Combination of SN-38 with gefitinib or imatinib overcomes SN-38-resistant small-cell lung cancer cells

NAGIO TAKIGAWA[1-3], MASAMI TAKEYAMA[2], TOSHIYUKI KOZUKI[3], TAKUO SHIBAYAMA[1,2],
AKIKO HISAMOTO[3], KATSUYUKI KIURA[3], ATSUHIKO TADA[1,2], KATSUYUKI HOTTA[3],
SHIGEKI UMEMURA[3], KADOAKI OHASHI[3], YOSHIRO FUJIWARA[3], SABURO TAKATA[3], EIKI ICHIHARA[3],
MASAHIRO OSAWA[3], MASAHIRO TABATA[3], MITSUNE TANIMOTO[3] and KIYOSHI TAKAHASHI[1,2]

[1]Department of Respiratory Medicine and [2]Clinical Research Instutute, National Hospital Organization
Minami-Okayama Medical Center, 4066 Hayashima, Okayama 701-0304; [3]Department of Respiratory
Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Abstract. Irinotecan is one of the effective anticancer agents for small-cell lung cancer (SCLC) and 7-ethyl-10-hydroxy-campthothecin (SN-38) is an active metabolite of irinotecan. Gefitinib and imatinib are tyrosine kinase inhibitors which have clinical activities in several malignancies and they are also potent inhibitors of breast cancer resistance protein (BCRP) transporter, which confers the resistance of topoisomerase I inhibitors including SN-38 and topotecan. The cytotoxicity of SN-38, gefitinib and imatinib for the SN-38-resistant cells (SBC-3/SN-38) from human SCLC cells, SBC-3, was evaluated using AlamarBlue assay. The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) for 96-h exposure was used to evaluate the cytotoxicity. BCRP expression was determined by Western blotting and immunofluorescence staining. Intracellular topotecan accumulation was evaluated by flow cytometry. No differences were observed in the IC_{50} values (mean ± SD) of the tyrosine kinase inhibitors between the SBC-3 cells and the SBC-3/SN-38 cells: 15±1.6 and 12±2.8 μM of gefitinib, respectively; 15±0.51 and 14±3.9 μM of imatinib, respectively. The SBC-3/SN-38 was 9.5-fold more resistant to SN-38 than the parental SBC-3. The SBC-3/SN-38 restored sensitivity to SN-38 when combined with 8 μM gefitinib or 8 μM imatinib, even though the IC_{50} values of SN-38 combined with gefitinib or imatinib in the SBC-3 cells did not change. BCRP was equally overexpressed in the SBC-3/SN-38 with and without gefitinib or imatinib. In addition, the BCRP expression on the SBC-3/SN-38 cell membrane with and without gefitinib seemed to be equal. Gefitinib increased intracellular accumulation of topotecan in the SBC-3/SN-38 cells. Gefitinib or imatinib reversed SN-38-resistance in these SCLC cells, possibly due to intracellular accumulation of SN-38 without any change in BCRP quantity. Irinotecan with gefitinib or imatinib might be effective for SCLC refractory to irinotecan.

Introduction

Gefitinib, an epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI), showed antitumor activity in several cancers, especially in non-small-cell lung cancer (NSCLC) (1). Imatinib is also a TKI and it has demonstrated clinical efficacy in Bcr-Abl-expressing chronic myeloid leukemia and c-Kit-expressing gastrointestinal stromal tumors (2). Breast cancer resistance protein (BCRP) is a transporter, which contributes to a reduced accumulation of topoisomerase I inhibitors in the cells by an enhanced efflux of them (3,4). Recently, gefitinib and imatinib have been reported to be potent inhibitors of BCRP and reverse the BCRP-mediated resistance (5).

A combination of irinotecan and cisplatin is one of the standard chemotherapy regimens in the treatment of extensive disease small-cell lung cancer (SCLC) (6). 7-ethyl-10-hydroxy-campthothecin (SN-38) is an active metabolite of irinotecan. We have already established an SN-38-resistant subline (SBC-3/SN-38) from a human SCLC cell line, SBC-3 (7). In the present study, the usefulness and the mechanism of the combination of either SN-38 with gefitinib or imatinib for the SBC-3/SN-38 cells were evaluated.

