Gefitinib (''Iressa'', ZD1839), an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, Reverses Breast Cancer Resistance Protein/ABCG2–Mediated Drug Resistance

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Abstract

Gefitinib ("Iressa", ZD1839) is an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor, and the single agent is clinically effective in non–small cell lung cancer. Although gefitinib combined with various cytotoxic agents has been reported to enhance cytotoxicity *in vitro* and in mouse models, the mechanism remains undetermined. Here, to explore the mechanism with topoisomerase I inhibitors, we focused on the efflux pump of the breast cancer resistance protein (BCRP/ABCG2), and then examined whether gefitinib restored drug sensitivity in multidrug-resistant cancer cells overexpressing BCRP. We used PC-6 human small cell lung cancer cells and multidrug-resistant PC-6/SN2-5H cells selected with SN-38 of the active metabolite of irinotecan, and BCRP-overexpressing MCF-7/MX cells selected with mitoxantrone and BCRP cDNA transfectant MCF-7/clone 8 cells. Drug sensitivity against anticancer drugs was determined by tetrazolium dye assay, and intracellular topotecan accumulation by FACScan. The topotecan transport study was done using the plasma membrane vesicles of PC-6/SN2-5H cells. The resistant PC-6/SN2-5H cells overexpressed BCRP but not epidermal growth factor receptor mRNA. Ten micromoles of gefitinib reversed topotecan, SN-38, and mitoxantrone resistance, and increased the intracellular topotecan accumulation in the resistant cells but not in the parental cells. Furthermore, gefitinib inhibited the topotecan transport into the vesicles, and the K_i value was 1.01 $\pm$ 0.09 μmol/L in the Dixon plot analysis, indicating direct inhibition of BCRP by gefitinib. However, gefitinib was not transported into the vesicles with the high-performance liquid chromatography method. These results indicate that gefitinib reverses BCRP-mediated drug resistance by direct inhibition other than competitive inhibition as a BCRP substrate. Combination of gefitinib and topoisomerase I inhibitors could be clinically effective in cancers expressing BCRP. (Cancer Res 2005; 65(4): 1541-6)

Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with specific tyrosine kinase activity, and it serves to regulate proliferation and differentiation of epidermal cells (1, 2). In human solid tumors, overactivation and/or dysregulation of EGFR promotes tumor progression, including invasion, angiogenesis, metastasis, and treatment resistance with blocking apoptosis (3–6). Because EGFR is important in epithelial tumor biology, EGFR-targeted cancer therapy has been developed (7). Gefitinib ("Iressa", ZD1839, AstraZeneca. Co., Macclesfield, United Kingdom) is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells (8). Although gefitinib combined with various cytotoxic agents, such as taxanes, platinums, and topotecan, has been reported to enhance cytotoxicity *in vitro* and *in vivo*, the exact enhancement mechanisms still remain undetermined (9–15).

Transporter proteins belonging to the ATP-binding cassette superfamily pump out drugs using the energy from ATP hydrolysis, leading to resistance of cancer cells against multiple anticancer drugs (16). Among them, P-glycoprotein and the multidrug resistance protein family have been extensively investigated (16). The breast cancer resistance protein (BCRP/ABCG2) of an ATP-binding cassette half-transporter was isolated from atypical multidrug-resistant MCF-7 human breast cancer cells selected with doxorubicin and verapamil (17). BCRP-overexpressing cells show cross-resistance to mitoxantrone and DNA topoisomerase I inhibitors of topotecan and SN-38, suggesting strongly that BCRP plays an important role in acquiring resistance against these drugs (16, 18, 19). We have already shown that BCRP efficiently transports SN-38 with a high affinity *in vitro* (20). Interestingly, CI1033 of the HER family tyrosine kinase inhibitors was reported to enhance the cytotoxicity of topotecan and SN-38 through inhibition of BCRP-mediated drug efflux in cancer cells (21). The HER family of receptors includes EGFR, HER-2, HER-3, and HER-4. Therefore, we hypothesized that gefitinib would reverse drug resistance in cancer cells overexpressing BCRP. Here, using plasma membrane vesicles, we report that gefitinib reverses BCRP-mediated resistance against topotecan, SN-38, and mitoxantrone through direct inhibition of BCRP in multidrug-resistant human small cell lung cancer and breast cancer cells.

