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A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2)

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Abstract

The fluorescent compounds rhodamine 123, LysoTracker Green DMD-26, mitoxantrone, and BODIPY-prazosin were used with the antagonist fumitremorgin C (FTC) in order to develop functional assays for the half-transporter, MXR/BCRP/ABCP1. A measure of FTC-inhibitable efflux was generated for each compound in a series of MXR-overexpressing drug-selected cell lines and in ten unselected cell lines which were used to determine if the four fluorescent compounds were sensitive enough to detect the low MXR levels found in drug-sensitive cell lines. FTC-inhibitable efflux of mitoxantrone and prazosin was found in four of the ten cell lines, SF295, KM12, NCI-H460, and A549, and low but detectable levels of MXR mRNA were also observed by Northern analysis in these cells. FTC-inhibitable mitoxantrone and prazosin efflux in both selected and unselected cell lines was found to correlate well with MXR levels as determined by Northern blotting, $r^2 = 0.89$ and $r^2 = 0.70$ respectively. In contrast, rhodamine and LysoTracker were not able to reliably detect MXR. Cytotoxicity assays performed on two of the four unselected cell lines confirmed increased sensitivity to mitoxantrone in the presence of FTC. FTC was found to be a specific inhibitor of MXR, with half-maximal inhibition of MXR-associated ATPase activity at 1 μM FTC. Short term selections of the SF295, KM12, NCI-H460 and A549 cell lines in mitoxantrone resulted in a small but measurable increase in MXR by both Northern blot and functional assay. These studies show that flow cytometric measurement of FTC-inhibitable mitoxantrone or prazosin efflux is a sensitive and specific method for measuring the function of the MXR half-transporter in both selected and unselected cell lines.

Keywords: MXR/ABCP1/BCRP; Mitoxantrone; Adenosine 5′-triphosphate-binding cassette transporter; Drug resistance

1. Introduction

In cancer cells, the multidrug resistance phenotype is often associated with overexpression of members of the ATP-binding cassette (ABC) transporter family of proteins. P-glycoprotein (Pgp) and the multidrug resistance-associated protein (MRP) are two of the most extensively studied ABC transporters and have been shown to actively transport a wide variety of cytotoxic agents [1,2]. More recently, an ABC half-transporter protein, the mitoxantrone resistance protein, MXR, was cloned from colon cells selected in high levels of mitoxantrone [3]. Except for minor amino acid differences, MXR is identical to the

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breast cancer resistance protein (BCRP), which was found to be highly overexpressed in breast cancer cells selected in adriamycin in the presence of verapamil [4]; and to the placental ABC protein (ABCP1) which was found by examination of expressed sequence tag databases [5]. MXR/BCRP/ABCP1 confers high levels of resistance to mitoxantrone as well as to the anthracyclines, the camptothecins, topotecan, and SN-38 [3,4,6–8]. Since MXR is a half-transporter, it is thought that dimerization is required for activity [9]. It has high homology with the *Drosophila* white gene which encodes a half-transporter involved in the transport of precursors to eye pigment. The Human Gene Nomenclature Committee has proposed that MXR/BCRP/ABCP1 be renamed ABCG2 (http://www.gene.ucl.ac.uk/users/hester/abc.html).

The ability of ABC transporters to actively transport compounds against a concentration gradient across the cell membrane has allowed the development of a number of functional assays to measure the level and function of transporter present. The efflux of fluorescent compounds from cells expressing ABC proteins can be quickly and easily measured by flow cytometry. Many fluorescent compounds have been used to characterize Pgp, although rhodamine 123 seems to be one of the most widely used [10,11]. When used in conjunction with a Pgp blocker such as cyclosporin A or PSC 833, rhodamine 123 efflux has been shown to correlate well with Pgp expression in unselected cell lines [12]. MRP-mediated transport in cells has been measured similarly by using calcein AM [13,14]. These assays have been most widely developed in leukemia where functional measures of Pgp and MRP have correlated well with mRNA expression levels [15,16].

A fluorescent substrate specific for MXR alone has yet to be identified. Rhodamine 123 efflux has been previously shown in cell lines which express MXR, and cells transfected with the BCRP gene have been shown to transport rhodamine as well [5]; however, it is not known how sensitive rhodamine 123 is in detecting low levels of MXR. A marked decrease in mitoxantrone accumulation has also been shown in cells expressing high levels of MXR [3,4,8]. A fluorescent aza-anthrapyrazole, BBR 3390, was also recently shown to be a substrate of BCRP [17]. In a search for specific substrates for MXR, we have also noted decreased accumulation of the acidic organelle probe LysoTracker Green DND-26, and BODIPY-prazosin in MXR-overexpressing cells [8]. Recently, fumitremorgin C (FTC) [18,19], an extract of *Aspergillus fumigatus*, and GF120918 [20], a compound first developed as a Pgp inhibitor, have been shown to inhibit MXR. Based on other flow cytometry models used for detection of Pgp and MRP, it should be possible to develop a functional assay for MXR.

We sought to develop a flow cytometric assay which would accurately reflect the high levels found in drug selected cell lines, as well as be sensitive enough to detect low levels of MXR such as would be found in unselected cell lines and clinical samples. We assessed the ability of four fluorescent compounds – rhodamine 123 (rhodamine), LysoTracker Green DND-26 (LysoTracker), BODIPY-prazosin (prazosin), and mitoxantrone – used in combination with FTC to detect MXR in unselected cell lines as well as several drug-selected cell lines expressing varying amounts of MXR. Additionally, we wished to determine if efflux of these fluorescent compounds could be correlated with MXR expression at the RNA level.

2. Materials and methods

Fumitremorgin C was synthesized by Thomas McCloud, Developmental Therapeutics Program, Natural Products Extraction Laboratory, National Institutes of Health (Bethesda, MD). Mitoxantrone and rhodamine 123 were purchased from Sigma (St. Louis, MO, USA). BODIPY-prazosin and LysoTracker Green DMD-26 were obtained from Molecular Probes (Eugene, OR, USA).

