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# ANALYSIS OF *MRP* mRNA IN MITOXANTRONE-SELECTED, MULTIDRUG-RESISTANT HUMAN TUMOR CELLS

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**Abstract**—*MRP*, a gene recently isolated from a non-P-glycoprotein-mediated multidrug-resistant small cell lung cancer cell line, is a candidate multidrug-resistance gene. Mitoxantrone, an anthracenedione antitumor agent, frequently selects for non-P-glycoprotein-mediated multidrug resistance in *in vitro* models. To determine whether mitoxantrone-selected multidrug resistance was due to overexpression of *MRP*, we examined the expression of *MRP* in four mitoxantrone-selected, multidrug-resistant human tumor cell lines, using a reverse transcriptase/polymerase chain reaction assay. Results from these experiments suggest that overexpression of *MRP* does not appear to play a primary role in mitoxantrone-selected multidrug resistance in these cell lines, and that other novel drug-resistance mechanisms are likely.

*Key words: MRP*; PCR; mitoxantrone; drug resistance; mRNA; human tumor cell lines

Drug resistance is a major impediment to the successful treatment of cancer. Cells selected for resistance to one agent often become resistant to a wide variety of structurally and mechanistically unrelated compounds. One mechanism by which cells may manifest multidrug resistance is through the overexpression of the *mdr*1 gene and its cognate protein, PGP‖ [1]. This mechanism has proven to be clinically relevant; however, PGP itself is not sufficient to explain all acquired multidrug resistance. For instance, while some drug-resistant tumors overexpress PGP (e.g. multiple myeloma and lymphoma), other tumors often do not (e.g. small cell and non-small cell lung cancer) [2].

A gene isolated from a non-PGP multidrug-resistant cell line, designated *MRP*, has been suggested to be a multidrug-resistance gene [3]. This gene is an appealing candidate because it is overexpressed in at least four different non-PGP multidrug-resistant human tumor cell lines [3–5]. Each of these cell lines was selected with doxorubicin, and to date there has been little analysis of *MRP* expression in other drug-resistance models.

Mitoxantrone is an anthracenedione antitumor agent that is structurally and functionally similar to doxorubicin. Mitoxantrone has shown clinical promise in both hematologic and solid tumors and is less cardiotoxic than doxorubicin. Like doxorubicin, mitoxantrone is a substrate for PGP-mediated efflux; however, a number of studies have shown that mitoxantrone, when used as the selecting agent, most often results in a non-PGP-mediated multidrug resistance [6–10]. This unique mitoxantrone-selected multidrug-resistant phenotype frequently involves reduced drug accumulation that may be an energy-dependent process [6, 9]; therefore, overexpression of *MRP*, a member of the ABC transporter gene superfamily, may be a possible mechanism for this non-PGP drug-resistant phenotype.

Using a newly developed RT/PCR assay, we examined the level of *MRP* expression in four different mitoxantrone-selected multidrug-resistant human tumor cell lines. The drug-resistant cells were derived from both epithelial and hematopoietic cell lines: WiDr, a human colon cancer cell line; MCF7, a human breast cancer cell line; 8226, a human multiple myeloma cell line; and K562, a human chronic myelogenous leukemia cell line. H69 and H69AR, human small cell lung cancer cell lines used to clone *MRP*, were used as negative and positive controls, respectively.

Basal expression of *MRP* was detected in all cell lines examined. Mitoxantrone-selected cells derived from K562, MCF7, and WiDr did not overexpress *MRP*. A small increase (2- to 4-fold) in *MRP* expression was observed in a series of increasingly drug-resistant mitoxantrone-selected 8226 cells; however, the increase was considerably less than that seen in comparison with H69AR (i.e. 40-fold), or other cell lines reported to overexpress *MRP* [3–5]. Results from this study suggest that overexpression of *MRP* is not important in mitoxantrone-selected multidrug resistance, and that other mechanisms are likely responsible for mitoxantrone-selected multidrug resistance.

