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ORIGINAL ARTICLE

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BCRP/ABCG2 levels account for the resistance to topoisomerase I inhibitors and reversal effects by gefitinib in non-small cell lung cancer

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Abstract *Purpose*: Breast cancer resistance protein (BCRP) confers resistance against topoisomerase I inhibitors in cancer cells. Very recently, we reported that gefitinib reverses BCRP-mediated drug resistance by direct inhibition. However, it remains undetermined how much BCRP contributes to the resistance to topoisomerase I inhibitors in non-small cell lung cancer (NSCLC). The present study was designed to examine whether BCRP levels in NSCLC cells are correlated with the resistance to topoisomerase I inhibitors and the reversal effect by gefitinib. *Methods*: BCRP levels and its function were evaluated by Western blotting and flowcytometry, respectively. Gefitinib-insensitive NSCLC cells expressed various levels of BCRP, which were closely correlated not only with the IC_{50} values of SN-38 ($r = 0.874$, $P < 0.05$) and those of topotecan ($r = 0.968$, $P < 0.001$), but also with the reversal effects of 1 μM gefitinib on SN-38 resistance ($r = 0.956$, $P < 0.001$) and topotecan resistance ($r = 0.977$, $P = 0.0001$). *Results*: BCRP levels accounted for between 80 and 90% of the variation in the resistance to topoisomerase I inhibitors and the reversal effects by gefitinib. Also, gefitinib increased intracellular topotecan accumulation in proportion to the BCRP levels. *Conclusions*: These findings suggest that BCRP is the most important molecule responsible for topoisomerase I inhibitor resistance, and that the development of BCRP inhibitors is an effective approach for overcoming this resistance. In addition, the examination of BCRP levels in NSCLC tissues may identify an optimal patient population for treatment with topoisomerase I inhibitors alone or in combination with BCRP inhibitors.

Keywords Breast cancer resistance protein · Topoisomerase I inhibitors · Gefitinib · Non-small cell lung cancer · Multidrug resistance · Molecular-targeted therapy

Abbreviations ABC: ATP-binding cassette · NSCLC: Non-small cell lung cancer · BCRP: Breast cancer resistance protein · EGFR: Epidermal growth factor receptor · SN-38: 7-Ethyl-10-hydroxycamptothecin (the active metabolite of irinotecan) · MRP: Multidrug resistance protein

Introduction

The overexpression of the ATP-binding cassette (ABC) transporter proteins protects cells from cytotoxic drugs due to drug efflux, and is a major mechanism responsible for multidrug-resistance [3]. Breast cancer resistance protein (BCRP) of the ABC half-transporter was previously isolated from adriamycin-resistant MCF-7 breast cancer cells [6], and its overexpression was found to promote resistance to topoisomerase I inhibitors including SN-38, an active metabolite of irinotecan, in vitro [13, 18, 20]. The development of BCRP inhibitors is an important strategy in overcoming resistance to topoisomerase I inhibitors on chemotherapy. Recently, we as well as other groups showed that gefitinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR), inhibits BCRP and helps to overcome the

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resistance to topoisomerase I inhibitors in vitro [19, 30] Thus, gefitinib is probably a promising BCRP inhibitor.

Irinotecan, a topoisomerase I inhibitor, is clinically effective against non-small cell lung cancer (NSCLC) [17], but this response is not observed in all patients with NSCLC. Previously, we found that *BCRP* mRNA is expressed in 22% of resected chemotherapy-naive NSCLC tissues [14]. BCRP expression may be an important factor involved in the resistance to topoisomerase I inhibitors in NSCLC. However, it remains undetermined how much BCRP contributes to the resistance to topoisomerase I inhibitors in NSCLC. The present study was designed to examine whether BCRP expression levels are correlated with the resistance to topoisomerase I inhibitors and help to overcome the resistance induced by gefitinib in NSCLC cells. Here, we report that BCRP levels account for between 80 and 90% of the variation of the resistance to topoisomerase I inhibitors and reversal effects by gefitinib in NSCLC cell panels.

