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# 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/NR1and 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

ABC[2] 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 MRP1 (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 recently been 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 MRP1. 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 MRP1 (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 concentration 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

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[2] The abbreviations used are: ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer-resistant protein; MXR, mitoxantrone resistance; ABCP, placenta-specific ABC transporter; MDR, multidrug resistance; topo, topoisomerase; DNP, 2,4-dinitrophenol; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

human adenocarcinoma cell line HeLa were grown in DMEM supplemented with 10% FCS and 420 $\mu$g/ml of kanamycin. The human lung cancer cell line PC-13 was grown in RPMI 1640 supplemented with 10% FCS and 420 $\mu$g/ml of kanamycin. The NB-506-resistant LY cell lines were selected by continuous stepwise exposure to NB-506. The exposure of cells was started at 0.1 $\mu$M NB-506 for 2 weeks and then at 0.3 $\mu$M for 3 weeks. Finally, two clonal cell lines that grew in the presence of 0.3 $\mu$M 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 $\mu$M NB-506. These cell lines were further selected by culture with 20 $\mu$M 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 $2 \times 10^6$ in 25-cm$^2$ culture flasks were incubated in medium supplemented with 25 mM HEPES or HBSS buffer containing various concentrations of [$^{14}$C]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 $2000 \times g$ 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 $\mu$M [$^{14}$C]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 $\mu$M [$^{14}$C]J-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 for ~30,000 genes were used for hybridizations (Mu11KsubA, subB, Mu19KsubA, subB, and subC; Affymetrix, Santa Clara, CA). Hybridization and the array wash procedure were performed according to the Affymetrix Fluidics Station Mu11K and Mu19K protocols.

**Microarray Data Analysis.** Each gene, either full-length or EST, is represented by 20 probes, 25 mer oligonucleotide, on the GeneChip. The expression levels of genes were measured by comparing the signal intensities of hybridization of these probe sets to the one-base, mismatch oligonucleotide probes by GeneChip software (average difference). Expression of the genes was scored as presence when the perfect match signal exceeded mismatch counterpart, and this parameter was called "absolute call." Experimental data were compared with those of parental cell lines as the baseline file in Comparison Analysis on the GeneChip software to evaluate the change. The data in this article represent the data sets that met the following criteria: (*a*) fold change value compare with the parental cell line >4.0; (*b*) genes with presence call on absolute call on NR2 lines; and (*c*) genes with given score of increase on differential call.

**Northern Blots.** Total RNAs were extracted from each cell line with ISOGEN (Nippon Gene, Toyama, Japan), and mRNAs were isolated by oligodeoxythymidylate columns. Samples of 0.8 $\mu$g of poly(A)$^+$ RNA or 8 $\mu$g of total RNA were separated on 1% agarose gel and transferred to nylon membranes. The membranes were probed with a $^{32}$P-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′-CAAAAAGCTTAAGACCGAGCTCTATTAAGC and 5′-GAATTAAGGGGAAATTTAAGAAT, which were based on the AF103796 sequence with an added *Hin*dIII 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 Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequence of the clone was deposited in GenBank under accession no. AB051855. For the analysis of nucleotide sequences of BCRP from human tissue, human *BCRP* was amplified by PCR with the primers 5′-CAAAAAGCTTAAGACCGAGCTCTATTAAGC and 5′-AGAGATCGATGCCCTGCTTTACCA 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 Dyedeoxy 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 **t**he 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 radiolabeled NB-506 was measured in the NB-506-resistant LY cells (Fig. 1*A*). 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. 1*B*). 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

