Expression of Multidrug Resistance-associated Protein in NIH/3T3 Cells Confers Multidrug Resistance Associated with Increased Drug Efflux and Altered Intracellular Drug Distribution

Lisa M. Breuninger, Saptarshi Paul, Kathleen Gaughan, Toru Miki, Andrew Chan, Stuart A. Aaronson, and Gary D. Kruh

Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [L. M. B., S. P. K., G. D. K.]; Laboratory of Cellular and Molecular Biology, NIH, Bethesda, Maryland 20892 [T. M.]; and The Derrald H. Rutenberg Cancer Center, New York, New York 10029 [A. C. S. A. A.]

ABSTRACT

Multidrug resistance is a major obstacle to cancer treatment. Using an expression cDNA library transfer approach to elucidating the molecular basis of non-P-glycoprotein-mediated multidrug resistance, we previously established that expression of multidrug resistance protein (MRP), an ATP-binding cassette superfamily transporter, confers multidrug resistance (G. D. Krut et al., Cancer Res., 54: 1649–1652, 1994). In the present study, we generated NIH/3T3 MRP transfectants without using chemotherapeutic drugs to facilitate the pharmacological analysis of the MRP phenotype. MRP transfectants displayed increased resistance to several lipophilic drugs, including doxorubicin, daunorubicin, etoposide, actinomycin D, vincristine, and vinblastine. However, increased resistance was not observed for Taxol, a drug for which transfection of MDR1 confers high levels of resistance. Verapamil increased the sensitivity of MRP transfectants relative to control transfectants, but reversal was incomplete for doxorubicin and etoposide, the drugs for which MRP conferred the highest resistance levels. For the latter two drugs, MRP transfectants, which were ~6- and ~10-fold more sensitive than control cells in the absence of verapamil, exhibited 3.8- and 3.3-fold relative sensitization with 10 μM verapamil, respectively, but remained ~2 and ~3-fold more resistant than control cells. Analysis of drug kinetics using radiolabeled daunorubicin revealed decreased accumulation and increased efflux in MRP transfectants. Confocal microscopic analysis of intracellular daunorubicin in MRP transfectants was consistent with reduced intracellular drug concentrations, and also revealed an altered pattern of intracellular drug distribution characterized by the initial accumulation of drug in a perinuclear location, followed by the development of a punctate pattern of drug scattered throughout the cytoplasm. This pattern was suggestive of a process of drug sequestration, possibly followed by vesicle transport. Both increased drug efflux and perinuclear drug accumulation are consistent with the reported localization of MRP in plasma and cytosolic membranes (N. Krishnamachary and M. S. Center, Cancer Res., 53: 3658–3663, 1993; M. J. Fens et al., Cancer Res., 54: 4557–4563, 1994). These results thus indicate that the drug specificity of MRP is quite similar to that of MDR1, but also suggest potential differences in Taxol specificity and the level of verapamil sensitivity. In addition, these results indicate that MRP functions to extrude drug from the cell, but additionally suggest the intriguing possibility that drug sequestration contributes to drug resistance by protecting cellular targets and/or contributing to drug efflux.

INTRODUCTION

Drug-resistant cancer cells are a major obstacle in the treatment of disseminated malignancies. Most disseminated cancers are either inherently resistant to cytotoxic drugs or, after initial response, become resistant during treatment. Broad spectrum resistance to cytotoxic drugs, termed multidrug resistance, involves simultaneous resistance to a wide array of functionally distinct natural product agents. Multidrug resistance has reduced the promise of combination chemotherapy, the classic approach to overcoming drug resistance (1).

