Table 2. No. of patients with drug-related adverse events that occurred in ≥20% of patients receiving lapatinib

	Dose (mg/day)[a]												No. of patients (%)
	900			1200			1600			1800			
Common terminology criteria grade	1	2	3	1	2	3	1	2	3	1	2	3	
Any adverse events	3	3	0	4	2	0	1	4	1	2	2	2	24 (100)
Gastrointestinal	1	1	0	4	0	0	2	3	1	3	1	2	18 (75)
Diarrhea	1	1	0	4	0	0	2	1	1	3	1	2	16 (67)
Stomatitis	0	0	0	1	0	0	1	2	0	1	0	0	5 (21)
Skin	4	2	0	3	1	0	4	2	0	4	2	0	22 (92)
Rash	1	0	0	4	0	0	1	2	0	3	2	0	13 (54)
Dry skin	5	0	0	2	0	0	1	0	0	0	0	0	8 (33)
Seborrheic dermatitis	3	1	0	0	0	0	0	0	0	1	0	0	5 (21)
Paronychia	0	1	0	0	1	0	2	0	0	1	0	0	5 (21)
Metabolism and nutrition	1	0	0	1	0	0	2	0	0	4	0	0	8 (33)
Anorexia	0	0	0	1	0	0	1	0	0	3	0	0	5 (21)
Investigations	2	1	0	3	2	0	3	1	0	3	1	1	17 (71)
Decreased lymphocyte count	0	1	0	1	1	0	0	1	0	1	0	0	5 (21)

[a]Six patients at each dose level.

patients at each dose level. The majority of events was mild (Grade 1–2); the most common events were skin reactions (mostly rash and dry skin) observed in 22 patients (92%) and gastrointestinal disorders (mostly diarrhea) in 18 patients (75%). The most severe drug-related adverse events were Grade 3 diarrhea observed in one patient at 1600 mg dose level and two patients at 1800 mg dose level. One of these also had Grade 3 γ-GTP increase. All diarrhea resolved with routine symptomatic treatment during or after withdrawal of lapatinib therapy, γ-GTP increase resolved without further treatment after completion of lapatinib therapy.

Grade 1/2 drug-related nausea and vomiting were experienced only by patients at higher dose levels of lapatinib [1/6 (17%) at 1600 mg/day and 3/6 (50%) at 1800 mg/day], with Grade 2 symptoms only seen at the 1800 mg dose level.

For other adverse events, no clear drug relation was found. The most frequent events included decreased body weight and serum alkaline phosphatase increase, each observed in 10 patients (42%). Grade 1 drug-related decreases in left ventricular ejection fraction were found in three of the six patients at the 1200 mg dose level. No clinically relevant changes in vital signs, 12-lead electrocardiogram or echocardiography were noted.

Hypoxemia and pneumonia were reported at the 900-mg dose level in another patient with NSCLC on Day 35. After hypoxemia occurred, the patient continued to receive study drug medication until Day 40. We attributed hypoxemia to bronchostenosis caused by the primary disease. Oxygen inhalation and erythromycin were given and hypoxemia improved while the pneumonia was resolved on Day 41 before the patient died from progression of primary disease 3 months after the events were resolved. Chest X-rays and CT findings for this patient were inconsistent with those for interstitial pneumonia associated with other tyrosine kinase inhibitors; therefore a drug relation with lapatinib was denied.

Maximum Tolerated Dose

Dose escalation was stopped at 1800 mg/day, where two patients experienced DLT (Grade 3 diarrhea). One of these patients also experienced Grade 3 γ-GTP increase. Thus, 1800 mg/day was determined as the MTD.

Pharmacokinetics

Table 3 shows the PK parameters derived from data on 23 patients (data from one patient received lapatinib for only 19 days and are not included).

Serum concentrations of lapatinib at each dose level on Days 1 and 21 are shown in Fig. 1. Repeated doses of lapatinib (900–1800 mg/day) for 21 days resulted in dose-related increases in mean C_{max} (range, 1715–3111 ng/ml) and mean $AUC_{0\ 24}$ (range, 25 680–51 099 ng·h/ml) (Table 3). Large inter-patient variations were found in mean C_{max} and mean $AUC_{0\ 24}$. After a single dose of lapatinib, t_{max} was ~4 h, although values varied greatly among patients. After 21 days of treatment, t_{max} values were similar to those observed after the single dosing on Day 1.