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‖ Abbreviations: ATCC, American Type Culture Collection; cDNA, complementary DNA; PCR, polymerase chain reaction; PGP, P-glycoprotein; RT, reverse transcriptase; and 1 × TBE, 0.089 M Tris, 0.089 M boric acid, 0.001 M EDTA, pH 8.3.

## MATERIALS AND METHODS

*Cell culture.* All cell lines were grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (Gemini, Calabasas, CA), penicillin (1 U/mL), and glutamine (2 mM). Cells were incubated at 37° in a 5% $CO_2$–95% air atmosphere and were passaged once every 6 days. Drug-resistant cells were cultured in the presence of mitoxantrone, except for H69AR which was cultured in the presence of doxorubicin.

The derivation and characterization of MCF7/Mitox (MCF7/MR), a drug-resistant variant of MCF7, has been reported [6]. WiDr, and WiDr/resistant were gifts of Dr. R. Wallace (Lederle Laboratories, Pearl River, NY) and have been characterized previously [7]. H69 and the drug-resistant variant H69AR were gifts of Dr. S. P. C. Cole (Queen's University, Kingston, Ontario), and were characterized previously [3, 11]. The cell line K562 was purchased from ATCC, and a drug-resistant variant, K562/MR, was created by continuous exposure of cells to stepwise increases in the concentration of mitoxantrone (from $10^{-8}$ M to $2 \times 10^{-7}$ M). The cell line 8226 was purchased from ATCC, and drug-resistant variants, 8226/MR4, MR10, MR20, and MR40, were created by continuous exposure to stepwise increases in the concentration of mitoxantrone (from $0.5 \times 10^{-8}$ M to $4 \times 10^{-7}$M). The final selection concentration of mitoxantrone in the culture medium of 8226/MR4 was $4 \times 10^{-8}$ M, 8226/MR10 was $1 \times 10^{-7}$ M, 8226/MR20 was $2 \times 10^{-7}$ M, and 8226/MR40 was $4 \times 10^{-7}$ M.

*In vitro* cytotoxicity was measured using a tetrazolium-based semiautomated colorimetric assay [12].

*Nucleic acid manipulations.* Total cellular RNA was isolated by guanidium isothiocyanate cell lysis and cesium chloride purification [13]. RNA was quantitated from spectrophotometric absorbance measurements at 260 nm.

A single large scale cDNA reaction for use in the different gene-specific amplifications was prepared as follows. A 100-μL first strand cDNA reaction comprised of 1 × PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$); 1 mM each dATP, dCTP, dGTP, and TTP; 500 pmol random hexamer; 100 U RNasin; 60 U Avian Myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN); and 500 ng of total cellular RNA was incubated at 42° for 60 min, then at 99° for 10 min, followed by a 4° quick chill.

*MRP*-specific PCR was performed by adding 80 μL of amplification reaction buffer (1 × PCR buffer, 25 pmol of *MRP*-specific amplimers, and 2 U of *Taq* DNA polymerase) to the equivalent of 100 ng starting RNA template (20 μL of the large scale cDNA reaction), followed by incubation at 94° for 5 min, then 29 cycles of 94° for 1 min, 58° for 15 sec, 72° for 15 sec, a final extension at 72° for 5 min, and a quick chill to 4°. The amplimer sequences for *MRP* correspond to nucleotides 4109–4128 and 4381–4400 of the cDNA sequence obtained from Genbank (accession number L05628). The *MRP* amplimer sequences are as follows:

*MRP* upstream:
5′-GGACCTGGACTTCGTTCTCA-3′
*MRP* downstream:
5′-CGTCCAGACTTCCTTCATCCG-3′

PCR parameters and amplimer sequences for *mdr1* and histone *H3.3* are as described [14] except that 29 cycles of PCR was performed.

Radioactive tracer was incorporated into all PCR products by the addition of [α-$^{32}$P]dCTP (3000) Ci/mmol) to the PCR.

Analysis was performed by fractionation of PCR products through a non-denaturing 1 × TBE–6% polyacrylamide gels at 100 V for 2 hr, or 1 × TBE–3% agarose gels at 75 V for 2 hr. Gels with radiolabeled PCR products were dried down, quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and autoradiographed for figures.