Materials and methods

Cell lines and chemicals

Drug-unselected NSCLC cells of the cell lines H23, H358, H441, H460, H522, and H1299 were obtained from the American Type Culture Collection (Rockville, MD, USA), and PC-6 cells without BCRP expression of a small cell lung cancer (SCLC) cell line were kindly provided by Dr. Akiko Tohgo of Daiichi Pharmaceutical Co. (Tokyo, Japan) [12]. BCRP-overexpressing PC-6/SN2–5H cells were selected by continuous exposure to SN-38 as previously reported [13], and showed cross-resistance to topotecan and mitoxantrone [14]. All cells were cultured at 37°C in RPMI 1640 medium (Gibco BRL, Gland Island, NY) supplemented with 10% FCS and L-glutamine in a humidified atmosphere of 5% CO_2. Gefitinib was kindly provided by AstraZeneca Co. (Macclesfield, UK), and SN-38 was obtained from Yakult Honsha, Co. (Tokyo). Topotecan and vanadate were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA) and Sigma Chemical Co. (Tokyo), respectively.

Drug sensitivity assay

The sensitivities of the cells to anticancer drugs were determined using a tetrazolium dye assay as previously described [27]. The anti-proliferative effects of the drugs were evaluated by determining the 50% cell growth inhibition values (IC_{50}), and the reversal effects of drug resistance caused by gefitinib were calculated as (IC_{50} in the absence of gefitinib)/(IC_{50} in the presence of gefitinib).

Reverse transcription-PCR of *EGFR* mRNA

Total RNA from cultured cells was extracted using the guanidium–isothiocyanate method (ISOGEN, Nippongene, Tokyo) and was reverse-transcribed using a random hexamer according to the manufacturer's protocol (THERMOSCRIPT™ RT-PCR System, Invitrogen Corp., Carlsbad, CA, USA). The PCR primer sets were as follows: *EGFR*, forward 5′-CTCACGCAGTTGGGCACTTT-3′ and reverse 5′-TCATGGGCAGCTCCTTCAGT-3′ for a 261-bp product; and *GAPDH*, forward 5′-GTAAGGTCGGAGTCAACGGATTT-3′ and reverse 5′-CATGTGGGCCATGAGGTCCACCAC-3′ for a 983-bp product. The reaction conditions were 30 cycles at 94°C for 60 s, 60°C for 30 s, and 72°C for 30 s for *EGFR*. The PCR products were separated by 1% agarose gel electrophoresis and then visualized by ethidium bromide staining as previously reported [27].

Western blot analysis of BCRP

Whole cell proteins were separated on 7.5% SDS-polyacrylamide gel, and then electro-transferred to PVDF membranes. Using BXP-21 (Kamiya Co., Seattle, WA, USA) of anti-human BCRP antibody (1:500) and the enhanced Chemiluminescence detection system (Amersham Co., Bucks, United Kingdom), immunoblotting was performed as previously described [13]. The density of each band was measured using a densitometer, and the relative BCRP expression levels were calculated as (density of the band for a sample)/(density of the band for PC-6/SN2-5H as a positive control).

Measurement of BCRP-ATPase activity

Membrane BCRP-ATPase activity was measured by colorimetric assay as previously described with several modifications [24, 25]. Human BCRP-expressing membrane was refined from PC-6/SN2-5H cells. The ATPase activity was estimated by measuring the difference in inorganic phosphate liberation between the absence and presence of 500 μM vanadate. Membrane protein suspensions of 2 μg were incubated at 37°C in medium containing 50 mM Tris–Mes (pH 6.8), 50 mM KCl, 2 mM dithiothreitol, 2 mM EGTA and 5 mM Na-azide. The ATPase reaction was started by the addition of Mg-ATP, and stopped by the addition of 5% trichloroacetic acid. The liberated inorganic phosphate was measured at a wavelength of 620 nm in a Biotrak (Amersham Bioscience).

