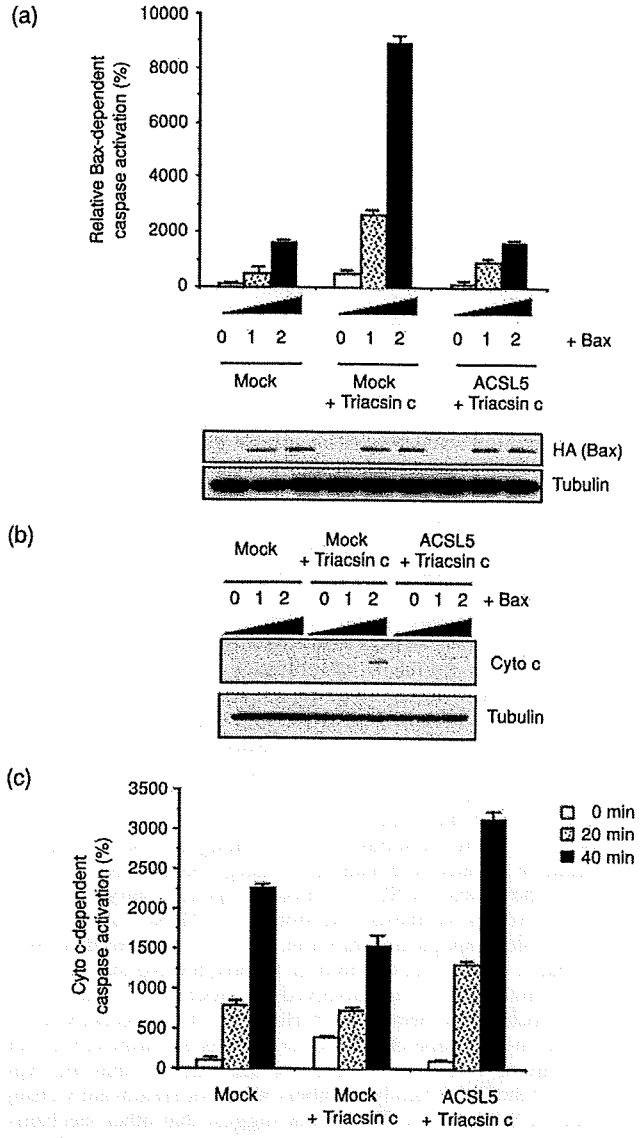

Fig. 3. Potentiating the mitochondrial apoptosis pathway by acyl-CoA synthetase (ACS) inhibition. (a) To estimate Bax-induced caspase activation, mock- and ACSL5-transduced SF268 cells were seeded in six-well plates and transiently transfected with pCGBL-HA-Bax (0, 0.1, and 0.2 μg/well) and pGVC, a luciferase-expressing construct (0.4 μg/well). At 6 h after transfection, cells were left untreated or were treated with 1 μM Triacsin c for an additional 24 h. Each cell lysate was prepared and caspase activity measured as described in 'Materials and Methods'. The expression of Bax (HA) was examined by western blot. The expression of α-tubulin was analyzed as a loading control. (b) Cells were transiently transfected with the Bax plasmid vector and then were treated with Triacsin c as in (a). Cytochrome c release from the mitochondria to cytoplasm was monitored by western blot analysis. (c) Mock- and ACSL5-transduced SF268 cells were left untreated or treated with 1 μM Triacsin c for 24 h. Cytosolic extracts were prepared and incubated with 10 μM cytochrome c and 1 mM dATP for 0–40 min. After the incubation, caspase activity was measured, as described in 'Materials and Methods'. In (a) and (c), data are mean values of three independent experiments. Error bars show standard deviations.

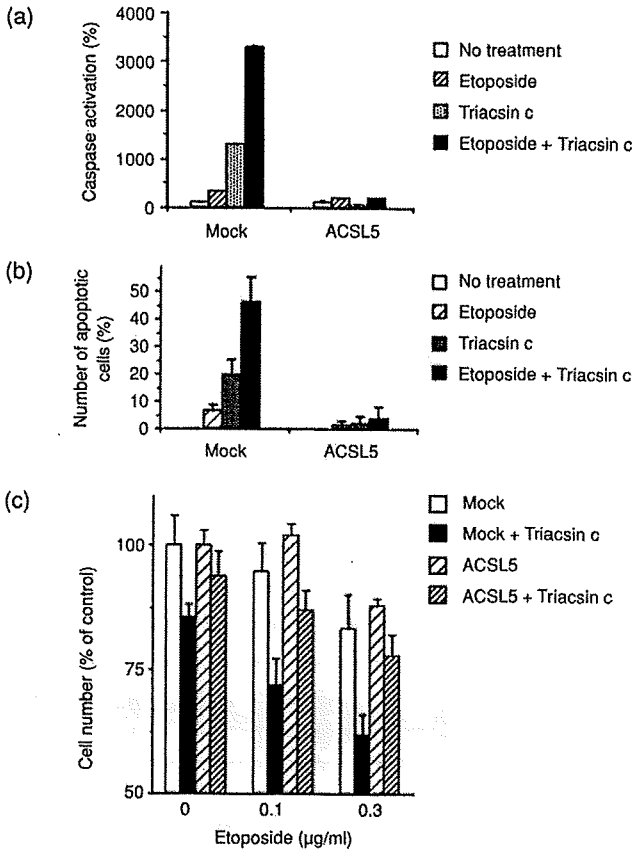

Fig. 4. Potentiating etoposide-induced cell death by acyl-CoA synthetase (ACS) inhibition. (a) Enhanced activation of caspase by etoposide in combination with Triacsin c. Mock- and ACSL5-transduced SF268 cells were left untreated or were treated with 0.3 μg/mL of etoposide, 1 μM Triacsin c or 0.3 μg/mL of etoposide and 1 μM Triacsin c for 48 h. Caspase activity was measured as described in 'Materials and Methods'. (b) Potentiation of apoptosis by etoposide in combination with Triacsin c. Mock- and ACSL5-transduced SF268 cells were treated as in (a), and apoptotic cells were evaluated and counted. (c) Effect of ACS inhibition on etoposide-induced cytotoxicity. Mock- and ACSL5-transduced SF268 cells were left untreated or were treated with the indicated concentrations of etoposide in the absence or presence of 1 μM Triacsin c for 48 h. Cell viability was measured, using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method. Data are mean values of three independent experiments. Error bars show standard deviations.

activated 3.5-fold or 13-fold, respectively, compared to non-treated cells (Fig. 4a). Co-treatment with etopiside and Triacsin c significantly enhanced the caspase activation (33-fold). This synergism in caspase activation by etoposide and Triacsin c was quenched by ACSL5 overexpression. Correspondingly, the sublethal dose of Triacsin c (1 μM) significantly potentiated etoposide-induced apoptosis (Fig. 4b) and the loss of cell numbers (Fig. 4c). Again, these effects of Triacsin c were canceled by ACSL5 overexpression. These results indicate that inhibiting ACS activity would be a rational strategy to potentiate etoposide-induced cytotoxicity.

To test whether ACS inhibition could increase the antitumor efficacy of etoposide, we developed a tumor xenograft model in nude mice. Because SF268 cells could not form stable tumors in nude mice (our unpublished observation), we chose another implantable glioma cell line, U251, for this *in vivo* study. Mice were treated with saline (control), etoposide, Triacsin c, or etoposide in combination with Triacsin c. Under the limited dose conditions in Figure 5(a), etoposide or Triacsin c alone did not show apparent antitumor effects. Strikingly, however, cotreatment with etoposide and Triacsin c significantly retarded tumor growth. During the treatment, no toxic death or significant body weight change was observed (Fig. 5b). These results indicate

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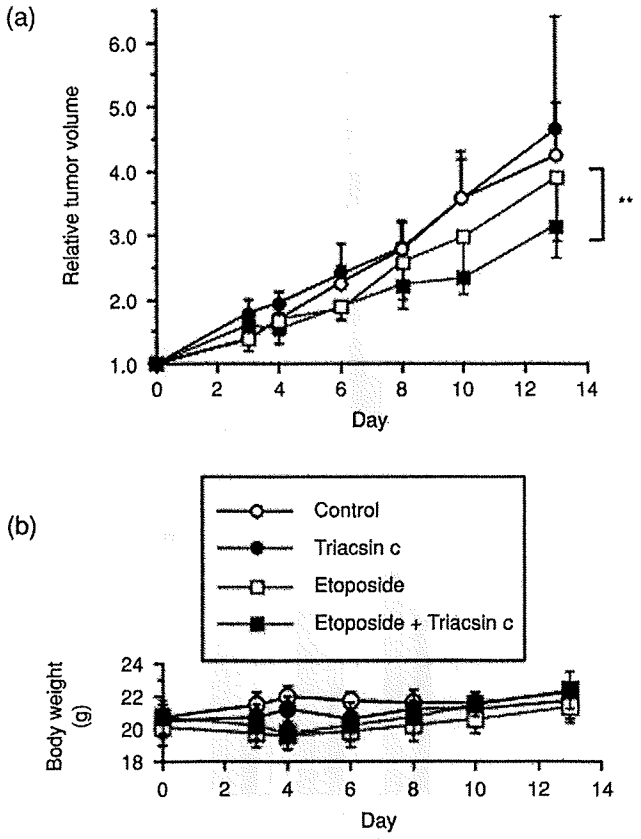

Fig. 5. Triacsin c enhances the efficacy of etoposide *in vivo*. Therapeutic experiments (five mice per group) were started (day 0) when U251 tumors reached 90–170 mm³. Etoposide (12 mg/kg/day) was administrated i.v. on days 0, 1, and 2. Triacsin c (4 mg/kg/day) was administrated by intratumoral injection in 40 μL of saline on days 0, 1, and 2. Control mice received the same volume of saline. Relative tumor volumes and body weight changes of the mice are shown in (a) and (b), respectively. Data are mean values for five mice, and error bars show standard deviations. Statistical evaluations were performed as described in 'Materials and Methods'. $^{**}P < 0.01$.

that the ACS inhibition potentiates the antitumor effect of etoposide *in vivo* with minimal side effects in the mice.

Discussion

ACS catalyzes a critical step in both the anabolic and catabolic pathways of fatty acid metabolism. In our present study, we showed that ACS enzyme activity is a critical factor for cancer cell survival and apoptosis inhibition. When cancer develops, excessive mitogenic signals, due to oncogenic activation or uncontrolled cell cycle progression, are coupled with constitutive activation of the intrinsic apoptosis machinery.[29] Under these conditions, expressions of antiapoptotic factors are requisite for cancer cell survival, and these factors could be the cells' Achilles heel. In fact, agents or strategies that suppress antiapoptotic proteins or directly activate the mitochondria-dependent apoptosis pathway (apoptosome pathway) selectively induce tumor cell death or potentiate the chemosensitivity of tumor cells.[13,14,16,30,31] Our present results suggest that ACS could be one such factor essential for cancer cell survival and whose inhibition induces tumor-selective cell death.

ACS inhibition potentiated Bax- and etoposide-induced apoptosis (Figs 3 and 4). Consistently, the overexpression of ACSL5 suppressed apoptosis induced by etoposide (Fig. 4a,b). These data indicate that ACS could act as an apoptosis inhibitor in tumor cells.

We have shown that ACSL5 nearly completely suppressed Triacsin c–induced cell death. Since small molecules could have off-targets, we cannot exclude the possibility of an ACS-unrelated, off-target effect of Triacsin c. However, such effect would be minor, if any, in our experimental settings, because the catalytically inactive ACSL5 mutant did not suppress the Triacsin c–induced cell death. Thus, Triacsin c could induce apoptosis mainly through ACS inhibition. Meanwhile, because ACS have five isoforms, it is difficult to perform gene-knockdown experiments or to examine the effect of dominant-negative mutants.

