Identification of Breast Cancer Resistant Protein/Mitoxantrone Resistance/Placenta-Specific, ATP-binding Cassette Transporter as a Transporter of NB-506 and J-107088, Topoisomerase I Inhibitors with an Indolocarbazole Structure

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Abstract

The antitumor drugs NB-506 and J-107088 are potent topoisomerase I inhibitors with an indolocarbazole structure. To clarify the factors involved in resistance to these drugs, we established two NB-506-resistant mouse fibroblast cell lines (LY/NR1 and LY/NR2), a human colon carcinoma cell line (HCT116/NR1), and a lung cancer cell line (PC13/NR1). These cell lines were highly resistant to NB-506 and J-107088, and LY/NR2 cells showed markedly reduced accumulation and strong efflux of NB-506, suggesting activation of a drug efflux pump in the resistant cells. To identify the molecules responsible for efflux of NB-506, we compared the gene expressions of the mouse resistant LY/NR1 cells, LY/NR2 cells, and their parental cells by oligonucleotide microarray. Of 34,020 genes analyzed, we found that an ATP-binding cassette transporter BCRP/MXR/ABCP (BCRP) gene showed the highest increase in the expression, 31-fold higher in the LY/NR2-resistant cells than in their parental cells. The selective overexpression of this gene was also detected in the two human resistant cell lines, suggesting the involvement of breast cancer resistant protein (BCRP) in the resistance and efflux of these drugs. Finally, a PC-13 cell line transfected with BCRP expression vector displayed 22- and 17-fold resistance to NB-506 and J-107088 and enhanced efflux activity of J-107088. However, the transfectants were not resistant to mitoxantrone or topotecan, the drugs previously thought to be the substrates of BCRP. Thus, our study presents a novel mechanism of drug resistance mediated by BCRP.

Introduction

ABC2 transporters are a large superfamily of proteins that transport a wide variety of substrates (1, 2). Some members of the family are thought to play an important role in the host-defense mechanism to xenobiotics as well as in the drug resistance of cancer cells to anticancer agents. Cancer cells expressing such ABC transporters exhibit decreased intracellular concentrations of drug because of active efflux by the transporters, which causes drug resistance.

Two members of this family, P-gp (3, 4) and MRPI (5), are well characterized and have been shown to be involved in resistance to multiple anticancer drugs, which is known as multidrug resistance. Both molecules transport a variety of anticancer drugs with unrelated structures and functions. In addition, increasing numbers of ABC proteins have been recently identified as associated with the drug resistance.

One such transporter, BCRP, is a half-transporter and has the highest homology to the Drosophila white gene family, which transports eye pigment precursor. This gene was isolated independently from human placenta (6), from a cell line selected with doxorubicin and verapamil (7), and from a cell line selected with mitoxantrone (8), and the genes were designated as ABCP, BCRP, and MXR, respectively. A minute variation of amino acid sequences was observed in these clones. BCRP was reported to confer resistance to mitoxantrone (7), and some cell lines with overexpression of this gene showed cross-resistance to camptothecin derivatives (9, 10). BCRP is localized in the plasma membrane (11, 12) and thus is thought to be a novel type of drug efflux pump with a substrate spectrum different from those of P-gp and MRPI. However, the spectrum of cross-resistance varied, depending on the cell line and experimental system (7, 9), which suggests that complex mechanisms determine the substrate specificity.

Indolocarbazole compounds are a novel class of anticancer agents with a unique structure. These agents include the topo I inhibitor NB-506 (13) and J-107088 (14) and protein kinase inhibitors such as UCN-01 (15). They show strong antitumor activity, and J-107088 and UCN-01 are currently under clinical evaluation. Moreover, study of NB-506 suggested that it is not a substrate of P-gp or MRPI (13, 16), implying that the indolocarbazole topo I inhibitors are effective in multidrug-resistant cells.

