Int. J. Cancer: **66,** 506–514 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

RESISTANCE TO THE CHEMOSENSITIZER VERAPAMIL IN A MULTI-DRUG-RESISTANT (MDR) HUMAN MULTIPLE MYELOMA CELL LINE

Mohammad R. ABBASZADEGAN[1,2,3], Nils E. FOLEY[1,2], Mary C. GLEASON-GUZMAN[1,2] and William S. DALTON[1,2,3,4]

[1]*Bone Marrow Transplant Program,* [2]*Arizona Cancer Center and* [3]*Department of Pharmacology/Toxicology, University of Arizona, Tucson, AZ, USA.*

Inhibitors of P-glycoprotein (P-gp) or chemosensitizers, such as verapamil, are used to reverse multi-drug resistance (MDR) in cancer patients. Clinical studies in patients with myeloma have shown that some patients with P-gp-positive cancer cells respond to the chemosensitizing effect of verapamil. However, this response is short-lived and tumor cells ultimately become resistant to chemosensitizers. To study mechanisms of resistance to chemosensitizers, a human myeloma cell line, 8226/$MDR_{10}V$, was selected from a P-gp-positive cell line, 8226/Dox_{40}, in the continuous presence of doxorubicin and verapamil. $MDR_{10}V$ cells are consistently more resistant to MDR drugs than parent cells, Dox_{40}. Chemosensitizers, including verapamil and cyclosporin A, were less effective in reversing resistance in $MDR_{10}V$ compared with Dox_{40} cells. Verapamil and cyclosporin A were only partially effective in blocking P-gp drug efflux in $MDR_{10}V$ compared to Dox_{40} cells. Despite higher resistance to cytotoxic agents, $MDR_{10}V$ cells express less P-gp in the plasma membrane than do its parent cells, Dox_{40}. [^{3}H]Azidopine photoaffinity labeling of P-gp and its binding competition with unlabeled verapamil showed similar affinity for P-gp between Dox_{40} and $MDR_{10}V$ cell lines. Non-P-gp-mediated mechanisms of drug resistance, including over-expression of MRP and alterations in topoisomerase II, were not different for $MDR_{10}V$ cells compared with Dox_{40} cells.

Tumor cells in cancer patients acquire drug resistance as a result of chemotherapy. One type of "acquired" drug resistance is multi-drug resistance (MDR) caused by P-glycoprotein (P-gp) efflux pump. MDR cells exhibit a cross-resistance to multiple drugs that have little similarity in structure and in mechanisms of action. These cells have reduced intracellular drug concentration compared to their drug-sensitive parental cells, through over-expression of a 170 kDa P-gp that is dependent on ATP hydrolysis. The role of P-gp in clinical drug resistance, particularly in hematologic malignancies (Dalton and Sikic, 1994), necessitates its circumvention to overcome MDR. A major development in the field was the finding of Tsuruo *et al.* (1981) that verapamil, a calcium channel blocker, can sensitize vincristine-resistant P388 leukemia cells to the cytotoxic actions of vincristine and vinblastine. They also showed the MDR-reversal effect of verapamil in mice bearing vincristine-resistant Ehrlich ascites tumors (Tsuruo *et al.,* 1981). Furthermore, Tsuruo *et al.* (1982) reported that verapamil and other P-gp antagonists, such as phenothiazines, enhance anti-cancer drug cytotoxicity by increasing their accumulation and retention in P388 leukemia MDR cells. Since these findings, a wide variety of agents have been identified to reverse P-gp-mediated MDR (Beck, 1991).

Studies conducted in patients with hematopoietic malignancies have been the most promising in demonstrating the potential for reversing clinical MDR. High-dose infusion verapamil in combination with vincristine/doxorubicin/dexamethasone (VAD) has reversed MDR in patients with myeloma who had progressed on VAD alone (Dalton *et al.,* 1989; Salmon *et al.,* 1991). Responses in these studies, however, were short-lived, and toxicity due to verapamil was high. More recent studies, using high-dose infusion of cyclosporin A (CsA) as a chemosensitizer, have shown promising results in patients with drug-resistant myeloma and AML (Sonneveld *et al.,* 1992; Marie *et al.,* 1993; List *et al.,* 1993). Despite these encouraging preliminary results, relapses following the use of chemosensitizing agents are common, suggesting that tumor cells become resistant to chemosensitizers.

The present study examines the possibility that treatment with chemosensitizing agents (in this case, verapamil) and cytotoxic agents (doxorubicin) selects for a cell population which is resistant to the chemosensitizing effects of verapamil. A human myeloma cell line, 8226/$MDR_{10}V$, was selected from a P-gp-positive cell line, 8226/Dox_{40}, in the continuous presence of 0.1 μM doxorubicin and 22 μM verapamil. Selection of this cell line mimics the current use of chemosensitizing agents to reverse P-gp-mediated resistance in the clinic. Analysis of this cell line should assist in defining mechanisms by which cells become resistant to chemosensitizers.

MATERIAL AND METHODS

Reagents

All reagents were purchased from Sigma (St. Louis, MO) unless specifically indicated. Reagents were analytical grade or of the highest grade available.

Cell lines, drug selection

The RPMI 8226 human multiple myeloma cell line was obtained from the ATCC (Rockville, MD). 8226/Dox_{40} was selected from drug-sensitive 8226 (8226/S) by continuous exposure to stepwise increasing concentrations of doxorubicin (10–400 nM) (Dalton *et al.,* 1986). 8226/$MDR_{10}V$ was selected from 8226/Dox_{40} by continuous culture in 10 μg/ml verapamil (22 μM, racemic) and doxorubicin (Fig. 1). The addition of verapamil to doxorubicin in the selection pressure of 8226/Dox_{40} required a reduction in the doxorubicin concentration from 400 to 10 nM. Over the course of 1 year, the doxorubicin concentration was increased to 100 nM. All cell lines were maintained in RPMI 1640 media (GIBCO, Grand Island, NY), which was supplemented with 5% FCS, 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml) and 1% (v/v) L-glutamine (GIBCO). Cell cultures were incubated in a 37°C incubator with saturated humidity and an atmosphere of 95% air and 5% CO_2. Cell lines were sub-cultured every 7 days.

Cytotoxicity assay

Cytotoxicity of drugs was measured *in vitro* using the MTT assay (Alley *et al.,* 1988). The assay relies on the conversion of a soluble tetrazolium salt (3,4,5-dimethylthiazole-2,5 diphenyl tetrazolium bromide) into an insoluble formazan product by mitochondrial succinate dehydrogenases. The insoluble product was dissolved in DMSO and measured by recording optical density at 540 nm. Cells were plated at 8,000–10,000 cells/well in 96-well microtiter plates, drug was added and the plate was placed in a tissue culture incubator for 4 days. MTT dye (50 μl

[4]To whom correspondence and reprint requests should be sent, at Arizona Cancer Center, 1515 North Campbell Ave., Tucson, AZ 85724, USA. Fax: (520) 626-2415.

Received: October 6, 1995 and in revised form December 7, 1995.

