Table 2 Cox proportional hazards model

Variable	Hazard ratio	95% CI	P-value
Regimen			
CPT-P/VDS-P	0.80	0.60–1.07	0.1341
CPT/VDS-P	0.88	0.66–1.16	0.3557
Stage (IV/IIIB)	1.70	1.32–2.19	0.0001
PS (2/1/0)	1.40	1.08–1.80	0.0098
Gender (male/female)	1.29	0.96–1.73	0.0922
Weight loss (≥5%/<5%)	2.00	1.53–2.60	0.0001
Albumin (<4.0 g dl^{-1}/≥4.0 g dl^{-1})	1.53	1.17–1.98	0.0016
LDH (≥400 IU l^{-1}/<400 IU l^{-1})	1.54	1.17–2.04	0.0021

CI=confidence interval; CPT=irinotecan; P=cisplatin; VDS=vindesine; PS=performance status; LDH=lactic acid dehydrogenase.

Table 3 Objective response

	n	CR	PR	NC	PD	NE	ORR (%)
CPT-P	126	3	52	51	17	3	43.7
VDS-P	120	1	37	54	25	3	31.7
CPT	127	1	25	66	33	2	20.5

n=number of assessable patients, CR=complete response, PR=partial response, NC=no change, PD=progressive disease, NE=not evaluable, ORR=objective response rate, CPT=irinotecan, P=cisplatin, VDS=vindesine.

Table 4 Major adverse reactions

	CPT-P (n=126)	VDS-P (n=120)	CPT (n=127)
Leucopenia (grade 4)	8 (6%)	4 (3%)	2 (2%)
Neutropenia (grade 4)	46 (37%)	65 (54%)	10 (8%)
– Febrile neutropenia	13 (10%)	13 (11%)	8 (6%)
Thrombocytopenia (grade 4)	4 (3%)	1 (1%)	0 (0%)
Anaemia (grade 4)	2 (2%)	1 (1%)	1 (1%)
GOT↑ (grades 3 and 4)	0 (0%)	1 (1%)	0 (0%)
GPT↑ (grades 3 and 4)	1 (1%)	1 (1%)	0 (0%)
BUN↑ (grades 3 and 4)	2 (2%)	2 (2%)	0 (0%)
Creatinine↑ (grades 3 and 4)	0 (0%)	1 (1%)	0 (0%)
Diarrhoea (grades 3 and 4)	15 (12%)	4 (3%)	19 (15%)
Nausea/vomiting (grades 3 and 4)	41 (33%)	27 (23%)	12 (9%)
Infection (grades 3 and 4)	4 (3%)	1 (1%)	6 (5%)

CPT=irinotecan; P=cisplatin; VDS=vindesine; GOT=glutamic oxaloacetic transaminase; GPT=glutamic pyruvic transaminase; BUN=blood urea nitrogen.

Two patients died of causes whose relation to the chemotherapy treatment could not be completely ruled out. A patient who had received VDS-P died because of bleeding from the digestive tract after recovery from myelosuppression. Another patient who had received CPT died owing to subsequent infection associated with severe diarrhoea after recovery from myelosuppression. There were no cases of treatment-related death in the CPT-P arm.

QOL

A total of 82 patients in the CPT-P arm, 65 patients in the VDS-P arm and 74 patients in the CPT arm completed at least one QOL questionnaire. Compliance with the QOL questionnaire was 65% on CPT-P, 54% on VDS-P and 58% on CPT. We could not adequately evaluate QOL for the three arms because compliance with the QOL questionnaire was low. Merely crude analysis suggested no difference among the three arms in terms of QOL.

Second-line treatment

Among patients with stage IIIB disease, 45% on CPT-P, 61% on VDS-P and 59% on CPT were subsequently treated with thoracic irradiation.

Among patients with stage IV disease, 25% on CPT-P, 26% on VDS-P and 40% on CPT were subsequently treated with other chemotherapeutic regimens. In particular, seven patients on CPT-P, nine patients on VDS-P and 10 patients on CPT were subsequently treated with docetaxel (DTX) monotherapy or DTX-containing regimens.

DISCUSSION

Since 1990, newer agents such as CPT-11, paclitaxel (PTX), DTX, gemcitabine (GEM) and vinorelbine (VNB) have shown response rates of 20% or more when used alone in previously untreated NSCLC (Bunn and Kelly, 1998).

Except for CPT-11, all four chemotherapeutic agents have been examined in many phase III trials in patients with advanced NSCLC and their roles have been mostly elucidated. No trial has been conducted, however, in order to establish the role of CPT-11 in advanced NSCLC. The present clinical trial was the first phase III study of CPT-11 in patients with advanced NSCLC. We planned this phase III trial in order to verify (1) whether CPT-P significantly prolongs the survival time and (2) whether CPT alone is not inferior in terms of the survival time, as compared with VDS-P, one of the most commonly used chemotherapeutic regimen that has been used in advanced NSCLC.

The first results showed that CPT-P did not significantly prolong the survival time compared with VDS-P. Previous studies of PTX (Belani *et al*, 1998; Giaccone *et al*, 1998; Bonomi *et al*, 2000), DTX (Kubota *et al*, 2002), GEM (Crino *et al*, 1999) and VNB (Le Chevalier *et al*, 1994; Martoni *et al*, 1998) were for comparison of a platinum compound plus a new agent *vs* an old platinum-based chemotherapy. Only the studies by Le Chevalier *et al* (1994) and Kubota *et al* (2002) reported significant differences in terms of survival based on the analysis scheduled in the protocol. Similar to the present trial, there was no significant prolongation of the survival time in other studies. There were meta-analyses of these results that reported the superiority of the regimen of platinum compounds plus a new agent (Baggstrom *et al*, 2002; Yana *et al*, 2002).

The second results showed that CPT-11 alone was not inferior to VDS-P in terms of the survival time. In a study of Le Chevalier *et al* (1994) that compared three groups (VNB and CDDP *vs* VDS and CDDP *vs* VNB alone), similar to the present trial, the survival was also similar between VNB alone and VDS-P. Toxicity on CPT alone was mild except for slightly frequent diarrhoea. Based on these results, chemotherapy with the new single agent may be considered for patients who are unable to receive CDDP-containing regimens because it can produce the similar survival to conventional platinum-based combinations, and toxicity is relatively mild.

In the subgroup analyses according to the stage, in stage IV patients, the MST was 50.0 weeks on CPT-P and 36.4 weeks on VDS-P, with the CPT-P being superior to the VDS-P with respect to survival prolongation (one-sided, log-rank test: $P=0.004$). CPT-11 alone also significantly prolonged the survival time in patients with stage IV NSCLC compared with VDS-P. Among stage IV patients, there was no bias in terms of background factors including PS (the ratio of PS 0–1 patients was 96% on CPT-P, 92% on VDS-P and 94% on CPT), gender (male ratio was 69% on CPT-P, 79% on VDS-P and 68% on CPT) and the rate of weight loss at baseline (the ratio of patients without weight loss was 63% on CPT-P, 58% on VDS-P and 55% on CPT). There was no bias in the content of second-line treatment among the treatment arms. These are just the results of the subgroup analyses and their significance should be shown in another research.

The results of subgroup analyses differed between stage IIIB and stage IV patients. Between-arm differences in the ratio of patients receiving second-line chest irradiation might, in part, lead to such differences. In the present trial, there was no eligibility criterion to restrict stage IIIB patients for whom thoracic irradiation was indicated. Therefore, the possibility could not be ruled out that the present trial included patients who were not suitable for research purely comparing chemotherapeutic agents as in the case of this trial. This might be one reason for different results between stages IIIB and IV patients in the subgroup analyses. In future research, the inclusion of stage IIIB patients should be limited to patients for whom definitive thoracic radiation is not indicated.

Comparing survival between CPT-P and CPT-11 alone, although this was not planned, no significant difference was shown between the two arms (one sided, log-rank test: $P = 0.587$). The hazard ratio for CPT *vs* CPT-P was 1.03 (95% CI: 0.79 – 1.34).

The overall response rate in this study was 43.7% on CPT-P, 31.7% on VDS-P and 20.5% on CPT (two-sided, χ^2 test: $P < 0.001$). This significant difference in the response rate did not lead to significant differences in the survival time. Similar results have been frequently reported in comparative studies of chemotherapeutic agents in advanced NSCLC. Various known and unknown prognostic factors were involved in this result. Of them, the major factor is that complete response (CR) is obtained only in small numbers of NSCLC patients receiving chemotherapy. In the present trial, the CR rate was just 2.4% (three out of 126) in the CPT-P arm, 0.8% (one out of 120) in the VDS-P arm and 0.8% (one out of 127) in the CPT arm.

The present trial allowed including patients with PS 2. This resulted in the inclusion of approximately 6% of PS 2 patients in each treatment arm. In a large-scale ECOG study (E1594) by Schiller *et al* (2002), the accrual of patients with PS 2 was discontinued owing to the high rate of serious adverse events. This fact was disclosed in May 1999 (Johnson *et al*, 1999), and our trial completed patient registration before this time, so it was impossible to exclude PS 2 patients. Inclusion of PS 2 patients had no significant impact on the results of the present trial because the number of such patients was small and they were distributed evenly to the three arms according to baseline demographic factors.

Neutropenia and neurotoxicity were more frequent in the non-CPT-containing VDS-P arm, than in the CPT-containing CPT-P and CPT arms. Nausea/vomiting was more frequent in the CDDP-containing CPT-P and VDS-P arms, than in the non-CDDP-containing CPT arm. Diarrhoea was more frequent in the CPT-containing arm than in the VDS-P arm. In the present trial, there were no deaths associated with the treatment in the CPT-P arm, but one patient in the CPT arm developed diarrhoea after recovery from myelosuppression and subsequently died from infection. In this patient, it was not clear whether the source of infection was the bowel or the tip of the catheter. In 10 patients treated with the regimen containing CPT-11 who developed grade 3 or higher infection, seven patients developed diarrhoea coincidentally with myelosuppression, which suggested enteritis as the source of infection. Infection was avoided in many patients by taking proactive measures against aggravation of diarrhoea and taking early anti-infection measures.

