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The Role of Half-Transporters in Multidrug Resistance

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ATP-binding cassette proteins comprise a superfamily of transporter proteins, a subset of which have been implicated in multidrug resistance. Although P-glycoprotein was described over 15 years ago, the recent expansion in the number of transporters identified has prompted renewed interest in the role of drug transporters in clinical drug resistance. These newly identified transporters include additional members of the MRP family, ABC2, and a new half-transporter, MXR/BCRP/ABCP1. This half-transporter confers high levels of resistance to mitoxantrone, anthracyclines, and the camptothecins SN-38 and topotecan. At 72 kDa, MXR localizes to the plasma membrane in cells which highly overexpress the protein either through gene amplification or though gene rearrangement. Future studies will be aimed at identifying an inhibitor, and attempting to translate recognition of this new transporter into a target for anticancer treatment.

KEY WORDS: ABCG2; mitoxantrone; ABC transporter; drug resistance gene.

INTRODUCTION

ATP-binding cassette (ABC) transporter genes comprise a large superfamily, the protein products of which transport a wide range of compounds including sugars, amino acids, peptides, salts, and xenobiotics (Higgins, 1992). Recently, there has been a rapid increase in the number of ABC transporter genes identified, most in the last 2 years (Klein *et al.*, 1999). Classification of known human ABC genes into families, based upon homology of the ATP-binding domain, generates seven families (Klein *et al.*, 1999). For several of the genes, a role in multidrug resistance has been hypothesized.

P-glycoprotein, product of the *MDR1* gene, has been under intense study for over 15 years as a mediator of multidrug resistance. MDR1/Pgp is able to confer resistance to the anthracyclines, vinca alkaloids, taxanes, and epipodophyllotoxins, as well as to a broad range of other structurally unrelated compounds (Lee *et al.*, 1994; Ling, 1997). Overexpression of MDR1/Pgp has been implicated as a mechanism of resistance in leukemia, breast cancer, myeloma, and in sarcoma (Malayeri *et al.*, 1996; Trock *et al.*, 1997; van den Heuvel-Eibrink *et al.*, 2000). However, conflicting results have been reported, presumably owing to difficulties in detection methodology (Beck *et al.*, 1996). In acute leukemia, for which the data are the most convincing, expression of Pgp is correlated with reduced complete response rate and increased relapse rate. Although still ongoing with newer and more potent antagonists, clinical trials with Pgp inhibitors have not provided convincing evidence for multidrug resistance reversal (Ling, 1997; Sandor *et al.*, 1998). One potential explanation is the coexistence of other ABC transporters. Lending support to this possibility is the identification of a number of new transporters also able to reduce drug accumulation.

The multidrug resistance associated protein (MRP1) was the second ABC transporter discovered with a potential role in multidrug resistance (Cole *et al.*, 1992; Hipfner *et al.*, 1999). Initial studies demonstrated that MRP could confer resistance to anthracyclines, vinca alkaloids, and epipodophyllotoxins, thus overlapping in substrate specificity with MDR1/Pgp. It was subsequently recognized that MRP1 was able to transport drugs conjugated to

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glutathione, glucuronide, and sulfate (Jedlitschky *et al.*, 1996). Recent studies suggest that for GSH, conjugation is not required; rather, there is strong evidence for co-transport (Borst *et al.*, 1999; Hipfner *et al.*, 1999). Other MRP family members have been identified, including the hepatocyte transporter of bilirubin glucuronide, cMOAT (MRP2), and the less well-characterized MRPs 3–6 (Kool *et al.*, 1997). Interestingly, MRP1 and MRP3 have been recently shown to transport methotrexate, extending the range of compounds potentially involved in the multidrug resistance phenotype (Kool *et al.*, 1999; Zeng *et al.*, 1999). Two other transporters have been identified, the hepatocyte bile salt transporter, sPgp (sister of Pgp) or BSEP, which has been shown to transport paclitaxel; and ABC2, which was found to be overexpressed in estramustine-resistant cells (Childs *et al.*, 1998; Laing *et al.*, 1998; Strautnieks *et al.*, 1998). Many of these transporters are expressed in the normal liver and are likely to be involved in drug disposition.

Among the recently discovered genes with a potential role in drug resistance is an ABC half-transporter known as the breast cancer resistance protein (BCRP) (Doyle *et al.*, 1998b); also known as ABC transporter expressed in placenta (ABCP1) (Allikmets *et al.*, 1998); and in our laboratory, as the mitoxantrone resistance gene (*MXR*) (Miyake *et al.*, 1999). BCRP was first reported in abstract form at the American Association for Cancer Research annual meeting in April 1998 (Doyle *et al.*, 1998a). However, the sequence information did not become available for comparison until after the gene was separately cloned in our laboratory and by Allikmets *et al.* It will be referred to as MXR in this manuscript, although the Human Genome Nomenclature Committee (HUGO) recently recommended that the gene be renamed *ABCG2* (www.gene.ucl.ac.uk/users/hester/abc.html) (Klein *et al.*, 1999).

