Advances in Brief

Overexpression of Multidrug Resistance-associated Protein (MRP) Increases Resistance to Natural Product Drugs[1]

Caroline E. Grant, Gunnar Valdimarsson, David R. Hipfner, Kurt C. Almquist, Susan P. C. Cole, and Roger G. Deeley[2]

Cancer Research Laboratories [C. E. G., G. V., D. R. H., K. C. A., S. P. C. C., R. G. D.] and Department of Pathology [D. R. H., K. C. A., S. P. C. C., R. G. D.], Queen's University, Kingston, Ontario, Canada K7L 3N6

Abstract

Amplification of the gene encoding multidrug resistance-associated protein (MRP) and overexpression of its cognate mRNA have been detected in multidrug-resistant cell lines derived from several different tumor types. To establish whether or not the increase in MRP is responsible for drug resistance in these cell lines, we have transfected HeLa cells with MRP expression vectors. The transfectants display an increase in resistance to doxorubicin that is proportional to the levels of a M_r 190,000, integral membrane protein recognized by anti-MRP antibodies. The transfectants are also resistant to vincristine and VP-16 but not to cisplatin. The results demonstrate that MRP overexpression confers a multidrug resistance phenotype similar to that formerly associated exclusively with elevated levels of P-glycoprotein.

Introduction

Multidrug resistance severely limits the effectiveness of chemotherapy of a variety of common malignancies. In experimental models, this type of resistance is characterized by reduced sensitivity to a spectrum of structurally diverse chemotherapeutic agents with multiple subcellular targets. These agents are predominantly natural products such as the anthracyclines, the *Vinca* alkaloids, the epipodophyllotoxins, and their semisynthetic congeners. Despite the structural diversity of these compounds, overexpression of a single gene has been demonstrated to be capable of conferring a multidrug resistance phenotype (1, 2). The gene responsible, designated *MDR1*, encodes a 1280-amino acid transmembrane phosphoglycoprotein, termed Mdr1 or P-glycoprotein. The human *MDR1* gene is closely linked on chromosome 7 at q21.1 to another gene in the P-glycoprotein family whose protein product displays 75% amino acid identity with Mdr1 (3). Despite this structural similarity, only Mdr1 has been shown to confer multidrug resistance (4, 5).

Overexpression of P-glycoprotein occurs in numerous *in vitro* drug-selected cell lines and some clinical situations. However, it has become evident that alternative mechanisms of multidrug resistance exist. Some malignancies, such as lung cancer, frequently display either acquired or intrinsic multidrug resistance without elevated levels of Mdr1 (6). A number of multidrug-resistant tumor cell lines, such as the human small cell lung cancer cell line H69AR, have also been described which do not overexpress Mdr1 (7–10). H69AR cells display a cross-resistance profile very similar to cells which overexpress the *MDR1* gene (7, 11) but contain no more Mdr1 than parental

NCI-H69 (H69) cells (7, 12). Furthermore, multidrug resistance in these cells is poorly reversed by chemosensitizers that are effective in cells overexpressing Mdr1 (13, 14).

In a search for proteins responsible for the multidrug resistance of H69AR cells, we isolated and sequenced cloned cDNAs[3] corresponding to a mRNA that is highly expressed in the resistant cells but not in drug-sensitive parental or revertant cells (15). The mRNA encodes a protein of 1531 amino acids that we have named MRP. The predicted primary sequence and secondary structure of MRP indicate that it is a member of the ATP-binding cassette superfamily of membrane transport proteins (16). This superfamily encompasses a broad range of functionally diverse transport proteins. These include the P-glycoproteins of plants and animals, the cystic fibrosis transmembrane conductance regulator, the MHC class II-linked peptide transporters, and a variety of peptide and small molecule transporters from lower eukaryotes and bacteria. MRP is very distantly related to all currently characterized members of the superfamily (15). For example, the amino acid identity of MRP with human Mdr1 and the cystic fibrosis transmembrane conductance regulator is only 14 and 19%, respectively, and is confined predominantly to the predicted ATP-binding domains of the three proteins.

