

Verapamil-mediated Sensitization of Doxorubicin-selected Pleiotropic Resistance in Human Sarcoma Cells: Selectivity for Drugs Which Produce DNA Scission¹

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ABSTRACT

The effects of verapamil on the cytotoxicity and accumulation of multiple drugs were studied in a model of pleiotropic resistance generated by doxorubicin (DOX) selection of the human sarcoma cell line MES-SA. The *in vitro* sensitivity of the DOX-resistant variant (named Dx5), which is 50- to 100-fold resistant to DOX compared to MES-SA, was enhanced approximately 7-fold by verapamil (3 µg/ml). In addition, the cytotoxicity of several agents to which the Dx5 line displays cross-resistance, *i.e.*, daunorubicin, dactinomycin, mitoxantrone, and etoposide, was also enhanced 2- to 14-fold by verapamil. These agents share the properties of DNA intercalation and/or interaction with topoisomerase II. In contrast, verapamil did not alter the sensitivity of Dx5 to several other agents to which cross-resistance had been demonstrated, *i.e.*, vincristine, vinblastine, colchicine, mitomycin C, and melphalan; nor did verapamil enhance the cytotoxicity of DOX or other agents against the DOX-sensitive parent, MES-SA.

The sensitizing effect of verapamil did not correlate well with its effects on intracellular drug accumulation. [¹⁴C]DPX accumulation was increased by 30-40% in Dx5 but not in MES-SA cells in the presence of verapamil. [³H]Vinblastine accumulation was increased by 24-72% in both MES-SA and Dx5 cells in the presence of verapamil, although cytotoxicity of the *Vinca* alkaloids was not affected.

In this human sarcoma model of DOX-selected pleiotropic resistance, verapamil partially reversed the resistance to DOX, as well as four of the nine drugs for which cross-resistance had been demonstrated in Dx5. The potentiation by verapamil of the cytotoxicity of some but not all of these antitumor agents suggests that factors other than altered drug transport may be responsible. The pattern of sensitization, restricted to agents which produce DNA strand scission by interaction with topoisomerase II, suggests that verapamil may be acting to promote the formation or inhibit the repair of such DNA strand breaks.

INTRODUCTION

The anthracycline antibiotic DOX⁵ is a clinically valuable chemotherapeutic agent which possesses a broad spectrum of antitumor activity (1, 2). The development of resistance to DOX represents one of the major obstacles to effective therapy with this agent (3-6). Mammalian cells which have been selected for resistance to DOX and its sister compound daunorubicin *in vitro* typically display resistance to multiple chemo-

therapeutic agents (pleiotropic drug resistance), including the *Vinca* alkaloids and dactinomycin (7-17). Most of these resistant lines are thought to be resistant on the basis of decreased drug retention as a result of enhanced efflux of drug from the resistant cells (10-16, 18-23).

Recent studies have identified several calcium antagonists and calmodulin inhibitors which can enhance the cytotoxic effects of DOX and daunorubicin in mammalian cells selected for resistance to these agents (24-30). The enhanced cytotoxicity of DOX in the presence of the calcium antagonist verapamil has been associated with increased cellular accumulation and retention of DOX in some models. The ability of verapamil to promote responsiveness in drug resistant cells *in vitro* has led to the design of clinical trials utilizing verapamil in combination with standard chemotherapy (28).

In this study, we have examined the effect of verapamil on intracellular DOX accumulation and cytotoxicity in variants of the human sarcoma cell line, MES-SA, which were selected for resistance to DOX. In addition, we have studied the ability of verapamil to enhance the cytotoxicity of the various agents to which the DOX-resistant cell line displays cross-resistance.

MATERIALS AND METHODS

Cell Culture. The DOX-resistant MES-SA/Dx5 cell line (hereafter referred to as Dx5) was derived from the human uterine sarcoma cell line MES-SA and displays 50- to 100-fold resistance to DOX compared to MES-SA (11, 31). Monolayer cultures of both the DOX-sensitive and -resistant lines were grown in W/M medium (Grand Island Biological Company, Grand Island, NY) supplemented with penicillin (100 µg/ml), streptomycin (100 µg/ml), insulin (5 µg/ml), and 7.5-15% NBCS.

