Enhanced Drug-Induced Apoptosis Associated with P-Glycoprotein Overexpression Is Specific to Antimicrotubule Agents

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Purpose. We have reported that overexpression of *mdr1* P-glycoprotein (Pgp) is associated with a higher sensitivity to paclitaxel-induced apoptosis (1,2). The present study examined the substrate specificity of this phenomenon.

Methods. Two Pgp substrates (vincristine and doxorubicin) and three nonsubstrates (cisplatin, camptothecin, and 5-fluorouracil) were studied. Serum deprivation, known to induce apoptosis, was used as a comparison.

Results. The Pgp nonsubstrates and serum deprivation showed similar overall cytotoxicity and apoptosis in human breast MCF7 cells (with negligible Pgp expression) and its *mdr1*-transfected subline BC19 cells (with nine-fold higher Pgp expression). In contrast, the overall cytotoxicity and apoptosis of the two Pgp substrates was higher in MCF7 cells. Cotreatment with a Pgp inhibitor, verapamil, abolished the difference in intracellular accumulation of doxorubicin as well as the differences in apoptosis between MCF7 and BC19 cells. This finding confirms that the lower apoptosis of doxorubicin in BC19 cells, in the absence of verapamil, was a result of lower intracellular drug accumulation secondary to high Pgp expression in BC19 cells. In contrast, abolishing the difference in intracellular vincristine concentration by verapamil cotreatment resulted in significantly higher apoptosis in BC19 cells. This finding is identical to our previous finding with paclitaxel, where equal intracellular drug concentration resulted in greater apoptosis in the Pgp-rich BC19 cells.

Conclusions. These data, together with the opposite effects of paclitaxel and vincristine on microtubules (i.e., polymerization versus depolymerization), indicate that the enhanced apoptosis in Pgp-rich cells is specific for antimicrotubule agents but is not related to the polymerization of microtubules.

KEY WORDS: P-glycoprotein; apoptosis; antimicrotubules.

INTRODUCTION

We observed, in patient tumors, a positive correlation between the expression of *mdr1* P-glycoprotein (Pgp) and the extent of paclitaxel-induced apoptosis (1). This finding was unexpected and counterintuitive, given the fact that Pgp overexpression reduces intracellular paclitaxel accumulation. To investigate the relationship between Pgp expression and paclitaxel-induced apoptosis without confounding factors such as the intra- and intertumor heterogeneity associated with patient tumors, we evaluated the pharmacodynamics of the apoptotic and antiproliferation effects of paclitaxel at equal intracellular drug concentrations in two cell lines that differ in their expression of Pgp, i.e., human breast MCF7 carcinoma cells, which display insignificant Pgp expression, and its *mdr1*-transfected variant BC19 cells, which show a nine-fold higher Pgp level and >13-fold higher *mdr1* expression (2). The results confirm that Pgp overexpression is associated with a high sensitivity to paclitaxel-induced apoptosis, an effect that is unrelated to the Pgp-mediated drug efflux.

We have initiated a series of studies to determine the mechanisms underlying the higher apoptosis associated with *mdr1* transfection and P-glycoprotein overexpression. The purpose of the present study was to determine whether this phenomenon is limited to paclitaxel or is generally applicable to other antimicrotubule agents or other Pgp substrates. Five drugs, including two Pgp substrates (doxorubicin and vincristine) and three nonsubstrates (cisplatin, camptothecin, 5-fluorouracil), were studied. These drugs represent the major classes of anticancer drugs, i.e., antimicrotubule agents (vincristine), inhibitors of topoisomerase I and II (camptothecin, doxorubicin), DNA cross-linking agents (cisplatin), and antimetabolites (5-fluorouracil). As a reference, serum deprivation, which is known to induce cytotoxicity and apoptosis, was also evaluated. The results showed that the association between Pgp overexpression and higher apoptosis was observed only for antimicrotubules but was not related to the polymerization of microtubules.

MATERIALS AND METHODS

Chemicals

Doxorubicin was purchased from Hande Tech (Houston, TX), cefotaxime sodium from Hoechst-Roussel (Somerville, NJ), gentamicin from Solo Pak Laboratories (Franklin Park, IL), other tissue culture supplies from GIBCO Laboratories (Grand Island, NY), and vincristine, cisplatin, 5-fluorouracil, camptothecin, verapamil, phenylmethylsulfonyl fluoride, sulforhodamine B (SRB), and propidium iodide from Sigma Chemical Co (St. Louis, MO). All chemicals and reagents were used as received.