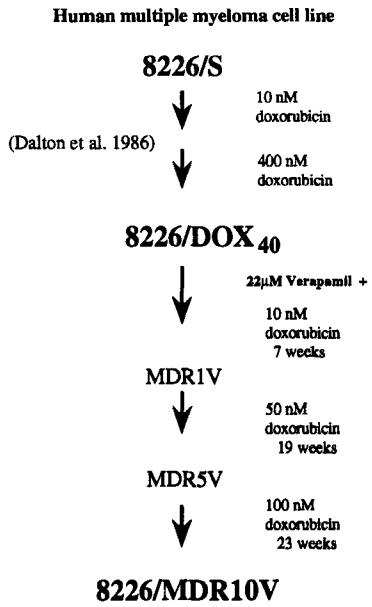

FIGURE 1 – The drug selection scheme used to generate 8226/$MDR_{10}V$, a cell line resistant to chemosensitizers. Four $MDR_{10}V$ clones were also isolated, and their characteristics were similar to the selected $MDR_{10}V$ cell line.

of a 1 mg/ml solution) was added, and plates were incubated for an additional 4 hr. Plates were centrifuged at 300 × *g* for 5 min, the supernatant was aspirated and DMSO was added to the pelleted cells/formazan dye. Plates were agitated for 5 min and assayed on an automated spectrophotometer; IC_{50} values were calculated by linear regression analysis.

The results of MTT assays were confirmed by a 2-layer, soft agar culture system (Dalton *et al.,* 1986). Drug exposure was either for 1 hr prior to plating or continuously by incorporating the agent into the agar. Cells were plated in triplicate at a concentration of 2.0×10^5 cells/35-mm tissue culture dish (Falcon, Oxnard, CA). Tumor cell colonies were evaluated using inverted microscopy 10–14 days after plating. Percentage of survival was based upon the ratio of plating efficiency of treated to control cells. The IC_{50} for each particular drug was defined as the concentration of the drug which reduces colony formation to 50% of untreated control cells. A relative resistance index was expressed as the ratio of the IC_{50} of resistant cells to the IC_{50} of sensitive (8226/S) cells.

Drug-accumulation analysis

Cell lines were analyzed for retention of the P-gp substrate doxorubicin. Intracellular doxorubicin fluorescence was used as a measure of drug accumulation. Cells were washed with PBS and resuspended at a concentration of 1×10^6 cells/ml. One milliliter of this cell suspension was transferred to 15-ml conical tubes and incubated as a suspension in tubes submerged in a water bath at 37°C. Some tubes received a chemosensitizer, verapamil or cyclosporin A, at various concentrations ranging from 1 to 200 μM. After a 15-min incubation, 10 μM doxorubicin was added to each tube, except that containing control cells. Samples were incubated for 1 hr at 37°C, with intermittent mixings every 10 min. Samples were then washed twice in cold PBS and resuspended in 1 ml cold PBS. Samples were kept in the dark and on ice and analyzed immediately on a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer. Fluorescence from doxorubicin was measured at 585 nm. Cells with no exposure to doxorubicin were used as a control for autofluorescence.

Verapamil uptake

Intracellular accumulation of [^{3}H]verapamil in $MDR_{10}V$ cells was compared with Dox_{40} cell line. Cells were washed in PBS and resuspended in media at a concentration of 1×10^6 cells/ml; 200 μl of the cell suspension (2×10^5 cells) were transferred into each well of a 96-well plate in multiples of 5 wells for each cell line; 20 μl of a working solution of verapamil containing 0.1 μM of [N-methyl-^{3}H]-verapamil hydrochloride (NEN, Boston, MA) plus 9.9 μM of unlabeled verapamil was added to each well. The 96-well plate was incubated at 37°C for 15 min (this time point was chosen to be consistent with the doxorubicin uptake studies). Following incubation, cells were harvested onto filter papers using a cell harvester. Filters were washed with saline, air-dried and transferred to scintillation tubes. Cells were digested in 100 μl of 1 N NaOH and neutralized with 100 μl of 1 N HCl. Radioactivity was measured in scintillation cocktail by a scintillation counter. [^{3}H]verapamil uptake was expressed as cpm/μg of protein for each cell line. Protein determination was performed on a total of 2×10^5 cells in triplicate wells of a 96-well plate using the BioRad (Richmond, CA) assay of protein determination.

Immunoblot analysis for P-gp

Plasma membranes from 2×10^8 cells were purified according to the method of Riordan and Ling (1979). The membrane-enriched fractions from 8226/S, Dox_{40} and $MDR_{10}V$ cell lines were separated in a 7% SDS/polyacrylamide gel by electrophoresis and transferred to nitrocellulose. Blots were probed with the C219 monoclonal antibody (MAb; Signet, Dedham, MA), JSB1 (Boehringer-Manheim, Indianapolis, IN) or C494 (Signet), followed by reaction with ^{125}I-conjugated goat-anti-mouse antibody. Quantitation of the 170-kDa P-gp band was performed on blots using a phosphor-imaging system.

Immunofluorescence and flow cytometer analysis of P-gp

Cells (1×10^6) were incubated with 5 μg of MRK16 (Kamiya, Thousand Oaks, CA) or IgG_{2a} control (Dako, Carpiuteria, CA) in 200 μl of PBS + 2% FBS for 30 min at 25°C; 5 μg of MRK16 were used because it yielded maximal staining for P-gp and minimal non-specific staining of isotypic control samples. Cells were washed with PBS + 2% FBS, then incubated with FITC-conjugated goat anti-mouse Ig (1:50) for 15 min on ice and kept in the dark. Cells were then washed and analyzed immediately using a FACScan flow cytometer with excitation at 488 nm and emission measured at 515 nm for FITC. Fluorescence ratios were calculated by dividing the mean fluorescence value of the MRK16-stained population by the mean fluorescence value of the isotypic control (IgG_{2a})-stained population.

Photoaffinity labeling of P-gp

Plasma membrane vesicles from exponentially growing cells were prepared as described above and photo-labeled with [^{3}H]azidopine (Safa *et al.,* 1987); 50 μg purified plasma membranes were photo-labeled in 40 mM potassium phosphate buffer (pH 7.5) containing 10 μM $CaCl_2$, 20 μM [^{3}H]azidopine (Amersham, Aylesbury, UK) in a final volume of 50 μl. This mixture was incubated in the dark for 1 hr at 25°C in the absence or presence of non-radioactive competing verapamil (10–200 μM), then irradiated for 20 min at 25°C with a UV lamp equipped with 2 15-W self-filtering 302-nm lamps (model XX-15; Ultra-violet products, San Gabriel, CA). Photo-labeled membranes were analyzed by 7% SDS-PAGE and fluorography (Safa *et al.,* 1986). Quantitation of radiolabeled protein bands was accomplished by densitometer readings using Ambis (San Diego, CA) image acquisition and analysis software. The least squares regression line of the area O.D. by log $base_{10}$ of verapamil concentration was calculated for each cell line. Analysis of co-variance was done to test for

differences in verapamil competition of [^{3}H]azidopine binding between Dox_{40} and $MDR_{10}V$ cell lines.

Topoisomerase II assays

Nuclear extracts were prepared from exponentially growing cells as previously described (Wolverton *et al.,* 1989); 3 to 4 × 10^8 cells were initially washed twice with PBS, then pelleted and resuspended in 15 ml of buffer A (0.15 M NaCl, 10 mM KH_2PO_4). Cells were washed a second time in buffer A, then resuspended in 10 ml of buffer B (5 mM KH2PO$_4$, 2 mM $MgCl_2$, 4 mM DTT, 0.1 mM Na_2EDTA) and allowed to swell for 30 min on ice. Cells were then Dounce-homogenized for 10 strokes and the released nuclei collected at 2,500 *g* for 15 min. Nuclei were resuspended in 4 ml buffer C (buffer B + 0.25 M sucrose) and layered over 1.2 ml of buffer D (buffer B + 0.6 M sucrose). This sucrose gradient was centrifuged in an L8-70 centrifuge (Beckman, San Ramon, CA) using a swinging bucket rotor (SW28) for 20 min at 2,000 *g*. The nuclear pellet was resuspended in 300 μl of buffer E (5 mM KH_2PO_4, 4 mM DTT, 1 mM Na_2EDTA) and the total volume measured. An equal volume of buffer F (40 mM Tris [pH 7.5], 2 M NaCl, 4 mM DTT) was added and the solution placed on ice for 1 hr. After adjusting to 10% glycerol, the solution was centrifuged at 100,000 *g* for 1 hr and the supernatant divided into aliquots and stored at −70°C. To minimize proteolysis, all procedures were performed at 4°C, and 1.0 mM Pefabloc (Boehringer Mannheim) was added to buffers A–F. In addition, antipain, aprotinin, leupeptin and pepstatin A (each at 20 μg/ml) were added to buffers B–F. Protein concentrations were determined with the BioRad protein assay kit, according to the manufacturer's instructions. For immunoblotting, the nuclear extract was used immediately, while frozen preparations could be used for up to 30 days to assay for topoisomerase II activity.