Drugs. DOX, DNR, dactinomycin, mitoxantrone, VBL, vincristine, etoposide, bleomycin, 5-fluorouracil, carmustine, melphalan, mitomycin C, and methotrexate were obtained from the Pharmaceutical Resources Branch, National Cancer Institute, NIH. Verapamil hydrochloride (Calan) was a generous gift of Searle Pharmaceuticals, Inc., Skokie, IL. Colchicine was obtained from Sigma Chemical Co., St. Louis, MO. [¹⁴C]DOX (doxorubicin HCl-14-¹⁴C; 13.4 µCi/mg) was synthesized by Morris Leaffer at SRI International (Menlo Park, CA) and made available through Dr. Robert Engle of the Pharmaceutical Resources Branch, National Cancer Institute. [³H]Vinblastine (12.5 mCi/mg) was purchased from Amersham Corp., Arlington Heights, IL. The radioactivity of the [¹⁴C]DOX and [³H]vinblastine was >95% as determined by thin layer and high performance liquid chromatographic assay (32, 33).

***In Vitro* Drug Sensitivity.** The drug concentrations required for 50% inhibition of colony formation by DOX, verapamil, and other drugs were determined using a soft agar clonogenic assay as described previously (31). MES-SA or Dx5 cells were exposed to various concentrations of the drugs for 1 h at 37°C in the presence or absence of verapamil. Following drug treatment, the cells were washed twice with HBSS + 10% NBCS and plated in triplicate in W/M medium containing 0.3% agar. The plating efficiencies of cells which had been exposed to drug alone, verapamil alone, or drug plus verapamil were compared to the plating efficiencies of untreated control cells for each line.

DOX and VBL Transport Studies. Uptake and efflux of [¹⁴C]DOX and [³H]VBL in MES-SA and Dx5 were studied in cells in mid-logarithmic growth phase. For uptake studies, cells were harvested by

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⁵ The abbreviations used are: DOX, doxorubicin; W/M, Waymouth's McCoy's 5A medium (1:1); NBCS, newborn calf serum; Dx5, the MES-SA/Dx5 subline; HBSS, Hanks' balanced salt solution; VBL, vinblastine; PBS, Dulbecco's phosphate buffered saline.

exposure for 3–5 min to 0.06 M EDTA, pH 7.4, washed twice with HBSS + 10% NBCS, and resuspended in W/M medium containing 7.5% NBCS, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and insulin (5 µg/ml) at 37°C, pH 7.4 (hereafter called transport medium). DOX uptake was initiated by the addition of [¹⁴C]-DOX to the cell suspension (5 × 10⁶ cells/ml) at a final DOX concentration of 1.0 µg/ml. [³H]VBL uptake was studied at VBL concentrations of 0.1 and 1.0 µg/ml. Both DOX and VBL uptake were studied in the presence or absence of verapamil at a concentration of 1.0 µg/ml (2.04 µM). For those studies, verapamil was added to the cells just prior to the addition of labeled drug. At various time points after the addition of labeled drug, 0.5-ml aliquots containing 1.0 × 10⁶ cells were removed, added directly to microcentrifuge tubes containing 0.6 ml of ice-cold Dulbecco's phosphate buffered saline (Grand Island Biological Co.), and centrifuged at 12,000 × g for 1 min to remove the cells from the drug containing medium. The cell pellets were then washed again with cold PBS, and the medium was aspirated. The tube tips containing cell pellets were then cut off, and tip contents were solubilized in 0.2 N NAOH for 12 h at 60°C. The tube contents were then neutralized, scintillation fluor was added, and radioactivity was determined.

For studies of cellular retention of [¹⁴C]DOX and [³H]VBL cells were harvested, washed twice with HBSS + 10% NBCS, and resuspended in transport medium at 37°C. Cells were allowed to accumulate labeled drug for 60 min at a [¹⁴C]DOX concentration of 1.0 µg/ml or at [³H]-VBL concentrations of 0.1 and 1.0 µg/ml, following which the cells were washed twice with cold (4°C) PBS and then resuspended in medium free of labeled drug at 37°C. The effect of verapamil on efflux of DOX and VBL from MES-SA and Dx5 was assessed by suspending drug loaded cells in medium with and without verapamil (1 µg/ml). At various points after the resuspension of the drug loaded cells into drug free medium, 0.5-ml aliquots containing 1 × 10⁶ cells were removed, washed twice with cold PBS, and processed for scintillation counting as described above.