Cell Culture Conditions

Two human breast cancer cell lines, MCF7 and its *mdr-1*-transfected variant BC19 cells, were gifts from Dr. Kenneth Cowan (National Cancer Institute, Bethesda, MD). Some studies used MCF7 cells purchased from American Type Culture Collective (Manassas, VA). The culture medium was RPMI 1640 medium supplemented with 9% fetal bovine serum, 2 mM L-glutamine, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime sodium. Cells were incubated with complete medium at 37°C in a humidified atmosphere of 5% CO_2 in air. For experiments, cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating. The cell cycle time, or the doubling time in exponentially growing cells, was 24 h for both cell lines.

Measurement of Overall Drug Effect

Stock solutions were prepared by dissolving the drugs in water (5-fluorouracil, 10 mM), physiologic saline (cisplatin and vincristine, 1 mM), dimethyl sulfoxide (doxorubicin, 1 mM), or ethanol (camptothecin, 1 mM; verapamil, 1 mg/ml).

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ABBREVIATIONS: IC_{50}, concentration needed to produce 50% reduction in cell number; Pgp, p-glycoprotein; SRB, sulforhodamine B.

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Stock solutions were aliquoted and stored frozen at –20°C until use.

Drug treatment and evaluation of drug effect were as previously described (2). Briefly, cells were seeded in 96-well microtiter plates (3,000–5,000 cells in 0.2 ml per well) overnight and then treated with drugs. Treatment with cisplatin was for 48 h, whereas treatments with other drugs were for 96 h. Six replicates were used for each drug concentration. The overall drug effect, which represents the combination of the antiproliferative and apoptotic effects of individual drug, was measured using the SRB assay, as previously described (3).

The drug concentration–effect relationship was analyzed by computer-fitting a sigmoidal pharmacodynamic function to the experimental data using nonlinear least-squares regression (NLIN; SAS, Cary, NC), as previously described (4). The pharmacodynamic function takes into account the residual unaffected cell fraction.

Measurement of Drug-Induced Apoptosis

Apoptosis was quantified by two methods. The first method was used for 5-fluorouracil, doxorubicin, camptothecin, vincristine, and serum deprivation. This method monitored the amount of DNA–histone complex released to the cytoplasm using the Cell Death Detection ELISA kit (Boehringer Mannheim, Germany), as previously described (2). Briefly, cells were lyzed in lysis buffer, and the cytoplasmic fractions of the lysates were placed in a plate precoated with mouse antihistone primary antibody and mouse anti-DNA antibody conjugated to peroxidase. The peroxidase substrate 2,2′-azido-di-3-ethylbenzthiazoline sulfonate, was applied, and the absorbance at 405 nm was measured. The time points for apoptosis measurements were selected based on the kinetics of drug-induced apoptosis. A pilot study showed peak apoptosis at 48 h for all drugs except doxorubicin, which showed a peak at 72 h.

The second method was used for cisplatin. In this method, the number of apoptotic cells with externalized phosphatidylserine was quantified using the ApoAlert Annexin V assay (Clontech Lab., Palo Alto, CA), as described previously (2). Briefly, cells were suspended in binding buffer, incubated with FITC-labeled Annexin V and propidium iodide for 5–15 min in the dark, and examined by fluorescence microscopy using a dual filter at 488 nm. The numbers of labeled cells, including Annexin V-labeled cells, which were in the early stage of apoptosis, and cells that were doubly labeled with Annexin V and propidium iodide, which were in the middle and late stages of apoptosis, were quantified.

Intracellular Drug Accumulation

Cells were plated at a density of 10^5–10^6 cells/ml per well in six-well plates. One day after seeding, 1 ml of medium containing mixture of doxorubicin plus [^{14}C]doxorubicin (final specific activity, 0.058 to 58 Ci/mol) or vincristine plus [^{3}H]vincristine (final specific activity, 0.0072 to 0.36 Ci/mol) was added. A pilot study indicated that apparent steady-state conditions between intracellular and extracellular concentrations in both MCF7 and BC19 cells were achieved at 4 h for doxorubicin or vincristine. Subsequent studies used 4 h incubation. After incubation at 37°C for 4 h, cells were washed with 0.5 ml of ice-cold versene twice, harvested using trypsinization, and diluted 20-fold with Isotone (Coulter Electronics Inc., Hialeah, FL). Cell number was then determined using a Coulter Counter (Coulter Electronics Inc). To determine the amount of doxorubicin and vincristine, 0.5 ml of cell suspension was dissolved in 1 ml of Solvable tissue gel Solubilizer (Packard, Groningen, The Netherlands), mixed with 15 ml of Atomlight, and processed by liquid scintillation counting. All experiments were performed in triplicates.