For immunoblotting, 50 μg of fresh nuclear extract from the cell lines were separated on a 7% SDS-polyacrylamide gel, transferred to PVDF and blocked as described above. The blot was probed with a polyclonal antibody kindly provided by Dr. M. Danks (Memphis, TN). This antibody recognizes both 170 and 180 kDa (alpha and beta, respectively) isozymes of topoisomerase II (Wolverton *et al.,* 1989). Following incubation with the primary antibody, the blot was washed and probed with ^{125}I-labeled goat anti-rabbit IgG (specific activity 6.20 μCi/μg; NEN). The membrane was washed and dried and autoradiography performed.

Catalytic activity was measured as the decatenation of networks of kinetoplast DNA (kDNA) isolated from *Crithidia fasciculata* in the following manner. Various amounts of nuclear extracts were added to a microfuge tube containing 0.5 μg kDNA and topoisomerase II reaction buffer (50 mM Tris [pH 7.5], 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM Na_2EDTA, 30 μg/ml BSA, 1 mM ATP). Samples were incubated for 30 min at 30°C and reactions terminated by the addition of 5 μl 0.05% bromophenol blue, 2% SDS and 50% glycerol. Samples were then electrophoresed (1% agarose gel) for 150 min at 75 v, and DNA was visualized using ethidium bromide staining. Agarose gel electrophoresis of released minicircles was used to determine topoisomerase II activity.

The ability of another topoisomerase II-interactive drug, etoposide (VP-16), to stimulate the formation of covalent topoisomerase II–DNA complexes was examined by the K^+/SDS precipitation assay (Liu *et al.,* 1983; Nelson *et al.,* 1984). Nuclear extract proteins (50 ng) isolated from log-phase cells were incubated with linearized, ^{32}P-end-labeled pBR322 in the presence of varied concentrations of VP-16 (0–50 μM). Drug-induced topoisomerase II–DNA complexes were then precipitated by the addition of SDS and KCl. The amount of topoisomerase II–DNA complex was quantitated and expressed as the percent precipitation of available ^{32}P-labeled pBR322.

RT-PCR analysis of MRP

A single large-scale cDNA reaction for use in different gene-specific amplifications was prepared. A 60-μl reverse-transcription reaction mixture, prepared at 25°C and containing 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$); 1 mM each dATP, dGTP, dCTP and dTTP; 500 pmol random hexamer; 100 units RNasin; 60 units AMV-RT and 300 ng of total cellular RNA, was incubated at 42°C for 45 min, then 99°C for 5 min, followed by a 4°C quick chill.

MRP amplification was performed as previously described (Abbaszadegan *et al.,* 1994) by adding 80 μl of the PCR reaction mixture (1× PCR buffer, 25 pmol of MRP-specific amplimers and 2 units of Taq DNA polymerase) to the equivalent of 100 ng starting RNA template (20 μl of the large-scale cDNA reaction) followed by incubation at 94°C for 1 min, then 29 cycles of 94°C for 15 sec, 58°C for 15 sec, 72°C for 15 sec, a final extension at 72°C for 1 min and a quick chill to 4°C in a 9600 thermocycler. Twenty-nine cycles of PCR was in the exponential range of amplification for *MRP,* which was determined previously using RNA from the low-level-expressing cell line H69 and the over-expressing variant H69AR (Futscher *et al.,* 1994). H69, H69AR and lung cells were kindly provided by Dr. S.P. Cole (Kingston, Ont.). Ten percent of PCR products were separated on an ultrapure 3% agarose gel and the gel was stained with ethidium bromide for visualization. Amplimer sequences for *MRP* were as described previously (Abbaszadegan *et al.,* 1994).

To ensure RNA integrity and to provide a gene expression reference point, a cell cycle–independent, ubiquitously expressed histone gene, *H3.3* (Wells *et al.,* 1987; Wells and Kedes, 1985), was amplified from the same cDNA reaction. Like *MRP,* amplification of *H3.3* was performed for 29 cycles, but cDNA from the equivalent of 50 ng of input RNA was amplified. Ten percent of the respective PCR products were separated on an ultrapure 3% agarose gel and stained with ethidium bromide. PCR parameters and amplimer sequences for histone *H3.3* were as described previously, except that 29 cycles of PCR were performed, which is still in the exponential range of amplification for *H3.3* (Futscher *et al.,* 1993).

RESULTS

Selection and characterization of the $MDR_{10}V$ cell line

A human myeloma cell line, 8226/$MDR_{10}V$ ($MDR_{10}V$), was selected from a P-gp-positive MDR cell line, 8226/Dox_{40} (Dox_{40}), by continuous exposure to verapamil (racemic) and a stepwise increase in doxorubicin concentration (Fig. 1). Twenty-two micromoles of verapamil were chosen for selection of the $MDR_{10}V$ cell line because this concentration was the highest non-cytotoxic dose used to completely reverse P-gp drug efflux and MDR in 8226 doxorubicin-resistant cell lines (Bellamy *et al.,* 1988). The cell line selected in the presence of doxorubicin and verapamil did not differ from the parent lines in doubling time (approximately 32 hr) or percent S-phase of the cell cycle (40–46%) as determined by propidium iodide and flow cytometry. Four $MDR_{10}V$ subclones of the parent $MDR_{10}V$ cell line selected by limiting dilution assay demonstrated characteristics identical to those observed for the parent $MDR_{10}V$ cell line.

The pattern and degree of drug resistance in the $MDR_{10}V$ cell line were analyzed. Figure 2 shows the results of one MTT cytotoxicity assay of the $MDR_{10}V$ and its parent, Dox_{40}, exposed to the natural products vincristine and doxorubicin. Despite reducing the concentration of doxorubicin in the selection process from that used to select the parent cell line, Dox_{40}, $MDR_{10}V$ cells were shown to be approximately 13-fold more resistant to doxorubicin and approximately 4-fold more resistant to vincristine than Dox_{40} cells. Repeated experiments produced similar results, showing that $MDR_{10}V$ cells were