On the other hand, there are unknown mechanisms controlling the intracellular accumulation of indolocarbazole topo I inhibitors. Analysis of the cytotoxicity of NB-506 against various cell lines suggested that the tumor cell lines showed different levels of accumulation of NB-506 and that the accumulation levels were associated with the cellular sensitivity to the drug (13). In addition, some NB-506-resistant cell lines showed reduced accumulation of NB-506 (17). Therefore, we assumed that the putative transporter protein(s) is involved in the control of intracellular concentration of NB-506. To identify this molecule(s), we established several NB-506-resistant cell lines showing reduced accumulation of NB-506 and investigated changes in expression of thousands of genes by oligonucleotide microarray. In this way, we were able to identify BCRP as a transporter of these indolocarbazole drugs.

Materials and Methods

Materials. NB-506 and J-107088 were synthesized in our institute as previously described (13, 14). Topotecan was synthesized in our institute. Doxorubicin, mitoxantrone, and camptothecin were purchased from Sigma Chemical Co. (St. Louis, MO); Vincristine, paclitaxel, and etoposide were purchased from Wako Pure Chemicals (Osaka, Japan). Labeled NB-506 and J-107088 were purchased from Daiichi Pure Chemical Co. (Tokyo, Japan).

Cell Culture and Isolation of the NB-506-Resistant Cell Line. The mouse fibroblast cell line LY, the human colon carcinoma cell line HCT116, and the...
human adenocarcinoma cell line HeLa were grown in DMEM supplemented with 10% FCS and 420 μg/ml of kanamycin. The human lung cancer cell line PC-13 was grown in RPMI 1640 supplemented with 10% FCS and 420 μg/ml of kanamycin. The NB-506-resistant LY cell lines were selected by stepwise exposure to NB-506. The exposure of cells was started at 0.1 μM NB-506 for 2 weeks and then at 0.3 μM for 3 weeks. Finally, two clonal cell lines that grew in the presence of 0.3 μM NB-506 were isolated and designated as LY/NR1 and LY/NR2, respectively. The NB-506-resistant HCT116 and PC-13 cell lines were obtained by continuous exposure to 1.1 μM NB-506. These cell lines were further selected by culture with 20 μM NB-506, and the isolated cell lines were designated as HCT116/NR1 and PC-13/NR13, respectively.

Drug Sensitivity Test. The cytotoxicity of anticancer drugs was determined as previously described (13) using a sulforhodamine B dye-staining method (18).

Drug Accumulation Assay. For measurement of drug accumulation in the cells, the cells seeded at a density of 2 × 10^6 in 25-cm² culture flasks were incubated in medium supplemented with 25 mM HEPES or HBSS buffer containing various concentrations of [3H]NB-506 at 37°C. For measurement of the accumulation in energy-depleted cells, the cells were pretreated with HBSS containing 1 mM DNP for 15 min, and then incorporation of NB-506 was performed for an additional 60 min in the presence of 1 mM DNP. After incorporation of the drug, the cells were washed with ice-cold PBS and treated with trypsin on ice. The suspended cells were counted, washed again with PBS, and solubilized in 0.2% Triton X-100. The lysates were centrifuged at 2000 × g for 10 min, the supernatants were collected, and remaining pellets were solubilized in 2 N NaOH to lyse the nuclei. The radioactivities of supernatant fractions and nuclear lysates were counted in a liquid scintillation counter. The values of the two fractions were added and normalized to the cell numbers to determine the amount of NB-506 accumulated per 10^7 cells.

Drug Efflux Assay. For analyses of LY cell lines, the cells were preloaded with 50 μM [3H]NB-506 in the presence of DNP for 60 min. For analyses of BCRP-transfected PC-13 cells, the vector transfectant and the BCRP transfectants were preloaded with 10 and 75 μM [3H]NB-107088, respectively, for 120 min. The cells were rapidly chilled on ice, washed with PBS after incorporation of the drugs, and incubated in the medium supplemented with 10% FCS for 15 min on ice to recover the cells from an energy-depleted condition. Then the cells were incubated in fresh medium supplemented with 10% FCS for the indicated times. After incubation, the cells were collected by trypsinization on ice, and the radioactivity remaining in the cells was measured as described above.

