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Reversal of multidrug resistance in murine fibrosarcoma cells by thioxanthene flupentixol

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Summary

The purpose of this study was to identify calcium channel and calmodulin antagonists effective in increasing the cytotoxic effects of several chemotherapeutic drugs against UV-2237 murine fibrosarcoma MDR cells. Among 8 compounds tested at nontoxic concentrations, flupentixol, a piperazine-substituted thioxanthene, was the most potent in enhancing the cytotoxicity of anticancer drugs commonly associated with the multidrug resistant (MDR) phenotype, such as Adriamycin, actinomycin D, vinblastine, and vincristine, but not 5-fluorouracil, a drug usually unaffected by MDR. The chemosensitizing effects of flupentixol were produced by increasing intracellular drug accumulation via a mechanism unrelated to the binding of the plasma membrane P-glycoprotein.

Introduction

The cross-resistance of tumor cells to Adriamycin and certain natural anticancer drugs has been considered to mark the development of the multidrug resistance (MDR) phenotype [1,2]. MDR has been shown to be associated with an amplification of the *mdr*1 gene and overexpression of its product, P-glycoprotein [3–5]. P-glycoprotein is a plasma membrane-bound energy-dependent drug efflux pump that prevents the intracellular accumulation of certain anticancer drugs [6]. In addition to overexpression of P-glycoprotein, factors implicated in MDR include topoisomerase II and tubulin mutation [5], altered cellular calcium and calmodulin levels [7], formation of double minute chromosomes [8], P-glycoprotein-independent facilitated drug efflux [9], enhanced sodium pump activity [10], overexpression of the 22-kDa sorcin [11,12], amplification of episomes [13,14], and elevated levels of protein kinase C (PKC) activity [15–17].

An increasing body of evidence demonstrates that the MDR phenotype can be circumvented, at least *in vitro*, by the use of calcium channel and calmodulin antagonists such as verapamil and trifluoperazine [18–21]. However, clinical studies to evaluate the potential therapeutic utility of verapamil in reducing tumor cell resistance to anticancer drugs *in vivo* have not been encouraging due to undesirable hemodynamic side effects [22]. For this reason, the use of compounds such as phenothiazine [23,24] and cyclosporine [25] that reverse the MDR phenotype with less severe side effects has attracted attention as alternative strategies for therapeutic reversal of resistance in cancer chemotherapy [21].

The MDR phenotype in tumor cells shares similarities with the chloroquine-resistance phenotype in the malarial parasite *Plasmodium falciparum* [26]. Resistance to chloroquine in malaria is mediated by a plasma membrane protein with sequence homology to the mammalian P-glycoprotein [27], and resistance can be modu-

lated by the same repertoire of calcium channel and calmodulin antagonists that circumvent drug-resistant tumor cells [28]. The purpose of the present study was to examine whether compounds that influence intracellular calcium can alter drug sensitivity. We report that a piperazine-substituted thioxanthene, flupentixol, potentiates the activity of several anticancer drugs against which MDR resistance is known to develop.

Materials and methods

Cell lines and culture conditions

The Adriamycin (ADR)-sensitive UV-2237 parent line is a UV-induced fibrosarcoma syngeneic to C3H/HeN [29]. ADR-resistant variants were selected from the parental line by continuous exposure to the drug. The UV-2237-R1 and UV-2237-R10 [15] are established cell lines that are maintained in culture medium containing 1 and 10 μg/ml of ADR, respectively. All cells were maintained on plastic in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, L-glutamine, pyruvate, nonessential amino acids, and vitamins (GIBCO, Long Island, NY). All cell cultures were free of mycoplasma, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocyte choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus.

Chemicals and reagents

Bepridil, diltiazem, flunarizine, flupentixol, lidoflazine, R-56865, sabeluzole, and verapamil were supplied by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). A stock solution of each compound was prepared in deionized distilled water or in dimethyl sulfoxide (DMSO) and stored at –70°C for up to 30 days. ADR (NDC 0013-1086-91, Adria Laboratories, Columbus, OH), actinomycin D (NDC 0006-3298-22, Merck Sharp & Dohme, West Point, PA), vinblastine sulfate (NDC 53906-091-10, Cetus Corporation, Emeryville, CA), and vincristine sulfate (NDC 51309-201-02, QUAD Pharmaceuticals, Indianapolis, IN) were dissolved in 0.9% sodium chloride, stored in 0.2-ml aliquots at –70°C, and used within 30 days. Fluorouracil (NDC 39767-012-10, SoloPak, Franklin Park, IL) was dissolved in 0.9% sodium chloride, stored in 0.2-ml aliquots at room temperature, and used within 30 days. Tetrazolium (MTT, M2128) was purchased from Sigma Chemical Co. (St. Louis, MO), and a stock solution was prepared by dissolving 2.5 mg of MTT in 1 ml of PBS and filtering the solution to remove particulates. The solution was protected from light, stored at 4°C, and used within 30 days. [14-^{14}C]Adriamycin hydrochloride (CFA.615, specific radioactivity 55 mCi/mmol), and [^{3}H]azidopine (TRK.821, specific radioactivity 47 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL. Fluorescein isothiocyanate (FITC)-labeled and non-labeled monoclonal antibodies to P-glycoprotein (P-glycoCHEK-C219) were purchased from Centocor, Inc., Malvern, PA. Protein A-Sepharose CL-4B was purchased from Pharmacia, Piscataway, NJ. Unless otherwise specified, reagents for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA.