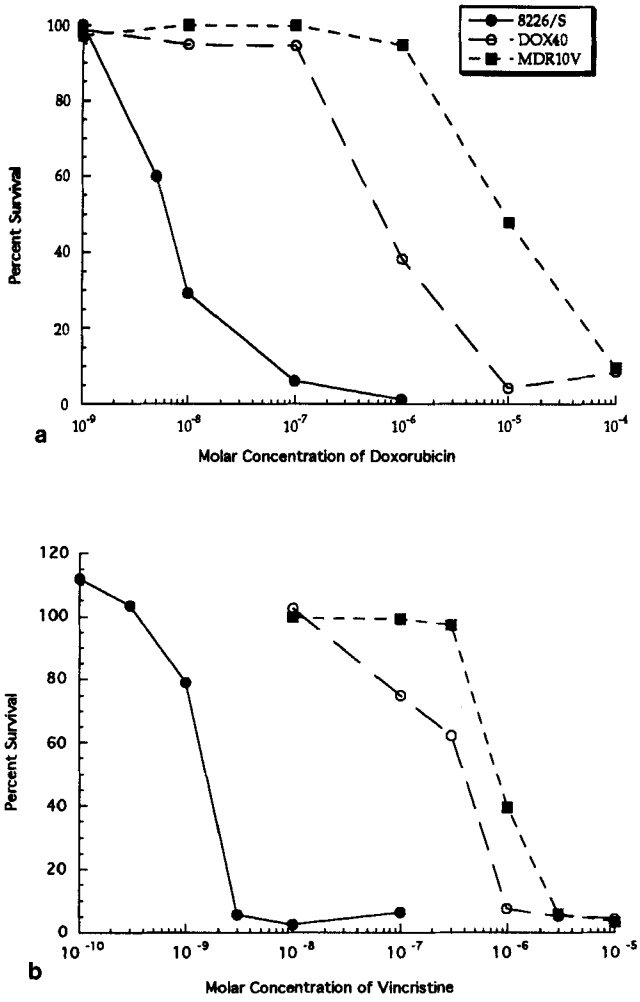

FIGURE 2 – Effect of cytotoxic agents on 8226/$MDR_{10}V$ and its parent cell line, 8226/Dox_{40}. Cytotoxicity was measured by MTT assay. *(a)* Doxorubicin cytotoxicity: IC_{50} for 8226/S = 7.9×10^{-9}, Dox_{40} = 8.1×10^{-7} (100), $MDR_{10}V$ = 1.1×10^{-5} (1322). *(b)* Vincristine cytotoxicity: IC_{50} for 8226/S = 4.3×10^{-9}, Dox_{40} = 2.5×10^{-7} (58), $MDR_{10}V$ = 9.1×10^{-7} (212). Numbers in parantheses are degrees of resistance, which show the ratio of the IC_{50} of 8226/resistant cells over the IC_{50} of 8226/sensitive cells.

consistently more resistant to doxorubicin and vincristine than Dox_{40} cells.

Table I shows the drug-resistance profile of $MDR_{10}V$ and its parent cell line, Dox_{40}, exposed to a number of other chemotherapeutic agents with varying mechanisms of action. The $MDR_{10}V$ cell line has a resistance profile similar to its P-gp-positive parent, Dox_{40}; however, the degree of resistance is consistently higher in $MDR_{10}V$ cells.

The effectiveness of various chemosensitizers to reverse drug resistance in Dox_{40} and its verapamil-selected cell line, $MDR_{10}V$, was examined using MTT cytotoxicity assay. The dose-modifying factor for each cytotoxic agent was calculated as the IC_{50} of that agent in the absence of a chemosensitizer divided by its IC_{50} in the presence of a chemosensitizer. Both verapamil and CsA were less effective in reversing resistance to doxorubicin and vincristine in the $MDR_{10}V$ cell line compared with the Dox_{40} cell line (Table II).

Drug accumulation

Drug accumulation in the $MDR_{10}V$ and Dox_{40} cell lines was measured by intracellular fluorescence of doxorubicin using

TABLE I – DRUG-RESISTANCE PROFILE OF 8226/$MDR_{10}V$ AND ITS PARENT CELL LINE, 8226/DOX_{40}

Class/agent	8226/Dox_{40}	8226/$MDR_{10}V$
Anthracyclines		
Doxorubicin	138[1]	1,245
Topo II inhibitors		
Mitoxantrone	95	118
Etoposide	48	201
Vinca alkaloids		
Vincristine	269	1,149
Vinblastine	85	107
Alkylating agents		
Melphalan	3.4	2.3
Anti-metabolites		
Methotrexate	1.1	1.5
Ara-C	1.4	1.3
Decadron	0.4	1.0

[1]Degree of drug resistance = IC_{50} 8226/resistant/IC_{50} 8226/sensitive, calculated by linear regression analysis.

TABLE II – EFFECTIVENESS OF VARIOUS CHEMOSENSITIZERS ON DRUG-RESISTANT 8226 CELL LINES

Agent	Dox_{40}	$MDR_{10}V$
Doxorubicin		
Verapamil (6 μM)	45[1]	25
Cyclosporin (1 μM)	16	7
Vincristine		
Verapamil (6 μM)	72	31

[1]Dose modifying factor = IC_{50} Doxorubicin alone/IC_{50} Doxorubicin + chemosensitizers.

flow cytometry. The level of intracellular doxorubicin accumulation at 1 hr was very low for both resistant cell lines compared with the drug-sensitive cell line 8226/S. Increasing doses of either verapamil or CsA were only partially effective in blocking doxorubicin efflux in the $MDR_{10}V$ cells compared with the Dox_{40} cell line (Fig. 3). A least-squares linear regression analysis of the area O.D. by log $base_{10}$ of the verapamil concentration was calculated for each cell line. This analysis showed a significant difference ($p < 0.01$) between slopes, indicating that verapamil is less effective in reversing P-gp doxorubicin efflux in $MDR_{10}V$ compared with Dox_{40} (Fig. 3*a*). Similar results were observed for CsA (Fig. 3*b*).

Intracellular accumulation of [^{3}H]verapamil in $MDR_{10}V$ cells was compared with Dox_{40} cells. Intracellular radioactivity was measured by scintillation counting and expressed as cpm/μg of protein for each cell line. The level of [^{3}H]verapamil uptake in 8226/S (52 ± 10 cpm/μg of protein) was twice that in the drug-resistant cell lines Dox_{40} and $MDR_{10}V$. Intracellular uptake of [^{3}H]verapamil was equal in both Dox_{40} (28 ± 3 cpm/μg of protein) and $MDR_{10}V$ (26 ± 2 cpm/μg of protein) cell lines.

Analysis of P-gp

The amount of P-gp on the cell surface membranes of $MDR_{10}V$ compared to Dox_{40} cells was recorded by immunoblot analysis. Cell membrane-enriched fractions from 8226/S, Dox_{40} and $MDR_{10}V$ cell lines were separated by SDS/PAGE, transferred to nitrocellulose and probed with the C219 MAb. Surprisingly, $MDR_{10}V$ had approximately 40% lower membrane-bound P-gp compared with its parent cell line Dox_{40}, which was determined by densitometer quantitation of P-gp bands (Fig. 4*a*). This observation was repeated in experiments using other MAbs against internal epitopes of P-gp (C494 and JSB-1).

To confirm the immunoblot results, the amount of P-gp on the surface of cells was analyzed by FACS using the MRK16 MAb. This antibody recognizes an external epitope of P-gp

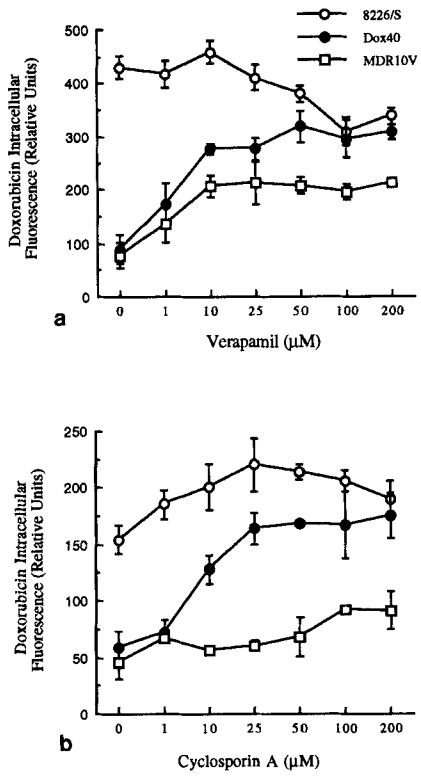

FIGURE 3 – Intracellular accumulation of doxorubicin in 8226-sensitive and its drug-resistant variant cell lines *(a)* in the absence or presence of verapamil and *(b)* in the absence or presence of cyclosporin A. Intracellular fluorescence compared between cell lines by Kolmogorov-Smirnov statistics.