Expression of MDR1, a member of the ATP-binding cassette superfamily of transporters, is known to confer multidrug resistance to natural product agents (2–4). P-glycoprotein, the M, 170,000 protein product of the MDR1 gene, is an energy-dependent efflux pump that reduces the intracellular concentrations of chemotherapeutic agents by transporting them across the plasma membrane. It is apparent that other mechanisms can also contribute to multidrug resistance since multidrug-resistant cell lines that do not overexpress P-glycoprotein have been described (5–11). Recently, MRP, another member of the ATP-binding cassette transporter superfamily, was isolated from H69AR, a non-Pgp, small cell lung cancer cell line (12). Several multidrug-resistant cell lines selected for high level resistance to natural product agents have subsequently been shown to overexpress MRP (13–16).

The analysis of in vitro drug-selected cells lines that overexpress MRP has raised several questions pertaining to the pharmacology associated with MRP expression. Some MRP-overexpressing cell lines have been reported to have altered drug accumulation and/or efflux (6, 9, 10, 17), suggesting that MRP might function as a drug efflux pump similar to Pgp. However, the highly resistant H69AR cell line from which MRP was initially isolated does not display altered drug accumulation or efflux (5), and this has led to speculation that MRP might confer resistance by a mechanism that does not involve altered drug kinetics. In addition, the analysis of daunorubicin fluorescence in some non-Pgp cell lines that have subsequently been shown to overexpress MRP has suggested that alterations in the intracellular distribution of the drug might also contribute to MRP-mediated resistance (18–20). Finally, the observation that MRP-overexpressing cell lines are only partially sensitive to verapamil (6, 10, 21, 22), the prototypic Pgp-reversing agent (23), has led to consideration of the possibility that MRP might be substantially less sensitive to this reversing agent than Pgp. Since these observations regarding the pharmacology of MRP were made in cell lines obtained by stepwise selection in drug, a technique that is known to be associated with the development of multiple collateral resistance mechanisms (24), they are subject to some degree of uncertainty. Indeed, several MRP-overexpressing cell lines have been shown to have alterations in topoisomerase II activity (5, 25–28).

We previously established that MRP confers multidrug resistance (29) by applying an expression cDNA transfer strategy involving transfection of drug-sensitive NIH/3T3 cells with an expression cDNA library prepared from the non-Pgp multidrug-resistant HL-60/ADR cell line (7). The resulting transfectants had highly amplified MRP gene copies and possibly harbored collateral resistance mechanisms as a result of the cytotoxic drug selection that was part of the cDNA transfer methodology. In the present study, we addressed the...
pharmacology associated with MRP expression by analyzing drug sensitivity, verapamil sensitization, drug kinetics, and intracellular drug distribution in MRP transfectants generated in the absence of chemotherapeutic drug selection. These transfectants permit the analysis of the MRP phenotype without interference from the multiple resistance mechanisms that can arise in stepwise selected cell lines.

MATERIALS AND METHODS

Isolation of MRP cDNA Clones. An MRP probe consisting of nucleotides 3547–4254 (12) was prepared by PCR using our previously reported HL-60/ADR cDNA library as template (29). This probe was used to isolate several overlapping MRP cDNA clones by plaque hybridization. A 6.5-kb clone containing the full-length MRP coding sequence was assembled from two overlapping cDNA clones. Nucleotide sequence analysis of both DNA strands of the 6.5-kb clone was performed using the dideoxy chain termination method (sequenase kit; U. S. Biochemical Corp.). This clone was inserted into the retroviral based expression vector pSRαMSVtkneo (30) to create pSRα-MRP. The neo selection marker of this vector is driven by the thymidine kinase promoter, and is located downstream of the MRP cDNA but within the 5' and 3' long terminal repeats.

Transfection of NIH/3T3 Cells. Cells were seeded at 1.5 × 10^5 cells/dish in DMEM supplemented with 10% FCS, and after overnight growth were transfected with 10 μg of expression vector DNA using the calcium phosphate precipitation method, as previously described (31). Twenty-four h after transfection, the cells were washed and then grown in medium containing 750 μg/ml G418 for 2 to 3 weeks. Individual G418-resistant colonies were isolated using the cloning cylinder technique and expanded for further analysis.