ACS shows various subcellular localizations.[24] The ACS inhibitor–induced apoptosis was suppressed by ACSL5, which localizes on the mitochondria and in the nuclei. Moreover, the ACSL5 mutant lacking nuclear localization still inhibited the ACS inhibitor–induced cell death. These data indicate that the nuclear ACS is not required for cancer cell survival and suggest that the organelle ACS could be a critical factor for the survival. To be sure of the role of the organelle ACS, however, further studies with an ACSL5 mutant that lacks the organelle localization could be required. Given the fact that ACSL5 is the only known ACS isozyme that localizes to the mitochondria and is frequently overexpressed in human cancers, it is suggested that ACSL5 could play an important role in tumorigenesis or malignant transformation by means of inhibiting mitochondrial apoptosis pathway. Meanwhile, we should take into account additional unknown mitochondrial ACS enzymes that are involved in this mechanism, since a Triacsin c–sensitive ACS activity is known to exist on rat mitochondria in spite of the fact that ACSL5 is Triacsin c–resistant.[23]

How could the organelle ACS function as an antagonist of apoptosis? Mitochondrial ACS was thought to be involved in β-oxidation of fatty acid leading to energy production.[5] Recently, it was shown that ACSL5 partitions exogenous fatty acids toward triacylglycerol synthesis and storage.[32] These pathways could play a role in apoptosis inhibition. We have reported that Triacsin c reduces the level of cardiolipin, a phospholipid that is localized on the mitochondria and involved in cytochrome c anchorage on the mitochondria membrane.[10] However, it was recently reported that down-regulation of cardiolipin alone is not sufficient to induce cytochrome c release.[33] Here, we also showed that the expressions of the Bcl-2 family members were not significantly changed after ACS inhibition. These data suggest that other mechanisms could also be involved in the potentiation of cytochrome c release by ACS inhibition.

In the present study, we demonstrated that ACS inhibition enhances the etoposide-induced cell death of human glioma cells. We examined combinations of several agents with Triacsin c and also found a synergistic effect of the ACS inhibitor with SN-38 (data not shown). Lipid metabolism is selectively activated in a wide variety of cancers, and ACS is overexpressed in such cancers as glioma, colon cancer, and hepatocellular cancer.[7–9,34] Our data suggest that ACS inhibition would be a rational strategy to potentiate chemosensitivity of cancer. Additional studies, including the combinational effect of ACS inhibition with other antitumor agents, could further clarify the importance of ACS as a new therapeutic target for cancer.

Acknowledgments

We thank the National Cancer Institute as well as Takao Yamori for providing us human glioma cell lines. We also thank members in our laboratory for helpful discussions. This work was supported by grants-in-aid for Cancer Research on Priority Areas (T. Mashima, T. Tsuruo, H. Seimiya) and Young Scientists (T. Mashima) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Abbreviations

ACS	Acyl-CoA synthetase
DAPI	4,6-diamino-2-phenylindole
DEVD-MCA	Acetyl-Asp-Glu-Val-Asp-(4-methyl-coumaryl-7-amide)
EGFR	Epidermal growth factor receptor
FASN	Fatty acid synthase
Hsp	Heat shock protein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PARP	Poly(ADP-ribose) polymerase
Z-VAD-fmk	Z-Val-Ala-Asp(OMe)-CH2F

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Acyl-CoA synthetase (ACS) enzyme activity of the delta L mutant. ACS activity in mock-, ACSL5-, and delta L–transduced SF268 cells was measured as described in 'Materials and Methods'.

Fig. S2. Localization of the ACSL5 delta L mutant in SF268 cells. The delta L mutant of ACSL5 (FLAG) and a mitochondria marker, cytochrome c (Cyto c), were detected by indirect immunofluorescence staining of delta L–transduced SF268 cells with anti-FLAG M2 (red) and anticytochrome c (green) antibodies, respectively. DAPI staining of DNA is shown in blue.

Fig. S3. Effect of acyl-CoA synthetase (ACS) inhibition on mitochondria-dependent apoptosis pathway regulators. Mock- and ACSL5-transduced SF268 cells were left untreated or were treated with 1-μM Triacsin c in the absence or presence of a 50 μM caspase inhibitor, Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD), for 36 h. Expression of proteins that regulate cytochrome c release from mitochondria (Bcl-2, Bcl-XL, and Bax) was analyzed by western blot.

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Pharmacological Interplay between Breast Cancer Resistance Protein and Gefitinib in Epidermal Growth Factor Receptor Signaling

KAZUHIRO KATAYAMA[1*], KAZUHIKO SHIBATA[1*], JUNKO MITSUHASHI[1,2], KOHJI NOGUCHI[1] and YOSHIKAZU SUGIMOTO[1,2]

[1]*Division of Chemotherapy, Graduate School of Pharmaceutical Sciences, Keio University, Tokyo;*
[2]*Division of Gene Therapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan*

Abstract. *Background: It has been previously shown that gefitinib reverses breast cancer resistance protein (BCRP)-mediated drug resistance. Here, the impact of BCRP on gefitinib-mediated inhibition in epidermal growth factor receptor (EGFR) signaling is evaluated. Materials and Methods: Sensitivity to gefitinib was determined by growth inhibition assay, and intracellular gefitinib levels were measured with HPLC. Western blotting was performed to detect EGFR signaling molecules. Results: BCRP reduced intracellular gefitinib levels and attenuated inhibitory activities of gefitinib to EGF-dependent EGFR signalings including downstream MAPK and Akt pathways in gefitinib-sensitive PC-9 cells. However, gefitinib did not inhibit MAPK and Akt signalings in KB-3-1 and HCT-116 cells, and BCRP-mediated gefitinib-resistance shown in PC-9 cells was not observed in gefitinib-insensitive KB-3-1 and HCT-116 cells. Conclusion: BCRP transports gefitinib and suppresses its inhibitory effects on EGFR phosphorylation. However, effects of BCRP on gefitinib activity in the EGFR signaling and on gefitinib-resistance were limited in the gefitinib-sensitive cells only.*

*Both authors contributed equally to this work.

Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; ATP, adenosine triphosphate; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HPLC, high performance liquid chromatography; NSCLC, non-small cell lung cancer.

Correspondence to: Yoshikazu Sugimoto, Division of Chemotherapy, Graduate School of Pharmaceutical Sciences, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Tel: +81 3 5400 2670, Fax: +81 3 5400 2669, e-mail: sugimoto-ys@pha.keio.ac.jp

Key Words: BCRP/ABCG2, gefitinib, HPLC, EGFR.

ATP-binding cassette (ABC) transporters, including breast cancer resistance protein (BCRP)/ABCG2, P-glycoprotein (P-gp)/ABCB1 and multidrug resistance-related protein 1 (MRP1)/ABCC1, are involved in multidrug resistance phenotypes (1). These proteins function by pumping out various structurally unrelated agents using ATP hydrolysis energy. BCRP is a half-molecule ABC transporter with an NH_2-terminal ATP-binding site and a COOH-terminal transmembrane domain (2-6). BCRP forms homodimers *via* disulfide bridges between Cys603, a residue on the third outer-membrane domain of the BCRP monomer (7, 8). Homodimeric BCRP acts as an efflux pump for various anticancer agents, including 7-ethyl-10-hydroxycamptothecin (SN-38), 9-aminocamptothecin and mitoxantorone. BCRP prevents intracellular accumulation of such compounds and thereby decreases their cytotoxic effects (5, 9-11). BCRP is expressed in various normal human tissues and cells, including the placenta, liver, kidney and small intestine, and exports natural compounds, including sulfated estrogens and flavonoids (12-15).

Gefitinib is an epidermal growth factor receptor (EGFR) inhibitor that functions by competitively binding to the ATP-binding domain, and is clinically used for treating non-small cell lung cancer (NSCLC) patients (16, 17). In particular, this drug is more effective against tumor growth in NSCLC harboring deletions in exon 19 (del E746-T753) and/or point mutations in exon 21 (L858R and L861Q) of EGFR (16, 17). Gefitinib markedly inhibits epidermal growth factor (EGF)-mediated autophosphorylation of EGFR in various EGFR-expressing human cancer cell lines and xenografts, and effectively suppresses important signal transduction pathways that are implicated in the proliferation and survival of tumor cells (16, 17).

In a previous study, it has been shown that gefitinib reverses the BCRP-mediated anticancer drug resistance phenotype (18). In addition, it was shown that *BCRP*-transduced human lung cancer PC-9 (PC-9/BCRP) cells show gefitinib resistance, whereas *BCRP*-transduced human myelogenous leukemia K562

(K562/BCRP) cells do not (19). To better understand the mechanisms underlying gefitinib resistance by BCRP, the effects of BCRP upon gefitinib uptake and efflux and the consequences of this for the inhibition of EGFR downstream signaling were examined. It is demonstrated that BCRP-expressing cells show lower accumulation and higher efflux of gefitinib than their parental cells regardless of the cell types tested. However, the data show that BCRP-expressing cells show gefitinib resistance only when the cells are sensitive to gefitinib.

Materials and Methods

Reagents. Gefitinib was kindly provided by AstraZeneca UK Ltd. (London, UK). EGF was obtained from Sigma (St. Louis, MO, USA). Rabbit anti-BCRP polyclonal antibody 3488 was prepared in the laboratory as described previously (7). Other primary antibodies were purchased as follows: mouse anti-MDR1+3 monoclonal antibody (C219) was sourced from Zymed (South San Francisco, CA, USA), mouse anti-GAPDH monoclonal antibody was obtained from Chemicon (Temecula, CA, USA), mouse anti-EGFR monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit anti-p44/p42, anti-phospho-p44/p42 (Thr202/Tyr204), anti-Akt and anti-phospho-Akt (Ser473) polyclonal antibodies, and mouse anti-phospho-EGFR (Tyr1068) monoclonal antibody were supplied by Cell Signaling Technology (Danvers, MA, USA).

Cells and drug sensitivity assay. Human NSCLC PC-9, human epidermoid carcinoma KB-3-1 and human colorectal tumor HCT-116 cells were cultured in DMEM supplemented with 7% fetal bovine serum at 37°C in 5% CO_2. PC-9/BCRP, KB/BCRP and HCT-116/BCRP cells were established by the transduction of PC-9, KB-3-1 and HCT-116 cells, respectively, with a HaBCRP retrovirus harboring a Myc-tagged human *BCRP* cDNA in the Ha retrovirus vector as described previously (7). The effects of anticancer agents on the cells were evaluated by measuring cell growth inhibition after incubation at 37°C for 5 days in presence of various concentrations of the drugs. Cell numbers were determined with a Coulter counter. The IC_{50} values (the dosage of drugs at which a 50% inhibition of cell growth was achieved) were determined from the growth inhibition curve.

Western blot analysis. Western blot analysis was performed as reported previously (7, 20). Briefly, cell lysates were solubilized with sample buffer (2% SDS, 50 mmol/L Tris-HCl (pH 8.0), 0.2% bromophenol blue, 5% 2-mercaptoethanol) with boiling for 10 min at 70°C (to evaluate ABC transporters) or for 5 min at 100°C (to assess EGFR signaling). The lysates were then separated by SDS-PAGE using 5-20% gradient gel, and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences Corp., Piscataway, NJ, USA). Bands were visualized using the ECL (enhanced chemiluminescence) Plus detection kit (Amersham Biosciences Corp.).