Materials and Methods

Cell Lines and Reagents. PC-6 and PC-6/SN2-5 human small cell lung cancer cell lines were kindly provided by Dr. Akiko Tohgo (Daiichi Pharmaceutical, Co., Tokyo, Japan). SN-38-resistant PC-6/SN2-5 cells were selected from the parental PC-6 cells by continuous exposure to 4.8 nmol/L

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Table 1. Reversal of drug resistance in PC-6 and MCF-7 cell panels by gefitinib

Drugs	Cells	IC_{50} nmol/L (RR)*	IC_{50} nmol/L (RR)* + gefitinib†
Topotecan	PC-6	7.27 ± 0.93	7.62 ± 0.27
	PC-6/SN2-5H	369 ± 37.2 (50.8)	11.0 ± 0.92 (1.44)
	MCF-7	120 ± 9.11	81.2 ± 12.6
	MCF-7/MX	4,143 ± 161 (34.5)	716 ± 118 (8.82)
	MCF-7/clone 8	5,806 ± 907 (48.4)	309 ± 5.88 (3.81)
SN-38	PC-6	4.97 ± 0.44	4.10 ± 0.23
	PC-6/SN2-5H	153 ± 2.20 (30.8)	5.03 ± 0.26 (1.23)
	MCF-7	89.4 ± 15.4	70.3 ± 3.35
	MCF-7/MX	1,139 ± 79.3 (12.7)	116 ± 18.2 (1.65)
	MCF-7/clone 8	845 ± 63.9 (9.45)	59.7 ± 1.05 (0.85)
Mitoxantrone	PC-6	27.0 ± 1.82	20.2 ± 1.92
	PC-6/SN2-5H	903 ± 5.94 (33.4)	24.2 ± 0.57 (1.20)
	MCF-7	10.5 ± 1.98	10.1 ± 0.25
	MCF-7/MX	1,210 ± 289 (115)	178 ± 16.3 (17.6)
	MCF-7/clone 8	9,870 ± 346 (940)	150 ± 31.4 (14.9)
Doxorubicin	PC-6	23.0 ± 1.92	19.2 ± 0.66
	PC-6/SN2-5H	30.3 ± 0.88 (1.32)	25.8 ± 2.25 (1.34)
	MCF-7	86.6 ± 11.1	79.0 ± 1.65
	MCF-7/MX	273 ± 9.29 (3.15)	74.0 ± 12.6 (0.94)
	MCF-7/clone 8	2,638 ± 17.1 (30.5)	103 ± 9.92 (1.30)
Vincristine	PC-6	1.01 ± 0.02	1.12 ± 0.03
	PC-6/SN2-5H	1.20 ± 0.05 (1.19)	1.08 ± 0.08 (0.96)
	MCF-7	1.12 ± 0.10	1.08 ± 0.13
	MCF-7/MX	1.99 ± 0.12 (1.78)	1.82 ± 0.12 (1.69)
	MCF-7/clone 8	4.24 ± 0.66 (3.79)	4.24 ± 0.40 (3.93)
Etoposide	PC-6	423 ± 21.2	495 ± 8.95
	PC-6/SN2-5H	435 ± 7.36 (1.03)	485 ± 36.9 (0.98)
	MCF-7	1,254 ± 249	1,201 ± 318
	MCF-7/MX	4,152 ± 442 (3.31)	4,153 ± 82.9 (3.46)
	MCF-7/clone 8	10,425 ± 905 (8.31)	10,161 ± 609 (8.46)

NOTE: Data represent mean ± SD.
*RR, resistance ratio = [(IC_{50} in resistant cells) / (IC_{50} in respective parental cells)].
†Gefitinib (10 and 2 μmol/L) were added in PC-6 and MCF-7 cell panels, respectively.

SN-38 (22). Highly resistant PC-6/SN2-5H cells were further selected from PC-6/SN2-5 cells by continuous exposure to 25 nmol/L SN-38 in our laboratory. These cells have no mutation at codon 482 of BCRP (23), which determines the specificity of BCRP substrates (16, 18). MCF-7 human breast cancer cells and mitoxantrone-resistant MCF-7/MX cells were provided by Dr. Masayuki Nakagawa (Kagoshima University, Kagoshima, Japan; ref. 24). MCF-7/MX cells have no mutation at codon 482 of BCRP (25). MCF-7/BCRP clone 8 cells, transfectants of the vector pcDNA3-BCRP construct, were kindly provided by Dr. Douglas D. Ross (University of Maryland, Baltimore, MD; ref. 17). The transfectants have a mutant form of BCRP (R482T; ref. 25). These cells were cultured in RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Inc., Grand Island, NY) and 50 mg/L kanamycin sulfate (Meiji Seika, Co., Tokyo) in a humidified incubator containing 5% CO_2 at 37°C.

