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The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2)

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SUMMARY

Mechanisms of drug resistance other than P-glycoprotein are of increasing interest as the list of newly identified members of the ABC transport family has grown. We sought to characterize the phenotype of the newly discovered ABC transporter encoded by the mitoxantrone resistance gene, *MXR*, also known as *ABCP1* or *BCRP*. The pharmacodynamics of mitoxantrone and 12 other fluorescent drugs were evaluated by confocal microscopy in four multidrug-resistant human colon (S1) and breast (MCF-7) cancer cell lines. We utilized two sublines, MCF-7 AdVp3000 and S1-M1-80, and detected overexpression of MXR by PCR, immunoblot assay and immunohistochemistry. These *MXR* overexpressing sublines were compared to cell lines with P-glycoprotein- and MRP-mediated resistance. High levels of cross-resistance were observed for mitoxantrone, the anthracyclines, bisantrene and topotecan. Reduced levels of mitoxantrone, daunorubicin, bisantrene, topotecan, rhodamine 123 and prazosin were observed in the two sublines with high *MXR* expression. Neither the P-glycoprotein substrates vinblastine, paclitaxel, verapamil and calcein-AM, nor the MRP substrate calcein, were extruded from MCF-7 AdVp3000 and S1-M1-80 cells. Thus, the multidrug-resistant phenotype due to *MXR* expression is overlapping with, but distinct from, that due to P-glycoprotein. Further, cells that overexpress the MXR protein seem to be more resistant to mitoxantrone and topotecan than cells with P-glycoprotein-mediated multidrug resistance. Our studies suggest that the ABC half-transporter, MXR, is a potent, new mechanism for conferring multiple drug resistance. Definition of its mechanism of transport and its role in clinical oncology is required.

Key words: Atypical multidrug resistance, Mitoxantrone, MXR, ABC half-transporter, Confocal microscopy, Cancer

INTRODUCTION

In cancer treatment, a major problem to be overcome is the resistance of tumor cells to anticancer drugs. An intensively studied type of cellular drug resistance is the multidrug resistance phenotype, which is characterized by a reduced intracellular drug level and overexpression of individual members of the ABC (ATP binding cassette) superfamily of membrane transporters (Allikmets et al., 1996; Dean and Allikmets, 1995), including the well-characterized members P-glycoprotein (P-gp), encoded by the *MDR1* gene (Gottesman and Pastan, 1993) and MRP (multidrug resistance associated protein) (Müller et al., 1994). However, several atypical multidrug-resistant tumor cell lines have been described that lack overexpression of P-gp and MRP, but nevertheless have reduced intracellular drug levels, suggesting the presence of other drug transport mechanisms (Dietel et al., 1990; Mülder et al., 1993, 1994; Scheffer et al., 1995; Chen et al., 1990; Lee et al., 1997). Several of these atypical multidrug-resistant sublines were selected in mitoxantrone, including the gastric carcinoma cells EPG85-257RN (Dietel et al., 1990; Kellner et al., 1997), the human HL-60 leukemia cell line HL-60/MX2 (Harker et al., 1989), various sublines of the human breast carcinoma cell line MCF-7 (Taylor et al., 1991; Nakagawa et al., 1992; Dexter et al., 1998), and the colon carcinoma cell lines WiDr/R (Dalton et al., 1988; Fox and Smith, 1995), and S1-M1-80 (Rabindran et al., 1995). Our laboratory recently identified a mitoxantrone resistance-associated gene, *MXR* (GenBank accession numbers AF093771 and AF093772), in a human colon cancer subline, S1-M1-80 (Miyake et al., 1999). This gene encodes a predicted 655-amino-acid protein with six transmembrane domains; a proposed model of MXR is shown in Fig. 1A. The highest primary structure similarity was found to the half-transporter members of the ABC superfamily, including the *Drosophila white* gene (O'Hare et al., 1984); a phylogenetic tree constructed with the distance matrix using the neighbor-joining method (Saitou and Nei, 1987) illustrates the close relationship to the *white* family of ABC half-transporters, as well as to other ABC proteins, some of which are involved in drug resistance (Fig. 1B). Localized to

Table 1. The panel of six cell lines described in the study

	Cell line					
	S1 wt	S1-B1-20	S1-M1-80	MCF-7 wt	MCF-7 VP-16	MCF-7 AdVp3000
Selecting agent (concentration in culture)		Bisantrene (20 μM)	Mitoxantrone (80 μM)		VP-16 (4 μM)	Adriamycin (5.5 μM) Verapamil (5.5 μM)
RR to selecting agent	1	>400*	21,600	1	25	164
Resistance mechanism		MDR	MXR		MRP	MXR

RR, relative resistance compared to wild type (wt).
*More than 400-fold resistant to bisantrene as compared with the wild type. IC_{50} for S1-B1-20 was not reached even at 100 μM bisantrene. It was not possible to achieve higher bisantrene concentrations in the medium due to limited solubility of the drug.

chromosome 4, *MXR* is amplified in the MCF-7 AdVp3000 cells, and overexpressed in S1-M1-80 cells. *MXR* is identical to the *ABCP1* (Allikmets et al., 1998) and *BCRP* (Doyle et al., 1998) genes.

We have examined the phenotype of two *MXR*-overexpressing cell lines by confocal microscopy, with the aim of relating intracellular drug accumulation patterns to the expression of specific transport proteins. The two sublines, MCF-7 AdVp3000 and S1-M1-80, which do not overexpress *MDR1* or *MRP*, demonstrated a parallel phenotype with similar patterns of cross-resistance, matching drug distribution profiles and energy-dependent mitoxantrone efflux. This mechanism of resistance, while most prominent for mitoxantrone, extends to numerous other anticancer agents and appears to be as potent as P-gp in conferring multidrug resistance.

MATERIALS AND METHODS

Tissue culture

All cell lines were cultured in IMEM supplemented with glutamine (2 mM), fetal calf serum (10%), penicillin (100 i.u./ml) and streptomycin (100 μg/ml). Drug-resistant cells were maintained in the presence of the selecting drugs (Table 1). The S1-B1-20 and S1-M1-3.2 (established by Greenberger and colleagues; Rabindran et al., 1995) lines were derived from the S1 clone of LS-180 colon carcinoma cells. S1-M1-80 cells were advanced to higher levels of resistance by exposure to increasing concentrations of mitoxantrone. The cells were continuously maintained in the selecting agent, which was 20 μM bisantrene for S1-B1-20 and 80 μM mitoxantrone for S1-M1-80. The MCF-7 resistant sublines were derived by stepwise selection from parental MCF-7 breast carcinoma cells. The MCF-7 VP-16 cells, a gift from Dr Ken Cowan, were maintained in 4 μM VP-16. The MCF-7 AdVp3000 cells were independently selected from MCF-7 parental cells and were cultured in 5.5 μM adriamycin plus 5.5 μM verapamil (Lee et al., 1997). For the transfection experiment, MCF-7 cells were transfected with full-length MXR cDNA by the calcium phosphate precipitation method, and subsequently selected in G418 (1 mg/ml)-containing medium, whereas control MCF-7 cells were transfected with the empty pcDNA3 vector before selection in G418 (Doyle et al., 1998).