Fluorescence-Activated Cell Sorting (FACS). The expression levels of BCRP on cell surfaces were determined by FACS analysis as described before (20). In brief, cells were incubated with or without a biotinylated human-specific monoclonal antibody raised against BCRP (eBioscience, San Diego, CA, USA) (100 μg/mL). These cells were then washed and incubated with R-phycoerythrin-conjugated streptavidin (400 μg/mL; Becton Dickinson and Company, Franklin Lakes, NJ, USA). Fluorescence staining levels were detected using FACSCalibur instrument (Becton Dickinson and Company).

High performance liquid chromatography (HPLC) analysis. Trypsinized cells (3×10^6) were incubated with 0.5 μmol/L gefitinib for 2, 5 or 10 min at 37°C for uptake experiments and then washed twice with ice-cold PBS. For efflux experiments, the cells were incubated with 0.5 μmol/L gefitinib for 10 min, washed twice with ice-cold PBS, further incubated in gefitinib-free flesh normal growth medium for 2, 5 or 10 min at 37°C and immediately washed twice with ice-cold PBS. Cells were lysed with ethanol, vortexed and centrifuged at 14,000 rpm for 20 min at 4°C. The cell extracts were chromatographed on a 4.6 mm x 250 mm ID Inertsil ODS3 column (GL Sciences Inc., Tokyo, Japan) with 80% acetonitrile: 20% aqueous ammonium acetate at a flow rate of 1 mL/min. A Shimadzu SPD-20A mass spectrometer was used for subsequent detection of gefitinib at a measuring wavelength of 332 nm.

Results

Characterization of BCRP-transduced PC-9, KB-3-1 and HCT-116 cells. It has been previously reported that gefitinib reverses BCRP-mediated anticancer drug resistance (18). To examine whether BCRP directly transports gefitinib in the current study, three different cell lines and BCRP-transduced cells derived from them were used. The expression levels of EGFR protein in these cell lines were confirmed by Western blotting. PC-9, PC-9/BCRP, KB-3-1 and KB/BCRP cells expressed significant amounts of EGFR, however, HCT-116 and HCT-116/BCRP cells expressed only marginal levels of EGFR (Figure 1A). BCRP transduction did not affect EGFR expression in any cell types. The expression of BCRP was then confirmed by both Western blotting and FACS (Figure 1B and C). Further, P-gp expression was also confirmed by Western blotting in each cell line (Figure 1B). BCRP was not detectable in the parental PC-9, KB-3-1 and HCT-116 cells, whereas PC-9/BCRP, KB/BCRP and HCT-116/BCRP cells expressed significant amounts of exogenous BCRP. The BCRP expression levels of PC-9/BCRP and HCT-116/BCRP cells were higher than those of the KB/BCRP cells, but none of these cell types expressed P-gp. Furthermore, FACS analysis revealed that BCRP was expressed on the cell surface in the transduced cells only.

PC-9/BCRP but not KB/BCRP or HCT-116/BCRP cells are resistant to gefitinib. Drug sensitivity assays were performed for topotecan in PC-9/BCRP, KB/BCRP and HCT-116/BCRP cells (Table I). All of the *BCRP*-transduced cells showed much higher resistance to topotecan compared with the parental cells, indicating that BCRP is active in these three transduced cell lines. Their sensitivity to gefitinib was then examined. The IC_{50} values of PC-9 and PC-9/BCRP cells to gefitinib were determined to be 4 and 27 nmol/L, respectively, and therefore PC-9/BCRP cells showed an approximately 7-fold higher resistance to gefitinib than the parental PC-9 cells. In contrast,

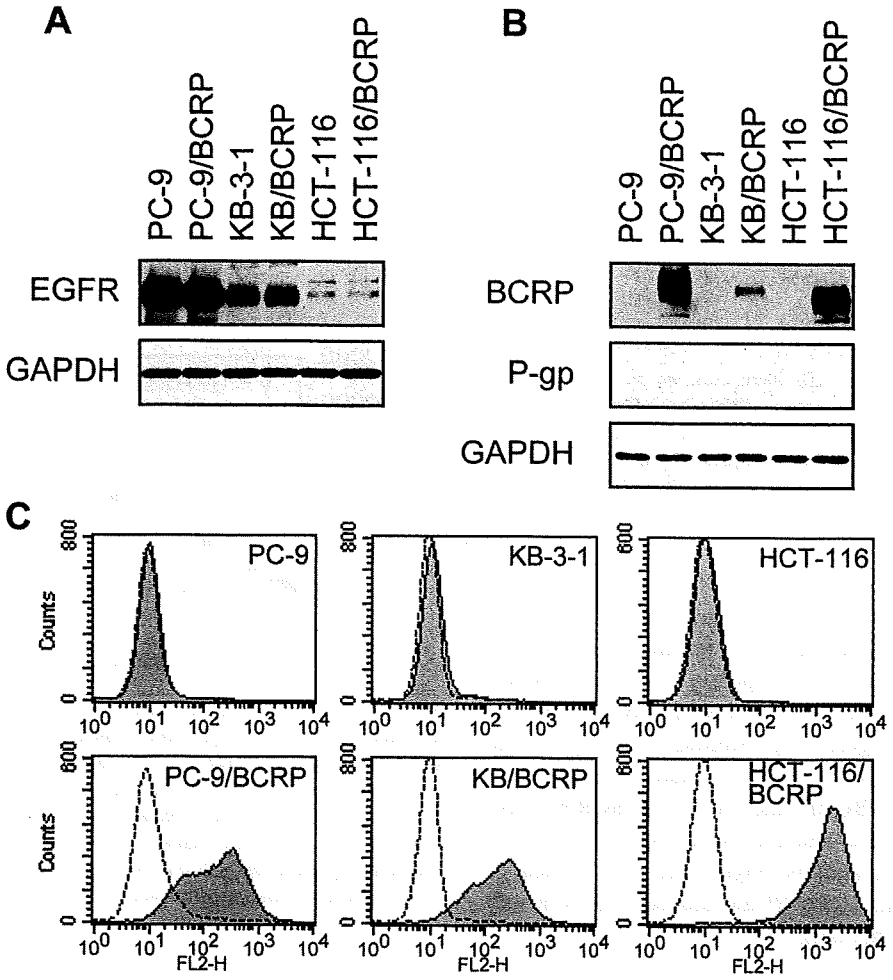

Figure 1. *Analysis of the expression levels of EGFR, BCRP and P-gp in BCRP-transduced cells. (A, B) Cell lysates (10 μg/lane) were resolved by SDS-PAGE and expression levels of EGFR or GAPDH were detected by Western blotting using anti-EGFR polyclonal antibody or anti-GAPDH monoclonal antibody, respectively (A). The expression levels of BCRP, P-gp or GAPDH were detected by anti-BCRP (3488), anti-P-gp (C219) or anti-GAPDH antibodies, respectively (B). (C) Cells were harvested with trypsin, washed with PBS and incubated with (closed areas) or without (open areas) a biotinylated anti-BCRP antibody. The cells were incubated with R-phycoerythrin–conjugated streptavidin. BCRP expression levels were determined using FACSCalibur instrument.*

KB/BCRP and HCT-116/BCRP cells did not show any resistance to gefitinib compared with their respective parental cells. The IC_{50} values for KB-3-1 and HCT-116 cells were approximately 2 and 3 μmol/L respectively, and were much higher than those for PC-9 cells. These data indicate that BCRP confers gefitinib-resistance in gefitinib-sensitive PC-9 cells while it did not in gefitinib-insensitive KB-3-1 or HCT-116 cells.

Lower accumulation of Gefitinib in BCRP-transduced cells. Both the uptake and efflux of gefitinib were examined in *BCRP*-transduced cells and their parental cells. HPLC was used to determine intracellular gefitinib levels with a calibration curve (Figure 2). In the uptake experiments, cells were incubated with 0.5 μmol/L gefitinib for 2, 5 or 10 min. Intracellular gefitinib was then extracted from the cells and quantified by HPLC (Figure 3A-C). The intracellular gefitinib levels almost reached a plateau phase at 2 min after treatment with this drug in each cell line. Significantly, the intracellular gefitinib levels in each *BCRP*-transduced cell line were much lower than those in the corresponding parental cells. Actually, at 10 min incubation period, the intracellular gefitinib levels in the PC-9/BCRP and KB/BCRP cells were approximately 2-fold lower than those in the PC-9 and KB-3-1 cells, respectively (Figure 3A and B). The intracellular levels of gefitinib in the HCT-116/BCRP cells were approximately two-thirds of those in the HCT-116 cells (Figure 3C). In the efflux experiments, the cells were incubated with 0.5 μmol/L gefitinib for 10 min, washed and

Table I. *Drug resistance characteristics of BCRP-transduced cells*.*

Cell line	Topotecan		Gefitinib	
	IC_{50} (nmol/L)	RR#	IC_{50} (nmol/L)	RR#
PC-9	11.4±0.42		3.67±0.56	
PC-9/BCRP	206±17.4	18.0	26.8±4.4	7.3
KB-3-1	21.4±0.01		2310±72	
KB-/BCRP	84.1±0.55	3.9	2200±140	0.95
HCT-116	3.67±0.15		3140±410	
HCT-116/BCRP	49.9±0.98	13.6	3030±410	0.96

*Parental or *BCRP*-transduced cells were cultured for 5 days with increasing concentrations of topotecan or gefitinib. Cell numbers were counted with a Coulter counter, and IC_{50} values were determined. #Relative resistance. These values were obtained by dividing the IC_{50} values of the BCRP-transduced cells by the IC_{50} values of the corresponding parental cells.

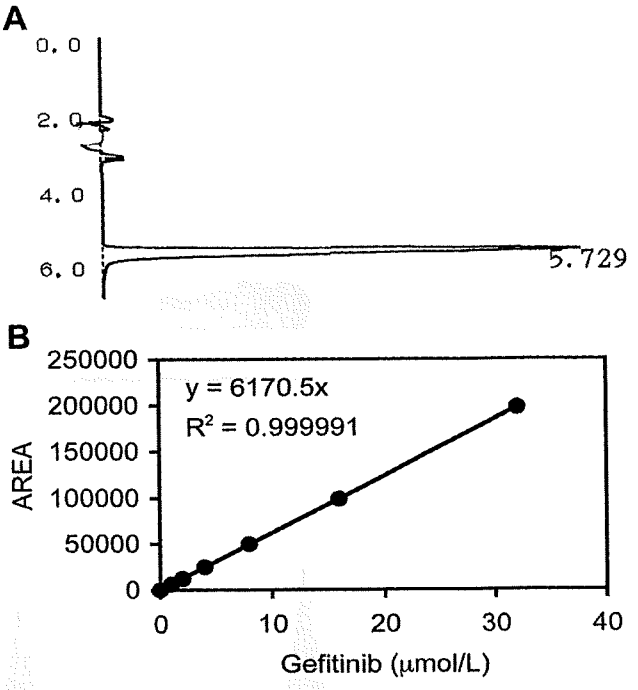

Figure 2. *Detection and quantification of gefitinib by HPLC. (A) Retention time of eluted gefitinib is shown in Y-axis, and eluted gefitinib peak is shown in X-axis in the chromatographic pattern. (B) A calibration curve plotted using the indicated concentrations of gefitinib and the corresponding areas obtained by chromatography. Each dose of gefitinib was prepared using a two-fold dilution series and chromatographed as described in Materials and Methods. The data was shown to be a representative subset.*

then incubated in gefitinib-free normal growth medium for 2, 5 or 10 min. After 2 min incubation in gefitinib-free medium, 49% of the gefitinib that had incorporated into PC-9/BCRP cells was released, whereas this figure was only 11% in the parental PC-9 cells (Figure 3D). Similarly, during 2 min incubation 72% and 61% of the accumulated gefitinib was released from the KB/BCRP and HCT-116/BCRP cells, respectively, whereas these amounts were 46% and 38% in the corresponding parental cells (Figure 3E and F). Hence, lower uptake of gefitinib in *BCRP*-transduced cells is due to an increased efflux of this drug.