Flowcytometric detection of topotecan-efflux function

Flowcytometric analysis was performed as previously described [14, 19, 27]. Cultured cells at 2×10^6/ml were exposed to 30 μM topotecan for 15 min at 37°C with or without 10 μM gefitinib, and were then washed twice in ice-cold PBS. The fluorescence of topotecan was then analyzed with a FACscan flowcytometer (Becton Dickinson, Mountain View, CA, USA). Topotecan-derived

fluorescence of 10,000 events was measured through a 488 nm bandpass filter at an excitation wavelength of 585 nm, and the accumulation after incubation for 15 min was expressed in fluorescence units. In all fluorescence assays, parallel samples were stored on ice to control for non-specific binding of the drugs to plasma membranes, and cells without topotecan were included as a control for autofluorescence.

Statistical analysis

The data were presented as means ± standard deviation. Dose–response relationship was evaluated by Spearman correlation analysis. Correlations between BCRP levels and drug sensitivity were analyzed by linear regression, and a coefficient of determination (r^2) was used to assess the contribution of BCRP levels to the variability in drug sensitivity. A two-tailed $P < 0.05$ was considered to indicate significance. The data were analyzed using StatView software Version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Expression of BCRP in NSCLC cells

Various protein levels of BCRP were expressed in H460, H441, H358, H23 cells, but not in H1299 and H522 cells (Fig. 1). Another transporter of *MDR1* mRNA was not detected in NSCLC cells used in this study (data not shown), as previously reported [14]. In contrast, NSCLC cells expressed *EGFR* mRNA (data not shown); however, IC_{50} values of gefitinib were more than 18 μM, and the NSCLC cells used here were gefitinib-insensitive.

Correlations between BCRP levels and resistance to topoisomerase I inhibitors

Non-small cell lung cancer cells showed various IC_{50} values for SN-38, topotecan and gefitinib (Table 1). The BCRP expression levels in NSCLC cells were closely correlated with the IC_{50} values of SN-38 ($r = 0.874$, $r^2 = 0.764$, $P < 0.05$) and those of topotecan ($r = 0.968$, $r^2 = 0.937$, $P < 0.001$) as shown in Fig. 2. Accordingly, these levels accounted for 76.4 and 93.7% of the variation in the resistance to SN-38 and topotecan, respectively.

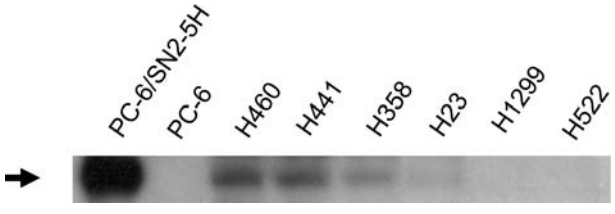

Fig. 1 Western blot analysis of BCRP in SCLC cell lines (PC-6 and PC-6/SN2-5H) and NSCLC cell lines (H460, H441, H358, H23, H1299, and H522). BCRP migrated to approximately 70 kDa position (*arrow*). The relative BCRP expression levels of PC-6/SN2-5H, PC-6, H460, H441, H358, H23, H1299, and H522 cells were 1, 0, 0.50, 0.28, 0.14, 0.05, 0, and 0, respectively

Table 1 Sensitivity to topoisomerase I inhibitors and gefitinib in NSCLC cells

	TPT (nM)	SN-38 (nM)	Gefitinib (μM)
H460	598 ± 25.2	908 ± 14.2	25.3 ± 3.41
H441	189 ± 5.14	56.8 ± 4.62	18.8 ± 0.48
H358	106 ± 1.73	4.84 ± 0.32	33.3 ± 0.72
H23	21.9 ± 0.59	3.89 ± 0.17	31.0 ± 1.10
H1299	9.58 ± 0.60	2.69 ± 0.46	23.5 ± 0.88
H522	8.87 ± 0.43	3.66 ± 0.49	20.5 ± 0.32