Overexpression of the half-transporter has been found in a series of multidrug-resistant cell lines characterized by high levels of mitoxantrone resistance and cross-resistance to anthracyclines, without resistance to vinca alkaloids or taxanes, and without evidence of overexpression of the well-characterized multidrug resistance genes, *MDR1* or *MRP1* (Ross *et al.*, 1999). Reduced accumulation of mitoxantrone or anthracyclines was observed in these cell lines; this reduced accumulation could be reversed by incubation of cells in ATP-depleting conditions. Several of the sublines were derived in independent selections from the MCF-7 human breast cancer cell line either in mitoxantrone (Nakagawa *et al.*, 1992; Taylor *et al.*, 1991) or in adriamycin (Lee *et al.*, 1997). Other sublines with a similar phenotype include those derived from 8226 human myeloma cells (Hazlehurst *et al.*, 1999), S1 (LS174T) colon cancer cells (Rabindran *et al.*, 1998), WiDr colon cancer cells (Dalton *et al.*, 1988; Wallace *et al.*, 1987), and EPG85–287 gastric cancer cells (Dietel *et al.*, 1990). Recently, a topotecan-selected human ovarian carcinoma subline with reduced topotecan accumulation and overexpression of MXR was described (Ma *et al.*, 1998; Maliepaard *et al.*, 1999). Interestingly, when Allen and co-workers used doxorubicin, mitoxantrone, or topotecan to derive resistant sublines from immortalized mouse fibroblast cells in which the orthologue for both MDR1 and MRP was deleted, MXR overexpression resulted for each selection (Allen *et al.*, 1999).

MXR belongs to the *White* subfamily of ABC transporters. The *white* locus was the first genetic marker described in *Drosophila*. The White protein forms heterodimers with the related Brown or Scarlet protein to generate a functional ABC transporter for guanine or tryptophan, respectively, precursors for eye pigment (Ewart and Howells, 1998). Although precise localization studies have never been performed, it has been thought that the White heterodimer is localized to the plasma membrane, with the ATP-binding domain localized in the cytoplasm, while guanine and tryptophan binding must occur on the extracellular surface to allow transport into the cell. The organization of most full transporters has the transmembrane (TM) domain 5′ to the ATP-binding domain: TM-ATP-TM-ATP. In contrast, the organization of MXR and the other members of the White family is the reverse: ATP-TM.

Studies in our laboratory have been aimed at characterizing substrates and inhibitors for MXR, the mechanism of overexpression, and the protein localization. While many questions remain, our studies taken together with those of others suggest that the substrate specificity is almost as broad as that for Pgp, and that there is considerable overlap with that of Pgp. We have studied a series of selected cell lines. As shown in Table I, the MCF-7 AdVp cells were selected in adriamycin in the presence of verapamil from the human breast cancer cell line MCF-7

Table I. Resistant Cell Lines Used in MXR Studies

Parent cell line	Selecting agent	Subline
MCF-7	Adriamycin (3000 ng/mL) Verapamil (5 μg/mL)	MCF-7 AdVp3000
	Adriamycin (2000 ng/mL)	MCF-7 Ad2000
	Mitoxantrone (8 nM)	MCF-7 MX8
	Mitoxantrone (100 nM)	MCF-7 MX100
	Adriamycin (2000 nM)	MCF-7 AD2000
S1[a]	Mitoxantrone (80 μM)	S1-M1-80
	Bisantrene (20 μM)	S1-B1-20

[a] S1-M1-3.2 cells selected from S1 clone of LS174T.

in order to prevent overexpression of P-glycoprotein. The MCF-7 MX8 and MX100 cells were separately selected in mitoxantrone, independently from previously published mitoxantrone-resistant MCF-7 sublines. The S1-M1-80 cells were derived from S1, a clone of the LS 180 human colon cancer cell line, and advanced in mitoxantrone concentration from S1-M1-3.2 cells obtained from Lee Greenberger at Wyeth-Ayerst (Rabindran *et al.*, 1998). All four sublines are MXR-overexpressing. Two sublines with Pgp overexpression were used as controls in several experiments. MCF-7 Ad2000 cells were obtained from MCF-7 cells by stepwise selection in adriamycin. S1-B1-20 cells, also derived from S1 cells, were selected in bisantrene by Lee Greenberger (Zhang *et al.*, 1994).