Materials and methods

Chemicals and reagents. SN-38 and topotecan were provided by Yakult Honsha Co., and SmithKline Beecham Co., Tokyo, Japan, respectively. Gefitinib and imatinib were purchased from AstraZeneca, Osaka and Novartis Pharma, Tokyo, Japan,

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respectively. The drugs were dissolved in dimethylsulfoxide and the drug solutions were stored at -20˚C. AlamarBlue (UK-Serotec Ltd., Oxford, UK) was purchased from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan.

Cell culture. The parent cell line, SBC-3 was established from bone marrow aspirate of a previously untreated patient with SCLC (8). The growth medium (RPMI-FBS) was RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The SN-38-resistant subline (SBC-3/SN-38) was established by the continuous exposure of the SBC-3 cells to increasing concentrations of SN-38 (7).

Assay of drug sensitivity. Drug sensitivity was determined using an AlamarBlue assay (9). Briefly, 50 μl of RPMI-FBS containing serial concentrations of each chemotherapeutic agent was prepared in 96-well flat-bottomed microplates (Coster 3596, Corning Inc., Corning, NY, USA). Next, 50 μl of RPMI-FBS containing 500 cells for SBC-3 and 1500 cells for SBC-3/SN-38 were added to each well. The cells were then incubated at 37˚C for 96 h in a highly humidified incubator with 5% CO_2 and 95% air. Next, 10 μl of AlamarBlue was added to each well. After incubation at 37˚C for 5 h, the fluorescence of each well was measured using Fluoroskan Ascent (Labsystems Inc., Franklin, MA, USA) with 544-nm excitation and 590-nm emission. The fluorescence of a well without chemotherapeutic agents was used as the control and a well containing only RPMI-FBS and AlamarBlue was used to determine the background. The percentage of surviving cells was calculated using the following formula: [(mean fluorescence in 4 test wells - fluorescence in background wells)/ (mean fluorescence in control wells - fluorescence in background wells)] x 100. The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of the drug concentration versus the percentage of surviving cells. Determinations were carried out in quadruplicate in each experiment, and the results were confirmed by 3 or more separate experiments.

Western blotting. The cells were cultured for 96 h in the absence or presence of 2 or 8 μM of gefitinib or imatinib in RPMI-FBS. The cells were lysed in a radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA and ß-mercaptoethanol plus protease and phosphatase inhibitors. Aliquots of cell lysates (14 μg protein per lane) were electrophoresed on a 10% Readygels J (Bio-Rad, Tokyo, Japan) and then were transferred to PVDF membrane. The membrane was blocked in 5% non-fat dry milk in 20 mM Tris-HCl, pH 8.0, 150 mM and 0.05% Tween-20 at room temperature for 1 h. The membrane was then incubated with an appropriate dilution of the primary antibody at 4˚C overnight. Following washing, a secondary antibody, was diluted at 10000-fold for 1 h at room temperature. Anti-BCRP monoclonal antibody (BXP-21) from Kamiya Co. (Seatle, WA, USA) (1:500) and anti-actin monoclonal antibody (MAB1501) from Chemicon International Inc. (Temecula, CA, USA) (1:1000) as the primary antibodies and the enhanced chemiluminescence detection system (Amersham Co., Bucks, UK) were used.

Table I. IC_{50} values (μM; mean ± SD) of gefitinib and imatinib in the parent (SBC-3) and SN-38-resistant subline (SBC-3/SN-38).

	IC_{50}	
	Gefitinib	Imatinib
SBC-3	15±1.6	15±0.51
SBC-3/SN-38	12±2.8	14±3.9

IC_{50}, 50% inhibitory concentration; SD, standard deviation.

Immunofluorescence. The cells were incubated in RPMI-FBS with and without 8 μM gefitinib for 1 and 4 h at a cell density of 1x10^6/ml in a 37˚C/5% CO_2 incubator. At the end of each time period, the cells were collected and washed twice with phosphate-buffered saline (PBS) at 4˚C. The location of BCRP was visualized by staining the cells using anti-BCRP monoclonal antibody (sc-18841) (1:50) and goat anti-mouse IgG-FITC (sc-2781) (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using a confocal laser-scanning microscope (Zeiss LSM 510, Tokyo, Japan). The excitation of fluorescent dye was performed at 488 nm for IgG-FITC.