The data represent the means ± standard deviation of IC_{50} values

Correlations between BCRP levels and the reversal effects of gefitinib

Gefitinib is an inhibitor of BCRP, and non-toxic doses, including the clinically achievable plasma concentration of 1 μM, overcame the drug resistance to SN-38 and topotecan in a dose-dependent manner (Fig. 3). BCRP levels were closely correlated with the reversal effects of gefitinib on SN-38 resistance (1 μM: $r = 0.956$, $r^2 = 0.914$, $P < 0.001$; 10 μM: $r = 0.959$, $r^2 = 0.92$, $P < 0.001$; Fig. 4) and topotecan resistance (1 μM: $r = 0.977$, $r^2 = 0.955$, $P = 0.0001$; 10 μM: $r = 0.977$, $r^2 = 0.955$, $P = 0.0001$; Fig. 5). As well as predicting the resistance to topoisomerase I inhibitors, the BCRP levels accounted for 91.4 and 95.5% of the variation of the reversal effects of 1 μM gefitinib on the resistance to these drugs, respectively.

The effects of gefitinib on the BCRP function

First, we confirmed whether gefitinib directly interacts with BCRP using a highly BCRP-expressing membrane refined from PC-6/SN2-5H cells. Gefitinib increased the ATPase activity in the membrane system in a dose-dependent manner ($P < 0.05$) as shown in Fig. 6. Next, we examined whether gefitinib inhibits the function of BCRP as an efflux transporter, using a topotecan flowcytometric assay. Gefitinib increased topotecan accumulation in BCRP-expressing NSCLC cells, whereas accumulation was not observed in H522 cells not BCRP-expressing (Fig. 7a). BCRP levels were also correlated with the reversal of the topotecan fluorescence shift by gefitinib ($r = 0.976$, $r^2 = 0.953$, $P = 0.0001$; Fig. 7b), as well as the reversal effects of resistance to topoisomerase I inhibitors.

Discussion

The present study found that the resistance to topoisomerase I inhibitors in NSCLC cells was mainly

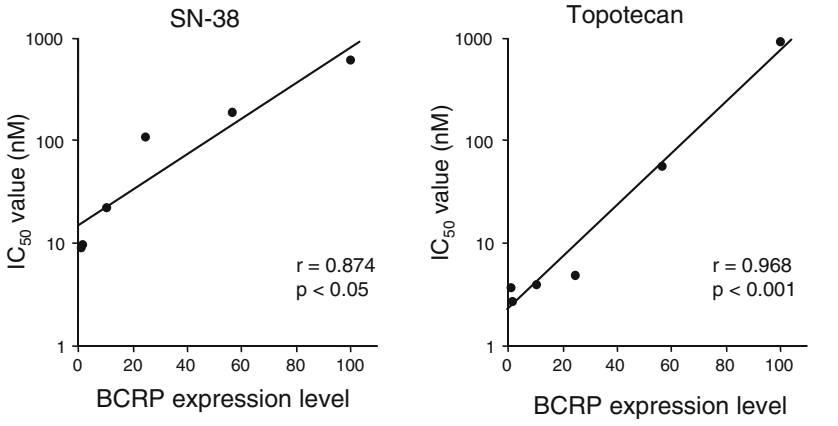

Fig. 2 Correlations between BCRP expression and IC_{50} values of SN-38 and topotecan in NSCLC cell lines determined by linear regression analysis

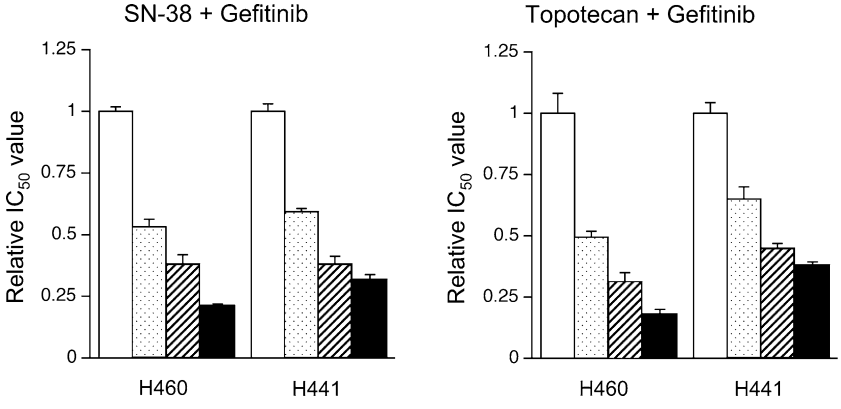

Fig. 3 Reverse resistance to SN-38 and topotecan by gefitinib. IC_{50} values of SN-38 and topotecan in the absence (*white*) or presence of 1 μM (*dot*), 5 μM (*border*), and 10 μM (*black*) gefitinib in NSCLC cells. The IC_{50} values are relative to those in the absence of gefitinib