Effects of BCRP expression on the inhibition of EGFR downstream signaling by gefitinib. Next, examination on whether the inhibitory effects of gefitinib upon EGFR signaling were affected by BCRP expression was performed. To test this, PC-9 and PC-9/BCRP cells were treated with various concentrations of gefitinib followed by EGF treatment in absence of serum (Figure 4A). The levels of phosphorylated EGFR were reduced over 0.1 nmol/L gefitinib in PC-9 cells but were unaffected until 30 nmol/L in PC-9/BCRP cells. Consistently, gefitinib downregulated EGF-dependent phosphorylation of extracellular signal-regulated kinase (ERK) at a dose of 0.1 nmol/L in PC-9 cells but only did so in PC-9/BCRP cells at dose of over 300 nmol/L. The downregulation of Akt phosphorylation by gefitinib in PC-9 and PC-9/BCRP cells was observed at concentrations of 0.1 and 100 nmol/L, respectively. BCRP thus confers resistance to the inhibitory effects of gefitinib on EGFR signaling in PC-9/BCRP cells.

The EGFR signaling status of KB-3-1, KB/BCRP, HCT-116 and HCT-116/BCRP cells after gefitinib exposure in a concentration range of 0.1-30,000 nmol/L (Figures 4B and C) was also examined. Regarding phosphorylated EGFR levels, KB/BCRP and HCT-116/BCRP cells were found to be resistant to gefitinib compared with the corresponding parental cells. However, exogenous BCRP expression did not confer resistance to the effects of gefitinib upon the EGFR downstream signaling pathways, including ERK and Akt activities in KB/BCRP and HCT-116/BCRP cells. The phosphorylated ERK and Akt levels were unaffected by a much higher concentration of gefitinib (~30 μmol/L) in both *BCRP*-transduced and parental cells. Taken together, the presented data therefore indicate that BCRP exports gefitinib from all cell types but BCRP-mediated gefitinib-resistance is acquired in gefitinib-responsive cells only.

Discussion

It has been previously demonstrated that gefitinib reverses BCRP-mediated anticancer drug resistance in K562 and murine lymphocytic leukemia P388 cells, suggesting gefitinib as a competitor for other BCRP substrates including SN-38 and mitoxantrone (18). In addition, it has been shown that PC-9/BCRP cells show gefitinib resistance but K562/BCRP cells do not (18, 19). K562 cells are not a suitable for these studies because they do not express EGFR.

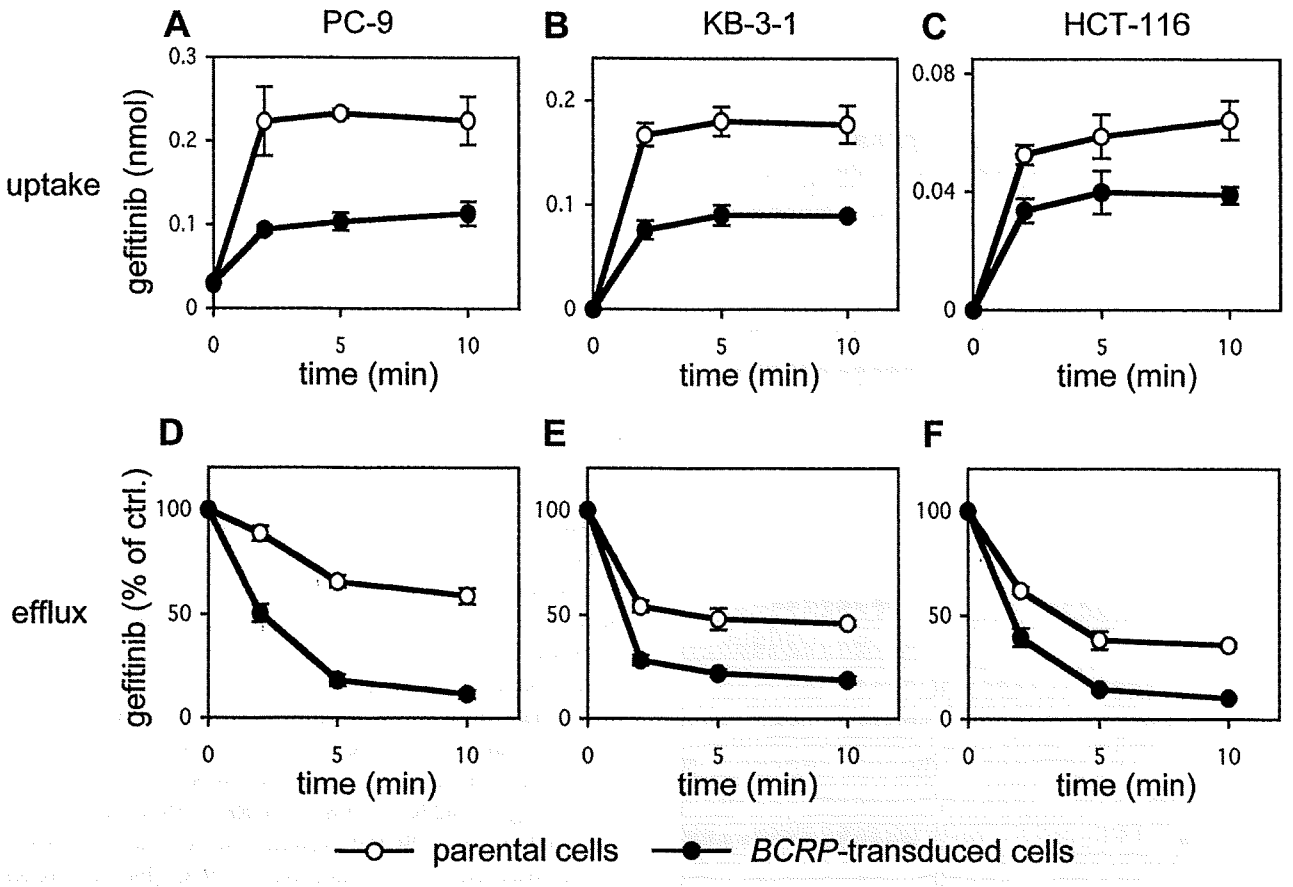

Figure 3. *Reduction of intracellular gefitinib concentration in BCRP-transduced cells. (A-C) Uptake of gefitinib. The BCRP-transduced cells (closed circles) or their respective parental cells (open circles) were treated with 0.5 μmol/L gefitinib for 2, 5 or 10 min at 37°C. At each time point, cells were lysed with ethanol, and cell extracts were chromatographed by HPLC to detect intracellular gefitinib. Intracellular gefitinib concentration is shown as the levels per 3×10^6 cells and was calculated from a pre-determined calibration curve. Data points are measurements of the mean±SD from triplicate determinations. (D-F) Efflux of gefitinib. The BCRP-transduced cells (closed circles) or their respective parental cells (open circles) were treated with 0.5 μmol/L gefitinib for 10 min at 37°C. Cells were further incubated in gefitinib-free normal growth medium for 2, 5 or 10 min. Cells were lysed with ethanol, and cell extracts were chromatographed as described above. The data shown are the relative amounts of gefitinib compared with the control (treatment with gefitinib only at the 0 time point) and are the mean±SD from triplicate determinations.*

In the present study, three cancer cell lines that express EGFR (Figure 1A) and their respective *BCRP*-transduced cells were used to further examine the mechanisms of BCRP-dependent gefitinib resistance. PC-9 cells were highly sensitive to gefitinib with an IC_{50} of approximately 4 nmol/L (Table I). It has been demonstrated that gefitinib appreciably inhibits EGFR mutants harboring deletions in exon 19 or point mutations in exon 21, when compared with the wild-type protein (16, 17). *In vitro* studies have indicated that gefitinib may exert much higher inhibitory effects against mutant EGFR variants (16, 17, 21). Consistently, PC-9 cells harbor a deletion in EGFR (del E746-A750) (22), and PC-9 cells are highly gefitinib-sensitive. BCRP was found to suppress the intracellular accumulation of gefitinib by promoting its efflux in all three cell lines tested in the present study (Figure 3). The low levels of accumulation of gefitinib in PC-9/BCRP cells will reduce cytotoxic effects against EGFR downstream signaling compared with PC-9 cells. Since EGFR downstream signalings, MAPK and Akt pathways, were also highly sensitive to gefitinib in PC-9 cells (Figure 4A), BCRP would be able to confer resistance to gefitinib in this gefitinib-sensitive cells.

On the other hand, KB-3-1 and HCT-116 cells were less sensitive to gefitinib with IC_{50} values of approximately 2 and 3 μmol/L, respectively (Table I), and their gefitinib-insensitivities were no longer affected by BCRP. Unlike PC-9 cells, cells that are marginally responsive to gefitinib, including KB-3-1 and HCT-116 cells which harbor wild-type EGFR, are not dependent on EGFR signaling for cell growth (23). Actually, cell growth and survival signaling such as

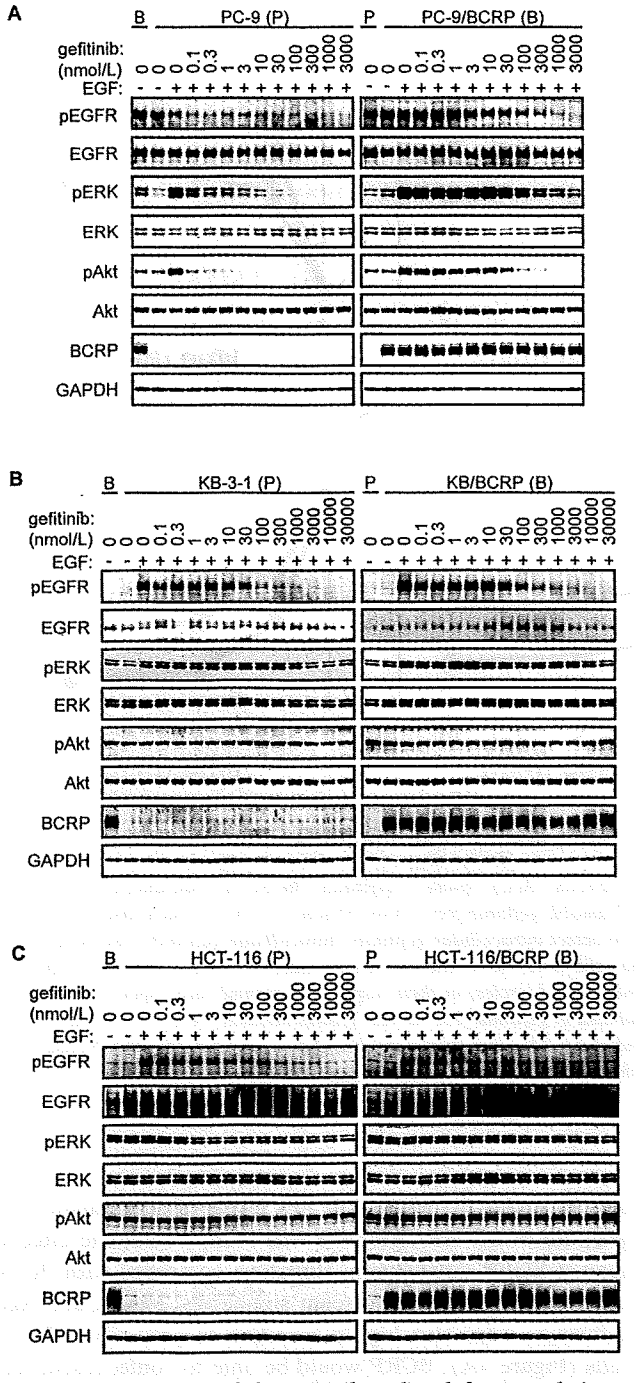

Figure 4. *Suppression of the gefitinib-mediated down-regulation of EGFR signaling in the BCRP-transduced cells. PC-9 and PC-9/BCRP cells (A), KB-3-1 and KB/BCRP cells (B), or HCT-116 and HCT-116/BCRP cells (C) were cultured in medium without serum for one hour and then treated with the indicated concentrations of gefitinib for 3 h under conditions of serum starvation. The cells were then treated with 100 μg/L of EGF for 15 min and harvested immediately. Cell extracts were used in Western blotting with anti-phospho-EGFR (Tyr1068), anti-EGFR, anti-phospho-ERK (Thr202/Tyr204), anti-ERK, anti-phospho-Akt (Ser473), anti-Akt, anti-BCRP or anti-GAPDH antibodies. P, parental cells; B, BCRP-transduced cells.*

