Verapamil Reversal of Doxorubicin Resistance in Multidrug-resistant Human Myeloma Cells and Association with Drug Accumulation and DNA Damage

William T. Bellamy, William S. Dalton, Jenai M. Kailey, Mary C. Gleason, Thomas M. McCloskey, Robert T. Dorr, and David S. Alberts

Department of Medicine and Cancer Pharmacology Program, Arizona Cancer Center, Tucson, Arizona 85724

ABSTRACT

Verapamil reversed resistance to doxorubicin in a human multiple myeloma cell line selected for multiple drug resistance. The drug-resistant cell line 8226/DOX40 is known to have reduced intracellular drug accumulation associated with the overexpression of P-glycoprotein when compared to the sensitive parent cell line 8226/S. Verapamil alone was minimally cytotoxic in both cell lines, but reversed doxorubicin resistance in a dose-related manner in 8226/DOX40. A similar dose-response relationship was observed for verapamil in increasing intracellular doxorubicin accumulation. This increased net accumulation was secondary to block of enhanced doxorubicin efflux by verapamil from resistant cells. In contrast, verapamil did not alter initial doxorubicin accumulation over the first 60 s when incubated with resistant cells. Addition of verapamil to the 8226/DOX40 cells enhanced the formation of doxorubicin-induced DNA single strand breaks, double strand breaks, and DNA-protein cross-links. Verapamil had no effect on these lesions in the drug-sensitive cells. In addition, verapamil did not affect chemotherapeutic cytotoxicity or transport in the drug-sensitive cell line. Verapamil appears to reverse doxorubicin resistance in this human myeloma cell line by blocking enhanced drug efflux, leading to increased drug accumulation and enhanced DNA damage.

INTRODUCTION

The development of multidrug resistance in multiple myeloma has been documented. Although the majority of patients respond to initial chemotherapy, essentially all patients become drug refractory and die of their disease (1–3). Recently, multiple agent chemotherapy which includes vincristine and doxorubicin has been reported to be more effective than single agent doxorubicin (4–6) and in some cases can induce responses in drug-refractory disease. In addition, doxorubicin did not affect chemotherapeutic cytotoxicity or transport in the drug-sensitive cell line. Verapamil appears to reverse doxorubicin resistance in this human myeloma cell line by blocking enhanced drug efflux, leading to increased drug accumulation and enhanced DNA damage.

glycoprotein (9, 10). The exact function of P-glycoprotein is unknown; however, recent studies have demonstrated drug binding to this protein, suggesting that it is involved in drug transport (11, 12).

Recent pharmacological studies have reported the ability of several calcium channel blockers, including verapamil and calmodulin inhibitors such as trifluoperazine, to reverse the resistance of natural product chemotherapeutic drugs in vitro and in vivo (13–16). The circumvention of this resistance is associated with increased drug accumulation and retention; however, the mechanism by which this occurs is unknown. In this study, we evaluated the activity of verapamil and possible mechanisms by which it reverses the resistance to both doxorubicin and vincristine in a human multiple myeloma cell line. This cell line has previously been characterized as having a classical multidrug-resistant phenotype and overexpression of P-glycoprotein (17).

MATERIALS AND METHODS

Cells and Culture Conditions. The RPMI 8226 human myeloma cell line and its multidrug-resistant counterpart (8226/DOX) was selected as described previously (17). The particular resistant cell line used in the present study, 8226/DOX40, was grown in medium containing 4 × 10−7 M DOX, a concentration 40 times the original concentration of doxorubicin used in the selection process. The resistant line usually demonstrates a 40- to 50-fold resistance to doxorubicin in soft agar.

Drugs and Materials. Doxorubicin was obtained from Adria Laboratories (Columbus, OH). [14C]Doxorubicin (specific activity, 42.0 μCi/mg) was obtained from SRI International (Menlo Park, CA). Verapamil was obtained from Knoll Pharmaceuticals (Whippany, NJ).