Intracellular drug concentration was expressed in molar terms by dividing the amount of drug per cell by the cell volume. The cell volumes of MCF7 and BC19 cells are 2.09 and 1.96 μl per 10^6 cells, respectively (5).

Effect of Verapamil on Intracellular Drug Accumulation and Drug-Induced Cytotoxicity and Apoptosis

The effect of verapamil, a Pgp efflux inhibitor, on the intracellular accumulation and cytotoxicity of doxorubicin and vincristine in MCF7 and BC19 cells was investigated. Verapamil (5 μM) was administered together with doxorubicin or vincristine. A pilot experiment showed that this concentration of verapamil did not produce cytotoxicity in MCF7 or BC19 cells, whereas cytotoxicity was observed at higher concentrations (i.e., 10% cytotoxicity for treatments with 10 μM for 96 h). Other experimental procedures are as described above.

RESULTS

Drug-Induced Overall Cytotoxicity

Figure 1 shows the overall drug cytotoxicity, and Table I summarizes the drug concentrations needed to produce 50% reduction in cell number (IC_{50}). The results previously obtained for paclitaxel are included for comparison. The Pgp nonsubstrates, i.e., cisplatin, camptothecin, and 5-fluoroura-

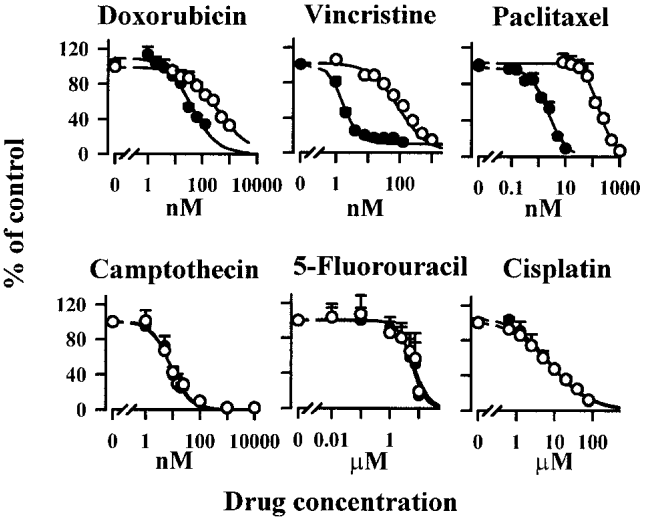

Fig. 1. Reduction of cell numbers by drug treatment. Cells were treated with doxorubicin, vincristine, camptothecin, and 5-fluorouracil for 96 h and cisplatin for 48 h. Cell numbers for MCF7 *(closed circles)* and BC19 *(open circles)* cells were determined using the SRB assay. Lines are computer-fitted to experimental data. Mean ± S.D. of two to five experiments. Paclitaxel data, described in a previous publication (2) with permission for reproduction, are included for comparison.

Table I. Cytotoxic Effects of Anticancer Agents[a]

Drug (concentration)	MCF7	BC19	Resistance factor
Pgp substrates			
Paclitaxel (nM)	3.22 ± 0.78	116.5 ± 20.4*	36
Doxorubicin (nM)	35.8 ± 4.1	317.2 ± 70.7*	9
Vincristine (nM)	2.0 ± 0.1	109.3 ± 11.6*	53.6
Pgp nonsubstrates			
Camptothecin (nM)	8.3 ± 2.3	8.5 ± 0.9	1.0
5-Flurouridine (μM)	4.1 ± 1.4	4.6 ± 1.5	1.1
Cisplatin (μM)	7.2 ± 0.7	8.3 ± 0.2	1.2

* $p < 0.05$ compared to MCF7 cells.