Oligonucleotide Microarray Analysis. Total RNA was isolated using a RNeasy total RNA isolation kit (QIAGEN, Valencia, CA). Total RNA was biotin-labeled with T7 RNA polymerase (Enzo Diagnostics, Farmingdale, NY; Refs. 19–21). Five oligonucleotide microarrays containing mouse probes were selected by Northern blot analysis. These microarrays were hybridized with biotin-labeled RNA or 8 μg of poly(A)−RNA or 8 μg of total RNA were separated on 1% agarose gel and transferred to nylon membranes. The membranes were probed with a 32P-labeled 425-bp fragment of mouse BCRP cDNA (1230–1654 of AF140218) or 269-bp fragment of human BCRP cDNA (161–429 of AF103796).

Cloning of BCRP cDNA from HeLa Cells. The entire coding sequence of BCRP cDNA was generated by RT-PCR from total RNA of HeLa cells. The PCR amplification of the cDNA was performed with LA-Taq polymerase (Takara, Tokyo, Japan) using primers: 5′-CAAAAAGCTTAAGCGAGCTTCTATTAGC-3′ and 5′-GAATTAGGGAATATTAGAAAT-3′, which were based on the AF103796 sequence with an added HindIII linker. These fragments were cloned into pCDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA).

Nucleotide Sequencing Analysis of Human BCRP. The coding region of a cDNA clone was completely sequenced with a Dydeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequence of the clone was deposited in GenBank under accession no. AB518555. For the analysis of nucleotide sequences of BCRP from human tissue, human BCRP was amplified by PCR with the primers 5′-CAAAAAAGCTTAAGCGAGCTTCTATTAGC-3′ and 5′-AGAGATCATGTCCTGCTTTACCA-3′ from human placenta cDNA, which was a pool of samples from seven individuals, and human kidney cDNA, which was a pool of samples of eight individuals, in Multiple Tissue cDNA Panels (CLONTECH, Palo Alto, CA). The PCR fragments were directly sequenced using a Dydeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). We also performed direct sequencing analysis of PCR fragments derived from BCRP transcripts from HCT116/NR1 and PC-13/NR13 cells as described above.

Transfection Studies. PC-13 cells were transfected with the expression vector pcDNA3.1/V5-His-TOPO containing the full-length BCRP cDNA or vector alone using Effecten Transfection Reagent (QIAGEN). After selection by culture in medium containing 0.2 mg/ml of G418, the emerging colonies were isolated with cloning rings. Clones with high-level expressions of BCRP were selected by Northern blot analysis.

Results

Drug Sensitivities of NB-506-resistant Cell Lines. To obtain NB-506-resistant cell lines with reduced drug accumulation, we selected mouse fibroblast LY cells, the human colon carcinoma cell line HCT116, and the human lung cancer cell line PC-13, by culture in increasing concentrations of NB-506. Mouse LY/NR1 cells showed relatively low resistance to NB-506 and J-107088, 12- and 17-fold resistance, respectively, whereas LY/NR2 cells showed high resistance to NB-506 and J-107088, 64- and 210-fold resistance, respectively, compared with the parental cells (Table 1). The human resistant cell line, HCT116/NR1 displayed 2400- and 230-fold resistances to NB-506 and J-107088, respectively, and PC13/NR13 cells displayed more than 4300- and 140-fold resistances to NB-506 and J-107088, respectively, compared with the parental cells (Table 1). These cell lines showed low cross-resistances to topotecan and mitoxantrone.

Reduced Intracellular Concentration and Enhanced Efflux of NB-506 in Resistant Cells. The intracellular concentration of radio-labeled NB-506 was measured in the NB-506-resistant LY cells (Fig. 1A). The accumulation of NB-506 was considerably reduced in both resistant cell lines, and the accumulations in LY/NR1 and LY/NR2 were ~50 and 14%, respectively, of that in parental cells. Thus, an increase in relative resistance appeared to be associated with a decrease in drug accumulation. The accumulation of J-107088 was also reduced in these two resistant cells in a manner similar to that of NB-506 (data not shown). Additional analysis suggested that reduced drug accumulation in the resistant cells was attributable to enhancement of energy-dependent efflux (Fig. 1B). First, NB-506 incorporation in the energy-depleted condition achieved by incubation with DNP reversed the accumulation of NB-506 in LY/NR1 and LY/NR2 to almost the same level as that in parental cells. Then, when the cells with incorporated NB-506 were incubated in drug-free media, 70 and 20% of the drug were effluxed within 15 min from LY/NR2 cells and LY/NR2 cells, respectively, whereas 100% of intracellular NB-506 remained in the parental cells. Therefore, the putative efflux pump for NB-506 seemed to be activated in the LY/NR2 cells. Reduced accumulation and enhanced efflux of NB-506 were also detected in the
human resistant cell lines HCT116/NR1 and PC-13/NR13 (data not shown).