Assay for in vitro *cytostasis*

Since the seeding density of target cells in a 38-mm^2 culture well can influence the results of a cytostasis assay, the optimal conditions to measure growth inhibition of the UV-2237 cells were initially determined to be between 2×10^3 and 5×10^3 cells/well in 0.2 ml of medium. After 4 days growth, this plating density allowed for ready quantitation but did not produce confluency. In all assays, tumor cells were seeded into 38-mm^2 wells of flat-bottomed 96-well plates in quadruplicate and allowed to adhere overnight. The cultures were then washed and refed with medium (negative control), medium containing ADR or other chemotherapeutic drugs (positive control), or medium containing test agents with or without chemotherapeutic drugs. After 4 days, tumor cell growth was determined by the MTT assay, which monitors the number of metabolically active cells [30]. After incubation for 2–4 hours in medium containing 0.42 mg/ml of MTT, the cells were lysed in DMSO. The conversion of MTT to formazan by

metabolically viable cells was monitored by a MR-5000 96-well microtiter plate reader at 750 nm (Dynatech, Inc., Chantily, VA). Growth inhibition was calculated by the formula:

$$\text{cytostasis (\%)} = [1-(A/B)] \times 100,$$

where A is the absorbance of treated cells, and B is the absorbance of the control cells.

Adriamycin accumulation

The effects of the calcium channel antagonists on intracellular accumulation of ADR was evaluated [30]. Cells (1×10^6) were plated into 35-mm tissue culture dishes. Eighteen hours later, the cells were treated with test compounds, such as trans-(E)-flupentixol, at 0.5 μg/ml for various times. The medium was removed and the cultures were reincubated in 2 ml of fresh medium containing [^{14}C]ADR (specific activity 27.5 mCi/mmol) at 37°C with or without the test compound. After 15 min, 1 hour, 4 hours, and 6 hours, the cultures were washed four times with ice-cold PBS. An independent zero time control was used to monitor nonspecific labeling of the cells by incubating the cells in ice-cold medium containing [^{14}C]ADR at 4°C for 6 hours. To determine drug retention, cells were first incubated with [^{14}C]ADR for 4 hours with or without the test compound, then washed with ice-cold PBS, and incubated in ADR-free medium at 37°C for various times. In all assays, the cells were lysed for 10 min in 1 N NaOH at 60°C. The radioactivity of the lysates was monitored in a liquid scintillation counter.

Expression of immunoreactive P-glycoprotein

The level of P-glycoprotein in tumor cells was determined by the FITC-labeled murine monoclonal antibody C219, which reacts with a P-glycoprotein epitope expressed on the internal surface of the plasma membrane [31]. Single-cell suspensions were prepared from semiconfluent cultures by a 1-min treatment with 0.25% trypsin and 0.02% EDTA. The cells were washed with ice-cold PBS, fixed in 70% methanol for 7 min at –20°C, and then washed again with ice-cold PBS. The cells were resuspended to 5×10^6 cells/ml in ice-cold PBS containing 1% bovine serum albumin (BSA). P-glycoCHEK reagents were reconstituted in 1 ml of deionized water, and 5 μl of the FITC-C219 or control antibody was added to 100-μl samples of cells at 4°C. One hour later, the cells were washed twice and resuspended in PBS-1% BSA. The samples were analyzed with an Epics Profile flow cytometer (Coulter Corp., Hialeah, FL). Cell-bound FITC-C219 fluorescence was determined, and the expression of the plasma membrane P-glycoprotein was calculated by normalizing its relative mean channel fluorescence with that of the FITC-irrelevant antibody [30].