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

| Drug | $IC_{50}$ ($\mu$M)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | HCT116 | HCT116/NR1 | PC13 | PC13/NR13 | LY | LY/NR1 | LY/NR2 |
| NB-506 | 0.13 ± 0.005 | 300 ± 36 (2400) | 0.23 ± 0.08 | >1000 (>4300) | 0.12 ± 0.03 | 1.4 ± 0.51 (12) | 7.7 ± 2.6 (64) |
| J-107088 | 0.0034 ± 0.00007 | 0.78 ± 0.06 (230) | 0.013 ± 0.006 | 1.8 ± 0.94 (140) | 0.0017 ± 0.0003 | 0.029 ± 0.008 (17) | 0.36 ± 0.18 (210) |
| Camptothecin | 0.0094 ± 0.0006 | 0.017 ± 0.005 (1.8) | 0.038 ± 0.009 | 0.029 ± 0.006 (0.76) | 0.046 ± 0.006 | 0.058 ± 0.006 (1.3) | 0.38 ± 0.25 (8.2) |
| Topotecan | 0.034 ± 0.018 | 0.14 ± 0.0071 (4.1) | 0.075 ± 0.014 | 1.1 ± 0.75 (15) | 0.069 ± 0.022 | 0.25 ± 0.07 (3.6) | 2.9 ± 1.59 (42) |
| Etoposide | 1.1 ± 0.48 | 3.4 ± 0.57 (3.1) | 1.2 ± 0.04 | 3.0 ± 2.0 (2.5) | 0.28 ± 0.08 | 0.52 ± 0.19 (1.9) | 2.0 ± 1.1 (4.7) |
| Doxorubicin | 0.025 ± 0.002 | 0.059 ± 0.01 (2.3) | 0.028 ± 0.008 | 0.069 ± 0.050 (2.5) | 0.043 ± 0.002 | 0.067 ± 0.007 (1.6) | 0.19 ± 0.03 (4.5) |
| Vincristine | 0.0020 ± 0.0001 | 0.0093 ± 0.001 (4.7) | 0.013 ± 0.003 | 0.0070 ± 0.0020 (0.56) | 0.015 ± 0.011 | 0.011 ± 0.002 (0.77) | 0.025 ± 0.014 (1.7) |
| Paclitaxel | 0.0011 ± 0.0002 | 0.0021 ± 0.0005 (1.9) | 0.0020 ± 0.0003 | 0.0018 ± 0.0012 (0.92) | 0.041 ± 0.007 | 0.057 ± 0.009 (1.4) | 0.081 ± 0.036 (2.0) |
| Mitoxantrone | 0.0070 ± 0.0023 | 0.067 ± 0.035 (9.6) | 0.033 ± 0.004 | 0.16 ± 0.14 (4.9) | 0.0045 ± 0.0029 | 0.0071 ± 0.0047 (2.2) | 0.057 ± 0.06 (17) |

[a] Values are means ± SE of three independent experiments, with relative resistances indicated in parentheses, which were determined by dividing the $IC_{50}$ value of the resistant cell line by that of the parental cell line.

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. 3*B*). 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. 3*B*). 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. 3*A*) and from a human placenta cDNA (Fig. 3*B*). 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

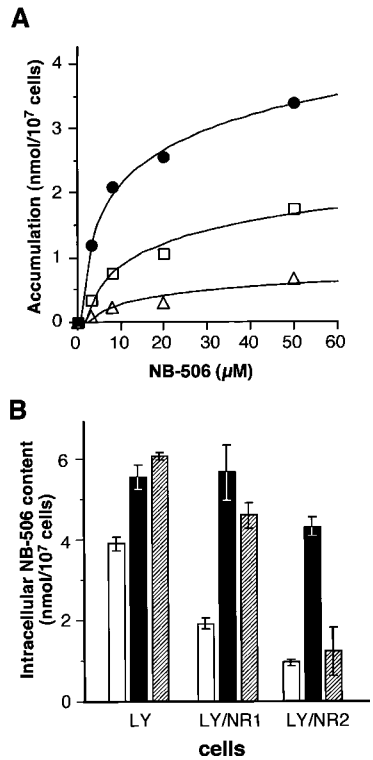



Fig. 1. Accumulation and efflux of NB-506 in resistant LY cell lines. *A*, dose-dependent accumulation of NB-506 in LY cell lines. Each cell line was incubated in medium containing various concentrations of [$^{14}$C]NB-506 for 120 min. ●, LY cells; □, LY/NR1 cells; △, LY/NR2 cells. *B*, effect of energy depletion on the accumulation and efflux of NB-506 in drug-resistant LY cells. For study of the effect of energy depletion on the accumulation, 50 $\mu$M NB-506 was incorporated into cells for 60 min in the absence (□) or presence (■) of DNP as described in "Materials and Methods." For determination of drug efflux from the cells, they were incubated with 50 $\mu$M [$^{14}$C]NB-506 for 60 min in energy-depleted conditions, washed, and then incubated in drug-free medium for 15 min (▨). *Bars*, SE from three independent experiments.