Immunoblot Analysis. Lysates were prepared from NIH/3T3 transfectants and HL-60/ADR cells using RIPA lysis buffer (32), and 100-μg samples were separated by electrophoresis through 6.0% SDS-polyacrylamide gels (33). After transfer to nitrocellulose filters (S&S, Keene, NH), MRP was detected using an anti-MRP monoclonal antibody (MTPmAb-1) and chemiluminescence (ECL kit; Amersham). Monoclonal antibody MRPmAb-1 was generated against a recombinant immunogen consisting of amino acids 609–761 of the protein.

Analysis of Drug Sensitivity. Drug sensitivities of NIH/3T3 transfectants (pSRα-MRP or the control vector) were determined for doxorubicin, daunorubicin, vincristine, and vinblastine, using the colony formation assay. Cells were plated in triplicate at a density of 3.1 × 10^3 cells/well in 96-welled culture dishes. After overnight growth in DMEM containing 10% FCS, various concentrations of drugs were added. After 8 days of growth in the presence of the drug, dishes were washed with PBS, and the cells were fixed and stained with 0.5% crystal violet solution containing 20% ethanol. Colonies consisting of >20 cells were enumerated using light microscopy, and IC_{50} values were obtained from standard survival curves.

Analysis of Drug Uptake and Efflux. NIH/3T3 transfectants were seeded at a density of 8 × 10^2 cells/well in 96-welled culture dishes. After overnight growth in DMEM containing 10% FCS, the medium was replaced with fresh growth medium containing various concentrations of [3H]daunorubicin (3.7 Ci/mmol; New England Nuclear, Boston, MA). At various time points the growth medium was removed, and the cells were washed three times with cold PBS. The cells were then lysed with 0.2 ml PBS containing 0.5% SDS, and the radioactivity was counted in a liquid scintillation counter. For drug efflux studies, NIH/3T3 transfectants were incubated for 1.5 h with various concentrations of [3H]daunorubicin, after which time the medium was replaced with drug-free growth medium. At various time points the medium was removed, and the cells were washed three times with cold PBS. The cells were then lysed with 0.2 ml PBS containing 0.5% SDS, and the radioactivity was counted in a liquid scintillation counter.

Analysis of Intracellular Daunorubicin Distribution. NIH/3T3 cells (5 × 10^5) were seeded on sterilized glass coverslips in 35-mm culture dishes. After overnight growth, the cells were incubated in medium containing 20 μM daunorubicin for 1.5 h. The cells were then washed and analyzed immediately, or after an additional 1.5 h of growth in drug-free medium. Daunorubicin fluorescence was detected by confocal imaging using a Bio-Rad MRC-600 imaging system (Bio-Rad Microscience Ltd., Richmond, CA) equipped with an argon krypton laser coupled to a Nikon Optiphot II fluorescence microscope and a 60X Plan Apo oil objective. Successive images were rendered using Voxel view ultra-2.1 software (Vital Images, Airfield, IA).

RESULTS

Molecular Cloning and Nucleotide Sequence Analysis of the HL-60/ADR MRP cDNA. We isolated MRP cDNA clones from our previously described HL-60/ADR cDNA library (29) by plaque hybridization, and assembled a clone containing the full-length coding sequence from two overlapping cDNA clones. The resulting 6.5-kb clone contained 118 bp of 5' untranslated sequence, and ~1.5 kb of 3' untranslated sequence. Nucleotide sequence analysis indicated that the coding region of the HL-60/ADR MRP cDNA differed at five nucleotide positions from the previously reported H69AR cDNA sequence (12). Two of these differences, T → C substitutions at nucleotide positions 1021 and 1258, were silent. However, C → T, T → C, and A → G substitutions at nucleotide positions 546, 2250, and 3149 resulted in Thr → Met, Leu → Ser, and Asn → Asp changes at amino acid positions 117, 685, and 985, respectively. The predicted amino acid differences were confirmed by nucleotide sequence analysis of three other independently isolated HL-60/ADR MRP cDNA clones. Interestingly, Ser-685 is located at the eighth amino acid position of the Walker A motif (GXXXXGKSITI') and is therefore consistent with the Walker A motif (GQVGCGBKST), whereas Leu-685 of the H69AR cDNA is not. Met-117 and Asp-985 are located at or near the junctions of transmembrane segments 1 and 9, respectively. The integrity of the MRP coding sequence in the 6.5-kb cDNA clone was confirmed by in vitro protein translation, which revealed the expected Mr ~190,000 product (data not shown).