MAPK and Akt pathways of KB-3-1 and HCT-116 cells were gefitinib-insensitive and looked to be independent of EGFR phosphorylation (Figures 4B and C). Therefore, it is presumed that BCRP-mediated gefitinib efflux and restoration of EGFR phosphorylation would not confer gefitinib-resistance in gefitinib-insensitive cells.

ERK1/2 and Akt are central molecules during EGF-mediated cell growth and survival. EGF activates ERK1/2 and Akt in a phosphorylation-dependent manner *via* EGFR activation (24). However, the status of MAPK and Akt pathways will be different in each cell type, which may be due to the presence of *EGFR* gene mutations and the dependency of a particular cell type upon EGFR signaling for their survival and growth. The activities of these factors are therefore important parameters when monitoring gefitinib therapy.

In addition, these studies reveal that BCRP expression would modulate gefitinib sensitivity in highly gefitinib-sensitive cancer cells. Concerning BCRP activity, single nucleotide polymorphisms (SNPs) in the *BCRP* gene have been reported to determine its expression levels and transport activities (25-27). The expression levels of *BCRP* gene products harboring a C421A (Q141K) SNP are 5-fold lower than those of the wild-type gene, and the resistance of cells with a C421A *BCRP* SNP to SN-38 is also 5-fold lower than those with wild-type BCRP (25, 27). Cells containing a T623C (F208S) *BCRP* cDNA express only marginal levels of BCRP protein, and resistance to SN-38 is not observed (27). In addition, T1291C (F431L) BCRP-transfectants express two BCRP products of 65 kDa and 70 kDa, and resistance to SN-38 in these cells is significantly lower than wild-type *BCRP*-transfectants (27). Hence, SNPs affect the BCRP protein expression levels and thereby *BCRP* SNP(s) may also affect gefitinib transport and resistance to it. Indeed, Cusatis *et al.* reported that C421A *BCRP* SNP was associated with a high incidence of diarrhea in gefitinib-treated patients (28). It will therefore be important to evaluate *BCRP* SNPs in any future gefitinib therapy designs.

Overall, it has been hereby clarified that BCRP transports gefitinib. In cells that depend on EGFR signaling for their growth, the expression of BCRP was able to confer resistance to gefitinib-mediated cytotoxicity and inhibitory effects on EGFR signaling. It is reasoned that BCRP expression will affect the pharmacokinetics and pharmacodynamics of anticancer agents, and that BCRP is an important determining factor in the development and design of gefitinib-responsive cancer therapies.

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Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy☆

Kohji Noguchi [a], Kazuhiro Katayama [a], Junko Mitsuhashi [a], Yoshikazu Sugimoto [a,b,*]

[a] Division of Chemotherapy, Faculty of Pharmacy, Keio University, Tokyo, Japan
[b] Laboratory of Gene Therapy, Cancer Chemotherapy Center, Japanese Foundation of Cancer Research, Tokyo, Japan

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ABSTRACT

The breast cancer resistance protein, BCRP/ABCG2, is a half-molecule ATP-binding cassette transporter that facilitates the efflux of various anticancer agents from the cell, including 7-ethyl-10-hydroxycamptothecin, topotecan and mitoxantrone. The expression of BCRP can thus confer a multidrug resistance phenotype in cancer cells, and its transporter activity is involved in the *in vivo* efficacy of chemotherapeutic agents. Thus, the elucidation of the substrate preferences and structural relationships of BCRP is essential to understanding its *in vivo* functions during chemotherapeutic treatments. Single nucleotide polymorphisms (SNPs) have also been found to be key factors in determining the efficacy of chemotherapeutics, and those therapeutics that inhibit BCRP activity, such as the SNP that results in a C421A mutant, may result in unexpected side effects of the BCRP- anticancer drugs interaction even at normal dosages. In order to modulate the BCRP activity during chemotherapy, various compounds have been tested as inhibitors of this protein. Estrogenic compounds including estrone, several tamoxifen derivatives in addition to phytoestrogens and flavonoids have been shown to reverse BCRP-mediated drug resistance. Intriguingly, recently developed molecular targeted cancer drugs, such as the tyrosine kinase inhibitors imatinib mesylate, gefitinib and others, can also interact with BCRP. Since both functional SNPs and inhibitory agents of BCRP modulate the *in vivo* pharmacokinetics and pharmacodynamics of its substrate drugs, BCRP activity is an important consideration in the development of molecular targeted chemotherapeutics.

Contents

1. BCRP and Cancer

Cancer drug resistance is a major problem in clinical chemotherapy such that overcoming multidrug resistance to functionally and structurally unrelated anti-cancer agents is of critical importance for future treatments using these molecules and their derivatives. Various

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* Corresponding author. Keio University Faculty of Pharmacy, 1-5-30, Shibakoen, Minatoku, Tokyo 105-8512, Japan. Tel.: +81 3 5400 2698; fax: +81 3 5400 2669.
E-mail address: sugimoto-ys@pha.keio.ac.jp (Y. Sugimoto).

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mechanisms, such as reduced drug uptake, the efflux of intracellular drugs, the activation of DNA repair pathways, and the induction of anti-apoptotic machineries can confer multidrug resistance in cancer cells [1]. Among these mechanisms, the ABC transporter proteins, particularly P-glycoprotein (ABCB1), have been extensively examined as key components in pathways that result in a multidrug resistant phenotype [2–4]. A second ABC transporter protein, the breast cancer resistance protein (BCRP/ABCG2), is a 655 amino acid protein that contains an ATP-binding domain and six transmembrane domains, and it is a half transporter member of the ABCG subfamily [5]. BCRP was originally identified in anticancer drug-resistant human cancer cell lines obtained by *in vitro* selection [6–8].

In a similar fashion to the well-studied multidrug resistant protein P-glycoprotein, which is known to play important roles in the multidrug resistant phenotype of clinical cancer cells [9,10], the overexpression of BCRP renders cancer cells resistant to various chemotherapeutic drugs such as the topoisomerase I inhibitor topotecan and the antifolate agent methotrexate [8,11]. In a previous study from our laboratory, we observed that BCRP-transduced human myelogenous leukemia K562 cells (K562/BCRP) show a 24-fold higher resistance to SN-38, a 10-fold higher resistance to mitoxantrone, and a 10-fold higher resistance to topotecan [12]. However, the P-glycoprotein substrates vinblastine, paclitaxel, and verapamil are not eliminated by BCRP. As a half transporter, BCRP functions as a homodimeric/oligomeric efflux pump [13,14], and in a manner that is similar to other ABC transporters, BCRP eliminates a variety of compounds such as sulfated hormone metabolites, the chlorophyll metabolite pheophorbide A, fluorescent dyes such as Hoechst 33342 and BODIPY-prazosin, cimetidine, various flavonoids, some antibiotics, and various cytotoxic agents [15,16]. Typical BCRP substrates such as irinotecan and SN-38 are detoxified by glucuronidation via the activity of UDP-glucuronyltransferase, although BCRP can eliminate SN-38-glucuronide even though this substrate has a much lower affinity for BCRP than SN38 itself [17]. Interestingly, BCRP can also transport another of the glucuronideconjugates, 17-β-estradiol 17-(β-D-glucuronide) in addition to the sulfated-conjugates estrone-3 sulfate and dehydroepiandrosterone [18,19]. Our previously reported results of a membrane vesicle assay using ^{3}H-labeled compounds indicated that the ^{3}H-labeled estrone sulfate, but not the ^{3}H-labeled estrone or estradiol, was imported by membrane vesicles prepared from K562/BCRP cells in an ATP-dependent manner [20]. Hence, BCRP appears to transport xenobiotics and endogenous metabolites of steroids that are either sulfated or glucuronidated.

It has been suggested that the expression of BCRP is associated with a poor response to cancer chemotherapy and may be responsible for clinical drug resistance [21–23]. Moreover, BCRP is widely expressed in normal cells and tissues including the capillary endothelial cells, the hematopoietic stem cells [24,25], the maternal–fetal barrier of the placenta and the blood-brain barrier [26]. BCRP also appears to play a protective role against xenobiotics and their metabolites [15,19]. The apical localization of BCRP in the intestinal epithelium and in the bile canalicular membrane also suggests the intestinal absorption and hepatobiliary excretion of BCRP substrates [26–29]. Thus, BCRP may restrict the bioavailability of orally administered anticancer agents that are BCRP-substrates such as topotecan (and its metabolite SN-38), irinotecan, camptotecin derivatives, methotrexate, and flavopiridol, in addition to other compounds [16]. In this regard, the dual inhibitor of BCRP and P-glycoprotein, GF120918, has been reported to increase the oral bioavailability of topotecan through the inhibition of BCRP function [30]. Since undesired toxic effects of chemotherapeutic drugs on the digestive organs are a significant problem during clinical cancer chemotherapy, the functional activity of BCRP is an important consideration for BCRP-transportable drug pharmacokinetics in patients.

It has been suggested that BCRP underlies the drug resistance observed in clinical samples of different cancers such as acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) [21–23,31–34]. In addition, BCRP expression in solid tumors has been examined [35]. However, there is some controversy surrounding the precise roles of BCRP as functional correlations between BCRP expression and clinical drug resistance have not yet been definitively demonstrated. Since P-glycoprotein expression is also an important factor in the drug resistance levels of clinical leukemia, a larger scale analysis that assesses both BCRP and P-glycoprotein will be required to properly delineate the involvement of BCRP expression in the drug resistance of cancer patients. Furthermore, in addition to expression profiling, recent advances in ABC transporter research have highlighted that the activity of these factors is affected by both posttranslational regulation [36,37] and genetic polymorphisms [38–40]. Thus, in order to elucidate the relationship between the functions of BCRP and the clinical outcomes following chemotherapy, new and more sensitive methods for testing specific pharmacological inhibitors, and also immunological and genetic probes, will be required.