Increased concentrations of MRP mRNA have been detected in multidrug-resistant cell lines derived from a variety of tissues (15, 17–19). Several of these cell lines have also been shown to contain multiple copies of the MRP gene as a result of amplification and translocation of a region of chromosome 16 spanning the MRP gene at band p13.1 (17). In H69AR cells, the MRP gene is amplified 40–50 fold. The involvement of this type of mechanism in overexpression of the MRP gene, combined with the lack of structural similarity between MRP and the P-glycoproteins, raises the possibility that the MRP gene may not confer multidrug resistance but may simply be coamplified with the gene that does. In view of the multistep selection procedures used to derive these cell lines, it also remains possible that overexpression of the MRP gene is only one component of a set of alterations required to confer multidrug resistance. We have examined these possibilities by constructing an MRP expression vector and assessing its ability to increase the relative drug resistance of transfected cell populations.

Materials and Methods

Vector Construction and Transfection. A DNA fragment containing the complete coding region of MRP mRNA was assembled in the vector pBluescript II KS[+] (Stratagene, La Jolla, CA) using overlapping cDNA clones or polymerase chain reaction products generated from these clones. The fidelity of the MRP sequence was confirmed by DNA sequence analysis before moving the intact MRP fragment to the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA). The integrity of the MRP fragment in the expression

vector was assessed by detailed restriction mapping and DNA sequence analysis of the cloning sites. In the pRc/CMV vector, MRP expression is under the control of the enhancer/promoter sequence from the immediate early gene of human cytomegalovirus. The MRP transcript also contains part of the 3' untranslated region and the polyadenylation signal from bovine growth hormone mRNA which is provided by the vector. Thus, the pRc/CMV-MRP construct generates a transcript of 5.2 to 5.3 kilobases that includes the entire coding sequence of MRP, approximately 260 nucleotides of 5' untranslated sequence (86 nucleotides of which are derived from MRP mRNA sequence) and approximately 250 nucleotides of 3' untranslated sequence (32 nucleotides of which are derived from MRP mRNA sequence).

HeLa cells were transfected with the pRc/CMV vector or the vector containing the MRP coding sequence using a standard calcium phosphate transfection procedure (20). Approximately 50,000 cells in each well of a 6-well tissue culture plate were exposed for 16 h to 10 μg of supercoiled DNA in a calcium phosphate precipitate. After 48 h, the growth medium was changed to include G418 at 200 μg/ml which selected for cells that expressed the neomycin resistance gene encoded by the pRc/CMV vector. Three weeks later, six independently transfected populations of cells were tested for resistance to doxorubicin using a tetrazolium salt microtiter plate assay (11). Those populations demonstrating increased relative resistance to the drug were expanded for testing for cross-resistance to other cytotoxic drugs and analysis of MRP mRNA and protein levels. At this time, the level of G418 in the growth medium was increased to 400 or 800 μg/ml without any noticeable effect on the growth rate of cells transfected with either the parental vector or the vector containing the MRP coding sequence. Transfected populations have been grown continuously for up to 4 months in G418-containing medium without any change in the level of resistance to doxorubicin.

RNA and Protein Blot Analyses. Poly(A)$^+$ RNA was isolated using the Micro-FastTrack RNA isolation kit (Invitrogen, San Diego, CA). The RNA was subjected to electrophoresis on a formaldehyde-agarose gel and transferred to Zetaprobe membrane (Bio-Rad, Hercules, CA). The blots were hybridized with ^{32}P-labeled cDNA fragment probes complementary to the mRNAs for MRP (15), MDR1 (21), topoisomerase II α, topoisomerase II β (22), annexin II (23), and a region of the pRc/CMV vector encoding part of the 3' untranslated region and polyadenylation signal from the bovine growth hormone gene. Hybridization of the probes was quantified by densitometry of the autoradiographs on a Molecular Dynamics Computing Densitometer (Sunnyvale, CA). Care was taken to compare autoradiographic exposures that were within the linear range of the film. In addition, variations in loading of RNA on the gels were estimated by probing blots with a ^{32}P-labeled GAPDH cDNA fragment (ATCC/NIH #57090) and by densitometric scanning of the ethidium bromide-stained ribosomal RNA bands on photographic negatives of the RNA gels.