Confocal Microscopy in Cell Lines with MXR Overexpression

To evaluate drug accumulation and localization, confocal microscopy was performed in MCF-7 and S1 parental cells and resistant sublines. Representative results are shown in Fig. 1. As expected, reduced accumulation of mitoxantrone, daunorubicin, and topotecan was observed in the Pgp-overexpressing MCF-7 Ad2000 and S1-B1-20 sublines. Reduced accumulation of the three substrates was also observed in the MXR-overexpressing MCF-7 AdVp3000 and S1-M1-80 cells. In the S1-M1-80 cells, although the overall accumulation is reduced, hot spots consistent with accumulation in acidic vesicles are observed. Colocalization studies with lysotracker have confirmed localization of mitoxantrone in acidic vesicles in both parental S1 cells and resistant sublines (Litman *et al.*, 2000).

Substrates for MXR-Mediated Transport

The phenotype conferred by MXR overexpression is characterized by high levels of resistance to mitoxantrone, topotecan, and SN-38 (the active metabolite of CPT-11); moderate resistance to the anthracyclines including daunorubicin, doxorubicin, and epirubicin; and lack of resistance to cisplatin, paclitaxel, and vinblastine (Brangi *et al.*, 1999; Doyle *et al.*, 1998b; Litman *et al.*, 2000; Maliepaard *et al.*, 1999; Rabindran *et al.*, 1998; Ross *et al.*, 1999; Yang *et al.*, 1995). In addition, the azaanthrapyrazole BBR3390 and etoposide were also demonstrated to be substrates in mitoxantrone-resistant 8226 multiple myeloma cells (Hazlehurst *et al.*, 1999). A Venn diagram which shows the overlapping substrate specificity for MXR, MRP1, and MDR1 is shown in Fig. 2, summarizing data in the literature combined with our findings by confocal microscopy. By flow cytometry and by confocal microscopy the fluorescent compounds rhodamine, BODIPY-prazosin, and lysotracker were also shown to be substrates (de Bruin *et al.*, 1999; Litman *et al.*, 2000). Studies with transfected cells have confirmed that this phenotype is mediated by MXR. First, transfection confers a cross-resistance pattern similar to that found in cells overexpressing MXR (Doyle *et al.*, 1998b). Figure 3 shows flow cytometry results with rhodamine in cells transfected with a cDNA containing BCRP, which has minor sequence differences from the sequence reported when MXR was cloned (Doyle *et al.*, 1998b; Miyake *et al.*, 1999). Efflux of rhodamine can be demonstrated in the transfected cells, with fluorescence levels increased in the presence of the MXR inhibitor fumitremorgin C (FTC) described below.

Inhibitors of MXR Activity

Several Pgp antagonists have been examined in an effort to identify inhibitors of MXR. Verapamil, PSC 833, and a new Pgp antagonist XR9576 failed to increase drug accumulation or cytoxicity in cells overexpressing MXR (Ma *et al.*, 1998). However, GF120918, a Glaxo compound, was able to increase accumulation of both compounds in the cells (de Bruin *et al.*, 1999). Higher concentrations were required to increase accumulation in the S1-M1-80 cells than in Pgp-overexpressing cells. GF120918 at 1 μM increased cytotoxicity of mitoxantrone and topotecan in MXR-overexpressing cells. By inhibiting both Pgp and MXR, GF120918 serves as a model for a multispecific antagonist. Depending upon the tumor type, or the antineoplastic agent being administered, it may be preferable to have an antagonist able to block one or more transporters.

An apparently specific MXR antagonist was reported by Rabindran *et al.*, derived from *Aspergillus fumigatus* (Hazlehurst *et al.*, 1999; Rabindran *et al.*, 1998). FTC increased mitoxantrone and doxorubicin accumulation in MXR-overexpressing cells, but not in Pgp-overexpressing cells. In addition to sensitization of sublines selected for drug resistance, MCF-7 cells transfected with BCRP cDNA are sensitized to mitoxantrone, topotecan, and doxorubicin by FTC (Hazlehurst *et al.*, 1999; Rabindran *et al.*, 2000). Figure 4 shows flow cytometry results of accumulation and efflux studies, with rhodamine and topotecan as substrates. The Pgp inhibitor PSC 833 increased accumulation of rhodamine and topotecan in Pgp-expressing AD2000 cells (dashed line), but was unable to increase accumulation in MXR-overexpressing MCF-7 AdVp3000 cells. However, FTC (dotted line) is able to return levels of accumulation to parental levels in the MXR-overexpressing cells.