2. BCRP structure and anti-cancer drugs

Structural and functional studies of BCRP and its substrates have provided valuable insights into the molecular mechanisms underlying BCRP-mediated multidrug resistance. Intriguingly, the cloning of BCRP cDNAs from drug-selected cells and normal tissues have also uncovered functional variations associated with amino acid substitutions in the BCRP protein resulting in an alteration in substrate preferences. BCRP proteins expressed in drug-selected cells such as those of the S1-M1-80 and MCF7/AdVp3000 cell lines were unexpectedly found to be mutant forms, and several unique mutations at amino acid position 482 in BCRP have been identified [41]. MCF7/AdVp3000 and S1-M1-80 cells expressing R482T and R482G variants of BCRP, respectively, are highly resistant to both mitoxantrone and doxorubicin. Anthracyline resistance and a rhodamine efflux ability are also unique phenotypes in these two cell lines when they are overexpressing BCRP [41,42]. Subsequently, we have learned that substitutions of Arg with either Gly or Thr at position 482 in BCRP confer an additional efflux activity against rhodamine 123, doxorubicin, and other anthracyclins, which are not substrates for wild-type BCRP [41,43]. Moreover, the BCRP variants R482G and R482T lose their methotrexate-transporting activity but at the same time confer increased mitoxantrone resistance [18,42,44,45]. These findings suggest that structural variations of BCRP can strongly influence its drug efflux functions and substrate preference. A positively charged Arg at position 482 affects the interaction between BCRP and the drug, and therefore the COOH-terminus of the transmembrane (TM) 3 region that is in close proximity to position of amino acid 482 appears to be involved in the substrate-binding pocket interface of BCRP [45–47].

In the case of P-glycoprotein, extensive mutagenesis studies and recent three-dimensional structural analyses have suggested that its transmembrane domains are involved in influencing its substrate-specificity [9,48,49]. Hence, in order to further elucidate the structural features of BCRP and how these features relate to substrate recognition, systematic mutational analysis of its TM regions would likely be of great benefit and provide valuable information regarding the molecular mechanisms underlying multidrug interactions. In an analogous manner to studies of P-glycoprotein, we performed such mutational analysis of BCRP using 32 mutants of this protein and found that Glu 446 in TM2, Arg 482 in TM3, Asn 557 in TM5, and His 630 in TM6 alter its drug resistant phenotype [50]. These findings confirmed that the transmembrane region of BCRP plays important roles in its activity. Moreover, murine fibroblast PA317 cells expressing E446 mutant BCRPs did not show drug resistance to either mitoxantrone or SN-38.

Furthermore, in a manner similar to that observed in S1-M1-80 and MCF7/AdVp3000 cells, 13 variant BCRPs harboring an amino acid substitution at R482 (R482N, C, M, S, T, V, A, G, E, W, D, Q and H, but not Y or K) conferred strong resistance to doxorubicin and mitoxantrone in

PA317 cells. Mutations in BCRP at positions N557 and H630 however severely affected this resistant phenotype. Cells expressing either the N557D or the H630E BCRP mutant displayed a lower resistance to SN-38, although the mitoxantrone-resistance of these cells was comparable to that observed for the wild-type BCRP-expressing cells. Consistently, recent structural studies using three-dimensional homology modeling of BCRP have suggested that the transmembrane domains of BCRP function as a drug-recognition interface [46,47]. These data, coupled with other numerous studies regarding BCRP functions, indicate that the drug efflux activity of this protein appears to be influenced by various mutations that will necessarily affect the clinical efficacy of BCRP-transportable anticancer drugs.

3. Effects of BCRP SNPs upon drug resistance

As mentioned in the previous section, amino acid variations in BCRP may be associated with its drug-transporter function. In addition, a variety of germ-line mutations in the *BCRP* gene have been found in ethnically diverse populations [39,51–53]. Such variations, particularly of the single nucleotide polymorphisms (SNPs) in the *BCRP* genomic region should be evaluated to estimate the possible effects of BCRP among different patients.

3.1. C421A (Q141K) BCRP SNP

In a previous study, we screened for BCRP SNPs among a population of Japanese individuals and in human cancer cell lines where we identified three variant *BCRP* cDNAs harboring the following substitutions: G34A (V12M), C421A (Q141K) and an amino acid deletion of residues 944-949 that lacks Ala-315 and Thr-316 (Δ315-6) [54]. The G34A and C421A variations were determined to be SNPs, and we have subsequently determined that the C421A *BCRP*-transfected murine fibroblast PA317 (PA/Q141K) cells show lower exogenous BCRP protein levels than the wild-type *BCRP*-transfected cells [54]. The intracellular topotecan accumulation in PA/Q141K cells was also found to be higher compared with other *BCRP* transfectants, indicating that the C421A (Q141K) SNP influences BCRP function. This polymorphism is located within the functionally important ATP-binding region between the Walker A and B motifs of BCRP and likely affects its ATPase activity levels, since the ATPase activity of the membrane of C421A *BCRP*-transduced insect Sf9 cells were 1.3-fold lower than that of the wild-type BCRP transduced cells [38,55]. Regarding the Q141K BCRP SNP, there are conflicting reports on its effects upon expression levels, localization, and functionality [38,54,56–58]. Additional studies will be required to clarify the mechanism by which the Q141K mutation reduces the protein expression levels of BCRP. In contrast to the above results, the G34A *BCRP*-transfected PA317 (PA/V12M) cells showed comparable protein expression levels and drug resistance levels to the wild-type *BCRP*-transfected cells.

Our earlier studies on the frequency of the C421A SNP in a normal Japanese population showed that 57 of 124 samples possessed the A421 allele and that 9 of these were homozygous for this polymorphism [39,59]. These data indicate that some Japanese individuals likely express low amounts of BCRP. Furthermore, the C421A SNP is of some importance as the allelic frequency of this variant differs greatly between diverse populations. This SNP appears to be very common in Asian populations, with reported allelic frequencies between 27% and 34% [38,54,57]. In contrast, the C421A SNP is rare in sub-Sahara African and in African American populations, with a frequency of <5% [60]. Its frequency in Caucasian populations is approximately 10% [61]. The physiological significance of the C421A-*BCRP* SNP has also now been evaluated in relation to the pharmacokinetics of diflomotecan, a new anticancer agent that is a derivative of camptothecin, during a phase I study [62]. In this analysis, 5 patients who were heterozygous for the A421 allele showed much higher plasma levels of diflomotecan after intravenous administration compared with 15 wild type individuals who were homozygous for the allele (mean values of 138 ngxh/mLxmg^{-1} versus 46.1 ngxh/mLxmg^{-1}, respectively). The findings from this clinical study indicate that the expression levels and functions of the BCRP derived from the C421A-*BCRP* allele are adversely perturbed in comparison to the wild-type allele. Hence, the C421A SNP is considered to be one of the most important BCRP variations in terms of cancer chemotherapy and drug resistance.

3.2. C376T (Q126stop)-BCRP SNP

We have identified another SNP within the *BCRP* gene, C376T, which substitutes a stop codon for Gln-126 (Q126stop) and is present at a low frequency in samples from healthy Japanese individuals as a heterozygote (reported frequencies of 3/124 and 2/120 in two studies, respectively) [54,60]. Similar stop codon SNPs have been reported in the *MRP2* gene and are linked to the rare hyperbilirubinemia associated with Dubin-Johnson syndrome [63]. Although the frequency of the T376 allele of *BCRP* is low and has not been observed in Caucasian or African American groups, a combination of the C376T and C421A SNPs would be expected to occur at a significant rate in the Japanese population. Since these SNPs are each anticipated to have negative effects on BCRP activity, the combined C376T/C421A variants would be expected to show severely reduced BCRP activity. Such individuals may thus be hypersensitive to anticancer agents.

3.3. Additional BCRP SNPs

The *BCRP* SNPs identified to date are summarized in Table 1. These polymorphisms include G34A, G151T, C376T, C421A, C458T, C496G, A616C, T623C, T742C, G1000T, T1291C, T1465C, A1768T and G1858A, all of which generate amino acid substitutions. However, additional mutations such as T114C, C369T, C474T, A564G, G1098A, and A1425G have been identified in the coding region of *BCRP*. Among these SNPs, with the exception of C376T and C421A, only a few have been studied

Table 1
Identified SNPs within the BCRP gene

Variation	Effect	Domain
A-1379G	-	-
Δ-654/-651	-	-
G-286C	-	-
T-476C	-	-
Δ-235A	-	-
A-113G	-	-
A-29G	-	-
G34A	V12M	N-terminal
T114C	No change	N-terminal
G151T	G51C	N-terminal
C369T	No change	NBD
C376T	Q126stop	NBD
C421A	Q141K	NBD
C458T	T153M	NBD
C474T	No change	NBD
C496G	Q166E	NBD
A564G	No change	NBD
A616C	I206L	NBD
T623C	F208S	NBD
T742C	S248P	Linker
G1000T	E334stop	Linker
G1098A	No change	Linker
T1291C	F431L	TMD
A1425G	No change	TMD
T1465C	F489L	TMD
A1768T	N590Y	TMD
G1858A	D620N	TMD
G2237T	-	
G2393T	-	

NBD, nucleotide-binding domain; TMD, transmembrane domain.

in association with the protein expression levels and function of BCRP. The G34A SNP generating an amino acid substitution at position 12 (V12M) has been observed in the Japanese population [54]. The highest allele frequency for this polymorphism is observed in Mexican-Indians, and there are significant differences in the frequencies of this SNP between Caucasian, Japanese and Swedish populations [56,64]. Transfection studies of the V12M BCRP have shown, however, that the expression levels and drug-resistance associated with this variant are comparable to the wild type BCRP and therefore that this SNP has no significant impact on the BCRP protein activity [54]. It is noteworthy, however, that, although the physiological and pathological significance of the G34A SNP in the *BCRP* gene is unclear, a recent report has suggested a possible association between this polymorphism and alternative splicing event of the BCRP mRNA, specifically in the splicing of the liver-specific polymorphic exon 2 of these transcripts [65]. Polymorphic and differential expression of alternatively spliced BCRP mRNA involving exon 1b is also observed in the liver [66] and appears to be associated with lower BCRP expression. Approximately 90% of the cases characterized by a G34A BCRP SNP display exon 2 skipping in the liver that may suggest that the lower expression of BCRP transcripts in this organ may be associated with this SNP in the Hispanic population [65].

4. BCRP and anticancer kinase inhibitors

Recent advances in molecular targeted therapy have resulted in the development of various anticancer drugs with unique pharmaceutical properties [1]. In particular, a growing number of small-molecule protein kinase inhibitors have been brought into clinical use and have shown great potential as anticancer drugs [67,68]. Imatinib mesylate was the first protein kinase inhibitor to be approved as an anticancer drug and targets BCR-ABL, the platelet-derived growth factor receptor (PDGFR), and stem cell factor/c-kit [69]. Imatinib is highly effective against chronic myeloid leukemia and other cancers associated with deregulation of kinase pathways. Resistance to this drug is typically conferred by mutations arising in the target kinase within the drug-kinase-interaction region [70–73]. Interestingly, another mechanism leading to imatinib resistance has been proposed and involves a correlation with P-glycoprotein expression [74,75]. As shown in Table 2, a number of recent studies have indicated a possible interaction of several kinase inhibitory drugs with ABC transporters, including P-glycoprotein and BCRP [76–81]. Among members of the ABC transporter family, BCRP seems to have a strong tendency to interact with clinically important kinase inhibitors including imatinib [78–80,82–85], nilotinib [86], gefitinib [52,87–93], canertinib [94], erlotinib [95,96], and lapatinib [97]. Imatinib and nilotinib are both inhibitors of BCR-ABL, whereas gefitinib, erlotinib, canertinib, and lapatinib target the HER family. Functional and pharmaceutical interactions between BCRP and imatinib or gefitinib have been extensively examined, and these data indicate that, although these kinase inhibitors are substrates for BCRP, they exhibit potent inhibitory activity against this ABC transporter when used at relatively high concentrations [86].