2.1. Cell lines

The cell lines comprising the National Cancer Institute Anticancer Drug Screen were obtained as described previously [21]. The human colon carcinoma cell line S1 and its MXR-overexpressing subline S1-M1-3.2 were obtained from Dr. Lee M. Greenberger (Wyeth-Ayerst) [18]. The S1-M1-80 subline was generated in our lab by exposing the S1-M1-3.2 cell line to increasing concentrations of mitoxantrone [3]. The S1-M1-3.2 and S1-M1-80 cell lines

were maintained in 3.2 μM and 80 μM of mitoxantrone, respectively. The Pgp-overexpressing line S1-B1-20 was also obtained from Dr. Greenberger and was maintained in 20 μM bisantrene [22].

Several MXR-overexpressing MCF-7 derivative cell lines were also used in our study. The MCF-7 AdVp3000 cell line was derived by stepwise selection and was maintained in 3000 ng/ml adriamycin respectively, in the presence of 5 μg/ml verapamil; the MCF-7 AdVp10 cell line was an early selection step maintained in 10 ng/ml adriamycin and 5 μg/ml verapamil [23]. The MCF-7 MX8 cells were selected in a single step and maintained in 8 ng/ml mitoxantrone. The MCF-7 MX20, MX40, MX80 and MX100 sublines were independently selected in our laboratory in a stepwise manner. The sublines are carried in 20, 40, 80 and 100 ng/ml mitoxantrone respectively. The MRP-overexpressing MCF-7/VP cell line was obtained from Dr. Kenneth Cowan [24], and was maintained in 4 μM etoposide.

The drug screen cells as well as S1 and derivative sublines were maintained in RPMI; MCF-7 cells and resistant sublines were maintained in IMEM; and both were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Resistant cell lines were maintained in the selecting drug at the indicated concentrations and all cells were kept at 37°C in 5% CO_2.

2.2. Microsomal membrane preparation

Microsomal membranes were prepared as previously described [25]. Briefly, cells were washed with PBS before being resuspended in 10 ml lysis buffer (HEPES-Tris, 10 mM; EDTA, 5 mM; EGTA, 5 mM; dithiothreitol, 2 mM; pH 7.4) containing protease inhibitors (phenylmethylsulfonyl fluoride, 2 mM; aprotinin, 5 μg/ml; pepstatin, 10 μg/ml; leupeptin, 10 μg/ml). This and all subsequent steps were performed at 4°C. Cells were lysed by nitrogen cavitation (Parr Instrument, Moline, IL, USA) at 1200 psi for 20 min. Nuclei were sedimented by centrifugation at $300\times g$ for 10 min, mitochondria were removed by centrifugation at $4000\times g$ for 10 min and in a final centrifugation ($45\,000\times g$, 60 min), the microsomal fraction was sedimented. The pellet was resuspended in 1 ml of lysis buffer and homogenized by aspiration ten times through a 27 gauge syringe. Aliquots of 150 μl (containing 1–2 mg protein/ml) were stored at −80°C until analysis.

2.3. ATPase assay

MXR-associated ATPase activity was determined according to Borgnia et al. [26], by quantitating the release of inorganic phosphate from ATP, using a sensitive, colorimetric assay originally described by Chifflet et al. [27] and later modified by Doige et al. [28]. The microsomes were thawed on ice before diluting to a protein concentration of 20 μg/ml in ice-cold ATPase assay medium (ATP, 3 mM; KCl, 50 mM; $MgSO_4$, 2.5 mM; dithiothreitol, 3 mM; Tris-HCl, 25 mM; pH 7.0) containing 0.5 mM EGTA (to inhibit Ca-ATPases), 2 mM ouabain (to inhibit the (Na+K)-ATPase), and 3 mM azide (to inhibit mitochondrial ATPase). Each series of experiments was carried out in a 96-well microtiter plate, with reaction volumes of 50 μl/well corresponding to 1 μg protein/well. Incubation with FTC at various concentrations was started by transferring the plate from ice to 37°C for 1 h, and terminated by addition of 200 μl ice-cold stopping medium (ammonium molybdate, 0.2% (w/v); sulfuric acid, 1.3% (v/v); SDS, 0.9% (w/v); trichloroacetic acid, 2.3% (w/v); freshly prepared ascorbic acid, 1% (w/v)) to each well. After 30 min incubation at room temperature, the released phosphate was quantitated colorimetrically in a microplate reader (Bio-Tek Instruments, VT, USA) at 620 nm. Background and control experiments with ATPase assay buffer alone, samples incubated on ice, and samples incubated with 500 μM vanadate (inhibiting the MXR ATPase) were obtained in parallel and subtracted from the measurements.

2.4. Quantitative PCR and Northern blot

RNA was prepared using RNA STAT-60 according to the manufacturer's instructions (Tel-Test, Friendswood, TX, USA). Northern blotting was performed as previously described [3]. Quantitative PCR was performed as previously described [29] using the following primers: 5′ primer: 5′-TGC CCA GGA CTC AAT GCA AC-3′; 3′ primer: 5′-GAC TGA AGG GCT ACT AAC C-3′.