RESULTS

In preliminary studies, verapamil at concentrations up to 50 µg/ml (1.02 × 10⁻⁴ M) was found to be noncytotoxic and did not inhibit the proliferation of MES-SA or Dx5 cells (data not shown). The effects of DOX alone or in combination with verapamil (0.05–10 µg/ml) on cell colony formation by MES-SA or Dx5 cells in soft agar are shown in Fig. 1. In this experiment, the Dx5 cells were 50-fold resistant to DOX, compared to MES-SA cells. The addition of verapamil enhanced the cytotoxicity of DOX for the Dx5 cells by a factor of 1.6-, 2-, 4-, and 7-fold at verapamil concentrations of 0.05, 0.1, 1.0, and 3.0 µg/ml, respectively. There was no further enhancement of DOX cytotoxicity by verapamil concentrations

above 3.0 µg/ml (5.0 and 10.0 µg/ml). As can be seen in Fig. 1, DOX resistance in Dx5 cells was only partially reversed by verapamil, with the Dx5 cells maintaining 7-fold resistance to DOX, relative to MES-SA cells, even at the highest verapamil concentrations. Verapamil had no effect on the sensitivity of MES-SA cells to DOX at verapamil concentrations up to 20 µg/ml (data not shown).

The effects of verapamil (3 µg/ml) on the sensitivity of Dx5 to agents for which cross-resistance had been demonstrated previously are shown in Table 1. The cytotoxicity of the anthracycline antibiotic daunorubicin was enhanced approximately 14-fold by verapamil. The activities of dactinomycin and mitoxantrone were enhanced 8.7- and 8.3-fold, respectively, by verapamil, whereas the activity of etoposide was enhanced only 2.5-fold in the presence of verapamil. There did not appear to be any correlation between the agent's molecular weight and the level of verapamil enhancement of cytotoxicity. With one exception, dactinomycin, there did appear to be a direct correlation between the level of cross-resistance and the degree to which the cytotoxicity of the agents was increased by verapamil. Of note as well was the fact that the four agents for which the greatest degree of enhancement was demonstrated—doxorubicin, daunorubicin, dactinomycin, and mitoxantrone—are all felt to act at least in part via intercalation of DNA. At the verapamil concentrations utilized for these studies (3 µg/ml), there was no evidence that verapamil enhanced the cytotoxicity of the bifunctional alkylating agents melphalan and mitomycin C or the microtubular poisons vincristine and vinblastine. In fact, there was no alteration in the pattern of Dx5 sensitivity to vinblastine despite cell exposure to verapamil concentrations reaching 50 µg/ml. Only at this high verapamil concentration (50 µg/ml) was there any evidence of enhanced vincristine cytotoxicity (2½- to 3-fold enhancement) not noted previously at lower verapamil concentrations (up to 10 µg/ml).

The effects of verapamil on cellular accumulation and retention of [¹⁴C]DOX and [³H]VBL were also studied. In Fig. 2, the effect of verapamil on [¹⁴C]DOX accumulation in MES-SA and Dx5 cells is shown. As demonstrated previously, cellular levels of DOX were 2- to 3-fold higher in MES-SA than in Dx5 at DOX concentrations of 1 µg/ml (11). DOX levels in Dx5 cells

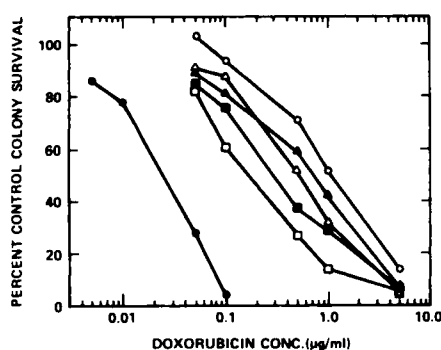


Fig. 1. Effects of verapamil on DOX cytotoxicity in MES-SA/Dx5 cells. Cells in mid-logarithmic phase of growth were exposed to various concentrations of DOX for 1 h in the presence or absence of verapamil. The verapamil concentrations utilized were 0 (○), 0.05 (▲), 0.1 (△), 1.0 (■), and 3.0 (□) µg/ml. The cells were then washed free of DOX and verapamil and seeded in a soft agar bilayer as described in "Materials and Methods." Tumor cell colonies were counted 14 days after plating. Each point is the mean of triplicate determinations. The DOX sensitivity of the MES-SA cell line (●) is also shown for comparison.