In Vitro Drug Assays. Two methods were used to assess doxorubicin cytotoxicity. A colorimetric assay utilizing the tetrazolium salt, MTT, was used to assess cytotoxicity of a 1-h exposure to doxorubicin in the presence or absence of verapamil (18). Data were expressed as the percentage of survival of control cells calculated from the absorbance (540 nm) corrected for background absorbance. The surviving fraction of cells was determined by dividing the mean absorbance values of the doxorubicin-treated samples by the mean absorbance values of untreated control samples.

In addition, a standard two-layer, soft-agar culture system was used for drug sensitivity testing (19). Cells were continuously exposed to drug by incorporating it into the upper layer of medium and agar. Cells were plated in triplicate at a concentration of 2 × 10⁴ cells/35-mm tissue culture dish. Tumor cell colonies greater than 60 μm in diameter were counted using an automated inverted microscope (FAS-II Omnicon; Bausch and Lomb, Rochester, NY) 10–14 days after plating. The percentage of survival was based upon the ratio of the plating efficiency of treated to control cells.

Drug Accumulation, Influx, and Efflux Studies. Unidirectional and net intracellular drug accumulation was determined in 8226/S and 8226/DOX40 cells by using the silicone oil method of cell separation according to the method of Vistica et al. (20). Net intracellular accumulation of drug was determined by incubating cells with 0.5 μM [14C]Doxorubicin in RPMI 1640 medium containing 2% fetal bovine serum and 1% L-glutamine at 37°C. After 1 h of incubation, the cells were diluted with 10 volumes of ice-cold PBS, pelleted, and washed once again in cold PBS. Following the PBS wash, 1 × 10⁶ cells were layered

Received 3/21/88, revised 6/14/88; accepted 8/18/88.

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1This work was supported in part by National Cancer Institute Grants CA43043, CA27032, and CA17094.

21987–1988 Sterling-Winthrop Pharmacology/Toxicology Fellow.

2To whom requests for reprints should be addressed, at Cancer Pharmacology Program, Arizona Cancer Center, Tucson, AZ 85724.

The abbreviations used are: MDR, multidrug resistance; DOX, doxorubicin; MTX, 3-(4,5-dimethylthiazol-2)-5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline (5 mM KH₂PO₄;150 mM NaCl, pH 7.4); HPLC, high-performance liquid chromatography; SSB, single strand breaks; DSB, double strand breaks; DPC, DNA-protein cross-links.

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on silicone oil (SF 1250; General Electric Co., Inc., Waterford, NY) in microcentrifuge tubes.

In addition to the radiolabeled doxorubicin accumulation studies, accumulation was also studied by reverse-phase high-pressure liquid chromatography. Cells were incubated with doxorubicin for 1 h at 37°C in the presence or absence of verapamil. At the end of the incubation period, cells were washed twice with cold PBS, pH 7.4, and lysed by sonication. The cell lysates were prepared for HPLC analysis by the method of Peng (21). Daunorubicin was added to each sample as an internal standard prior to HPLC analysis. The HPLC analysis was performed on a Waters Associates Model 600A solvent delivery system and a C18 Bondapak reversed phase column (Waters Associates, Milford, MA). Compounds were eluted isocratically with a mobile phase consisting of 25% acetonitrile and 75% 0.2 M ammonium acetate, pH 4.0. Solvent flow rate was 2.0 ml/min. Fluorescent measurements of doxorubicin and daunorubicin were made with a Perkin-Elmer (Perkin-Elmer, Oak Brook, IL) LS-1 fluorescence detector (excitation, 480 nm; emission, 550 nm). Quantiﬁcation was performed by the internal standard method using peak areas of doxorubicin and daunorubicin.

Initial influx of doxorubicin in the presence and absence of verapamil was also investigated using resistant cells. In this case, 1 x 10^6 cells were incubated with 1.0 ml of medium containing 0.5 μM [14C]doxorubicin overlayed on silicone oil. In some cases the medium also contained 3 μg/ml of verapamil. After incubation intervals from 0 to 60 s, the cells were pelleted immediately by centrifugation at 12,000 rpm for 30 s using a Beckman microfuge to separate cells from medium. In determining the zero time point, cells, medium, and drug were maintained at 4°C to measure nonspeciﬁc membrane binding. Cell pellets were then solubilized and radioactivity determined.