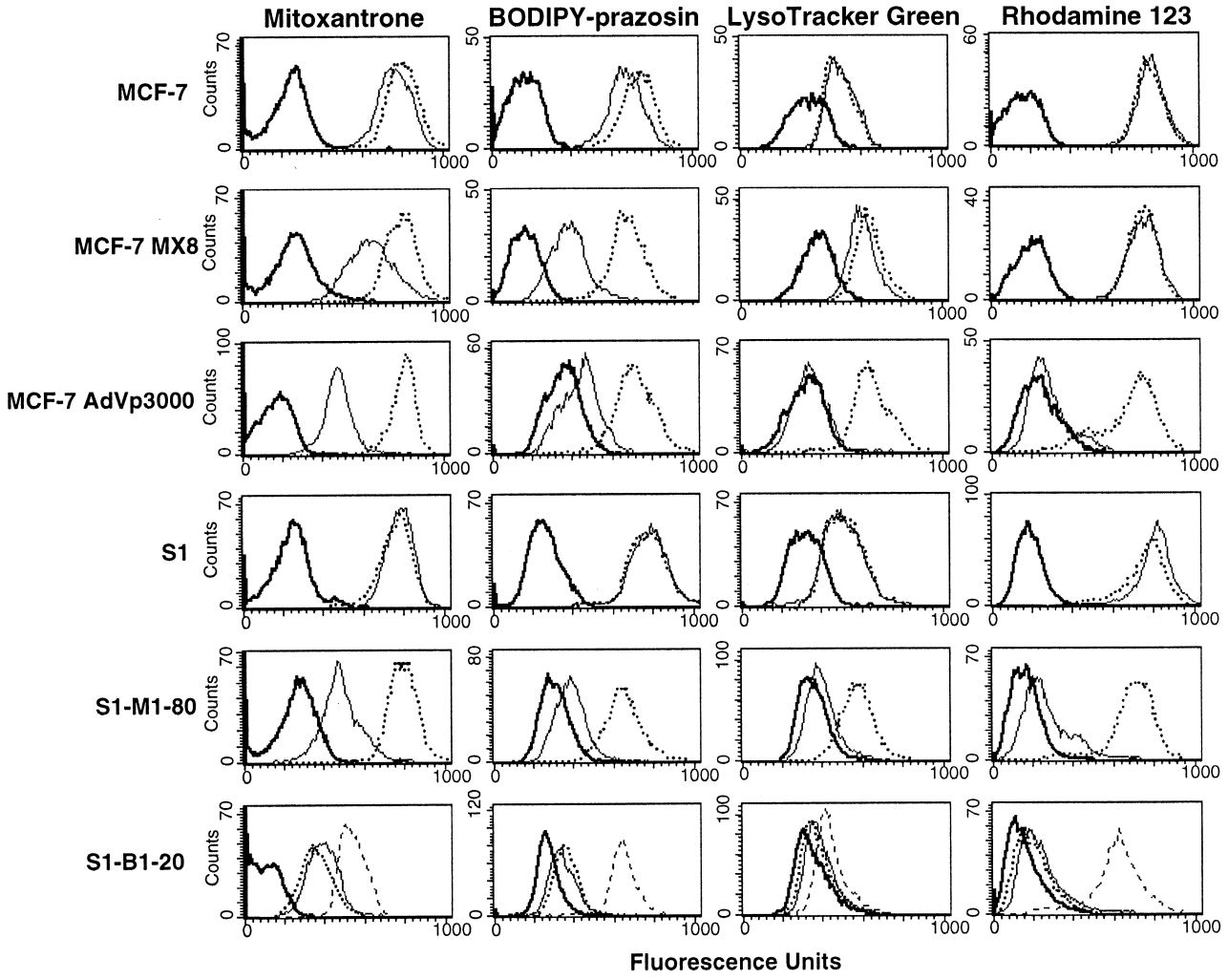

Fig. 1. Functional assay for MXR expression in selected cell lines. Efflux assays were performed with the four fluorescent compounds in each of the selected cell lines. Representative histograms are shown for four of the selected cell lines. Cells were incubated for 30 min with medium alone (heavy solid line); incubated with 0.5 μg/ml rhodamine, 100 nM LysoTracker, 250 nM prazosin, or 20 μM mitoxantrone, washed, then allowed to incubate for 60 min in medium alone (thin solid line); or incubated with 0.5 μg/ml rhodamine, 100 nM LysoTracker, 250 nM prazosin, or 20 μM mitoxantrone in the presence of 10 μM FTC, washed, then allowed to incubate for 60 min in medium containing 10 μM FTC (dotted line). For the S1-B1-20 cell line, cells were additionally incubated with 0.5 μg/ml rhodamine, 100 nM LysoTracker, 250 nM prazosin, or 20 μM mitoxantrone in the presence of 3 μg/ml PSC 833, washed, then allowed to incubate for 60 min in medium containing 3 μg/ml PSC 833 (dashed line).

Autoradiograms or ethidium-stained gels were captured and quantitated using the IP Lab Gel program v 2.2 (Scanalytics, Fairfax, VA, USA).

2.5. *Efflux assays*

The efflux assays were based on those previously described with minor modifications [12]. Suspensions of log phase cells were obtained by trypsinization and transferred to round-bottom 96-well plates. The cells were resuspended in complete medium alone (phenol red-free IMEM with 10% FCS), or complete medium containing the desired fluorescent compound (0.5 μg/ml rhodamine 123, 100 nM LysoTracker Green DMD-26, 250 nM BODIPY-prazosin, or 20 μM mitoxantrone) with or without 10 μM FTC and incubated at 37°C in 5% CO_2 for 30 min. Cells in complete medium alone yielded the Blank

