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

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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.

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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,

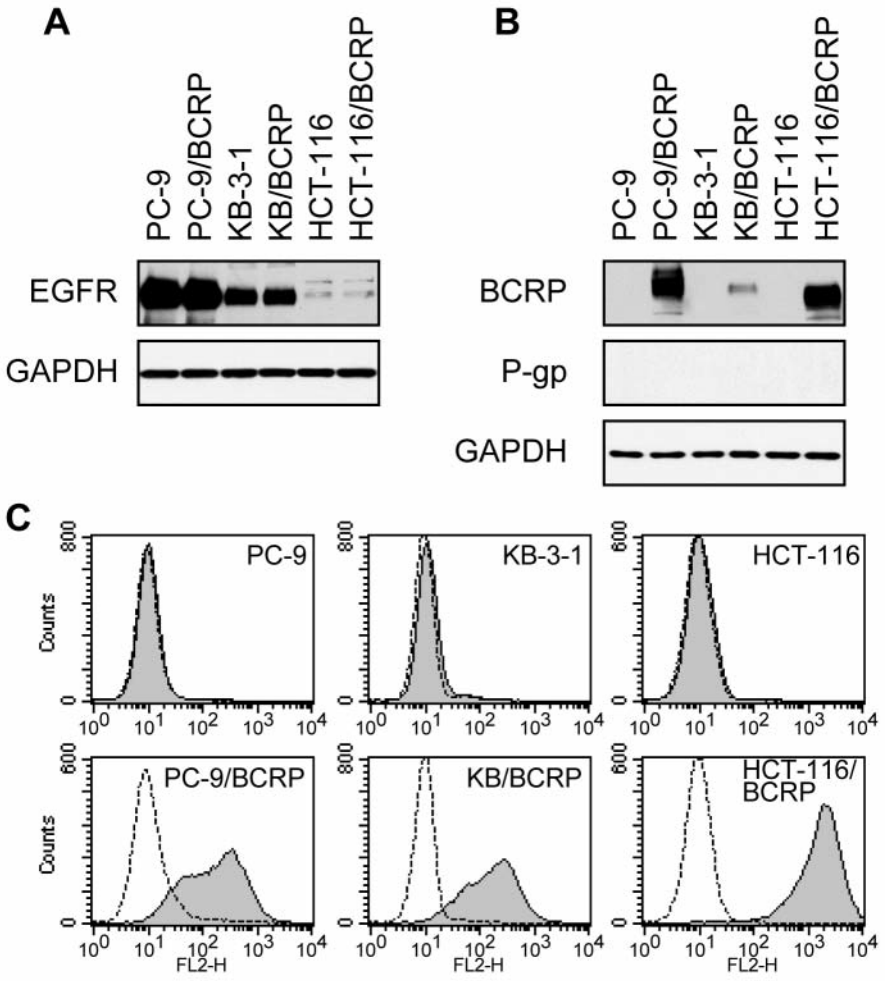

Figure 1. *Analysis of the expression levels of EGFR, BCRP and P-gp in BCRP-transduced cells. (A, B) Cell lysates (10 μg/lane) were resolved by SDS-PAGE and expression levels of EGFR or GAPDH were detected by Western blotting using anti-EGFR polyclonal antibody or anti-GAPDH monoclonal antibody, respectively (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.