Table 2
Functional interaction between tyrosine kinase inhibitors and BCRP

	BCRP	P-glycoprotein	Ref
Imatinib	+	+	[78,79,82]
Nilotinib	+	+	[86]
Dasatinib	ND	+	[102]
INNO-406	ND	-	[101]
Gefitinib	+	+	[78,87,90,92,93]
Erlotinib	+	+	[95,96,107]
Canertinib	+	ND	[94]
Flavopiridol	+	+	[109]

ND, not determined.

4.1. BCR-ABL kinase inhibitors

Imatinib mesylate is a tyrosine kinase inhibitor of BCR-ABL, PDGFR, and c-Kit that is now a widely used anticancer drug. In terms of the functional interaction between this agent and ABC transporters, contentious observations were reported in initial studies regarding whether or not imatinib is in fact a substrate of these efflux proteins. Recent analyses by Brendel et al. [86] have further shown that BCRP-expression confers imatinib-resistance and reduces imatinib accumulation in K562 cells, effects that are abrogated by the BCRP inhibitor fumitremorgin C (FTC). However, this previous study also demonstrated that imatinib directly interacts with BCRP at the substrate-interacting region and can stimulate its ATPase activity. Intriguingly, this study suggests that imatinib-transportation by BCRP may be concentration dependent as the efflux of this drug by BCRP was facilitated when imatinib was at low concentrations (<1 μM). At relatively high concentrations (μM level), imatinib has also been observed as a potent BCRP inhibitor that reverses the BCRP-mediated drug resistance to SN-38 and topotecan [79] and increases mitoxantrone accumulation in BCRP-expressing CD34+ cells [84]. Furthermore, *in vivo* studies indicate that BCRP, together with P-glycoprotein, appears to regulate the penetration of imatinib into brain tissue. Imatinib brain penetration in *Bcrp1* knockout mice was found to increase [80], whereas the inhibition of BCRP and P-glycoprotein activities significantly improved the brain penetration of imatinib in wild-type mice [98]. These observations support the notion that imatinib can function as both a substrate for and inhibitor of BCRP.

Nilotinib is a newly developed BCR-ABL kinase inhibitor with improved selectivity and potency [99,100]. BCRP-expressing K562 cells showed nilotinib resistance over a narrow range of concentrations (10 to 25 nM) [86] such that nilotinib resistance by BCRP may not be a significant phenomenon in the clinical setting. However, in this same study, BCRP expression was found to reduce the intracellular accumulation of nilotinib, a compound that binds to BCRP and stimulates its ATPase activity [86]. In addition, nilotinib reverses the BCRP-mediated Hoechst 33342 dye exclusion and therefore appears also to be both a substrate and a potent inhibitor of BCRP. Functional interactions between P-glycoprotein and the novel BCR-ABL inhibitors dasatinib and INNO-406 have also been reported [101,102], but the effects of these agents against BCRP have not yet been examined.

4.2. EGFR/HER kinase inhibitors

Gefitinib is an orally active, selective epidermal growth factor receptor-tyrosine kinase inhibitor used in the treatment of patients with advanced non-small cell lung cancer [103,104]. Human epidermoid carcinoma A431 cells and human non-small cell lung cancer PC-9 cells, both of which are highly sensitive to gefitinib, were found to become resistant to gefitinib at nanomolar concentrations upon transduction with BCRP [52]. Consistent with this observation, Elkind et al. [90] have also reported that the expression of BCRP confers gefitinib-resistance in A431 cells. However, it should be noted from these studies that BCRP expression did not confer ectopic gefitinib-resistance to naturally occurring gefitinib-insensitive cells as the efflux function of BCRP itself seems to be suppressed by relatively high concentrations (~μM level) of this drug. In this regard, studies from our laboratory and others have demonstrated that BCRP overexpression in the less gefitinib-sensitive cell lines K562, P388, and MCF7 was not in fact a determinant of gefitinib sensitivity [88,91]. In contrast, we have shown from our analyses that gefitinib can reverse the SN-38 resistant phenotype in human leukemia K562/BCRP and mouse leukemia, P388/BCRP cells, and suppress the ATP-dependent transport of estrone sulfate in membrane vesicles prepared from K562/BCRP cells [88]. Co-treatments with gefitinib have also been shown to induce the intracellular accumulation of topotecan in K562/BCRP cells, and the combination of irinotecan with gefitinib resulted in a potent

enhancement of irinotecan cytotoxicity in multiple tumor models [87,88,91]. These results indicate that gefitinib can inhibit the transporter activity of BCRP and thus reverse the BCRP-mediated drug resistance when used at relatively high concentrations in the micromolar range.

With regard to possible interactions between BCRP and gefitinib *in vivo*, Stewart et al. [87] have demonstrated that the oral bioavailability of irinotecan is affected by the oral administration of gefitinib. BCRP has been detected at the blood-brain and blood-cerebrospinal barriers, where it restricts xenobiotic penetration of the brain [16,105]. Zhuang et al. [106] have further shown that the oral administration of gefitinib increases topotecan penetration into the brain extracellular fluid but conversely decreases the ventricular cerebrospinal fluid penetration of topotecan. BCRP is also expressed in the intestinal epithelial cells, and Cusatis et al. [92] demonstrated that diarrhea, an adverse event related to oral gefitinib administration, is linked to genetic polymorphisms of BCRP, most notably C421A (Q141K). The resulting C421A variant of BCRP reduces its efflux activity [52] such that orally administrated gefitinib (presumably at submicromolar concentration *in vivo*) is also thought to inhibit BCRP function. Collectively, these observations strongly indicate that gefitinib at low concentrations (nM) is recognized as a substrate by BCRP, and that BCRP is one of the important mediators of gefitinib sensitivity both *in vitro* and *in vivo*.

Analogous to gefitinib, other small molecular kinase inhibitors of the HER-family members have been found to interact with BCRP. Erlotinib, a tyrosine kinase inhibitor with similarities to gefitinib, has been shown to interact with BCRP [95,107]. At relatively high concentrations, this drug shows antagonistic activity toward both BCRP and P-glycoprotein and can reverse the multidrug resistance by inhibiting the drug efflux activity of BCRP. In addition, BRCP expression was shown to decrease the intracellular accumulation of both erlotinib and gefitinib, and our unpublished findings indicate that lower concentrations of erlotinib are likely to be transported by BCRP. In studies of drug interactions *in vivo*, two polymorphic loci were identified in the BCRP promoter, −15622C/T and 1143C/T, which cause lower protein expression and are associated with improved pharmacokinetic parameters for erlotinib [96]. Hence, BCRP appears to recognize erlotinib as a substrate when this drug is administered at relatively low concentrations.

Canertinib also appears to be a substrate for BCRP since it has been shown to be transported by BCRP [94]. The intracellular accumulation of canertinib was shown to be reduced by the overexpression of BCRP and treatment with this agent was found to sensitize BCRP-expressing cells to SN-38 and topotecan via an increased intracellular accumulation of these drugs. Lapatinib is another newly developed kinase inhibitor that targets the HER-family that is a substrate and inhibitor of BCRP and P-glycoprotein [97]. *In vivo* studies show that the efflux transporters at the blood-brain barrier influence lapatinib penetration into the brain. However, BCRP appears to have little impact upon the intestinal absorption of lapatinib because systemic exposure to this drug by oral dosing was unchanged even when BCRP and P-glycoprotein are absent from the gastrointestinal tract.

4.3. Interactions between kinase inhibitors and BCRP

In addition to clinical kinase inhibitors, a variety of kinase inhibitory compounds appear to interact with ABC transporters [76,101,108–113]. Most of the recently developed protein kinase inhibitors are designed to compete with ATP binding to the kinase domain, thereby exerting their suppressive effects [114]. Typical of such molecules, imatinib and gefitinib also have inhibitory activity against ABC transporters that contain ATP-binding domains. Most BCRP-interactive kinase inhibitors used at relatively high concentrations were thus initially predicted to block the ATPase activity of this protein. However, Saito et al. [115] have demonstrated that gefitinib binds to ATP-bound BCRP, indicating that the as yet to be determined gefitinib-binding site in BCRP is not the ATP-binding domain. Photo affinity labeling using ^{125}I-labeled iodoarylazidoprazosin, a typical substrate for P-glycoprotein and BCRP, has been widely used for competition experiments with sample compounds at ABC transporter substrate-binding sites. Using this technique, Brendel et al. showed that imatinib and nilotinib bind to BCRP at the substrate-interaction site [86], but Shi et al. demonstrated that erlotinib has little ability to compete with iodoarylazidoprazosin at the substrate-binding sites of BCRP and P-glycoprotein [95]. In regard to a drug-BCRP interaction model, some interesting studies have proposed the presence of multiple drug binding sites on this ABC transporter [116,117] that may include kinase inhibitors. Further studies will be needed to properly elucidate the modes of interaction between kinase inhibitors and BCRP.

5. Other BCRP inhibitors

BCRP inhibitors may have important clinical applications as modulators of the efficacy of cancer drugs that are BCRP substrates. Co-administration of such inhibitors may overcome BCRP-mediated drug resistance in some tumor cells and will necessarily affect the pharmacokinetics and pharmacodynamics of BCRP-substrates in tissues. This may however have consequences in terms of the increased toxicity of specific anticancer agents. Various compounds, including Fumitremorgin C, have been found to reverse drug resistance through the inhibition of BCRP function [105]. The placenta synthesizes and secretes estrogens and BCRP is highly expressed in the syncytiotrophoblasts of the placenta [26]. Other than protein kinase inhibitors, we anticipated in our early work that estrogens would interact with BCRP as a physiological substrate or as an inhibitor. We have since found that estrone and 17βestradiol can restore drug sensitivity in *BCRP*-transduced human myelogenous leukemia K562 (K562/BCRP) cells [118]. In addition, we have also examined estrogen agonists, antagonists, and their derivatives as potential BCRP-reversing agents [12]. Although neither tamoxifen nor toremifene was found to have any effects upon topotecan uptake in K562/BCRP cells, diethylstilbestrol showed strong BCRP-reversing activity. Diethylstilbestrol enhances the cellular accumulation of topotecan and reverses the resistance to SN-38 and mitoxantrone in K562/BCRP cells without affecting the parental K562 cells. Further screening identified TAG-139, a derivative of tamoxifen, as a strong BCRP inhibitory agent. TAG-139 reversed both SN-38 and mitoxantrone-resistance in K562/BCRP cells with a 5-fold greater potency than estrone. Intriguingly, the dose-dependent characteristics of drug resistance reversal by TAG-139 and estrone appear to be similar, suggesting that tamoxifen derivatives and estrone may interact with the same binding site of BCRP.

Some phytoestrones and flavonoids have weak estrogenic activity, and we have shown in our laboratory that some of these compounds, including genistein, naringenin, acacetin, kaempferol and some glycosylated flavonoids, are effective BCRP-inhibitors and potentiate the cytotoxicity of SN-38 and mitoxantrone in K562/BCRP cells [119,120]. However, genistein and naringenin were unable to reverse either P-glycoprotein-mediated vincristine resistance or MRP1-mediated etoposide resistance. Our findings have indicated that genistein is a naturally occurring substrate of BCRP and competitively inhibits BCRP-mediated drug efflux.