Photoaffinity labeling of P-glycoprotein

Plasma membrane fractions were prepared by homogenization and differential centrifugation of tumor cells harvested from semiconfluent cultures. Protein concentration was determined by the bicinchoninic acid (BCA) method. Plasma membrane vesicles (50 μg of protein) in 40 mM potassium phosphate buffer (pH 7.0) containing 4% (vol/vol) DMSO [30] were incubated at room temperature with 0.25 μM [^{3}H]azidopine (2.5 μCi) for 30 min in the absence or presence of sensitizing agents. The samples were irradiated with ultraviolet light at 366 nm for 20 min at room temperature and then solubilized in 200 μl of deoxycholate buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.5% deoxycholate, 1 mM phenyl-methylsulfonylfluoride) for 30 min at 4°C. To immunoprecipitate the labeled P-glycoprotein, solubilized samples were incubated overnight at 4°C with 10 μg of specific anti-P-glycoprotein C219 antibody (Centocor, Inc.). To bind the antibodies, protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) was added to the mixture and incubated for 30 min at 4°C. The protein A-Sepharose-antibody pellets were washed five times with deoxycholate buffer. To release the proteins, the final pellets were resuspended in Laemmli sample buffer [32]. The Sepharose beads were removed by centrifugation, and the labeled proteins were separated by electrophoresis under reducing conditions on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel using a discontinuous buffer system of Laemmli [32].