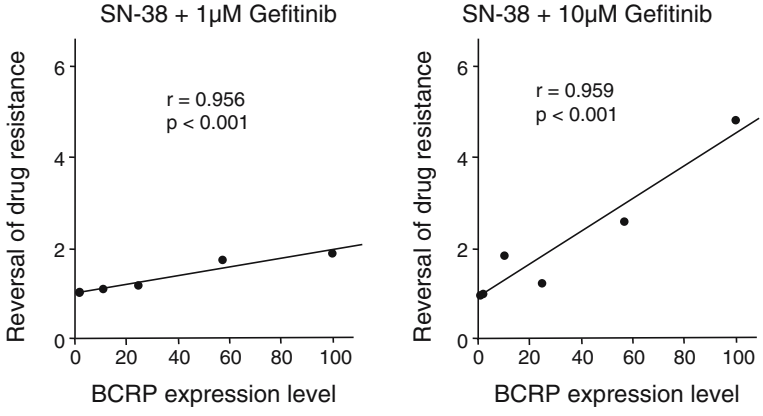

Fig. 4 Correlations between BCRP expression levels and the reversal effects of 1 μM gefitinib on drug resistance to SN-38 and topotecan in NSCLC cell lines determined by linear regression analysis. The reversal effects of gefitinib were calculated as (IC_{50} in the absence of gefitinib)/(IC_{50} in the presence of gefitinib)

implicated in BCRP, and that gefitinib reversed BCRP-mediated drug resistance in proportion to the BCRP expression levels in NSCLC cells. The examination of BCRP levels in cancer tissues could lead to the improvement of chemotherapies for patients with NSCLC.

Drug transporters confer drug resistance in lung cancer. Among the ABC transporters, BCRP is involved in drug resistance against mitoxantrone, SN-38, and topotecan in cell lines selected with topoisomerase I inhibitors or transfectant cells [1]. Previously we reported that the levels of *BCRP* mRNA expression in NSCLC cell lines were significantly correlated with BCRP function, and that 22% of untreated NSCLC tissues expressed functional levels of BCRP mRNA levels, but not examined at the protein levels [14]. Then, the present study found that the protein levels of BCRP accounted for between 80 and 90% of the resistance to topoisomerase I inhibitors in NSCLC cells. Topoisomerase I inhibitors are key drugs in the treatment of NSCLC [7, 17, 22]. Also, it is reported that BCRP appears to be a predictor of survival in patients with

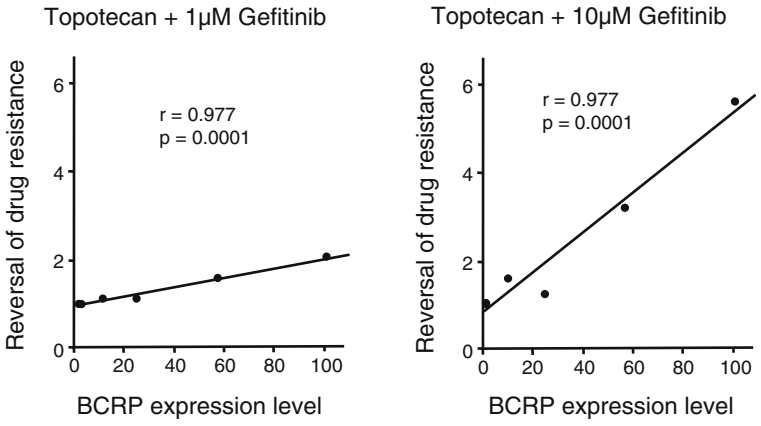

Fig. 5 Correlations between BCRP expression levels and the reversal effects of 10 μM gefitinib on drug resistance to SN-38 and topotecan in NSCLC cell lines determined by linear regression analysis. The reversal effects of gefitinib were calculated as described in Fig. 4