Table 1 Effect of verapamil on the sensitivity of doxorubicin-resistant MES-SA cells to various chemotherapeutic agents

Drug	Cross-resistance ^a	IC ₅₀ ^b (µg/ml) in Dx5 cells ^c		Verapamil sensitization ratio ^d
		-Verapamil	+Verapamil ^e	
Daunorubicin	160	0.65	0.046	14.1
Dactinomycin	1200	5.4	0.62	8.7
Mitoxantrone	60	0.05	0.006	8.3
Etoposide	30	20.0	8.0	2.5
Melphalan	8	0.60	0.45	1.3
Mitomycin C	10	0.09	0.07	1.3
Colchicine	27	0.9	0.75	1.2
Vinblastine	103	20.0	20.0	1.0
Vincristine	238	54.0	54.0	1.0

^a Degree of cross-resistance in the Dx5 line expressed as a ratio of the drug concentration required for 50% inhibition of colony formation (IC₅₀) versus MES-SA, or IC₅₀ (Dx5)/IC₅₀ (MES-SA).

^b IC₅₀ concentration of drug required for 50% inhibition of colony formation.

^c IC₅₀ values represent the average of at least three determinations.

^d Degree of sensitization of the Dx5 line by verapamil expressed as a ratio of the drug IC₅₀ with and without verapamil.

^e The verapamil concentration utilized for these experiments was 3 µg/ml.

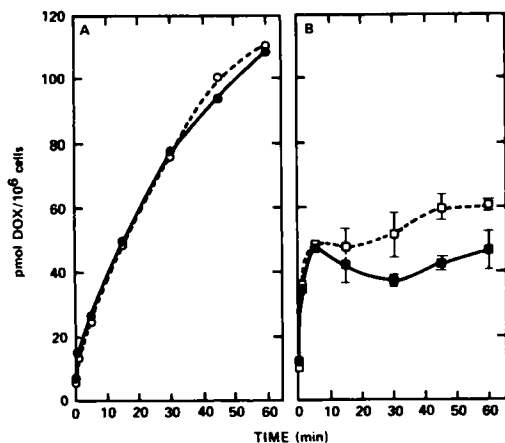


Fig. 2. Representative time course of [^{14}C]DOX (1 $\mu\text{g}/\text{ml}$) accumulation in the absence (●, ■) or presence (○, □) of verapamil (1 $\mu\text{g}/\text{ml}$) in (A) MES-SA and (B) MES-SA/Dx5. Bars, SD. In B, differences in DOX accumulation at the 30-, 45-, and 65-min time points are significant at $P < 0.05$, Student's t -test.

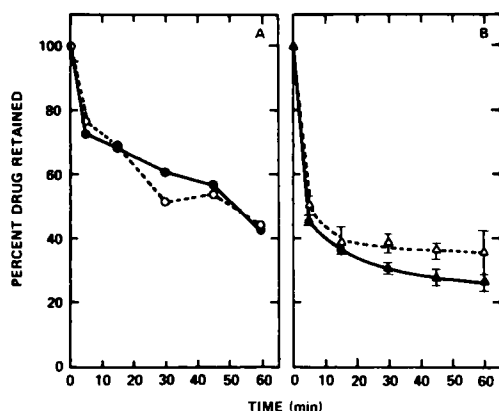


Fig. 3. Effect of verapamil on [^{14}C]DOX retention in (A) MES-SA and (B) MES-SA/Dx5 (○, △, with verapamil; ●, ▲, without verapamil). Points represent the mean of triplicate determinations. Bars, SD. In B, differences in DOX retention at the 30- and 45-min time points are significant at $P < 0.05$, Student's t -test.

exposed to DOX in the presence of verapamil (1 $\mu\text{g}/\text{ml}$) were 30–40% higher than in cells exposed to DOX alone over the 60-min period of observation (Fig. 2B). Verapamil had no measurable effect, however, on DOX accumulation in the MES-SA cells (Fig. 2A).

The effect of verapamil on [^{14}C]DOX retention in MES-SA and Dx5 is shown in Fig. 3. In MES-SA cells, retention of DOX was similar in the presence and absence of verapamil (1 $\mu\text{g}/\text{ml}$) (Fig. 3A). Exposure to verapamil resulted in a 30–50% greater DOX retention in Dx5 cells when compared to Dx5 cells exposed to DOX without verapamil (Fig. 3B).