The relative amounts of MRP protein were assessed by immunoblot analysis of total cell extracts and membrane-enriched fractions. Cell pellets were resuspended at 5 × 10^7 cells/ml in buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 1.5 mM MgCl$_2$, and protease inhibitors (2 mM phenylmethylsulfonylfluoride, 50 μg/ml antipain, 2 μg/ml aprotinin, 200 μg/ml EDTA, 200 μg/ml benzamidine, and 1 μg/ml pepstatin). After 10 min on ice, cells were homogenized with approximately 80 strokes of a Tenbroeck homogenizer. The homogenate was adjusted to 250 mM in sucrose before remaining, intact cells and nuclei were removed by centrifugation at 800 × g at 4°C for 20 min. To prepare a membrane-enriched fraction, the supernatant was centrifuged at 100,000 × g at 4°C for 20 min in a Beckman TL-100 ultracentrifuge, and the pellet was resuspended in 10 mM Tris-HCl (pH 7.4), 125 mM sucrose, and the protease inhibitors listed above. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, appropriate amounts of protein were mixed 1:1 with solubilizing buffer (final concentration of 4 M urea, 0.5% sodium dodecyl sulfate, and 50 mM dithiothreitol). Samples were loaded without heating onto a 7% resolving gel with a 4% stacking gel. Proteins were transferred to Immobilon-P PVDF membranes (Millipore) using 50 mM 3-(cyclohexylamino)-1-propanesulfonate (pH 11.0). For detection of MRP, blots were incubated with an affinity-purified, rabbit polyclonal antibody raised against a synthetic peptide, the sequence of which was predicted from that of the cloned MRP cDNA and which is not found in any other known protein (15). Antibody binding was visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminesence detection (Amersham, Arlington Heights, IL). The affinity-purified anti-MRP antibody recognizes a glycosylated, integral membrane protein with an apparent mo-

lecular weight of 190,000. In its deglycosylated form, the molecular weight of the protein decreases to 165,000 to 170,000 which is in agreement with the molecular weight of 171,000 predicted from the primary amino acid sequence of MRP (24).

Results and Discussion

A DNA fragment corresponding to the complete coding region of MRP mRNA plus 86 nucleotides of 5' and 32 nucleotides of 3' untranslated sequence was assembled and transferred into the expression vector pRc/CMV under the control of the human cytomegalovirus promoter. This vector also contains the bacterial aminoglycoside 3' phosphotransferase gene which confers resistance to geneticin (G418). HeLa cells were transfected with either the parental vector or the vector containing the MRP coding region. Integration of these vectors into genomic DNA has the potential to alter the expression of endogenous genes that might adventitiously increase drug resistance. Consequently, chemotherapeutic drugs were not used as selecting agents, and populations of transfected cells were selected solely by their ability to grow in the presence of G418. Since cells overexpressing MRP do not display increased resistance to this antibiotic, variable levels of expression of MRP were expected in the transfected cell populations.

The relative resistances to doxorubicin of two examples of G418-resistant cell populations transfected with the MRP expression vector (T2 and T5) as well as untransfected HeLa cells and a population transfected with the parental vector (C1) are shown in Fig. 1. One of the populations transfected with the MRP expression vector (T2) displayed little change in doxorubicin resistance whereas the resistance of the other (T5) was increased 15-fold. In addition, several clones from the resistant population were grown in the presence of G418, and their degree of doxorubicin resistance was determined. A dose-response curve for one of the clones (T5–5) which displayed a 5-fold increase in resistance is also shown in Fig. 1. The two populations of transfectants (T2; T5) and the resistant clone (T5–5) were then compared to determine whether their relative resistance to doxorubicin correlated with the concentrations of MRP mRNA.