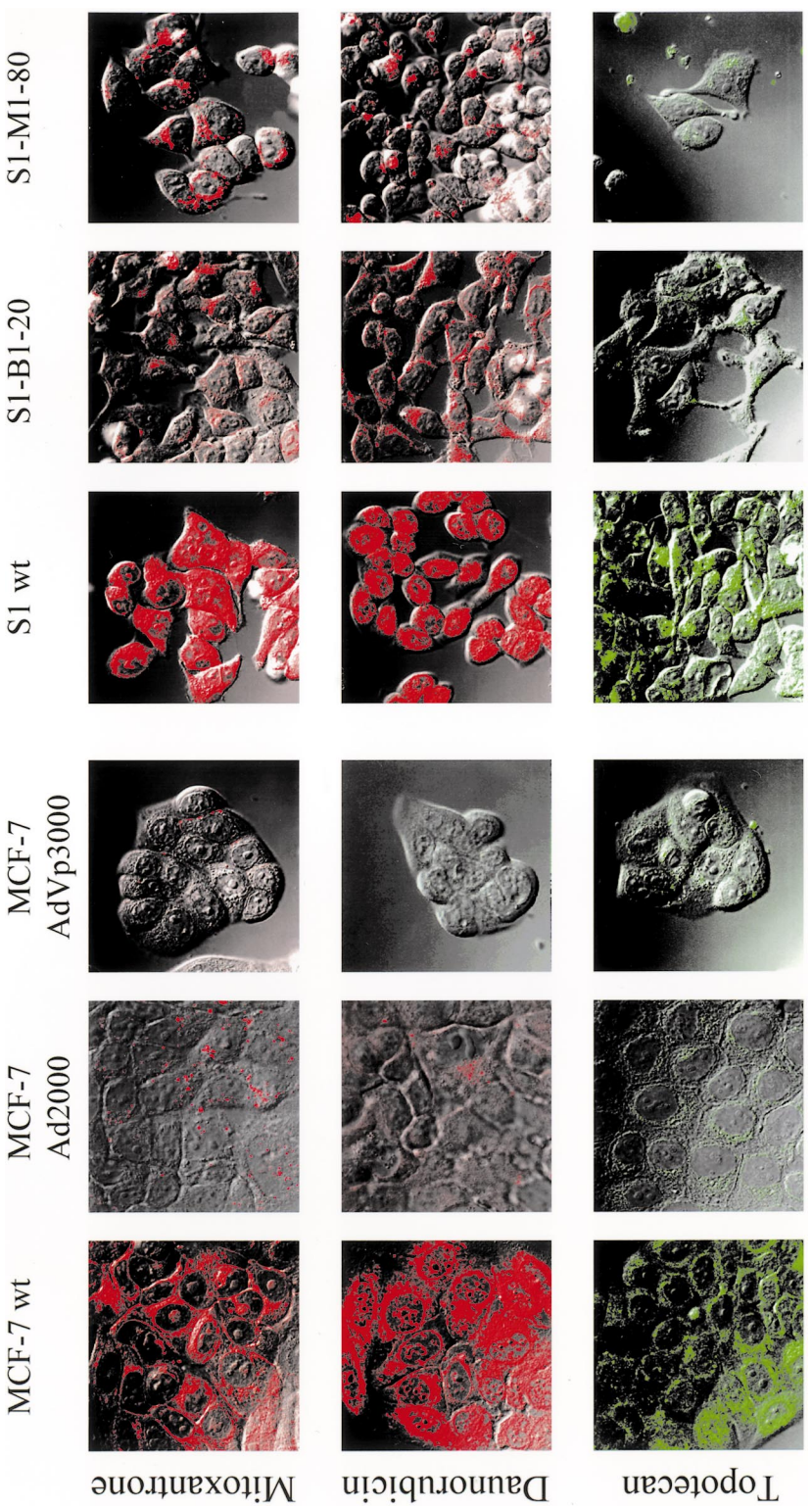

Fig. 1. Accumulation of MXR substrates in parental cell lines and multidrug-resistant sublines. Confocal microscopic images show reduced accumulation of mitoxantrone, daunorubicin, and topotecan in MDR1-overexpressing MCF-7 Ad2000 and S1-B1-20 cells and in MXR-overexpressing MCF-7 AdVp3000 and S1-M1-80 cells. To achieve steady-state concentrations, cells were incubated in 5 μM drug for 2 h at 37°C.