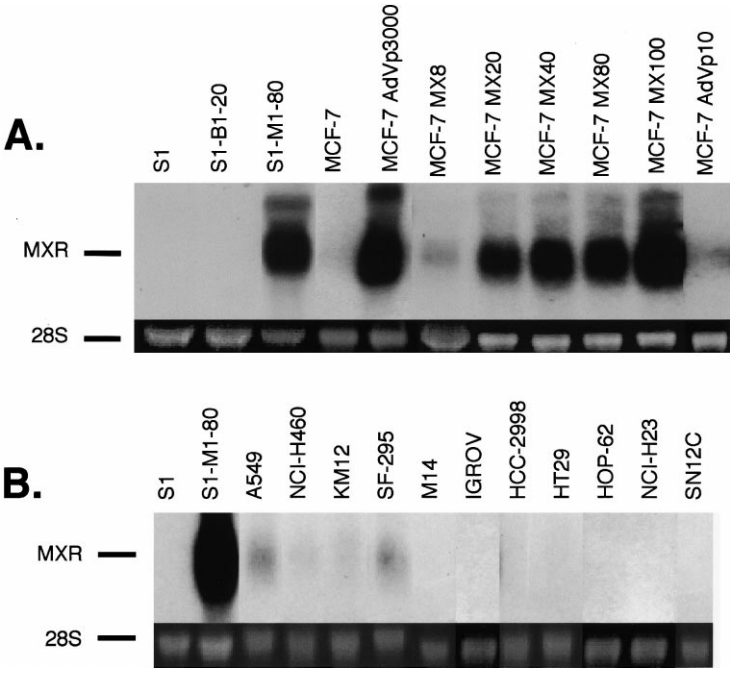

Fig. 2. Expression of MXR in parental and drug-selected cell lines. Northern blot analysis of MXR expression in (A) drug-selected cell lines and (B) the 11 unselected cell lines was performed as outlined in Section 2.

histogram which is a measure of cell autofluorescence, while cells in complete medium with a fluorescent compound or complete medium with a fluorescent compound and FTC generated the Control and FTC histograms, respectively. After the 30 min incubation period, the cells were washed with ice-cold complete medium and either placed on ice in the dark, or were resuspended in complete medium, continuing with or without 10 μM FTC and allowed to incubate for 1 additional hour at 37°C in 5% CO_2. The Efflux histogram was generated from cells which were incubated 30 min with a fluorescent compound and then allowed to efflux for 1 h in complete medium alone; the FTC/Efflux histogram was obtained from cells which were incubated 30 min with a fluorescent compound and FTC, then incubated in complete medium and FTC for 1 h. The cells were then washed in cold DPBS and placed on ice. A FACSort flow cytometer with a 488 nm argon laser and 530 nm bandpass filter was used to detect rhodamine, LysoTracker, and prazosin fluorescence, while a FACSCalibur flow cytometer equipped with a 635 nm red diode laser and 670 nm bandpass filter was used to detect mitoxantrone fluorescence. At least 10 000 events were collected. Debris was eliminated by gating on forward versus side scatter and dead cells were excluded based on propidium iodide staining. The mean channel number for each histogram was used as the measure of fluorescence for calculation of efflux values.

2.6. Cytotoxicity assay

The cytotoxicity assays were performed using the sulforhodamine method previously described [30]. Briefly, cells were plated in flat-bottom 96-well plates at a density of 2000 cells per well and allowed to attach for 24 h at 37°C in 5% CO_2. Mitoxantrone at various concentrations with or without FTC at a concentration of 5 μM was added to the cells and allowed to incubate at 37°C in 5% CO_2 for 96 h. After incubation, the cells were fixed in 50% TCA and stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid). Optical densities were read on a Bio-Rad plate reader at an absorbance of 540 nm. Each concentration was tested in triplicate and controls were done in replicates of eight.

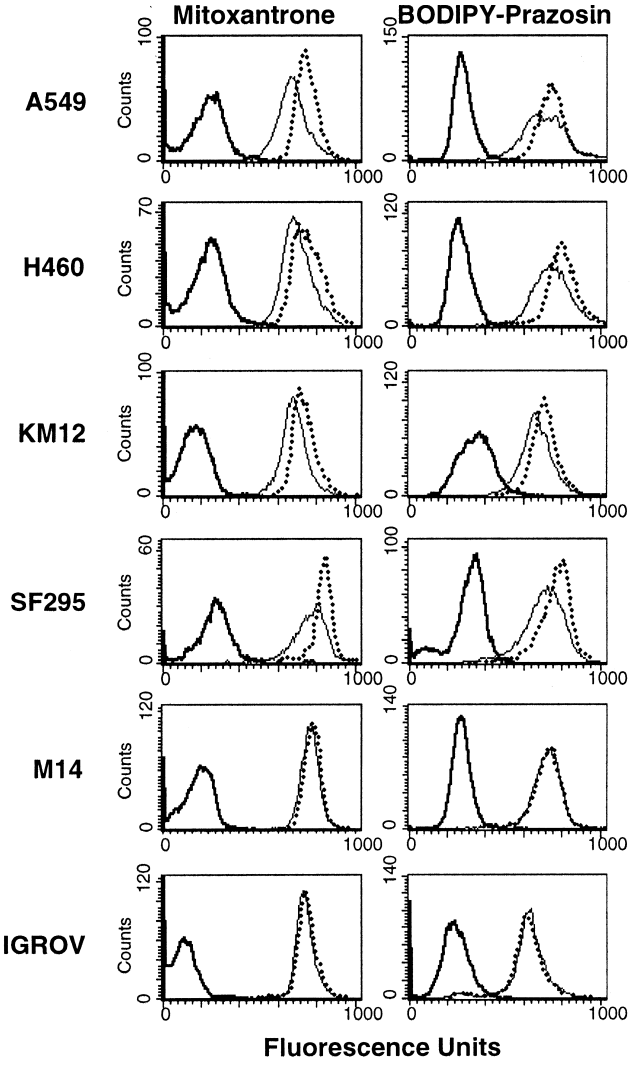

Fig. 3. Functional assay for MXR expression in unselected cell lines. Efflux assays were carried out on the 11 unselected cell lines with the four fluorescent compounds as outlined in Fig. 1: heavy solid line, Blank; thin solid line, Efflux; dotted line, FTC/Efflux.