Gefitinib was from AstraZeneca, topotecan from SmithKlineBeecham, Co. (Tokyo), SN-38 from Yakult Honsha, Co. (Tokyo), mitoxantrone hydrochloride from Takeda Yakuhin Kogyo, Co. (Osaka, Japan), and doxorubicin from Kyowa Hakko Kogyo, Co. (Tokyo). Vincristine, etoposide, and novobiocin sodium salt were purchased from Sigma Chemical, Co. (St. Louis, MO).

RNA Extraction and Reverse Transcription-PCR Analysis. Total RNA extraction from culture cells and reverse transcription-PCR were done as previously described (19). The first-strand cDNA was 2-fold diluted from 2.5- to 320-fold in reverse transcriptase buffer. Target sequences for the EGFR and glyceraldehyde-3-phosphate dehydrogenase genes were separately amplified in 5-fold dilutions for 28 and 26 cycles, respectively. The amplifying conditions were 94°C for 90 seconds, 63°C for 60 seconds, and 72°C for 60 seconds in a 9600 Thermocycler (Perkin-Elmer Cetus, Norwalk, CT). Preliminary runs were done to determine the maximum number of cycles that could be carried out with cDNA derived from A431 human lung adenocarcinoma cells of positive control cells for EGFR. The set of primers for the EGFR gene was as follows: forward 5′-CTCACGCAGTTGGGCACTTT-3′ (accession no. AF125253, nucleotide: nt 357-376) and reverse 5′-TCATGGGCAGCTCCTTCAGT-3′ (nt 638-657), for a 261-bp product. The set of primers for the glyceraldehyde-3-phosphate dehydrogenase gene was described previously (19). Ten microliters of PCR products were separated by 2% agarose gel electrophoresis and were then visualized by ethidium bromide staining.

Anticancer Drug Sensitivity Assay. The sensitivity of the cells to each anticancer drug was determined using the tetrazolium dye assay as reported previously (19). Briefly, cells (7,500 cells per well) were seeded in 96-well plates in medium containing 10% FCS. Cells were incubated for 96 hours, and then WST-1 reagent (Boehringer Mannheim GmbH, Mannheim, Germany) was added, and they were subsequently incubated for 4 hours. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The drug concentration producing 50% cell growth inhibition (IC_{50}) was calculated for each drug from linear regression analysis of the linear portion of the growth curves. Each experiment was done at least in triplicate.

Flow Cytometric Detection of Intracellular Topotecan. Cells (2×10^6) were exposed to 30 μmol/L topotecan for 15 minutes at 37°C with or without 0.05 to 10 μmol/L gefitinib or 300 μmol/L novobiocin, and then washed twice in ice-cold saline, as described previously (26). Novobiocin of the relatively specific BCRP inhibitor was used as a positive control (26). Fluorescence of topotecan was analyzed with a fluorescence-activated cell sorting scan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15-mW argon laser. The topotecan-derived fluorescence of 30,000 events was measured through a 488 nm band-pass filter at an excitation wavelength of 585 nm. Topotecan accumulation after incubation for 15 minutes was expressed in fluorescence units. In all fluorescence assays, parallel samples were stored on ice to control for nonspecific binding of the drugs to plasma membranes, and cells without topotecan were included as a control for autofluorescence.