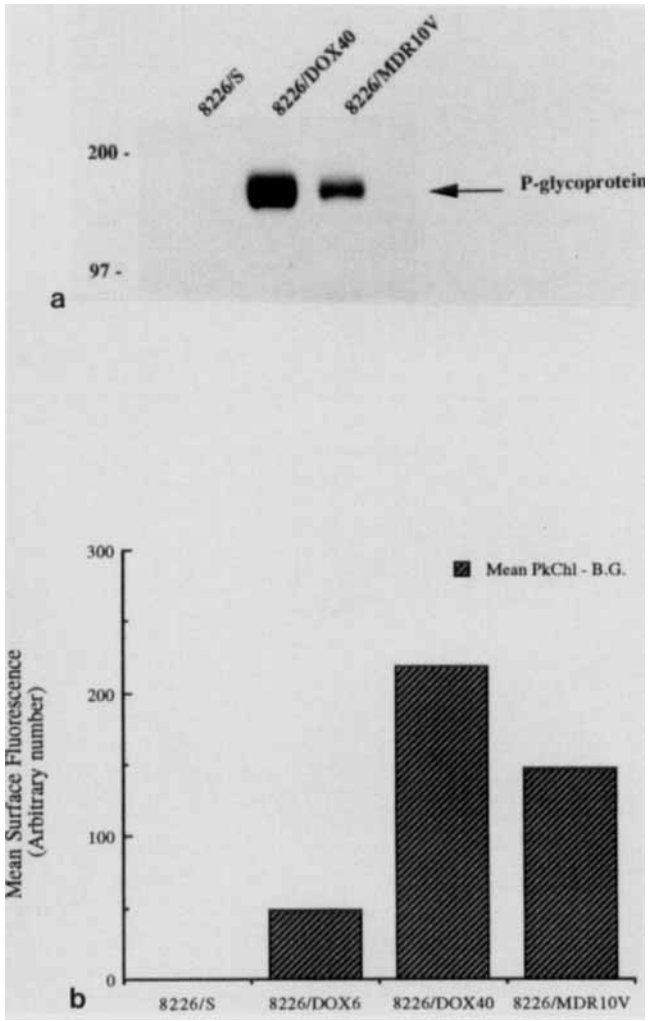

FIGURE 4 – Immunoblot *(a)* and FACS *(b)* analyses of P-glycoprotein.

and allows for analysis of live cells (Hamada *et al.,* 1990). Titration of MRK16 MAb against P-gp in the Dox_{40} cell line was performed to determine the concentration of antibody (5 μg) which yielded maximal staining for P-gp and minimal non-specific staining of isotypic control samples. Kolmogorov-Smirnov statistics were used for comparison of 10,000 events in each cell line. $MDR_{10}V$ was found to have significantly less P-gp on the surface of cells (approximately 33%) compared with Dox_{40} cells and confirms results from the immunoblot analysis (Fig. 4*b*).

Binding affinity of verapamil to P-gp was analyzed by [^{3}H]azidopine photoaffinity labeling of P-gp and inhibition of binding with unlabeled verapamil (Fig. 5). Consistent with the results of immunoblot and FACS analyses of P-gp, the absolute values for [^{3}H]azidopine binding of P-gp in plasma membrane preparations was less in $MDR_{10}V$ compared with Dox_{40} cells ($p < 0.01$). A verapamil dose-related inhibition of azidopine binding to P-gp was observed for both cell lines. A 3-way ANOVA comparing slopes calculated from least squares regression lines showed no difference between the cell lines for azidopine binding affinity to P-gp. Thus, the relative binding affinity for verapamil to P-gp is not different for $MDR_{10}V$ compared to Dox_{40} measured by this indirect technique.

Non-P-gp-mediated MDR mechanisms

Topoisomerase II. The results of immunoblot analysis for topoisomerase II protein demonstrated similar levels of topoisomerase II expression in both $MDR_{10}V$ and Dox_{40} cell lines (Fig. 6*a*). The role of topoisomerase II in the phenotype of $MDR_{10}V$ was further examined by measuring the decatenation activity of this enzyme. Catalytic activity was measured as the decatenation of networks of kinetoplast DNA (kDNA) isolated from *C. fasciculata.* No difference in topoisomerase II activity was found between Dox_{40} and $MDR_{10}V$ cell lines (Fig. 6*b*). Additionally, the ability of another topoisomerase II-interactive drug, etoposide (VP-16), to stimulate the formation of covalent topoisomerase II–DNA complexes was examined by the K^+/SDS precipitation assay (Liu *et al.,* 1983; Nelson *et al.,* 1984). Nuclear extract protein (50 ng) isolated from log phase cells was incubated with linearized, ^{32}P-end-labeled pBR322 in the presence of varied concentrations of VP-16. Drug-induced topoisomerase II–DNA complexes were then precipitated by the addition of SDS and KCl. The amount of topoisomerase II–DNA complex was quantitated and expressed as the percent precipitation of available ^{32}P-labeled pBR322. Results of these experiments showed no differences between Dox_{40} and $MDR_{10}V$ (Fig. 7). However, the levels of topoisomerase II–DNA complexes in both drug-resistant cell lines of 8226 were less than that in their drug-sensitive parent cell line 8226/S. These results show that topoisomerase II-mediated drug resistance contributes to the overall drug-resistance phenotype of both cell lines but do not explain the increased MDR and decreased sensitivity to verapamil observed in the $MDR_{10}V$ cell line compared to the Dox_{40} cell line.

Multi-drug resistance protein (MRP). We also examined the cell lines for over-expression of the *MRP* gene using RT/PCR

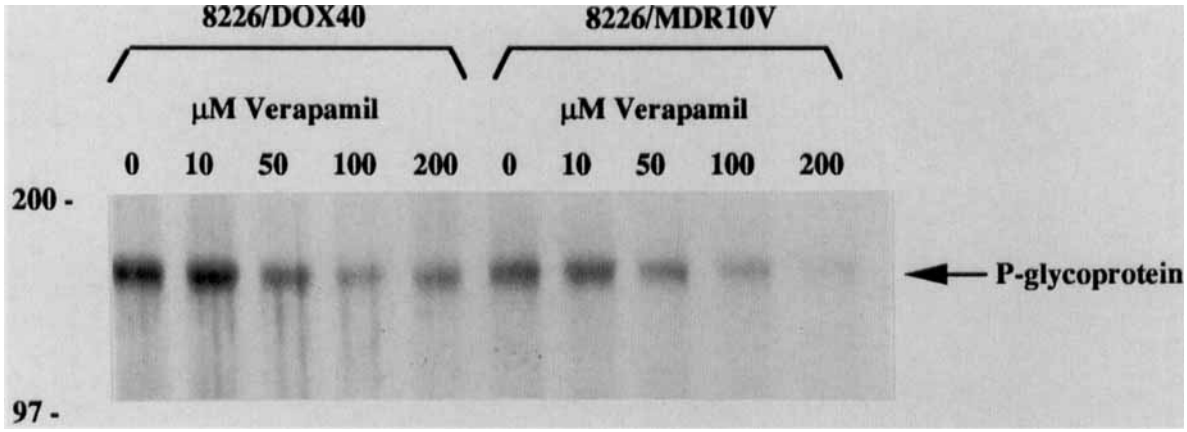

FIGURE 5 – Tritiated-azidopine photoaffinity labeling of P-glycoprotein; 50 μg purified plasma membranes were photolabeled with [^{3}H]azidopine at 25°C in the absence or presence of non-radioactive competing verapamil (10–200 μM).