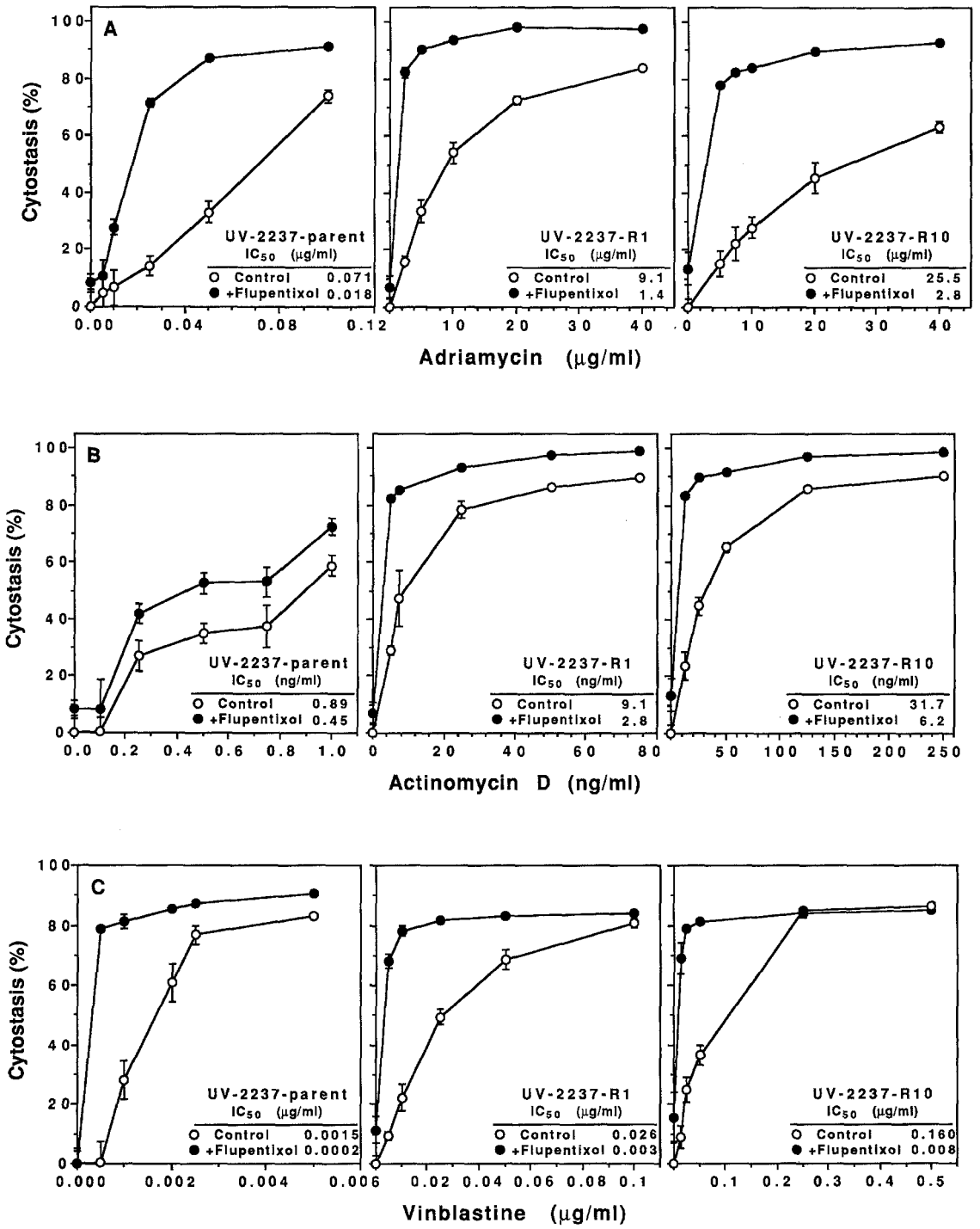

Fig. 1. Chemosensitization of the UV-2237 murine fibrosarcoma parental and MDR variants to ADR by flupentixol (A), actinomycin D (B), vinblastine (C), vincristine (D), and 5-fluorouracil (E). Cells were seeded at 2–5 × 10^3 cells/38 mm^2 well in quadruplicate. After an attachment period of 16 hours, quadruplicate samples were treated for 4 days with a concentration range of drugs with (●) or without (○) 0.5 μg/ml of flupentixol. Cell growth was determined by the MIT assay. These are representative data from one of 3 experiments. The values are the mean ± S.D.

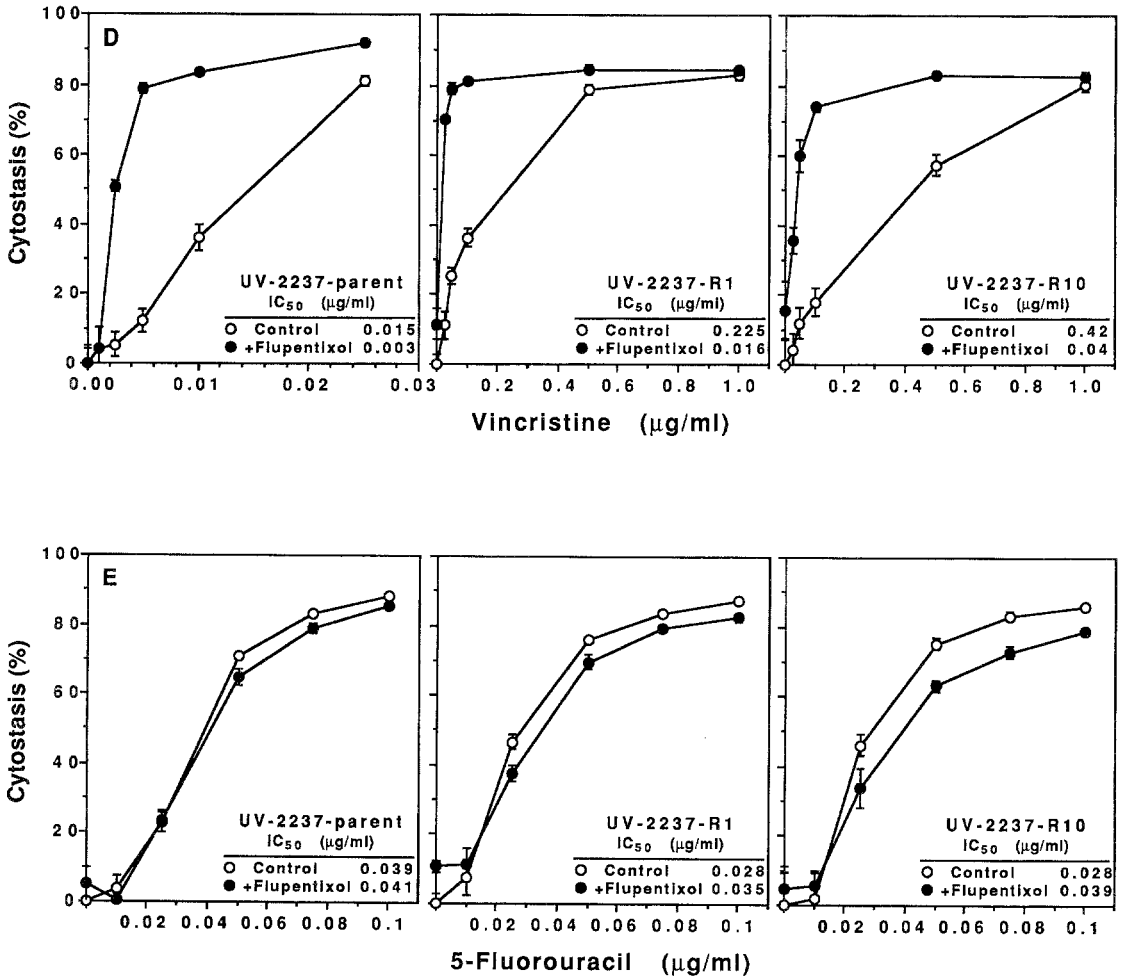

Fig. 1d,e.