To compare the efflux of [14C]doxorubicin from 8226/S and 8226/DOX cells, 1 x 10^6 cells were incubated with 0.5 μM [14C]doxorubicin for 1 h as described above. Following this preincubation period, the cells were washed once in PBS and then resuspended in doxorubicin-free medium and placed in a 37°C water bath. To evaluate the effect of verapamil on drug efflux, 8226/DOX40 cells were incubated in medium with and without verapamil (3 μg/ml). At intervals, 1 x 10^6 cells were centrifuged through silicone oil and radioactivity determined.

DNA Alkaline Elution. Sensitive and resistant 8226 cells were labeled with [3H]thymidine (0.1 μCi/ml; 55 mCi/mmol; ICN Radiochemical, Irvine, CA) and exposed to 0.5 μM doxorubicin for 1 h at 37°C. In some instances, 5 μg/ml of verapamil were included with the doxorubicin exposure. The formations of DNA SSB and DPC were measured according to the procedure of Kohn et al. (22). A "neutral elution" assay (pH 9.6) was used to measure DNA DSB according to the bound-to-one-terminus model of Ross et al. (24). In addition to the radiolabeled doxorubicin accumulation studies, accumulation was also studied by reverse-phase high-pressure liquid chromatography. Cells were incubated with doxorubicin for 1 h at 37°C in the presence or absence of verapamil. At the end of the incubation period, cells were washed twice with cold PBS, pH 7.4, and lysed by sonication. The cell lysates were prepared for HPLC analysis by the method of Peng (21). Daunorubicin was added to each sample as an internal standard prior to HPLC analysis. The HPLC analysis was performed on a Waters Associates Model 600A solvent delivery system and a C18 Bondapak reversed phase column (Waters Associates, Milford, MA). Compounds were eluted isocratically with a mobile phase consisting of 25% acetonitrile and 75% 0.2 M ammonium acetate, pH 4.0. Solvent flow rate was 2.0 ml/min. Fluorescent measurements of doxorubicin and daunorubicin were made with a Perkin-Elmer (Perkin-Elmer, Oak Brook, IL) LS-1 fluorescence detector (excitation, 480 nm; emission, 550 nm). Quantiﬁcation was performed by the internal standard method using peak areas of doxorubicin and daunorubicin.

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Quantitation of DNA Lesions. The DPC lesions were converted to rad equivalents using the bound-to-one-terminus model of Ross et al. (24)

\[
P_e = [(I - r)^{-1} - (I - r_0)^{-1}] \times P_b
\]

where \(P_b\) is the frequency of DNA single strand breaks produced by 30 Gy, and \(r\) and \(r_0\) are the fractions of DNA eluting in the slow elution phase in the presence (\(r\)) or absence (\(r_0\)) of drug. The degree to which \(r\) exceeds \(r_0\) is a measure of DPC.

DNA SSB and DSB in sensitive and resistant cells were quantitated as rad equivalents, defined as the dose of radiation which produces an equivalent number of DNA strand breaks in control experiments with radiation alone (22).

RESULTS

In Vitro Cytotoxicity Studies. As shown in Fig. 1, verapamil had a marked effect on the sensitivity of 8226/DOX40 cells to the cytotoxic action of doxorubicin. Such effects were related to the concentration of verapamil. In the absence of verapamil, there was little doxorubicin-induced cytotoxicity in the 8226/DOX40 cells. The addition of 3 μg/ml of verapamil to the cells resulted in a significant shift of the concentration-survival curve to the left, indicating an increased sensitivity to doxorubicin. This concentration of verapamil did not, however, completely reverse doxorubicin resistance in these cells. A concentration of 5 μg/ml of verapamil completely reversed doxorubicin resistance in the 8226/DOX40 cells. Verapamil had little effect on the cytotoxicity of doxorubicin in the sensitive parent cell line 8226/S. Verapamil by itself was relatively nontoxic in both cell lines, reducing the percentage of survival from 100% to approximately 85% with 5 μg/ml of verapamil (data not shown). Fig. 2 demonstrates that the ability of verapamil to reverse doxorubicin resistance was related to the dose of verapamil. A dose of verapamil as low as 0.1 μg/ml had a demonstrable effect on doxorubicin cytotoxicity in the 8226/DOX40 subline, decreasing the percentage of survival...
from 50% to 12% when the dose of doxorubicin was held constant at 0.5 μM.