(Abbaszadegan *et al.,* 1994). Drug-sensitive, low *MRP* expresser (H69, a small cell lung carcinoma cell line) and drug-resistant, high MRP expresser (H69AR) were used as controls (Cole *et al.,* 1992). Complementary DNA reaction products from the equivalent of 100 ng of input RNA from the various drug-sensitive and drug-resistant cell lines were used for analyses of MRP expression. Following PCR of the cDNA reaction products, 10% of the respective PCR products were separated on an ultrapure 3% agarose gel and stained with ethidium bromide (Fig. 8). Expression of *MRP* in $MDR_{10}V$ and Dox_{40} cell lines was similar to the level of MRP mRNA in the drug-sensitive H69 line. These results show that *MRP* does not play a role in the drug resistance phenotype of $MDR_{10}V$ compared with Dox_{40} cells.

DISCUSSION

Clinical protocols have used chemosensitizers in combination with cytotoxic drugs to reverse clinical drug resistance. Pre-clinical studies by Tsuruo *et al.* (1981, 1982, 1985) demonstrated that chemosensitizers, such as the calcium channel blocker verapamil, potentiated the anti-tumor activities of vincristine and adriamycin in MDR cells both *in vitro* and *in vivo.* This potentiation of anti-tumor activity was through an increased intracellular concentration of cytotoxic agents in the tumor cells (Tsuruo *et al.,* 1985). These results provided a rationale for using chemosensitizers such as verapamil in combination with cytotoxic agents in clinical treatment of cancer patients (Dalton *et al.,* 1989; List *et al.,* 1993; Sonneveld *et al.,* 1992; Sikic, 1993).

Two widely used chemosensitizers for circumventing clinical drug resistance are verapamil and CsA (Dalton, 1993). Chemosensitizers bind to a 170-kDa drug transporter protein, P-gp, to inhibit its function and increase the intracellular accumulation of cytotoxic drugs in tumor cells (Safa, 1988; Twentyman *et al.,* 1987). Limited success has been achieved in reversing clinical drug resistance using chemosensitizers, particularly in hematologic malignancies (Salmon *et al.,* 1991; Miller *et al.,* 1991; List *et al.,* 1993). Cancer patients who respond to this treatment ultimately relapse, however, with the emergence of a population of drug-resistant malignant cells that do not respond to chemosensitizers. To date, mechanisms of resistance to the effects of chemosensitizers are unknown. Elucidating these mechanisms may provide a means of improving current chemotherapeutic regimens.

The human multiple myeloma cell line $8226/Dox_{40}$, known to over-express P-gp, was used to select a new cell line, $8226/MDR_{10}V$, by growing cells in the continuous presence of verapamil plus doxorubicin. The concentration of doxorubicin in the selection scheme of $MDR_{10}V$ was decreased from that used for the selection of its parent, Dox_{40}, to compensate for the chemosensitizing effect of verapamil. Despite this reduction in the concentration of doxorubicin in the selection of $MDR_{10}V$, this cell line exhibited a greater resistance to MDR drugs. The chemosensitizers verapamil and CsA were less effective in reversing drug resistance in the $MDR_{10}V$ cell line (Table II). This was due to the inability of these chemosensitizers to block drug efflux by P-gp (Fig. 3).

Mutations in the *MDR1*/P-gp gene may alter the binding and efflux of drugs known to be substrates for P-gp (Safa *et al.,* 1990; Gros *et al.,* 1991). These mutations may cause a preferential resistance to selecting agents (Choi *et al.,* 1988; Safa *et al.,* 1990; Devine *et al.,* 1992). Kajiji *et al.* (1994) demonstrated that site-directed mutagenesis in TM11 of mouse P-gp alters the interaction of P-gp with structurally different P-gp chemosensitizing agents (Kajiji *et al.,* 1994). It is possible that the selection pressure of doxorubicin plus verapamil has selected for a mutation in P-gp, which in turn enhances doxorubicin resistance and reduces the effectiveness of verapamil. Although we found no difference in intracellular verapamil accumulation or verapamil binding to P-gp by indirect measurements using [^{3}H]azidopine binding in Dox_{40} and $MDR_{10}V$ cell lines, doxorubicin efflux from $MDR_{10}V$ cells compared with Dox_{40} cells was relatively unabated by the presence of verapamil or CsA. A mutation in the *MDR1*/P-gp gene which increases doxorubicin binding and efflux but reduces binding of these chemosensitizing agents might explain the observed phenotype of $MDR_{10}V$. Future mutational analysis of the *MDR1* gene is necessary to evaluate this possible mechanism.

DNA topoisomerase II is the intracellular target for a structurally diverse group of anti-cancer agents (anthracyclines, m-AMSA, ellipticines). MDR associated with alterations in DNA topoisomerase II is termed "at-MDR" (altered topoisomerase) (Danks *et al.,* 1988). Resistance to topoisomerase-directed agents may be mediated by quantitative or qualitative alterations in the cellular target. Alterations in topoisomerase II may occur by changes in content, activity, point mutation in the gene and/or depletion of nuclear matrix topoisomerase II (Fernandes *et al.,* 1993). Reduced expression of the topoisomerase II enzyme leads to decreased drug–target interaction and, thus, renders cells resistant to cytotoxic drugs (Beck, 1989). We found no differences in topoisomerase II content and activity between $MDR_{10}V$ and Dox_{40} cell lines. The increased resistance to MDR drugs and non-responsive-

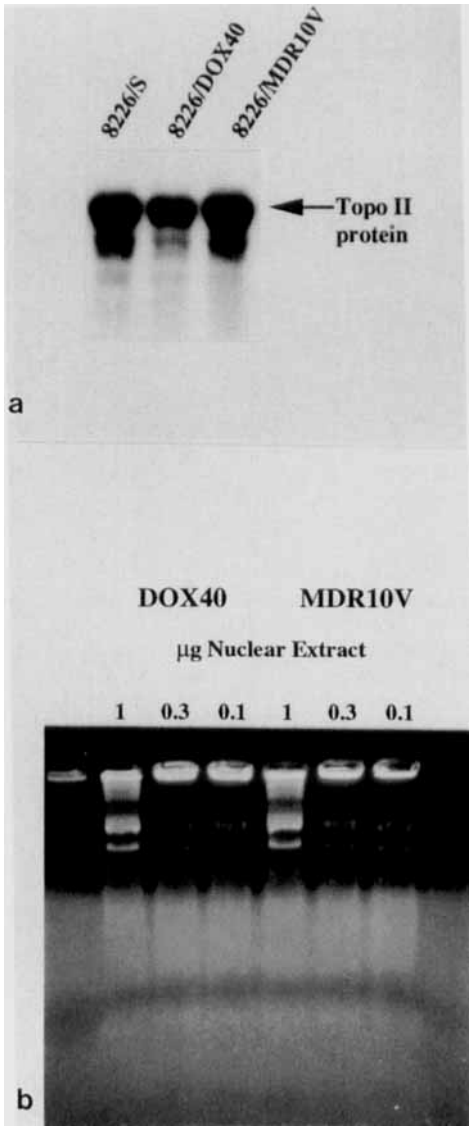

FIGURE 6 – Immunoblot analysis and catalytic activity of topoisomerase II. The activity of topo II was determined as the release of minicircle DNAs (the second band in each lane).