Competitive binding of the test compound against [^{3}H]azidopine to P-glycoprotein was visualized by fluorography [33]. After they were stained with Coomassie blue and destained, the gels were soaked in Amplify (Amersham, Arlington Heights, IL) for 30 min and dried under a vacuum at 75°C. The dried gels were exposed to Hyperfilm-MP autoradiography films (Amersham) for 3 to 15 days at –70°C and developed. The competition of the most effective sensitizing agent, trans-(E)-flupentixol, against [^{3}H]azidopine for functional binding to P-glycoprotein was determined by comparison to the control.

Results

Cytotoxic effects of calcium channel and calmodulin antagonists

Although many of the compounds tested in this study have been approved for clinical use, it was necessary to exclude any direct *in vitro* cytotoxicity against the target cells. Tumor cells were incubated with different concentrations of the test agents for 4 days and their effects on cell growth determined by the MTT assay. At concentrations of up to 0.05 µg/ml, none of the compounds examined were toxic. At 0.5 µg/ml, only Sabeluzole was toxic, and then only against the UV-2237-R10 cells (Table 1).

Table 1. Chemosensitization of UV-2237 murine fibrosarcoma cells to ADR by various calcium channel blockers

		Cytostasis (%)[a]					
		UV-2237		UV-2237-R1		UV-2237-R10	
Agents	μg/ml	–ADR	+ADR	–ADR	+ADR	–ADR	+ADR
Adriamycin[b]			8.7		13.8		13.2
Bepridil	0.005	1.5	27.4	0.0	6.1	–5.3	21.7
	0.05	6.8	30.5	–1.2	5.0	6.1	24.1
	0.5	9.1	30.8	0.7	3.7	11.2	29.5
Verapamil	0.005	–0.4	25.2	–13.3	–7.6	2.8	19.8
	0.05	5.1	30.1	–4.6	–1.5	6.2	28.7
	0.5	6.3	56.3	–2.1	29.4	17.2	48.1
Lidoflazine	0.005	3.1	24.8	–4.7	12.4	–0.5	20.6
	0.05	6.9	30.0	–4.3	13.9	–2.2	20.1
	0.5	8.6	34.5	–4.3	17.1	3.2	22.5
Sabeluzole	0.005	2.9	20.8	–2.0	5.3	5.4	29.3
	0.05	6.4	22.8	0.1	12.7	8.9	32.8
	0.5	14.3	37.4	9.7	31.3	23.9	59.6
R-56865	0.005	6.2	23.7	–1.8	18.1	11.7	24.6
	0.05	10.9	24.0	–3.9	19.8	7.3	25.8
	0.5	17.5	26.4	0.7	27.5	9.3	26.7
Flupentixol	0.005	–6.6	22.1	–15.0	8.5	–14.1	20.0
	0.05	7.5	32.0	–9.6	23.4	–0.3	31.3
	0.5	12.8	53.7	–3.1	71.4	1.5	76.7
Diltiazem	0.005	–0.5	10.1	–6.2	14.5	–1.9	24.7
	0.05	6.8	18.9	–11.0	15.6	5.0	26.4
	0.5	7.1	42.3	–8.1	19.7	6.7	28.1
Flunarizine	0.005	3.7	27.9	–11.1	8.7	–0.3	25.0
	0.05	8.3	21.5	–7.8	16.8	5.1	28.3
	0.5	12.1	53.3	–0.1	31.1	15.1	52.8

[a]UV-2237 cells (2–5 × 10^3 cells/38 mm^2 well in quadruplicate) were allowed to attach for 16 h, then washed and refed with fresh medium with or without the indicated concentrations of drugs. The cells were incubated for an additional 4 days and growth was measured by the MTT procedure. The values are the mean of three experiments. SEM did not exceed 13.5%.
[b]The respective concentrations of ADR were 0.02 μg/ml for the UV-2237 parental cells; 2 μg/ml for UV-2237-R1 cells; and 10 μg/ml for UV-2237-R10 cells.

Enhanced effects of calcium channel and calmodulin antagonists on Adriamycin

To determine whether any of the 8 test compounds could enhance tumor cell sensitivity to ADR, tumor cells were incubated with suboptimal cytostatic concentrations of ADR over a dose range of 0.005 to 0.5 μg/ml. All 8 compounds enhanced the cytotoxicity of ADR against the parental cells (Table 1). The trans-(E)-flupentixol, a piperazine-substituted thioxanthene, enhanced ADR-mediated cytotoxicity against the drug-sensitive parental UV-2237 cells and the two MDR variants (Table 1). Therefore, flupentixol at the concentration of 0.5 μg/ml was used in control studies.