Cytotoxicity assay

The IC_{50} values (the drug concentration at which 50% of colony formation was inhibited) were determined in 96-well plates using the colorimetric assay described by Skehan et al. (1990). Plating concentrations varied from 2,000 cells per well (S1) to 10,000 cells per well (MCF-7 AdVp3000), due to differences in doubling time for the different cell lines. Cells were exposed to increasing concentrations of drug for 4 days, then fixed in 10% TCA (by addition of 50 μl 50% TCA to an incubation volume of 200 μl), washed and air-dried. After staining with 0.4% (w/v) sulforhodamine B in 1% (v/v) acetic acid, and washing four times in 1% (v/v) acetic acid, bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5). The number of cells per well was determined by measuring the absorbance at 540 nm in a microplate reader (Bio-Rad model 450, Bio-Rad, CA, USA).

Mitoxantrone uptake

Uptake of [^{3}H]mitoxantrone by whole cells was assayed as described by Fojo et al. (1985). The incubation medium (low glucose MEM, no bicarbonate or serum, buffered with 10 mM Hepes to pH 7.3) contained 20 nM [^{3}H]mitoxantrone. The cells were incubated in 6-well cell culture plates for 60 minutes at 37°C, washed twice with ice-cold phosphate-buffered saline, and subsequently trypsinized. The cell count per well was determined in a Coulter Counter (Coulter Electronics, Hialeah, FL, USA). Accumulated [^{3}H]mitoxantrone was determined by liquid scintillation counting (Packard 2000CA TRI-CARB Scintillation Analyzer, Packard, IL, USA).

mRNA extraction, northern blots and PCR

RNA was extracted by the RNASTAT-60^{TM} method, according to the manufacturers' directions (TelTest Inc., Friendswood, TX, USA). Northern blotting was performed as previously described (Miyake et al., 1999). Hybridization was performed by standard procedures. For the quantitative PCR analysis, which has been described previously by Zhan et al. (1997), the following primers were used: MDR1: 5′-end primer: ^{529}5′-GCC TGG CAG CTG GAA GAC AAA TAC ACA AAA TT ^{560}, 3′-end primer: ^{814}5′-CAG ACA GCA GCT GAC AGT CCA AGA ACA GG ACT ^{783}. MRP: 5′-end primer: ^{792}5′-CGG AAA CCA TCC ACG ACC CTA ATC C ^{816}, 3′-end primer: ^{1086}5′-ACC TCC TCA TTC GCA TCC ACC TTG G ^{1062}. MRP2: 5′-end primer: ^{4072}5′-CTG CCT CTT CAG AAT CTT AG ^{4091}, 3′-end primer: ^{4312}5′-CCC AAG TTG CAG GCT GGC C ^{4294}. MXR: 5′-end primer: ^{1979}5′-TGC CCA GGA CTC AAT GCA ACA G ^{2000}, 3′-end primer: ^{2540}5′-GAC TGA AGG GCT ACT AAC C ^{2522}.

The expression of MDR1, MRP, MRP2 and MXR is provided in units based on densitometric measurements of the ethidium-stained PCR gels and is normalized to the expression level in wild-type (wt) cells, which is arbitrarily set at 10 units. The MXR PCR product for MCF-7 wt was readily detectable from 61.25 ng RNA amplified 30 cycles.

Immunoblot analysis of proteins

Microsomal membrane fractions (50 μg of protein, without boiling) were loaded onto an 8% (w/v) SDS-polyacrylamide gel and subjected to electrophoresis (Laemmli, 1970). After electrotransfer onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) the blots were blocked with Tris-buffered saline containing 0.05% (v/v) Tween-20 and 5% (w/v) non-fat dry milk. Probing was performed overnight at 4°C with the polyclonal anti-MXR antibody 87405 (dilution 1:2000), which we have raised against an 18-mer peptide of the ATP-binding region of MXR (manuscript in preparation). A secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Affinity Bioreagents, Golden, CO, USA) was applied for 1 hour (dilution 1:1000), followed by enhanced chemiluminescence

Table 2. Cross resistance patterns of various cell lines

	Cell line									
	S1 wt	S1-B1-20		S1-M1-80		MCF-7 wt	MCF-7 VP16		MCF-7 AdVp3000	
Drug	IC_{50}	IC_{50}	RR	IC_{50}	RR	IC_{50}	IC_{50}	RR	IC_{50}	RR
Mitoxantrone	0.006±0.002 (6)	0.667±0.178 (6)	111	222±45 (5)	35,106	0.028±0.007 (7)	0.044±0.009 (4)	1.5	322±36 (6)	11,326
Daunorubicin	0.156±0.056 (5)	6.00±2.48 (4)	38	3.48±0.95 (5)	22	0.070±0.027 (3)	0.163±0.037 (3)	2.3	3.67±0.67 (3)	52
Bisantrene	0.260±0.051 (5)	>100 (5)	>350	28.3±12.6 (4)	109	0.278±0.062 (5)	0.232±0.06 (6)	0.8	>100 (4)	>350
Vinblastine	0.0010±0.0004 (4)	0.163±0.080 (4)	167	0.0004±0.0001 (4)	0.4	0.002±0.001 (5)	0.004±0.001 (6)	1.6	0.029±0.017 (5)	13
Paclitaxel	0.0017±0.0003 (4)	0.460±0.060 (5)	285	0.0028±0.0005 (4)	1.6	0.0025±0.0003 (3)	0.003±0.001 (3)	1.2	0.118±0.022 (3)	47
Topotecan	0.0047±0.001 (5)	0.016±0.003 (4)	3.4	3.20±1.69 (3)	680	0.045±0.014 (5)	0.052±0.018 (2)	1.2	52.4±16.9 (4)	1164

IC_{50}, 50% inhibitory concentration (μm) as determined by the sulforhodamine B assay. Values are means ± s.e.m. (number of experiments are given in parentheses).

RR, relative resistance, i.e. the ratio of IC_{50} for the drug-resistant cell line to that of the drug-sensitive cell line (MCF-7 wt or S1 wt).

For drug concentrations, see Materials and Methods and Table 1.

detection (NEN, Boston, MA, USA) with exposure on Kodak X-OMAT AR autoradiography film to visualize immunoreactive bands.

Confocal microscopy

The cells (10^5/ml) were grown in phenol red-free IMEM for 48 hours in 35 mm glass bottom (poly-d-lysine coated) microwells (MatTek Corporation, Ashland, MA, USA) before analysis. A Zeiss LSM 410 confocal laser scanning microscope equipped with a 150 mW Omnichrome Ar-Kr laser exciting at 488 nm (all dyes except mitoxantrone) and 568 nm (mitoxantrone) was used. In our confocal microscopy of mitoxantrone we experimented with both a 568 nm and a 647 nm excitation wavelength. We chose to use the more powerful 568 nm line, as this yielded the highest signal-to-noise ratio (data not shown). Emitted light passed through a 515-540 nm band-pass filter (all dyes except mitoxantrone and daunorubicin) or a 590 nm long-pass filter (mitoxantrone, daunorubicin). Images were stored online to a Panasonic optical disk drive and analyzed off-line in Paint Shop Pro v.4.14 (Jasc Inc. Eden Prairie, MN, USA).

Immunohistochemistry

Formalin-fixed paraffin embedded tissue blocks were cut into 5 μm sections. The sections were baked at 60°C, deparaffinized in xylene and ethanol, microwaved for 5 minutes until boiling in Citra Plus (BioGenex), and then microwaved for another 15 minutes at low power. Sections were quenched in 3% H_2O_2, and subsequently incubated in 5% goat serum for 20 minutes. Anti-MXR polyclonal antibody (87405, dilution 1:2500) was applied to the sections, and the slides incubated for 2 hours at room temperature. A biotinylated secondary antibody was next applied and slides were incubated for 30 minutes at room temperature. Finally, the antibody binding was detected with DAB.