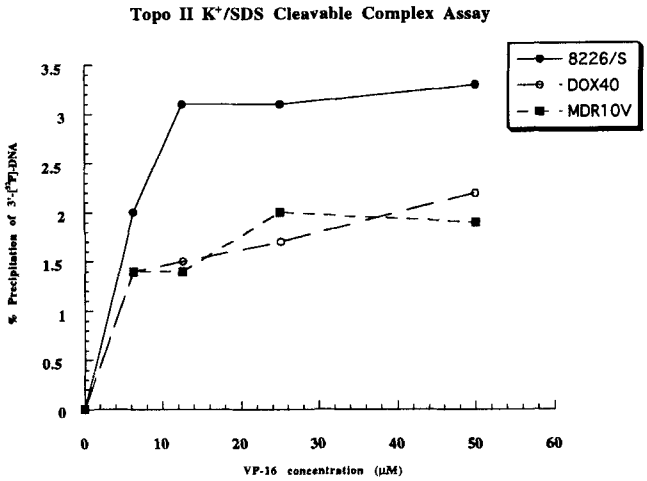

FIGURE 7 – Topoisomerase II activity measured by K^+/SDS precipitation assay.

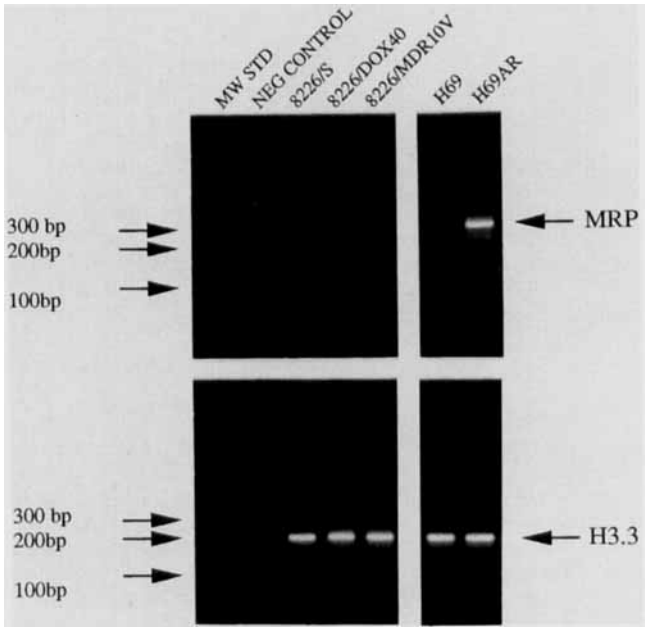

FIGURE 8 – Relative expressions of *MRP* mRNA (top) and *H3.3* mRNA (bottom). *MRP* expression was measured in 8226/S and its drug-resistant variants and compared with control cell lines H69 and H69AR.

ness to chemosensitizers observed for the $MDR_{10}V$ cell line cannot be explained by alterations in topoisomerase II activity.

MRP, a novel drug transporter, has been identified and demonstrated to cause MDR in drug-selected cell lines (Cole *et al.,* 1992; Grant *et al.,* 1994). Over-expression of MRP, a new member of the ATP-binding cassette transporter gene superfamily, has been shown to be sufficient to confer a non-P-gp-mediated drug resistance (Grant *et al.,* 1994; Deeley *et al.,* 1994). We examined $MDR_{10}V$ cells for the over-expression of MRP and compared it with Dox_{40} cells. MRP was not over-expressed in either cell line and, therefore, does not play a role in the MDR phenotype for these cell lines. It is still possible that another, as yet undiscovered, drug transport protein may be contributing to the increased resistance to MDR drugs and ability to resist chemosensitizers in the $MDR_{10}V$ cells.

The $MDR_{10}V$ cell line selected for resistance to both doxorubicin and verapamil is characterized by a high level of resistance to natural product agents and relative resistance to the chemosensitizing effects of verapamil and CsA. The resistance to chemosensitizers is due to the inability of the chemosensitizers to block the enhanced efflux of cytotoxic drugs. Future studies will compare the structure/function of P-gp in $MDR_{10}V$ cells compared with Dox_{40} cells. This cell line should prove to be a valuable model for studying the response of human tumor cells to a combination treatment of cytotoxic drugs and chemosensitizers and for determining the mechanism by which MDR-positive tumor cells become resistant to chemosensitizers.

ACKNOWLEDGEMENT

Supported in part by NCI grants 17094 and 43043 to W.D.

REFERENCES

ABBASZADEGAN, M.R., FUTSCHER, B.W., KLIMECKI, W.T., LIST, A. and DALTON, W.S., Analysis of multidrug resistance-associated protein (MRP) messenger RNA in normal and malignant hematopoietic cells. *Cancer Res.,* **54,** 4676–4679 (1994).

ALLEY, M.C., SCUDIERO, D.A., MONKS, A., HURSEY, M.L., CZERWINSKI, M.J., FINE, D.L.A., MAYO, J.G., SHOEMAKER, R.H. and BOYD, M.R., Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.,* **48,** 589– 601 (1988).

BECK, W.T., Unknotting the complexities of multidrug resistance: the involvement of DNA topoisomerases in drug action and resistance. *J. nat. Cancer Inst.,* **81,** 1683–1685 (1989).

BECK, W.T., Modulators of P-glycoprotein-associated multidrug resistance. *In:* R.F. Ozols (ed.), *Molecular and clinical advances in anticancer drug resistance,* pp. 151–170, Kluwer, Boston (1991).

BELLAMY, W.T., DALTON, W.S., KAILEY, J.M., GLEASON, M.C., MCCLOSKEY, T.M., DORR, R.T. and ALBERTS, D.S., Verapamil reversal of doxorubicin resistance in multidrug-resistant human myeloma cells and association with drug accumulation and DNA damage. *Cancer Res.,* **48,** 6365–6370 (1988).

CHOI, K.H., CHEN, C.J., KRIEGLER, M. and RONINSON, I.B., An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the *mdr1* (P-glycoprotein) gene. *Cell,* **53,** 519–529 (1988).

COLE, S.P.C., BHARDWAJ, G., GERLACH, J.H., MACKIE, J.E., GRANT, C.E., ALMQUIST, K.C., STEWART, A.J., KURZ, E.U., DUNCAN, A.M.V. and DEELEY, R.G., Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science,* **258,** 1650–1654 (1992).

DALTON, W.S. Overcoming the multidrug-resistant phenotype. *In:* V. DeVita, Jr., S. Hellman and S.A. Rosenberg (eds.), *Cancer: principles and practice of oncology,* pp. 2655–2666, J.B. Lippincott, Philadelphia (1993).

DALTON, W.S., DURIE, B.G.M., ALBERTS, D.S., GERLACH, J.H. and CRESS, A.E., Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. *Cancer Res.,* **46,** 5125–5130 (1986).

DALTON, W.S., GROGAN, T.M., MELTZER, P.S., SCHEPER, R.J., DURIE, B.G.M., TAYLOR, C.W., MILLER, T.P. and SALMON, S.E., Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J. clin. Oncol.,* **7,** 415–424 (1989).

DALTON, W.S. and SIKIC, B.I., The multidrug-resistance gene (*MDR1*) represents a potential target for reversing drug resistance in human malignancies. (Controversies in science: multidrug resistance). *J. NIH Res.,* **6,** 54–61 (1994).