Intracellular topotecan accumulation. The cells were incubated in RPMI-FBS with drugs (50 or 100 μM topotecan with and without 8 μM gefitinib) at a cell density of 1x10^6/ml in a 37˚C/5% CO_2 incubator for 15 min. At the end of each time, the cells were collected and washed twice with PBS at 4˚C. Topotecan was detected with 488-nm excitation and 585-nm emission by FACS Calibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). The data were analyzed according to the ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Results

The mean values for IC_{50} of gefitinib and imatinib for SBC-3 and SBC-3/SN-38 cells ranged from 12 to 15 μM (Table I). The resistant cells retained their sensitivity to gefitinib and imatinib at the same level as that observed in the parent cells. The combination effect of SN-38 with gefitinib or imatinib is shown in Table II. When the SBC-3 cells were simultaneously treated with gefitinib or imatinib (0.5, 2 and 8 μM), the IC_{50} values of SN-38 were approximately 9.4-11 μM. In contrast, the IC_{50} values of SN-38 for the SBC-3/SN-38 declined from 95 to 12 or 13 μM with gefitinib or imatinib, respectively, in a dose-dependent manner. SN-38 sensitivity in the SBC-3/SN-38 cells was restored by adding 8 μM gefitinib or imatinib.

The overexpression of BCRP in SBC-3/SN-38 is shown in lanes 2 and 8 in Fig. 1. Neither imatinib nor gefitinib affected the BCRP levels in SBC-3/SN-38 (lanes 4, 6, 10 and 12). The BCRP was located on cell membrane in SBC-3/SN-38 and seemed equivalent both with and without gefitinib (Fig. 2). There was no difference in the expression on the cell membrane between 1- and 4-h treatment of gefitinib. Fig. 3 shows the

Table II. IC$_{50}$ values (nM; mean ± SD) for SN-38 with several concentrations of gefitinib or imatinib in the parent (SBC-3) and SN-38-resistant subline (SBC-3/SN-38).

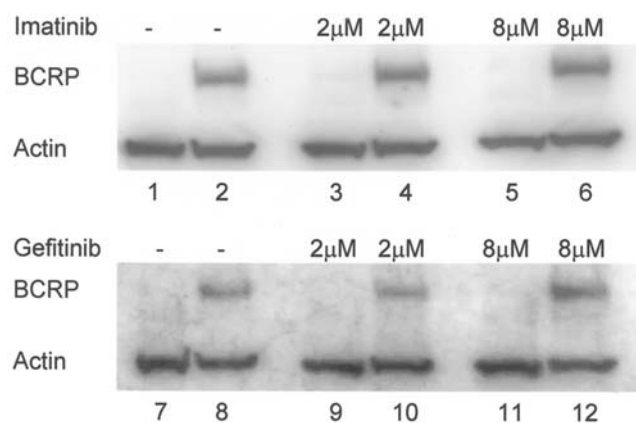|	Gefitinib (μM)	IC$_{50}$ for SN-38	Imatinib (μM)	IC$_{50}$ for SN-38
SBC-3	0	10±0.11		
	0.5	10±0.48	0.5	9.6±0.35
	2	9.4±0.30	2	11±0.35
	8	10±1.4	8	11±0.38
SBC-3/SN-38	0	95±4.3		
	0.5	40±1.5	0.5	42±0.83
	2	22±2.7	2	22±2.0
	8	12±0.52	8	13±1.3

IC$_{50}$, 50% inhibitory concentration; SD, standard deviation.

effects of gefitinib on the intracellular accumulation of topotecan. In a dose-dependent manner, topotecan was accumulated in the SBC-3 cells equally irrespective of adding gefitinib.

There were no differences in the cellular fluorescence of SBC-3/SN-38 cells without gefitinib. However, gefitinib increased the intracellular accumulation of topotecan in the SBC-3/SN-38 in a dose-dependent manner.