[a] Cells were treated with drugs for 96 h (except cisplatin, where treatment was for 48 h) and then processed with the SRB assay. Mean ± S.D. of two to five experiments, six replicates per data point for each experiment. The previously reported paclitaxel data (2) are included for comparison.

cil, showed similar activity in MCF7 and BC19 cells, whereas the Pgp substrates, i.e., doxorubicin, vincristine, and paclitaxel, showed greater activity in MCF7 cells.

Drug-Induced Apoptosis

Figure 2 shows the apoptosis induced by Pgp nonsubstrates (i.e., 5-fluorouracil, camptothecin, cisplatin) and serum deprivation. For all three drugs, there were no differences in the apoptosis between MCF7 and BC19 cells. For 5-fluorouracil, the amount of cytoplasmic DNA–histone complex increased above the background level at 10 nM and continued to increase with drug concentration up to the highest concentration of 10,000 nM, or 2.5 times the IC_{50} for cytotoxicity. For camptothecin, apoptosis was evident at 10 nM (approximate equal to the IC_{50} for cytotoxicity), reached a peak level at 1,000 nM, followed by a decline at 10,000 nM. For cisplatin, the fraction of apoptotic cells increased above the baseline level at 10 μM (about twice the IC_{50} for cytotoxicity) and reached above 70% at 100 μM. The apoptosis induced by serum deprivation became evident at 48 h and reached a peak level at 96 h, followed by a decline at 120 h.

Figure 3 compares the apoptotic effect of doxorubicin and vincristine. For both drugs, the apoptosis in MCF7 cells was higher than that in BC19 cells. However, these drugs showed different pharmacodynamics. For doxorubicin, induction of apoptosis in MCF7 cells required a 10-fold lower drug concentration than for BC19 cells (i.e., 100 nM versus 1,000 nM). These apoptotic doxorubicin concentrations were about three times the IC_{50} for cytotoxicity. In both cells, the doxorubicin-induced apoptosis peaked at 1,000 nM, followed by a decline at 5,000 nM. In contrast, the apoptosis in MCF7 cells increased with vincristine concentration but reached plateau values at 1,000 nM drug concentration, with no significant increases when the drug concentration was further increased to 5,000 nM. In BC19 cells, these values continued to increase with drug concentration without reaching a plateau. The drug-induced apoptosis in MCF7 cells exceeded the apoptosis in BC19 cells at extracellular concentrations up to 1,000 nM, whereas the reverse was observed at 5,000 nM.

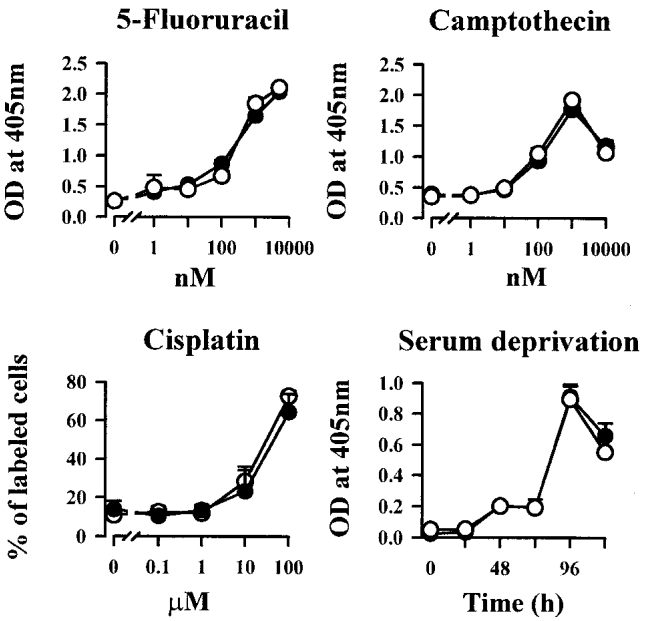

Fig. 2. Apoptosis induced by 5-fluorouracial, camptothecin, cisplatin, and serum deprivation. MCF7 *(closed circles)* and BC19 *(open circles)* were treated with 5-fluorouracil, camptothecin, and cisplatin for 48 h, and serum deprivation for up to 120 h. Note the different x-axes for treatment with drugs versus serum deprivation. Apoptosis induced by cisplatin was measured as Annexin V-labeled cells, whereas apoptosis induced by the other drugs was measured as release of DNA–histone complex from the nucleus to the cytoplasm. Mean ± S.D. (n = 3 experiments).