3. Results

3.1. Functional assay for MXR in selected cell lines

We first examined the intracellular fluorescence of four fluorescent compounds by flow cytometry in a series of multidrug-resistant cell lines. Previous studies by confocal microscopy had demonstrated reduced intracellular accumulation of mitoxantrone, prazosin, LysoTracker and rhodamine, among others, in two selected sublines with MXR overexpression [8]. Each cell line was tested at least twice with each compound; histograms in four drug-selected cell lines are shown in Fig. 1. Cells were incubated, as described in Section 2, with or without the addition of FTC during the accumulation period and then washed and continued with or without FTC for an efflux period. Data were collected following both the accumulation period and the efflux period and comparable results were obtained with both; only the post-efflux histograms are presented in Fig. 1. The difference between the mean channel number for the FTC/Efflux histogram (dotted line) and the Efflux histogram (solid line) yielded the most reproducible values. This difference in mean channel numbers was termed 'FTC-inhibitable efflux' and was used in subsequent correlations with MXR expression. In the MXR-overexpressing MCF-7 AdVp3000 and S1-M1-80 cells, a high level of FTC-inhibitable efflux is noted with all of the compounds. In the MCF-7/MX8 cells, which overexpress MXR at a slightly lower level, mitoxantrone and prazosin efflux was detected, while efflux of LysoTracker and rhodamine was not. In the parental cell line, S1, no efflux was noted, and in the parental MCF-7 cells a slight but reproducible amount of FTC-inhibitable mitoxantrone and prazosin efflux was observed. In the Pgp-overexpressing line S1-B1-20, the Pgp antagonist PSC 833 (dashed line) was able to inhibit efflux of all of the compounds except LysoTracker; however, there was no effect of FTC. Likewise, no FTC-inhibitable efflux was noted in the MRP-overexpressing subline MCF-7/VP with any of the compounds studied (data not shown). There was significant quenching of LysoTracker which may account for the small values of FTC-inhibitable LysoTracker efflux in some of the cell lines.

3.2. MXR mRNA expression in selected and unselected cell lines

With the aim of correlating MXR levels with the functional assay, MXR mRNA expression was measured by Northern blot analysis in the selected cell lines with the results presented in Fig. 2A. Extending previous reports, high levels were found in S1-M1-80 and MCF-7 AdVp3000 cells. High levels were also noted in MCF-7/MX100 cells, intermediate levels

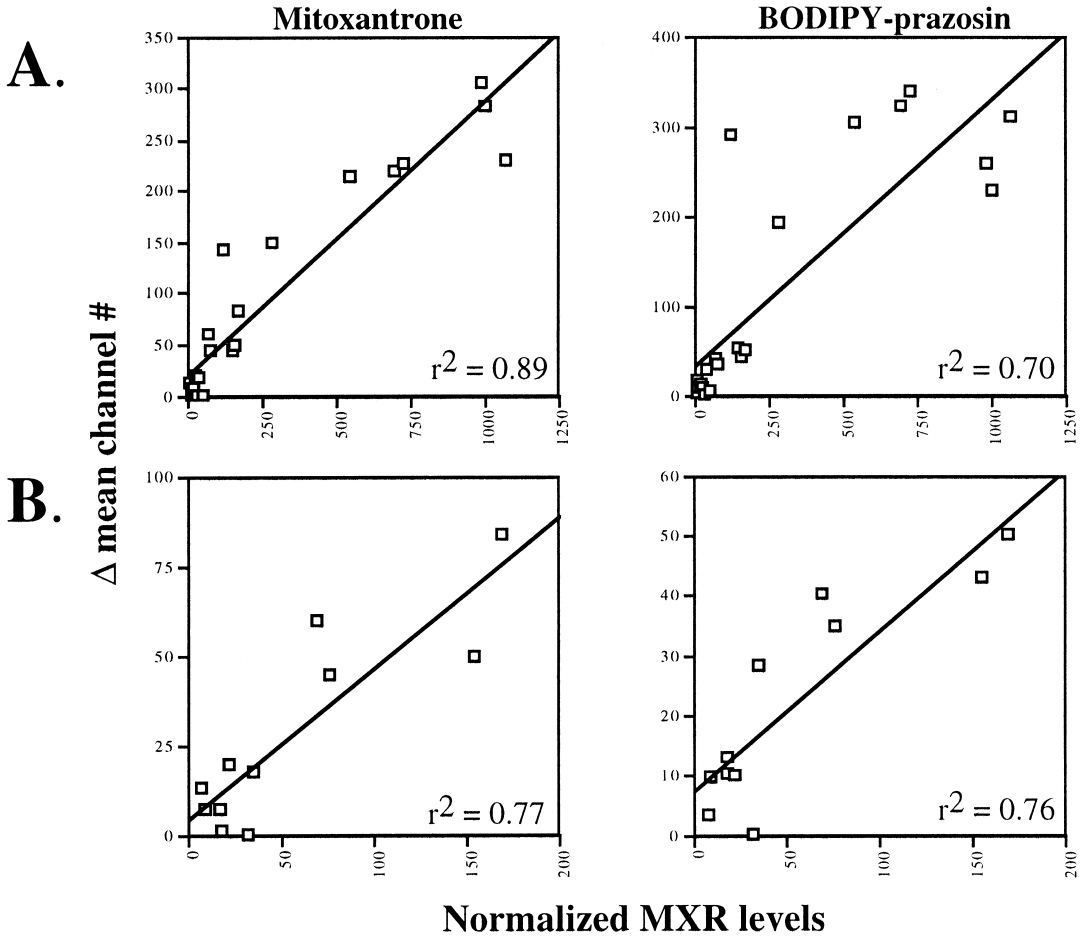

Fig. 4. Correlation of MXR expression with efflux of the two fluorescent compounds. The difference in mean channel number between the FTC/Efflux and Efflux histograms for mitoxantrone and prazosin was calculated for each cell line studied and plotted against normalized MXR expression values as determined by Northern blot in (A) all cell lines studied or (B) the 11 unselected cell lines alone.

were found in MCF-7 MX8 and MCF-7 AdVp10 cells, a very low level was seen in MCF-7 parental cells and no MXR was detected in S1 or S1-B1-20 cells.