In conclusion, the response rate was significantly higher in patients with CPT-P therapy compared with VDS-P, and the survival time and toxicity were comparable, although this therapy failed to demonstrate significant survival prolongation. Based on these results, a large-scale randomised phase III trial comparing CPT-P as the control regimen with three new drugs (NVB, PTX, GEM) containing platinum-based doublets is currently underway in Japan. CPT-11 monotherapy produced survival result that was not inferior to that obtained with VDS-P, but produced less toxicity. Further studies are necessary to determine the significance of CPT-11 monotherapy in the treatment of NSCLC. This randomised phase III trial has demonstrated that the regimen containing CPT-11 is one of the most active and well tolerated in the treatment of advanced NSCLC.

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APPENDIX

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Original article

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Phase I pharmacokinetic trial of the selective oral epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa', ZD1839) in Japanese patients with solid malignant tumors

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Background: This phase I dose-escalating study investigated the tolerability and toxicity of the selective epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa', ZD1839) in Japanese patients with solid tumors. Thirty-one patients were included.

Patients and methods: Patients initially received a single oral dose of gefitinib followed by 10–14 days of observation. Oral gefitinib was subsequently administered on 14 consecutive days, every 28 days. Dose escalation was from 50 mg/day to a maximum of 925 mg/day or dose-limiting toxicity (DLT).

Results: Most adverse events were mild (grade 1/2); the most frequent were an acne-like rash and gastrointestinal effects. Two of six patients at 700 mg/day had DLT; no further dose escalation occurred. C_{max} was reached within 3–7 h and exposure to gefitinib increased with dose. Mean terminal half-life following multiple dosing was 50.1 h (range 27.8–79.7 h). A partial response (duration 35–361 days) was observed in five of the 23 patients with non-small-cell lung cancer over a range of doses (225–700 mg/day), and seven patients with a range of tumors had disease stabilization (duration 40–127 days).

Conclusions: In conclusion, gefitinib showed a favorable tolerability profile in Japanese patients. The safety profile, pharmacokinetic parameters and antitumor activity observed in our study are comparable to those observed in patients from the USA and Europe.

Key words: efficacy, EGFR inhibitor, gefitinib, 'Iressa', phase I trial, tolerability, ZD1839

Introduction

Specific inhibition of epidermal growth factor receptor (EGFR) function is an attractive therapeutic target in anticancer treatment. Potential new therapies are under development that modulate the activation of this signal transduction pathway, resulting in inhibition of mitogenesis and other cancer-promoting processes [1]. The extracellular ligand-binding region of the EGFR has been targeted by monoclonal antibodies such as cetuximab [2], while agents that target the intracellular tyrosine kinase region include small-molecule tyrosine kinase inhibitors (TKIs) such as gefitinib ('Iressa', ZD1839) [3] and erlotinib [4]. Advantages of these compounds compared with standard chemotherapy include their ability to inhibit specific deregulated pathways in cancer cells with minimal effects on normal cell function. This class of agents may therefore offer antitumor activity with a better-tolerated adverse event profile than traditional agents.

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The rationale for targeting the EGFR comprises several key points. Activation of the EGFR tyrosine kinase has been found to be a key factor in cell proliferation and has been implicated in the control of cell survival, decreased apoptosis and increased metastasis [5]. Furthermore, the EGFR is expressed or highly expressed in a wide variety of human solid tumors, and high-level expression has been associated with advanced disease, development of a metastatic phenotype and poor prognosis [6, 7].

Gefitinib is an orally active, selective EGFR-TKI that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting cancer cell growth. Early preclinical studies indicated that, *in vitro*, gefitinib potently inhibited EGFR tyrosine kinase activity at low concentrations that did not significantly affect other kinases tested [8]. Preclinical toxicology studies showed gefitinib to have a favorable tolerability profile over 6 months of oral dosing in animals, with mechanism-based, dose-dependent reversible effects on the skin, cornea, kidney, liver and ovary [9]. This range of toxicity is explained by the fact that EGFR signal transduction is involved in the normal physiology of these organs. Gefitinib has been shown to inhibit growth of a range of human tumor cell lines expressing EGFR (IC_{50} 0.2–0.4 μmol/l) when used as a single

agent, and to potentiate the activity of cytotoxic agents [10]. It also showed antitumor activity in various xenograft models [10, 11]. Gefitinib was well tolerated in healthy volunteers and demonstrated a terminal half-life of 28 h, which suggests that once-daily oral administration is appropriate [12].

Our study was performed to investigate the safety and pharmacokinetics of gefitinib in Japanese patients and to enable a comparison between Japanese patients and non-Japanese patients participating in a parallel study in the USA and Europe [13] in accordance with regulatory requirements. It was principally designed to evaluate the tolerability of increasing oral doses of gefitinib in Japanese patients with solid malignant tumors, using an intermittent dosing schedule to ensure the patients' safety.

Patients and methods

Trial design

We conducted an open, multicenter, non-randomized, phase I, dose-escalating study, recruiting patients at four centers in Japan. Our primary objective was to investigate the tolerability and toxicity of single and multiple oral doses of gefitinib in patients with solid malignant tumors. Secondary objectives included assessment of pharmacokinetics and antitumor activity. In addition, we compared our pharmacokinetic results with those from patients taking part in a parallel study in the USA and Europe.

Patient eligibility

We enrolled patients with solid malignant tumors that were resistant to standard therapies or for which the investigator believed no appropriate treatment was available. Tumors were among those known to commonly express or overexpress EGFR, but patients were not selected on the basis of individual EGFR status. Patients aged 20–74 years with a life expectancy of ≥3 months and World Health Organization (WHO) performance status of ≤2 were eligible for inclusion. Exclusion criteria have been described previously [13].

Prior to initiation of the study, we recorded information on each patient's background and treatment history and conducted assessments, including a physical examination, vital signs, performance status, clinical laboratory tests (hematology, blood biochemistry, urinanalysis, fecal test), ophthalmologic assessments and 12-lead ECG with measurement of PR intervals. All patients gave written informed consent and the study was conducted in accordance with 'Good Clinical Practice for Trials on Drugs' [14] and the 'Declaration of Helsinki' [15].

Treatment

In the first part of the trial, patients received a single oral dose of gefitinib, followed by 10–14 days of observation. If drug exposure was well tolerated, patients progressed to the second part of the study and received the same dose repeated daily for 14 days, followed by 14 days of observation (one cycle), based on advice from the Efficacy and Safety Evaluation Committee. A parallel study in the USA and Europe was conducted in a similar dose-escalation manner (except that the single dose was given only at the 50 mg dose level) and we compared, on an ongoing basis, pharmacokinetic data following single and multiple dosing in this study with data from our study to determine whether similar dose dependency was observed in Western and Japanese patients and to consider whether prior single dosing to establish safety was necessary at each dose level.

The 50 mg starting dose was chosen on the basis of preclinical animal toxicology studies and two clinical studies in healthy volunteers. The Western volunteer studies at single doses up to 100 mg/day showed that the maximum plasma concentration (C_{max}) and the area under the plasma concentration–time curve from 0 to 24 h (AUC_{0-24}) increase linearly with dose. Provided the disposition of gefitinib in our patient population is similar to that in the healthy Caucasian volunteers, C_{max} and AUC_{0-24} following the initial single 50 mg dose will be ~17 ng/ml and 220 ng·h/ml, respectively. These values represent approximately one-third and one-fifth, respectively, of the exposure at the no-effect dose in rats, the most sensitive species, in the 28-day toxicology studies (2 mg/kg/day). Following multiple dosing at 50 mg for 14 consecutive doses, predictions from the Western volunteer data suggest that the steady-state C_{max} and AUC_{0-24} will be ~35 ng/ml and 500 ng·h/ml, respectively. These values represent approximately one-fifteenth and one-tenth, respectively, of the exposure at the NOAEL (no observed adverse effect level; 10 mg/kg/day) in the 28-day toxicology studies in rats. The single dose of 50 mg/day caused no clinically significant adverse effects in the volunteers.

We planned to escalate the dose to 100, 150, 225, 300, 400, 525, 700 and 925 mg/day, with the option to omit dose levels following consideration of the results of the parallel USA/European study. We initially entered four patients at each dose level, but if National Cancer Institute-Common Toxicity Criteria (NCI-CTC 2.0) grade 3 or 4 drug-related toxicity occurred in one of these patients, we enrolled two additional patients. Dose-limiting toxicity (DLT) was defined during the first treatment period as any grade 3/4 drug-related adverse effect, significant corneal epithelial change or PR interval (measured by 12-lead ECG) prolongation attributed to gefitinib (as prolongation of the PR interval was noted in preclinical animal toxicity studies). The dose at which DLT occurred in more than two patients was defined as the maximum tolerated dose (MTD). Dose escalation took place following a review of safety data when all patients at a dose level reached day 28 or had been removed from the trial due to drug-related toxicity, and following consideration of the results from the Western study.

Appropriate supportive care measures and symptomatic treatment were permitted, as was prophylactic use of antiemetics during the second and subsequent cycles, but not during the first 28 days. Any grade 3/4 nausea that was not readily managed with antiemetics was classed as DLT.

Following completion of the first 14 days of treatment and 14 days of observation, patients demonstrating clear clinical benefit could remain on gefitinib (14-day treatment period, every 28 days) if there was no drug-related DLT and if they fulfilled eligibility criteria. Gefitinib treatment was discontinued in the event of ocular toxicity, cardiac conduction defects, disease progression, any DLT, withdrawal of consent or if it was in the patient's best interest to discontinue treatment. Following withdrawal, patients were monitored for 30 days for reversibility of drug-related adverse effects or occurrence of new adverse effects. No intra-patient dose escalation or reduction was allowed.

Safety assessments

We recorded the incidence, type and severity of adverse effects at each dose level. If grade 3/4 myelosuppression was observed, we carried out hematology assessments at least every 2 days until values returned to grade 1/2. All clinical laboratory tests were conducted at screening, pre-first dose, 24 h after the single dose, on days 1, 3, 8, 15, 22 and 29, and at withdrawal. Ophthalmologic assessments, including slit-lamp examination, were carried out at screening, 48 h after the single dose, on days 8, 15 and 22 and at withdrawal. The other safety parameters were also reassessed periodically throughout the trial.

Pharmacokinetic analysis

During the first cycle, plasma concentrations of gefitinib were determined from blood samples (4 ml) using liquid–liquid extraction and high-performance liquid chromatography with mass spectrometric detection [16]; pharmacokinetic parameters were calculated by standard methods. Blood samples were taken pre-first dose, and at 1, 3, 5, 7, 12, 24, 48, 72, 96, 120 and 144 h after administration of the single dose. The same sampling times were used following the last dose of the multiple dosing administration (i.e. day 14) and samples were also taken pre-dose on days 1, 3, 7 and 10 during multiple dosing.

