Resistance to Mitoxantrone in Multidrug-resistant MCF7 Breast Cancer Cells: Evaluation of Mitoxantrone Transport and the Role of Multidrug Resistance Protein Family Proteins

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ABSTRACT

We examined the role of multidrug resistance protein (MRP) 1 (ABCC1) in the emergence of mitoxantrone (MX) cross-resistance in a MCF7 breast cancer cell line selected for resistance to etoposide. The resistant cell line, MCF7/VP, expresses high levels of MRP1, whereas the parental cell line, MCF7/WT, does not. MCF7/VP cells are 6–10-fold cross-resistant to MX when compared with MCF7/WT cells. Drug transport studies in intact MCF7/VP cells revealed that MX resistance is associated with reduced MX accumulation due to enhanced MX efflux. MX efflux is ATP dependent and inhibited by sulfipyrazone and cyclosporin A. Inhibition of MX efflux with these agents sensitizes cells to MX cytotoxicity and partially reverses MX resistance in MCF7/VP cells. Whereas resistance is partially attributable to increased MX efflux in MRP1-expressing MCF7/VP cells, we found no evidence for glutathione or other conjugates of MX in these cells. Moreover, glutathione depletion with buthionine sulfoximine had no effect on MX transport or sensitivity in MCF7/VP cells. MRP1 substrates are generally amphiphilic anions such as glutathione conjugates or require the presence of physiological levels of glutathione for MRP1-mediated transport. Therefore we conclude that MRP1 overexpression is unlikely to be responsible for increased MX efflux and resistance in MCF7/VP cells. In considering the potential involvement of other MRP family isoforms, a 3-fold increase in the expression of MRP5 was observed in MCF7/VP cells. However, stable expression of a transduced MRP5 expression vector in MCF7/WT cells failed to confer MX resistance. Because other transporters known to be associated with MX resistance, including P-glycoprotein and BCRP/MXR [ABCG2 (11, 16)], are not expressed in MCF7/VP cells, we conclude that increased MX efflux and resistance in MCF7/VP cells is attributable to a novel transport mechanism or that MX represents a novel class of cationic, glutathione-independent MRP1 substrates.

INTRODUCTION

The emergence of resistance to the anticancer drug MX has been associated with several alternative mechanisms including altered topoisomerase II activities (1–5), decreased drug accumulation due to overexpression of the drug efflux pump P-glycoprotein/MDR1 (6, 7), and, more recently, overexpression of the ABC half transporter BCRP/MXR [ABCG2 (11, 16)], and the role of this protein in MX efflux is unclear. We observed 6–10-fold cross-resistance to MX in a MCF7 cell line selected for resistance to etoposide (16, 17). This derivative cell line, MCF7/VP, does not express P-glycoprotein as shown by RNase protection assay and Western blot analysis (16). Moreover, Western and Northern blot analyses demonstrated that BCRP/MXR [ABCG2] is not overexpressed in MCF7/VP relative to parental MCF7/WT cells (11, 18). The major phenotypic change in the resistant MCF7/VP cells compared with the parental MCF7/WT cells is the high-level expression of MRP1 found only in MCF7/VP cells (16, 17, 19). A small decrease in topoisomerase II activity is also observed in MCF7/VP cells (16). Whereas reduced topoisomerase II activity has been associated with MX resistance (16), the level of decrease observed in MCF7/VP cells is insufficient to fully account for the 6–10-fold resistance to MX.

The goals of this study were to determine whether MX resistance is associated with and hence partially attributable to decreased drug accumulation, and, if so, whether decreased drug accumulation is due to increased efflux mediated by MRP1. MRP1-mediated transport is energy dependent and inhibited by a number of compounds including organic anions and CsA (20, 21). Additionally, most MRP1 substrates fulfill one of the following criteria: they are either (a) amphiphilic anions (e.g., conjugates of glutathione, glucuronide, or sulfate; Refs. 22–26) or (b) neutral or cationic lipophilic drugs that require physiological concentrations of glutathione to effect MRP1-mediated transport (26–28). The present study shows that whereas MX resistance in MCF7/VP cells is associated with increased ATP-dependent, sulfipyrazone- and CsA-inhibitable drug efflux, the data indicate that MX does not fulfill the other criteria for a MRP1 substrate in these MCF7/VP cells. The expression and potential roles of other MRP family isoforms in MX resistance are considered.