We have developed a number of new inhibitors of BCRP in our laboratory, including a novel acrylonitrile derivative YHO13351 and its parent compound YHO13177. Both of these molecules enhance the *in vitro* cytotoxicity of SN-38 in the human lung cancer cell lines NCI-H460 and NCI-H23, the leukemia cell line RPMI8226, and the pancreatic cancer cell line AsPC-1. All of these cell lines express BCRP, and the effects of YHO13351 and YHO13177 were dose dependent. YHO13177 was found *in vitro* to reverse SN-38-, mitoxantrone-, and topotecan-resistance in *BCRP*-transduced HCT-116 cells, but it showed little effect upon P-glycoprotein-mediated paclitaxel resistance. Moreover, YHO-13351 markedly reduced HCT-116/BCRP tumor

growth in a xenograft model when combined with half of the maximal tolerated dose of CPT-11. These findings suggest that YHO-13351 may be a clinically useful drug that can reverse BCRP-mediated drug resistance to CPT-11, mitoxantrone, or topotecan.

6. Perspectives

A number of studies now strongly suggest that BCRP expression is associated with the clinical efficacy of a specific class of anticancer drugs. The accelerated development of anticancer drugs continues to produce a growing number of novel molecular targeted agents such as the small molecule protein kinase inhibitors [67,121]. Molecular analyses of the functional interactions between such novel drugs and the ABC transporter BCRP suggest their usefulness as indicators of the clinical efficacy of these anticancer agents in individual patients. In addition, the increased risk of adverse effects resulting from the use of putative BCRP substrates needs to be evaluated when considering combinations of protein kinase inhibitors, even at clinically relevant dosages.

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ORIGINAL ARTICLE

Promotion of glioma cell survival by acyl-CoA synthetase 5 under extracellular acidosis conditions

T Mashima[1], S Sato[2], Y Sugimoto[3,4], T Tsuruo[5] and H Seimiya[1]

[1]Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan; [2]Division of Experimental Chemotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan; [3]Department of Chemotherapy, Faculty of Pharmacy, KEIO University, Tokyo, Japan; [4]Division of Gene Therapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan and [5]Director's Room, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan

Extracellular acidosis (low pH) is a tumor microenvironmental stressor that has a critical function in the malignant progression and metastatic dissemination of tumors. To survive under stress conditions, tumor cells must evolve resistance to stress-induced toxicity. Acyl-CoA synthetase 5 (ACSL5) is a member of the ACS family, which converts fatty acid to acyl-CoA. ACSL5 is frequently overexpressed in malignant glioma, whereas its functional significance is still unknown. Using retrovirus-mediated stable gene transfer (gain of function) and small interfering RNA-mediated gene silencing (loss of function), we show here that ACSL5 selectively promotes human glioma cell survival under extracellular acidosis. ACSL5 enhanced cell survival through its ACS catalytic activity. To clarify the genome-wide changes in cell signaling pathways by ACSL5, we performed cDNA microarray analysis and identified an ACSL5-dependent gene expression signature. The analysis revealed that ACSL5 was critical to the expression of tumor-related factors including midkine (MDK), a heparin-binding growth factor frequently overexpressed in cancer. Knockdown of MDK expression significantly attenuated ACSL5-mediated survival under acidic state. These results indicate that ACSL5 is a critical factor for survival of glioma cells under acidic tumor microenvironment, thus providing novel molecular basis for cancer therapy.

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Correspondence: Dr H Seimiya, Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan.
E-mail: hseimiya@jfcr.or.jp
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Introduction

Enhanced lipid biosynthesis occurs selectively in tumor cells and is closely linked with tumorigenesis (Menendez and Lupu, 2007). In tumor cells, the supply of cellular fatty acid is highly dependent on *de novo* synthesis, and several enzymes in the lipid biosynthesis pathways are involved in tumor cell survival (Brusselmans *et al.*, 2005; Hatzivassiliou *et al.*, 2005; Kuhajda, 2006). These observations suggest that mediators of lipid metabolism are newly recognized molecular targets to induce selective tumor cell death.

Acyl-CoA synthetases (ACSs) are enzymes that convert long-chain fatty acids to acyl-CoA. This reaction is a critical step in several lipid metabolic pathways, including phospholipid biosynthesis, lipid modification of cellular proteins and β-oxidation (Coleman *et al.*, 2002). ACSs are overexpressed in a variety of cancers (Cao *et al.*, 2000, 2001; Yamashita *et al.*, 2000; Sung *et al.*, 2003, 2007; Gassler *et al.*, 2005; Liang *et al.*, 2005; Yeh *et al.*, 2006). Moreover, our recent screening identified an ACS inhibitor as a tumor-selective inducer of apoptosis (Mashima *et al.*, 2005; Mashima and Tsuruo, 2005). These data suggest that ACSs are predominantly involved in tumor cell survival.

Acyl-CoA synthetase 5 (ACSL5) is a unique isozyme among the ACS members, as it is the only known ACS isozyme that localizes on mitochondria (Lewin *et al.*, 2001; Coleman *et al.*, 2002). In human glioma, aberrations occur on chromosome 10q25.1–q25.2, on which the ACSL5 gene is located, and ACSL5 is frequently overexpressed (Yamashita *et al.*, 2000). These observations strongly suggest potential functions of the enzyme in the growth or malignancy of glioma. At present, however, the precise functions of ACSL5 in cancer have not been elucidated.

Extracellular acidosis (low pH) is a tumor microenvironmental stressor (Vaupel *et al.*, 1989). Solid tumors are commonly characterized by a unique pathophysiologic microenvironment (Tannock and Rotin, 1989; Vaupel *et al.*, 1989; Tomida and Tsuruo, 1999). This hostile microenvironment activates several intracellular signaling pathways that promote malignant progression and metastatic dissemination (Harris, 2002;

Rofstad *et al.*, 2006; van den Beucken *et al.*, 2006). On the other hand, to survive under such stress conditions, tumor cells must also develop resistance to the microenvironmental stress-induced cytotoxicity (Graeber *et al.*, 1996), although the underlying mechanisms remain unclear.

Midkine (MDK) is a basic heparin-binding growth factor of low molecular weight, a member of the neurite growth-promoting factor family (Kadomatsu and Muramatsu, 2004). MDK shows highly increased expression in a number of malignant tumors (Nakagawara *et al.*, 1995; O'Brien *et al.*, 1996; Mishima *et al.*, 1997; Ye *et al.*, 1999; Ikematsu *et al.*, 2000; Jia *et al.*, 2007; Maeda *et al.*, 2007) and enhances tumor progression by promoting survival, growth, migration and angiogenic activity (Kadomatsu *et al.*, 1997; Takei *et al.*, 2001; Kadomatsu and Muramatsu, 2004; Mirkin *et al.*, 2005; Tong *et al.*, 2007). In human brain tumors, especially MDK is overexpressed during tumor progression, and patients whose tumors express a higher level of MDK have a worse prognosis (Mishima *et al.*, 1997).

In this study, we examined the function of ACSL5 in glioma cell survival under extracellular acidosis conditions. Moreover, the ACSL5-regulated gene signature was analysed. The analysis revealed that ACSL5 is a critical regulator of tumor-related genes including MDK.

Results

ACSL5 promotes human glioma cell survival under extracellular acidosis conditions

To clarify the function of ACSL5 in glioma cell survival, we examined the effect of its overexpression on cell survival under various tumor-related stress conditions. We initially examined the expression of endogenous ACSL5 in human glioma cell lines. As a result, we found two cell lines with low levels of ACSL5, SF268 and U251, and two cell lines with relatively high amounts of ACSL5, SNB78 and A1207 (data not shown; see Figure 2a). We stably transduced SF268 cells with a retroviral vector harboring a human ACSL5 gene with a FLAG tag at its carboxy end. Overexpression of FLAG-tagged ACSL5 in the transduced cells (SF268/ACSL5) was confirmed by immunoblot analysis (Figure 1a). Under normal culture conditions, both SF268/mock and SF268/ACSL5 cells showed similar growth rates (Supplementary Figure 1a). By contrast, SF268/ACSL5 showed markedly enhanced survival under extracellular acidosis conditions (pH 6.5) (Figures 1b and c). Similar results were obtained in another human glioma cell line, U251, when it was stably transduced with ACSL5 (data not shown). The major source of proton ion *in vivo* is lactic acid. Therefore, we also examined cell survival under low pH conditions (pH 6.3–6.5) that were generated by lactic acid. As a result, we found that ACSL5 expression also promoted cell survival under lactic acid-based low pH conditions (Supplementary Figure 1b). Extracellular acidosis (range pH 5.8–7.6) is known as one of the pathophysiologic microenvironmental stresses that are characteristically observed in solid tumors (Tannock and Rotin, 1989; Vaupel *et al.*, 1989; Tomida and Tsuruo, 1999). ACSL5-mediated promotion of survival was selective under acidosis conditions, as SF268/ACSL5 did not show apparent survival advantage under other tumor-related stresses such as hypoxia and low serum conditions (Figure 1d).

We have shown earlier that inhibition of total cellular ACS induces cell death through the activation of caspases, the cysteine proteases that have a central function in apoptosis induction (Mashima *et al.*, 2005). To characterize the molecular mechanisms of the reduced cell viability under low pH, we next examined the involvement of a caspase-mediated pathway. As shown in Supplementary Figure 2a, treatment with a specific caspase inhibitor, Z-VAD-fmk, did not recover the reduced SF268 cell viability under low pH. Consistently, caspase protease activity was not elevated in the cells exposed to extracellular acidosis and neither was it affected by ACSL5 expression (Supplementary Figure 2b). Flow cytometric analysis further revealed that the loss of viability under low pH did not accompany the emergence of the sub-G1 population, a characteristic of apoptotic cells (Supplementary Figure 2c). These results indicate that the reduced cell viability under acidosis is caspase-independent and non-apoptotic.

To confirm the function of ACSL5 under acidic conditions, a loss-of-function study was performed using the small interfering RNA (siRNA) against endogenous ACSL5. We found two ACSL5-overexpressed glioma cell lines, SNB78 and A1207 (Figure 2a), and used these cell lines for the loss-of-function study. When SNB78 cells were transfected with ACSL5-siRNAs (si1 and si2), the level of ACSL5 mRNA was clearly reduced in the ACSL5 siRNA-transfected cells (Supplementary Figure 3a). Consistently, the ACSL5 protein was decreased in the SNB78 cells treated with ACSL5 siRNAs (Figure 2b). We found that the inhibition of ACSL5 expression significantly reduced cell viability under the acidic state (pH 6.5) (Figure 2c, right), whereas it did not influence cell survival under normal conditions (pH 7.3) in SNB78 cells (Figure 2c, left and Supplementary Figure 3b). We observed similar results in A1207 cells (Figures 2b and d), except for slight suppression of A1207 cell growth under normal conditions (pH7.3) by one of the ACSL5 siRNAs (siRNA 1). The growth inhibition by siRNA1 could result from its off-target effect, as the other ACSL5 siRNA (siRNA 2) did not show any growth inhibitory effect under normal conditions. By contrast, the inhibition of ACSL5 expression did not reduce cell viability under low serum conditions (Supplementary Figure 3c). To clarify the function of overexpressed ACSL5 in *in vivo* growth of tumor, we further tested the effect of ACSL5 siRNA treatment on ACSL5-overexpressed tumor. For this study, we chose human glioma A1207 cells, as they overexpress endogenous ACSL5 and are tumorigenic in nude mice (Mishima *et al.*, 2001). As a result, we found that *in vivo* treatment