Derived pharmacokinetic parameters included the maximum plasma drug concentration (C_{max}), time to C_{max} (t_{max}), area under the plasma drug concentration–time curve from 0 to 24 h (AUC_{0-24}) and to ∞ ($AUC_{0-\infty}$) and terminal half-life ($t_{1/2}$).

Efficacy assessments

We assessed tumor response in accordance with the 'criteria for direct response of solid tumors to chemotherapy' of the Japan Society for Cancer Therapy, which are very similar to those used by the WHO. Measurable lesions were assessed in the same manner before and after dosing, by X-ray, computed tomography (CT) scan, magnetic resonance imaging or echogram. Complete response, partial response or no change in disease status had to be confirmed by a second assessment after 4 weeks.

Serum tumor markers including carcinoembryonic antigen (CEA), prostate-specific antigen, CA 125, CA 19-9, squamous-cell carcinoma-related antigen and thyroglobulin were recorded in patients with relevant tumor types, at screening, on days 1, 8, 15 and 29, and at withdrawal.

Results

Patients

We recruited 31 patients, all of whom had received prior chemotherapy. The median age was 61 years and all patients had a WHO performance status of 0–1 (Table 1). Most patients (74%) had advanced non-small-cell lung cancer (NSCLC) and had been pretreated with chemotherapy (1–4 regimens); 70% of these had received platinum-based regimens.

Of the 31 patients, 30 completed one cycle of treatment (28 days); eight completed ≥2 cycles and six ≥3 cycles. One patient (50 mg group) was withdrawn due to grade 3 atrial fibrillation on day 2 of cycle 1 (due to respiratory failure associated with disease progression); therefore, another patient was added to this group in order that we could collect data on plasma gefitinib concentrations from four patients.

Table 1. Patient demographics

No. of patients	31
Male/female	19/12
Median age (range), years	61 (40–73)
<65	21
≥65	10
WHO performance status	
0	9
1	22
Prior chemotherapy	31
Prior hormonal therapy	0
Prior radiotherapy	13
Prior chemotherapy and radiotherapy	13
Tumor type	
Non-small-cell lung	23
Adenocarcinoma	18
Squamous	4
Poorly differentiated adenocarcinoma	1
Colorectal	5
Head and neck	2
Breast	1

Based on the safety data from the parallel USA/European study, it was judged unnecessary to repeat the 150 and 300 mg dose levels in Japan. In our study, patients in the 50, 100 and 225 mg dose-level groups received the single dose plus the 14-day daily dose. Following comparison of our data with those from the Western study, the pharmacokinetic and adverse effect profiles were found to be similar in the two populations; therefore, the initial single dose was omitted for patients in the 400, 525 and 700 mg dose-level groups.

Dose-limiting toxicity

The highest dose administered was 700 mg/day with two of six patients experiencing DLT [grade 3 diarrhea (one patient) and grade 3 elevation of alanine aminotransferase (ALT; one patient)]. Grade 3 drug-related adverse effects were observed in two additional patients: elevated transaminases in one patient each at 225 and 525 mg/day. Consequently, two additional patients were enrolled, so that a total of six patients were treated at these dose levels. No dose level other than 700 mg/day had more than one of six patients with DLT. No additional DLT occurred.

Tolerability

All 31 patients were evaluable for tolerability and safety. The majority of adverse effects were mild (grade 1/2) and reversible on cessation of treatment. The most frequently reported adverse effects included gastrointestinal tract disorders (77.4%; including diarrhea, nausea, vomiting, anorexia), skin reactions (74.2%; including acne-like rash, seborrhea, dry skin) and increased hepatic enzymes.

Drug-related adverse effects occurring in ≥10% of the patient population are detailed by dose level in Table 2. The most common drug-related adverse effects were grade 1/2 acne-like rash and seborrhea, observed in 32.3 and 22.6% of patients, respectively. Acne-like rash (or folliculitis) covers descriptions such as maculopapular and pustular. These skin disorders tended to occur more often with higher doses of gefitinib and were resolved without treatment or with symptomatic treatments. They tended to recur after the start of treatment with gefitinib in subsequent cycles and often disappeared on cessation of treatment.

Drug-related diarrhea was observed in 19.4% of patients. At doses up to 525 mg/day it was grade 1/2 (loose stools occurring 2–3 days a week) and manageable with routine treatment. Diarrhea generally occurred within the first 2 weeks of a treatment cycle. Drug-related nausea and vomiting were observed in six and four patients, respectively, with most adverse effects resolving on the day of onset or within a few days after treatment cessation with or without adverse effect management.

Drug-related increases in the hepatic transaminases ALT and aspartate aminotransferase (AST) were each seen in 19.4% of patients, and drug-related increases in alkaline phosphatase were seen in 16.4% of patients.

Table 2. Number of patients with drug-related adverse events that occurred in ≥10% of the patient population in all cycles

Gefitinib dose (mg/day)	50		100		225		400		525		700		All		Total	%
No. of patients	5		4		6		4		6		6		31			
NCI-CTC grade	1/2	3/4	1/2	3/4	1/2	3/4	1/2	3/4	1/2	3/4	1/2	3/4	1/2	3/4		
Adverse event																
Acne-like rash	0	0	1	0	0	0	2	0	4	0	3	0	10	0	10	32.3
Seborrhea	0	0	0	0	0	0	2	0	1	0	4	0	7	0	7	22.6
Diarrhea	0	0	0	0	0	0	2	0	1	0	2	1	5	1	6	19.4
Anorexia	0	0	0	0	0	0	1	0	2	0	3	0	6	0	6	19.4
Nausea	0	0	1	0	0	0	1	0	2	0	2	0	6	0	6	19.4
AST/SGOT increased	0	0	0	0	0	1	1	0	2	1	1	0	4	2	6	19.4
ALT/SGPT increased	0	0	0	0	0	1	1	0	2	1	0	1	3	3	6	19.4
Alkaline phosphatase increased	0	0	0	0	0	0	1	0	1	0	3	0	5	0	5	16.1
Vomiting	0	0	0	0	1	0	0	0	2	0	1	0	4	0	4	12.9

ALT, alanine aminotransferase; AST, aspartate aminotransferase; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

Following frequent and specific ophthalmologic tests, adverse effects were reported in 14 patients (45.2%). Five patients had conjunctivitis (recorded as conjunctivitis, conjunctival congestion and conjunctival epithelial disorder), four had corneal disease (corneal epithelial disorder, corneal erosion and abnormal Rose Bengal staining), keratitis and blepharitis were reported in three patients each and two patients had eye disorders. All ophthalmological events were mild (grade 1/2). In six patients the adverse effects were considered to be drug-related; they resolved in five cases without intervention and in one case following treatment with eye drops (ofloxacin and flavin adenine dinucleotide). Drug-related hematological toxicity occurred in four patients (12.9%) over a range of doses (100–400 mg/day) and was limited to anemia, leukopenia and eosinophilia, all mild in nature (grade 1/2). One patient (gefitinib 400 mg/day) had drug-related prolongation of the PR interval (grade 1), which recovered without intervention. One patient (gefitinib 525 mg/day) had mild alopecia, which also resolved without intervention.

Grade 3/4 adverse effects

No grade 4 adverse effects were observed. A total of 19 grade 3 adverse effects was reported in eight patients. Six of these, in four patients, were considered to be drug related; elevated AST and ALT in one patient each at the 225 and 525 mg/day dose levels, elevated ALT in one patient at 700 mg/day and diarrhea in one patient at 700 mg/day. The grade 3 diarrhea appeared 5 days after withdrawal of treatment. The grade 3 drug-related increases in hepatic enzymes resolved, either with or without treatment. In two of these patients onset of increased hepatic enzymes occurred on day 8 of the second treatment cycle; one (gefitinib 225 mg/day) recovered to normal range within 3 weeks while the other (gefitinib 525 mg/day) recovered 5 days after gefitinib treatment was withdrawn. In the third patient (gefitinib 700 mg/day), onset of increased hepatic enzymes occurred on day 21 of the first cycle (during the treatment interval) and recovered within 1 week. The grade 3 adverse effects in the remaining four patients were considered to be disease related.

Withdrawals

In addition to patients withdrawn from the trial due to progressive disease, four patients were withdrawn due to adverse effects. A female patient with NSCLC with multiple lung micrometastases receiving the 525 mg/day dose was withdrawn due to grade 3 increased transaminases (AST and ALT) on day 8 of cycle 2. AST returned to normal levels without medical intervention, as did serum ALT 54 days after withdrawal. These events were considered to be related to gefitinib. A male patient with rectal cancer, receiving 700 mg/day, withdrew due to grade 2 diarrhea during cycle 3 (day 7), which resolved after 3 days. The patient experienced grade 3 diarrhea 2 days later and, after a further 2 days, had dehydration associated with grade 2 diarrhea. He was hospitalized 8 days after withdrawal and treated with fluid replacement; the diarrhea and dehydration resolved within 2 days. Diarrhea in this patient was judged to be drug related. A male patient with NSCLC was withdrawn due to grade 3 atrial fibrillation judged to be caused by increased heart burden due to respiratory failure associated with progression of primary NSCLC. A male patient with colorectal cancer receiving gefitinib 225 mg/day was withdrawn due to grade 1 infectious keratoconjunctivitis during cycle 2, which was considered by the investigator to be related to adenovirus infection. In this trial, a total of seven patients remained on study for ≥3 months and three for ≥12 months.

Pharmacokinetics

The derived pharmacokinetic parameters following a single dose and multiple dosing of gefitinib are given in Table 3. At the starting dose of 50 mg/day, absorption of gefitinib was moderately slow, median t_{max} being 3 h from the single dose (range 3–5 h). For the dose range 50–225 mg/day, the mean $t_{1/2}$ was similar for all

Table 3. Derived pharmacokinetic parameters following single dose (50, 100 and 225 mg) and 14 days of multiple dosing of gefitinib

Gefitinib dose (mg/day)	No. of patients	Geometric mean (% CV) C_{max}, ng/ml	Median (range) t_{max}, h	Geometric mean (% CV) $AUC_{0-\infty}$, ng·h/ml	Geometric mean (% CV) AUC_{0-24}, ng·h/ml	Mean (SD) $t_{1/2}$, h
Single dose						
50	5	31 (42)	3 (3–5)	948 (85)	378 (51)	38 (11)
100	4	43 (69)	4 (3–7)	1228 (54)	531 (52)	35 (7)
225	6	150 (92)	4 (3–12)	4623 (43)	1986 (58)	30 (5)
Multiple doses						
50	4	60 (89)	6 (5–7)	3070 (145)	1021 (89)	52 (19)
100	4	105 (35)	5 (5–7)	5258 (58)	1860 (41)	45 (18)
225	6	341 (61)	5 (3–7)	13 868 (77)	5191 (61)	40 (8)
400	4	779 (54)	3 (3–7)	29 691 (138)	11 399 (68)	45 (21)
525	6	850 (71)	5 (3–7)	65 055 (82)	16 350 (65)	59 (10)
700	6	1156 (48)	5 (3–7)	75 620 (66)	21 580 (49)	55 (14)

CV, coefficient of variation; SD, standard deviation.

single doses (30–38 h). Single doses higher than 225 mg/day were not given.