MATERIALS AND METHODS

Drugs and Chemicals. MX, etoposide, BSO, sulfipyrazone, CsA, sulfodiamine B, sodium azide, and 2-deoxy-d-glucose were obtained from Sigma Chemical Co. (St. Louis, MO). Drug stock solutions were prepared and stored at −80°C as described previously (17). DMEM and FCS were from Life Technologies, Inc. (Gaithersburg, MD). [3H]MX (1.5 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Cell Culture, Transductions, and Cytotoxicity Determinations. MCF7/VP and MCF7/WT cells have been described previously (16, 17, 19). MCF7/VP cells are a multidrug-resistant variant of parental MCF7/WT cells obtained by chronic exposure of MCF7/WT cells to etoposide (16). MCF7/VP cells express high levels of MRP1, whereas MCF7/WT cells do not. Neither cell line expresses P-glycoprotein/MDR1 or BCRP/MXR [ABCG2 (11, 16)]. Unless otherwise noted, MCF7 cells were grown in DMEM containing 10% FCS at 37°C in 5% CO2. HepG2 hepatocarcinoma cells were grown in DMEM:Ham’s F-12 containing 10% FCS.

Forced overexpression of MRP5 was achieved by stable transduction of MCF7/WT cells with a retroviral expression vector, pLNCX-mrp5. This vector contained a human MRP5 cDNA inserted into the IRES site of pLNCX (29). The MRP5 insert was prepared by PCR amplification of a first-strand cDNA synthesized from human liver mRNA (Clontech, Palo Alto, CA) using avian myeloblastosis virus reverse transcriptase (30). A 4330-bp fragment containing the entire coding region of MRP5 was amplified using the Expand High Fidelity DNA polymerase mixture (Roche, Indianapolis, IN) according to the protocol for the Expand High Fidelity DNA polymerase mixture.
To determine whether MCF7 cells produce MX-SG metabolites, 5–7 × 10^6 cells were plated in 100-mm tissue culture dishes and incubated 24 h later for 3 h in 4 ml of DMEM/1% FCS containing 50 μM MX (or vehicle). Medium was collected, and ascorbic acid added to a final concentration of 100 mM to prevent further oxidation (35). Cells were scraped in 50% acetonitrile, ascorbic acid was added to 100 mM (total volume, 1.17 ml), and the mixture was disrupted by sonication. Cell lysates were centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant was saved. Medium and lysate samples (100 μl) were analyzed by HPLC as described above.

Biochemical Assays. Cellular levels of glutathione and ATP were determined according to the method of Tietze (36) and Lust et al. (37), respectively.

Analysis of Proteins, DNA, and RNA. Western blot analysis of membrane protein preparations for MRPI and MRFP5 expression was accomplished as described previously (38). Blots were examined for MRPI expression and MRFP5 expression using QCR-1 antibody (38) and M51-1a antibody kindly provided by Drs. S. P. C. Cole and Rik Sheper, respectively.

Northern and Southern blot analyses were accomplished as described previously (39) using total cellular RNA and genomic DNA from MCF7 and HepG2 cells. Probes were derived from cDNAs encoding the 5′ 2629 bp of human MRPI (40), the 3′ 606 bp of human MRFP5 (41, 42), and the 3′ 646 bp of human MRFP6 (43). DNA inserts were radiolabeled with [α-32P]dCTP by random priming (30).