Effects of Verapamil on Doxorubicin Accumulation and Efflux. We have previously reported that human myeloma cells with the MDR phenotype have decreased net drug accumulation compared to sensitive cells and that this is primarily due to enhanced efflux of drug from the resistant cell (17). We therefore examined the effects of verapamil on net intracellular DOX accumulation in sensitive and resistant cells. As observed with cytotoxicity assays, there is a clear dose-response effect for verapamil in increasing net intracellular DOX accumulation in the resistant cell line only (Fig. 3). With the use of 5 μg/ml of verapamil the resistant cells accumulated a similar net amount of doxorubicin as that seen in the sensitive cell line, 65.9 ± 8.4 versus 59.7 ± 2.8 pmol/10⁶ cells, respectively. This difference was not statistically significant (P < 0.05) according to Student's t test for paired analysis. In contrast, verapamil did not increase DOX accumulation in the sensitive cell line even at the highest dose of verapamil. Results obtained with [14C]-doxorubicin revealed identical findings (data not shown). The effects of verapamil on the accumulation of doxorubicin during the first 60 s of drug exposure were examined in 8226/DOX40. As shown in Fig. 4, there were no differences in initial drug accumulation over the first 60 s of [14C]DOX incubation for the

Fig. 3. Dose-effect analysis of verapamil on the net accumulation of doxorubin in 8226 myeloma cells. Cells were exposed to 0.5 μM doxorubicin for 1 h at 37°C in the presence or absence of varying concentrations of verapamil. Net drug accumulation was determined by HPLC analysis as described in "Materials and Methods." Columns, mean of three experiments; bars, SD.

Fig. 4. Initial [14C]DOX accumulation in 8226/S (●) and 8226/DOX40 with (△) and without (○) verapamil (3 μg/ml). There was no statistical difference in rate of initial accumulation over the first 60 s for any of the cell lines as determined by comparing slopes using linear regression analysis.

Fig. 5. Efflux of [14C]DOX from 8226/S (○) and 8226/DOX40 with (△) and without (●) verapamil (3 μg/ml). Cells were exposed to [14C]DOX (5 x 10⁻⁷ M) for 1 h at 37°C, washed with PBS, and then resuspended in drug-free medium at 37°C. Each point represents the mean of six replicates. The coefficient of variation for each point was less than 10%.

Verapamil also did not change the initial uptake of doxorubicin into resistant cells during this time period. Thus, the increase in net accumulation of doxorubicin in resistant cells associated with concurrent verapamil exposure was not due to increased influx of doxorubicin.

Efflux of [14C]DOX was greater in drug-resistant cells compared to sensitive cells. This enhanced drug efflux resulted in decreased net intracellular [14C]DOX accumulation in the drug-resistant cells. To determine if verapamil increased net doxorubicin accumulation in resistant cells by blocking this enhanced efflux, the amount of [14C]DOX retained in cells was measured in the presence and absence of verapamil. Fig. 5 shows that, in both the sensitive and resistant cells, there was a rapid loss of [14C]DOX within the first 5 min of placement in doxorubicin-free medium. The sensitive cells lost approximately 40% of the drug within the first 5 min but retained the remaining 60% over the next 2 h. In contrast, over 60% of the drug was rapidly lost in the resistant cells, and there was a continuous but slower loss of drug, such that the cells retained only 12% of the original doxorubicin at the 2-h time point. When the resistant cells were exposed to 3 μg/ml of verapamil, however, this enhanced efflux was blocked, and the pattern of efflux more closely resembled that of the sensitive cells. Linear regression analysis and comparison of the terminal slopes (25) demonstrated that the rate of elimination of doxorubicin was significantly greater (P < 0.01) in the resistant cells compared to the sensitive cells, but that this difference disappeared when verapamil was added to the resistant cells. Thus, it would appear that the increase in net accumulation of doxorubicin seen with verapamil in the drug-resistant cells was due to the blocking of enhanced drug efflux.

