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ORIGINAL ARTICLE

A phase II trial of cisplatin and irinotecan alternating with doxorubicin, cyclophosphamide and etoposide in previously untreated patients with extensive-disease small-cell lung cancer

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Abstract
Purpose The aim of this trial was to investigate the efficacy and safety of cisplatin (P) and irinotecan (I) (PI) alternating with doxorubicin (A), cyclophosphamide (C) and etoposide (E) (ACE) in patients with extensive-disease small-cell lung cancer (ED-SCLC).
Patients and Methods Patients with previously untreated ED-SCLC were enrolled in this trial. In the first, third and fifth cycles, PI (P: 60 mg/m^2 on day 1; I: 60 mg/m^2/day on days 1, 8 and 15) was administered, whereas ACE (A: 50 mg/m^2 on day 1; C: 750 mg/m^2 on day 1; E 80 mg/m^2/day on days 1–3) was given in the second, fourth and sixth cycles. Each cycle was repeated every 4 weeks. At the end of six cycles, patients who had obtained a complete response were given prophylactic cranial irradiation.
Results In total, 28 patients were enrolled, of whom 27 were assessable for efficacy and safety. Objective responses, including 4 (15%) complete responses, were observed in 25 patients (93%). Median survival time was 12.9 months. The principal toxicity was myelosuppression; grade 4 neutropenia and thrombocytopenia were observed in 89 and 4%, respectively. Febrile neutropenia occurred in 30% of patients. Diarrhea was mild (grade 3–4; 4%). All toxicities were reversible and there were no treatment-related deaths. The mean percentage of the delivered doses, relative to the projected doses, of PI and ACE were 84.6 and 91.1%, respectively.
Conclusions These results indicate the PI–ACE regimen to have promising activity against ED-SCLC with moderate toxicities.

Keywords Small-cell lung cancer · Alternating chemotherapy · Irinotecan

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Introduction

Standard treatment for previously untreated extensive-disease small-cell lung cancer (ED-SCLC) is currently considered to be systemic chemotherapy consisting of cisplatin and etoposide (PE) [1]. However, the majority of responders relapse and the long-term survival rate is still quite low. To improve outcomes, several treatment strategies have been investigated. Alternating chemotherapy based on the Goldie–Coldman hypothesis was evaluated mainly in the late 1980s [2]. Fukuoka et al. [3] conducted a phase III trial comparing cyclophosphamide, doxorubicin, and vincristine

(CAV), PE with alternation of CAV and PE, in 300 SCLC patients to clarify whether rapid alternation of these two regimens produced superior therapeutic results as compared with either regimen alone. They showed a trend toward longer survival with alternating therapy as compared with the standard PE regimen.

Roth et al. [4] also conducted a phase III trial of the same three regimens in 437 patients with ED-SCLC. They found no significant differences in treatment outcomes among the regimens in terms of response rate and overall survival. Thus, alternating chemotherapy did not definitely improve the survival of SCLC patients as compared to standard treatments. One possible explanation for the negative results might be that these two regimens, CAV and PE, were partially cross-resistant [3, 4]. In addition, the outdated chemotherapy regimen, CAV, might have resulted in the failure of alternating chemotherapy to provide a survival advantage.

Recently, there have been several advances in chemotherapy for SCLC. First, a Japanese randomized phase III study comparing cisplatin and irinotecan (PI) with standard PE in previously untreated patients with ED-SCLC, demonstrated a significant survival benefit of PI with a median survival time of 12.8 versus 9.4 months [5]. Furthermore, Ando et al. [6] demonstrated that patients who relapsed after receiving a combination of platinum and etoposide responded well to subsequent PI chemotherapy with an overall response rate of 80%, which might suggest that irinotecan and etoposide are not cross-resistant. Second, Bunn et al. [7] demonstrated that a three-drug combination of doxorubicin, cyclophosphamide and etoposide (ACE) yielded a significant survival benefit in patients with ED-SCLC, as compared with the CAV regimen, in a randomized phase III trial.

There have been no investigations of alternating chemotherapy using new chemotherapeutic agents such as irinotecan. Given these background factors, we aimed to reappraise alternating chemotherapy in a prospective phase II trial using PI and ACE regimens. The primary endpoint of this trial was objective response rate, and secondary endpoints were toxicity and overall survival.