Effects of flupentixol on the MDR phenotype

To determine if flupentixol influenced the MDR phenotype, tumor cells were treated with flupentixol and one of the following drugs: ADR, actinomycin D, vinblastine, vincristine, or 5-fluorouracil. Flupentixol enhanced the cytotoxicity of ADR, actinomycin D, vinblastine, and vincristine against both drug-sensitivity and MDR cells (Figs. 1A–1D) but not 5-fluorouracil, a cytotoxic drug unrelated to the MDR phenotype (Fig. 1E, Table 2). These observations, coupled with the reduced equitoxic ratios (IC_{50} of a given drug to the resistant cells/IC_{50} of the same drug to the parental cells) and the increased chemo-

Table 2 Equitoxic ratios of chemotherapeutic drugs and enhancement indices of the UV-2237 murine fibrosarcoma cells treated with flupentixol

	Equitoxic ratios[a]								
	Minus flupentixol			Plus flupentixol			Enhancement indices[b]		
Drug	Parent	R1	R10	Parent	R1	R10	Parent	R1	R10
Adriamycin	1	128	359	1	77	155	4	7	9
Actinomycin D	1	10	36	1	6	14	2	3	5
Vinblastine	1	17	107	1	15	40	8	9	20
Vincristine	1	15	28	1	5	13	5	14	11
5-Fluorouracil	1	0.7	0.7	1	0.9	0.9	1	0.8	0.7

[a]Equitoxic ratio = IC_{50} of a drug to the resistant cells/IC_{50} of the same drug to the parental cells. A larger value indicates a higher degree of resistance to the drug.
[b]Enhancement index = IC_{50} of a drug to the drug-treated cells/IC_{50} of the same drug to the drug- and flupentixol-treated cells. A larger value indicates a greater enhancement of cytotoxicity by flupentixol.

sensitization (IC_{50} of a given drug to drug-treated cells/IC_{50} of the same drug- and flupentixol-treated cells) of the drug-resistant variants suggest that flupentixol reversed the MDR phenotype in the UV-2237 murine fibrosarcoma cells (Table 2).

Effects of flupentixol on intracellular accumulation of ADR

Since cells expressing an MDR phenotype often exhibit decreased intracellular accumulation of anticancer drugs [6], the accumulation of ADR in UV-2237 cells treated with flupentixol was measured. Cells were pretreated with 0.5 μg/ml of flupentixol for 16 hours after which [^{14}C]ADR was added in the presence of the same concentration of flupentixol for various times. After 6 hours incubation, flupentixol increased the intracellular concentration of [^{14}C]ADR in the target cells (Fig. 2). However, the [^{14}C]ADR retention rate was similar in cells growing with or without flupentixol in the medium after preincubation with [^{14}C]ADR for 4 h (Fig. 2).

Effect of flupentixol on plasma membrane P-glycoprotein expression

We next determined whether treatment of tumor cells with flupentixol altered the expression of P-glycoprotein as assayed by flow cytometry using the FITC-C219 antibody. The relative mean channel fluorescence units (RFUs) of FITC-C219 binding to P-glycoprotein in the UV-2237-R10 (110 RFU) and the UV-2237-R1 (48 RFU) cells was 7.3- and 3.2-fold higher than that of the drug-sensitive parental cell (15 RFU) (Fig. 3A). Treatment with flupentixol did not change the FITC-C219 binding profiles. The RFU values for flupentixol-treated cells were 12, 47, and 102 for the parental, UV-2237-R1, and UV-2237-R10 cells, respectively (Fig. 3B).

Effect of flupentixol on the photoaffinity labeling of P-glycoprotein

To determine whether flupentixol bound to P-glycoprotein and thus interfered with drug efflux, we next measured the competitive binding of the photoaffinity labeling compound azidopine to P-glycoprotein. The chemosensitizing effect produced by flupentixol was independent of binding on the plasme membrane P-glycoprotein since flupentixol did not change the photoaffinity binding of [^{3}H]azidopine to P-glycoprotein (Fig. 4).

