Atypical Multidrug Resistance: Breast Cancer Resistance Protein Messenger RNA Expression in Mitoxantrone-Selected Cell Lines

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Background: Human cancer cell lines grown in the presence of the cytotoxic agent mitoxantrone frequently develop resistance associated with a reduction in intracellular drug accumulation without increased expression of the known drug resistance transporters P-glycoprotein and multidrug resistance protein (also known as multidrug resistance-associated protein). Breast cancer resistance protein (BCRP) is a recently described adenosine triphosphate-binding cassette transporter associated with resistance to mitoxantrone and anthracyclines. This study was undertaken to test the prevalence of BCRP overexpression in cell lines selected for growth in the presence of mitoxantrone. Methods: Total cellular RNA or poly A + RNA and genomic DNA were isolated from parental and drug-selected cell lines. Expression of BCRP messenger RNA (mRNA) and amplification of the BCRP gene were analyzed by northern and Southern blot hybridization, respectively. Results: A variety of drug-resistant human cancer cell lines derived by selection with mitoxantrone markedly overexpressed BCRP mRNA; these cell lines included sublines of human breast carcinoma (MCF-7), colon carcinoma (S1 and HT29), gastric carcinoma (EPG85-257), fibrosarcoma (EPF86-079), and myeloma (8226) origins. Analysis of genomic DNA from BCRP-overexpressing MCF-7/MX cells demonstrated that the BCRP gene was also amplified in these cells. Conclusions: Overexpression of BCRP mRNA is frequently observed in multidrug-resistant cell lines selected with mitoxantrone, suggesting that BCRP is likely to be a major cellular defense mechanism elicited in response to exposure to this drug. It is likely that BCRP is the putative “mitoxantrone transporter” hypothesized to be present in these cell lines. [J Natl Cancer Inst 1999;91:429–33]
lines. This study was undertaken to test the prevalence of BCRP overexpression in mitoxantrone-selected cell lines.

**Materials and Methods**

**Cell lines.** The cell lines used and the conditions under which they were cultured are given in Table 1 and references listed therein. The S1M1-3.2 colon carcinoma cells were derived from S1 cells (a subclone of human colon carcinoma cell line LS174T) by selection for growth in increasing concentrations of mitoxantrone until a final concentration of 3.2 μM was achieved. The HL-60/MX2 leukemia cells were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in culture as described previously (12).

**Northern blot hybridization.** Total cellular RNA was used for northern blot hybridization in all cases except for H209 or H69 cells, where poly A⁺ RNA was used. RNA extraction and northern blotting were performed by standard techniques as described previously (11). A 795-base-pair (bp) fragment of the 3' end of the 2418-bp BCRP cDNA (GenBank database accession number AF098951) was used as the hybridization probe after labeling with [32P]deoxyctydine triphosphate (“Prime-a-Gene” labeling kit; Promega Corp., Madison, WI) as described previously (11). To control for variations in sample loading, the blots were stripped, then rehybridized with 32P-labeled β-actin or 18S RNA probes as described previously (11). The levels of messenger RNA (mRNA) expression in different cell lines were compared by an arbitrary grading system (Table 1) based on visual determination of signal intensities.

**Southern blot hybridization.** Genomic DNA was isolated from the MCF-7 cell lines, digested with EcoRI or BamHI, and separated by 0.8% agarose gel electrophoresis. After staining with ethidium bromide, the DNA was transferred and fixed to a nitrocellulose filter by use of standard techniques (13). The filter was hybridized with the 32P-labeled 795-bp BCRP probe described above for northern blot analysis.

Northern or Southern blots were prepared by collaborating authors (S. P. C. Cole, W. S. Dalton, M. Dietel, H. Lage, and E. Schneider) who maintain multidrug-resistant cell lines in their laboratories; the blots were then probed for BCRP expression in the laboratories of D. D. Ross and L. A. Doyle at the University of Maryland Greenebaum Cancer Center.

**Results**

The drug-resistant cell lines used and their characteristics with respect to the degree of resistance that they exhibit and expression (relative to parental cells) of mRNAs encoding the multidrug resistance transporters Pgp, MRP, and BCRP are summarized in Table 1.