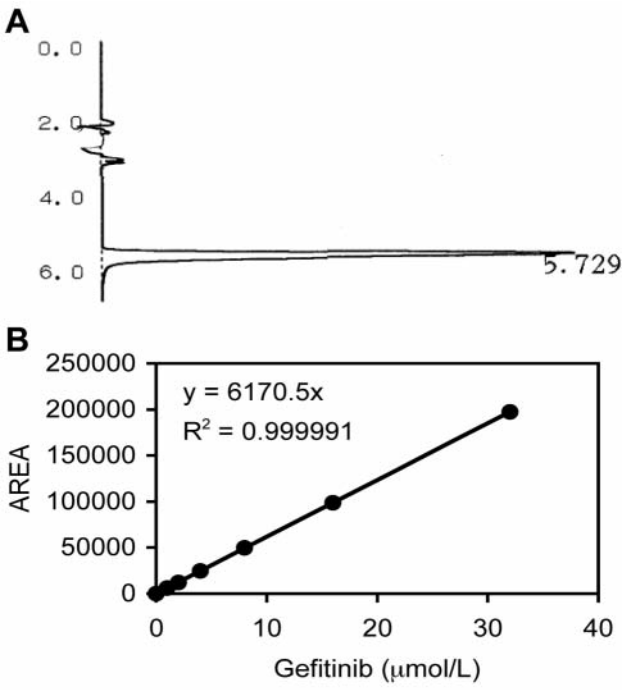

Figure 2. *Detection and quantification of gefitinib by HPLC. (A) Retention time of eluted gefitinib is shown in Y-axis, and eluted gefitinib peak is shown in X-axis in the chromatographic pattern. (B) A calibration curve plotted using the indicated concentrations of gefitinib and the corresponding areas 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.