Effect of Verapamil on Doxorubicin-induced DNA Lesions. Sensitive and resistant 8226 cells were exposed to 0.5 μM doxorubicin for 1 h and the formation of DNA lesions was assessed as described. The results of these studies are presented in Table 1. In the absence of verapamil there is a significant decrease (P < 0.001; Student's t test) in the formation of SSB, DSB, and DPC lesions in the 8226/DOX40 cell line compared to the sensitive 8226/S cell line. The addition of 5 μg/ml of verapamil to these cells resulted in a dramatic increase in the number of all three lesion types. For example, in the absence of verapamil there are 49 ± 11 rad equivalents of single strand breaks in the 8226/DOX40 cells as compared to 252 ± 53 rad equivalents in its presence. This compares with 274 ± 27 rad
equivalents in the 8226/S line. This same pattern holds true for the DSB and DPC lesions as well. Verapamil had no effect on the formation of these lesions in the 8226/S cells (data not shown). Thus, in the presence of verapamil we observe an equivalent amount of doxorubicin-induced DNA lesions in the sensitive and resistant cells, presumably due to the increase in drug accumulation in the resistant cells. As demonstrated in Fig. 6, the addition of 5 μg/ml of verapamil resulted in equivalent intracellular doxorubicin accumulation, identical DNA damage, and cytotoxicity in the two cell lines.

**DISCUSSION**

A major mechanism of acquired multidrug resistance has been attributed to enhanced drug efflux, resulting in reduced intracellular drug accumulation (26, 27). The M, 170,000 glycoprotein, P-glycoprotein, has been causally related to the development of this resistance although the exact function of this protein remains to be elucidated (9, 10, 28). It has been speculated that the P-glycoprotein may function as an energy-dependent export pump for various natural products, including anticancer drugs. Support for the possibility that P-glycoprotein is directly involved in the transport of drugs is provided by the vinblastine photoaffinity labeling of P-glycoprotein in multidrug-resistant cells (11, 12). Recently, Gerlach et al. reported a striking similarity in the structure of P-glycoprotein and a bacterial hemolysin transport protein, HlyB protein (29). Based on this structural homology, they hypothesized that the P-glycoprotein serves as a drug transport system by actually transporting a carrier protein to which various drugs are bound. This hypothesis is appealing in that it unifies the mechanism of tumor cell resistance to multiple drugs.

We have previously demonstrated that human myeloma cells exhibit the multidrug-resistant phenotype and overexpress P-glycoprotein (17). Consequently, the resistance to doxorubicin and Vinca alkaloids is associated with decreased drug accumulation secondary to enhanced efflux. These observations suggested that, if drug efflux could be inhibited, then drug resistance to these particular agents could be overcome. The effectiveness of certain calcium channel blockers in reversing drug resistance has been demonstrated in both murine and human tumor cell lines (13, 30). Although it is generally agreed that this reversal is due to increased drug accumulation, it is not clear whether this is due to altered uptake (31), distribution (32), or efflux (33) of drug in resistant cells. Reports also vary as to whether calcium channel blockers are pleiotropic in their ability to reverse resistance or more selective, reversing resistance to only certain classes of drugs (34, 35). In this paper, studies were undertaken to evaluate the efficacy and possible mechanisms by which the calcium channel blocker, verapamil, circumvents doxorubicin resistance in multidrug-resistant human myeloma cells.