All of the mitoxantrone-selected sublines derived from human breast carcinoma MCF-7 cells overexpressed BCRP mRNA relative to its expression in parental MCF-7 cells. BCRP-positive cell lines included MCF-7/Mitox cells (3) and two sublines of MCF-7/MX (MCF-7/MX<sub>R5250</sub> and MCF-7/MX<sub>R5600</sub>) (Mitox = MX = RNOV [see below] = mitoxantrone) that were reselected with higher concentrations of mitoxantrone (6) (Fig. 1, B; lanes 4–6). The MCF-7/MX cells (Fig. 1, B; lanes 4–6) appeared to express amounts of BCRP mRNA comparable to or greater than those expressed by the MCF-7/AdrVp1000 cells (Fig. 1, B; lane 10) from which BCRP was originally isolated (Adr = Adriamycin® = doxorubicin, and Vp = verapamil) (11). In contrast to BCRP mRNA expression in the mitoxantrone-selected cell line, BCRP mRNA was not overexpressed in MCF-7 cells selected with methotrexate [MCF-7/MTX (13)], etoposide [MCF-7/VP (14)], or doxorubicin [MCF-7/Adr (15)], which derive resistance, at least in part, from the overexpression of dihydrofolate reductase (DHFR), MRP, or Pgp, respectively (Fig. 1, B; Table 1). Mitoxantrone-selected human breast carcinoma MDA-MB-231RNOV cells, which are less resistant to mitoxantrone than the MCF-7 sublines, demonstrated only slightly elevated levels of BCRP mRNA compared with the levels seen in the parental cell line (Fig. 1, C; lanes 3 and 4).

**Table 1.** Characteristics of selected multidrug-resistant human cancer cell lines

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Tumor origin</th>
<th>-fold resistance to selecting agent</th>
<th>Selecting agent</th>
<th>Messenger RNA expression levels relative to parental cell line</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/Mitox</td>
<td>Breast</td>
<td>–</td>
<td>Mitoxantrone</td>
<td>– +/− ++</td>
<td>(3)</td>
</tr>
<tr>
<td>MCF-7/MX&lt;sub&gt;R5250&lt;/sub&gt;</td>
<td>Breast</td>
<td>–</td>
<td>Mitoxantrone</td>
<td>– +/− ++</td>
<td>(5)</td>
</tr>
<tr>
<td>MCF-7/MX&lt;sub&gt;R5600&lt;/sub&gt;</td>
<td>Breast</td>
<td>–</td>
<td>Mitoxantrone</td>
<td>– – +/−</td>
<td>(6)</td>
</tr>
<tr>
<td>MCF-7/AdrVp1000</td>
<td>Breast</td>
<td>–</td>
<td>Doxorubicin, verapamil</td>
<td>– – +/−</td>
<td>(9,10)</td>
</tr>
<tr>
<td>MCF-7/VP</td>
<td>Breast</td>
<td>28</td>
<td>Etoposide</td>
<td>– – +/−</td>
<td>(14)</td>
</tr>
<tr>
<td>MCF-7/Adr</td>
<td>Breast</td>
<td>192</td>
<td>Doxorubicin</td>
<td>– – –</td>
<td>(15)</td>
</tr>
<tr>
<td>MCF-7/MTX</td>
<td>Breast</td>
<td>1000</td>
<td>Methotrexate</td>
<td>– – –</td>
<td>(13)</td>
</tr>
<tr>
<td>MDA-MB-231RNOV</td>
<td>Breast</td>
<td>93</td>
<td>Mitoxantrone</td>
<td>– – +/−</td>
<td>Lage H: unpublished data</td>
</tr>
<tr>
<td>8226/MR20</td>
<td>Myeloma</td>
<td>36</td>
<td>Mitoxantrone</td>
<td>– – –</td>
<td>(4)</td>
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<tr>
<td>S1M1-3.2</td>
<td>Colon</td>
<td>1435</td>
<td>Mitoxantrone</td>
<td>– – +/−</td>
<td>(7)</td>
</tr>
<tr>
<td>HT29RNOV</td>
<td>Colon</td>
<td>100</td>
<td>Mitoxantrone</td>
<td>+/− –</td>
<td>Lage H: unpublished data</td>
</tr>
<tr>
<td>H209/MX2</td>
<td>Small-cell lung</td>
<td>2</td>
<td>Mitoxantrone</td>
<td>– – –</td>
<td>Cole SP: unpublished data</td>
</tr>
<tr>
<td>H209/MX4</td>
<td>Small-cell lung</td>
<td>4</td>
<td>Mitoxantrone</td>
<td>– – –</td>
<td>Cole SP: unpublished data</td>
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<tr>
<td>H209/V6</td>
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<td>22</td>
<td>Etoposide</td>
<td>– – –</td>
<td>(16)</td>
</tr>
<tr>
<td>H69/AR</td>
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<td>Doxorubicin</td>
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<td>(17)</td>
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<td>EPG85-257RNOV</td>
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<td>457</td>
<td>Mitoxantrone</td>
<td>– – +/−</td>
<td>(8,18)</td>
</tr>
<tr>
<td>EPG85-257RDB</td>
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<td>1857</td>
<td>Daunorubicin</td>
<td>+/− –</td>
<td>(8,18)</td>
</tr>
<tr>
<td>EPP85-181RNOV</td>
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<td>27</td>
<td>Mitoxantrone</td>
<td>– – –</td>
<td>Lage H: unpublished data</td>
</tr>
<tr>
<td>EPP85-181RDB</td>
<td>Pancreatic</td>
<td>846</td>
<td>Daunorubicin</td>
<td>+/− –</td>
<td>(19)</td>
</tr>
<tr>
<td>EPP86-079RNOV</td>
<td>Fibrosarcoma</td>
<td>7</td>
<td>Mitoxantrone</td>
<td>– – –</td>
<td>Lage H: unpublished data</td>
</tr>
<tr>
<td>HL-60/MX2</td>
<td>Leukemia</td>
<td>35</td>
<td>Mitoxantrone</td>
<td>– – –</td>
<td>(12)</td>
</tr>
</tbody>
</table>