Discussion

The emergence of drug resistance in tumor cells during chemotherapy presents a major obstacle to the successful treatment of cancer. The discovery

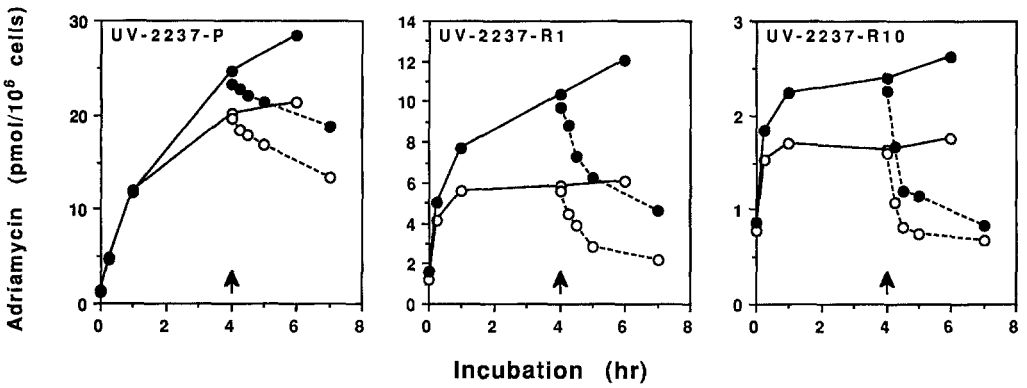

Fig. 2. Effects of flupentixol on intracellular accumulation and retention of ADR. Fibrosarcoma cells were seeded at 1×10^6 cells in 35-mm tissue culture dish in triplicate. The cells were pretreated for 16 hours with 0.5 μg/ml of flupentixol. Fresh medium containing 0.5 μg/ml of flupentixol and [^{14}C]ADR was added to the cultures. At various times, the medium was removed, the cells washed, and the intracellular radioactivity monitored. (–○–) Accumulation of ADR without flupentixol. (–●–) Accumulation of ADR with flupentixol. (...○...) Retention of ADR without flupentixol. (...●...) Retention of ADR with flupentixol. Arrow, the time that cells were washed and returned to ADR-free medium.

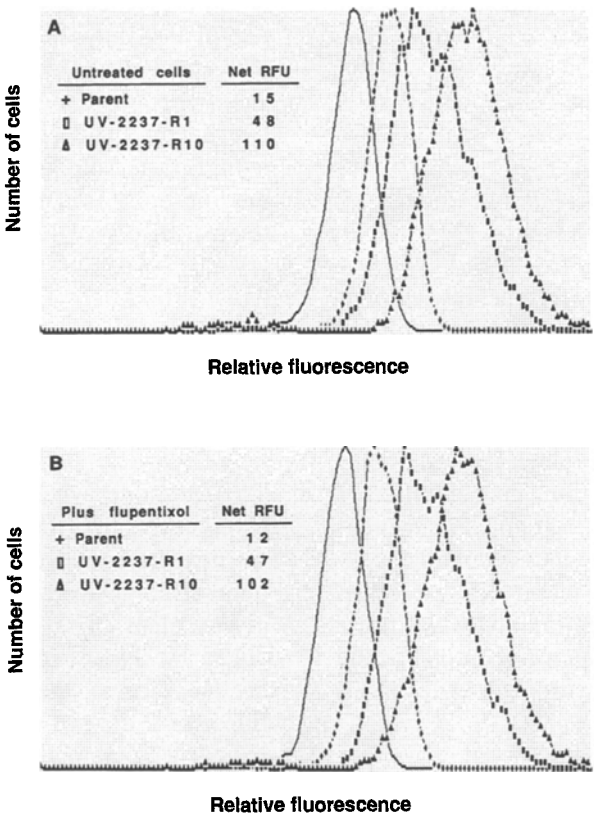

Fig. 3. Effect of flupentixol on the flow cytometric labeling profiles of UV-2237 cells by murine monoclonal antibody FITC-C219 directed against a cytoplasmic epitope of plasma membrane P-glycoprotein. Single-cell suspensions were prepared from subconfluent cultures incubated for 24 hours in medium with (B) or without (A) 0.5 μg/ml of flupentixol. The cells were fixed in 70% methanol, washed in PBS containing 1% BSA, and reacted with the FITC-C219 or FITC-negative antibodies. The samples were analyzed by an Epics Profile flow cytometer. The net mean channel relative fluorescence units (RFU) are shown minus the negative control fluorescence (first profile from the left).