Comparison of Gene Expression Profiles with the Oligonucleotide Microarray in Resistant and Parental Cells. To identify the gene responsible for enhanced drug efflux in the LY/NR2-resistant cells, we searched for the genes whose expression was selectively elevated in the resistant cells, using oligonucleotide microarrays. We compared the expressions of genes in the parental LY cells, modestly resistant LY/NR1 cells, and strongly resistant LY/NR2 cells, using a Mu11K and a Mu19K set representing about 30,000 murine genes and ESTs. The results from these chips were compiled and sorted on the basis of an increase in the LY/NR2-resistant cells compared with those in the parental cells (Table 2). Among the 34,020 genes surveyed, 12 showed more than 4-fold higher expression in LY/NR2 cells, and only 2 of these showed more than 10-fold higher expression. The gene whose expression was most prominently elevated in LY/NR2 cells was BCRP, an ABC transporter involved in mitoxantrone resistance. The expression of this gene was 31-fold higher in the LY/NR2-resistant cells and was strikingly high as determined by the average difference change. Furthermore, BCRP was expressed 6.0-fold higher than in LY/NR1 cells, which showed an intermediate level of resistance and drug accumulation. Thus, the expression of BCRP was associated with the indolocarbazole resistance of NB-506-resistant LY cells.

This analysis also enabled us to compare the expressions of most of the known ABC transporters. Among the transporters thought to be involved in drug resistance, Mdr1a, MRP1, MRP2, and MRP3 were not expressed in these cell lines. Although Mdr1b was expressed in the two resistant lines and the parental cell lines, the expression levels were the same in these three cell lines. Thus, the involvement of these ABC transporters in the NB-506 resistance of these cell lines was excluded.

Overexpression of BCRP mRNA in the Resistant Cell Lines. To confirm the results obtained by microarray analysis, we performed Northern blot analysis of the BCRP gene in the LY parental and resistant cells. As seen in Fig. 2, BCRP showed a correlation of expression with resistance, as detected in the microarray. We also examined the expression in the human resistant cell lines HCT116/NR1 and PC-13/NR13 and found that human BCRP was selectively overexpressed in both NB-506-resistant cell lines compared with their parental cells. The fact that BCRP was overexpressed in all of the resistant cell lines strongly suggested that this gene is involved in NB-506 and J-107088 resistance.

Nucleotide Sequence of BCRP cDNA. The whole coding region of BCRP was isolated by PCR amplification from mRNA of HeLa cells, and its complete nucleotide sequence was determined. The predicted amino acid sequence of the coding region was identical to that of the BCRP previously reported from Doyle et al. (7) except at codon 482. Codon 482 of our clones was AGG and encoded Arg, whereas the same codon of the BCRP clone reported by Doyle et al. was ACG, thus encoding Thr (Fig. 3B). Differences in codon 482 were observed in clones isolated in different laboratories, and the clone previously isolated from placenta by Allikmets et al. (6) also encoded Arg at this position (Fig. 3B). To investigate which amino acids are found at codon 482 in BCRP of normal human tissues, we performed direct sequencing analysis of the BCRP transcript from human kidney and placenta. We found that codon 482 was AGG, encoding Arg, in BCRP from a human kidney cDNA sample, which was a pool of samples from eight individuals (Fig. 3A) and from a human placenta cDNA (Fig. 3B). These results suggested that the BCRP transcript carrying Arg at codon 482 was expressed in human tissues. We also analyzed the nucleotide sequences of codon 482 of the BCRP transcripts expressed in the NB-506-resistant cell lines.