Our next step was to determine whether any of the fluorescent compounds would be sensitive enough to detect low levels of MXR such as would be found in unselected cell lines. MXR levels were determined by quantitative PCR (data not shown) in 54 of the 60 cell lines which comprise the National Cancer Institute Anticancer Drug Screen. From this initial screening ten cell lines were identified that had MXR mRNA levels above the mean, including SF-295, M14, SN12C, KM12, HCC-2998, HT29, HOP-62, NCI-H460, A549, and NCI-H23 cells. One cell line without detectable MXR expression, IGROV, was also included as a negative control. A Northern analysis of these cell lines is shown in Fig. 2B. In the 11 drug screen cell lines, MXR expression was detectable by Northern in SF295, KM12, NCI-H460 and A549 cells. S1 parental and S1-M1-80 cells are shown for comparison.

3.3. Functional assay for MXR in unselected cell lines

Functional assays with the four compounds were performed in the 11 drug screen cell lines. Fig. 3 presents histograms for mitoxantrone and prazosin for the SF295, KM12, NCI-H460, A549, M14, and IGROV cell lines. A notable and reproducible difference in mean channel values for cells allowed to efflux in the presence or absence of FTC was seen with

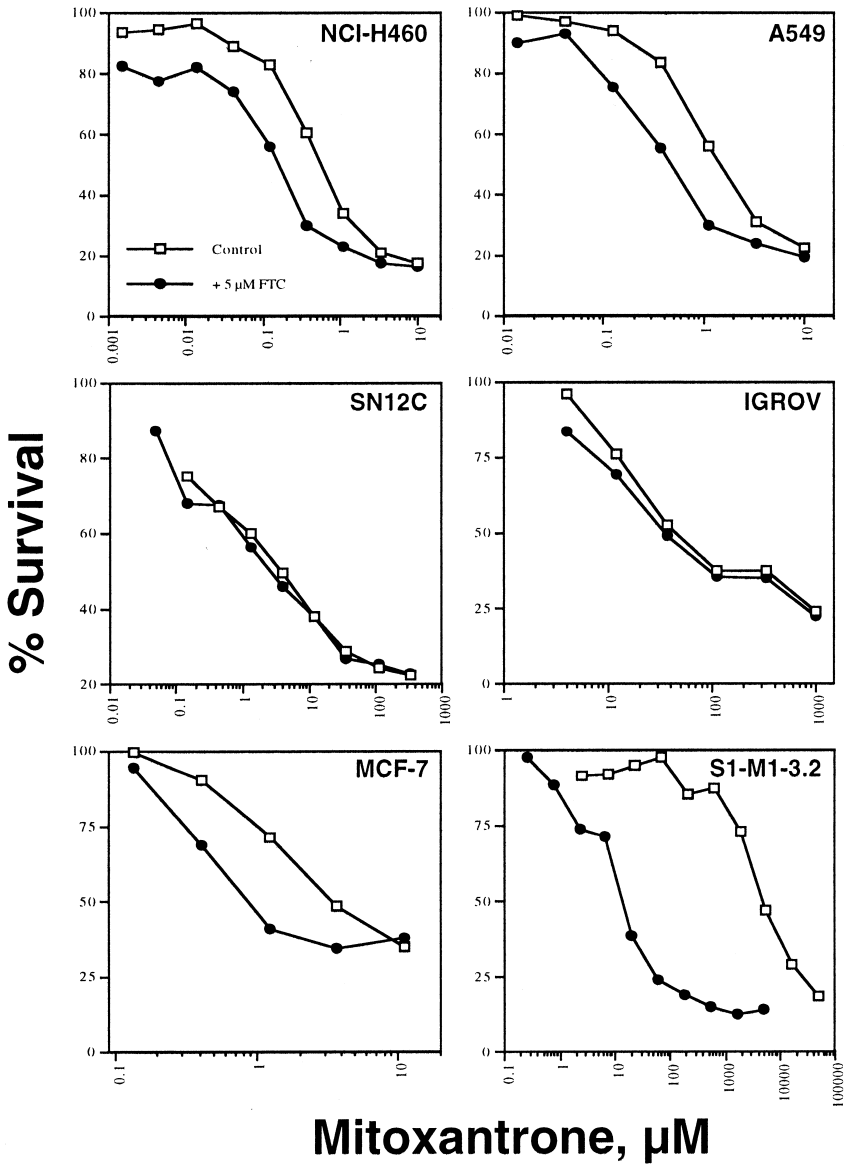

Fig. 5. Sensitization of unselected cell lines to mitoxantrone by FTC. Cytotoxicity assays were performed using mitoxantrone alone (□) or mitoxantrone in the presence of 5 μM FTC (●) on the NCI-H460, A549, SN12C, IGROV, MCF-7 cell lines. S1-M1-3.2 cells are shown for comparison.

mitoxantrone and prazosin in the unselected cell lines SF295, KM12, NCI-H460 and A549, but not in the remaining cell lines which is in agreement with MXR mRNA expression levels in these cells.

3.4. Correlation of MXR expression with efflux of fluorescent compounds

To determine whether the extent of FTC-inhibitable efflux could be related to MXR expression, the difference in mean channel numbers between the FTC/Efflux and Efflux histograms was correlated with MXR mRNA expression values obtained from Northern blots from all drug-selected and unselected cell lines shown in Fig. 2. When mitoxantrone efflux was plotted against MXR expression in both selected and unselected cell lines, a good correlation ($r^2 = 0.89$) was obtained; similar results were obtained with prazosin efflux ($r^2 = 0.70$) (Fig. 4A). FTC-inhibitable LysoTracker and rhodamine did

not correlate well in the selected cell lines, and were unable to detect low levels of MXR in the unselected cells (data not shown). In fact, some of the lines with high MXR levels did not exhibit any rhodamine efflux at all. As shown in Fig. 4B, FTC-inhibitable mitoxantrone and prazosin efflux also correlated well with MXR expression in the unselected cell lines alone, with $r^2 = 0.77$ and $r^2 = 0.76$, respectively. LysoTracker and rhodamine were not able to detect the low levels of MXR in these cells, $r^2 = 0.07$ and $r^2 = 0.03$ respectively (data not shown).