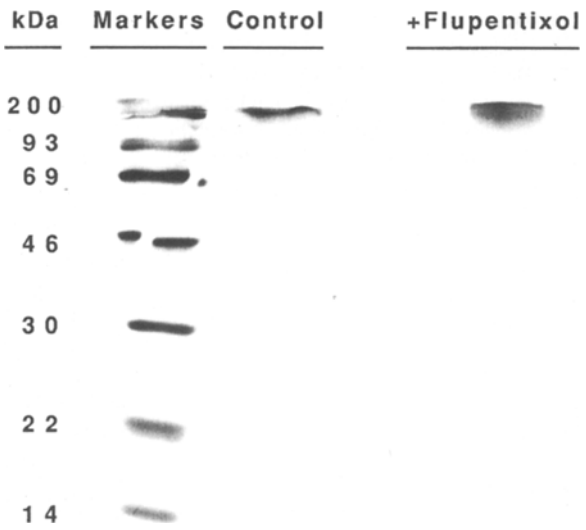

Fig. 4. Effect of flupentixol on photoaffinity labeling of UV-2237-R10 cells' P-glycoprotein by azidopine. Cell lysates (50 μg/ml of protein) prepared from UV-2237-R10 cells were photoaffinity-labeled by 0.25 μM [^{3}H]azidopine in the absence or presence of 0.5 μg/ml of flupentixol for 30 min at room temperature prior to UV irradiation at 366 nm. The labeled proteins were first immunoprecipitated by C219 and then separated on a 15% SDS-polyacrylamide gel. The labeled proteins in the dried gel were detected by fluorography with an exposure time of 2 to 7 days on Hyperfilm-MP at –70°C.

that the MDR phenotype can be reversed by calcium channel blockers [18,20], which influence the function of membrane P-glycoprotein, has prompted an active search for therapeutic approaches to reverse MDR.

Some antipsychotic phenothiazines have been shown to modify MDR [24], possibly by affecting the calcium messenger system. Indeed, active phenothiazine are known to inhibit voltage-dependent calcium channels [34], calmodulin [7], and protein kinase C [35].

Similar to previous observations in which flupentixol reversed ADR resistance in MCF-7/DOX cells [24], we found that at nontoxic doses, flupentixol could sensitize the UV-2237 murine fibrosarcoma MDR variants to ADR, actinomycin D, vinblastine, and vincristine, which are natural anticancer drugs commonly associated with the MDR phenotype. However, flupentixol did not alter cytotoxicity mediated by 5-fluorouracil, whose activity is independent of the MDR phenotype. The chemosensitizing effects of flupentixol are mediated by an increase in the intracellular accumulation of drugs such as ADR. However, flupentixol did not change the expression of the energy-dependent efflux pump P-glycoprotein nor compete with azidopine for its binding to the P-glycoprotein. The partial reversal of the MDR phenotype in the fibrosarcoma cells produced by flupentixol may be mediated via a mechanism unrelated to its effects on P-glycoprotein expression.

The finding that flupentixol also enhanced the chemosensitivity, although to a lesser degree, of the parental cells was of obvious concern. However, since the heterogeneous nature of tumor and cancer metastasis was conceptualized, it should be obvious that MDR is a resultant clinical outcome manifested by successful cancer cells endowed with multiple mechanisms for survival. Like other vital traits of neoplasms, MDR should be conceived as a phenotype marked by a collection of independent or collateral modification, overexpression and/or amplification of endogenous molecules interplay with distinct normal cellular pathways. Those cancer cells that presumably exist employing single and especially unique oncogenic mechanism, would have been eliminated by host defense during the progression of cancer or by conventional cancer therapy and would be without further clinical manifestation [21]. Hence, drug resistance (sensitivity) and its reversal (enhancement) remain as relative conditions between multiple sets of cell-growth within a wide spectrum of reactivity to drugs instead of an all-or-none phenomenon. The UV-2237 murine fibrosarcoma system that we used in this study is considered to express a high degree of intrinsic resistance to many drugs and biomolecules. The parental cells possess high levels of P-glycoprotein and its MDR variant can proliferate in the presence of 10 μg/ml (clinical peak plasma level ca. 1 μg/ml) of Adriamycin. It will be optimal but difficult to develop any compound specific only in reversing drug resistance of the MDR cells but sparing that of the parental cells. Therefore, a successful MDR reversal agent may be more a product of sensible pharmacology, such as proper dosing and scheduling, than specificity.

Although it is the general consensus that P-glycoprotein plays a central role in MDR, atypical MDR phenotypes unrelated to the presence of P-glycoprotein [36] have been documented. One possible mechanism for the specific effects of

flupentixol on the murine UV-2237 fibrosarcoma cells may be mediated via a facilitated drug efflux [9] or the sodium pump [10], which are believed to be independent of P-glycoprotein. However, the possibility that flupentixol may affect other functional aspects of P-glycoprotein than binding, such as ATP hydrolysis, cannot be ruled out. Several calcium channel antagonists have been used clinically to reverse MDR in cancer cells. Unfortunately, the use of the drugs verapamil [22] and trifluoperazine [37,38] has been associated with cardiotoxicity and neurotoxicity, respectively. In contrast, other clinical uses of flupentixol has been shown to be relatively nontoxic [39]. For this reason, future *in vivo* studies are warranted to determine the value of flupentixol in reversing the MDR phenotype of tumor cells growing *in vivo*.

Acknowledgements

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