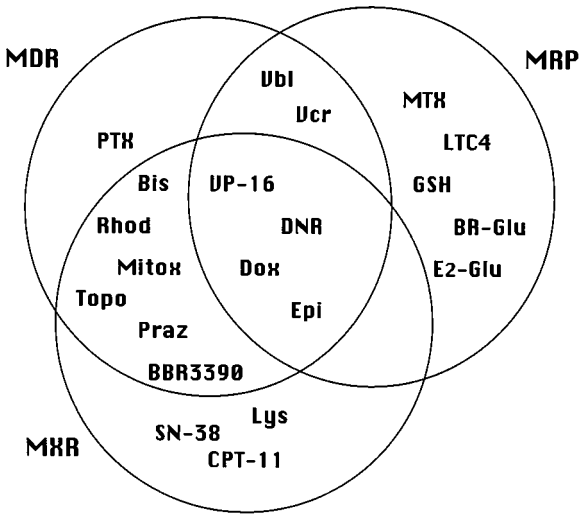

Fig. 2. Venn diagram showing overlapping substrate specificity for MDR1/Pgp, MRP, and MXR. Assignment was derived from published cross-resistance and drug accumulation data, as well as confocal microscopy data in cell lines shown in Fig. 1 (see text for references). Abbreviations: PTX, paclitaxel; Vbl, vinblastine; Vcr, vincristine; MTX, methotrexate; LTC4, leukotriene C4; GSH, glutathione; BR-Glu, bilirubin glucuronide; E2-Glu, 17β-glucuronosyl estradiol; VP-16, etoposide; DNR, daunorubicin; Dox, doxorubicin; Epi, epirubicin; Lys, lysotracker; CPT-11, irinotecan; SN-38, the active metabolite of CPT-11; Praz, BODIPY-prazocin; Topo, topotecan; Mitox, mitoxantrone; Rhod, rhodamine; Bis, bisantrene.

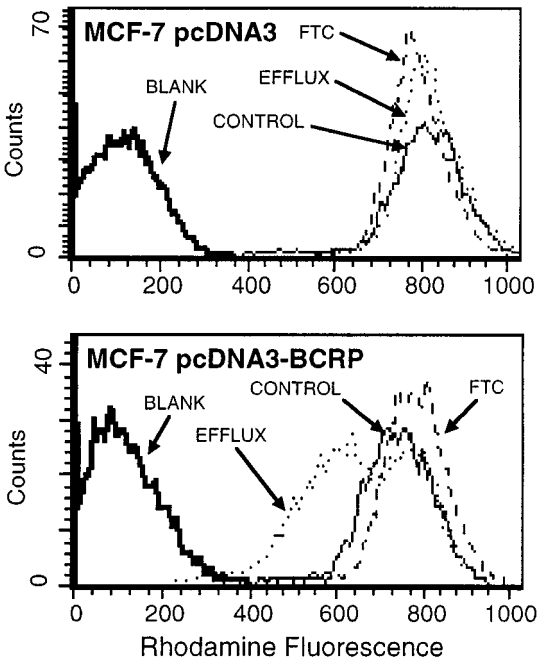

Fig. 3. Accumulation of rhodamine in BCRP-transfected and vector-control transfected MCF-7 cells. Following incubation for 30 min in media containing 0.5 μg/mL rhodamine 123, either without (Control, thin line) or with FTC, the cells were divided into two aliquots and either placed on ice (accumulation) or washed and resuspended in rhodamine-free medium for an additional 60 min at 37°C. These cells were continued either without (Efflux, dotted line) or with FTC (FTC, dashed line). Autofluorescence of cells incubated in media alone is also shown (Blank, thick line).

Mechanism of Overexpression of MXR

Southern analysis demonstrated amplification of MXR in the MCF-7 AdVp3000 cells, and no evidence of amplification in S1-M1-80 cells (Miyake *et al.*, 1999). Comparative genomic hybridization (CGH) confirmed a region of amplification in MCF-7 AdVp 3000 cells, in chromosome 4 (Knutsen *et al.*, 2000). This matched the assignment of an EST containing MXR (HUEST 157481) to chromsome 4q22–q23 (Allikmets *et al.*, 1996). Both whole chromsome paint and spectral karyotyping (SKY) demonstrated intact copies of chromosome 4 in both S1 and MCF-7 parent cell lines. However, in the MCF-7 AdVp3000 cells, whole chromosome 4 paint revelated multiple translocations of chromosome 4 in these cells, and a BAC probe containing MXR confirmed an amplification associated with one of the translocations. This amplification was located at the juncture of one translocation, t(4;5). Studies of the S1-M1-80 cells confirmed absence of amplification, but demonstrated a balanced translocation, t(4;17). The MXR BAC localized to 4q21–q22 in the normal chromosome 4, but to the breakpoint in the translocated 4. Figure 5 shows the results of CGH, whole chromosome paint, and BAC hybridization studies in the two cell lines. Amplification of the chromosome 4q21–q22 region can be seen in the CGH derived from MCF-7 AdVp3000 cells. With the BAC probe, an amplified region is labeled in the MCF-7 AdVp3000 cells while the breakpoint of the balanced translocation is labeled in the S1-M1-80 cells. These studies suggest that rearrangement of MXR results in overexpression through either loss of an inhibitor or that MXR comes under control of a more active promoter. Studies evaluating this question are underway.