Intracellular Accumulation of Pgp Substrates

Table II shows the intracellular accumulation of doxorubicin and vincristine in MCF7 and BC19 cells. At extracellular

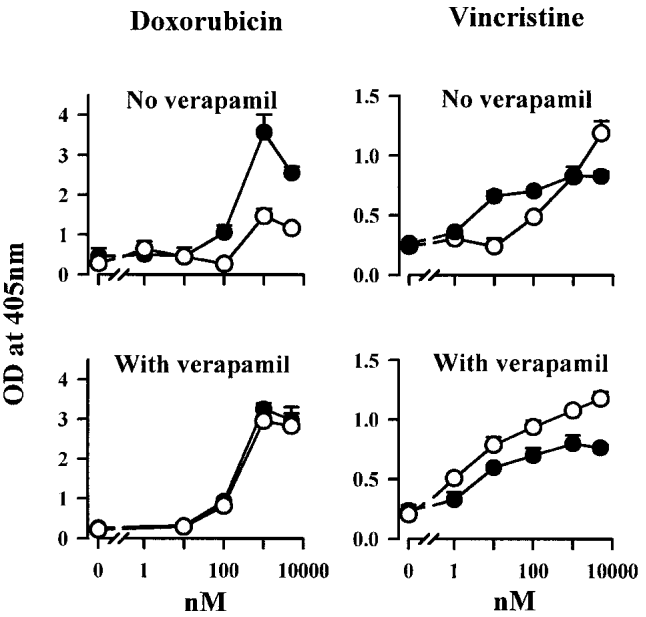

Fig. 3. Apoptosis induced by doxorubicin and vincristine, and effect of verapamil cotreatment. MCF7 *(closed circles)* and BC19 *(open circles)* cells were treated with doxorubicin or vincristine, with or without verapamil (5 μM). Treatment was 72 h for doxorubicin and 48 h for vincristine. Apoptosis was measured as release of DNA–histone complex from the nucleus to the cytoplasm. Mean ± S.D. (n = 3 experiments).

Table II. Intracellular Accumulation of Doxorubicin and Vincristine[a]

Extracellular drug concentration (nM)	Intracellular doxorubicin concentration (μM)						Intracellular vincristine concentration (μM)					
	No verapamil			With verapamil (5 μM)			No verapamil			With verapamil (5 μM)		
	MCF7	BC19	Ratio	MCF7	BC19	Ratio	MCF7	BC19	Ratio	MCF7	BC19	Ratio
10	1.54 ± 0.07	1.29 ± 0.13	1.20 ± 0.08	1.67 ± 0.16	1.66 ± 0.17	1.01 ± 0.05	0.25 ± 0.03	0.11 ± 0.01	2.22 ± 0.29	0.39 ± 0.03	0.41 ± 0.03	0.96 ± 0.07
100	13.3 ± 1.3	10.8 ± 1.54	1.24 ± 0.16	15.2 ± 1.58	15.4 ± 1.28	0.98 ± 0.04	2.78 ± 0.23	1.49 ± 0.14	1.82 ± 0.25	4.31 ± 0.35	4.50 ± 0.59	0.90 ± 0.14
1,000	123 ± 6	102 ± 4.58	1.22 ± 0.08	135 ± 6.18	141 ± 7.25	0.96 ± 0.03	12.2 ± 1.12	8.26 ± 0.98	1.51 ± 0.17	15.1 ± 1.17	14.8 ± 1.83	1.03 ± 0.10
5,000	571 ± 36	458 ± 21	1.25 ± 0.06	679 ± 50.2	697 ± 65.4	0.98 ± 0.07	29.6 ± 3.08	23.7 ± 1.44	1.23 ± 0.04	36.4 ± 4.93	32.9 ± 4.93	1.14 ± 0.06

[a] Cells were treated with doxorubicin or vincristine for 4 h and collected by trypsinization. Intracellular drug concentrations were calculated based on cell number and cell volume. Mean ± S.D. of three to five experiments, three replicates per data point for each experiment. Ratio is the MCF7-to-BC19 ratio.

concentrations between 10 and 5,000 nM, the intracellular oxorubicin accumulation in MCF7 cells was consistently about 20% higher than that in BC19 cells. In contrast, the differences between the intracellular vincristine concentrations in MCF7 and BC19 cells were inversely related to the extracellular concentrations, declining from a ratio of 2.22 at 10 nM to 1.23 at 5,000 nM.