Expression of MRP in NIH/3T3 Cells Confers Resistance to Doxorubicin. To generate MRP transfectants, the 6.5-kb MRP cDNA clone was inserted into the retroviral based expression vector pSRαMSVtkneo to create pSRα-MRP. NIH/3T3 cells were transfected with pSRα-MRP or the control vector (denoted pSRα), and marker selected cells were obtained by growth in medium containing G418. Ten G418-resistant colonies were isolated using the cloning cylinder technique and expanded for further analysis. The transfectants were maintained in the absence of chemotherapeutic agents to prevent the development of collateral resistance mechanisms that might influence subsequent analyses. We first compared the doxorubicin sensitivity of the MRP transfectants to control transfectants using colony formation assays. As shown in Table 1, each of the pSRα-MRP clones displayed substantially reduced doxorubicin sensitivity, with IC_{50} ranging from 7.6 to 18.8 nm compared to an IC_{50} of 2.0 nm for the control transfectants. This corresponded to resistance levels ranging from 3.8- to 9.4-fold.

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>IC_{50} (nm)</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSRα</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>pSRα-MRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td>10.4</td>
<td>5.2</td>
</tr>
<tr>
<td>-4</td>
<td>9.6</td>
<td>4.8</td>
</tr>
<tr>
<td>-5</td>
<td>15.0</td>
<td>7.5</td>
</tr>
<tr>
<td>-7</td>
<td>18.8</td>
<td>9.4</td>
</tr>
<tr>
<td>-8</td>
<td>12.7</td>
<td>6.3</td>
</tr>
<tr>
<td>-14</td>
<td>17.3</td>
<td>8.7</td>
</tr>
<tr>
<td>-16</td>
<td>13.7</td>
<td>6.8</td>
</tr>
<tr>
<td>-25</td>
<td>13.0</td>
<td>6.5</td>
</tr>
<tr>
<td>-27</td>
<td>7.6</td>
<td>3.8</td>
</tr>
</tbody>
</table>

a Drug concentration that inhibited colony formation by 50%.
b Fold resistance = IC_{50} of MRP transfectant/IC_{50} of control transfectant pSRα.
The prominent doxorubicin resistance displayed by the transfectants indicated that MRP was expressed from the pSRα-MRP expression vector. RNA blot analysis revealed appropriate-sized MRP transcripts in each of three representative MRP transfectants, pSRα-MRP-14, -16, and -32, but not in control transfectants or untransfected cells (data not shown). To confirm MRP protein expression, immunoblot analysis was performed using MRP-specific monoclonal antibody MRPmAb-1. As shown in Fig. 1, the expected M, ~190,000 MRP protein product (14, 15) was detected in each of the three MRP transfectants analyzed (Fig. 1, Lanes 2–4), but not in the control transfectants (Fig. 1, Lane 1). This protein migrated with similar mobility to the overexpressed MRP product of HL-60/ADR cells (Fig. 1, Lane 5; Ref. 15).