Protein Characterization

MXR is predicted to be 655 amino acids, with 6 transmembrane domains, and 1 ATP-binding domain. The predicted organization is ATP-TM. Peptide antibodies were made against regions in the ATP-binding domain. One of these, 87405, was used for immunoblot analysis, revealing a 72 kDa protein. Figure 6 shows the results of immunoblot analysis for MXR. High levels of expression are observed in protein generated from S1-M1-80 and in

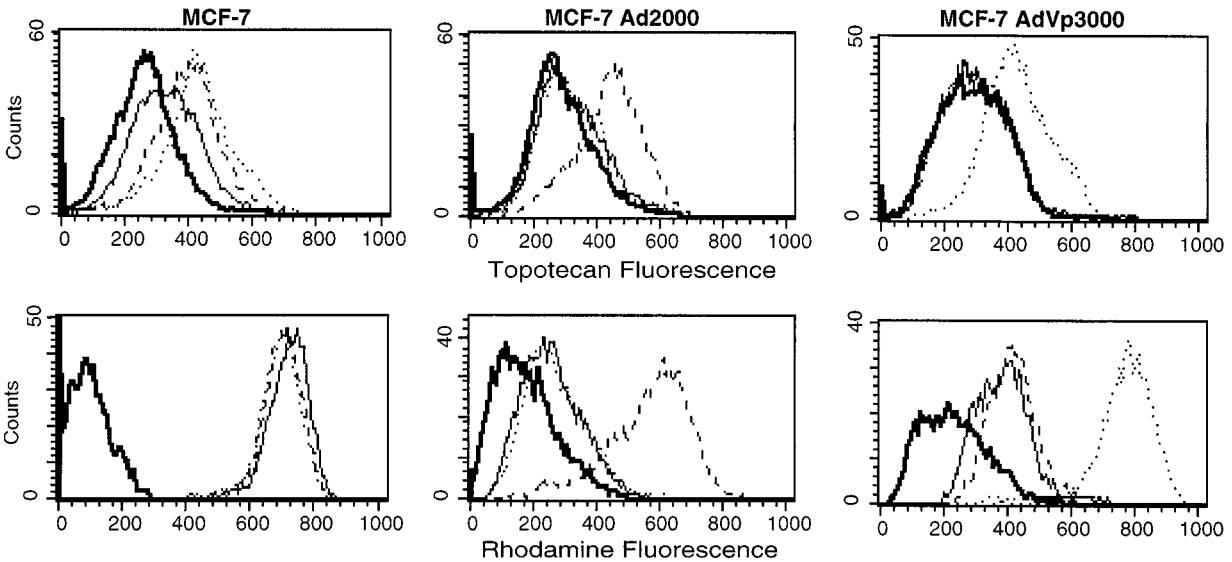

Fig. 4. Accumulation of rhodamine or topotecan in MCF-7 parental and drug-resistant sublines. Accumulation of the two substrates was performed as described in Fig. 3, except that only the histograms derived from the accumulation period are shown. Histograms are shown from cells incubated in media alone (thick line), in media containing rhodamine alone (thin line), in media containing rhodamine and the P-glycoprotein antagonist PSC 833 (dashed line), and in media containing rhodamine and the MXR-antagonist FTC (dotted line). Accumulation periods were 30 min at 37°C for all conditions.