Discussion

Gefitinib and imatinib reversed SN-38-resistance in the SBC-3/SN-38 overexpressing BCRP. Previous studies have indicated that gefitinib or imatinib reversed topoisomerase I inhibitor-resistance (10-14), while we showed that both TKIs were equally effective. Imatinib reversed BCRP-mediated resistance to SN-38 while also increasing the accumulation of topotecan in osteosarcoma cells and breast cancer cells overexpressing BCRP (10,11). The mechanism for overcoming resistance, however, remains unclear. Houghton *et al* showed that imatinib inhibited the function of BCRP but was not a substrate for the protein (10), while Burger *et al* revealed that it was a competitive substrate for BCRP (11). Other investigators showed that gefitinib reversed topoisomerase I inhibitor-resistance (12-14). Nakamura *et al* (13) and Yanase *et al* (12) suggested that the mechanism was not the competitive inhibition but the inhibition of the pump function of BCRP using an intravesicular transport assay. Recently, Nakanishi

Figure 1. The expression of BCRP in SBC-3 and SBC-3/SN-38 cells treated with gefitinib or imatinib. The overexpression of BCRP in SBC-3/SN-38 is shown in lanes 2 and 8. Imatinib or gefitinib did not affect the BCRP levels in SBC-3/SN-38 (lanes 4, 6, 10 and 12). SBC-3 cells did not have any detectable BCRP with and without imatinib or gefitinib.
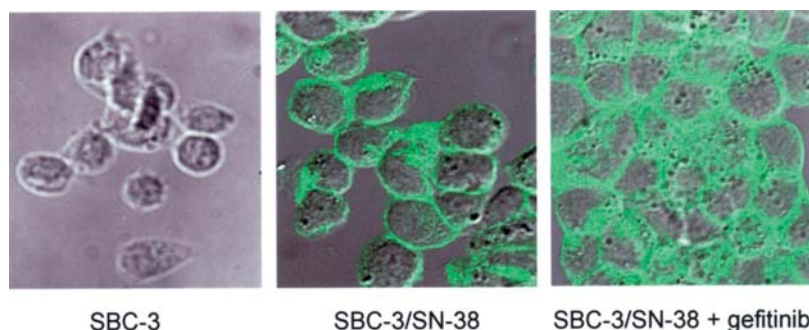

Figure 2. The expression of BCRP in SBC-3/SN-38 cells with 1-h treatment of gefitinib. The BCRP was located on cell membrane in SBC-3/SN-38 and seemed equivalent with and without gefitinib. There was no detectable BCRP expression in SBC-3.
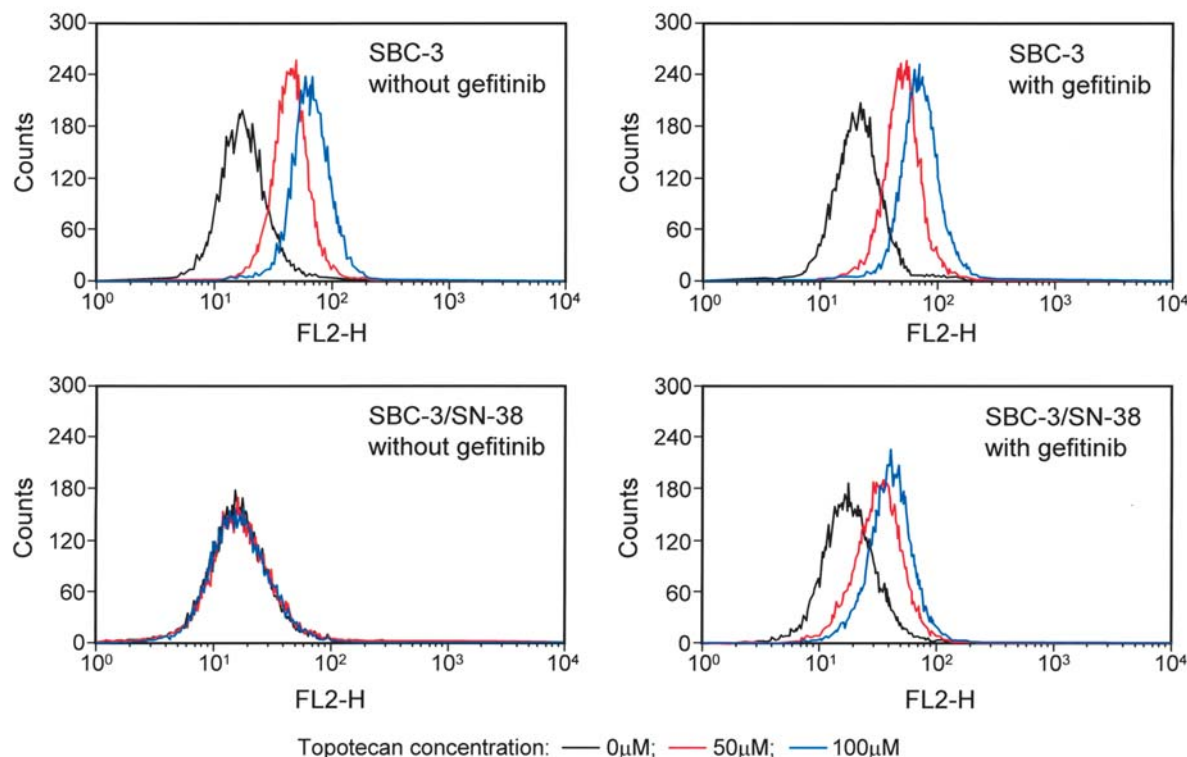
Figure 3. Effect of gefitinib on the intracellular topotecan accumulation. In a dose-dependent manner, topotecan was accumulated in the SBC-3 cells equally despite the addition of gefitinib. There were no differences in the cellular fluorescence of SBC-3/SN-38 cells without gefitinib. However, gefitinib increased the intracellular accumulation of topotecan in SBC-3/SN-38 in a dose-dependent manner.