Effect of Verapamil on Intracellular Drug Accumulation, Cytotoxicity, and Apoptosis

The effects of verapamil are shown in Figs. 3 and 4 and Table II. Cotreatment with 5 μM verapamil enhanced the intracellular doxorubicin concentrations in both MCF7 and BC19 cells, with greater increases in BC19 cells, such that the differences between the two cells were nearly completely abolished. Verapamil also abolished the difference in the doxorubicin-induced apoptosis and significantly reduced the differences in the drug-induced cytotoxicity between the two cells.

Verapamil cotreatment also enhanced the intracellular concentrations of vincristine in both MCF7 and BC19 cells, again with greater increases in BC19 cells, such that the differences between the two cells were nearly completely abolished. Verapamil also significantly reduced the differences in drug-induced cytotoxicity between the two cells. Verapamil did not affect the vincristine-induced apoptosis in MCF7 cells but significantly enhanced the apoptosis in BC19 cells. Contrary to the care of doxorubicin where verapamil treatment resulted in approximately equal apoptosis in MCF7 and BC19 cells, verapamil enhanced the vineristine-induced apoptosis in BC19 cells to a far greater extent than in MCF7 cells such that the apoptosis in BC19 cells was consistently higher than that in MCF7 cells at all extracellular vineristine concentrations between 1 and 5,000 nM.

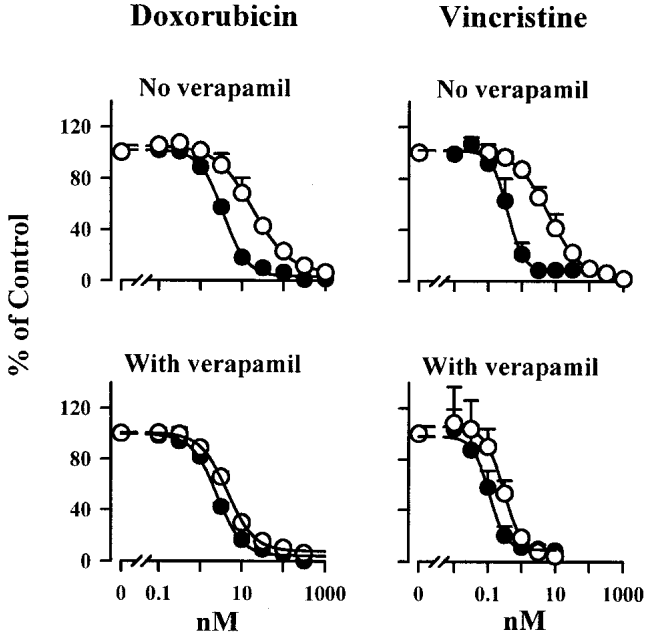

Fig. 4. Reduction of cell numbers by doxorubicin and vincristine and effect of verapamil cotreatment. MCF7 *(closed circles)* and BC19 *(open circles)* cells were treated with doxorubicin or vincristine, with or without verapamil (5 μM), for 96 h. Cell numbers were determined using the SRB assay. Lines are computer-fitted lines to experimental data. Mean ± S.D. (n = 3 experiments).

DISCUSSION

Results of the present study show that among the five anticancer drugs representing several drug classes, only the Pgp substrates (i.e., doxorubicin and vincristine) showed lower cytotoxicity and apoptosis in the Pgp-rich BC19 cells that were derived from *mdr1* transfection of MCF7 cells with negligible Pgp expression. The lower cytotoxicity of doxorubicin and vincristine in BC19 cells is in agreement with the lower intracellular accumulation of Pgp substrates in these cells. It is noted, however, that the decreases in drug activity greatly exceeded the decreases in intracellular drug accumulation.

The three Pgp nonsubstrates (5-fluorouracil, camptothecin, cisplatin) and serum deprivation produced identical cytotoxicity and apoptosis in MCF7 and BC19 cells, indicating that *mdr1* transfection and Pgp overexpression had no effect on the apoptosis induced by Pgp nonsubstrates and serum deprivation.