Table 3. Derived pharmacokinetic parameters of lapatinib (including 95% confidence intervals)

Dose (mg/day)[a]	Geometric mean C_{max} (ng/ml)		Mean CSS_{max} (ng/ml)	Median t_{max} (h)		Geometric mean AUC (h ng/ml)[b]		Median $t_{1/2}$ (h)	
	Day 1	Day 21	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21
900	1011 (694–1472)	1895 (1319–2721)	857 (386–1234)	4.0 (2.0–6.0)	4.0 (3.0–6.0)	17 577 (11 812–26 154)	29 272 (21 618–39 638)	12.9 (10.1–18.3)	23.1 (9.8–38.2)
1200	1027 (474–2227)	1715 (965–3048)	820 (226–1308)	3.5 (2.1–6.0)	3.6 (3.0–7.9)	15 441 (7410–32 176)	25 680 (13 728–48 038)	11.5 (10.1–19.5)	16.9 (15.1–34.3)
1600	1538 (1042–2268)	3111 (1937–4996)	1899 (818–4357)	4.0 (2.0–8.0)	5.1 (0.9–8.0)	26 361 (17 519–39 665)	51 099 (28 674–91 062)	13.9 (9.6–18.0)	26.2 (12.9–48.3)
1800	1227 (465–3242)	2333 (927–5870)	1528 (586–3393)	3.9 (3.0–8.0)	3.9 (3.0–7.3)	32 841 (18 884–57 114)	39 451 (14 909–104 391)	15.7 (11.0–133.1)	21.8 (18.5–104.5)

AUC, area under the plasma drug concentration–time curve; C_{max}, maximum serum concentration; CSS_{max}, mean steady state maximum serum concentration; t_{max}, time to reach C_{max}; $t_{1/2}$, terminal half-life.
[a]Six patients at 900, 1200 and 1600 mg/day and five at 1800 mg/day.
[b]Day 1, AUC from 0 to infinity; Day 21, AUC from 0 to 24 h.

Figure 1. Serum concentrations of lapatinib at each dose level as detected on (A) Day 1 and (B) Day 21.

Steady-state serum concentrations of lapatinib generally increased with dose, 820 ± 448 ng/ml at 1200 mg dose level and 1899 ± 1356 ng/ml at 1600 mg dose level (Table 3). Both concentrations exceeded the half maximal inhibitory concentration values for *in vitro* tumor growth (14). The median $t_{1/2}$ after repeat dose was 16.9 h (range, 15.1–34.3) at 1200 mg dose level and 26.2 h (range, 12.9–48.3) at 1600 mg dose level.

The fraction of urinary excretion of lapatinib was <0.1% of the dose, suggesting that none or negligible amount of drug is excreted in urine.

Comparison of on-treatment C_{max} and AUC_{0-24} values obtained in Japanese and western patients are shown in Fig. 2 (43,44).

Efficacy

Among 24 patients, the best overall response was assessed as partial response (PR) in two patients (8.3%), stable disease (SD) in 12 patients (50.0%), progressive disease in eight patients (33.3%) and indeterminate in two patients (8.3%).

Of the two patients with PR, the first was a 73-year-old man with NSCLC (squamous cell carcinoma) with prior docetaxel and gemcitabine treatment, who received lapatinib 900 mg/day. PR was assessed by CT scan with 41% shrinkage on Day 49. Time to progression was 191 days. The second patient was a 55-year-old woman with trastuzumab-resistant breast cancer (invasive ductal carcinoma; hormone receptor-negative, ErbB-2 3+). Disease progressed after doxorubicin and cyclophosphamide/docetaxel therapy, was

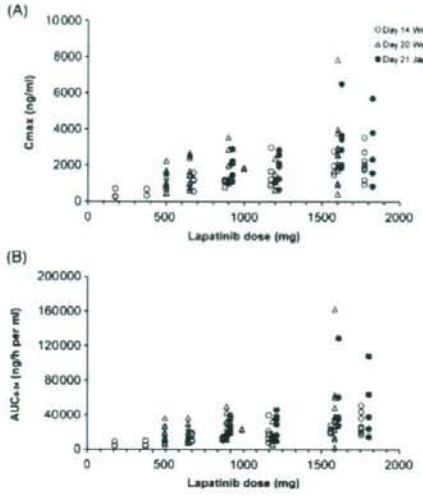

Figure 2. Relation between dose of lapatinib and exposure: comparison of (A) maximum serum concentration (C_{max}) and (B) area under the plasma drug concentration–time curve from 0 to 24 h (AUC_{0-24}) after dosing on Day 21 (our study, Japanese patients) and Days 14 and 20 (US studies, western patients).

stable with doxifluridine, and progressed with trastuzumab. Following treatment with lapatinib 1600 mg/day, the tumor shrank by 41% on Day 21. Time to progression was 133 days.