Table 1 Cross-resistance patterns of resistant cell lines selected for NB-506

<table>
<thead>
<tr>
<th>Drug</th>
<th>HCT116</th>
<th>HCT116/NR1</th>
<th>PC13</th>
<th>PC13/NR13</th>
<th>LY</th>
<th>LY/NR1</th>
<th>LY/NR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB-506</td>
<td>0.13 ± 0.005</td>
<td>300 ± 26 (2400)</td>
<td>0.23 ± 0.08</td>
<td>&gt;1000 (4300)</td>
<td>0.12 ± 0.03</td>
<td>1.4 ± 0.12 (12)</td>
<td>7.7 ± 2.6 (64)</td>
</tr>
<tr>
<td>J-107088</td>
<td>0.0034 ± 0.0007</td>
<td>0.001 ± 0.006</td>
<td>0.01 ± 0.006</td>
<td>1.8 ± 0.94 (140)</td>
<td>0.0017 ± 0.0003</td>
<td>0.029 ± 0.0088 (17)</td>
<td>0.36 ± 0.18 (210)</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>0.0094 ± 0.0006</td>
<td>0.017 ± 0.005 (1.8)</td>
<td>0.038 ± 0.009</td>
<td>0.029 ± 0.006 (0.76)</td>
<td>0.046 ± 0.006</td>
<td>0.058 ± 0.006 (1.3)</td>
<td>0.38 ± 0.25 (8.2)</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.034 ± 0.018</td>
<td>0.14 ± 0.071 (4.1)</td>
<td>0.075 ± 0.014</td>
<td>1.1 ± 0.75 (15)</td>
<td>0.069 ± 0.022</td>
<td>0.25 ± 0.07 (3.6)</td>
<td>2.9 ± 1.5 (49)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1.1 ± 0.48</td>
<td>3.4 ± 0.57 (3.1)</td>
<td>1.2 ± 0.04</td>
<td>3.0 ± 0.20 (2.5)</td>
<td>0.28 ± 0.08</td>
<td>0.52 ± 0.19 (1.9)</td>
<td>2.0 ± 1.1 (4.7)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.025 ± 0.002</td>
<td>0.059 ± 0.01 (2.3)</td>
<td>0.028 ± 0.008</td>
<td>0.069 ± 0.050 (2.5)</td>
<td>0.043 ± 0.002</td>
<td>0.067 ± 0.007 (1.6)</td>
<td>0.19 ± 0.03 (4.5)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.0020 ± 0.0001</td>
<td>0.0093 ± 0.001 (4.7)</td>
<td>0.013 ± 0.003</td>
<td>0.0070 ± 0.0020 (0.56)</td>
<td>0.015 ± 0.011</td>
<td>0.011 ± 0.0020 (0.77)</td>
<td>0.025 ± 0.014 (1.7)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0011 ± 0.0002</td>
<td>0.0021 ± 0.0005 (1.9)</td>
<td>0.0020 ± 0.0003</td>
<td>0.0018 ± 0.0012 (0.92)</td>
<td>0.041 ± 0.007</td>
<td>0.057 ± 0.0094 (1.4)</td>
<td>0.081 ± 0.036 (2.0)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.0070 ± 0.0023</td>
<td>0.067 ± 0.035 (9.6)</td>
<td>0.033 ± 0.004</td>
<td>0.16 ± 0.14 (4.9)</td>
<td>0.0045 ± 0.0029</td>
<td>0.0071 ± 0.0047 (2.2)</td>
<td>0.057 ± 0.06 (17)</td>
</tr>
</tbody>
</table>

*Values are means ± SE of three independent experiments, with relative resistances indicated in parentheses, which were determined by dividing the IC50 value of the resistant cell line by that of the parental cell line.
As detected in human tissues, codon 482 of BCRP expressed in the resistant cells encoded Arg (Fig. 3B).