Chemicals

BODIPY-vinblastine, BODIPY-verapamil, BODIPY FL-paclitaxel, BODIPY FL C5-ceramide, BODIPY FL-Brefeldin A, BODIPY-prazosin, and LysoTracker Green were obtained from Molecular Probes (Eugene, OR, USA). Mitoxantrone, topotecan, VP-16 and bisantrene were provided by the NCI drug screen. [3H]mitoxantrone was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). All other drugs were purchased from Sigma (St Louis, MO, USA).

RESULTS

Cross-resistance patterns

Table 2 shows the cytotoxicity profiles of the seven cell lines towards a panel of chemotherapeutic drugs. All drug-selected cell lines displayed resistance to mitoxantrone, the S1-M1-80 and MCF-7 AdVp3000 cells having the highest IC_{50} values (approximately 200-300 μM), corresponding to 35,100- and 11,300-fold relative resistance. The mitoxantrone resistance in MCF-7 AdVp3000 cells confirmed a previous report (Lee et al., 1997), but the level of resistance was about tenfold higher in the older study. Whether this is due to differences in plating density or to changes in the composition or purity of the mitoxantrone is unknown. The MCF-7 AdVp3000 and S1-M1-80 cells had very similar cross-resistance patterns, with high resistance towards mitoxantrone, daunorubicin, bisantrene and topotecan, while retaining sensitivity towards vinblastine and paclitaxel. Some paclitaxel and vinblastine resistance was observed with the MCF-7 AdVp3000 cell line, possibly due to overexpression of MRP2 or other drug resistance mechanisms. The P-gp overexpressing S1-B1-20 cell line showed cross resistance to all drugs tested, although its IC_{50} value for mitoxantrone was markedly lower (0.67 μM) than that of the corresponding S1-M1-80 line (222 μM). The MCF-7 VP-16 cell line, which overexpresses MRP, was twofold resistant to daunorubicin and slightly (1.6-fold) to vinblastine, but not to mitoxantrone, bisantrene, paclitaxel or topotecan. These data underline the parallel cross-resistance profiles of the S1-M1-80 and MCF-7 AdVp3000 sublines, and the overlap with the profile of P-gp mediated drug resistance.

Expression assayed by quantitative PCR

The expression levels of *MDR1*, *MRP*, *MRP2* and the mitoxantrone resistance gene, *MXR*, were assayed by quantitative PCR. As Table 3 shows, only the atypical cell lines, S1-M1-80 and MCF-7 AdVp3000, showed high expression of *MXR*, which is consistent with this protein being identified as the putative drug transporter in these cells. The absolute level of expression of *MXR* appears to be higher in the AdVp3000 cells (2361 units versus 1703) than in S1-M1-80 cells, a finding that is confirmed in the immunoblot results shown below. A tenfold increase in *MRP2* expression is noted in both the MCF-7 VP-16 and AdVp3000 sublines.

Northern blot, immunoblot and immunohistochemistry analysis

The expression of *MXR* was ascertained both at the gene level by northern blot (Fig. 2A), and at the protein level by immunoblotting with the MXR-specific polyclonal antibody 87405 (Fig. 2B) and by immunohistochemistry (Fig. 2C). High expression of MXR mRNA was observed only in the S1-M1-

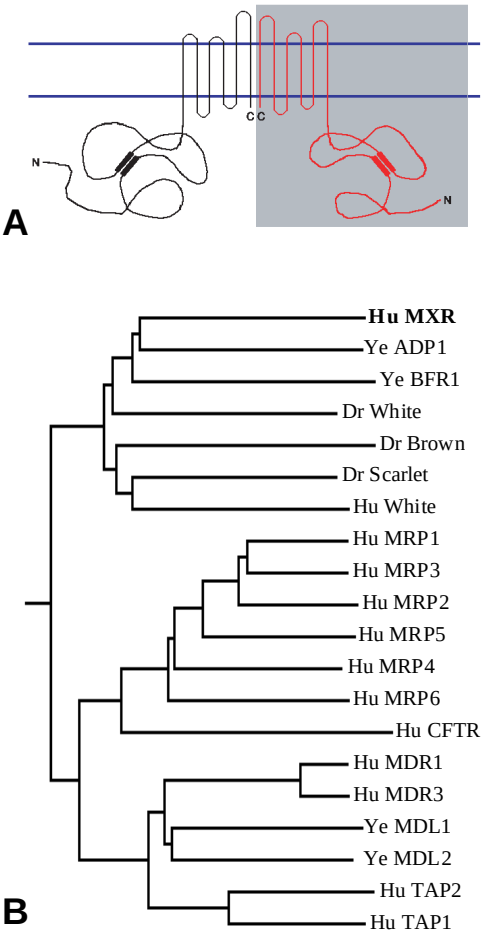


Fig. 1. (A) Schematic model of the predicted structure of MXR. The six transmembrane helixes and the ATP-binding fold (filled bars) are indicated. A putative dimerization partner for MXR is indicated in the gray box. (B) Rooted phylogenetic tree illustrating the primary structure similarity between MXR (also known as BCRP and ABCP1) and several members of the *white* gene branch of the ABC superfamily. The tree was constructed from a multiple alignment of the Walker A to Walker B regions (approx. 130 amino acids) of the proteins. Abbreviations (in alphabetical order, GenBank accession numbers in parenthesis): Dr Brown, *Drosophila melanogaster* Brown protein (P12428; Dr Scarlet, *Drosophila melanogaster* Scarlet protein (P45843); Dr White, *Drosophila melanogaster* White protein (P10090); Hu CFTR, Human Cystic Fibrosis Transmembrane Conductance Regulator protein (P13569; Hu MDR1, human multidrug resistance protein-1 gene product, P-glycoprotein (P08183); Hu MDR3, Human MDR3 gene product, phosphatidylcholine flippase (P21439); Hu MRP1, Human MRP1 gene product, multidrug resistance associated protein (P33527); Hu MRP2, Human MRP2 gene product, cMOAT (Q92887); Hu MRP3, Human MRP3 gene product (O15438); Hu MXR1, Human mitoxantrone resistance associated protein (AF093771); Hu White, Human White protein homolog (P45844); Hu TAP1, Human antigen peptide transporter 1 (Q03518); Hu TAP2, Human antigen peptide transporter 2 (Q03519); Ye ADP1, *Saccharomyces cerevisiae* probable ATP-dependent permease precursor (P25371); Ye BFR1, *Schizosaccharomyces pombe* Brefeldin A resistance protein (P41820); Ye MDL1, *Saccharomyces cerevisiae* ATP-dependent permease (P33310); Ye MDL2, *Saccharomyces cerevisiae* ATP-dependant permease (P33311); Ye YOL075, *Saccharomyces cerevisiae* probable ATP-dependent transporter (Q08234).

80 and MCF-7 AdVp3000 cells (Fig. 2A). As shown in Fig. 2B, a band of approximately 72 kDa, the expected molecular mass for MXR, is detected in S1-M1-80 and MCF-7 AdVp3000 cells. None of the other cell lines showed detectable MXR expression by immunoblotting. The immunohistochemistry micrographs (Fig. 2C) show cytoplasmic and plasma membrane localization of MXR in the S1-M1-80 and MCF-7 AdVp3000 cells, while the parent cells (S1 wt and MCF-7 wt) show no staining for MXR.

Mitoxantrone accumulation

Fig. 3 shows the steady state accumulation of 20 nM [^{3}H]mitoxantrone in wild-type (S1 and MCF-7) and mitoxantrone-resistant (S1-M1-80 and MCF-7 AdVp3000) cells. In both of the latter cell lines, the intracellular mitoxantrone levels were significantly lower (50% and 40%, respectively) than in wild type. This mitoxantrone accumulation defect was more thoroughly investigated by confocal microscopy, a study we extended to include a range of fluorescent drugs in order to define the substrate specificity of the putative new mechanism of drug resistance.