RNase protection was done as described using gel-purified [α-32P]UTP-labeled antisense RNA probes (30). Probes were transcribed using T3 or T7 RNA polymerase from pBluescript SK− (Stratagen) or pGEM3Z (Promega) plasmid templates containing cDNA inserts encoding the 781-bp 5′ fragment of human GAP (44, 45) and the 3′ MRPI and MRFP6 fragments described above. The MRPI and MRFP6 inserts were generated by reverse transcription-PCR (30) of human liver mRNA using the following oligonucleotide pairs: (a) MRPI, 5′-CCCTTCTCCGGTCGTTGGAATGTA-3′ and 5′-GGGAGGAGTCTGACCCCTGAC-3′; and (b) MRFP6, 5′-GAGGGCGGTTCCTTCCTAAGA-3′ and 5′-TGAGACGAGCCGTACTGACTT-3′. Riboprobes were annealed to total cellular RNA at 50°C, treated with RNases, and resolved by 6% PAGE as described previously (30).

RESULTS

MX Resistance of MCF7/VP Cells. Previous studies showed that MCF7/VP cells are resistant to MX when the drug is applied continuously (16, 17). Fig. 1 demonstrates that, compared with MCF7/WT cells, MCF7/VP cells are also ~7-fold resistant to a 1-h exposure to MX. Indeed, 6–10-fold resistance was consistently observed for the entire range of drug exposure times used, including 1, 4, and 24 h and continuous exposure (Fig. 1; Refs. 16 and 17; data not shown).

Resistance in MCF7/VP Cells Is Associated with Increased MX Efflux. MX transport in MCF7/VP versus MCF7/WT cells was examined to determine whether altered MX accumulation might contribute to MX resistance. As shown in Fig. 2A, MCF7/VP cells accumulate steady-state intracellular levels of MX that are only ~50% of those achieved in MCF7/WT cells. After preloading cells

**Fig. 1.** Relative sensitivities of MRPI-expressing MCF7/VP cells versus non-MRPI-expressing MCF7/WT cells to MX. A representative cytotoxicity profile of the sensitivities of MCF7/WT (●) and MCF7/VP (□) cells to 1 h of MX exposure is shown. Values represent the means of eight replicate determinations ± SD.
with equivalent levels of MX, the studies shown in Fig. 2B demonstrate that reduced accumulation is attributable to increased efflux of MX in MCF7/VP cells. The decreased accumulation of MX in MCF7/VP cells is ATP dependent, as demonstrated in Fig. 3. In these experiments, treatment of MCF7/VP cells with azide and 2-deoxy-D-glucose under conditions that result in 90–95% depletion of cellular ATP completely restored MX accumulation in MCF7/VP cells to the high levels observed in MCF7/WT cells. These data indicate that MCF7/VP cells contain an ATP-dependent efflux pump for MX. A smaller increase in MX accumulation was observed upon ATP depletion of MCF7/WT cells (data not shown). These data suggest that whereas MCF7/WT cells also support ATP-dependent MX transport, the level of transport activity is considerably less than that in resistant MCF7/VP cells.

The effect of the organic anion, sulfinpyrazone, and the cyclic peptide, CsA, on MX transport in MCF7/VP and MCF7/WT cells was examined. Whereas neither sulfinpyrazone nor CsA is entirely specific, both have been used to inhibit MRP1-mediated transport in membrane vesicles and intact cells (20, 21). As shown in Fig. 4, sulfinpyrazone treatment restores MX accumulation to MCF7/WT levels (Fig. 4A) by inhibiting MX efflux (Fig. 4B). Similarly, treatment of MCF7/VP cells with CsA increases MX accumulation in MCF7/VP to levels comparable with those of MCF7/WT cells (Fig. 5).
5A) by inhibiting MX efflux (Fig. 5B). Treatment of MCF7/WT cells with sulfinpyrazone and CsA also results in reduced efflux of MX in these cells. Whereas the magnitude of the decrease in efflux is much smaller in MCF7/WT cells than in MCF7/VP cells, sulfinpyrazone and CsA effect a ~41% and ~35% reduction, respectively, in MX efflux in MCF7/WT cells (data not shown).