Figure 1 shows the mean plasma concentration–time profile following single and multiple dose administration of 50, 100 and 225 mg/day gefitinib. Multiple dosing resulted in at least a two-fold increase in C_{max} compared with single dosing; for the 50 mg/day dose, exposure (C_{max}) to gefitinib increased two-fold following 14 days of administration compared with the single dose (60 versus 31 ng/ml, respectively) and median t_{max} was 6 h (range 5–7 h).

Multiple dosing with gefitinib (50–700 mg/day) for 14 days resulted in dose-related increases in mean C_{max} (from 60 to 1156 ng/ml) and mean AUC_{0-24} (from 1021 to 21 580 ng·h/ml). Figure 2 shows the mean plasma concentration–time profile following multiple dose administration of 225 and 525 mg/day gefitinib. Day-14 AUC_{0-24} values across the dose range indicate an increase in exposure to gefitinib with dose, with up to six-fold interpatient variability at each dose level (Figure 3). Steady-state plasma concentrations were achieved by days 7–10 at all doses (Figure 4). The mean $t_{1/2}$ across the range 50–700 mg/day was 50.1 h (range 27.8–79.7 h).

Pharmacokinetic parameters were comparable with results from the parallel study in the USA and Europe [13]; for example, Figure 5 compares multiple dose AUC_{0-24} values for the two patient populations. We did not analyze the relationship between pharmacokinetic parameters and toxicity due to the limited sample size.

Antitumor activity

We observed partial responses (duration 35–361 days) in five of the 23 patients with NSCLC. The five patients had adenocarcinoma histology (Table 4) and were receiving a range of gefitinib doses. The first patient, a 51-year-old woman receiving gefitinib 225 mg/day, had previously shown no change in disease status as best response, then progressed on platinum-based combination therapy for 3 months, then progressed following a best response of no change in disease status on cyclophosphamide/etoposide/tegafur–uracil for 1.5 months. Her partial response was observed at the end of cycle 1 and was sustained for 119 days. The second patient, a 63-year-old woman (400 mg/day), had previously had no change in disease status as best response, then progressed on treatment with bleomycin, cisplatin/etoposide and etoposide for 1 month each. Her partial response, initially observed at the end of cycle 2, had a response duration of 361 days. This patient also had a fall in CEA levels over 13 cycles. The third patient was a 70-year-old woman (gefitinib 525 mg/day) who had previously had progressive disease following cisplatin/etoposide/radiotherapy (3 months) followed by no change in disease status as best response, then progression on docetaxel treatment (6 months). This patient experienced a partial response from the end of cycle 1 that was sustained for 35 days. Also at this dose level, a 68-year-old woman who had previously had no change in disease status as best response, then progressed on vindesine/mitomycin C/cisplatin (10 months) followed by docetaxel (2 months) and irinotecan/docetaxel (3 months) had a partial response first observed at the end of cycle 2 (response duration 340 days). This patient demonstrated a partial response on a CT scan (Figure 6A), with a reduction in lesion size visible after 4 months of treatment, and also had a reduction in CEA levels over 12 cycles. This patient continued to receive gefitinib (500 mg/day) for a further 6 months in an open-label extension study (20 months on gefitinib in total). The fifth patient with a partial response (observed from cycle 3; response duration 307 days) was a 67-year-old man receiving gefitinib 700 mg/day, who had previously shown no change in disease status as best response, then progressed after 3 months of treatment with irinotecan/docetaxel. The CT scan demonstrating this partial response is shown in Figure 6B, with a reduction in tumor size visible after 3 months of treatment. This patient continued to receive gefitinib (500 mg/day) for a further 6 months in the open-label extension study (18 months on gefitinib in total).

An additional seven patients [colorectal cancer (three patients), NSCLC (two patients), head and neck cancer (one patient), breast cancer (one patient)] had no change in disease status as their best response (duration 40–127 days). Three of these patients remained

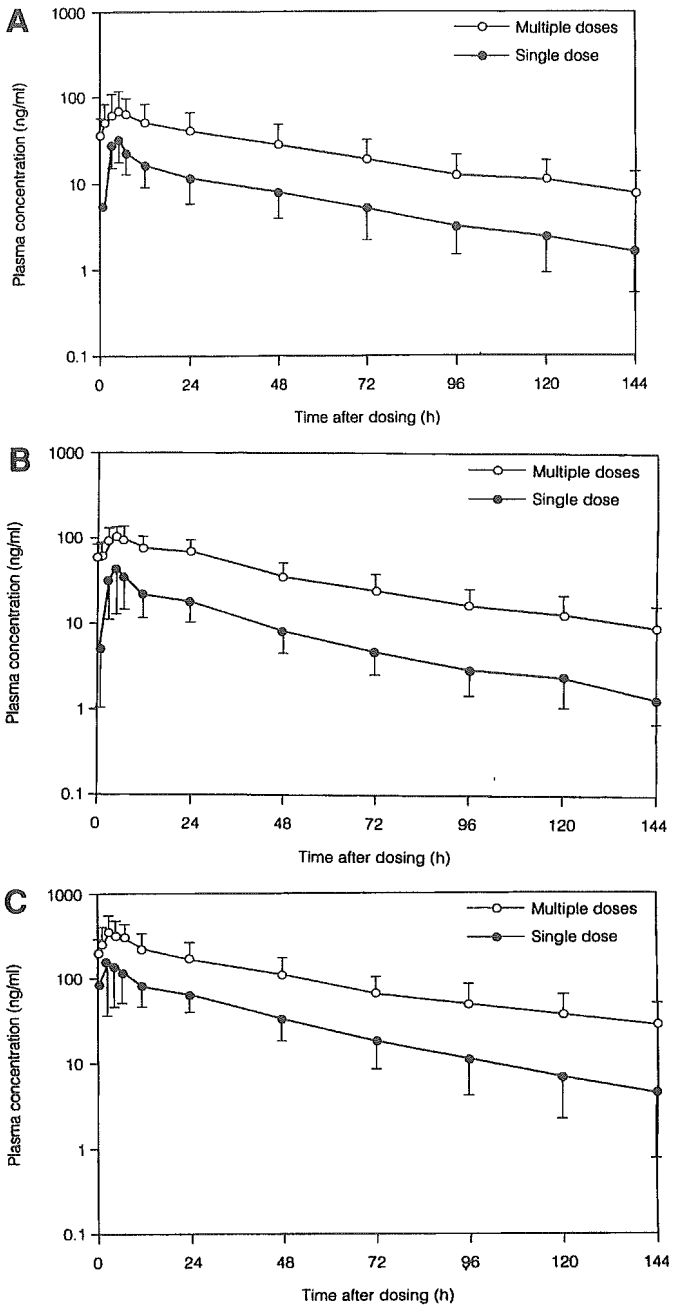

Figure 1. Mean plasma concentration–time profile for gefitinib single dose and multiple doses at (A) 50 mg, (B) 100 mg and (C) 225 mg.

on study for ≥3 months. One of the patients with colorectal cancer (gefitinib 700 mg/day) experienced a considerable fall in CEA and CA 19-9 levels over three cycles.

Discussion

Our study demonstrates that once-daily oral gefitinib, administered for 14 consecutive days every 28 days, has an acceptable tolerability profile in Japanese patients with solid malignant tumors. The safety profile observed in Japanese patients is comparable to that observed in patients from the USA/European phase I trial of gefitinib using similar dose administration and escalation

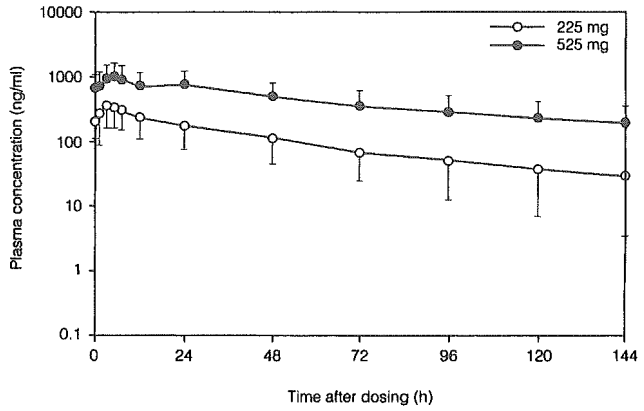

Figure 2. Mean plasma concentration–time profile for gefitinib multiple doses at 225 and 525 mg.

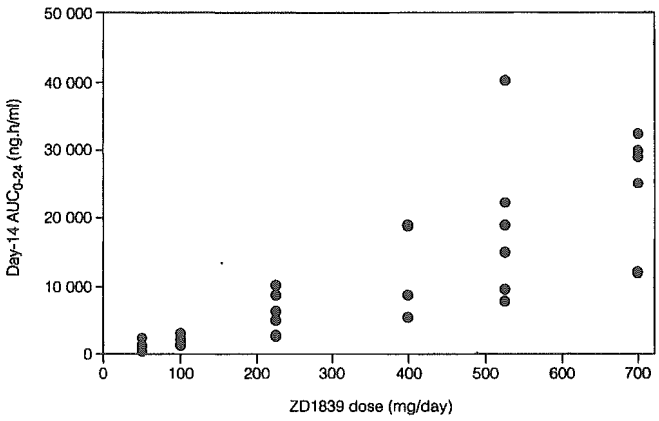

Figure 3. Relationship between exposure to gefitinib (AUC_{0-24}) and dose (mg/day).