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

| Probe ID | Gene Description | LY/NR2 | | LY/NR1 |
|---|---|---|---|---|
| | | Fold increase | Avg diff[a] change | Fold increase |
| aa616278 | BCRP/MXR/ABCP | 31.2 | 7189 | 6.0 |
| TC37566 | Hypothetical protein MnCb-2755 | 15.5 | 453 | 13.4 |
| TC30469 | EST | 6.2 | 4402 | NC[c] |
| m31419 | IFN activatable protein | 5.2 | 674 | NC |
| Msa.853.0 | IFN activatable protein | 4.7 | 557 | 2.3 |
| Msa.20701.0 | 3-$\beta$ hydroxylsteroid dehydrogenase | 4.6 | 228 | NC |
| M31418 | IFN activatable protein | 4.5 | 669 | 4.7 |
| TC32218 | EST | 4.5 | 685 | NC |
| aa571115 | Y13275, Meta-associated tetraspan molecule | 4.3 | 256 | 3.8 |
| m94179 | $M_r$ 19,000 glycoprotein autoantigen | 4.2 | 402 | 3.2 |
| TC15466 | Lipocortin | 4.1 | 4084 | NC |
| Msa.34934.0 | Y-box transcription factor | 4.0 | 432 | −1.6 |

[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.

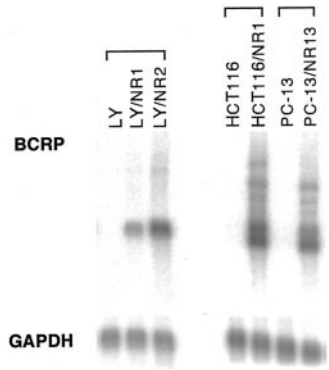



Fig. 2. Selective expression of *BCRP* mRNA in NB-506-resistant cell lines. Northern blots containing 0.8 $\mu$g poly(A)$^+$ RNA were first probed with a cDNA fragment of mouse *BCRP* (*left*) or human *BCRP* (*right*). The same blots were then probed with a cDNA fragment of GAPDH (*bottom*).

HCT116/NR1 and PC-13/NR13. As detected in human tissues, codon 482 of *BCRP* expressed in the resistant cells encoded Arg (Fig. 3*B*).

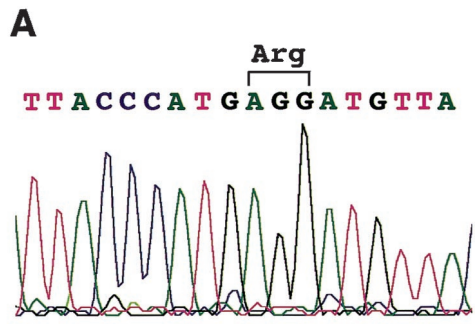
**Introduction of BCRP into PC-13 Cells Conferred Resistance to Indolocarbazole Compounds.** To examine whether expression of the *BCRP* gene can confer resistance to NB-506 and J-107088, we introduced expression plasmids encoding full-length human *BCRP* under the cytomegalovirus promoter into human 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. 4*B*, 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 ⅓ and ⅕, 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. 4*A*). 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 transfectant. 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. 4*C*). 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




B

| Origin | Amino acid | Accession No. |
|---|---|---|
| HeLa | Arg | |
| PC-13/NR13 | Arg | |
| HCT116/NR1 | Arg | |
| Human kidney | Arg | |
| Human placenta | Arg | |
| MCF-7/AdVp | Thr | AF098951 |
| S1-M1-80 | Gly | AF093771 |
| Human placenta | Arg | AF103796 |

Fig. 3. Nucleotide and amino acid sequences at codon 482. *A*, direct sequencing of the PCR product from human kidney cDNA. *B*, comparison of the putative amino acid at codon 482 of the human *BCRP* gene isolated from different cell lines or tissues. AF098951 (7), AF093771 (8), and AF103796 (6) were previously reported as *BCRP*, *MXR*, and *ABCP*, respectively.