The results further show two significant differences in the pharmacodynamics of apoptosis induced by doxorubicin and vincristine. First, for doxorubicin, the apoptosis in MCF7 cells was higher than in BC19 cells at all extracellular concentrations from 10 to 5,000 nM; the higher apoptosis in MCF7 cells can be explained by the higher intracellular drug accumulation in MCF7 cells. In contrast, vincristine showed higher apoptosis in MCF7 cells at the extracellular concentration range of 1 to 1,000 nM, but the opposite was found at 5,000 nM. This concentration-dependent difference in apoptosis between the two cells is consistent with the diminishing differences in intracellular vincristine accumulation with increasing extracellular concentrations (i.e., reducing from a MCF7:BC19 ratio of 2.22 at 10 nM to 1.23 to 5,000 nM). Second, inhibition of the Pgp-mediated efflux resulted in differences in the apoptosis induced by doxorubicin and vincristine. For doxorubicin, the simultaneous elimination of the differences in intracellular drug accumulation and apoptosis by verapamil indicates that the lower apoptosis in BC19 cells was related to the Pgp-mediated lowered drug accumulation in these cells. In contrast, for vincristine, abolition of the differences in intracellular drug accumulation by verapamil resulted in higher apoptosis in BC19 cells at all extracellular concentrations. These data indicate that the higher apoptosis in MCF7 cells observed at the lower vincristine concentrations (i.e., between 10 and 1,000 nM) was a result of higher intracellular drug accumulating in MCF7 cells, compared to BC19 cells. These results further indicate a greater intrinsic susceptibility of the Pgp-rich BC19 cells to apoptosis by vincristine.

The findings on vincristine are similar to those observed for paclitaxel (2). For paclitaxel, MCF7 cells showed higher apoptosis at extracellular drug concentrations of 1 to 100 nM, whereas BC19 cells showed higher apoptosis at extracellular concentration above 100 nM. In addition, the apoptosis in BC19 cells was consistently higher than that in MCF7 cells as the difference in intracellular drug accumulation was abolished.

The finding that verapamil enhanced the intracellular accumulation of doxorubicin and vincristine in MCF7 and BC19 cells is consistent with the Pgp inhibitory effect of verapamil, and the finding that verapamil enhanced the overall cytotoxicity of these drugs is consistent with the higher intracellular drug accumulation. On the other hand, verapamil can also enhance cytotoxicity of other chemotherapeutic agents including drugs that are Pgp substrates (i.e., doxorubicin and vincristine, refs. 6–8) and drugs that are not Pgp substrates (i.e., 5-fluorouracil, ref. 8). Hence, it is possible that the enhanced cytotoxicity of doxorubicin and vincristine by verapamil may be caused by other verapamil actions unrelated to Pgp-mediated drug efflux.

It is noted that verapamil treatment also elevated the intracellular drug concentrations in MCF7 cells (<20% for doxorubicin and ≤50% for vincristine). This may be a result of inhibition of the low levels of Pgp in MCF7 cells, as shown in our previous study (2). Verapamil was used in the present study because it is the most commonly used Pgp inhibitor. Further studies with additional Pgp inhibitors might yield insight into the nature or mechanisms by which microtubule-targeting agents induced more apoptosis in the Pgp-overexpressing cells. For the same reason, further studies with additional Pgp substrates that do not target microtubules are warranted.

Collectively, the findings of the present study and of our earlier study (2) indicate that the enhanced apoptosis in the Pgp-overexpressing BC19 cells is unique to antimicrotubule agents. Vinca alkaloids and paclitaxel have opposite effects on microtubules: vinca alkaloids increase depolymerization of microtubules, whereas paclitaxel increases polymerization. Hence, the higher apoptosis observed for these agents in Pgp-overexpressing cells is not related to the polymerization status of microtubules. It is noted that the effects of paclitaxel and vincristine on microtubules are highly concentration dependent; suppression of the dynamics of microtubules occurs at low concentrations (e.g., below 100 nM), whereas altered polymerization occurs at high concentrations (e.g., >100 nM) (9,10). It is not known whether these concentration-dependent effects on the dynamics and polymerization of microtubules are associated with the enhanced apoptosis associated with Pgp overexpression. Other studies on the mechanisms underlying the positive association between Pgp overexpression and sensitivity to apoptosis induced by antimicrotubule agents, including the levels of pro- and antiapoptosis proteins and intracellular drug distribution, are ongoing.

ACKNOWLEDGMENTS

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