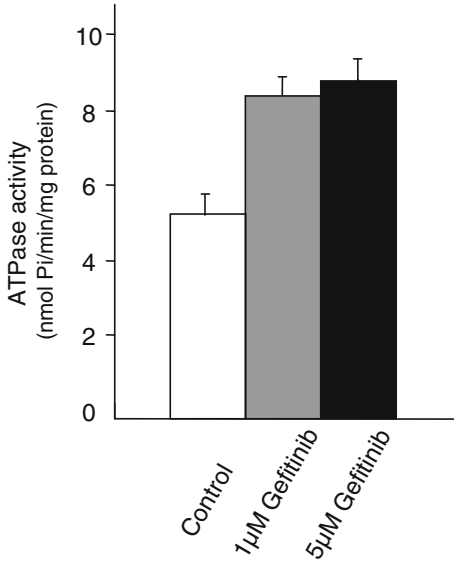

Fig. 6 ATPase activity of the BCRP-expressing membrane refined from PC-6/SN2-5H induced by gefitinib (untreated control, *white*; 1 μM, *shadow*; 5 μM, *black*)

advanced NSCLC [31]. Therefore, BCRP might be the most important target for overcoming drug resistance to topoisomerase I inhibitors in NSCLC.

Gefitinib, an EGFR tyrosine kinase inhibitor, has clinical antitumor activity which is expressed through signal transduction pathways downstream of mutant EGFR in patients with NSCLC [8, 21]. The combination of gefitinib with topoisomerase I inhibitors has synergistic antitumor effects both in vitro and in vivo [16, 28]. The present study is the first report demonstrating that gefitinib exhibited reversal effects on the topoisomerase I inhibitors in drug-unselected NSCLC cells, as well as SCLC as we reported previously [19]. In addition, the protein levels of BCRP accounted for between 80 and 90% of the reversal effects of gefitinib in NSCLC cells. To date, several potent BCRP inhibitors have been reported such as novobiocin, GF120918, and fumitremorgin C [5, 26, 27]. Among these, gefitinib is clinically available and reverses the resistance to topoisomerase I inhibitors at clinically achievable concentrations. Thus, gefitinib may be a promising BCRP inhibitor.

Our previous study using the membrane vesicle assay revealed that gefitinib inhibits the transport of the topoisomerase I inhibitor into the vesicles, and that gefitinib is not transported into the vesicles [19]. The kinetic parameters in the vesicle study showed that gefitinib reverses BCRP-mediated drug resistance by direct inhibition other than competitive inhibition as a BCRP substrate [19]. Other tyrosine kinase inhibitors such as imatinib are also reported to inhibit the function of BCRP [9]. In addition, we have recently demonstrated that gefitinib inhibits the function of p-glycoprotein/MDR1, another ABC transporter [15]. Gefitinib increases ATPase activity of pure MDR1-expressing membrane [15], indicating that the inhibition of transporter function is not due to the suppression of ATP hydrolysis. Tyrosine kinase inhibitors may have novel effects on the function of ABC transporters, and further biochemical studies including tridimensional structure of the transporters are required.

In the present study, however, the protein levels of BCRP could not completely explain the resistance to topoisomerase I inhibitors. Other mechanisms responsible for the resistance to topoisomerase I inhibitors may exist, such as 5′-diphosphoglucuronosyltransferase (UGT), single nucleotide polymorphisms (SNPs) in the BCRP gene or other ABC transporters. UGT is related to the metabolism and resistance of SN-38 [4, 23, 29], and that *UGT1A1* genotypes might be clinically useful for predicting severe toxicity in cancer patients receiving irinotecan [2]. Also, SNPs in the BCRP gene are associated with the drug resistance of topoisomerase I inhibitors [10]. Although some investigators reported that p-glycoprotein/MDR1 confers resistance to topoisomerase I inhibitors [11], *MDR1* mRNA was not detected in NSCLC cells examined in the present study. However, the existence of transporters other than p-glycoprotein/MDR1 and BCRP cannot be excluded.