Among the patients with SD, three (two with NSCLC and one with colorectal cancer) were stabilized for >6 months and three (two with NSCLC and one with cervical cancer) were stabilized for 3–6 months and therefore were considered as having a durable response.

DISCUSSION

The dual ErbB-1/-2 inhibitor lapatinib taken orally once daily for ≥21 days was well tolerated at doses of 900–1600 mg in Japanese solid tumor patients. Adverse events were mostly mild in nature, and only four grade ≥3 drug-related adverse events were noted, in three patients (three events of Grade 3 diarrhea and one Grade 3 γ-GTP increase). No NCI-CTC Grade 4 adverse events were observed. Grade 1–2 diarrhea occurred in some patients other than those who experienced Grade 3 diarrhea; for these, supportive therapy was given and fully recovered in all cases. Grade 1/2 drug-related nausea and vomiting were experienced only by patients at higher dose levels of lapatinib, with Grade 2 symptoms only seen at 1800 mg dose level.

The types and incidences of drug-related adverse events in Japanese patients were similar to those reported from studies conducted in healthy volunteers (18) and two overseas Phase I studies, the latter including a parallel study in western patients that used similar dose administration and dose-escalation schedules (43,44). In that study as well as in ours, diarrhea and rash were the most frequently noted drug-related adverse events. Adverse events were generally mild (Grade 1–2), transient and reversible on dose delay or interruption. Headache, which was common in western patients (18), was reported only by one patient at 1600 mg dose level. 1800 mg/day was considered as MTD, at which Grade 3 diarrhea and γ-GTP increase were observed.

Skin-related adverse events of lapatinib were similar to those reported for other agents that target ErbB-1; rash is also a common adverse event associated with the ErbB-1 tyrosine kinase inhibitors gefitinib (46–49) and erlotinib (7,50), as well as the anti-ErbB-1 antibody cetuximab (51). Patients who received these medications also experienced diarrhea (7,46–50). These adverse events occurred at a similar frequency in our study as in two overseas Phase I studies (43,44).

Apart from one event of γ-GTP increase, no Grade ≥3 abnormal laboratory test suggestive of liver dysfunction was noted. Therefore, drug-related liver abnormality was generally less frequently seen with lapatinib compared with gefitinib (48,49).

Hematologic toxicity was uncommon and limited to cases of anemia. This finding is similar to those of the Phase I biomarker study (44) and studies of gefitinib (48,49,52).

None of the patients developed interstitial lung disease, which is an adverse event reportedly associated with gefitinib (53,54) and occurs in 5.8% of Japanese patients (55). However, because of the limited number of patients in our study, further studies are required to assess safety of lapatinib in this regard.

Cardiotoxicity is a known adverse event associated with trastuzumab therapy and might be related to ErbB-2 inhibition (2,56); however, we found no evidence of drug-related cardiac dysfunction in our study.

PK parameters such as C_{max} and AUC_{0-24} in this study were analyzed and their means and 95% confidence intervals compared with those obtained at similar doses (900–1800 mg) in two overseas Phase I studies (43,44). As can be seen in Fig. 2, the values were comparable among the three studies. However, large inter-patient variations were noted, especially in Japanese patients, and these might have contributed to higher mean values. On the other hand, no clear pharmacokinetic differences were apparent between Japanese and non-Japanese subjects, suggesting that values obtained overseas can be extrapolated to the Japanese population.

The dose recommended for further clinical studies outside Japan, 1500 mg/day, can be used for Phase II studies in Japan. We base this recommendation on the similar PK profiles of lapatinib in Japanese and western patients, evidence of antitumor activity at doses of ≥900 mg/day, and an MTD of 1800 mg/day.