**Cytotoxic Drug Resistance Pattern of MRP Transfectants.** To determine the spectrum of drugs for which MRP confers resistance, we analyzed the sensitivity of MRP transfectants to a variety of natural product and synthetic chemotherapeutic agents. As shown in Table 2, pSRα-MRP-16 was resistant to a spectrum of lipophilic drugs, including daunorubicin, etoposide, vincristine, actinomycin D, and vinblastine. High levels of resistance were observed for doxorubicin (Table 1), daunorubicin, and etoposide, for which resistance factors ranged from 4.0 to 12.0. Moderate levels of resistance were observed for vincristine and actinomycin D, with resistance factors ranging from 2.0 to 4.0. Slightly increased resistance levels were observed for vinblastine, and MRP transfectants were as sensitive as control cells to Taxol. (The increase in the mitoxantrone resistance level was not statistically significant as determined by Student’s t test.) As expected, increased resistance was not observed for the alkylating agent cisplatin. A comparable pattern of drug sensitivity was observed for pSRα-MRP-32 (data not shown).

**Effect of Verapamil on MRP Transfectants.** The effect of verapamil on the multidrug resistance phenotype of MRP transfectants pSRα-MRP-16 was examined next. We first analyzed the doxorubicin sensitivity of pSRα-MRP-16 and control transfectant pSRα by colony formation assays. Similar sensitivity patterns were obtained in two other independent experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 pSRα</th>
<th>IC50 pSRα-MRP-16</th>
<th>Fold sensitivity</th>
<th>Relative sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1.8 ± 0.2</td>
<td>17.0 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.4×4</td>
<td></td>
</tr>
<tr>
<td>Etoposide</td>
<td>3.7 ± 1.0</td>
<td>42.2 ± 9.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.4×4</td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>5.6 ± 4.2</td>
<td>43.7 ± 13.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.8×3</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.3 ± 0.4</td>
<td>0.7 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3×3</td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>2.1 ± 1.1</td>
<td>3.2 ± 1.0</td>
<td>1.5×3</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>3.5 ± 0.3</td>
<td>13.4 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8×5</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>4.3 ± 1.5</td>
<td>5.1 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2×4</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>20.1 ± 3.0</td>
<td>26.5 ± 6.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3×5</td>
<td></td>
</tr>
<tr>
<td>Taxol</td>
<td>533 ± 175</td>
<td>480 ± 100</td>
<td>0.90×3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Drug concentration that inhibited colony formation by 50%.  
<sup>b</sup> IC50 of drug sensitization of pSRα-MRP-16 over pSRα.  
<sup>c</sup> Number of independent experiments, each performed in triplicate.  
<sup>d</sup> Significantly different from control transfectant as assessed by Student’s standard­ized t test (P < 0.05).
for the MRP transfectant compared to 3.9-fold for the control cells. This corresponded to a relative sensitization of 3.3. Increased relative sensitization was also observed for vincristine, but the fold resistance of pSRα-MRP-16 in the absence of verapamil was too low to permit a statistically significant evaluation of the magnitude of reversal. A modest degree of relative sensitization was observed for vinblastine, and no reversal was observed for cisplatin. Thus, increased relative sensitization was observed for each of the drugs for which the MRP transfectant exhibited increased resistance, but not the control drug cisplatin. Verapamil toxicity at all concentrations tested was <10% for both cell lines.

**MRP Transfectants Display Decreased Drug Accumulation and Increased Drug Efflux.** Kinetic studies of drug accumulation and efflux using radiolabeled daunorubicin were performed to examine the possibility that MRP might act as a drug transporter to reduce intracellular drug concentrations. To analyze drug accumulation, pSRα-MRP-16 and control transfectants were grown in the presence of 4.0 μM daunorubicin, and intracellular drug content was assayed at various time points. As shown in Fig. 3, the rate of drug accumulation in pSRα-MRP-16 was substantially reduced compared to control cells. The MRP transfectant accumulated 61% less drug than control cells at 45 min, and a difference in drug content was detectable at time points up to 6 h. Reduced accumulation was also observed at 0.2, 2.0, and 20.0 μM drug (data not shown). To examine drug efflux, pSRα-MRP-16 and control cells were grown in the presence of either 4.0 or 20.0 μM daunorubicin for 1.5 h, and cellular drug content was assayed at various time points after replacement with drug-free medium. As shown in Fig. 4, drug efflux was substantially increased in pSRα-MRP-16 cells at both drug concentrations. At 30 min, the MRP transfectant retained only 22 and 32% of the drug at 4.0 μM and 20.0 μM, respectively, compared to control cells which retained 74% of the drug at both concentrations.