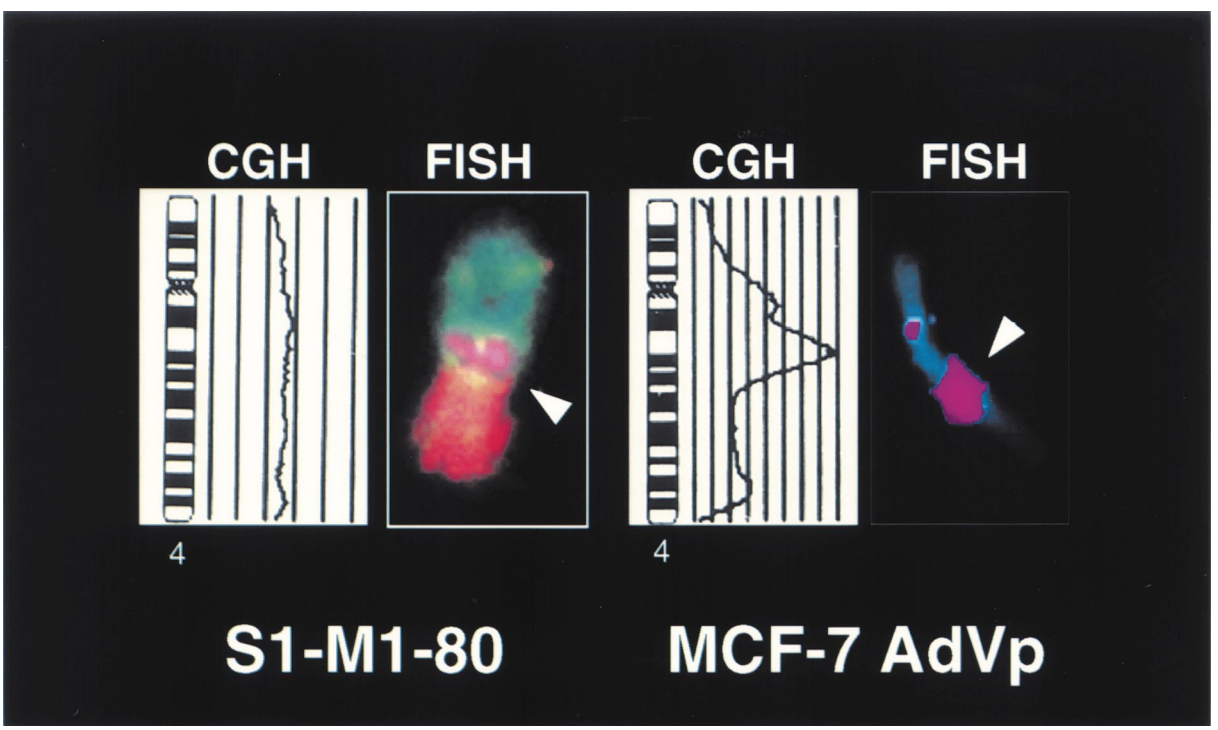

Fig. 5. Comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) studies in MXR-overexpressing S1-M1-80 and MCF-7 AdVp 3000 cells. DNA copy number changes are shown for chromosome 4 for the two sublines. Amplification peak at 4q21–q22 is noted in MCF-7 AdVp3000 cells. FISH performed with a BAC-derived MXR probe and whole chromosome paint shows the translocation in S1-M1-80. t(4;17)(q21-q22;p13); the MXR signal is split at the breakpoint. FISH with the BAC probe shows an amplified MXR signal in MCF-7 AdVp3000 cells (arrowhead).

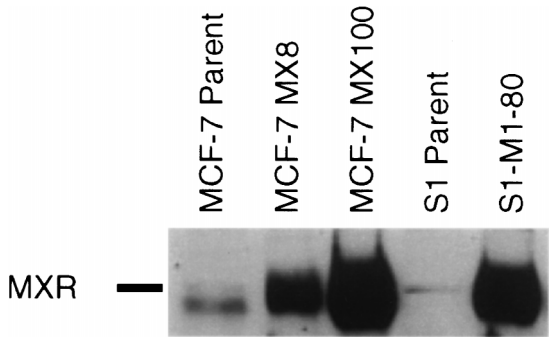

Fig. 6. Immunoblot analysis of MXR in resistant sublines. Using the 87405 polyclonal antibody to detect MXR, high levels of expression are observed in S1-M1-80 and MCF-7 MX100 cells, while MCF 7 MX8 cells have intermediate levels, MCF-7 parental cells have a low, detectable level, and S1 cells have no detectable level of MXR. Blots were incubated in a 1:3000 dilution of the polyclonal antibody.

MCF-7 MX100 cells, while an intermediate level of expression is observed in MX8 and a low level of expression in MCF-7 cells. No detectable expression is seen in the drug-sensitive S1 cell line. Immunohistochemical analysis using the 87405 antibody in S1-M1-80 and MCF-7 AdVp3000 localized the protein to the cell surface, as well as showed cytoplasmic staining (Litman *et al.*, 2000). Similar results were obtained by immunofluorescence using confocal microscopy in MCF-7 MX, a subline derived by stepwise selection in mitoxantrone by Schneider and co-workers (Nakagawa *et al.*, 1992; Rocchi *et al.*, 2000). Pre-adsorption of the antibody with peptide removed both cytoplasmic and plasma membrane staining, confirming specificity of the staining (not shown). Plasma membrane staining was also confirmed by Scheffer *et al.* (2000), using a monoclonal antibody raised in mice injected with MCF-7 MX cells. While these results do not exclude the possibility that MXR may be localized in a subcellular compartment in some cell types, they do show that in the drug-resistant cell lines used to date, MXR can be found on the cell surface.