To conclude, lapatinib, taken continuously as once-daily oral therapy at 900–1600 mg, was well tolerated in Japanese

patients with solid tumors. The safety and PK profiles shown in this study are similar to those in Phase I studies conducted in western patients. Phase II studies to determine the efficacy of lapatinib against a range of tumors are now in progress.

Acknowledgements

We thank all the patients who participated in this study, their families, and all the investigators (Dr K. Araki, Dr M. Fukuda, Dr M. Ikeda, Dr H. Kaneda, Dr T. Sato, Dr M. Tahara and Dr K. Tamura), research nurses, and study coordinators at study sites.

Funding

This study was sponsored by GlaxoSmithKline K.K.

Conflict of interest statement

The author, Hironobu Minami, receives honoraria from GlaxoSmithKline. The authors, Masayuki Kanezaki, Akihira Mukaiyama, and Yoshiyuki Minamide are employed by GlaxoSmithKline.

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Phase I/II Study of Docetaxel and S-1 in Patients with Previously Treated Non-small Cell Lung Cancer

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Introduction: The aim of this study was to determine and evaluate the recommended dose of docetaxel in combination with a novel oral 5-fluorouracil analogue S-1 and evaluate the efficacy and safety in patients with previously treated non-small cell lung cancer.

Methods: In phase I, patients with previously treated non-small cell lung cancer were treated with docetaxel (starting dose 40 mg/m^2) intravenously on day 1 and oral administration of S-1 at a fixed dose of 80 mg/m^2 on days 1 to14 every 3 weeks. The recommended dose was the dose level preceding the maximum tolerated dose; once determined, patients were enrolled in phase II.

Results: The recommended dose of docetaxel was 40 mg/m^2 in combination with S-1 80 mg/m^2/d. Of 30 patients enrolled in phase II part, 29 patients were eligible and analyzed. No complete response and 7 (24.1%) partial responses were observed, for an overall response rate of 24.1% (95% confidence interval, 10.3–43.5%). Median overall survival was 11.8 months. The 1-year survival rate was 42%. The grade 3 to 4 hematologic toxicities were neutropenia (34.5%), leukopenia (20.6%), and anemia (10.3%). The grade 3 to 4 nonhematological toxicities included fever 2 (6.9%), diarrhea 1 (3.4%), stomatitis 1 (3.4%), cerebral infarction 1 (3.4%), and pneumonitis 1 (3.4%). There was one treatment-related death due to relapse of drug induced pneumonitis.

Conclusions: This combination chemotherapy is highly active and well tolerated in previously treated patients with non-small cell lung cancer. These results are encouraging and warrant additional investigation.

Key Words: Phase I/II, Non-small cell lung cancer, Second-line chemotherapy, S-1, Docetaxel.

(*J Thorac Oncol.* 2008;3: 1012–1017)

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Supported by the National Hospital Organization Lung Cancer Study Group.
Disclosure: The authors declare no conflicts of interest.

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ISSN: 1556-0864/08/0309-1012

Lung cancer is the leading cause of tumor-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, and by 2002, there were 1.35 million new cases, representing 12.4% of all new cancers.[1] Surgery offers the best chance for cure in stage I and II NSCLC. However, most patients with NSCLC have advanced disease at diagnosis. Chemotherapy is the mainstay of management. The American Society of Clinical Oncology's clinical guidelines recognize that chemotherapy can prolong the survival of advanced NSCLC and is appropriate for those with good PS.[2] The use of doublet regimens has been widely adopted. The principal agents are platinum analogs, taxanes, gemcitabine, irinotecan, and vinorelbine.[3,4] First-line platinum-based chemotherapy is somewhat effective. However, all patients with advanced NSCLC will ultimately progress or relapse. Therefore, second-line chemotherapy is of importance in the clinical management of the patients who had previously received chemotherapy.