Table 1. Relative expression of *MRP* and *H3.3* in mitoxantrone-selected cell lines*

| Cell line | Resistance to mitoxantrone† | *MRP*‡ | *H3.3*§ | *MRP/H3.3* |
|---|---|---|---|---|
| 8226/MR4 | 10 | 1.8 | 1.0 | 1.8 |
| 8226/MR10 | 12 | 1.6 | 0.8 | 2.0 |
| 8226/MR20 | 36 | 1.6 | 1.0 | 1.6 |
| 8226/MR40 | 58 | 4.0 | 1.0 | 4.0 |
| K562/MR | 10 | 0.6 | 0.8 | 0.8 |
| MCF7/MR | 1208 | 0.4 | 0.9 | 0.4 |
| WiDr/R | 21 | 0.8 | 0.9 | 0.9 |
| H69AR | 16 | 36 | 0.9 | 40 |

* Expression was measured as the radiolabel incorporated into respective PCR products and was quantitated as arbitrary units by a PhosphorImager.

† Degree of resistance is relative to the parent cell line, and is calculated from the concentrations that inhibited growth by 50% in a modified tetrazolium-based assay. Values for MCF7/MR, WiDr/R, and H69AR were taken from Refs. 6, 7, and 11, respectively.

‡ *MRP*-resistant cells/*MRP*-sensitive parent.

§ *H3.3*-resistant cells/*H3.3*-sensitive parent.

## RESULTS

The mitoxantrone-selected human tumor cell lines used in this study are from diverse origins and display different spectrums of multidrug resistance, even though they were derived by similar selection schemes. Compared with the respective parental cell line, WiDr/R is resistant to mitoxantrone, doxorubicin and vinblastine [7]; MCF7/MR is resistant to a wide variety of agents including mitoxantrone, doxorubicin, vinblastine, and cis-platinum [6]; and K562/MR is resistant to many drugs including mitoxantrone, doxorubicin, and vinblastine. The 8226/MR series is resistant to mitoxantrone and doxorubicin but not vinblastine (Table 1 and data not shown).

PCR primers suitable for analysis of *MRP* mRNA were designed from the cDNA sequence found in Genbank. Since the *MRP* gene structure is not known, we designed primers separated by 252 bp because eukaryotic exons are rarely this long [15],