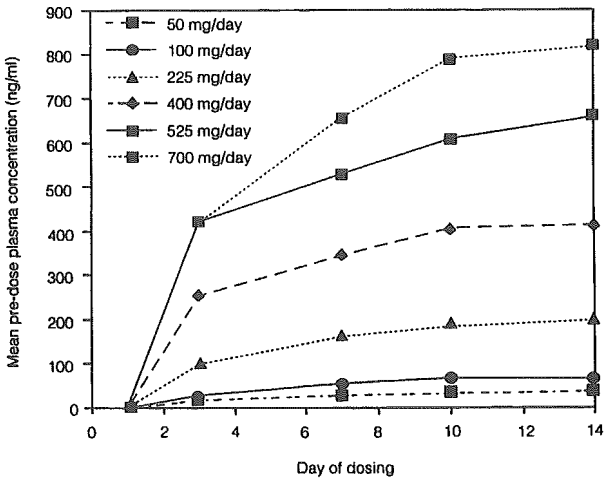

Figure 4. Mean pre-dose plasma concentrations of gefitinib during the multiple dosing phase.

schedules [13]. The incidence of reported drug-related adverse effects was similar in the two studies: 77.4% of patients in our study and 75% in the parallel study, with most adverse effects being grade 1 or 2 in severity. Toxicity increased with dose in both studies and dose escalation stopped at 700 mg/day, with grade 3 diarrhea and increased ALT being the DLTs at this dose.

Table 4. Patients with partial response (PR) or no change in disease status

Gefitinib dose (mg/day)	Tumor type	Response	Duration of PR (days)	Time to progression (days)
50	NSCLC (adeno)	No change	–	58
100	Colorectal	No change	–	43
225	NSCLC (adeno)	Partial response	119	144
	Colorectal	No change	–	57
	NSCLC (squamous)	No change	–	87
	Head and neck	No change	–	40
400	NSCLC (adeno)	Partial response	361	410
525	NSCLC (adeno)	Partial response	35	49[a]
	NSCLC (adeno)	Partial response	340	396[a]
700	NSCLC (adeno)	Partial response	307	362[a]
	Breast	No change	–	127
	Colorectal	No change	–	85

Adeno, adenocarcinoma; NSCLC, non-small-cell lung cancer.
[a]Patients with data cut-off.

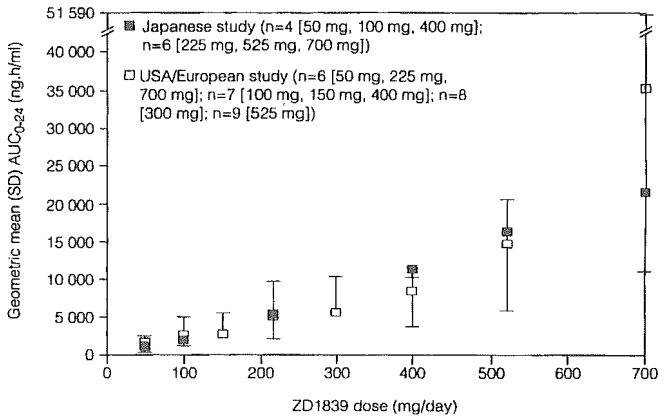

Figure 5. Relationship between dose and exposure in Japanese patients and in USA/European patients after the final dose of the first multiple dosing cycle.

In both our trial and the USA/European trial, among the most frequently reported drug-related adverse effects at doses ≤525 mg/day were an acne-like rash and gastrointestinal adverse effects. The incidence was similar in the two trials for these and other drug-related adverse effects. Results from studies evaluating chronic daily administration of gefitinib also support the conclusions of the present study, that gefitinib is generally well tolerated, with the most frequent adverse effects being grade 1 or 2 skin and gastrointestinal effects [17, 18]. In common with this study, an acne-like rash has been reported as the most common adverse effect of treatment with other EGFR-targeted treatments, including the anti-EGFR antibody cetuximab [19] and the EGFR-TKI erlotinib [4, 20–22], which was also associated with gastrointestinal effects. In our study, all skin-related adverse effects were grade 1/2 and manageable, in some cases without intervention. Similarly, hepatic enzyme elevations resolved with or without management. In our study there were no severe ophthalmological events and all adverse effects considered by the investigators to be possibly related to gefitinib were reversible. Further study will be required in more patients to determine whether any of the observed ophthalmological effects are due to gefitinib. With such intense monitoring in this elderly, ill population, the significance of these findings is not clear. Neither our study nor the parallel trial reported significant or consistent cardiac or renal toxicity. In the USA/European study, hematological toxicity was uncommon and limited to cases of anemia that showed no clear relation to gefitinib dose [13]. We observed a similar low incidence of hematologic effects, contrasting with the tolerability profile of cytotoxic agents. As the tolerability profile of gefitinib was acceptable with the intermittent dosing schedule used in these studies, subsequent studies have been conducted using continuous once-daily oral dosing.

Our study confirmed previous reports that gefitinib is orally bioavailable in both healthy volunteers and cancer patients and is suitable for once-daily dosing [12, 13]. The $t_{1/2}$ data following multiple-dose administration, observed in patients from the USA/European study (range 24–85 h) [13] and our study (range 27.8–79.7 h) were similar. Pharmacokinetic analysis of the data from the current study showed dose-related exposure to gefitinib, which is consistent with the results from the USA/European trial.

We observed very encouraging evidence of antitumor activity across a range of gefitinib doses that are well below the MTD. Interestingly, five of 23 patients (22%) with NSCLC (all five with adenocarcinoma) had a partial response. This supports results seen in the USA/European study, in which partial responses were observed in four of 16 patients with NSCLC, each of whom had received at least two prior chemotherapy regimens [13]. There was no tumor regrowth or symptomatic progression observed during the off-treatment period in the Japanese phase I study using the intermittent dosing schedule. This suggests that the efficacy of gefitinib treatment would be maintained in patients requiring treatment interruption for safety reasons during chronic continuous treatment with gefitinib.

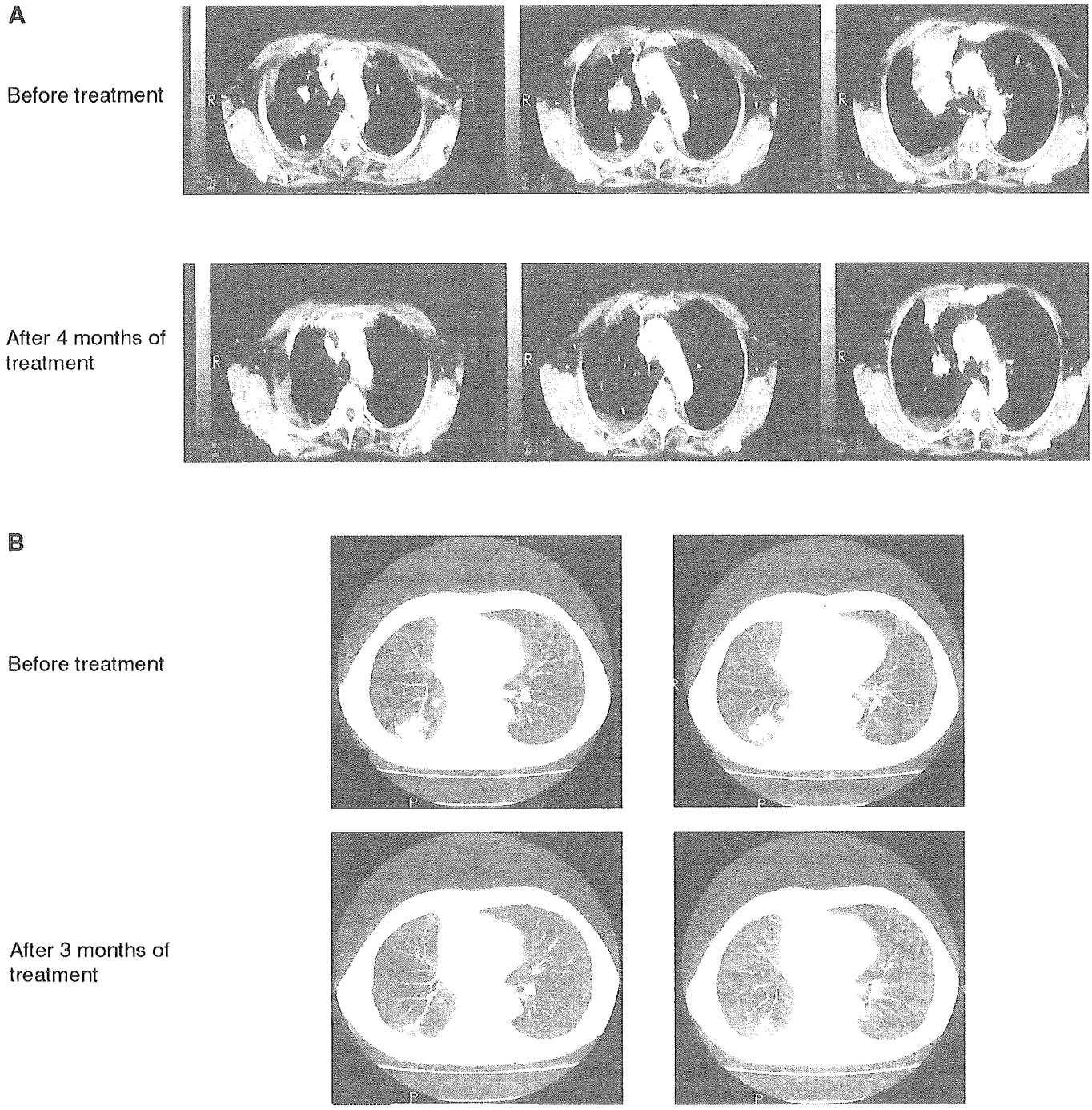

Figure 6. Computed tomography (CT) scan of patients with non-small-cell lung cancer (NSCLC) pre- and post-treatment with gefitinib (**A**) 525 mg/day and (**B**) 700 mg/day.

The findings of this study, in conjunction with those from other phase I studies [13, 17, 18], support the use of 250 and 500 mg/day doses for subsequent trials of gefitinib in advanced NSCLC. The 250 mg/day dose is higher than the lowest dose level at which objective tumor regression was seen, while 500 mg/day is the highest dose that was well tolerated when taken chronically in phase I trials. Two large-scale, randomized, double-blind, phase II studies, 'Iressa' Dose Evaluation in Advanced Lung cancer (IDEAL) 1 and 2, have been undertaken to evaluate the efficacy and tolerability of gefitinib monotherapy in patients with locally advanced or metastatic NSCLC who had previously received platinum-based chemotherapy. In both these studies, gefitinib was generally well tolerated and provided clinically significant antitumor activity [23, 24].