Patients and methods

Eligibility criteria

Patients were required to fulfill the following eligibility criteria: pathologically proven SCLC, extensive disease, no prior chemotherapy or thoracic irradiation, age $\leq$75 years, Eastern Cooperative Oncology Group performance status of 0 or 1, presence of measurable lesions, and adequate hematologic [white blood cell (WBC) count >4,000 per μl and platelet count >100,000 per μl], renal (creatinine clearance $\geq$60 ml per min), and hepatic (serum transaminases <1.5 × upper limit of normal range) functions. All participants provided written informed consent. Patients with massive pleural effusion, pericardial effusion or symptomatic brain metastases were excluded. The protocol was approved by the institutional review board of each participating institute. Baseline pretreatment evaluations included a complete history, physical examination, laboratory tests, chest radiograph and computed tomography (CT) scan of the chest. CT scan of the abdomen, magnetic resonance imaging of the brain and a radionuclide bone scan were also performed.

Assessments of antitumor activity and toxicity

Tumor response was assessed according to the World Health Organization criteria [8]. Complete response (CR) was defined as the disappearance of disease at all sites, partial response (PR) as a reduction of at least 50% in the sum of the products of the two largest perpendicular diameters of all measurable lesions, without progression in any other sites. No change was defined as a decrease of less than 50% or an increase of less than 25% in the sum of the products of the two largest perpendicular diameters of all measurable lesions for at least 4 weeks. Progressive disease was defined as an increase of 25% or more in the sum of the products of the two largest perpendicular diameters of all measurable lesions or the appearance of a new lesion. Tumor markers were not used to assess response. Response assessments were performed at the end of each cycle. A minimum duration of 4 weeks was required to document a response. The responses were finally confirmed by blinded extramural review.

All toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC, Version 2.0).

Treatment schedules and modifications

In the first cycle, PI was administered intravenously, and ACE was given intravenously in the second cycle (day 29 of the first cycle). Subsequently, PI and ACE were alternately administered and repeated every 4 weeks up to 6 cycles. PI consisted of cisplatin 60 mg/m^2 given on day 1 and irinotecan 60 mg/m^2/day on days 1, 8 and 15, same schedule as those in previous phase II and III trials [5, 9]. ACE was administered as cyclophosphamide 750 mg/m^2 on day 1, doxorubicin 50 mg/m^2 on day 1 and etoposide 80 mg/m^2/day on days 1–3. After completion of chemotherapy, prophylactic cranial irradiation was delivered at a dose of 30 Gy in 15 fractions to patients who had obtained CR. Each patient was pre-medicated with intravenous

dexamethasone (16 mg) and granisetron (3 mg). If grade 4 leucopenia, grade 4 neutropenia, or febrile neutropenia was noted, the use of granulocyte colony-stimulating factor (G-CSF) was permitted.

With the PI regimen, administration of irinotecan on days 8 or 15 was cancelled if the WBC count <3,000 per μl and/or the platelet count <100,000 per μl on the day of administration. For both the PI and the ACE regimens, initiation of the next cycle was delayed until recovery of the WBC count ≥ 4,000 per μl or the platelet count ≥ 100,000 per μl, and resolution of non-hematologic toxicities to ≤ grade 1. Patients were treated with at least two cycles of chemotherapy unless there was disease progression, unacceptable toxicity in the first cycle, or withdrawal of consent to participate in this study. Dose modification for the next cycle was defined as follows. If grade 4 leucopenia, neutropenia or thrombocytopenia was observed with the PI regimen, the dose of irinotecan in the next PI cycle was decreased by 10 mg/m^2. If the same toxicity occurred in the ACE regimen, doses of cyclophosphamide, doxorubicin and etoposide in the next ACE cycle were decreased by 100, 10 and 10 mg/m^2, respectively. For grade 3 diarrhea, the irinotecan dose in the next PI cycle was decreased by 10 mg/m^2. Irinotecan was discontinued for grade 4 diarrhea. In addition, the cisplatin dose in the next PI cycle was reduced to 40 mg/m^2 when creatinine clearance dropped to between 30 and 60 ml/min. A decrease to less than 30 ml/min required discontinuation of cisplatin.

The dose intensity of each drug was calculated, for each patient who received at least two cycles of chemotherapy, using the following formula: Dose intensity (mg/m^2/week) = Total milligrams of a drug in all cycles per body surface area/[(Total days of therapy)/7], where total days of therapy is the number of days from day 1 of cycle 1 to day 1 of the last cycle plus 28 days [10]. The mean dose intensity was then calculated.