Topotecan Transport Into Plasma Membrane Vesicles. The transport buffer [sucrose, Hepes/Tris (pH 7.4), $CaCl_2$, and $MgCl_2$ at 250, 10, 0.2, and 10 mmol, respectively] contained various concentrations of topotecan, 5 mmol ATP and the ATP-regenerating system (10 mmol creatine phosphate and 100 μg/mL creatine kinase). The transport reaction was initiated by adding 10 μL of the vesicle suspension (50 μg protein) to 70 μL of the transport buffer, preincubated for 2 minutes at 37°C. The substrate and ATP reactions were given in addition to 1 mL of ice-cold stop solution [sucrose, NaCl, Hepes/Tris (pH 7.4), and $CaCl_2$ at 250, 100, 10, and 0.2 mmol, respectively]. The stopped reaction mixtures were centrifuged at 16,000 × *g* for 30 minutes at 4°C, and then washed thrice with 1.5 mL of stop solution. The mixtures were then dissolved in 110 L of 0.05 mol/L NaOH, sonicated for 3 minutes and centrifuged at 16,000 × *g* for 2 minutes at 4°C. Next, the supernatants were subjected to high-performance liquid chromatography analysis to determine the uptake of topotecan into the plasma membrane vesicles. In the control experiments, uptake reaction was done at 0°C. The rates of net ATP-dependent transport were calculated by subtracting values obtained at 0°C, as a blank, from those at 37°C. For inhibition studies, topotecan transport was measured at 30 mol/L and 14 escalating concentrations of gefitinib (0-30 mol/L). For determination of kinetic constants, transport rates were measured at four independent concentrations of topotecan (5-30 mol/L). K_m and V_{max} values were determined as concentrations of topotecan at half-maximal velocity of transport under these conditions. For determination of the inhibition constant (K_i) for competitive inhibition kinetic constants in the Dixon method (27), transport rates were measured at each independent concentration of topotecan.

High-performance Liquid Chromatography Analysis of Gefitinib. The intracellular accumulation of gefitinib was measured by the modified high-performance liquid chromatography method reported by Warner et al. (28). The high-performance liquid chromatography system consisted of a Jusco PU-1580 pump, a Jusco 870-UV UV/VIS detector (Jusco Inc., Tokyo) and a Shimazu C-R4A integrator (Shimazu, Kyoto, Japan). Isocratic elutions were done using a Mightysil RP-18 (L) GP column (5 μmol/L, 4.6 × 150 mm; Kanto Chemical, Co., Tokyo) with a guard column (5 μmol/L, 4.6 × 5 mm). The excitation setting was 253 nm. The mobile phase, consisting of 0.1 mol/L triethylamine (pH 8.0)/acetonitrile/tetrahydrofuran (40:60:2, v/v/v), was used for the separation of gefitinib. The flow rate was 1.0 mL/min, and all separations were carried out at room temperature.

Statistical Analysis. Data in the drug sensitivity assay were presented as mean ± SD. Differences between groups were tested by the Student's *t* test. All *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

EGFR and BCRP Expression in Cell Lines. EGFR mRNA expression was not detected in PC-6 cells and PC-6/SN2-5H cells (data not shown). On the other hand, BCRP mRNA and protein were overexpressed in PC-6/SN2-5H cells but were not detectable in PC-6 cells, as reported previously (19, 20). Other transporter genes of MDR1, MRP2, and MRP3 mRNA were not detectable in PC-6 and PC-6/SN2-5H cells in reverse transcription-PCR analyses (19).

Reversal of Drug Resistance by Gefitinib. The IC_{50} values of each drug in the PC-6 and MCF-7 cell panels are shown in Table 1. Compared with the respective parental cells, PC-6/SN2-5H, MCF-7/MX, and MCF-7/BCRP clone 8 cells were resistant to topotecan, SN-38, and mitoxantrone. MCF-7/MX and MCF-7/BCRP clone 8 cells showed weak and moderate cross-resistance to doxorubicin, respectively, as reported previously (17, 24). In the preliminary experiments, the IC_{10} (the drug concentration producing 10% growth inhibition) of gefitinib was 11.8 ± 0.41, 10.4 ± 0.84, 2.45 ± 0.24, 2.45 ± 0.27, and 2.98 ± 0.94 μmol/L in PC-6, PC-6/SN2-5H, MCF-7, MCF-7/MX, and MCF-7/BCRP clone 8 cells, respectively. Based on these data, 10 and 2 μmol/L gefitinib, of a noncytotoxic maximum dose, were used for PC-6 and MCF-7 cell panels, respectively, in the present experiments (Table 1). In PC-6/SN2-5H cells, gefitinib completely reversed topotecan resistance from 50.8- to 1.44-fold ($P < 0.0001$). Furthermore, the degree of SN-38 and mitoxantrone resistance in the subline were decreased from 30.8- to 1.23-fold and from 33.4- to1.20-fold, respectively ($P < 0.0001$). In contrast, no effects were observed on sensitivity to doxorubicin, vincristine, and etoposide. On the other hand, in MCF-7/MX cells, gefitinib reversed topotecan, SN-38, mitoxantrone, and doxorubicin resistance from 34.5- to 8.82-fold ($P < 0.0001$), from 12.7- to 1.65-fold ($P < 0.0001$), from 115- to 17.6-fold