et al reported that imatinib decreased the BCRP level in the mitoxantrone-resistant K562/BCRP-MX10 cells overexpressing BCRP (15). To our knowledge, there have been no reports regarding the change of the BCRP expression level by gefitinib. We experimented using Western blotting and immunofluorescence in order to determine whether gefitinib could either decrease the total BCRP or induce an internalization of BCRP. As a result, gefitinib did not affect the BCRP expression level either in the cells or on the cell membrane. Meanwhile, the intracellular accumulation of topotecan increased in the SBC-3/SN-38 cells in a dose-dependent manner. Although we could not determine from our study whether gefitinib is a competitive inhibitor or not, it might therefore increase the SN-38 sensitivity in the SBC-3/SN-38 cells, not due to a decrease in BCRP but to pump dysfunction of BCRP.

The concentration of 8 μM of imatinib or gefitinib was considered to be relatively high in terms of the clinical settings. In the case of imatinib, this was a clinically achievable serum concentration with and without chemotherapeutic agents (16,17). Meanwhile, the pharmacologically achievable gefitinib concentration was 1 μM at most (18), although the maximum plasma concentration was 3.875 μg/ml (8.67 μM) in the child treated with 500 mg/m^2 of gefitinib (19). The mean concentration in breast tumor tissues was 16.7 μM (median, 14.3 μM; range, 0.2-25.8 μM) in the 19 breast cancer patients, which was 42 times higher than plasma (20). Eight μM of gefitinib may therefore be an achievable concentration in lung tissue.

The effectiveness of gefitinib for SCLC has only been previously reported in one case report (21). A single agent of gefitinib had effectiveness in NSCLC (1); however, the

addition of gefitinib to standard two-drug combinations such as cisplatin plus gemcitabine or carboplatin plus paclitaxel did not produce any survival advantage (22,23). Although imatinib had an antitumor activity for gastrointestinal stromal tumors expressing c-Kit (24), it did not show any effectiveness for SCLC, which commonly expresses c-Kit independently (25-27). In addition, a phase I study of imatinib with cisplatin and irinotecan in patients with untreated extensive SCLC showed increased toxicities (neutropenia, diarrhea and thrombosis) although 5 partial responses of 6 evaluable cases were noted (17). Monoclonal antibody against EGFR, cetuximab, combined with irinotecan was effective for irinotecan-refractory colorectal cancer (28). The two-drug combination of irinotecan with either gefitinib or imatinib may therefore be an interesting regimen for irinotecan-refractory SCLC.

In conclusion, gefitinib and imatinib similarly restore the SN-38 sensitivity in the SBC-3/SN-38 overexpressing BCRP. A combination of irinotecan with gefitinib or imatinib for irinotecan-refractory SCLC might thus be considered in clinical trials.

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