Statistical considerations

A Minimax two-stage design was used to test whether there was sufficient evidence to determine a response rate of at least 85% (i.e. clinically promising) versus at most 70% (i.e. clinically inactive), accepting a false-positive rate (α) ≤ 10% and a false-negative rate (β) ≤ 10%. In this two-stage design, accrual was stopped at the first stage for 22 patients if 16 or more patients did not respond to the treatment, otherwise it was continued to a total of 52 patients. This alternating chemotherapy was judged to be effective if more than 41 patients responded to the treatment.

Statistical analyses were performed using the StatView® 5.0 program (BrainPower Inc., Calabasas, CA, USA). The correlations between dose intensity and response or survival were assessed with Kruskal–Wallis test or Spearman's rank correlation coefficient. Overall survival curve was constructed using the Kaplan–Meier product-limit method. P-values less than 0.05 were considered statistically significant.

Results

Patient characteristics

Twenty-eight patients with previously untreated ED-SCLC were enrolled between November 1999 and November 2002 at 11 institutions in Japan. Initially, we aimed to accrue a total of 50 patients in this trial. However, a low accrual rate prompted termination of patient registration in 2003. Twenty-seven (96%) of the 28 patients were assessable for efficacy and toxicity analysis. The one remaining patient did not satisfy the eligibility criteria because of limited-disease. Characteristics of the 27 patients are listed in Table 1. The majority were male and had metastatic disease, but no weight loss, prior to the registration.

Objective response and survival

Of the 27 patients, 4 achieved CR (14.8%), 21 PR (77.8%), resulting in a total response rate of 92.6% (95% confidence interval; 75.7–99.1%), whereas disease stabilization was obtained in 2 patients (7.4%). Survival analysis was performed for all 27 patients. Twenty-five (93%) patients had died at the time of this analysis. Mean follow-up time for surviving patients was 14.7 months, and the median survival time of all patients was 12.9 months ranging from 3.5 to 34.5 months (Fig. 1).

Hematological toxicity

Myelosuppression was the principal toxicity experienced with this regimen. Among 27 patients, grades 3 and 4 neutropenia were seen in 2 (7%) and 24 (89%), respectively (Table 2). Of these, eight patients (30%) developed febrile episodes. However, these conditions were reversible with appropriate supportive care. Anemia and thrombocytopenia were relatively mild with grade 4 toxicities in 7 and 4% of patients, respectively. The PI regimen had less severe hematological toxicity than the ACE regimen; grade 3 and

Table 1 Demographics of the 27 patients

Age [median (range)]	67 (47–75)
Gender (male/female)	22 (81%)/5 (19%)
Performance status (0/1)	9 (33%)/18 (67%)
Body weight loss (<5/≥5%)	25 (93%)/2 (7%)
Stage (IIIB/IV)	4 (15%)/23 (85%)

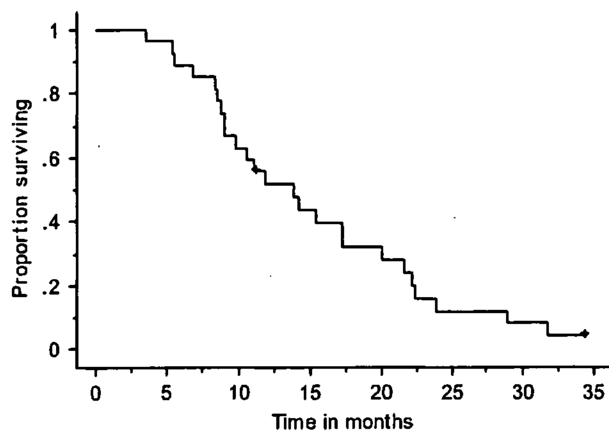

Fig. 1 Overall survival curve for the 27 patients

Table 2 Toxicity profiles for all cycles

Toxicity	No. of patients (%)			
	Grades			
	1	2	3	4
Leucopenia	0	5 (19%)	16 (59%)	6 (22%)
Neutropenia	1 (4%)	0	2 (7%)	24 (89%)
Febrile neutropenia			8 (30%)	
Anemia	2 (7%)	15 (56%)	8 (30%)	2 (7%)
Thrombocytopenia	10 (37%)	7 (26%)	5 (19%)	1 (4%)
Nausea/vomiting	10 (37%)	6 (22%)	4 (15%)	0
Diarrhea	6 (22%)	5 (19%)	1 (4%)	0
Nephrotoxicity	3 (11%)	1 (4%)	0	0
Hepatotoxicity	2 (7%)	2 (7%)	0	0
Constipation	8 (30%)	1 (4%)	0	0
Alopecia	11 (41%)	14 (52%)	–	–
Peripheral neuropathy	3 (11%)	1 (4%)	0	0