In conclusion, gefitinib is a novel agent designed to inhibit the EGFR signaling pathway, which is a relevant target in cancer biology. Gefitinib has a favorable tolerability profile and has demonstrated promising antitumor activity, especially in patients with NSCLC. The safety profile, pharmacokinetic parameters and antitumor activity observed in our study of Japanese patients are

comparable to those observed in patients from the USA and Europe. Therefore, an international phase II study of gefitinib can include Japanese patients. The potential for gefitinib monotherapy in the treatment of NSCLC has been confirmed by randomized phase II trials.

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ORIGINAL PAPER

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Ritterazine B, a new cytotoxic natural compound, induces apoptosis in cancer cells

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Abstract *Purpose*: Ritterazine B, one of the ritterazine analogues extracted from *Ritterella tokioka*, has been shown to be chemically similar to cephalostatin 1, and among the ritterazine derivatives is the most cytotoxic to P388 murine leukemia cells. The objective of this study was to determine the cytotoxicity of ritterazine B to non-small-cell lung cancer (NSCLC) cells in vitro and its effects on the cell cycle and apoptosis. *Methods*: The cytotoxicity of ritterazine B against PC14 NSCLC cells was investigated using a 4-day MTT assay. Morphological changes in cells after exposure to this compound were evaluated by phase-contrast microscopy. The effects on the cell cycle of HL-60 leukemia cells and PC14 cells were elucidated by flow cytometry and an in vitro CDK/cyclin kinase assay. Induction of apoptosis in HL-60 cells was assessed using the TUNEL assay and Hoechst 33342 staining. In addition, molecules involved in apoptosis were evaluated by Western blotting. *Results*: Ritterazine B exerted strong cytotoxic effects against PC14 cells with a mean GI_{50} of 75.1 n*M*. Cell cycle analysis showed that ritterazine B caused accumulation of HL-60 and PC14 cells at the G2/M checkpoint. Furthermore, ritterazine B-treated HL-60 cells became multinucleated, and at a concentration of 20 n*M* this resulted in the onset of apoptosis. Neither cleavage of caspase target molecules nor phosphorylation of bcl-2 were observed in ritterazine B-treated HL-60 cells. *Conclusions*: These results indicate that ritterazine B might be a potent inducer of apoptosis acting via a novel antimitotic mechanism.

Keywords Ritterazine · Cell cycle arrest
Apoptosis · Caspase

Abbreviations *CDK* Cyclin-dependent kinase · *DMSO* Dimethylsulfoxide · *ECL* Enhanced chemiluminescence · *FITC* Fluorescein isothiocyanate · GI_{50} 50% growth inhibition IC_{50} 50% enzyme inhibition · *MTT* 3-(4,5-Dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide · *NSCLC* Non-small-cell lung cancer · *PARP* Poly(ADP-ribose) polymerase · *PBS* Phosphate-buffered saline · *PI* Propidium iodide · *TUNEL* Terminal deoxynucleotidyl nick end-labeling

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Introduction

Much attention has been focused on natural products as potential sources of novel anticancer drugs over the decades [4]. The discovery of taxanes and camptothecins from natural sources has contributed to the recent progress in cancer treatment in the clinical setting. However, discovery of further active compounds from natural resources is needed to improve cancer chemotherapy.

Natural marine products have recently attracted attention because of their intriguing biological activities. For example, TZT-1027, which is now under clinical investigation, was synthesized from dolastatin 10, a marine product isolated from an Indian ocean sea hare *Dolabella auricularia* [20]. Another example is cephalostatin 1, isolated from an Indian ocean hemichordate *Cephalodiscus gilchristi*, which exhibits remarkable cytotoxic activity against P388 murine leukemia cells with GI_{50} values of 10^{-4}–10^{-6} ng/ml; however, the mechanism of action still remains unknown [8, 15].

In our search for cytotoxic substances originating from Japanese marine invertebrates, we have found that ritterazines extracted from *Ritterella tokioka* exert potent cytotoxic activities against P388 murine leukemia cells [6]. Among the ritterazine derivatives, we have found that ritterazine B exerts the most potent cytotoxic activity against P388 cells [7]. Ritterazines are dimeric steroidal alkaloids structurally related to cephalostatin 1. Furthermore, a COMPARE pattern-recognition analysis gave correlation coefficients of 0.93 between cephalostatin 1 and ritterazine B against NCI-10 cell lines, suggesting that ritterazine derivatives act by the same unknown mechanism as does cephalostatin 1 [12].

To evaluate the potential activity of ritterazine B, we examined the its cytotoxic effects in a NSCLC cell line, and its effects on the cell cycle and apoptosis in HL-60 leukemia cells. Ritterazine B showed potent cytotoxic activity against the NSCLC cell line, and cell death after exposure to ritterazine B was at least partly attributable to apoptosis, probably independently of caspase activation, and may affect cytokinesis. The increase in the G2/M population and multinucleated cells led us to consider that ritterazine B might be involved in cytokinesis.

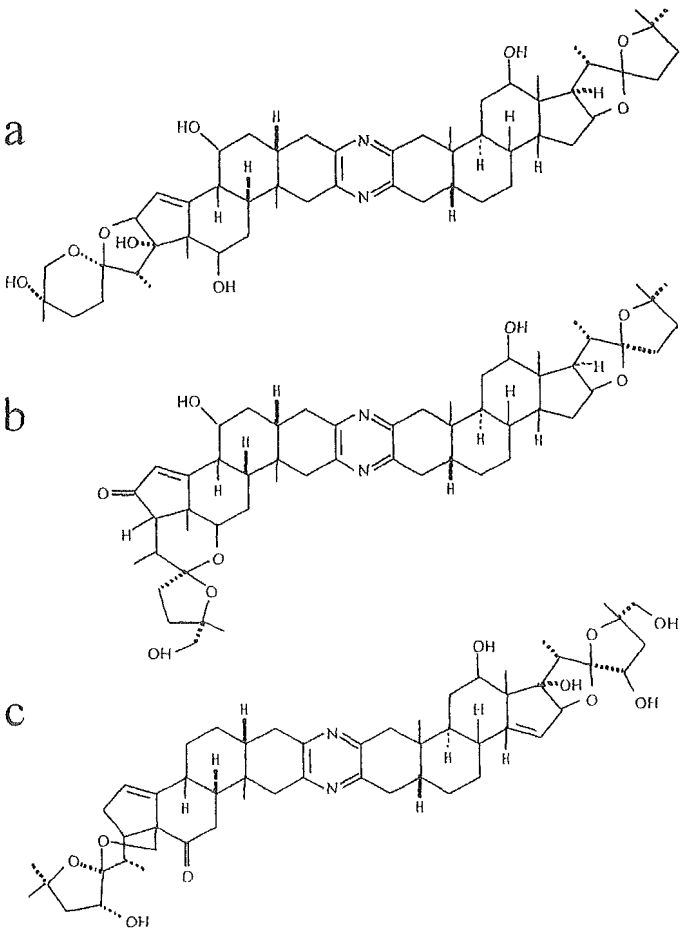

Fig. 1a–c Chemical structures of ritterazine B (a), compound 21 (b), and cephalostatin 1 (c)

Materials and methods

Cell culture

The NSCLC cell line PC14 was a generous gift from Dr. Nishio (National Cancer Center Research Institute, Tokyo). HL-60 (acute promyeloblastic leukemia) and PtK1 (normal kidney cells of the kangaroo rat *Potorous tridactylis*) were purchased from the American Type Culture Collection (Rockville, Md.). PC14 and HL-60 cells were cultured in RPMI-1640 medium (Sigma Chemicals, St Louis, Mo.) containing 10% fetal bovine serum and streptomycin (100 μg/ml) and penicillin (100 U/ml) in a humidified atmosphere containing 5% CO_2 at 37°C.

Materials

Ritterazine B and its less-active derivative compound 21 (Fig. 1) were isolated and purified as described previously. Stocks of these compounds were prepared at a concentration of 1 m*M* in DMSO, and stored at 70°C until use. Ritterazine B diluted in medium was used for the MTT assay and flow cytometric analysis at final concentrations ranging from 5 to 100 n*M*. Camptothecin, paclitaxel and jasplakinolide were purchased from Sigma Chemicals. Monoclonal anti-human PARP antibody (C2–10), anti-Bcl-2 antibody (clone 100, SC-509) and anti-caspase 3 antibody (clone 19) were purchased from BD Pharmingen (San Diego, Calif.), Santa Cruz Biotechnology (Santa Cruz, Calif.), and Transduction Laboratories (Lexington, Ky.), respectively. A 0.01% DMSO solution was used as the control for each experiment except for CDK inhibition assays, for which 10% DMSO was used.

Assessment of inhibition of cell growth and morphological changes

The growth-inhibitory effects of ritterazine B as well as other drugs on PC14 cells were evaluated using the MTT assay as described previously [21]. Cells were seeded into 96-well microplates (Corning, Corning, N.Y.) at a density of 500 or 1000 cells per well in culture medium. Drugs at various concentrations were added 24 h later. After the indicated times, cell viability was determined using the MTT (Sigma Chemicals) assay. Morphological changes in HL-60 and PC14 cells after treatment were evaluated by standard phase-contrast microscopy.

Cell cycle analysis

The effects of ritterazine B on the cell cycle were determined by flow cytometry. Briefly, cells exposed to ritterazine B were collected by centrifugation, washed twice with PBS, and then

resuspended in 0.5 ml PBS and fixed with 70% ethanol at 20°C for more than 24 h. Fixed cells were washed twice, resuspended in 0.5 ml PI/RNase solution (Phoenix Flow Systems, San Diego, Calif.) and incubated at room temperature for 30 min. The DNA content of the cells was determined by flow cytometry using a FACS Calibur system (Becton Dickinson, San Jose, Calif.) within 3 h [10]. CellQuest (Becton Dickinson) and ModFit (Verity Software House, Topsham, Me.) software was used.

CDK inhibition assays

The In vitro inhibitory effects of ritterazine B and its less-active analogue, compound 21 against CDK/cyclins were determined as described previously [11]. Briefly, baculovirus-expressed human CDK4/cyclin D1, CDK2/E, CDK2/A and CDC2/A complexes were mixed with the compounds in 40 µl kinase buffer and incubated at 30°C for 30 min. The kinase reaction was started by adding 400 ng glutathione S-transferase-retinoblastoma protein and 5 µCi [γ-^{32}P]ATP to the mixture and incubating at 30°C for 15 min. The labeled retinoblastoma protein was immunoprecipitated, and electrophoresed on 10% NuPAGE Bis-Tris gels (NOVEX, San Diego, Calif.), and detected by autoradiography.