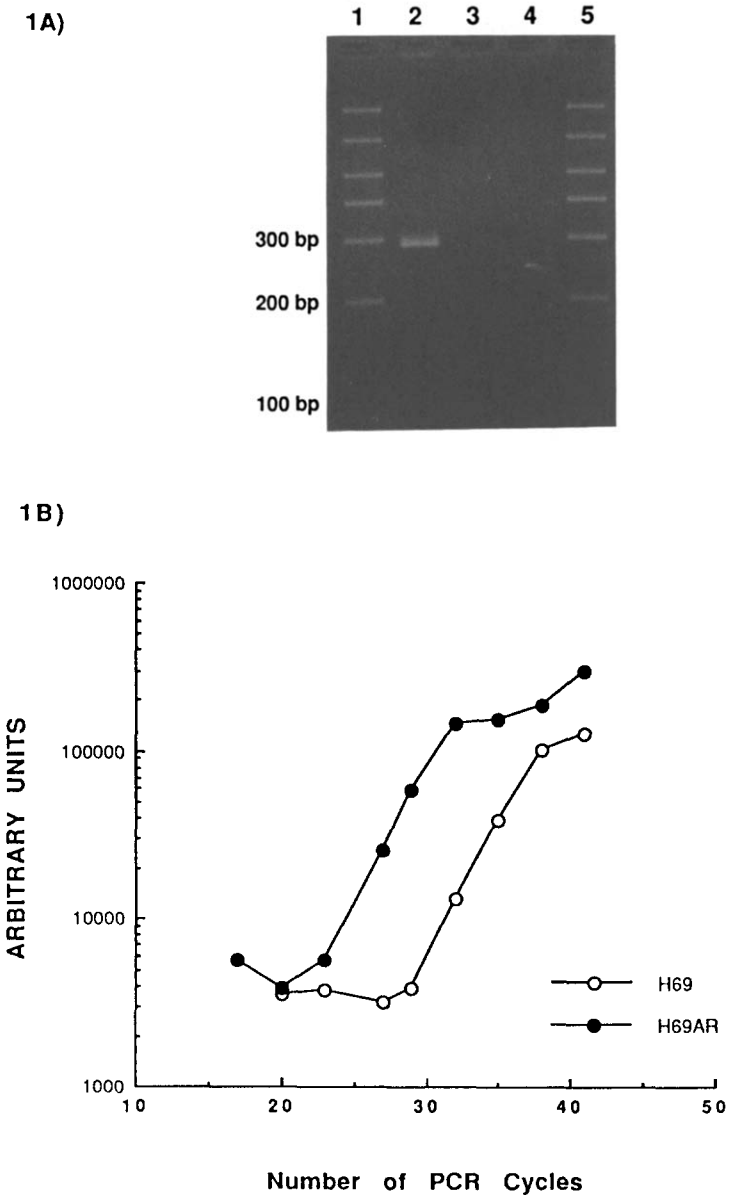


Fig. 1. (A) Specificity of *MRP* primers for *MRP* mRNA. One hundred nanograms of total RNA from H69AR cells was subjected to first strand cDNA reaction either in the presence or absence of reverse transcriptase, and then PCR for 35 cycles according to parameters described in the text. Ten percent of the PCR product was analyzed on a 1 × TBE–3% agarose gel and visualized by ethidium bromide fluorescence. Key: lanes 1 and 5, molecular weight standards; lane 2, is the PCR products generated from the cDNA reaction containing RT; lane 3, the PCR products from the cDNA reaction that had the RT omitted; and lane 4, the "mock" reaction. (B) Exponential range of amplification for *MRP*. PCR was performed on cDNA derived from 100 ng of total RNA from H69 or H69AR in the presence of [$\alpha$-$^{32}$P]dCTP for varying numbers of cycles. Following size fractionation by gel electrophoresis, the gel was dried and exposed to PhosphorImaging plates. Radiolabeled PCR products were quantitated on a PhosphorImager and plotted. The exponential range of amplification for H69AR was from approximately 20 to 32 cycles. The exponential range of amplification for H69 was from approximately 27 to 38 cycles.

and therefore the primers should be from different exons. To ensure that we were amplifying mRNA and not contaminating genomic DNA, RT/PCR was performed with and without the addition of reverse transcriptase to the cDNA reaction of H69/AR RNA. The 292 bp amplicon did amplify when reverse transcriptase was added to the cDNA reaction, but did not amplify when reverse transcriptase was omitted from the cDNA reaction (Fig. 1A). Using these primers, we also attempted to amplify *MRP*