DI dose intensity, *PI* cisplatin and irinotecan, *CDDP* cisplatin, *CPT* irinotecan, *ACE* doxorubicin, cyclophosphamide and etoposide, *DXR* doxorubicin, *CPA* cyclophosphamide, *ETP* etoposide

4 leucopenia: 37 versus 81%, grade 3 and 4 anemia: 19 versus 37%) (Table 3).

Non-hematological toxicity

Severe diarrhea was rare (Table 2); only one patient (4%) developed grade 3 diarrhea and none had no grade 4 diarrhea. The PI regimen produced diarrhea more frequently than the ACE regimen (all grades; 41 vs. 15%) (Table 3). Other toxicities were also generally mild and there were no treatment-related deaths.

Treatment delivery and dose intensity

In total, 126 cycles were administered. In the first, third and fifth cycles (total 68 cycles), 27, 24 and 17 cycles of PI were administered, while 27, 22 and 9 cycles of ACE were administered in the second, fourth and sixth (total 58 cycles). Median number of cycles of therapy was five ranging from two to six. Dose modification and/or treatment omission was undertaken in 30 (44%) of the 68 PI cycles and 31 (53%) of the 58 ACE cycles. However, the dose intensity of each drug was favorable (Table 4) and the mean percentages of the delivered doses relative to the projected doses of PI and ACE were 84.6 and 91.1%, respectively. The dose intensity correlated with neither objective response ($P = 0.7062$) nor overall survival ($P = 0.3132$).

Discussion

In this study, we obtained the following results: (1) PI–ACE alternating chemotherapy showed a promising antitumor activity with a response rate of 93% and median survival time of 12.9 months, comparable to those of PE chemotherapy (response rate of 44–68% and median survival time of 9.4–10.2 months) and PI chemotherapy (response rate of 48–84% and median survival time of 9.3–12.8 months) [5, 11], (2) toxicity was moderate and the main toxicity was myelosuppression, and (3) this regimen produced a favorable dose intensity.

In evaluating the efficacy of alternating chemotherapy, cross-resistance of the two regimens is an important consideration. Fukuoka et al. [3] reported a three-arm phase III trial of CAV, PE and CAV/PE in patients with SCLC. In their trial, only one (8%) of 13 patients responded to CAV after failing to respond to the PE regimen, which might suggest the CAV and PE regimens to be cross-resistant. However, nine (23%) of 39 patients who failed to respond to the initial CAV regimen responded to PE when they were crossed over, and in another study, patients who relapsed after receiving platinum plus etoposide chemotherapy responded to subsequent PI chemotherapy [6]. Thus, we initially considered that these findings appeared to point away from cross-resistance between PI and ACE regimens, and designed to investigate the combination chemotherapy of PI alternating with ACE. In the current trial, however, we could not evaluate whether PI and ACE were cross-resistant because the two regimens were rapidly alternated with a very short interval. Therefore, we could not assess the degree of cross-resistance between the PI and ACE regimens, which is one of the major limitations in our study.

Our favorable efficacy data might also be explained by the fact that combined use of topoisomerase I and II inhibitors has been demonstrated to be complementary in both preclinical and clinical studies [12, 13]; it was previously shown that development of cellular resistance to topoisomerase II inhibitors conferred an increased sensitivity to