The MRP mRNA produced from the expression vector has a predicted length of 5.2 to 5.3 kilobases allowing it to be distinguished from the 6.5–7-kilobase, endogenous MRP mRNA by Northern analysis. A blot of poly(A)$^+$ RNA from the cell populations shown in Fig. 1 that was hybridized with a cloned cDNA probe corresponding to part
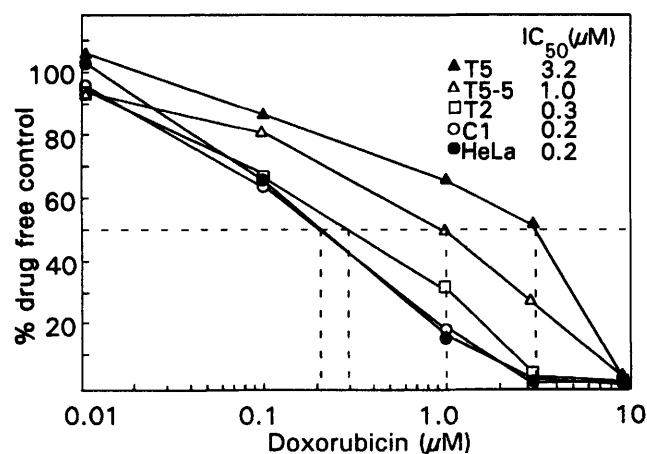

Fig. 1. Cytotoxicity of doxorubicin on MRP-transfected HeLa cells. ●, HeLa cells; ○, HeLa cells transfected with the expression vector pRc/CMV (*C1*); □ (*T2*) and ▲ (*T5*), HeLa cells transfected with the vector containing the MRP coding sequence; △ (*T5–5*), a clone isolated from the doxorubicin-resistant transfected T5 cells. *Points*, mean of triplicate determinations in a single experiment; SDs were <5%. Similar results were obtained in three additional experiments. The IC$_{50}$ is indicated on the figure and is defined as the concentration of doxorubicin required to decrease by 50% the values obtained with untreated cells.

of the MRP coding sequence revealed a relatively abundant mRNA of approximately 5.3 kilobases in the resistant transfectants and low levels of the longer endogenous MRP mRNA (Fig. 2A). The relative concentration of the 5.3-kilobase mRNA is 70- to 80-fold and 20- to 30-fold higher in the resistant cell population (T5) and clone (T5–5), respectively, than that of endogenous MRP mRNA present in the control population (C1). Expression of the 5.3-kilobase MRP mRNA
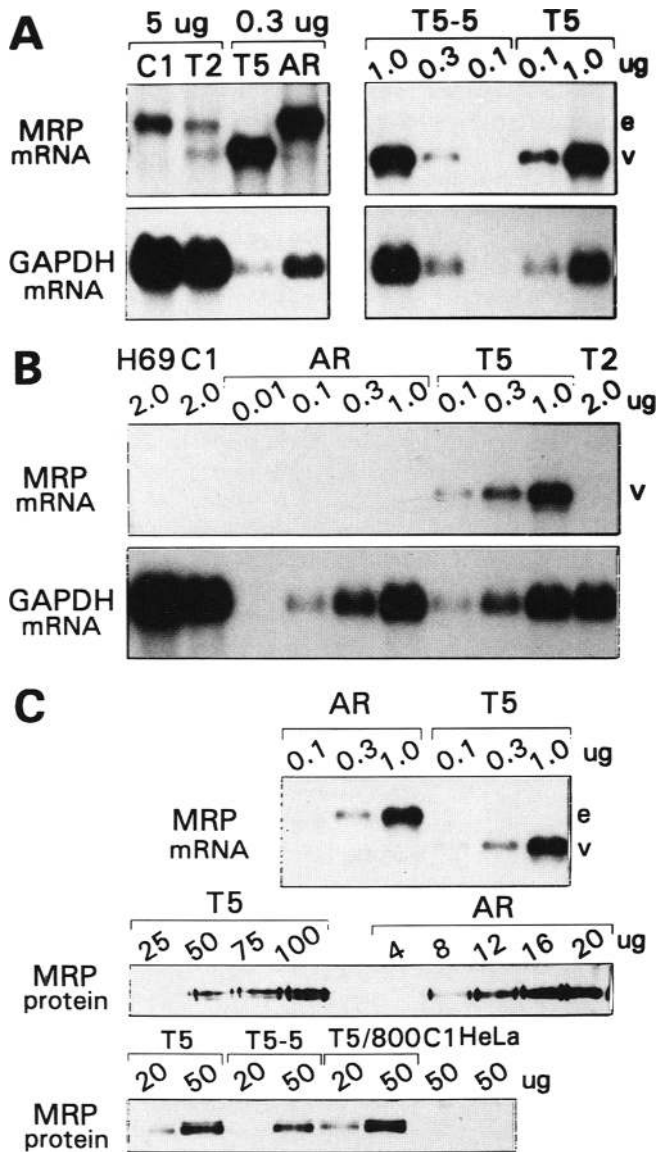