Introduction of BCRP into PC-13 Cells Conferred Resistance to Indolocarbazole Compounds. To examine whether expression of the BCRP gene can confer resistance to NB-506 and J-107088, we introduced expression plasmids encoding full-length human BCRP under the cytomegalovirus promoter into human cell lines. Stable cell lines with high expression of introduced BCRP were obtained from the PC-13 lung cancer cell line. As shown in Fig. 4B, clones 2-2 and 2-3 showed high expression of BCRP, whereas a vector transfectant did not express it. The expression levels of BCRP in clones 2-2 and 2-3 were about 1/3 and 1/5, respectively, of that in NB-506-selected resistant cell lines, PC13/NR13. The drug sensitivities of these clones were compared with that of a vector transfectant (Fig. 4A). Clones 2-2 and 2-3 showed 22- and 9-fold higher resistance to NB-506, respectively, than the vector-transfected cells. Similarly, 2-2 and 2-3 cells showed about 10-fold higher resistance to J-107088. On the other hand, these clones were not resistant to camptothecin, topotecan, or etoposide. Unexpectedly, both cell lines overexpressing BCRP were not resistant to mitoxantrone. Clones 2-2 and 2-3 showed 0.42- and 0.60-fold higher resistance, respectively, compared with the vector transfected. We also tested the sensitivities to doxorubicin and paclitaxel. The sensitivities of clones 2-2 and 2-3 were similar to those of vector transfectant (data not shown).

Active Efflux of J-107088 in the BCRP Transfectant. The J-107088 efflux from the PC-13 clone with introduced BCRP was examined. Clone 2-2, overexpressing BCRP, showed stronger efflux activity than the vector-transfected clone (Fig. 4C). The 60% of the intracellular J-107088 was transported out after a 30-min incubation in BCRP-transfected clone 2-2, whereas only 20% of the drug was transported out in vector-transfected cells. These results indicated the BCRP-mediated transport of J-107088.

Discussion

In this study we identified the transporter responsible for resistance to the indolocarbazole drugs NB-506 and J-107088, by oligonucleotide microarray.

To identify the putative transporter by gene expression analysis, we prepared three highly resistant cell lines with reduced accumulation and enhanced efflux. Of these cell lines, two mouse cell lines were selected for subsequent gene expression analysis using oligonucleotide microarrays, because the degrees of resistance of the mouse highly resistant LY/NR2 cells and moderately resistant LY/NR1 cells were correlated with their degrees of activation of efflux. Therefore, it was expected that they would reflect the expression levels of the BCRP/MXR/ABCP genes.

Table 2. Genes with elevated expressions in LY/NR2 cells

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Description</th>
<th>LY/NR2 Fold increase</th>
<th>Avg diffa change</th>
<th>LY/NR1 Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa612278</td>
<td>BCRP/MXR/ABCP</td>
<td>31.2</td>
<td>7189</td>
<td>6.0</td>
</tr>
<tr>
<td>TC37566</td>
<td>Hypothetical protein MaCh-2755</td>
<td>15.5</td>
<td>453</td>
<td>13.4</td>
</tr>
<tr>
<td>TC30469</td>
<td>IFN activatable protein</td>
<td>6.2</td>
<td>4402</td>
<td>NC</td>
</tr>
<tr>
<td>m31419</td>
<td>IFN activatable protein</td>
<td>5.2</td>
<td>674</td>
<td>NC</td>
</tr>
<tr>
<td>Msa.853.0</td>
<td>IFN activatable protein</td>
<td>4.7</td>
<td>557</td>
<td>2.3</td>
</tr>
<tr>
<td>Msa.20701.0</td>
<td>3-β hydroxysteroid dehydrogenase</td>
<td>4.6</td>
<td>228</td>
<td>NC</td>
</tr>
<tr>
<td>M31418</td>
<td>IFN activatable protein</td>
<td>4.5</td>
<td>609</td>
<td>4.7</td>
</tr>
<tr>
<td>TC32218</td>
<td>EST</td>
<td>4.5</td>
<td>685</td>
<td>NC</td>
</tr>
<tr>
<td>aa57115</td>
<td>Y13275, Meta-associated tetraspan molecule</td>
<td>4.3</td>
<td>256</td>
<td>3.8</td>
</tr>
<tr>
<td>m94179</td>
<td>M19,000 glycoprotein autoantigen</td>
<td>4.2</td>
<td>402</td>
<td>3.2</td>
</tr>
<tr>
<td>TC15466</td>
<td>Lipocortin</td>
<td>4.1</td>
<td>4084</td>
<td>NC</td>
</tr>
</tbody>
</table>

a The average difference (Avg diff) is an indication of hybridization to perfectly matched oligonucleotide probe sets versus hybridization to mismatched oligonucleotide probes.