Table 3 Toxicity profiles for all cycles stratified by treatment regimens

Toxicity	Cisplatin and irinotecan (PI $n = 27$)				Doxorubicin, cyclophosphamide and etoposide (ACE $n = 27$)			
	No. of patients (%)				No. of patients (%)			
	Grades							
	1	2	3	4	1	2	3	4
Leucopenia	2 (17%)	12 (44%)	10 (37%)	0	0	5 (19%)	16 (59%)	6 (22%)
Neutropenia	2 (17%)	0	11 (41%)	11 (41%)	1 (8%)	2 (17%)	1 (8%)	23 (85%)
Anemia	3 (25%)	17 (63%)	5 (19%)		3 (25%)	14 (52%)	8 (30%)	2 (7%)
Thrombocytopenia	11 (41%)	2 (7%)	3 (11%)	0	12 (44%)	7 (26%)	3 (11%)	1 (4%)
Diarrhea	6 (22%)	4 (15%)	1 (4%)	0	2 (7%)	2 (7%)	0	0
Nephrotoxicity	2 (7%)	1 (4%)	0	0	1 (4%)	0	0	0
Hepatotoxicity	2 (7%)	2 (7%)	0	0	1 (4%)	0	0	0

DI dose intensity, *PI* cisplatin and irinotecan, *CDDP* cisplatin, *CPT* irinotecan, *ACE* doxorubicin, cyclophosphamide and etoposide, *DXR* doxorubicin, *CPA* cyclophosphamide, *ETP* etoposide

Table 4 Dose intensity of each drug

Drug	Projected DI (mg/m^2 week)	Mean actual DI (mg/m^2/week) (range)	Mean percentage of projected DI (range)
PI	–	–	0.846 (0.608–1.201)
CDDP	15	14.1 (10.4–19.6)	0.943 (0.695–1.310)
CPT	45	33.7 (13.8–51.8)	0.749 (0.306-1.151)
ACE	–	–	0.911 (0.565–1.246)
DXR	12.5	11.3 (6.8–15.7)	0.902 (0.542–1.256)
CPA	187.5	173.4 (115.7–235.2)	0.925 (0.617–1.254)
ETP	60	54.4 (13.6–75.5)	0.906 (0.227–1.258)

topoisomerase I inhibitors [12]. The reverse effect, in which resistance to a topoisomerase I inhibitor enhanced sensitivity to topoisomerase II inhibitors, has also been reported [13]. In a clinical trial of topoisomerase I and II inhibitors, Masuda et al. [14] evaluated combination chemotherapy with irinotecan and etoposide in patients with relapsed SCLC, and the response rate of 71% far exceeded the response rates of 40–50% previously reported with PE for replased SCLC [15]. Our regimen was also designed to administer topoisomerase I (irinotecan) and II (etoposide) inhibitors alternately. This might have produced the favorable efficacy seen with our alternating chemotherapy in spite of the small sample size and the wide confidence intervals of response rate.

As to the toxicity profile, one of the advantages of our trial was that the incidence of diarrhea, a dose-limiting toxicity of irinotecan, was lower with this regimen than with the PI combination used in the aforementioned phase III trial (44 vs. 70%) [5]. On the contrary, there was a higher incidence of neutropenia in our trial than in the PI combination trial (96 vs. 62%) [5], leading to a higher incidence of febrile neutropenia (30%). However, toxicities were reversible with appropriate supportive care including antibiotics and G-CSF and there were no treatment-related mortalities. Thus, our combination chemotherapy appeared to be well-tolerated.

In conclusion, PI–ACE alternating chemotherapy showed promising antitumor activity, with moderate toxicities, in patients with ED-SCLC.

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

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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.

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Key words: gefitinib, imatinib, irinotecan, topotecan, small-cell lung cancer

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,

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 1×10^6/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 1×10^6/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

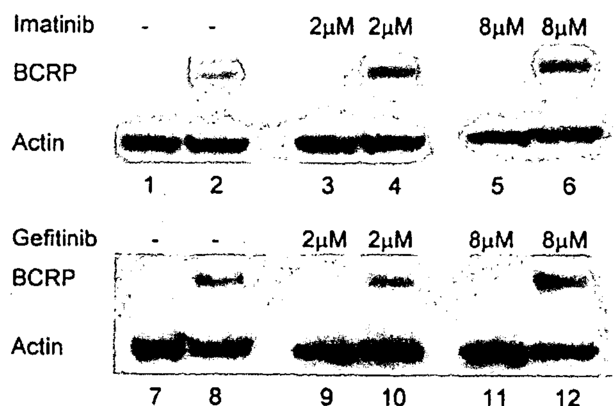

Figure 1. The expression of BCRP in SBC-3 and SBC-3/SN-38 cells treated with gefitinib or imatinib. The overexpression of BCRP in SBC-3/SN-38 is shown in lanes 2 and 8. Imatinib or gefitinib did not affect the BCRP levels in SBC-3/SN-38 (lanes 4, 6, 10 and 12). SBC-3 cells did not have any detectable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

