hydrophobic thioxanthene ring nucleus with a $-CF_3$ substitution at position 2, an exocyclic double bond in the *trans* configuration, and a piperazinyl amine with a *para*-methyl group, joined by a four-carbon alkyl bridge to the nucleus. Similar structural features were identified as important for modulating MDR in human leukemic cells in a recent study with derivatives of indole alkaloids (39).

Clinical trials of the antipsychotic effects of flupenthixol in humans showed that (*cis*)-flupenthixol was far more effective than (*trans*)-flupenthixol and that the latter was far less toxic (40). This observation may be explained by biochemical and crystallographic evidence that (*cis*)-flupenthixol is a potent antagonist of dopamine receptors (41, 42), whereas (*trans*)-flupenthixol, which displays the greater potency against MDR, has virtually no activity as a dopamine antagonist. This may explain the apparent lack of extrapyramidal side effects seen with this agent (43). Extrapyramidal side effects have proven to be dose limiting in phase I trials that combined trifluoperazine with bleomycin (44) or doxorubicin (45). Therefore, future studies of the *in vivo* toxicity and efficacy of (*trans*)-flupenthixol in combination with doxorubicin and other chemotherapeutic agents are indicated to elucidate the potential of this drug for clinical use against MDR tumors.

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References

1. Roufogalis, B. D. Specificity of trifluoperazine and related phenothiazines for calcium binding proteins, in *Calcium and Cell function* (W. Y. Cheung, ed.), Vol. III. Academic Press, New York, 129–159 (1982).
2. Pang, D. C., and F. N. Briggs. Mechanism of quinidine and chlorpromazine inhibition of sarcotubular ATPase activity. *Biochem. Pharmacol.* **25**:21–25 (1976).
3. Ruben, L., and H. Rasmussen. Phenothiazines and related compounds disrupt mitochondrial energy production by a calmodulin-independent reaction. *Biochim. Biophys. Acta* **637**:415–422 (1981).
4. Creese, I., and D. R. Sibley. Receptor adaptations to centrally acting drugs. *Ann. Rev. Pharmacol. Toxicol.* **21**:357–391 (1981).
5. Levin, R. M., and B. Weiss. Mechanism by which psychotropic drugs inhibit adenosine cyclic 3′,5′-monophosphate phosphodiecterase of brain. *Mol. Pharmacol.* **12**:581–589 (1976).
6. Rasmussen, C. D., and A. R. Means. Calmodulin is involved in regulation of cell proliferation. *EMBO J.* **6**:3961–3968 (1987).
7. Veigl, M. L., T. C. Vanaman, and W. D. Sedwick. Calcium and calmodulin in cell growth and transformation. *Biochim. Biophys. Acta* **738**:21–48 (1984).
8. Hait, W. N., and J. S. Lazo. Calmodulin: a potential target for cancer chemotherapeutic agents. *J. Clin. Oncol.* **4**:994–1012 (1986).
9. Ito, H., and H. Hidaka. Antitumor effect of a calmodulin antagonist on the growth of solid Sarcoma 180. *Cancer Lett.* **19**:215–220 (1983).
10. Wei, J. W., R. A. Hickie, and D. J. Klaassen. Inhibition of human breast cancer colony formation by anticalmodulin agents: trifluoperazine, W-7, and W-13. *Cancer Chemother. Pharmacol.* **11**:86–90 (1983).
11. Hait, W. N., L. Grais, C. Benz, and E. C. Cadman. Inhibition of growth of leukemic cells by inhibitors of calmodulin: phenothiazines and melittin. *Cancer Chemother. Pharmacol.* **14**:202–205 (1985).
12. Lee, G. L., and W. N. Hait. Inhibition of growth of C6 astrocytoma cells by inhibitors of calmodulin. *Life Sci.* **36**:347–354 (1985).
13. Hait, W. N., and G. L. Lee. Characteristics of the cytotoxic effects of the phenothiazine class of calmodulin antagonists. *Biochem. Pharmacol.* **34**:3973–3978 (1985).
14. Riordan, J. R., and V. Ling. Genetic and biochemical characterization of multidrug resistance. *Pharmacol. Ther.* **28**:51–75 (1985).
15. Inaba, M., and R. K. Johnson. Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. *Biochem. Pharmacol.* **27**:2123–2130 (1978).
16. Chen, C., J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson. Internal duplication and homology with bacterial transport proteins in the *mdr* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**:381–389 (1986).
17. Hamada, H., and T. Tsuruo. Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance: 170- to 180-kilodalton membrane glycoprotein is an ATPase. *J. Biol. Chem.* **263**:1454–1458 (1988).
18. Akiyama, S., M. M. Cornwell, M. Kuwano, I. Pastan, and M. M. Gottesman. Most drugs that reverse multidrug resistance also inhibit photoaffinity labeling of P-glycoprotein by a vinblastine analog. *Mol. Pharmacol.* **33**:144–147 (1988).
19. Ganapathi, R., and D. Grabowski. Enhancement of sensitivity to Adriamycin in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine. *Cancer Res.* **43**:3696–3699 (1983).
20. Fleckenstein, A. Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. *Ann. Rev. Pharmacol. Toxicol.* **17**:149–166 (1977).
21. Schatzman, R. C., B. C. Wise, and J. F. Kuo. Phospholipid-sensitive calcium-dependent protein kinase: inhibition by anti-psychotic drugs. *Biochem. Biophys. Res. Commun.* **98**:669–676 (1981).
22. Finlay, G. J., B. C. Baguley, and W. R. Wilson. A semiautomated microculture method for investigating exponentially growing carcinoma cells. *Anal. Biochem.* **139**:272–277 (1984).
23. Berenbaum, M. C. Criteria for analyzing interactions between biologically active agents. *Adv. Cancer Res.* **35**:269–335 (1981).
24. Finney, D. J. *Statistical Method in Biological Assay.* Charles Griffin and Co., London (1978).
25. Bliss, C. I. Confidence limits for measuring the precision of bioassays. *Biometrics* **12**:491–526 (1956).
26. Prozialeck, W. C., and B. Weiss. Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships. *J. Pharmacol. Exp. Ther.* **222**:509–516 (1982).
27. Roufogalis, B. D. Comparative studies on the membrane actions of depressant drugs: the role of lipophilicity in the inhibition of brain sodium and potas-