Steady state fluorescent drug distribution

Fig. 4 displays photomicrographs obtained by confocal microscopy of the steady state levels and localization of twelve drugs in the panel of six cell lines. Fig. 4A-I shows the distribution patterns of mitoxantrone (Fig. 4A) and seven other known P-gp substrates: daunorubicin (Fig. 4C), bisantrene (Fig. 4D), topotecan (Fig. 4E), rhodamine 123 (Fig. 4F), and fluorescent BODIPY derivatives of prazosin (Fig. 4G), vinblastine (Fig. 4H) and paclitaxel (Fig. 4I). Following incubation for 60 minutes at 37°C, the drug-sensitive parental cells (S1 and MCF-7) had the highest intracellular drug levels and intense nuclear staining with mitoxantrone (Fig. 4A) and daunorubicin (Fig. 4C). Mitoxantrone levels were decreased in the P-gp-overexpressing S1-B1-20 cells and strikingly decreased in the two mitoxantrone-resistant lines, S1-M1-80 and MCF-7 AdVp3000 (Fig. 4A). Fig. 4B shows that pretreatment with azide (15 mM) and deoxyglucose (50 mM) to deplete the cells of energy during mitoxantrone loading increases the level of mitoxantrone in the resistant sublines to that of the wild-type cells. Although not shown here, returning the cells to glucose-containing medium restores efflux and mitoxantrone levels in the

Table 3. Expression of *MDR1*, *MRP1*, *MRP2* and *MXR* in cell lines

	S1 wt	S1-B1-20	S1-M1-80	MCF-7 wt	VP16 MCF-7	MCF-7 AdVp3000
MDR1[a]	106	1486	135	0.09	<0.04[c]	0.08
MRP[a]	8.1	-	9.2	3.7	2690	7.8
MRP2[a]	0.29	-	0.09	12.2	215	152
MXR[b]	<0.04	<0.04	1703	10	7	2361

Expression was assayed by quantitative PCR.

[a]*MDR1*, *MRP* and *MRP2* levels are relative to the expression level, which is arbitrarily set to 10 units, in SW620 cells.

[b]*MXR* expression was not detectable in SW620 cells. Therefore, the levels are relative to the expression level, which is arbitrarily set to 10 units, in MCF-7 wt cells.

[c]Less than 0.04 units, which is the limit of detection.

-, Not assayed.

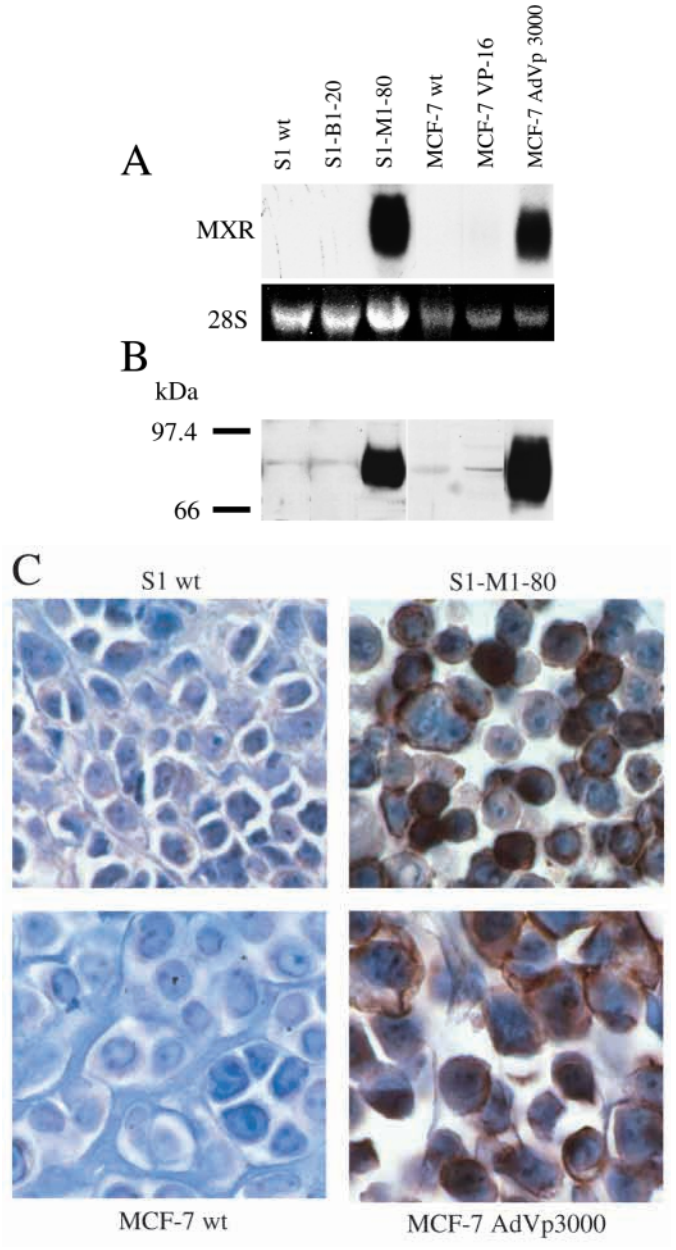


Fig. 2. (A) Northern blot (10 µg total RNA per lane) analysis showing the levels of *MXR* mRNA expression in the series of MCF-7 and S1 cell lines. Below the *MXR* bands are shown the control 28S rRNA bands. (B) Western blot analysis for MXR with the polyclonal antibody 87405 in MCF-7 and S1 cells. Each lane contains 50 µg protein, not boiled, solubilized in 2× SDS-sample buffer. (C) Immunohistochemistry showing MXR expression in MCV-7 AdVp3000 and S1-M1-80 cells compared to the wild type (wt). The MXR protein is localized mainly to the plasma membrane. An antibody dilution of 1:2500 was used.

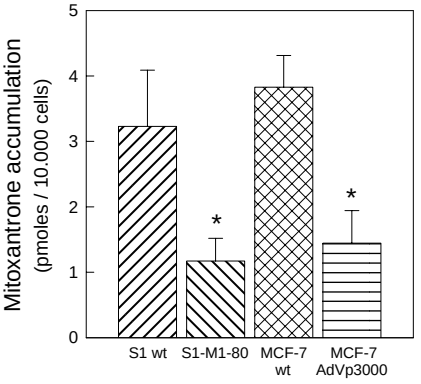


Fig. 3. Steady state accumulation of [^{3}H]mitoxantrone in sensitive (S1 wt and MCF-7 wt) and resistant (S1-M1-80 and MCF-7 AdVp3000) cells. Intracellular accumulation was measured after 60 minutes incubation at 37°C in 20 nM mitoxantrone. Values are means + s.e.m. (n=3). *P<0.05, Student's paired t-test.

resistant lines decrease to levels comparable to those found in untreated cells. Thus, energy-dependent drug efflux was confirmed in both P-gp-expressing (S1-B1-20) as well as in MXR-expressing (S1-M1-80 and MCF-7 AdVp3000) cell lines.