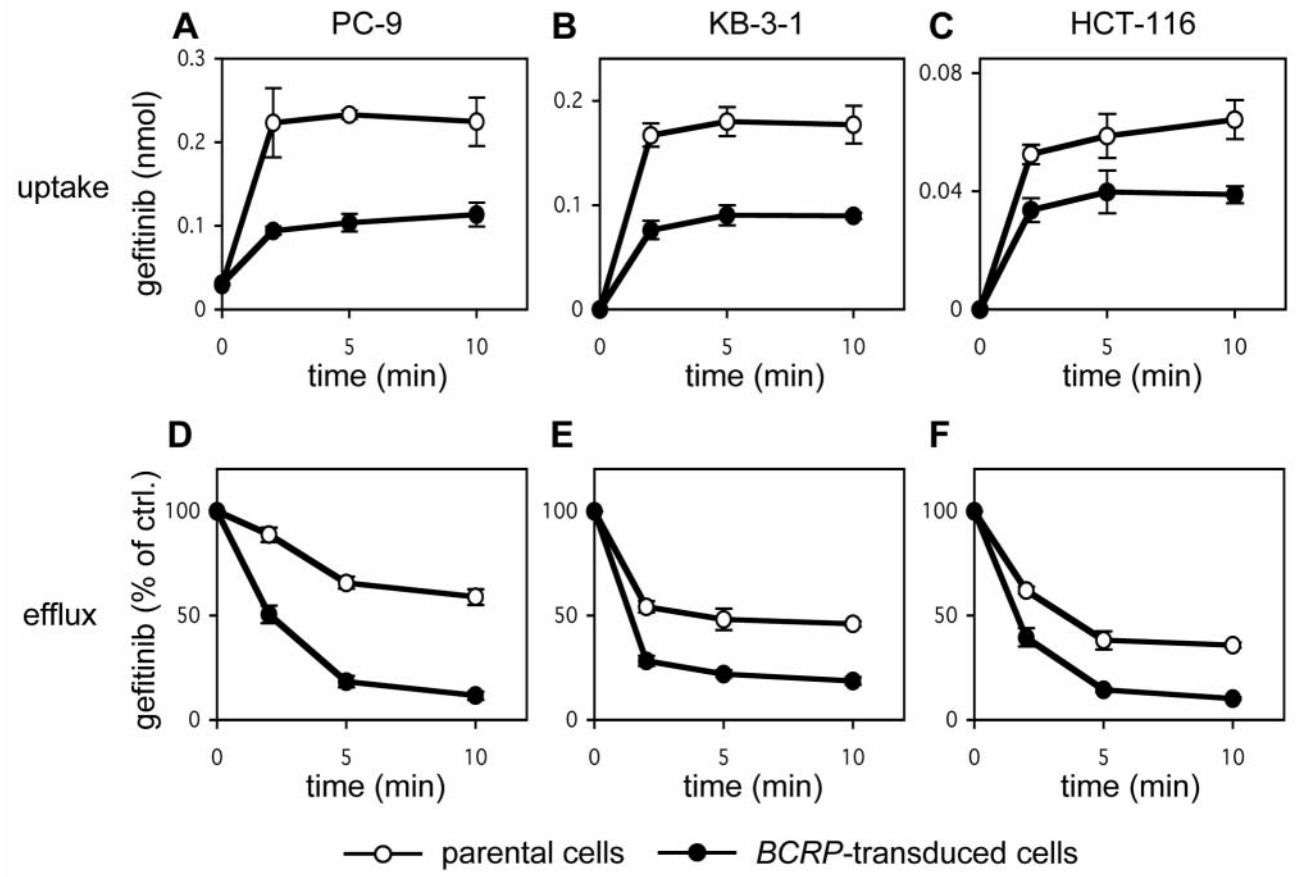

Figure 3. *Reduction of intracellular gefitinib concentration in BCRP-transduced cells. (A-C) Uptake of gefitinib. The BCRP-transduced cells (closed circles) or their respective parental cells (open circles) were treated with 0.5 μmol/L gefitinib for 2, 5 or 10 min at 37˚C. At each time point, cells 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

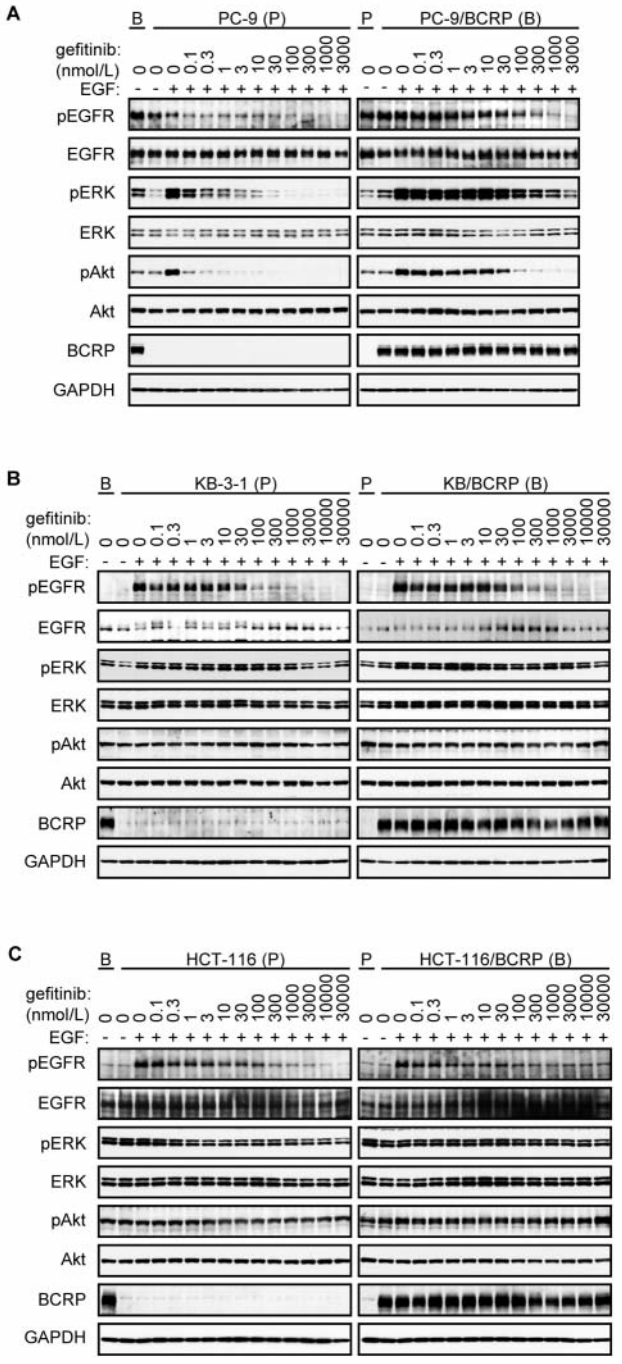

Figure 4. *Suppression of the gefitinib-mediated down-regulation of EGFR signaling in the BCRP-transduced cells. PC-9 and PC-9/BCRP cells (A), KB-3-1 and KB/BCRP cells (B), or HCT-116 and HCT-116/BCRP cells (C) were cultured in medium without serum for one hour and then treated with the indicated concentrations 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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