A

B

C

Fig. 2. Levels of MRP mRNA and protein in transfected cell lines. Northern blots and immunoblots were used to determine the relative abundance of MRP mRNA and protein in the H69 cell line and its multidrug-resistant derivative, H69AR (AR), as well as untransfected HeLa cells, HeLa cells transfected with the expression vector pRc/CMV (C1), HeLa cells transfected with the same vector containing the MRP coding sequence (pRc/CMV-MRP; T2 and T5), T5 cells maintained in selection media at 800 μg/ml G418 (T5/800), and a clone of the T5 cells (T5–5). To assess the relative abundance of the two mRNAs in different cell populations, various amounts of RNA were loaded on the gels. The relative levels of MRP mRNA stated in the text were determined by densitometry and normalization to the levels of GAPDH mRNA. A. Northern blots of poly(A)+ RNA were probed with a 4-kilobase MRP cDNA fragment which hybridizes with endogenous (e) MRP mRNA and an mRNA of the size (5.2 to 5.3 kilobases) expected for the MRP transcript encoded by the expression vector (v) pRc/CMV-MRP. B. The identity of the vector-encoded MRP transcript (v) was confirmed by hybridizing a Northern blot with a DNA fragment from the pRc/CMV vector that encodes the segment of the 3' untranslated region of the MRP transcript that is derived from bovine growth hormone mRNA (v). Low levels of the transcript were also detected in T2 cells after prolonged exposure of the film. C. Levels of the endogenous (e) MRP mRNA and protein in the H69AR (AR) cell line relative to the levels of the vector (v)-encoded MRP mRNA and protein in the doxorubicin-resistant transfected HeLa cell population (T5) were estimated by densitometry of Northern blots and immunoblots.
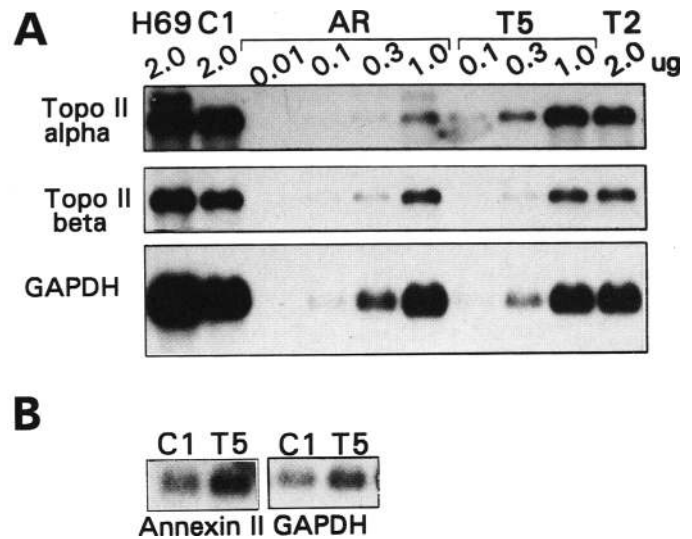
A

B

Fig. 3. Northern blot analyses of topoisomerase II and annexin II mRNA levels in MRP transfectants. A. A Northern blot of poly(A)+ RNA isolated from H69 cells, H69AR cells (AR), HeLa cells transfected with the vector pRc/CMV (C1), and HeLa cells transfected with the vector pRc/CMV-MRP (T2; T5) was hybridized with cDNA probes for topoisomerase II (Topo II) α and topoisomerase II β mRNAs. No significant differences in levels of either topoisomerase II isoform were detected in the HeLa cell transfectants (C1; T2; T5) after normalization to the levels of GAPDH mRNA. Decreases in the relative levels of topoisomerase II α and β in H69AR cells were consistent with those reported elsewhere. B. A Northern blot of approximately 2 μg poly(A)+ RNA from C1 and T5 cells was hybridized with a cDNA probes for annexin II mRNAs. Variation in the RNA loading was assessed by probing the blot with a cDNA probe for GAPDH mRNA. Following normalization to the levels of GAPDH mRNA, no significant change in levels of annexin II mRNA was detected in the HeLa cell transfectants (C1; T5).

in the transfected cell population which showed little change in resistance (T2) was only approximately one-half that of endogenous MRP mRNA. Similar RNA blots were also probed with a DNA fragment from the pRc/CMV plasmid that forms part of the 3' untranslated region of the vector-encoded MRP mRNA. This probe hybridized only with the 5.3-kilobase MRP mRNA, confirming that it was transcribed from the vector and did not result from the increased expression of an endogenous MRP-related gene (Fig. 2B). Thus in cells transfected with the MRP expression vector, the relative level of drug resistance increases with the concentration of MRP mRNA.