While the enhancement of DOX cytotoxicity in the Dx5 line by verapamil was associated with increased DOX accumulation and retention, it remained to be seen what effect, if any, verapamil would have on cellular accumulation of agents for which no enhancement of cytotoxicity could be demonstrated. We, therefore, examined the effect of verapamil on cellular accumulation of [^3H]VBL in the MES-SA and Dx5 cells. Cellular levels of VBL in MES-SA cells were 6- to 10-fold higher than in Dx5 cells following 30 min of exposure to VBL at a concentration of 0.1 or 1.0 $\mu\text{g}/\text{ml}$ (Table 2). In the presence of verapamil (1.0 $\mu\text{g}/\text{ml}$), increases in cellular levels of VBL (24–72%) were observed in both MES-SA and Dx5 cells (Table 2).

Table 2. Effect of verapamil (1 $\mu\text{g}/\text{ml}$) on the cellular accumulation of [^3H]VBL (0.1 or 1.0 $\mu\text{g}/\text{ml}$) in MES-SA and Dx5 cells

VBL in media ($\mu\text{g}/\text{ml}$)	Cell line	Cellular VBL content (pmol/ 10^6 cells)	
		VBL alone	VBL + verapamil
1.0	MES-SA	28.7 \pm 2.7 ^a	35.6 \pm 3.4 ^b
1.0	Dx5	2.75 \pm 0.39	4.72 \pm 0.35 ^b
0.1	MES-SA	1.55 \pm 0.07	1.93 \pm 0.22 ^b
0.1	Dx5	0.23 \pm 0.02	0.30 \pm 0.04 ^b

^a Mean \pm SD.

^b Significant at $P < 0.05$, Student's t -test.

DISCUSSION

Resistance to the anthracycline antibiotics doxorubicin and daunorubicin in MES-SA and other drug-resistant cells is associated with decreased drug accumulation, which is thought to result from enhanced drug efflux via a process shared by several classes of drugs, including anthracyclines, *Vinca* alkaloids, dactinomycin, emetine, and podophyllotoxins (10–23, 34). Drug accumulation in cells which exhibit this pleiotropic or multidrug resistance can be increased in the presence of the metabolic inhibitors dinitrophenol and sodium azide (18–21, 23, 29, 34–38). Previous reports have suggested that calcium antagonists such as verapamil may inhibit the energy dependent efflux of anthracyclines and *Vinca* alkaloids, thus decreasing cellular resistance to these classes of chemotherapeutic agents (28, 30, 39–43). Verapamil completely reversed DOX resistance and cross-resistance to vinblastine in a human ovarian carcinoma cell line, 1847^{AD}, which was 6-fold resistant to DOX compared to the parent line (A 1847) (28). Incubation of the 1847^{AD} cells with DOX in the presence of verapamil increased the intracellular DOX concentrations 3-fold, to levels approaching that found in A 1847 cells. Efflux of DOX from the resistant 1847^{AD} cells was markedly inhibited by verapamil. Verapamil did not completely restore sensitivity to DOX in another ovarian carcinoma cell line, 2780^{AD}, which was 150-fold more resistant to DOX than was the parent line (28). Indeed, several authors have reported that verapamil acts to only partially restore DOX sensitivity in these DOX-resistant lines, in association with enhanced DOX accumulation (26, 30, 39–44).

In the studies reported here, verapamil enhanced the cytotoxicity of DOX approximately 7-fold in the 50- to 100-fold DOX resistant subline (Dx5) of the human sarcoma cell line MES-SA but had no demonstrable effect on MES-SA. Enhancement of DOX cytotoxicity in Dx5 occurred at verapamil concentrations ranging from 0.1–3.0 $\mu\text{g}/\text{ml}$, but further enhancement was not seen at concentrations of verapamil above 3 $\mu\text{g}/\text{ml}$. Verapamil also enhanced the cytotoxicities of four other agents—daunorubicin, dactinomycin, mitoxantrone, and etoposide—to which cross-resistance had been demonstrated previously in the Dx5 line. There was no evidence that verapamil enhanced the cytotoxicity of five additional agents—vincristine, vinblastine, colchicine, mitomycin C, and melphalan—to which the Dx5 line also demonstrates cross-resistance, nor was there evidence of enhancement of the action of any of these agents in the DOX sensitive MES-SA line (Table 1). These results differ from those of Tsuruo *et al.* (42), who have reported that verapamil greatly enhanced the cytotoxicity of the *Vinca* alkaloid vincristine as well as doxorubicin for P388 murine leukemia cells selected for resistance to either vincristine or doxorubicin. Differences in experimental design might explain the discrepancies between the results from these two studies, in-