A punctuate, perinuclear, staining pattern of fluorescence was observed in all cell lines, suggesting accumulation of mitoxantrone in a vesicular compartment, such as lysosomes and/or the Golgi apparatus. The daunorubicin distribution pattern was very similar to that of mitoxantrone, the drug being concentrated mainly in a perinuclear zone in all cell lines (Fig. 4C). Again, the lowest daunorubicin accumulation was seen with the MCF-7 AdVp3000, S1-M1-80 and S1-B1-20 cells. The drug accumulation profiles were repeated with four other drugs known to be P-gp substrates, bisantrene (Fig. 4D), topotecan (Fig. 4E), rhodamine 123 (Fig. 4F) and prazosin (Fig. 4G), the compounds being eliminated from both the P-gp-expressing S1-B1-20 cells, and the MXR-expressing S1-M1-80 and MCF-7 AdVp3000 cells, but not from the MRP-overexpressing cell line MCF-7 VP-16. In contrast, the P-gp substrate vinblastine (Fig. 4H) appeared not to be a good transport substrate for the MXR sublines, but was apparently transported by the MRP subline MCF-7 VP-16, whereas paclitaxel (Fig. 4I) was excluded only from the P-gp-overexpressing S1-B1-20 cells. A fluorescent BODIPY analogue of the MDR reversing agent verapamil was clearly extruded only from the S1-B1-20 cells (Fig. 4J). Next we examined substrates for MRP. P-gp is known to efflux the non-fluorescent calcein-AM ester (Homolya et al., 1994). Once inside the cell, the AM ester group is cleaved by intracellular esterases; the resulting calcein is fluorescent and has been shown to be a transport substrate for MRP but not for P-gp (Versantvoort et al., 1995). Consistent with this, the P-gp-expressing S1-B1-20 cells, as well as the MRP-overexpressing MCF-7 VP-16 cells, were characterized by their very low and vesicular calcein accumulation (Fig. 4K). The UV-excitable compound monochlorobimane becomes fluorescent when bound to glutathione, and has been used to assess intracellular glutathione levels (Millis et al., 1997; van Luyn et al., 1998). We found that the steady state level of glutathione-bound monochlorobimane was lower only in the MRP-overexpressing MCF-7 VP-16 cells (data not shown), an observation which is compatible with the suggested role of MRP as a glutathione S-conjugate pump (Zhang and Wong, 1996). The MXR-expressing cells showed no evidence of transport of either calcein or of glutathione-conjugated monochlorobimane (data not shown). Finally, the distribution of three organelle-specific probes was examined: a fluorescent BODIPY derivative of Brefeldin A, which labels the endoplasmatic reticulum,

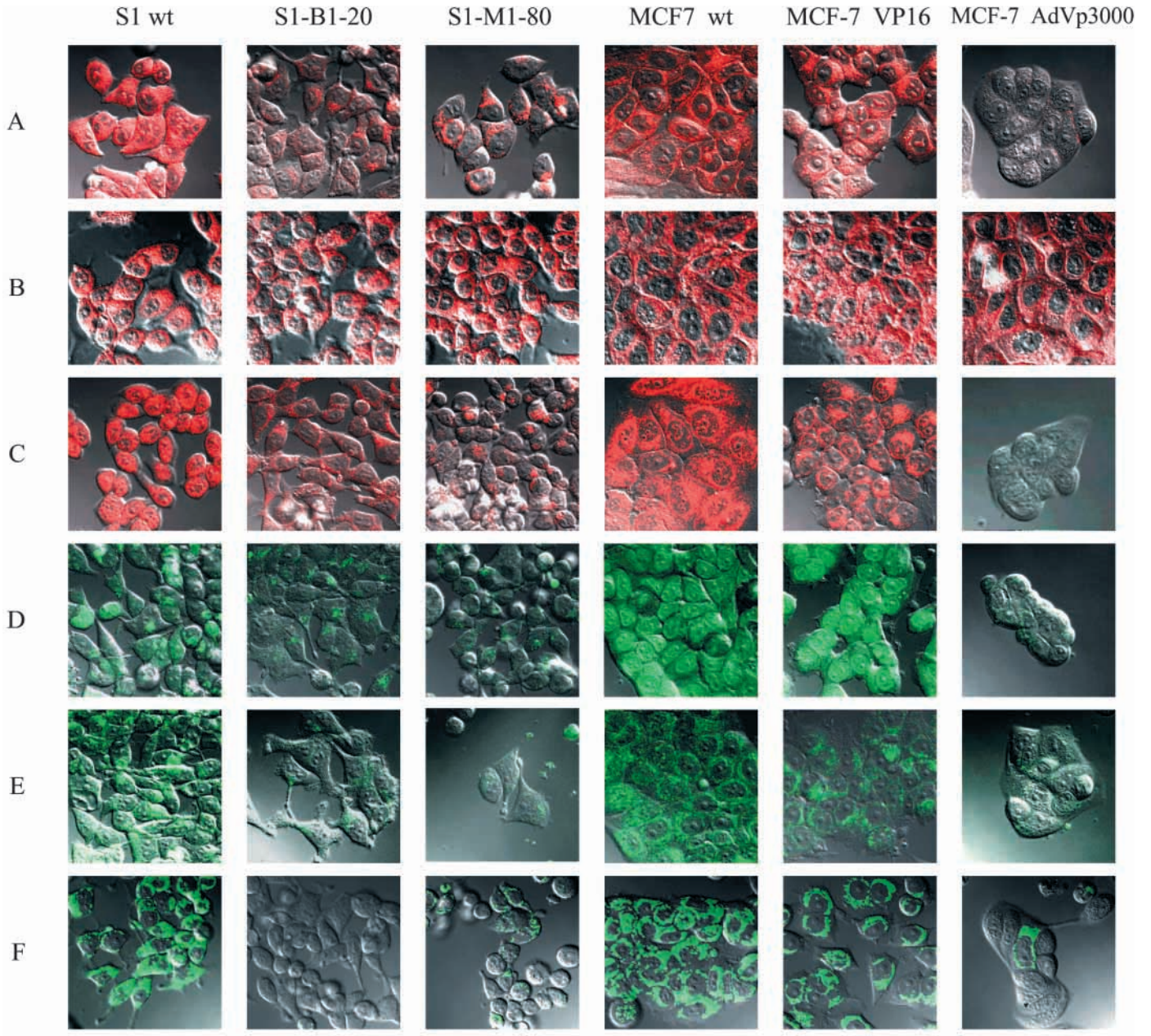
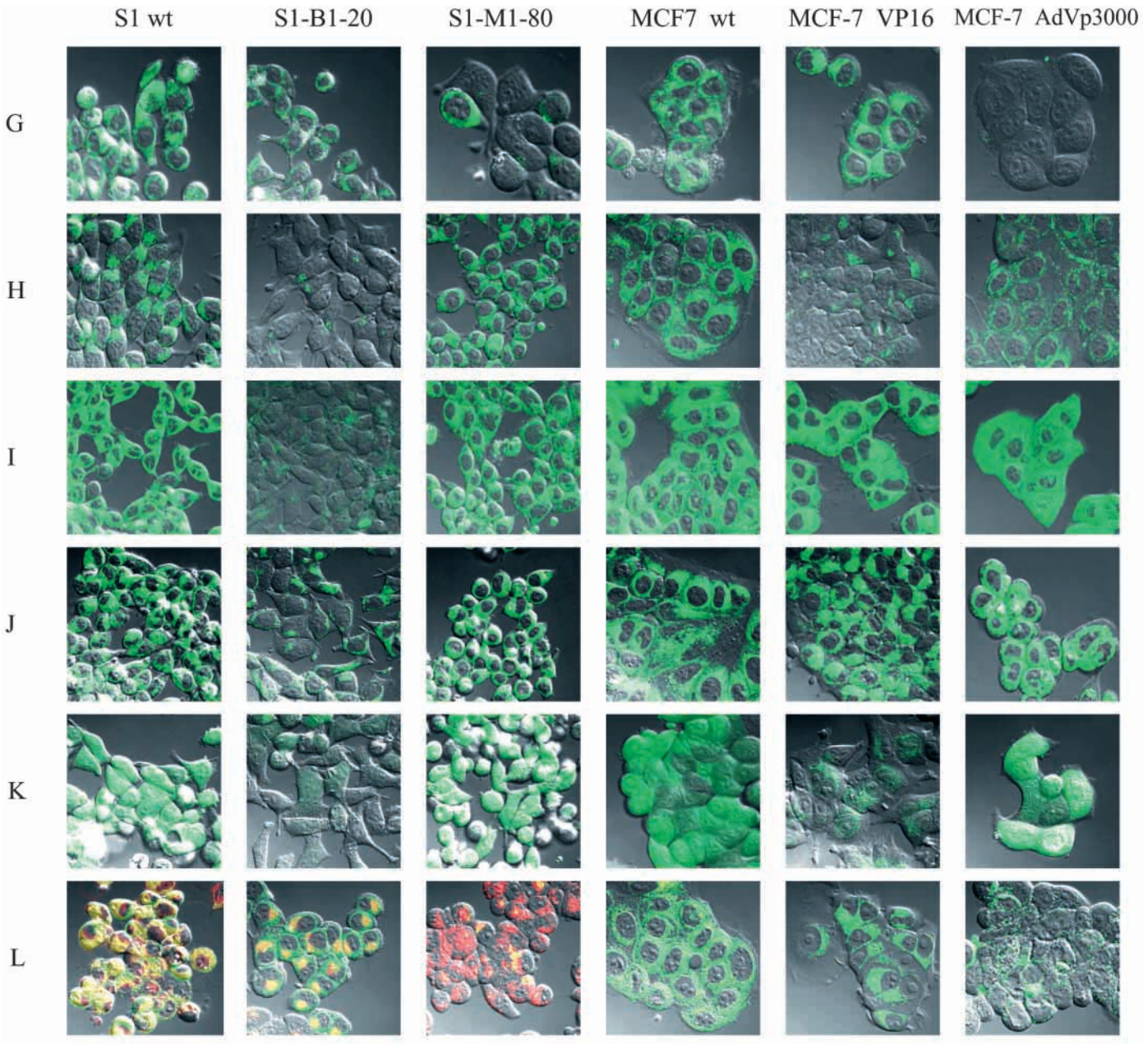