DANKS, M.K., SCHMIDT, C.A., CIRTAIN, M.C., SUTTLE, D.P. and BECK, W.T., Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cells selected for resistance to VM-26. *Biochemistry,* **27,** 8861–8869 (1988).

DEELEY, R.G., GRANT, C.E., ALMQUIST, K.C., HIPFNER, D.R., VALDIMARSSON, G., LOE, D.W., GALLEN, D.F., KUSS, B. and COLE, S.P.C., Multidrug resistance mediated by the 190 kD MRP, a novel member of the ATP-binding cassette transporter superfamily. *Proc. amer. Assoc. Cancer Res.,* **35,** 698–699 (1994).

DEVINE, S.E., LING, V. and MELERA, P.W., Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance. *Proc. nat. Acad. Sci. (Wash.),* **89,** 4564–4568 (1992).

FERNANDES, D.J., CATAPANO, C.V. and TOWNSEND, A.J., Topoisomerase-related mechanisms of drug resistance. *In:* B.A. Teicher (ed.), *Drug resistance in oncology,* pp. 479–498, M. Decker, New York (1993).

FUTSCHER, B.W., ABBASZADEGAN, M.R., DOMANN, F. and DALTON, W.S., Analysis of MRP mRNA in mitoxantrone-selected, multidrug-resistant human tumor cells. *Biochem. Pharmacol.,* **47,** 1601–1606 (1994).

FUTSCHER, B.W., BLAKE, L.L., GERLACH, J.H., GROGAN, T.M. and DALTON, W.S., Quantitative polymerase chain reaction analysis of *mdr1* mRNA in multiple myeloma cell lines and clinical specimens. *Anal. Biochem.,* **213,** 414–421 (1993).

GRANT, C.E., VALDIMARSSON, G., HIPFNER, D.R., ALMQUIST, K.C., COLE, S.P.C. and DEELEY, R.G., Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res.,* **54,** 357–361 (1994).

GROS, P., DHIR, R., CROOP, J. and TALBOT, F., A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse mdr1 and mdr3 drug efflux pumps. *Proc. nat. Acad. Sci. (Wash.),* **88,** 7289–7293 (1991).

HAMADA, H., MIURA, K., ARIYOSHI, K., HEIKEY, SATA, S., KAMEYAMA, K., KUROSAWA, Y. and TSURUO, T., Mouse-human chimeric antibody against the multidrug transporter P-glycoprotein. *Cancer Res.,* **50,** 3167–3171 (1990).

KAJIJI, S., DRESLIN, J.A., GRIZZUTI, K. and GROS, P., Structurally distinct MDR modulators show specific patterns of reversal against P-glycoproteins bearing unique mutations at serine939/941. *Biochemistry,* **33,** 5041–5048 (1994).

LIST, A.F., SPIER, C., GREER, J., WOLFF, S., HUTTER, J., DORR, R., SALMON, S., FUTSCHER, B., BAIER, M. and DALTON, W., Phase I/II trial of cyclosporine as a chemotherapy-resisance modifier in acute leukemia [see comments]. *J. clin. Oncol.,* **11,** 1652–1660 (1993).

LIU, L.F., ROWE, T.C., YANG, L., TEWEY, K.M. and CHEN, G.L., Cleavage of DNA by mammalian DNA topoisomerase II. *J. biol. Chem.,* **258,** 15365–15370 (1983).

MARIE, J.P., BASTIE, J.N., COLOMA, F., FAUSSAT SUBERVILLE, A.M., DELMER, A., RIO, B., DELMAS-MARSALET, B., LEROUX, G., CASASSUS, P., BAUMELOU, E., CATALIN, J. and ZITTOUN, R., Cyclosporin A as a modifier agent in the salvage treatment of acute leukemia (AL). *Leukemia,* **7,** 821–824 (1993).

MILLER, T.P., GROGAN, T.M., DALTON, W.S., SPIER, C.M., SCHEPER, R.J. and SALMON, S.E., P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high-dose verapamil. *J. clin. Oncol.,* **9,** 17–24 (1991).

NELSON, E.M., TEWEY, K.M. and LIU, L.F., Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-m-anisidide. *Proc. nat. Acad. Sci. (Wash.),* **81,** 1361–1365 (1984).

RIORDAN, J.R. and LING, V., Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J. biol. Chem.,* **254,** 12701–12705 (1979).

SAFA, A.R., Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. *Proc. nat. Acad. Sci. (Wash.),* **85,** 7187–7191 (1988).

SAFA, A.R., GLOVER, C.J., MEYERS, M.B., BIEDLER, J.L. and FELSTED, R.L., Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. *J. biol. Chem.,* **261,** 6137–6140 (1986).

SAFA, A.R., GLOVER, C.J., SEWELL, J.L., MEYERS, M.B., BIEDLER, J.L. and FELSTED, R.L., Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J. biol. Chem.,* **262,** 7884–7888 (1987).

SAFA, A.R., STERN, R.K., CHOI, K., AGRESTI, M., TAMAI, I., MEHTA, N.D. and RONINSON, I.B., Molecular basis of preferential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185 → Val-185 substitution in P-glycoprotein. *Proc. nat. Acad. Sci. (Wash.),* **87,** 7225–7229 (1990).

SALMON, S.E., DALTON, W.S., GROGAN, T.M., PLEZIA, P., LEHNERT, M., ROE, D.J. and MILLER, T.P., Multidrug-resistant myeloma: laboratory and clinical effects of verapamil as a chemosensitizer. *Blood,* **78,** 44–50 (1991).

SIKIC, B.I., Modulation of multidrug resistance: at the threshold [editorial; comment]. *J. clin. Oncol.,* **11,** 1629–1635 (1993).

SONNEVELD, P., DURIE, B.G.M., LOKHORST, H.M., MARIE, J.P., SOLBU, G., SUCIU, S., ZITTOUN, R., LOWENBERG, B. and NOOTER, K., Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON [see comments]. *Lancet,* **340,** 255–259 (1992).

TSURUO, T., IIDA, H., TSUKAGOSHI, S. and SAKURAI, Y., Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.,* **41,** 1967–1972 (1981).

TSURUO, T., IIDA, H., TSUKAGOSHI, S. and SAKURAI, Y., Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. *Cancer Res.,* **42,** 4730–4733 (1982).

TSURUO, T., KAWABATA, H., IIDA, H., KITATANI, Y., TSUKAGOSHI, S. and SAKURAI, Y., Potentiation of antitumor agents by calcium channel blockers with special reference to cross-resistance patterns. *Cancer Chemother. Pharmacol.,* **15,** 16–19 (1985).

TWENTYMAN, P.R., FOX, N.E. and WHITE, D.J.G., Cyclosporin A and its analogues as modifiers of adriamycin and vincristine resistance in a multi-drug resistance human lung cancer cell line. *Brit. J. Cancer,* **56,** 55–57 (1987).

WELLS, D., HOFFMAN, D. and KEDES, L., Unusual structure, evolutionary conservation of non-coding sequences and numerous pseudogenes characterize the human H3.3 histone multigene family. *Nucleic Acids Res.,* **15,** 2871–2889 (1987).

WELLS, D. and KEDES, L., Structure of a human histone cDNA: evidence that basally expressed histone genes have intervening sequences and encode polyadenylylated mRNAs. *Proc. nat. Acad. Sci. (Wash.),* **82,** 2834–2838 (1985).

WOLVERTON, J.S., DANKS, M.K., SCHMIDT, C.A. and BECK, W.T., Genetic characterization of the multidrug-resistant phenotype of VM-26-resistant human leukemic cells. *Cancer Res.,* **49,** 2422–2426 (1989).