cluding: (a) period of drug-verapamil exposure (72 h by Tsuruo, 1 h in these studies); and (b) assessment of cytotoxicity (growth inhibition by Tsuruo, clonogenicity in these studies). Attempts to resolve the differences between these two studies invariably lead to the question, "What action of verapamil is responsible for the enhancement of DOX cytotoxicity in these DOX resistance models?" Tsuruo *et al.* (45) have demonstrated a 50% increase in cellular calcium content of P388 cells selected for resistance to doxorubicin (P388/ADR). While their studies have suggested that decreased drug retention in models of pleiotropic drug resistance may be calcium dependent, further studies from these same investigators have failed to correlate the enhancement potency of agents such as verapamil with their potency as calcium antagonists (40). Furthermore, Kessel and Wilberding (26) were unable to demonstrate any calcium requirement for outward daunorubicin transport in P388 or P388/ADR leukemia cells. Neither the absence of Ca^{2+} in the medium nor addition of the calcium chelating agent ethylene-glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid affected drug accumulation in these cell lines. Kessel and Wilberding (46) have suggested one possible mechanism of verapamil enhancement of drug action with the observation of competition between verapamil and anthracyclines for outward transport in P388/ADR cells.

Models of multidrug cross-resistance based on a common drug efflux mechanism might predict that the cytotoxicity of all the cross-resistant agents would be enhanced by verapamil, if verapamil acted as a competitive inhibitor of efflux. Indeed, verapamil enhanced the cytotoxicity of both vincristine and doxorubicin in K562 myelogenous leukemia cells selected for resistance to vincristine and known to be cross-resistant to DOX (41). We, however, found no enhancement by verapamil of the cytotoxicity of the *Vinca* alkaloids vincristine and vinblastine, despite our demonstration that verapamil enhanced the accumulation of [^3H]VBL in the Dx5 line.

Ramu *et al.* (27) have demonstrated that perhexiline maleate, a calcium antagonist, could increase the sensitivity of P388/ADR cells to both doxorubicin and vinblastine. They suggested that this effect did not involve calcium antagonism but rather resulted from an interaction of perhexiline with the lipid domain of the cell which led to increased doxorubicin accumulation (27). These investigators have also shown that the triparanol analogues (tamoxifen, clomiphene) could increase the sensitivity of P388/ADR to doxorubicin and have speculated that the triparanol analogues altered the cell membrane lipid composition, resulting in an increased diffusion rate (47). Tsuruo *et al.* (48) have demonstrated that quinidine, an antiarrhythmic agent without calcium activity, increased the cytotoxicity of DOX in P388/ADR in association with increased intracellular DOX levels. It was proposed that the action of quinidine on the P388/ADR cells was to perturb the organization of membrane lipids, resulting in altered DOX accumulation. The exact mechanism by which these various agents affect DOX sensitivity, however, remains speculative.

Other potential explanations for the action of verapamil in enhancing the cytotoxicity of these chemotherapeutic agents would be potentiation of drug-mediated DNA damage or inhibition of DNA repair. Yalowich and Ross (49) have reported that verapamil potentiated etoposide-induced DNA single strand breaks in L1210 leukemia cells. This potentiation was associated with increased cellular accumulation of etoposide in these cells and appeared to correlate well with enhanced cytotoxicity when etoposide and verapamil were combined in soft agar assays (50). Their work suggested that verapamil was not

inhibiting DNA strand rejoining, however. Of note in our current studies is the fact that all of the drugs the cytotoxicity of which was enhanced by verapamil in the Dx5 line are capable of DNA binding and/or topoisomerase II mediated DNA strand scission (DOX, DNR, mitoxantrone, dactinomycin, and etoposide). The agents the cytotoxicity of which was not enhanced by verapamil act via mechanisms other than DNA strand scission (vincristine, vinblastine, colchicine, melphalan, and cisplatin). Studies to examine the effects of verapamil on DOX-related DNA scission and repair in DOX sensitive and resistant lines of MES-SA are ongoing.

In summary, we have demonstrated that the calcium channel blocking agent verapamil is capable of potentiating the cytotoxicity of doxorubicin in DOX-resistant variants of the human sarcoma cell line MES-SA. Although verapamil exposure resulted in increased cellular accumulation of doxorubicin and vinblastine, these increases did not correlate well with increased cytotoxicity. Thus, factors other than cellular drug levels might be important in explaining this action of verapamil. The demonstration in this model of pleiotropic resistance that verapamil enhances the cytotoxicity of agents which result in DNA strand breakage suggests that verapamil may act at the level of the generation or repair of such lesions in the nucleus of the cell.

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