Fig. 4. Confocal microscopy images showing steady state drug accumulation of fluorescent drugs after 60 minutes incubation at 37°C. Shown are the levels and localization of (A) mitoxantrone (5 μM); (B) mitoxantrone after 60 minutes preincubation with azide (15 mM) and deoxyglucose (50 mM); (C) daunorubicin (5 μM); (D) bisantrene (25 μM); (E) topotecan (5 μM); (F) rhodamine 123 (1 μM); (G) BODIPY-prazosin (100 nM); (H) BODIPY-vinblastine (2 μM); (I) BODIPY FL-paclitaxel (100 nM); (J) BODIPY-verapamil (3 μM); (K) Calcein-AM (1 μM); (L) BODIPY FL C_5-ceramide (100 nM). The overlayed colors indicate the fluorescence emission spectrum of the dyes: green (515-540 nm) and red (above 590 nm), respectively. BODIPY FL C_5-ceramide exhibits a shift in fluorescence emission maximum from green (515 nm) towards red (620 nm) when it forms excimers at high local concentration in the trans-Golgi network.

BODIPY FL C5-ceramide, a selective stain of the trans-Golgi network (Schindler et al., 1996; Demaurex et al., 1998), and LysoTracker Green, a weak base that will accumulate in acidic compartments such as lysosomes, due to trapping of the protonated and thus membrane-impermeable form of the dye (Haugland, 1996). All cell lines showed intense labeling of the ER with BODIPY Brefeldin A (data not shown). Five out of the six cell lines showed clear staining of the trans-Golgi with BODIPY FL C5-ceramide, the one exception being MCF-7 AdVp3000 (Fig. 4L), which showed only very faint fluorescence, suggesting that BODIPY FL C5-ceramide may be a substrate for the putative membrane transporter present in this cell line. A striking result was seen in S1-M1-80 cells in which the fluorescence emission of BODIPY FL C5-ceramide was strongly red-shifted, indicating a high local concentration of the dye in the Golgi of these cells. The acidic organelle probe LysoTracker Green (Fig. 7B) revealed an acidic compartment in all of the cell lines, again except for the MCF-7 AdVp3000 cells, as these seemed to efflux the dye rapidly.



Thus, cells that overexpress MXR show decreased accumulation of the P-gp substrates mitoxantrone, daunorubicin, bisantrene, topotecan, rhodamine 123 and prazosin, but not of taxol, verapamil, vinblastine and calcein, the latter being a substrate only of MRP. We have illustrated the overlapping substrate specificity of the three transporters in a Venn diagram (Fig. 5).

Mitoxantrone accumulation in MXR transfectants

To confirm that MXR alone could mediate the drug resistance phenotype described above, we examined mitoxantrone accumulation by confocal microscopy in cell lines transfected with the half-transporter. Since *MXR*, *BCRP* and *ABCP1* represent the same gene, we utilized the BCRP stable transfectants previously described (Doyle et al., 1998).

Fig. 6 shows confocal microscopy micrographs of the steady state level of mitoxantrone in MXR transfectants. Drug-sensitive MCF-7 cells, which have been transfected with full-length MXR cDNA (clone 8), accumulate less mitoxantrone than control cells (vector) transfected with the vector alone. This directly proves that functional expression of MXR mediates mitoxantrone resistance.

Intracellular localization of mitoxantrone

Colocalization studies with mitoxantrone and the above probes for intracellular compartments were performed to investigate whether sequestration plays a role in any of the resistance phenotypes, as suggested by Dietel et al. (1990) for the mitoxantrone-resistant carcinoma cell line EPG85-257. The green fluorescence of the lysosomal probe LysoTracker Green (Fig. 7B) was overlaid with the red emission signal of mitoxantrone (Fig. 7A) to obtain the composite images, where

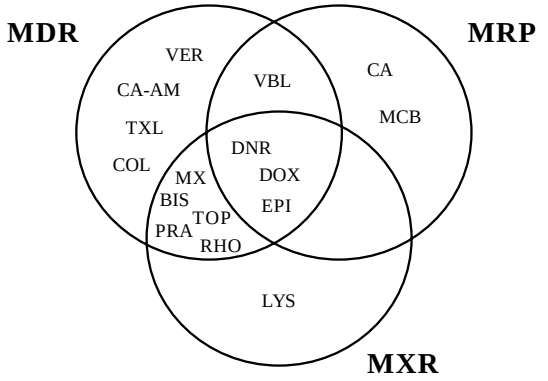


Fig. 5. Venn diagram showing the overlapping substrate specificity of MDR, MRP and MXR. BIS, bisantrene; CA, calcein; CA-AM, calcein-AM ester; COL, colchicine; DNR, daunorubicin; DOX, doxorubicin; EPI, epirubicin; LYS, LysoTracker; MCB, monochlorobimane; MX, mitoxantrone; PRA, prazosin; RHO, rhodamine 123; TXL, paclitaxel; TOP, topotecan; VBL, vinblastine; VER, verapamil. COL, DOX and EPI results also obtained by confocal microscopy (data not shown).

yellow indicates colocalization of mitoxantrone with the lysosomal marker (Fig. 7C). It appears that mitoxantrone normally accumulates in acidic vesicles in most cell types, as suggested by its colocalization with LysoTracker Green (Fig. 7C). The S1 cells displayed the most intense staining. This vesicular staining pattern persisted in S1-M1-80 cells, but the overall level of accumulation was reduced. It was not possible to see colocalization in MCF-7 AdVp3000 cells, due to marked efflux of both compounds. Similar results were obtained with daunorubicin (data not shown). Compartmentalization of drugs may thus represent an intrinsic protective mechanism upon which the drug efflux mechanism highly expressed in S1-M1-80 and MCF-7 AdVp3000 cells has been superimposed.