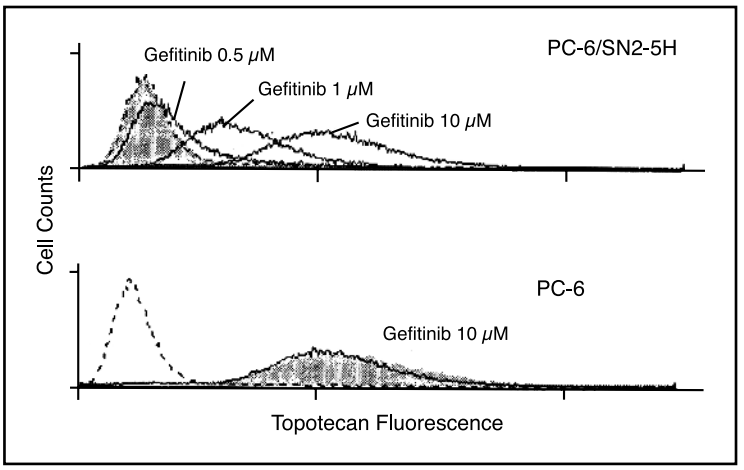

Figure 1. Intracellular topotecan accumulation in PC-6/SN2-5H and PC-6 cells in the presence (*solid line*) or absence (*shadow*) of gefitinib. *Dotted lines,* fluorescence without exposure to topotecan.