Cytotoxicity assays demonstrated that verapamil (5 μg/ml) reversed resistance to doxorubicin. When the dose-response relationship was examined for verapamil potentiation, we found the resistant myeloma cells to be very sensitive to the effects of verapamil with a dose as low as 0.1 μg/ml potentiating doxorubicin cytotoxicity. Previous studies from this laboratory have demonstrated the sensitivity of the 8226 myeloma cells to be very sensitive to the effects of doxorubicin cytotoxicity. Previous studies from this laboratory have suggested that, if drug efflux could be inhibited, then drug resistance to these particular agents could be overcome. The effectiveness of certain calcium channel blockers in reversing drug resistance has been demonstrated in both murine and human tumor cell lines (13, 30). Although it is generally agreed that this reversal is due to increased drug accumulation, it is not clear whether this is due to altered uptake (31), distribution (32), or efflux (33) of drug in resistant cells. Reports also vary as to whether calcium channel blockers are pleiotropic in their ability to reverse resistance or more selective, reversing resistance to only certain classes of drugs (34, 35). In this paper, studies were undertaken to evaluate the efficacy and possible mechanisms by which the calcium channel blocker, verapamil, circumvents doxorubicin resistance in multidrug-resistant human myeloma cells.

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A similar dose-response effect of verapamil on doxorubicin cytotoxicity was seen with net intracellular doxorubicin accumulation. Verapamil at 5 μg/ml increased doxorubicin intracellular accumulation in the drug-resistant cell line to a level seen in the sensitive cell line. As with the cytotoxicity assays, the effects of verapamil on drug accumulation were limited to the resistant cell line. Verapamil had no effect on initial doxorubicin accumulation in resistant cells, but did block the enhanced efflux of doxorubicin from resistant cells. It is also possible that verapamil may be altering the intracellular distri-
bution of doxorubicin in the 8226 cells. Such a phenomenon may be responsible for the large difference in the initial efflux of doxorubicin from the resistant cells. It would appear, therefore, that the ability of verapamil to overcome resistance is directly related to its effects on blocking enhanced drug efflux or altering drug distribution, thus leading to increased net intracellular drug. The relative contribution of these two possibilities remains to be determined in the 8226 cells.

Although the exact cytotoxic mechanism of doxorubicin is not fully agreed upon, one likely site of action is the cell nucleus. DNA single strand breaks, double strand breaks, and DNA-protein cross-links have all been detected following doxorubicin exposure (21, 37, 38). In our studies, we demonstrated that, in the absence of verapamil, there is an approximately 5-fold decrease in the formation of both SSB and DPC and a 2.5-fold decrease in DSB in the 8226/DOX40 cells. Such findings are not surprising given the differences in drug accumulation between the sensitive and resistant lines. In the presence of 5 μg/ml of verapamil, however, the differences in drug accumulation were removed, and this resulted in an equivalent number of DNA lesions between the two cell lines. This increase in DNA damage correlated well with the observed increase in doxorubicin cytotoxicity in the 8226/DOX40 cells under these conditions.

In a companion paper, we observed a discordance between doxorubicin cytotoxicity and drug accumulation in 8226/DOX40 cells (39). Although the extracellular concentration of doxorubicin was adjusted to achieve twice as much intracellular doxorubicin in the resistant cells at 1 h as compared to the sensitive cells, the resistant 8226/DOX40 cells maintained their resistance to doxorubicin. We hypothesized that with time the resistant cells were able to reduce intracellular doxorubicin concentrations to a sublethal concentration by active efflux, whereas the sensitive cell line, lacking the efflux mechanism, retained sufficient amounts of doxorubicin to be cytotoxic. The present study demonstrates that verapamil blocks drug efflux in the resistant cell line, resulting in prolonged retention of doxorubicin and enhanced cytotoxicity. Thus, with the addition of verapamil, the discordance between drug accumulation and cytotoxicity observed in the previous study is now removed, and a good correlation exists between drug accumulation, DNA damage, and cytotoxicity in the 8226/DOX40 line.

The clinical utility of these in vitro studies remains to be determined. Ozols and coworkers in a limited Phase I–II study failed to demonstrate a potentiating effect of verapamil in drug-resistant ovarian cancer (40). Whether these cases of refractory ovarian cancer possessed P-glycoprotein was not reported. The case for hematological tumors may be different, and in fact, we and a good correlation exists between drug accumulation, DNA damage and expression of P-glycoprotein in a variety of hematological tumor cell lines. The in vitro results reported in this study in addition to the case report have prompted us to investigate this possibility in a prospective clinical trial which is ongoing (41).

REFERENCES


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