3.5. Sensitization of unselected cell lines to mitoxantrone by FTC

Since the flow cytometry data showed that FTC was able to increase mitoxantrone accumulation in some of the unselected cell lines, the ability of FTC to sensitize cells to mitoxantrone in a 4 day cytotoxicity assay was evaluated. Cytotoxicity experiments were performed on three cell lines with FTC-inhibitable mitoxantrone efflux, A549, NCI-H460 and MCF-7, on one of the cell lines which showed no FTC-reversible mitoxantrone efflux, SN12C, and on the negative control cell line IGROV. As seen in Fig. 5, the NCI-H460, A549 and MCF-7 cell lines were sensitized 3–4-fold to mitoxantrone. The SN12C cell line showed no increased sensitivity to mitoxantrone in the presence of FTC which is in agreement with the mitoxantrone and prazosin efflux data. In the negative control cell line, IGROV, no further sensitization to mitoxantrone was seen. For comparison, results with the S1-M1-3.2 cells are presented, with FTC able to sensitize these cells 400-fold.

3.6. Kinetics of MXR inhibition by FTC

To evaluate the kinetics of the inhibition of MXR by FTC, the effects of FTC on ATPase activity in membranes isolated from S1-M1-80 cells were studied. As Fig. 6 demonstrates, FTC is able to inhibit MXR-associated ATPase activity, with a K_i, or concentration of FTC required for half-maximal inhibition of MXR, of 1 μM. No effect of FTC was observed in membranes prepared from parental S1 cells (data not shown).

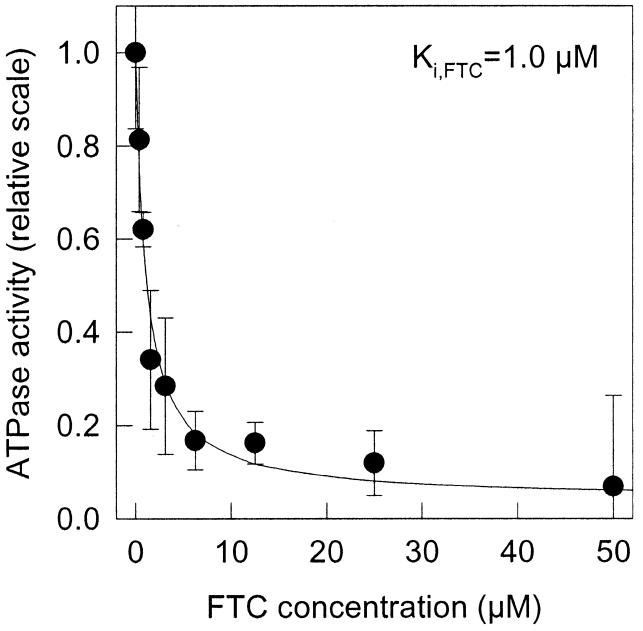

Fig. 6. FTC inhibits MXR-related ATPase activity. MXR ATPase activity was measured at various concentrations of FTC in microsomal membranes prepared from MXR-overexpressing S1-M1-80 cells. The K_i, or concentration required for half-maximal inhibition of MXR-related ATPase activity, was found to be 1 μM.

3.7. Upregulation of MXR in unselected cell lines treated with mitoxantrone

To determine whether MXR could be upregulated and thus detected more readily in unselected cells which already express low levels of MXR, the four cell lines showing the highest amount of mitoxantrone and prazosin efflux – SF295, KM12, NCI-H460 and A549 – were incubated in 10 nM mitoxantrone to develop the resistant sublines SF295 MX10, KM12 MX10, NCI-H460 MX10, and A549 MX10. Mitoxantrone efflux and MXR expression were then assayed. As shown in Fig. 7A, a reproducible increase in FTC-reversible mitoxantrone efflux is seen in three of the four mitoxantrone-resistant sublines compared to the parental lines. Additionally, MXR mRNA expression was upregulated in the A549 MX10, NCI-H460, KM12 MX10, and SF295 MX10 cells relative to the MXR mRNA levels in the parental lines as seen by the Northern blot in Fig. 7B. S1-M1-80 cells are shown as a positive control.