DISCUSSION

This report presents a comprehensive examination of the resistance phenotype of human colon (S1-M1-80) and breast (MCF-7 AdVp3000) cancer cells, which overexpress the mitoxantrone resistance associated gene *MXR* [GenBank accession numbers: AF093771 (MXR1) and AF093772 (MXR2)]. We recently identified MXR as an ABC half-transporter with strong homology to the *Drosophila* white gene product (Miyake et al., 1999). MXR is a member of the ABC superfamily of membrane transporters (Leveille-Webster and Arias, 1995; Higgins, 1992), one of the largest protein superfamilies known. This family comprises hundreds of members in both prokaryotic and eukaryotic organisms, most of which function as ATP-dependent active membrane transporters, translocating molecules across a cell membrane against a concentration gradient (Dean and Allikmets, 1995). While P-gp is the most well-characterized ABC transporter, in recent years new members of the ABC superfamily (Allikmets et al., 1996), such as MRP1 (Cole et al., 1992), MRP2 (cMOAT) (Elferink and Jansen, 1994), MRP6 (also known as ARA, anthracycline resistance associated protein) (Longhurst et al., 1996), MOAT-B (Lee et al., 1998), and BCRP (breast cancer resistance protein) (Doyle et al., 1998) have also been associated with drug resistance. The only slight variation of *MXR* from the *ABCP1* (Allikmets et al., 1998) and *BCRP* (Doyle et al., 1998) genes indicates that the three are indeed the same gene. Our goal in these studies was to characterize the phenotype of this newly described transporter.

Despite the different selection history of the S1-M1-80 and the MCF-7 AdVp3000 cell lines, a similar phenotype is displayed. First, the cross-resistance patterns seem to be almost identical for MCF-7 AdVp3000 and S1-M1-80 cells (Table 2), both having a high level of resistance to mitoxantrone, the anthracyclines and topotecan. Second, as visualized by confocal microscopy, there are similar intracellular drug distribution profiles, with reduced mitoxantrone, daunorubicin, bisantrene, topotecan, BODIPY-prazocin and rhodamine 123 accumulation. Neither the tubulin binding agents paclitaxel and vinblastine, nor the MRP substrate calcein, appear to be substrates, distinguishing the MXR resistance mechanism from that of both P-gp and MRP. Third, the mitoxantrone resistance correlated with that of an energy-dependent accumulation defect in both sublines. This was shown by restoration of mitoxantrone accumulation to the level in sensitive, parental cells after preincubation with azide (15 mM) and deoxy-glucose (50 mM). Efflux of mitoxantrone after restoration of energy was higher from the resistant cells than from the parental cell lines, extending previous findings of energy-dependent rhodamine efflux in these cells (Lee et al., 1997). It was recently shown that transfection of the *BCRP* gene conferred mitoxantrone resistance to parental MCF-7 cells, further confirming BCRP/MXR as the mechanism of resistance in these cells (Doyle et al., 1998). Extending that observation, we also demonstrate that these cells exhibit decreased mitoxantrone accumulation as compared to control cells transfected with the vector alone. Furthermore, in preliminary studies we have observed that transient transfection with MXR confers efflux of both rhodamine 123 and daunorubicin (data not shown). Since the MCF-7 AdVp3000 and S1-M1-80 cells overexpress MXR at high levels, we conclude that the displayed phenotype represents that of the product of the half-transporter gene. These studies suggest that overexpression of the MXR protein may allow homodimerization to form a functional transporter.

Neither the mechanism of transport by MXR nor its role in normal physiology is known. Considering that half-transporters are typically found on intracellular membranes (Townsend and Trowsdale, 1993), it can be speculated that MXR might reside on the endoplasmatic reticulum to facilitate drug metabolism. Indeed, glucuronidation of anticancer drugs such as mitoxantrone, the anthracyclines and SN-38 has been reported to occur in tumor cells (Burchell et al., 1991; Takahashi et al., 1997), and in our laboratory we have further demonstrated that MXR-expressing cells are capable of glucuronidating cytotoxic drugs (Brangi et al., 2000). In such a model, which requires experimental validation, the metabolite would require transport out of the ER, a potential physiologic role for MXR. In the case of the very high expression levels found in drug-resistant MCF-7 AdVp3000 cells the excess protein might redistribute to the plasma membrane. Indeed, by immunohistochemical analysis of MXR protein expression, we have observed both cytoplasmic and

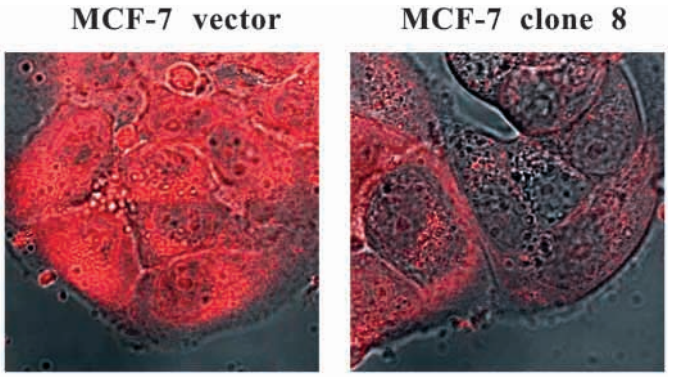


Fig. 6. Accumulation of mitoxantrone in MCF-7 cells transfected with full-length MXR cDNA (clone 8), and control cells transfected with the vector only (vector). Expression of MXR induces an accumulation defect for mitoxantrone. The reduced mitoxantrone accumulation in the MXR transfected cells is energy-dependent (as demonstrated with preincubation with sodium azide; results not shown).

plasma membrane staining in MCF-7 and AdVp3000 and S1-M1-80 cells, which is consistent with an intracellular localization with redistribution to the plasma membrane. It remains to be determined whether the plasma membrane is a physiological location for MXR in normal tissue.

Although MCF-7 AdVp3000 and S1-M1-80 cells both express MXR and have similar phenotypes, certain differences are detectable. The overexpressed MXR gene in the S1-M1-80 cells does not appear to be amplified, while amplification is present in the MCF-7 AdVp3000 cells (Miyake et al., 1999). Some compounds, including the lysosomal probe, LysoTracker Green, and the trans-Golgi probe, BODIPY FL C5-ceramide, appear to be better substrates for MCF-7 AdVp3000 than for S1-M1-80, as these dyes are practically eliminated from MCF-7 AdVp3000 cells. However, the possibility exists that the S1-M1-80 cells accumulate substrates into a vesicular compartment, as suggested by the BODIPY FL C5-ceramide data, rather than solely eliminating the probe from the plasma membrane. These differences may be related to the protein level, which is significantly higher in MCF-7 AdVp3000 than in S1-M1-80, as seen by immunoblot. A second possibility is that the MXR may be more efficiently located, e.g. in the plasma membrane in the MCF-7 AdVp3000 breast cancer cells. This differential localization would be analogous to the redistribution of MRP that has previously been described (Almquist et al., 1995). A third possibility is that the tenfold overexpression of MRP2 in MCF-7 AdVp3000 cells could account for the additional substrates. The same level of MRP2 overexpression is, however, found in MCF-7 VP-16 cells, which do not eliminate the noted compounds. Finally, it is possible that a difference in dimerization may confer a difference in transport efficiency for a particular compound. As a half-transporter, MXR should require dimerization in order to transport substrates. MXR may exist as a homo-dimer in the MCF-7 AdVp3000 cells in which the MXR gene is amplified, while it may form hetero-dimers with a hitherto unidentified partner in the S1-M1-80 cells, which do not show amplification of the *MXR* gene (Miyake et al., 1999). Whether homo- or hetero-dimers of MXR exist remains to be investigated.