*Pgp = P-glycoprotein; MRP = multidrug resistance protein; BCRP = breast cancer resistance protein; Mitox = MX = RNOV = MR = mitoxantrone resistant; Adr = Adriamycin® = doxorubicin; Vp = verapamil; VP = V = etoposide; RDB = daunorubicin resistant; MTX = methotrexate.
Amplification of the BCRP gene was detected in MCF-7/MXPR cells (Fig. 2; lanes 2 and 8), MCF-7/MXRS250 cells (Fig. 2; lanes 3 and 9), and MCF-7/MXRS600 cells (Fig. 2; lanes 4 and 10). No BCRP gene amplification was observed in etoposide-selected, MRP-overexpressing MCF-7/VP cells (Fig. 2; lanes 5 and 11) or in methotrexate-selected, DHFR-amplified MCF-7/MTX cells (Fig. 2; lanes 6 and 12).

Human myeloma 8226/MR20 cells are capable of sustained growth in 200 nM mitoxantrone (4) and also demonstrated overexpression of BCRP compared with BCRP expression in parental 8226 cells (Fig. 1, A; lanes 3 and 4). The expression of BCRP mRNA in 8226/MR20 cells was less than the amount of BCRP mRNA expressed in MCF-7/Mitox cells (Fig. 1, A; lane 2); however, this may be because the latter cell line is more resistant to mitoxantrone (1208-fold, Table 1) than is the former (36-fold, Table 1).

Overexpression of BCRP mRNA was also observed in human colon carcinoma S1/M1-3.2 cells (resistant to 3.2 μM mitoxantrone) (Fig. 1, B; lane 1), compared with parental S1 cells (Fig. 1, B; lane 2). Another human colon carcinoma cell line selected with mitoxantrone, HT29RNOV, displayed overexpression of BCRP compared with the BCRP expression in parental HT29 cells (Fig. 1, C; lanes 1 and 2).

None of the small-cell lung cancer cell lines tested overexpressed BCRP (data not shown), including those selected with mitoxantrone (H209/MX2 and H209/MX4) (Table 1). It is of note that these mitoxantrone-selected lines showed only a low degree of resistance to the drug and also have no demonstrable overexpression of Pgp or MRP. Etoposide-selected H209/V6 cells appear to derive their resistance from a mutated topoisomerase II (16); H69/AR cells, selected with doxorubicin, markedly overexpress MRP (17).

The human gastric carcinoma-derived resistant EPG85-257RNOV cell line was developed by stepwise selection with mitoxantrone (8). Compared with parental cells, EPG85-257RNOV cells have no detectable overexpression of Pgp or MRP; they display decreased mitoxantrone accumulation and cross-resistance to anthracyclines, but they retain sensitivity to cisplatin and vinca alkaloids (8,18). Northern blot analysis indicated elevated levels of BCRP mRNA in EPG85-257RNOV cells compared with the levels in the parental EPG85-257 cells (Fig. 1, C; lanes 7 and 8). In contrast, a Pgp-overexpressing subline selected with daunorubicin, EPG85-257RDB, did not overexpress BCRP relative to its expression in parental EPG85-257 cells (Fig. 1, C; lane 9).

BCRP mRNA was not overexpressed in human pancreatic carcinoma EPP85-181 cells selected in mitoxantrone (EPP85-181RNOV) or daunorubicin (EPP85-181RDB) (19) (Fig. 1, C; lanes 10–12). The baseline expression of BCRP in the parental EPP85-181 pancreatic carcinoma cells appeared to be lower than that in the parental EPG85-257 gastric carcinoma cells (Fig. 1, C; lanes 7 and 10).

Mitoxantrone-selected EP886-079RNOV human fibrosarcoma cells displayed overexpression of BCRP mRNA compared with the BCRP mRNA expression in parental EP886-079 cells (Fig. 1, C; lanes 5 and 6).