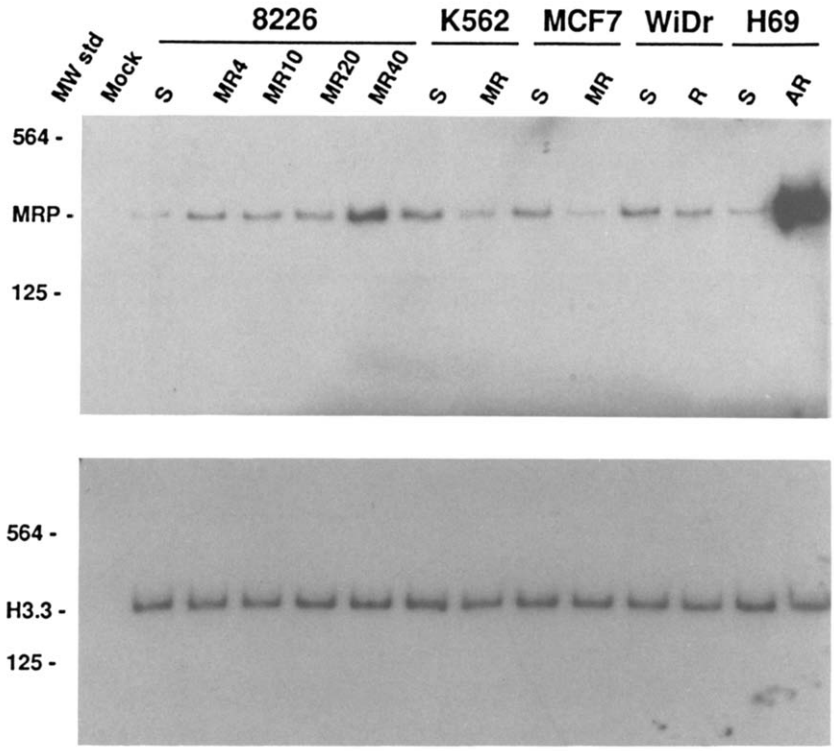


Fig. 2. Relative expression of *MRP* mRNA (*top*), and *H3.3* mRNA (*bottom*) in the various cell lines. cDNA derived from 100 ng (or 50 ng for *H3.3* amplification) of total RNA starting material underwent 29 cycles of PCR in the presence of [$\alpha$-$^{32}$P]dCTP. Ten percent of the respective PCR reaction products were separated on a non-denaturing 1 × TBE–6% polyacrylamide gel, dried, quantitated on a PhosphorImager, and autoradiographed. Results are tabulated in Table 1.

directly from genomic DNA, but were unsuccessful (data not shown). Together these data indicate that the primers described herein are specific for *MRP* mRNA.

To accurately estimate mRNA levels by PCR, the exponential range of amplification for *MRP* was determined using RNA from the low level expressing cell line H69 and the overexpressing variant, H69AR [3]. cDNA products from the equivalent of 100 ng of starting total cellular RNA from both cell lines were amplified, in the presence of [$\alpha$-$^{32}$P]dCTP, for varying numbers of cycles. Following amplification, PCR products were separated on non-denaturing 1 × TBE–6% polyacrylamide gels, dried and exposed to phosphorImaging plates. The relative amount of radioactivity incorporated into each *MRP* PCR product was quantitated on a Molecular Dynamics PhosphorImager. Figure 1B is a graphic representation of these results. From these results it can be seen that the overlap in the exponential range of *MRP* amplification for H69 and H69AR was between 27 and 32 cycles of PCR. In all subsequent experiments, 29 cycles of PCR were performed.

cDNA reaction products from the equivalent of 100 ng of input RNA from the various drug-sensitive and drug-resistant cell lines were used for analysis of *MRP* expression. Following PCR of the cDNA reaction products, 10% of the respective PCR products were separated on a non-denaturing 1 × TBE–6% polyacrylamide gel. The top panel of Fig. 2 is an autoradiograph of this gel, and Table 1 shows the expression of *MRP* in the drug-resistant cell line relative to the drug-sensitive parent cell line, as quantitated on a PhosphorImager. The results shown in Fig. 2 are representative of experiments done at least twice.

To ensure RNA integrity and to provide a gene expression reference point, a cell cycle independent, ubiquitously expressed histone gene, *H3.3* [16, 17], was amplified from the same cDNA reaction. Like *MRP*, amplification of *H3.3* was performed for 29 cycles, but cDNA from the equivalent of 50 ng of input RNA was amplified. PCR under these parameters was in the exponential range of amplification for *H3.3* (data not shown). Ten percent of the respective PCR products were separated on a non-denaturing 1 × TBE–6% polyacrylamide gel. The bottom panel of Fig. 2 is an autoradiograph of this gel, and Table 1 shows the expression of *H3.3* in the drug-resistant cell line relative to its drug-sensitive parent cell line.