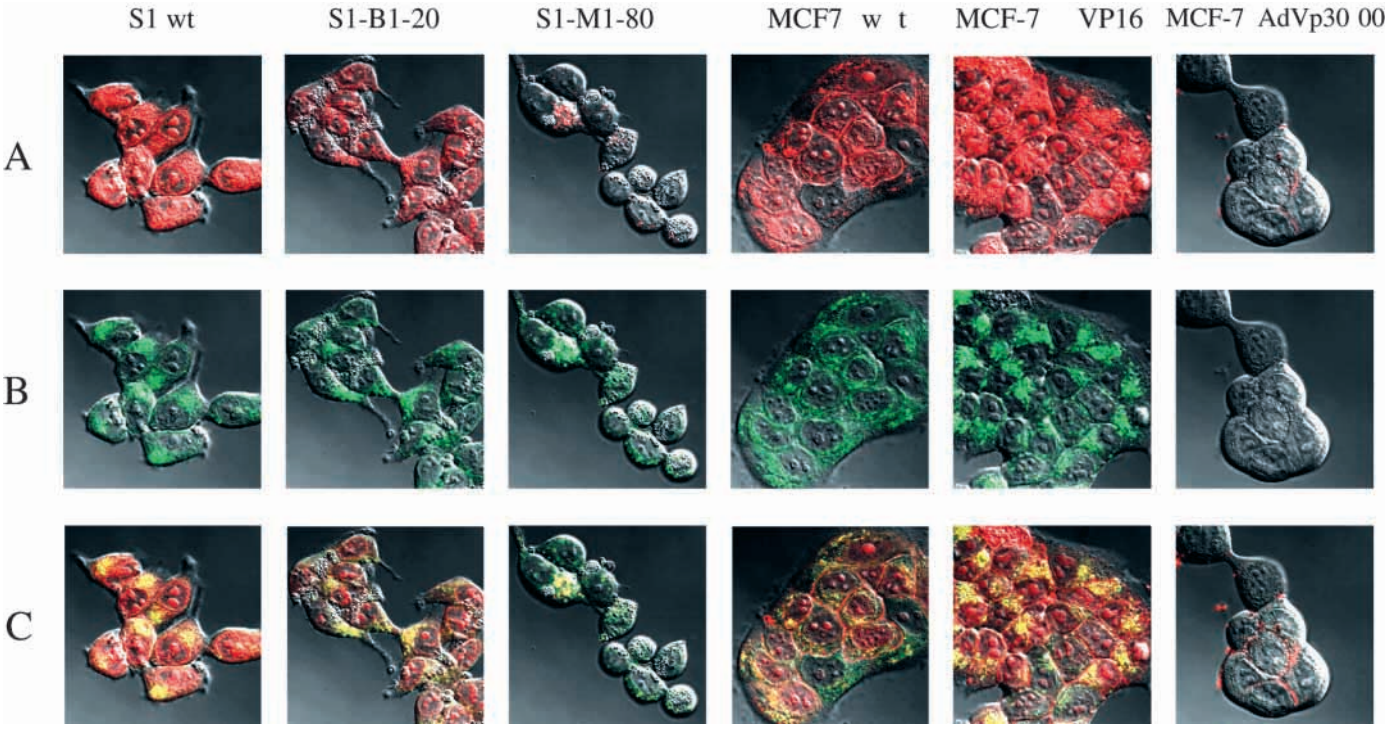


Fig. 7. Colocalization of mitoxantrone with LysoTracker Green. The red emission signal of mitoxantrone (A) was overlayed with the green fluorescence of LysoTracker Green (B) to obtain the composite images in (C), where yellow indicates colocalization of mitoxantrone with the lysosomal marker.

The confocal studies also allowed examination of drug compartmentalization, since it has been suggested that sequestration of mitoxantrone into membrane vesicles can contribute to drug resistance (Dietel et al., 1990; Fox and Smith, 1995). While a detailed examination of this question is beyond the scope of this paper, we noted cytoplasmic, mitoxantrone-containing vesicles in all the colon carcinoma (S1) cells. In the S1-M1-80 cells, colocalization studies indicated that the drug accumulated in acidic vesicles, the morphology of which did not appear to be different between the three colon cell lines. Although intracellular sequestration may play a role in intrinsic resistance to mitoxantrone, and may be a mechanism by which unselected cells dispose of the drug, this mechanism appears to be superceded by the MXR overexpression, which results in a profound drug accumulation defect.

The mitoxantrone accumulation defect as assessed by confocal microscopy seemed greater than that observed by accumulation of radioactive mitoxantrone. This could be due to the greater sensitivity of the radioactive method. Alternatively, the data could be explained by intracellular metabolism of mitoxantrone (Ehninger et al., 1990). Taking normal hepatic metabolism as a model, mitoxantrone is subject to glucuronidation (Wolf et al., 1986; Burchell et al., 1991; Blanz et al., 1991a), glutathione conjugation (Peters and Roelofs, 1992; Rekha and Sladek, 1997; Blanz et al., 1991b), or oxidation to its mono- or dicarboxylic acid derivatives (Richard et al., 1991). The latter primary metabolites of mitoxantrone are non-fluorescent (Bell, 1988) and would not be detectable by fluorescence microscopy. Thus, metabolism to an inactive metabolite prior to efflux in drug-resistant cells would be expected to result in a decrease in fluorescence emission, while not affecting the signal, in a study in which accumulation of radioactive mitoxantrone is measured. This could explain discrepancies in the literature on mitoxantrone-resistant cell lines, some studies reporting little or no decrease in intracellular mitoxantrone accumulation (Harker et al., 1989; Dalton et al., 1988; Bailly et al., 1997), with others reporting increased mitoxantrone efflux and a marked decrease in intracellular drug accumulation (Lee et al., 1997; Kellner et al., 1997; Taylor et al., 1991).

The fact that multiple transporters can affect individual anticancer agents has significant implications for studies attempting reversal of drug resistance. The anthracyclines, for example, are substrates for P-gp, MRP and MXR. Inhibition of one transporter may be thwarted by the presence of another. This observation is consistent with the profound drug resistance often found in clinical oncology and may explain why drug reversal trials in the clinic have not achieved dramatic results (Sandor et al., 1998; Tew, 1993). It is altogether likely that resistance mechanisms may yet encompass new, hitherto unidentified or uncharacterized members of the ABC superfamily (Allikmets et al., 1996; Ling, 1997; Müller, 1999). This further underscores both the complexity of multidrug resistance and the need for development of new and better reversers of clinical drug resistance.

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Note added in proof

The Human Gene Nomenclature Committee has proposed a new nomenclature for ABC transporters. In this nomenclature MXR/BCRP/ABCP would be replaced by ABCG2 (http://www.gene.ucl.ac.uk/users/hester/abc.html).