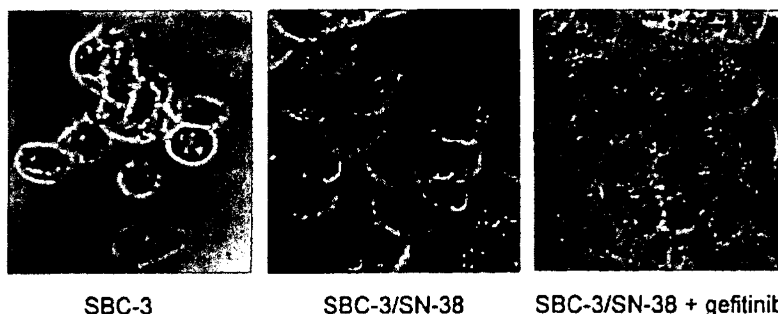

Figure 2. The expression of BCRP in SBC-3/SN-38 cells with 1-h treatment of gefitinib. The BCRP was located on cell membrane in SBC-3/SN-38 and seemed equivalent with and without gefitinib. There was no detectable BCRP expression in SBC-3.

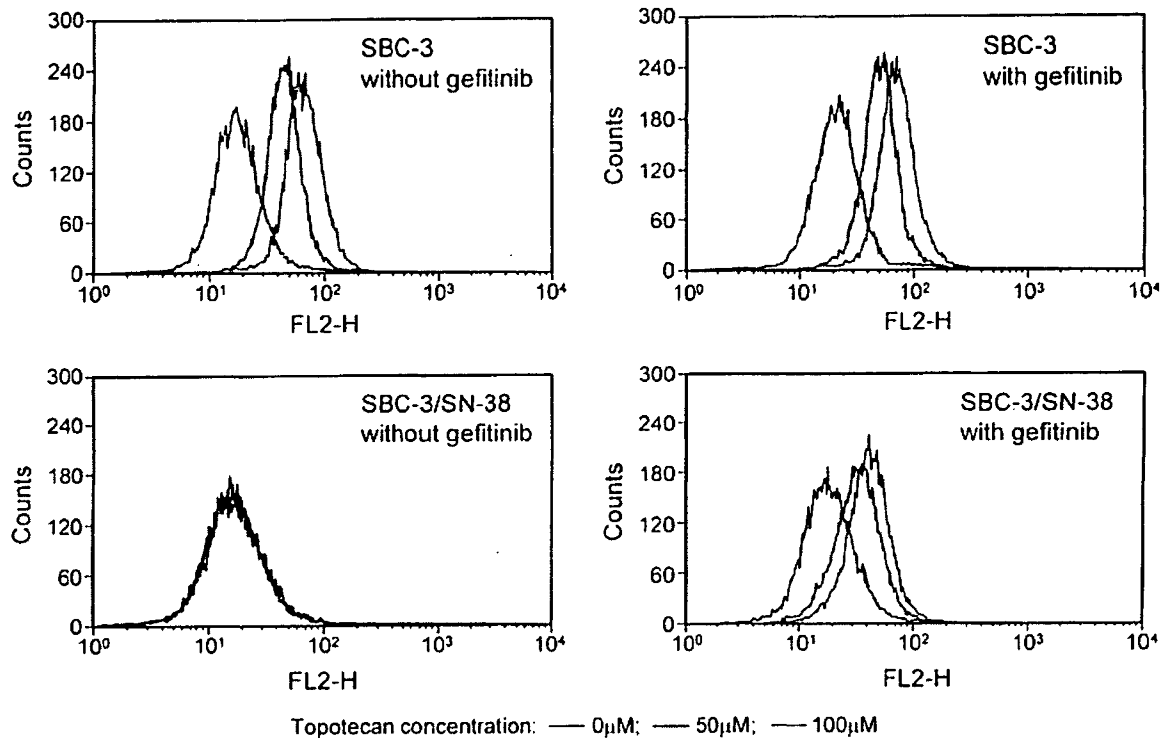

Figure 3. Effect of gefitinib on the intracellular topotecan accumulation. In a dose-dependent manner, topotecan was accumulated in the SBC-3 cells equally despite the addition of gefitinib. There were no differences in the cellular fluorescence of SBC-3/SN-38 cells without gefitinib. However, gefitinib 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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