Also shown in Table 1 is the degree of resistance of the various cell lines to mitoxantrone, as well as the relative expression of *MRP* in the various mitoxantrone-selected cell lines, when normalized to differences in *H3.3* expression. When examined by itself, or when compared with the expression of *H3.3*, no detectable increase in *MRP* expression was

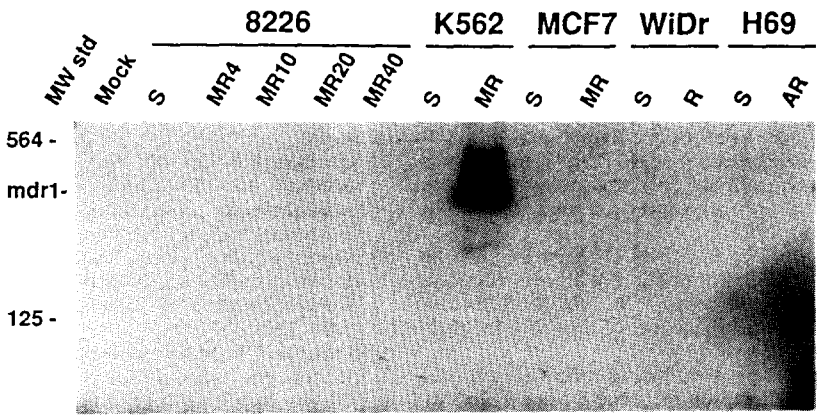


Fig. 3. Expression of *mdr*1 in the various cell lines. cDNA derived from 100 ng of total RNA starting material underwent 29 cycles of PCR in the presence of [$\alpha$-$^{32}$P]dCTP. Ten percent of the respective PCR reaction products were separated on a non-denaturing 1 × TBE–6% polyacrylamide gel and autoradiographed.

observed for mitoxantrone-selected K562, MCF7, or WiDr cells. The most highly resistant mitoxantrone-selected 8226 cells showed a modest increase in *MRP* expression (i.e. 4-fold); however, it was far less than that seen in the other *MRP* overexpressing cell lines reported to date [3–5]. In the control cell lines, H69AR expressed 40-fold more *MRP* relative to H69. This value is in good agreement with the value reported previously (i.e. 100-fold, Ref. 3), and the value we obtained by northern blot analysis (i.e. 25-fold, data not shown).

In addition to measuring *MRP* expression, we examined *mdr*1 expression in these cell lines, using an RT/PCR assay. Figure 3 is an autoradiograph of these results, and shows that only K562/MR expressed *mdr*1. Confirmation of PGP production in K562/MR was done by western blot analysis and showed PGP localized to the plasma membrane fraction of the cell (data not shown). In contrast to K562/MR, *mdr*1 expression was not detected in any of the other drug-sensitive or drug-resistant cell lines.

## DISCUSSION

Aside from the human mitoxantrone cell lines discussed in this report, other human cell lines have been selected for primary resistance to mitoxantrone [8–10]. With the exception of the K562 resistant cell line described in this report, the mitoxantrone-selected cell lines do not overexpress *mdr*1. In many of these reported cell lines, mitoxantrone resistance is associated with a decrease in intracellular drug accumulation that may be energy dependent [6, 7, 9]. From this information it is reasonable to predict that mitoxantrone-selected resistance may be mediated by *MRP*, a gene associated with non-PGP multidrug resistance which belongs to the ABC transporter gene superfamily. Although we were unable to associate mitoxantrone-selected drug resistance with *MRP* overexpression, we recently identified a 110 kDa protein in MCF7/MR and 8226/MR, using the monoclonal antibody LRP-56 that may be involved in imparting this multidrug-resistant phenotype [18].

In conclusion, our data demonstrated that a basal expression of *MRP* is found in a variety of different human tumor cell lines, and that overexpression of *MRP* appears not to be associated with mitoxantrone-selected multidrug resistance. Although one of the cell lines, 8226/MR40, the most drug-resistant cell line of the 8226/MR series, did overexpress *MRP* 4-fold, this level of overexpression was quite low when compared with other *MRP* overexpressing cell lines [3–5]. It is possible that this modest increase in *MRP* mRNA contributes to mitoxantrone resistance in cells of hematopoietic origin; however, the observed increase seen in *MRP* mRNA expression may simply be a non-specific change that occurred during the drug selection process. Analysis of *MRP* mRNA using the RT/PCR assay described in this report will be performed on human tumor specimens in an effort to elucidate the potential clinical relevance of *MRP*.

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