b NC, no change.
BCRP-resistant PC-13 cells (PC-13/NR13) were subjected to Northern hybridization with the M2 probe. Preloading with 10 and 75 nM J-107088 showed 22- and 17-fold resistance to NB-506 and J-107088, respectively. Although the NB-506-resistant cell lines HCT116/NR2 and PC-13/NR2 showed 9.6- and 4.9-fold mitoxantrone-resistance, respectively, the degree of resistance was limited compared with those to NB-506 and J-107088. Furthermore, the relative resistance of PC-13 transfector 2-2 cells to mitoxantrone was 0.42 despite their considerable expression of BCRP.

Several explanations are possible for this difference: (a) The difference in substrate specificity might be explained by the small difference in the amino acid sequences in BCRP. Our isolated BCRP differed from one isolated from MCF-7/AdVp by a single amino acid at codon 482. As previously reported, codon 482 varies in clones isolated in different laboratories (Fig. 3B). BCRP isolated from MCF-7/AdVp cells, which was previously shown to confer resistance to mitoxantrone, carries Thr at codon 482 (7). On the other hand, our BCRP clone carries Arg at codon 482, as detected in a clone from normal placenta (6). Codon 482 is located in the putative transmembrane 3-region (6), and analysis of MDR1 suggested that transmembrane regions of ABC transporters are likely to participate in substrate recognition (3). Therefore, it is possible that amino acid substitution at this position alters the substrate specificity of BCRP. Although these substitutions may reflect the occurrence of natural polymorphism, they may also be mutations occurring in the course of drug selection of cultured cells. Such mutations were reported in the case of MDR1 and were shown to affect substrate specificity (24). To clarify this point, we performed sequence analysis of the BCRP transcript in human tissues and demonstrated that the BCRP expressed in human tissue carries Arg at codon 482. Therefore, the characteristics of BCRP obtained in our analysis were not those of mutant protein and were likely to reflect the physiological function of this protein. (b) The different cross-resistance pattern is explained by putative cellular factors affecting the transport activity mediated by BCRP. Because the cell line with introduced BCRP was a lung cancer cell line in our study and a breast cancer cell line in a previous study, the different genetic background of the two cell lines are also likely to cause different cross-resistant patterns by the BCRP. This cellular factor might be either another half-transporter that functions by dimerizing with BCRP or endogenous small molecules to be cotransported with the substrates (25). (c) Cellular metabolism or modification of the drug is also a possible factor for the transport mechanism of the drug. MRP1 (25, 26) and MRP2 (27) are known to recognize the glutathione-conjugated compounds and glucuronidated compounds as substrates, respectively. Such metabolic pathways might contribute to the resistance mediated by BCRP in some cell lines.

Our results also did not show resistance to topotecan of BCRP-transfected PC-13 cell lines. This is consistent with a previous finding that the forced expression of BCRP in breast cancer cell lines resulted in little or no resistance to topotecan (28) and further supported the notion that BCRP alone is not sufficient for resistance to topotecan. In conclusion, we demonstrated involvement of the ABC half-transporter BCRP in the indolocarbazole topo I poisons, NB-506 and J-107088. Our results strongly suggested that BCRP is an efflux pump of NB-506 and J-1070788. Although the expression of drug efflux pumps causes resistance of tumor cells to chemotherapeutic drugs, they also play a physiological role in the host defense mechanism against toxic xenobiotics by preventing the entry of the drugs into important organs (29, 30). Thus, its expression determines the toxicity to the drugs as well as tumor sensitivity to the drug. The BCRP transcript was detected in various organs, although its expression was low, except in the placenta (7). On the other hand, expression of this protein in a panel of human tumor was very low or undetectable (11), although additional studies are required to conclude the expression status in human cancer. Considering these points, it is possible that BCRP contributes to reduction of the toxicity of indolocarbazole.
anticancer agents such as J-107088, which have a wide therapeutic window (31).

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References

Identification of Breast Cancer Resistant Protein/Mitoxantrone Resistance/Placenta-Specific, ATP-binding Cassette Transporter as a Transporter of NB-506 and J-107088, Topoisomerase I Inhibitors with an Indolocarbazole Structure

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