Inhibition of MX efflux by sulfinpyrazone (Fig. 6A) or CsA (Fig. 6B) results in significant sensitization of MCF7/VP cells to the cytotoxicity of MX, thus partially reversing MCF7/VP-associated resistance. MCF7/WT cells are also somewhat sensitized, presumably due to some inhibition of MX efflux by sulfinpyrazone and CsA also observed in these cells.

These data indicate that whereas both MCF7/WT and MCF7/VP cells express a sulfinpyrazone- and CsA-inhibitable MX efflux mechanism, MCF7/VP cells express higher levels of the MX efflux transporter.

**MX Resistance and Increased MX Efflux in MCF7/VP Cells Are Independent of Glutathione or Formation of MX-glutathione Conjugates.** MX has been shown to form glucuronides in vivo and to form conjugates with glutathione both in vitro and in some cells (34, 35, 46). Such conjugates render the parent drug, MX, which is a weakly cationic lipophile, an amphiphilic anion. These anionic compounds are typical MRP1 substrates (22–26). Accordingly, we examined the lysates (Fig. 7B, intracellular) and medium (Fig. 7C) of MCF7/VP cells treated for 3 h with 50 μM MX (+MX) or vehicle (DMSO; −MX). The cell lysates (B, intracellular) and medium (C) were examined by HPLC as described in “Materials and Methods.” The chromatographic elution times of the parent drug (MX) and its MX-SG and MX-SG2 derivatives are shown in A. Eluates were monitored by absorbance at 674 nm, and the absorbance profiles in B and C were expanded to the same arbitrary scale.

Other cationic or neutral substrates of MRP1 that do not form conjugates require the presence of physiological concentrations of glutathione to support their transport (26–28). However, depletion of >90% of intracellular glutathione by pretreatment with BSO had no significant effect on MX accumulation (Fig. 8) or cytotoxicity (Table 1) in MCF7/VP or MCF7/WT cells. In contrast, glutathione depletion sensitized MCF7/VP cells to etoposide cytotoxicity (Table 1), an
expression that has previously been shown for this known MRP1 substrate (47–49).

Expression of Other MRP Family Isoforms in MCF7/VP versus MCF7/WT Cells. We examined the expression of other MRP isoforms to determine whether they might be overexpressed in MCF7/VP cells and therefore be candidate MX efflux transporters. MRP1 and MRP6 are located adjacent to each other on chromosome 16 and are frequently coamplified in drug-selected cell lines (43). As shown in Fig. 9, both MRP1 and MRP6 genes are amplified in MCF7/VP cells (Fig. 9A). However, only MRP1 mRNA of the correct size is overexpressed in MCF7/VP cells (Fig. 9B). MRP6 RNA is barely detectable in MCF7/VP cells but is of an extremely large size indicative of defective RNA processing and maturation (Fig. 9B). In contrast, MRP5, which is not amplified (Fig. 9A), is ∼3-fold overexpressed at the mRNA level in MCF7/VP cells (Fig. 9B). The results of Northern analysis (Fig. 9B) were confirmed by RNase protection, which again shows a ∼3-fold increase in MRP5 in MCF7/VP versus MCF7/WT cells (Fig. 10). MRP6 RNA is only weakly expressed in MCF7 cells, and the predominant RNase protection fragment in MCF7/VP cells is detected as an aberrant band at 130 nt indicative of improper RNA processing (Fig. 10).

Table 1  Effect of glutathione depletion on cellular sensitivities to etoposide and MX cytotoxicities

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<th>Drug</th>
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a Fold sensitization is defined as etoposide or MX IC50 without glutathione depletion divided by IC50 after glutathione depletion. Glutathione depletion was accomplished by 48-h BSO treatment before exposure to cytotoxic drugs. Each value represents the mean ± SE determined from 5–12 independent experiments.