Detection of apoptosis

TUNEL assay

The TUNEL assay was carried out using an APO-BrdU kit (BD Pharmingen). Untreated or drug-treated cells were collected, washed, and resuspended in 0.5 ml PBS. Cells were fixed by adding 5 ml 1% paraformaldehyde/PBS for 30 min on ice. Cells were centrifuged, washed, resuspended in 0.5 ml PBS, and fixed by adding 5 ml 70% ethanol. Fixed cells were centrifuged, washed, resuspended in TdT enzyme and TdT buffer at 37°C for 1 h. Subsequently, FITC-labeled Br dUTP was added followed first by incubation at room temperature for 30 min in the dark, then by incubation with 0.5 ml PI/RNase solution at room temperature for 30 min in the dark. Finally, the cells stained with two colors were analyzed by flow cytometry using a FACS Calibur system (Becton Dickinson).

Hoechst 33342 staining

Drug-treated, unfixed HL-60 cells were stained with Hoechst 33342 (10 µ*M*, Molecular Probes, Eugene, Ore.) and analyzed under a non-confocal fluorescence microscope (Nikon Model Eclipse E800) with excitation at 360 nm (UV) [9]. Images were captured with a digital camera and obtained with the ×20 objective.

Western blotting

Western blotting for PARP was performed to detect its cleavage due to caspase 3 activation as described elsewhere [17]. Drug-treated cells were collected by centrifugation, washed with PBS, resuspended in NP40 lysis buffer (50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride, 1% NP40), disrupted by sonication on ice and then centrifuged at 15,000 rpm for 30 min at 4°C. A total of 50 µg protein was separated on 3–8% NuPAGE Tris-acetate gels (NOVEX, San Diego, Calif.), and electroblotted onto a nitrocellulose membrane. The membranes were blocked with TBS-T/5% blocking agent (Amersham Pharmacia Biotech, Arlington Heights, Ill.), exposed to a monoclonal antihuman PARP antibody at a dilution of 1:1000, followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham Pharmacia Biotech) at a dilution of 1:1500. Blotted bands were detected by an ECL assay (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Similarly, degradation of procaspase 3 and phosphorylated Bcl-2 were evaluated after electrophoresis on 12% Tris-glycine gels followed by immunoblotting with anti-caspase 3 and anti-Bcl-2 antibodies and visualized with an ECL assay.

Fluorescence staining for β-actin

PtK1 cells were seeded in chambered coverglasses (Nalge Nunc International, Naperville, Ill.) at a density of 2×10^4/ml, cultured for 3 days, and then exposed to ritterazine B or jasplakinolide at a concentration of 100 n*M* for 24 h. The coverglasses were washed twice with PBS, fixed with methanol at 20°C for 15 min, and permeabilized with acetone for 1 min. The cells were then washed twice, stained with 1 ml FITC-conjugated anti-β actin monoclonal antibody (Sigma Chemicals), and diluted at 1:250 with PBS at room temperature for 1 h in the dark. The coverglasses were washed twice, mounted on a slide with Slowfade antifade reagent (Molecular Probes, Eugene, Ore.) and examined under a Nikon Modl Eclipse E800 microscope [1].

Results

Cytotoxicity of ritterazine B

The cytotoxicity of ritterazine B against the NSCLC cell line was determined using a 4-day MTT assay (Table 1). Ritterazine B exerted potent growth-inhibitory activity against PC14 cells in a dose-dependent manner, with the GI_{50} being 75.1 n*M*. By comparison, the GI_{50} values of anticancer agents currently in use clinically range from 0.24 to 5.8 µ*M*, indicating that the cytotoxic activity of ritterazine B against PC14 cells was almost equipotent to those of commercially available anticancer agents.

Accumulation of HL-60 and PC14 cells at the G2/M checkpoint of the cell cycle following exposure to ritterazine B

After exposure of cells to ritterazine B at various concentrations for 24 h, significant changes were observed in the cell cycle distribution (Fig. 2). Flow cytometric analysis revealed that the percentage of cells staying at the G2/M checkpoint was significantly increased in both cell lines, while the percentage HL-60 cells in the G1 phase was decreased. Following treatment with 20 n*M* ritterazine B for 48 h, HL-60 cells underwent cell death as determined morphologically (data not shown). To determine the mechanism of cell cycle arrest, we inves-

Table 1 Growth inhibition of PC14 cells by anticancer drugs

Drug	GI_{50} (µ*M* ± SD)
Cisplatin	5.8 ± 10.85
Irinotecan	5.1 ± 0.96
Doxorubicin	0.24 ± 0.17
Paclitaxel	5.4 ± 1.3
Ritterazine B	0.075 ± 0.0063

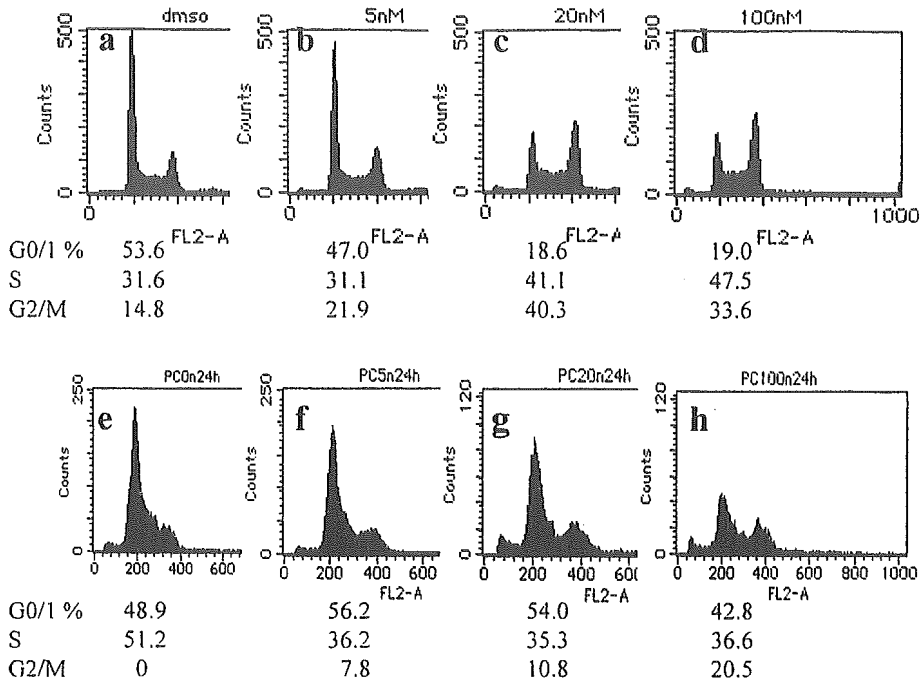

Fig. 2a–h Cell cycle analysis of HL-60 cells (**a–d**) and PC14 cells (**e–h**) 24 h after exposure to ritterazine B (**a**, **e** controls; **b**, **f** 5 n*M*; **c**, **g** 20 n*M*; **d**, **h** 100 n*M*). Ritterazine B treatment led to a significant increase in the G2/M population in both cell lines

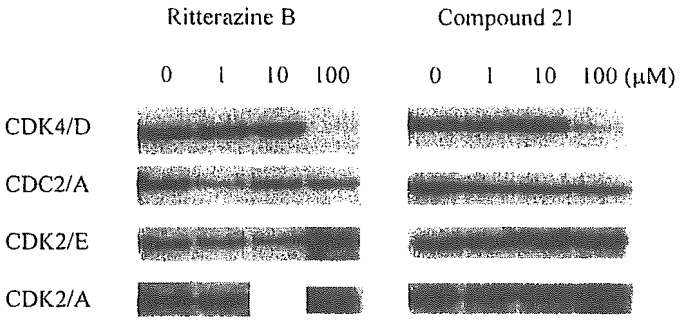

Fig. 3 CDK inhibition assays in vitro. The in vitro inhibitory effects of ritterazine B and the less-active derivative compound 21 against CDK/cyclins were investigated with baculovirus-expressed human CDK4/cyclin D1, CDK2/E, CDK2/A and CDC2/A complexes at concentrations in the range 1–100 μ*M*. Both ritterazine B and compound 21 moderately inhibited CDK4/D

tigated whether ritterazine B would exhibit CDK inhibitory activity in vitro. Both ritterazine B and its less-active analogue, compound 21, slightly inhibited CDK4/D1 with IC_{50} values of 80 and 70 μ*M*, respectively (Fig. 3). Neither compound inhibited CDK2/A, CDK2/E or CDC2/A even at concentrations up to 100 μ*M*.

Induction of apoptosis in HL-60 and PC14 cells by ritterazine B via morphological changes

In ritterazine B-treated PC14 and HL-60 cells, multinuclei were observed by phase contrast microscopy. Although typical apoptotic changes such as apoptotic bodies were not observed, Hoechst 33342 staining of HL-60 cells after ritterazine B treatment for 24 h disclosed extensive nuclear condensation and fragmentation (Fig. 4). Furthermore, in the TUNEL assay, a 24-h exposure to ritterazine B resulted in an increase in the percentage of TUNEL-positive cells in a time- and dose-dependent manner (Fig. 5). These findings indicated that cell death caused by ritterazine B treatment was mainly due to apoptosis.

Caspases are not activated in ritterazine B-treated HL-60 cells

To elucidate whether apoptosis would be affected by caspase activation, Western blot analysis was performed on PARP and pro-caspase 3 as the known reference substrates for caspases. Neither PARP nor pro-caspase 3 was cleaved or degraded in ritterazine B-treated HL-60 cells irrespective of the conditions (Fig. 6), indicating that caspase activation would not be required for ritterazine-mediated cell death. In addition, Western blot analysis for bcl-2 showed no evidence that phosphorylated bcl-2 was increased in ritterazine B-treated HL-60 cells.

An increase in the G2/M population and in the incidence of multinuclei in treated cells suggested that ritterazine B might arrest cells during cytokinesis. Accordingly, we investigated the possible influence of ritterazine B on the actin cytoskeleton, one of the major components involved in cytokinesis in PtK1 cells. However, fluorescence staining of PtK1 cells preincubated with ritterazine B did not show the appearance of F-actin, indicating disrupted actin formation; incidentally, however, a similar phenomenon was also observed in jasplakinolide-treated cells (data not shown).