A

| Drug | Vector $IC_{50}$ ($\mu$M) | PC-13/BCRP 2-2 RR | PC-13/BCRP 2-3 RR |
|---|---|---|---|
| NB-506 | 0.30 ± 0.07 | 22 | 9.0 |
| J-107088 | 0.012 ± 0.002 | 17 | 11 |
| Camptothecin | 0.043 ± 0.006 | 0.67 | 1.2 |
| Mitoxantrone | 0.052 ± 0.012 | 0.42 | 0.60 |
| Topotecan | 0.21 ± 0.023 | 0.90 | 0.46 |
| Etoposide | 1.0 ± 0.17 | 0.46 | 1.1 |

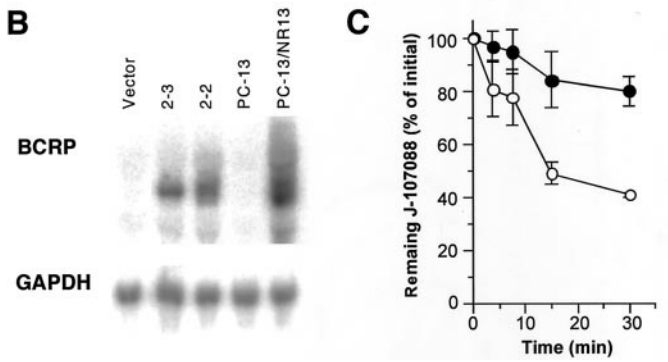



Fig. 4. Analysis of *BCRP*-transfected PC-13 cells. *A*, drug sensitivities of PC-13 stable clones transfected with *BCRP*. $IC_{50}$ values are means ± SE. Relative resistance (*RR*) was determined by dividing the $IC_{50}$ value of the *BCRP* transfected cell line by that of the vector transfected cell line. *B*, Northern blot analysis of PC-13 stable clones transfected with the expression vector containing *BCRP* cDNA. Samples of 8 $\mu$g of total RNA from the PC-13 cells (*PC-13*), the PC-13 clone transfected with vector (*Vector*), the PC-13 clones transfected with construct encoding full-length BCRP (*2-2* and *2-3*), and NB-506-resistant PC-13 cells (*PC-13/NR13*) were subjected to Northern hybridization with the *BCRP* cDNA fragment as a probe. GAPDH hybridization is also shown as a loading control. *C*, J-107088 efflux from the PC-13 stable clones transfected with BCRP. Vector transfectant (●) and BCRP transfectant clone 2-2 (○) were preloaded with 10 and 75 $\mu$M [$^{14}$C]J-107088, respectively, for 120 min. The J-107088 remaining in the cells after incubation is indicated by the percentage of the count at 0 time. *Bars*, SE from three independent experiments.

putative efflux pump. We found 31- and 6-fold elevations of *BCRP* expression in the highly and moderately resistant cells, respectively. The 31-fold increase of *BCRP* expression in the LY/NR2 cells was so high that it was easily distinguished from other up-regulated genes.

The involvement of this gene in NB-506 and J-107088 resistance was confirmed by its selective overexpression in human resistant cell lines and by analysis of *BCRP* transfected clones. The HCT116/NR1 and PC-13/NR13 cell lines showed high efflux of these compounds like mouse resistant cells (data not shown). Thus, the mechanism of the resistance is probably efflux of these compounds mediated by human BCRP.

The two resistant cell lines showed little or no cross-resistance to the topo I poison camptothecin and to substrates of P-gp or MRP1, such as doxorubicin, etoposide, paclitaxel, and vincristine (3, 5), suggesting little contribution of these known resistance factors to indolocarbazole resistance.

Analyses of *BCRP*-transfected PC-13 stable cell lines confirmed the involvement of BCRP in the resistance. The transfectant clone 2-2 showed 22- and 17-fold resistance to NB-506 and J-107088, respectively, and enhanced efflux activity of J-107088. These results strongly suggested that BCRP transports these indolocarbazole compounds and confers resistance by decreasing the intracellular concentrations of these compounds.

Our results demonstrated that *BCRP* expression did not confer resistance to mitoxantrone. This is in contrast with previous findings, because *BCRP* was reported to confer resistance to mitoxantrone when it was introduced into breast cancer cell lines (7) and because its overexpression was detected in several mitoxantrone-resistant cell lines (7, 8, 22, 23). Although the NB-506-resistant cell lines HCT116/NR1 and PC-13/NR13 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 transfectant 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. 3*B*). *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 endogeneous 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 indolocarbazle 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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# 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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