**Confocal Microscopic Analysis of Intracellular Drug Distribution in MRP Transfectants.** Although kinetic studies using radiolabeled drug are extremely sensitive for detecting alterations in drug efflux and accumulation, they do not provide information on the intracellular distribution of drug. We therefore analyzed the fate of daunorubicin in MRP transfectants by using confocal microscopy to detect the intrinsic fluorescence of this drug. pSRα-MRP-16 or control cells were treated for 1.5 h with 20 μM daunorubicin and analyzed immediately after treatment, or after 30 min of growth in drug-free medium. As shown in Fig. 5, immediately following daunorubicin treatment the cytoplasm and nuclei of control cells exhibited intense daunorubicin fluorescence (Fig. 5A). Somewhat less nuclear and cytoplasmic drug accumulation was observed for pSRα-MRP-16 (Fig. 5B). In addition, the cytoplasmic drug in pSRα-MRP-16 accumulated predominately in a perinuclear position. After 30 min of incubation in drug-free medium, a striking difference was observed in total drug content as well as in intracellular drug distribution. In contrast to control cells, in which nuclear and cytoplasmic fluorescence was largely unchanged (Fig. 5C), cytoplasmic and nuclear fluorescence was dramatically reduced in pSRα-MRP-16 cells (Fig. 5D), consistent with increased drug efflux. The complete absence of nuclear drug in the majority of pSRα-MRP-16 cells was particularly striking. In addition, both the location and appearance of the residual cytoplasmic drug in pSRα-MRP-16 was altered. Perinuclear drug was still present, but less pronounced, and the cytoplasmic fluorescence was now strikingly punctate and scattered throughout the cytoplasm. This appearance was suggestive of a process of drug sequestration in perinuclear structures, followed by vesicle transport. Similar drug distribution alterations were observed in MRP transfectants at daunorubicin concentrations as low as 0.2 μM (data not shown).

**DISCUSSION**

In this study, we sought to define the pharmacology of MRP expression in a setting in which the collateral resistance mechanisms associated with stepwise drug selection were absent. To accomplish this, stable NIH/3T3 MRP transfectants were developed without using chemotherapeutic drug selection, and several features of the resulting phenotype were analyzed. We found that MRP transfectants exhibited decreased drug accumulation and increased drug efflux, indicating that drug extrusion and reduced intracellular drug concentrations contribute to MRP-mediated resistance. Confocal microscopic analysis of the fate of daunorubicin was consistent with decreased intracellular drug concentrations in MRP transfectants. In addition, it revealed initial drug accumulation in perinuclear structure(s) and subsequent development of a scattered punctate pattern throughout the cytoplasm, possibly suggestive of a process of vesicle transport. Both
of these observations are consistent with the subcellular location of the MRP protein product, which was detected in both the plasma membrane and perinuclear structure(s) by immunofluorescence (36). Subcellular fractionation studies also demonstrated plasma and cytoplasmic membrane localization, but suggested that the majority of the protein is located in the latter compartment (15). Thus, the plasma membrane-associated protein could directly pump the drug out of the cell, resulting in drug efflux and reduced intracellular drug concentrations, and the cytoplasmic membrane-associated protein could account for the perinuclear drug accumulation and subsequent scattered pattern.