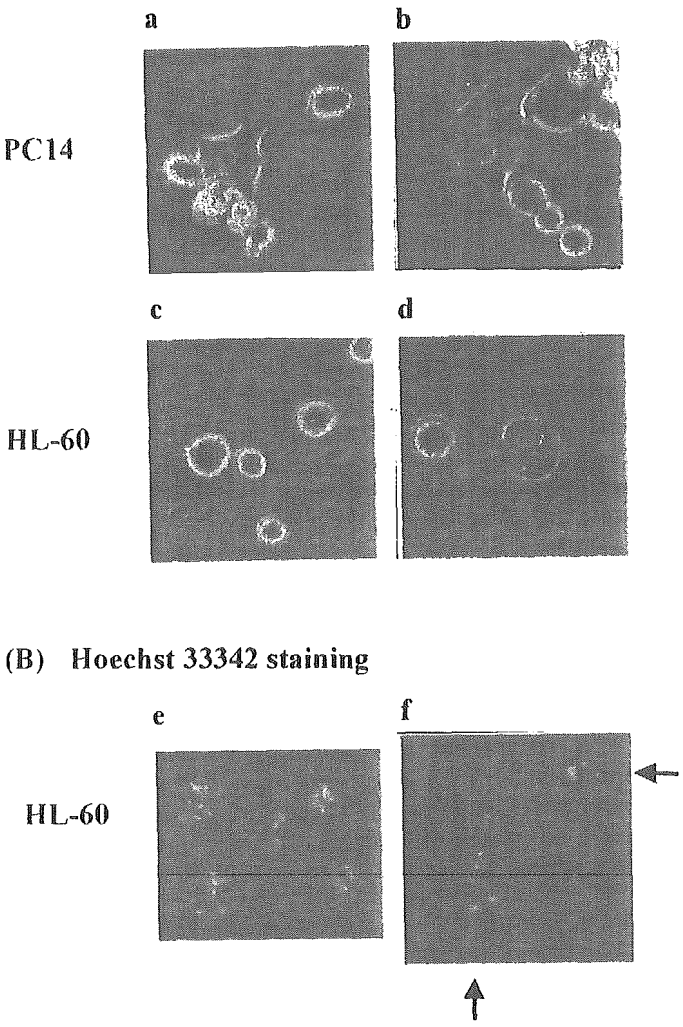

Fig. 4 A Phase contrast microscopy of PC14 (*a*, *b*) and HL-60 cells (*c*, *d*) 24 h after exposure to ritterazine B (*a*, *c* controls; *b* 200 n*M*; *d* 20 n*M*). B Hoechst 33342 staining of HL-60 cells (*e*, *f*) 24 h after exposure to ritterazine B (*e* control, *f* 20 n*M*; *arrows* apoptotic cells)

Discussion

The present study demonstrated that ritterazine B was a potent cytotoxic anticancer drug against NSCLC cells. In PC14 NCSLC cells, the MTT assay showed that the average GI_{50} of ritterazine B was 75.1 n*M*, indicating that this drug is as potent as many drugs currently used clinically. Death of HL-60 cells after treatment with ritterazine B was mainly due to apoptosis, which was confirmed by TUNEL and Hoechst 33342 staining. Apoptosis induced by ritterazine B appeared to be independent of the caspase pathway because neither cleavage nor degradation of caspase targets was observed. Although CDK4/cyclin D is known to be the major G1-regulating CDK/cyclin complex, the IC_{50} value inhibiting CDK4/cyclin D is approximately 1000 times the GI_{50} in cancer cells. Furthermore, cell cycle analysis revealed that ritterazine B kept the exposed cells at the G2/M checkpoint rather than in the G1 phase, thereby arresting many cells prior to the cytokinesis process.

The G2/M checkpoint is considered to be regulated by several molecules including tubulins. These molecules have been reported to play a role as the targets of drug-induced G2/M block. Of these targets, microtubules have been the most extensively investigated molecules, and are the targets of taxanes and vinca alkaloids. However, ritterazine B appears unlikely to act on microtubules because induction of phospho-Bcl-2 protein, a hallmark of tubulin-targeting drugs such as taxanes, was not observed [13]. Destruction of the actin cytoskeleton may also be responsible for drug-induced G2/M block mainly due to cytokinesis block. Several actin-targeting drugs such as jasplakinolide have been recently described [1]. Although we investigated the influence of ritterazine B on the actin cytoskeleton of PtK1 cells following findings indicative of cytokinesis block in ritterazine B-treated cells, fluorescence staining of PtK1 cells incubated with ritterazine B revealed no changes in the actin cytoskeleton (data not shown).

Other regulators of G2/M progression have been verified as the targets of several anticancer drugs. For example, cucurbitacin E [5], derived from plants with medicinal properties known since antiquity, has been identified as a sterol with potent growth-inhibitory activity against prostate carcinoma explants, besides being known to induce disruption of the vimentin as well as the actin cytoskeleton. Interestingly, cucurbitacin E potently inhibits proliferating human endothelia as compared to quiescent cells in vitro, implying its potential activity as an antiangiogenic agent. Also, inhibitors of kinesin motors known to regulate mitosis have been isolated [16]. By phenotype-based screening, Mayer et al. identified a small molecule inhibitor of mitotic spindle bipolarity, which was named monastrol [14]. Monastrol does not disrupt microtubules, actin cytoskeleton or chromosomes, but specifically inhibits the motility of the mitotic kinesin Eg5. Furthermore, several upstream molecules of CDC2/cyclin B involved in regulation of G2/M progression have been identified; accordingly, they might be candidate targets. Tamura et al. have reported that several small molecule inhibitors of Cdc25 phosphatases could block G2/M progression via negatively regulating CDC2/cyclin B [18, 19]. Also, inhibition of Chk1 by UCN-01 has been reported to cause G2 abrogation following cytotoxic chemotherapy [2, 3]. These novel molecules involved in G2/M progression still remain to be investigated in ritterazine B-treated cells.

The present study demonstrates that ritterazine B has cytotoxic activity against a human NSCLC cell line and can induce apoptosis via unknown antimitotic mechanisms. Further studies are needed to develop this encouraging drug for clinical use.

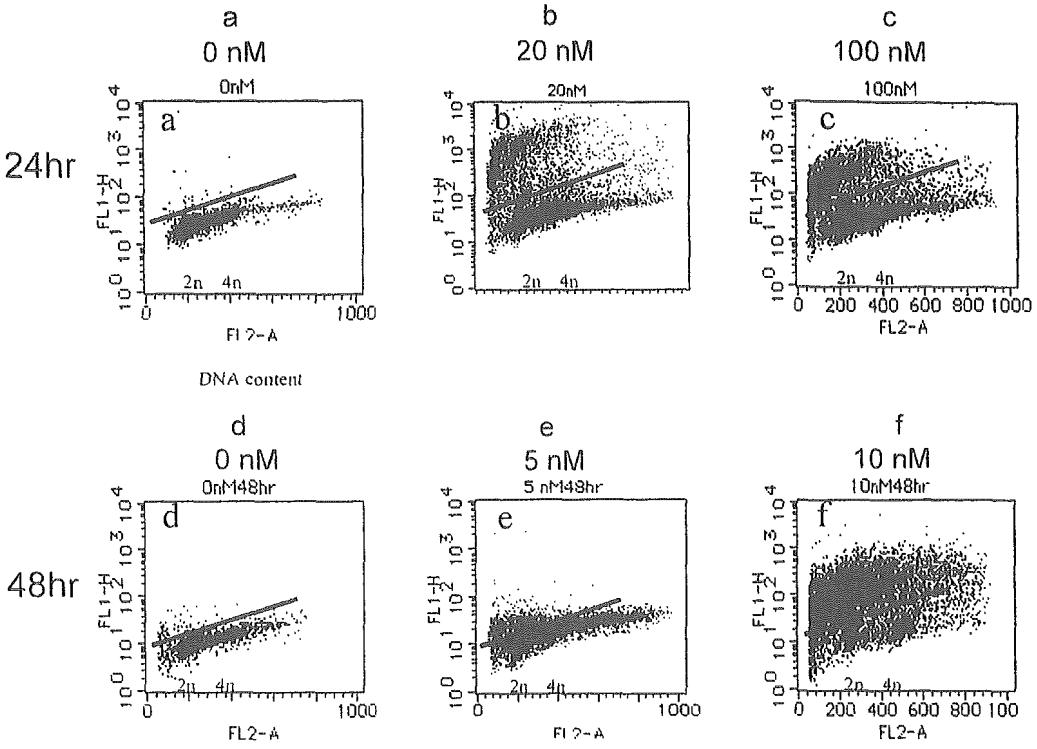

Fig. 5a–f TUNEL assays by flow cytometry. HL-60 cells were treated with 0–100 n*M* ritterazine B for 24 h (**a–c**) or 48 h (**d–f**), fixed and subjected to the TUNEL assay. Fluorescein intensity (TUNEL) is on the *y*-axis; *PI* intensity (DNA content) is on the *x*-axis. Cells whose TUNEL intensity was above the bars were considered TUNEL-positive

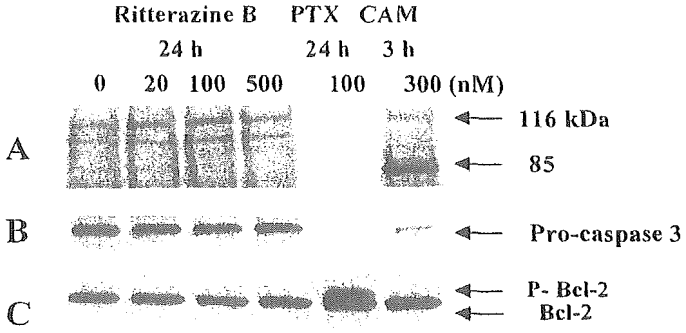

Fig. 6a–c Western blotting for apoptosis targets. Protein lysates from HL-60 cells treated with ritterazine B for 24 h were examined for cleaved bands of PARP (a 85 kDa), degradation of pro-caspase 3 (**b**), and phosphorylation of Bcl-2 (**c**). Neither PARP nor pro-caspase 3 was inactivated under any ritterazine B treatment condition (*PTX* paclitaxel, *CAM* camptothecin)

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