sium-stimulated ATPase. *J. Neurochem.* **24**:51–61 (1975).
28. Hidaka, H., M. Asano, and T. Tanaka. Activity-structure relationship of calmodulin antagonists. *Mol. Pharmacol.* **20**:571–578 (1981).
29. MacNeil, S., M. Griffin, A. M. Cooke, N. J. Pettett, R. A. Dawson, R. Owen, and G. M. Blackburn. Calmodulin antagonists of improved potency and specificity for use in the study of calmodulin biochemistry. *Biochem. Pharmacol.* **37**:1717–1723 (1988).
30. Norman, J. A., and A. H. Drummond. Inhibition of calcium-dependent regulator-stimulated phosphodiesterase activity by neuroleptic drugs is unrelated to their clinical efficacy. *Mol. Pharmacol.* **16**:1089–1094 (1979).
31. Ueda, K., C. Cardarelli, M. M. Gottesman, and I. Pastan. Expression of a full-length cDNA for the human *mdr*1 gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. USA* **84**:3004–3008 (1987).
32. 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).
33. Hamada, H., and T. Tsuruo. Characterization of the ATPase activity of the M_r 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res.* **48**:4926–4932 (1988).
34. Center, M. S. Mechanisms regulating cell resistance to Adriamycin. *Biochem. Pharmacol.* **34**:1471–1476 (1985).
35. Aftab, D. T., and W. N. Hait. Multiple forms of protein kinase C in multidrug resistant MCF-7 human breast cancer cells. *Proc. Am. Assoc. Cancer Res.* **29**:1428 (1988).
36. Palayoor, S. T., J. M. Stein, and W. N. Hait. Inhibition of protein kinase C by antineoplastic agents: implications for drug resistance. *Biochem. Biophys. Res. Commun.* **148**:718–725 (1987).
37. Fine, R. L., J. Patel, and B. A. Chabner. Phorbol esters induce multidrug resistance in human breast cancer cells. *Proc. Natl. Acad. Sci. USA* **85**:582–586 (1988).
38. Ferguson, P. F., and Y. Cheng. Transient protection of cultured human cells against antitumor agents by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res.* **47**:433–441 (1987).
39. Zamora, J. M., H. L. Pearce, and W. T. Beck. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol. Pharmacol.* **33**:454–462 (1988).
40. Johnstone, E. C., T. J. Crow, C. D. Frith, M. W. P. Carney, and J. S. Price. Mechanism of the antipsychotic effect in the treatment of acute schizophrenia. *Lancet* **1**:848–851 (1978).
41. Huff, R. M., and P. Molinoff. Assay of dopamine receptors with [α-^{3}H] flupenthixol. *J. Pharmacol. Exp. Ther.* **232**:57–61 (1985).
42. Post, M. L., U. Kennard, and A. S. Horn. Stereoselective blockade of the dopamine receptor and the X-ray structures of α- and β-flupenthixol. *Nature (Lond.)* **256**:342–343 (1975).
43. Nielsen, I. M., V. Pedersen, M. Nymark, K. F. Franch, V. Boeck, B. Fjalland, and A. V. Christensen. The comparative pharmacology of flupenthixol and some reference neuroleptics. *Acta Pharmacol. Toxicol.* **33**:353–362 (1973).
44. Hait, W. N., S. Morris, J. S. Lazo, R. J. Figlin, H. J. Durivage, K. White, and P. E. Schwartz. Phase I trial of combined therapy with bleomycin and the calmodulin antagonist, trifluoperazine. *Cancer Chemother. Pharmacol.* in press.
45. Miller, R. L., R. M. Bukowski, G. T. Budd, J. Purvis, J. K. Weick, K. Shepard, K. K. Midha, and R. Ganapathi. Clinical modulation of doxorubicin resistance by the calmodulin-inhibitor, trifluoperazine: a phase I/II trial. *J. Clin. Oncol.* **6**:880–888 (1988).

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