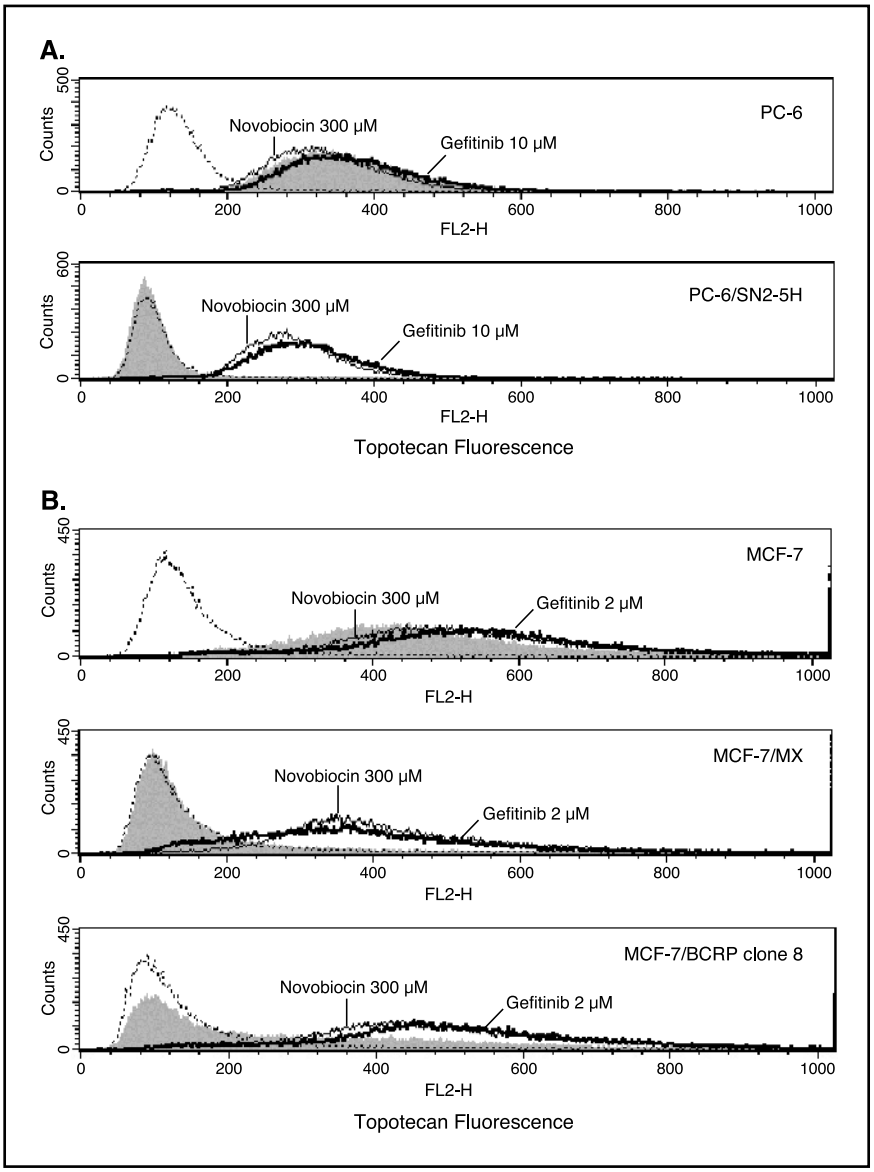

Figure 2. *A,* intracellular topotecan accumulation in PC-6 cells in the presence (*bold line*) or absence (*shadow*) of gefitinib. *Dotted lines,* fluorescence without exposure to topotecan; *thin lines,* topotecan fluorescence in the presence of novobiocin. *B,* intracellular topotecan accumulation in MCF-7 cells in the presence (*bold line*) or absence (*shadow*) of gefitinib. *Dotted lines,* fluorescence without exposure to topotecan; *thin lines,* topotecan fluorescence in the presence of novobiocin.

($P = 0.0035$), and from 3.15- to 0.94-fold ($P < 0.0001$), respectively. In MCF-7/BCRP clone 8 cells, gefitinib reversed these resistances from 48.4- to 3.81-fold ($P = 0.0005$), from 9.45- to 0.85-fold ($P < 0.0001$), from 940- to 14.9-fold ($P < 0.0001$), and from 30.5- to 1.30-fold ($P < 0.0001$), respectively.

Effects of Gefitinib on Intracellular Topotecan Accumulation. Intracellular topotecan-derived fluorescence was detectable in cells treated with topotecan in dose- and time-dependent manners. The intracellular topotecan accumulation was evaluated after a 15-minute incubation with 30 μmol/L topotecan (26). The topotecan accumulation in the absence of gefitinib was remarkably decreased in PC-6/SN2-5H cells, as compared with that in PC-6 cells. Conversely, 0.5 to 10 μmol/L gefitinib recovered the topotecan accumulation of PC-6/SN2-5H cells in a dose-dependent manner, and had no effects in PC-6 cells (Fig. 1). Next, we examined the effects of novobiocin and gefitinib on the intracellular topotecan accumulation in all cell lines. These agents increased the accumulation in the resistant cells and the transfectants but not in the parental cells (Fig. 2).

Inhibition of Topotecan Transport into Plasma Membrane Vesicles by Gefitinib, and ATP-Dependent Gefitinib Transport into Plasma Membrane Vesicles. We showed that topotecan was transported into the cellular membrane vesicles derived from PC-6/SN2-5H and that topotecan was a substrate for BCRP (20). These vesicles consisted of an inside-out cellular membrane, thus decreasing the intravesicle accumulation of topotecan, inhibiting the topotecan transportation out of PC-6/SN2-5H cells by BCRP. In our examination, the transport of 30 μmol/L topotecan into the PC-6/SN2-5H vesicles was measured in the presence of gefitinib from 0 to 30 μmol/L. We showed that topotecan transport was inhibited by 75% with 6.5 μmol/L of gefitinib and by 25% with 0.3 μmol/L (Fig. 3*A*). Based on these data, the transport rates at different concentrations of topotecan were measured from 0 to 6.5 mol/L gefitinib. Gefitinib (0-6.5 mol/L) inhibited topotecan transport with a K_m value of 48.2 ± 4.1 mol/L and a V_{max} value of 1,346 ± 72 pmol/mg protein, and the K_i value was calculated as 1.01 ± 0.09 mol/L in the Dixon plot analysis (Fig. 3*B* and *C*; ref. 27). These findings indicated that gefitinib interacted directly with BCRP.