Our findings suggest that BCRP is the most important molecule responsible for topoisomerase I inhibitors resistance and that the development of BCRP inhibitors is the most effective approach in overcoming these drugs

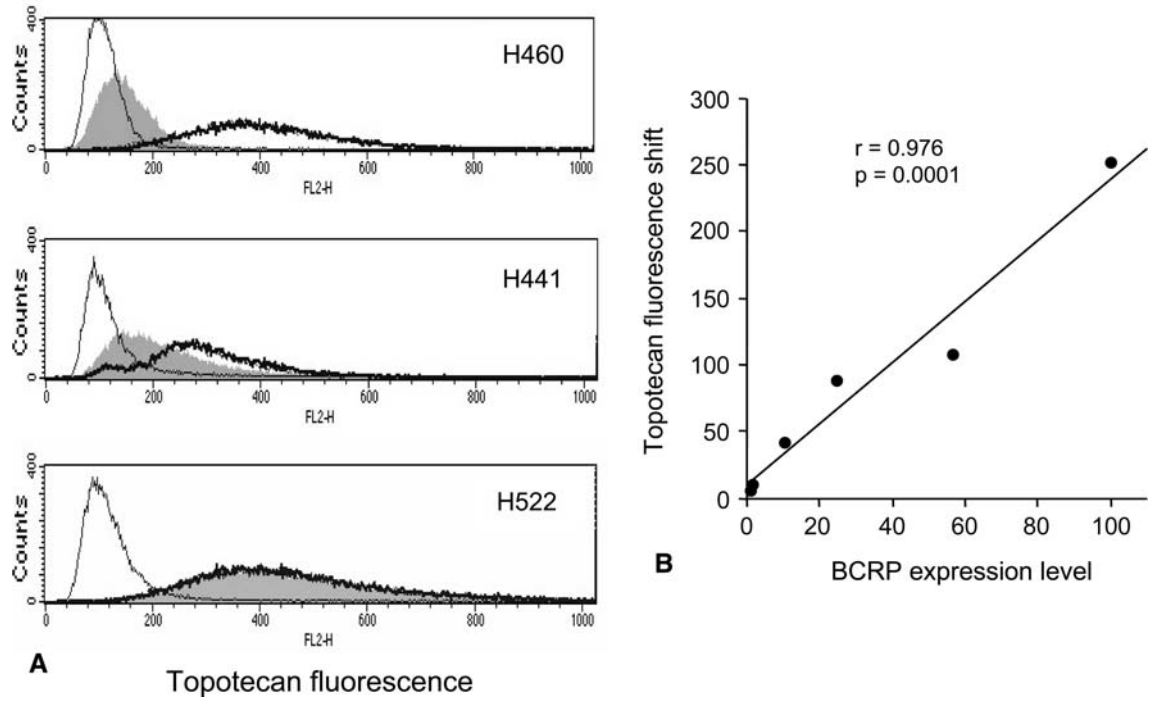

Fig. 7 **a** Intracellular topotecan accumulation in NSCLC cells in the presence (*thick line*) or absence (*shadow*) of 10 μM gefitinib. The blank (*thin line*) represents fluorescence without exposure to topotecan. **b** Correlations between BCRP expression levels and the reversal effects of 10 μM gefitinib on intracellular topotecan accumulation in NSCLC cell lines determined by linear regression analysis. The topotecan fluorescence shift in each cell line was calculated as (the mean fluorescence with gefitinib) − (the mean fluorescence without gefitinib)

resistance. In addition, the examination of BCRP levels in NSCLC tissues may clarify an optimal patient population for the treatment with topoisomerase I inhibitors alone or in combination with BCRP inhibitors, such as gefitinib.

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