The concentration of endogenous MRP mRNA in the multidrug-resistant H69AR cells is approximately 100-fold higher than in the H69 parental cells (15) and the relative resistances of the two cell lines to doxorubicin also differ by 50- to 100-fold (7, 11). Vector-encoded MRP mRNA levels in the T5 HeLa cell population are 70- to 80-fold higher than endogenous MRP mRNA levels in the parental cells. However, drug resistance is increased only 15-fold. To investigate why the relative increase in drug resistance was lower in the transfectants than in H69AR cells, we compared the levels of MRP mRNA and protein in the two different cell types. Northern analysis revealed that the levels of endogenous MRP mRNA in H69 cells and HeLa cells transfected with the pRc/CMV parental vector were very similar (data not shown). The relative abundance of vector-encoded MRP mRNA in the drug-resistant transfectant cell population (T5) was also comparable to that of endogenous MRP mRNA in H69AR cells (Fig. 2C). However, a protein blot probed with affinity-purified anti-MRP antibody indicated that the level of protein in the T5 HeLa cell transfectants was 5- to 8-fold lower than in H69AR cells (Fig. 2C). These findings are consistent with the 15-fold increase in resistance observed in the transfected T5 cells compared to the 50- to 100-fold resistance in H69AR cells. The lower level of protein in the transfected cells is most likely attributable to a difference in translational efficiency between the vector encoded and endogenous MRP mRNAs, although a
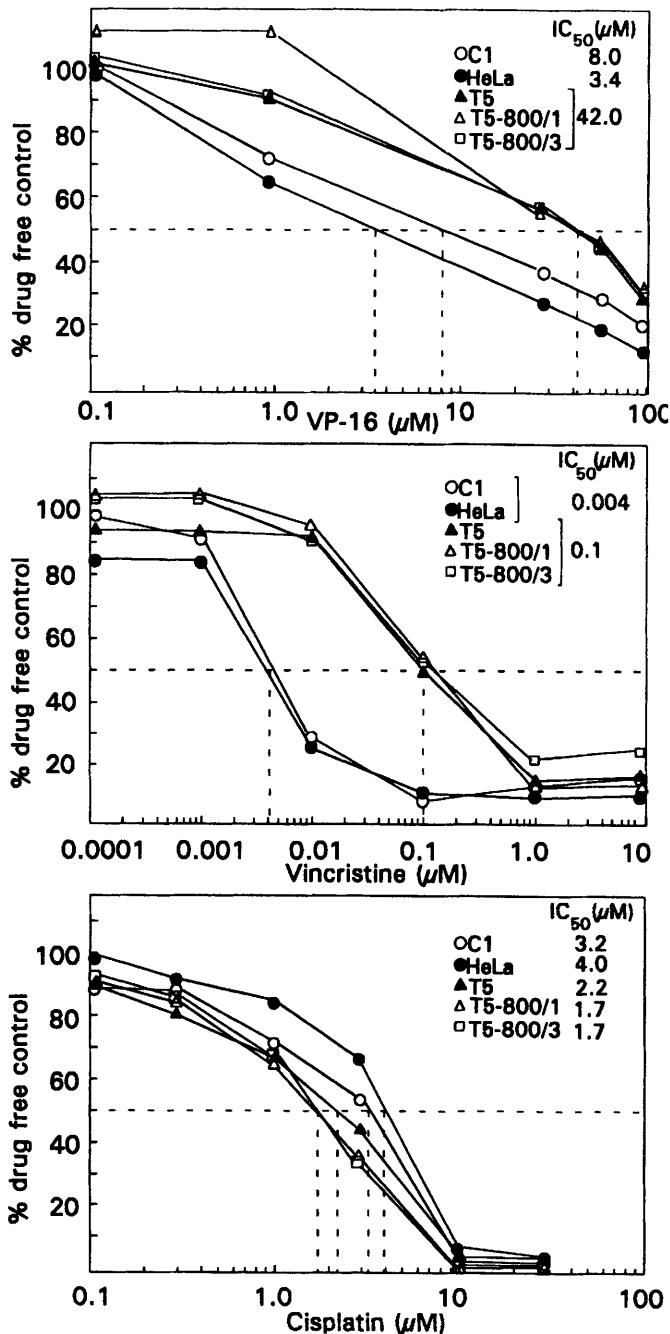
Fig. 4. Cross-resistance of doxorubicin-resistant MRP-transfected cell populations to the cytotoxic drugs VP-16, vincristine, and cisplatin. Cytotoxicity assays were performed on: ●, untransfected HeLa cells; ○, HeLa cells transfected with the expression vector pRc/CMV (C1); ▲, HeLa cells transfected with the expression vector pRc/CMV-MRP and maintained in G418 at 400 μg/ml for 4 months (T5); and T5 cells maintained at 800 μg/ml G418 for 1 month (T5–800/1; △) and 3 months (T5–800/3; □). Points, mean of triplicate determinations in a single experiment; SDs were <5%. Similar results with vincristine and VP-16 were obtained in two to three additional experiments. The IC_{50}s of the various cell lines are indicated on the figure and is defined as the concentration of doxorubicin required to decrease by 50% the values obtained with untreated cells.