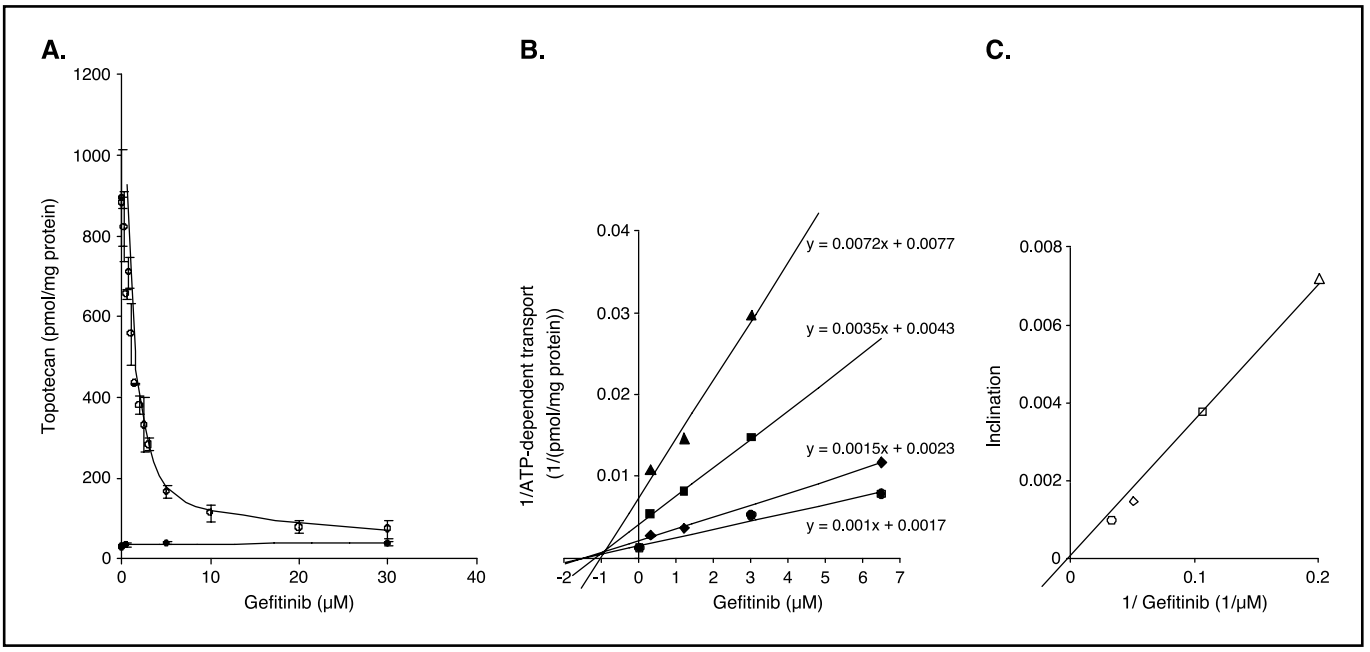

Figure 3. *A,* effects of gefitinib on topotecan transport. The amounts of ATP-dependent topotecan transport were determined in the membrane vesicles of PC-6/SN2-5H cells at 14 different gefitinib concentrations. The plasma membrane vesicles were incubated with 30 μmol/L topotecan in the presence (○) or absence (●) of 5 mmol ATP, and the topotecan concentration was determined as described in Materials and Methods. The amounts of topotecan transport were calculated by subtracting the values at 0°C, as a blank, from those obtained at 37°C. Data represent mean ± SD in at least three different experiments. *B,* the effects of gefitinib on the kinetics of topotecan transport in the membrane vesicles of PC-6/SN2-5H cells. The amounts of topotecan transport were analyzed in the presence of topotecan at 5 (▲), 10 (■), 20 (◆), and 30 (●) μmol/L. *Points,* mean (SD) from at least three different experiments. *C,* the inclinations of the topotecan transport graphs are plotted in *B*. Each inclination was obtained in the presence of topotecan at 5 (△), 10 (□), 20 (◇), and 30 (○) μmol/L, and the inhibition constant (K_i) was calculated by the Dixon analysis method.

ATP-dependent gefitinib transport into the membrane vesicles of P-C6/SN2-5H cells was examined over a 10-minute period (Fig. 4). Gefitinib was not transported into the vesicles with or without ATP, indicating that gefitinib was not transported by BCRP.

Discussion

The present study showed that gefitinib restored the drug sensitivity of multidrug-resistant PC-6/SN2-5H and MCF-7/MX cells overexpressing BCRP, and of BCRP cDNA transfectant MCF-7/BCRP clone 8 cells, with the increased intracellular accumulation of topotecan. No effects of gefitinib were observed in the parental cells. Moreover, using the plasma membrane vesicles of the cells, we found that gefitinib inhibited the topotecan transport into the vesicles in a dose-dependent manner (Fig. 3*A*), and that gefitinib itself was not transported into the vesicles (Fig. 4). The kinetic parameters in the vesicle study indicated that gefitinib directly inhibited BCRP function, but it was not a BCRP substrate. Thus, gefitinib is probably a BCRP inhibitor, resulting in its overcoming resistance to topoisomerase I inhibitors, although we could not exclude the effects of gefitinib on the EGFR signaling pathway completely.