SUMMARY

MXR/BCRP/ABCP1 has been cloned from both human and mouse mitoxantrone-resistant cell lines. Resistance is mediated by ATP-dependent drug transport, which reduces the intracellular accumulation of substrates including mitoxantrone, doxorubicin, daunorubicin, epirubicin, epidophyllotoxin, and the camptothecin analogues SN-38 and topotecan. A 72 kDa protein, MXR is localized to the plasma membrane in multidrug-resistant cells. The mechanism of overexpression was found to be gene amplification in two cell lines, and gene rearrangement in a third cell line.

FUTURE DIRECTIONS

As the first half-transporter implicated in multidrug resistance, much remains to be learned about MXR/BCRP/ABCP1. A central question is that of dimerization partners, which can be predicted based on the observation that other half-transporter families have multiple members. In the *Drosophila white* gene family, White dimerizes with the *brown* and *scarlet* gene products. Null mutations in *white* result in absence of eye pigment; mutations in *brown* or *scarlet* result in altered eye color because of lack of transport of either guanine or tryptophan, needed for eye pigment synthesis (Ewart and Howells, 1998). The human TAP ABC transporters TAP1 and TAP2 form heterodimers to transport peptides into the endoplasmic reticulum for loading onto the MHC type I complex for antigen presentation (Abele and Tampe, 1999). A loss of TAP gene expression results in impaired class I antigen presentation. The second human half-transporter family includes the adrenoleukodystrophy protein (ALDP) and the related proteins ALDR, PMP69, and PMP70 (Liu *et al.*, 1999). Mutations in ALDP result in abnormal oxidation of lipids because of the failure of the lipids to enter the peroxisome for beta-hydroxylation. The proteins are localized to the peroxisome, where it is known that they can heterodimerize, since overexpression of any family member can complement for loss of another. However, whether or not heterodimers occur under normal conditions is not known. For MXR, no partner has yet been discovered, although a mouse gene has been identified, *abcp2*, with significant homology to the *abcp1* mouse orthologue for MXR (Dean, manuscript submitted). Studies aimed at identifying a partner for MXR through a traditional co-immunoprecipitation strategy are underway.

A second major question for MXR is that of normal function. Localization in normal human tissues will help deduce the role for MXR. Expressed in the placenta at the surface of the chorionic villus (according to preliminary studies), it may be involved in transport across placental epithelium into fetal vessels if oriented according to the model for the *Drosophila white* gene family products (Ewart and Howells, 1998). Otherwise, it may play a role in elimination of fetal waste products, or protecting the fetus from potential toxins in the maternal bloodstream. Preliminary studies also suggest localization in the small intestine. If confirmed, then that localization would suggest a role akin to that described for P-glycoprotein in protection of normal tissues from xenobiotics.

A third question, and the most important from the perspective of multidrug resistance, is clinical relevance. Clinical investigators have suggested that "other mechanisms" of resistance explain the failure of P-glycoprotein antagonists to overcome clinical drug resistance. Expression of MXR in tumor tissue, and upregulation after treatment could easily confound the results of Pgp reversal trials, particularly since these studies have used substrates which the two transporters have in common (Ross, 2000). It will be important to use both protein and RNA methods to examine tumor tissue, and to look at tumor samples before and after treatment. Lessons learned from Pgp reversal trials can be applied to the development of MXR antagonists, avoiding problems which rendered many of the early trials difficult to interpret (Beck *et al.*, 1996; Sandor *et al.*, 1998). One major source of problems for the Pgp reversal trials was the disappointment that followed high expectation borne out of an overly enthusiastic assessment of the independent contribution of Pgp to clinical drug resistance. MXR may allow us to return some of that enthusiasm to the clinical investigation of multidrug resistance reversal.

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NOTE ADDED IN PROOF

Recent studies have shown that Mutations Resulting in an altered amino acid at position 482: R482G and R482T in S1-M1-80 and MCF-7AdVp3000, respectively, result in altered substrate specificity.

Rhodamine and daunorubicin are substrates for MXR containing G482 or T482, and not for the wild type R482. Reference: Honjo Y *et al.* Cancer Res 61: 6635–6639, 2001.

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