A mitoxantrone-selected subline of human acute myeloid leukemia HL-60 cells [HL-60/MX2 (12)] did not overexpress...
with deoxycytidine triphosphate of breast cancer resistance protein complementary DNA that was labeled and fixed to a nitrocellulose filter. The filter was probed with a 795-base-pair fragment of breast cancer resistance protein; MX = mitoxantrone; VP = etoposide; MTX = methotrexate; DHFR = dihydrofolate reductase; Kb = kilobase. Lanes 1 and 7 = MCF-7; lanes 2 and 8 = MCF-7/MXPR; lanes 3 and 9 = MCF-7/MXR_{2,3,5}; lanes 4 and 10 = MCF-7/MXR_{3,5,6,0,0}; lanes 5 and 11 = MCF-7/VP (overexpresses MRP); lanes 6 and 12 = MCF-7/MTX (overexpresses DHFR).

BCRP mRNA (data not shown). However, these resistant cells do not display the typical phenotype displayed by mitoxantrone-selected cells that overexpress BCRP, since HL-60/MX2 cells do not have diminished accumulation of mitoxantrone and are cross-resistant to etoposide. HL-60/MX2 cells have altered catalytic activity of DNA topoisomerase II and reduced levels of topoisomerase II alpha and beta proteins (12).

**DISCUSSION**

Elevated expression of the novel ABC transporter BCRP is a common feature of many cancer cell lines selected with mitoxantrone and is consistently associated with a phenotype that includes high-level resistance to mitoxantrone, lower resistance to anthracyclines, and sensitivity to vinca alkaloids, paclitaxel, and cisplatin. ATP-dependent export of mitoxantrone, anthracyclines, and rhodamine 123 has been observed in several BCRP-positive, mitoxantrone-resistant cell lines and BCRP-transfected breast cancer cells (7,11). Cross-resistance to topoisomerase I-directed agents has been reported in some mitoxantrone-resistant cell lines (20), but resistance to these agents has not yet been confirmed in BCRP-transfected cells.

The BCRP peptide has the characteristics of a “half-transporter,” having only a single ATP-binding domain and a single lipophilic region containing transmembrane domains (11). Hence, it is possible that the most efficient transmembrane conductance channel contains BCRP as a dimer or multimer either with itself or with another heretofore undescribed “half-transporter” (11). Although the transfection studies suggest that the enforced overexpression of BCRP cDNA is sufficient to confer drug resistance to the transfected cells (11), it is possible that another transporter(s) may be involved in a dimeric or a multimeric complex with BCRP, which may contribute to the resistance of the mitoxantrone-selected cell lines. The identification of other transporters that may participate in the BCRP transmembrane conductance channel is currently under active investigation in our laboratory.

The finding of elevated expression of BCRP mRNA in the human colon carcinoma S1M1-3.2 cells suggests that BCRP is the “non-Pgp, non-MRP” drug transporter manifested by this multidrug-resistant cell line. This is of particular importance because of the recent report (7) of a novel and specific inhibitor of the transporter identified in S1M1-3.2 cells. This inhibitor, fumitremorgin C, does not reverse resistance in cells that overexpress Pgp or MRP. In preliminary studies in our laboratory, fumitremorgin C is able to enhance the accumulation and inhibit the efflux by BCRP in BCRP-transfected MCF-7 cells [data not shown; (11)]. Further development of fumitremorgin C is warranted for use in functional assays of BCRP activity and possibly as a clinical adjunct to chemotherapy, should BCRP protein/activity levels be shown to be elevated in human cancers.

BCRP expression is associated with multidrug resistance in mitoxantrone-selected cell lines derived from human breast, gastric, and colon cancers, as well as from fibrosarcoma and multiple myeloma cells. High levels of BCRP expression are not associated with strong expression of MRP or Pgp in mitoxantrone-selected cell lines or in multidrug-resistant cell lines known to overexpress MRP or Pgp.

In conclusion, our study indicates that elevated expression of BCRP is frequently observed in mitoxantrone-selected cell lines derived from human multiple myeloma and a number of solid tumors. Considered together with results from BCRP transfection studies (11), these data demonstrate that BCRP is a novel ABC protein responsible for mediating resistance to mitoxantrone and other important chemotherapeutic agents in a wide variety of human cancer cell lines.

**REFERENCES**


(6) Yang CH, Cowan K, Schneider E. Reselection of a mitoxantrone-resistant...


NOTES

L. Greenberger owns stock in, and is an employee of, American Home Products, which fully owns Wyeth-Ayerst Research, the company that manufactures and sells mitoxantrone.

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