difference in rates of degradation of the protein between the two cell types cannot be excluded.

Since H69AR cells were obtained by multistep selection, it is possible that additional alterations have occurred which may, either independently or in concert with MRP, influence their degree of resistance to some drugs. H69AR cells have been shown to have decreased levels of topoisomerase II α and β mRNA and protein which could enhance their resistance to anthracyclines and epipodo-

phyllotoxins (12).[4] They have also been shown to overexpress annexin II which may affect the trafficking of membrane proteins (23). Annexin II has been shown to be involved in formation of fusogenic vesicles and in exocytosis (25). It is unknown to what extent these additional changes influence the degree of resistance of H69AR cells or whether they are linked in any way to overexpression of MRP. However, overexpression of MRP in the transfected cells does not alter the levels of mRNAs specifying either topoisomerase II isoform (Fig. 3A) or annexin II (Fig. 3B), nor do the transfected HeLa cells display any alterations in the level of Mdr1 mRNA (data not shown). These observations strongly support the conclusion that increased resistance to doxorubicin in the transfected cells is directly attributable to overexpression of MRP.

To determine whether the increased doxorubicin resistance of transfected cells was accompanied by increased resistance to other classes of chemotherapeutic drugs, the cells were tested for cross-resistance to vincristine (a Vinca alkaloid), VP-16 (an epipodophyllotoxin), and cisplatin (Fig. 4). Dose-response curves for several independently propagated cultures of MRP transfectants indicate that they are approximately 25-fold and 5- to 10-fold resistant to vincristine and VP-16, respectively, relative to untransfected HeLa cells or cells transfected with the parental vector (C1). The transfectants showed no increase in cisplatin resistance which is consistent with the pharmacological phenotype of H69AR cells (7) and which is also characteristic of cells that overexpress P-glycoprotein. These results demonstrate for the first time that this phenotype can be conferred by a member of the ATP-binding cassette superfamily of transporters that is structurally very different from the P-glycoproteins.

It is presently not known whether the mechanisms by which the P-glycoproteins and MRP confer resistance are the same. P-glycoprotein is located predominantly in the plasma membrane and is believed to confer resistance primarily by acting as an ATP-dependent drug efflux pump (26, 27). Recent studies on a multidrug-resistant leukemia cell line that overexpresses MRP suggest that a substantial fraction of the protein may be present in the endoplasmic reticulum as well as in the plasma membrane (19). Cell type-specific variations in MRP distribution between these two membrane compartments may explain why decreases in accumulation, as well as marked changes in intracellular drug distribution, have been observed in some cell lines which overexpress MRP (24, 28, 29).

Acknowledgments

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