The possibility that drug sequestration contributes to resistance by protecting cellular targets and/or contributing to drug efflux by a process of vesicle transport is intriguing. Whether cytoplasmic drug accumulation results from routine MRP trafficking to the plasma membrane or represents specific targeting to intracellular structures remains to be elucidated. Our observations in MRP transfectants are consistent with the increased drug efflux (6, 9, 10, 17) and altered intracellular drug distribution (19, 20) reported in MRP-overexpressing cell lines, including the HL-60/ADR cell line from which our MRP cDNA was isolated (17, 18). Perinuclear drug accumulation has also been reported for Pgp-overexpressing cell lines (37, 38). It is thus possible that this mechanism is not unique to MRP, but its contribution to resistance may differ for the two transporters.

It is tempting to speculate that mechanisms related to drug accumulation in cytoplasmic structures underlie the absence of increased drug efflux that has been reported for the MRP-overexpressing H69AR cell line (5), possibly by reducing the amount of free intracellular drug and thus diminishing the concentration gradient that favors direct drug extrusion by MRP located in the plasma membrane. The interplay between direct drug efflux and drug sequestration could be influenced by cellular factors that govern the rate of protein trafficking, and thus be cell-type specific. Alternatively, the absence of increased drug efflux in H69AR might reflect mutations in the MRP protein product. We found that the HL-60/ADR MRP cDNA differs in three amino acid residues from the previously reported H69AR sequence (12). Of note is that one of these amino acid differences occurs in the Walker A motif of the first ATP-binding domain, where Ser-685 of the HL-60/ADR MRP is consistent with the motif, whereas Leu-685 of the H69AR cDNA is divergent. The biological significance of these amino acid differences is currently unknown for MRP. However, the observation that mutations in MDR1 cDNA's isolated from drug-selected cell lines can influence biological activity (2) suggest that they might be significant.

We previously reported that doxorubicin-selected MRP transfectants obtained by expression cDNA library transfer were resistant to doxorubicin, etoposide, and vinblastine. In another report, resistance to doxorubicin, etoposide, and vincristine was observed in MRP transfectants (39). In this study, we extend the drug specificity of MRP to include daunorubicin and actinomycin D. Thus, the specific activity of MRP for lipophilic agents is quite similar to that of MDR1. This is surprising in view of the low level of amino acid identity shared by the two transporters. However, we also observed potential differences between MRP and Pgp. MRP transfectants did not exhibit increased resistance to Taxol, a drug that is usually considered part of the Pgp spectrum and for which transfection of MDR1 confers high levels of resistance (40, 41). This is consistent with our observation that even the highly resistant HL-60/ADR cell line exhibits only marginally increased Taxol resistance (2.7-fold) compared to 110- and 38-fold resistance to doxorubicin and vincristine, respectively.4 In addition, although MRP transfectants were sensitive to verapamil, reversal was incomplete for doxorubicin and etoposide, the two agents for which the transfectants exhibited the highest levels of resistance. Partial verapamil reversal has also been observed in several MRP-overexpressing cell lines (6, 10, 21, 22) and suggests that MRP may be less sensitive to this agent than Pgp. The availability of MRP transfectants should facilitate the analysis of other potential differences between MRP and Pgp.

Although it has been established that MRP confers multidrug resistance (29, 39), its clinical significance is largely unknown. The recent observations that MRP expression correlates with amplification and overexpression of N-myc in childhood neuroblastoma (42), and that MRP deletion correlates with clinical outcome in acute myelogenous leukemia with inversion 16 (43), suggest that MRP may be clinically important in some tumors. Insight into the clinical import of MRP will await correlative studies of MRP expression, chemosensitivity, and clinical outcome.

ACKNOWLEDGMENTS

We thank Dr. Ken Tew and Dr. Charles Smith for helpful discussions and Marija Helt and Cynthia Kuper for technical assistance.

4 L. M. Breuninger and G. D. Kruh, unpublished observations.
REFERENCES

Expression of Multidrug Resistance-associated Protein in NIH/3T3 Cells Confers Multidrug Resistance Associated with Increased Drug Efflux and Altered Intracellular Drug Distribution

Lisa M. Breuninger, Saptarshi Paul, Kathleen Gaughan, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/22/5342

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/55/22/5342.

Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.