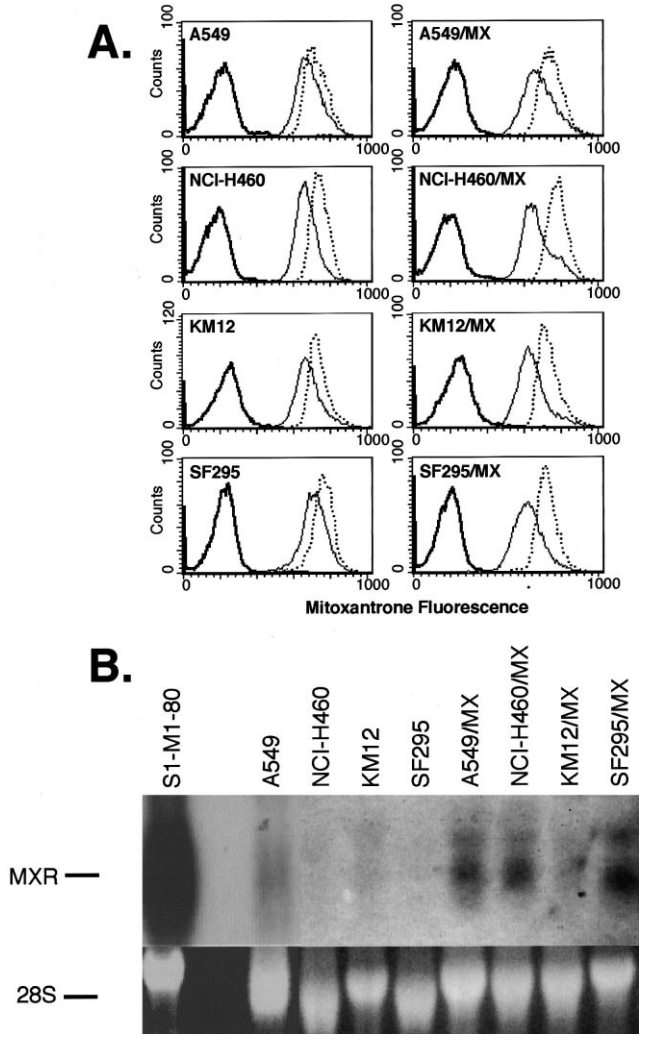

Fig. 7. Increased mitoxantrone efflux in mitoxantrone-selected cells. (A) The four drug screen cell lines having the highest amount of mitoxantrone efflux – A549, NIH-H460, KM12 and SF295 – were incubated in 10 nM mitoxantrone to generate the resistant sublines A549 MX10, NIH-H460 MX10, KM12 MX10, and SF295 MX10. The mitoxantrone efflux assay was performed as outlined in Fig. 1: heavy solid line, Blank; thin solid line, Efflux; dotted line, FTC/Efflux. (B) Northern blot analysis of the parental and mitoxantrone-selected drug screen cell lines. MXR-overexpressing S1-M1-80 cells are shown as a positive control.

4. Discussion

In the present study, flow cytometric measurement of FTC-inhibitable mitoxantrone or BODIPY-prazosin efflux proved to be a sensitive and specific method for detecting MXR in both highly resistant, drug-selected cell lines as well as unselected cell lines, and correlated well with MXR expression as determined by Northern blot. When cytotoxicity assays were performed with FTC on unselected cell lines in which FTC was able to increase mitoxantrone and prazosin accumulation, a 3-fold sensitization to mitoxantrone was noted. FTC-inhibitable efflux of mitoxantrone and prazosin was not seen in MDR or MRP positive cell lines, suggesting that FTC may be specific for inhibiting MXR. Additionally, the concentration of FTC required for half-maximal inhibition of MXR-related ATPase activity, or K_i, was determined to be 1 μM.

Our data are consistent with the findings of Rabindran et al., who described increased FTC-induced retention of [^{14}C]mitoxantrone and enhanced mitoxantrone cytotoxicity in S1-M1-3.2 cells which express increased levels of the MXR protein [18]. Additionally, FTC was shown to enhance retention of a fluorescent MXR substrate, BBR 3390, in BCRP-transfected MCF-7 cells [19].

As FTC-reversible mitoxantrone and prazosin efflux was detected in unselected cell lines, this method could be adapted to clinical applications. A number of flow cytometric assays have been developed to detect the multidrug resistance phenotype and have been extensively used in the clinical setting where often only a small sample volume can be obtained. Flow cytometry-based assays are also advantageous in that they provide functional as well as quantitative information about the multidrug resistance proteins present in a given sample. Rhodamine 123 in conjunction with a Pgp blocker has been used in clinical applications such as determining the effectiveness of Pgp blockers in patients [31,32] and has been used effectively as a measure of Pgp-mediated resistance in leukemia [15,33]. Calcein-AM has also been used with the Pgp blocker cyclosporin A and the MRP blocker probenecid to quantify Pgp- and MRP-mediated resistance in acute myeloid leukemia (AML) samples [16]. It has been suggested that MXR may be responsible for cyclosporin-resistant drug efflux which is not due to overexpression of Pgp, MRP or LRP in AML [33,34]. Further, blast cells from acute leukemia patients have recently been shown to express detectable levels of MXR [35]. The studies presented here thus form the basis for functional detection of MXR in clinical samples.

One interesting finding of the present study was

that rhodamine and LysoTracker efflux did not correlate well with MXR levels. In fact, some cell lines with very high levels of MXR did not display rhodamine efflux. This finding was surprising, since previous studies in our laboratory suggested that rhodamine 123 was a substrate for MXR [8]. Several potential explanations are possible. First, we noted quenching of the LysoTracker in many of the cell lines tested. Second, differing substrate specificities could result from different dimerization partners, since MXR is a half-transporter and is thought to dimerize for activity. Different partners confer different substrate specificities in the case of the *Drosophila* white gene product. When the white gene product complexes with the scarlet gene product, tryptophan is transported; when it complexes with the brown gene product, guanine is transported [9]. Third, polymorphisms or mutations in MXR could result in differing affinities for substrates. Polymorphisms in the rat *tap* gene result in differing substrate specificities [36], and a splice variant of the human *Tap2* gene, *Tap2iso*, was recently shown to encode for a protein having peptide presentation patterns different from the normal *Tap2* gene product [37]. Acquired mutations in Pgp have also been shown affect cross-resistance patterns in multidrug-resistant cells [38]. Recent studies in our laboratory suggest that amino acid variation at position 482 between the various published sequences for MXR correlates with the ability of the protein to efflux rhodamine.

We have shown that cells which express low endogenous levels of MXR are able to upregulate MXR when exposed to relatively low levels of mitoxantrone. A topotecan-resistant ovarian cancer cell line was reported to have reduced topotecan accumulation [39] and was found to overexpress MXR [40]. Recently, lung cancer cells exposed to SN-38 were also shown to overexpress MXR [6]. These data would suggest that MXR is an important mechanism of resistance in some models, and may explain drug resistance in mitoxantrone- or topotecan-resistant tumors which test negative for Pgp. Studies are currently underway to determine MXR expression in refractory tumors.

In conclusion, MXR appears to be an important new ABC transporter, and is expressed in unselected cells as well as in drug-selected cell lines. While its clinical relevance has yet to be determined, the diversity of its substrates and the high levels of cross-resistance which MXR can confer suggest it ought to be vigorously pursued.

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