Expression levels of MRP2, MRP3, and MRP4 RNA were poor or undetectable in MCF7 cells (50). There was no differential expression of any of these isoforms in MCF7/VP versus MCF7/WT cells.

Because a 3-fold increase in MRP5 expression (Figs. 9 and 10) was associated with MX resistance in MCF7/VP cells, we asked whether forced overexpression of MRP5 would confer MX resistance in MCF7/WT cells. MCF7/WT cells were stably transduced with a MRP5 expression vector. Three representative clones overexpressing MRP5 mRNA (Fig. 11A) and membrane-associated protein (Fig. 11B) were selected and tested for sensitivity to MX cytotoxicity. Despite considerable increases in membrane-associated MRP5 protein and 18–33-fold increases in MRP5 mRNA expression, none of these clones showed resistance to MX when compared with parental MCF7/WT cells (Table 2).

DISCUSSION

The present study demonstrates that MX resistance in MCF7/VP cells is associated with decreased drug accumulation due to increased MX efflux. Resistance is partially reversed by inhibiting MX efflux with sulfipyrazone or CsA. We conclude that this increase in energy-dependent MX efflux contributes significantly to MX resistance in MCF7/VP cells. MX efflux and resistance have been attributed to overexpression of P-glycoprotein/MDR1 and BCRP/MXR (ABCG2; Refs. 6–12). Because neither of these proteins is expressed in MCF7/WT cells (11, 16), a previously unrecognized MX transport system must be responsible for altered MX efflux in these cells.

Because MRP1 is highly overexpressed in MCF7/VP cells, we examined whether or not this protein was likely to contribute to MX efflux. Indeed, MX efflux in MCF7/VP cells shows some features of known MRP1 substrate pairs (20, 21, 51–53), including energy dependence and inhibition by sulfipyrazone and CsA. However, sulfipyrazone and CsA are not specific inhibitors for MRP1. Indeed, whereas the magnitude of sulfipyrazone and CsA inhibition of MX efflux is greater in MCF7/VP cells than in MCF7/WT cells, both sulfipyrazone and CsA significantly inhibit MX efflux in MCF7/WT cells (41% and 35%, respectively). These results indicate that both MCF7/WT and MCF7/VP cells express a MX efflux transport mech-
anism, but MCF7/VP cells express the efflux transporter at higher levels. Because MRP1 protein is undetectable in MCF7/WT cells, it appears unlikely that MRP1 is the mitoxantrone- and CsA-inhibitable MX transporter. Moreover, unlike other MRP1 substrates (26–28), MX is a weakly cationic lipophile whose transport is independent of glutathione levels. Additionally, stable conjugates of MX are not detected in MCF7/VP cells treated with MX. These considerations and data suggest either that MRP1 is unlikely to be responsible for MX transport and resistance in MCF7/VP cells or that a novel, thus far unrecognized mechanism for MRP1-mediated transport of MX exists.

These findings do not exclude the possibility that MX may form transient, labile conjugates in MCF7/VP cells that were not detectable in these studies. Moreover, it is likely that gluturonide and glutathione derivatives of MX, which are formed in other cells and tissues (34, 46), may be substrates of MRP1 or other MRP family members in these other cells.

We considered other potential transporters for MX in MCF7/VP cells and examined the expression of other MRP isofoms. MRP6 is amplified and overexpressed in MCF7/VP cells relative to MCF7/WT cells. However, the level of MRP6 is quite low, and most of the material expressed is aberrantly processed RNA that is presumably unrecognized mechanism for MRP1-mediated transport of MX exists.

Therefore, MX resistance in MRP1-overexpressing MCF7/VP cells is associated with reduced drug accumulation due to increased ATP-dependent, glutathione-independent MX efflux. Our data indicate that increased MX efflux in these multidrug-resistant cells is mediated by a novel transport mechanism, or, if MRP1 is involved, MX represents a novel class of cationic, glutathione-independent substrates for this transporter.

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