Gefitinib at 0.5 to 10 μmol/L dose-dependently increased the topotecan accumulation within PC-6/SN2-5H cells (Fig. 1), whereas 2 and 10 μmol/L gefitinib reversed drug resistance in three BCRP-expressing cell lines (Table 1). Because the 0.5 to 10 μmol/L gefitinib used here was in clinically achievable plasma concentrations (29), the reversal effects as shown in the present study would be observed *in vivo*. Ciardiello et al. (13) reported that gefitinib enhanced the cytotoxicity of paclitaxel and docetaxel in multidrug-resistant breast cancer cells overexpressing P-glycoprotein and EGFR *in vitro*. In a tumor-bearing mouse model, gefitinib has shown a synergistic effect in combination with various cytotoxic agents, resulting in prolonged survival, and the phenomenon was not associated with the EGFR expression status in the cancer cells (9–14). However, the enhancement mechanisms have not been explored, and interactions between gefitinib and the expression or function of ATP-binding cassette transporters were not analyzed in these prior reports. On the other hand, as shown in our study, the enhancement effect of gefitinib with topoisomerase I inhibitors is probably due to the direct inhibition of BCRP-mediated drug efflux.

To date, several BCRP inhibitors with different structures have been reported, but the exact inhibition mechanisms have not

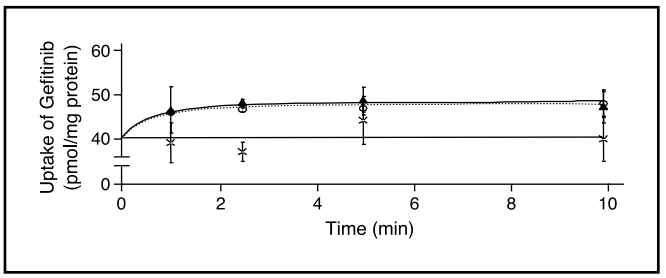

Figure 4. Uptake of 30 μmol/L gefitinib into the vesicles of PC-6/SN2-5H cells in the presence of 30 μmol/L topotecan. The plasma membrane vesicles were incubated with 30 μmol/L topotecan in the presence (○) or absence (▲) of 5 mmol ATP; (×) control level of 30 μmol/L gefitinib in the absence of ATP at 0°C. Gefitinib concentration was determined as described in Materials and Methods. The amounts of gefitinib transport were calculated by subtracting the values at 0°C, as a blank, from those obtained at 37°C. *Points,* mean from at least three different experiments; *bars,* SD.

been sufficiently investigated and explored (18). Very recently, we also showed that novobiocin of a coumermycin antibiotic reversed BCRP-mediated resistance using membrane vesicles (26). Fumitremorgin C of a specific BCRP inhibitor, a mycotoxin from *Aspergillus fumigatus*, and its structural analogues, are likely to reverse BCRP-mediated resistance through inhibition of BCRP-ATPase activity (30–32). The acridone carboxamide derivative GF120918 of an antagonistic P-glycoprotein inhibitor also inhibits BCRP-mediated drug efflux and resistance (33, 34). Thus, probable direct interactions between BCRP and gefitinib, as shown here, are of great interest, and experiments are extensively ongoing for the exploration of the reversal mechanisms of gefitinib.

When combined with gefitinib and BCRP substrate drugs in a clinical setting, gefitinib may affect the plasma concentrations of these drugs, leading to adverse effects because gefitinib may inhibit BCRP-mediated drug transport in normal cells. In fact, coadministration of the BCRP inhibitor GF120918 has significantly increased the plasma levels of topotecan in mice and humans (34, 35), especially when topotecan was given p.o. Thus, BCRP is probably involved in the regulation of drug uptake and excretion from the gastrointestinal tract because BCRP is expressed in the epithelium of the small intestine and colon (36). In addition, the effects of gefitinib on other ATP-binding cassette transporters would be of great interest. In our preliminary experiments, no modulating effects on MRP1/ABCC1 were observed, but there were moderate effects on P-glycoprotein (detailed data not shown).

In conclusion, the EGFR tyrosine kinase inhibitor gefitinib effectively reversed drug resistance through inhibition of drug efflux in three multidrug-resistant cancer cell lines overexpressing BCRP. In addition, gefitinib inhibited BCRP-mediated topotecan transport in the plasma membrane vesicles of these cells by mechanisms other than competitive inhibition. The cellular pharmacologic effect of combining gefitinib with topoisomerase I inhibitors in BCRP-overexpressing cells requires further clarification.

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