Docetaxel has been proven to show antitumor activity against various cancers, including NSCLC.[5–8] This anticancer agent is a mitotic spindle poison that promotes tubulin polymerization and inhibits the depolymerization of microtubules.[9] Docetaxel is one of the standard drugs in second-line chemotherapy. Two recent studies showed improved survival in patients with NSCLC previously treated with platinum in comparison to best supportive care or other drugs.[10,11]

S-1 is a new oral fluorinated pyrimidine. It is a combination drug consisting of a mixture of futraful, 5-chloro-2,4-dihydrozypyridine, and potassium oxonate (Oxo) in a molar ratio of futraful: 5-chloro-2,4-dihydrozypyridine: Oxo = 1: 0.4: 1, based on the biochemical modulation of 5-FU.[12] In phase II studies for advanced NSCLC conducted in Japan, favorable results of S-1 monotherapy or combination therapy have been reported. Kawahara et al. reported that S-1 monotherapy achieved an overall response rate of 22.0% and a median survival time (MST) of 10.2 months.[13] There were no irreversible, severe or unexpected toxicities. Ichinose et al. reported that S-1 plus cisplatin achieved a 47% response rate and a MST of 11 months.[14] Docetaxel and S-1 have shown synergy in human gastric, and breast cancer xenograft models.[15,16] The expression of thymidylate synthase and dihydrouracil dehydrogenase was lower than compared with con-

trol levels. In in vivo experiments using breast cancer xenografts, significant down-regulation of dihydrouracil dehydrogenase activity was observed in tumors treated with S-1, docetaxel and their combination.[16] However, thymidylate synthase activity was not significantly different from control. We hypothesized that the doublet combination chemotherapy using docetaxel and S-1 would have more effect against NSCLC as compared with the monotherapy of docetaxel. The rationale for this combination is that the drugs have different action mechanisms and safety profiles. To improve upon the efficacy of docetaxel alone as second-line treatment, we conducted a phase I/II study of doublet chemotherapy of docetaxel plus S-1.

PATIENTS AND METHODS

Eligibility

Eligible patients were required to have locally advanced or metastatic NSCLC and had failed one or more prior chemotherapy regimens and had at least one measurable lesion. Other main eligibility criteria were as follows: age 20 years or more; Eastern Cooperative Oncology Group performance status (PS) 0 or 1; estimated life expectancy $\geq$3 months; one or more prior chemotherapy regimens that did not include docetaxel or 5-FU and that was completed >4 weeks before entry; adequate bone marrow, hepatic, renal, and cardiac function [i.e., WBC count $\geq$4000/μl, absolute neutrophil count $\geq$2000/μl, platelet count $\geq$100,000/μl, hemoglobin $\geq$9.5 g/dl, serum bilirubine level <1.5 mg/dl, aspartate aminotransferase, and alanine aminotransferase within 2.5 times the upper limit of normal (ULN) for the institution, blood urea nitrogen <25 mg/dl, serum creatinine within the ULN, and creatinine clearance $\geq$60 ml/min]. Exclusion criteria included the presence of other concomitant or metachronous cancers, severe allergy to drugs, simultaneous infectious disease, interstitial pneumonia, or other serious underling medical conditions. The study was approved by the institutional review board of the participating center and all patients provided written informed consent.

Evaluation

All eligible patients who received any part of the treatment were considered assessable for response and toxicity. The complete blood cell counts and blood chemistry studies were measured weekly. The response was assessed based on weekly chest radiograph or computed tomography scan every 4 weeks findings that initially had been used to define tumor extent during the treatment period. The response was evaluated according to the criteria of response evaluation criteria in solid tumors. A complete response (CR) was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks. A partial response (PR) was defined as an at least 30% decrease in the sum of the longest diameters of the target lesions for more than 4 weeks with no new area of malignant disease. Progressive disease (PD) indicated at least a 20% increase in sum of the longest diameter of the target lesions or a new malignant lesion. Stable disease (SD) was defined as insufficient shrinkage to qualify for PR and insufficient increase to qualify for PD. The best response achieved during the treatment course was reported. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.[17]

Study Design and Treatment Schedule

This was an open-label multicenter, single-arm phase I/II study in patients with previously treated NSCLC. The objective of the phase I part was to determine the dose-limiting toxicity (DLT), maximum-tolerated dose (MTD), and recommended dose (RD) of docetaxel plus a fixed dose of S-1. In the phase II part, the primary objective was to estimate the overall response rate of this combination at the RD. Secondary objectives were to assess overall survival, 1-year survival rate, adverse events, and progression-free survival (PFS).

In the phase I part of this study, patients received variable doses of docetaxel administered as a 1-hour infusion on day 1 and oral S-1 administered at a fixed dose of 80 mg/m on days 1 to 14 every 3 weeks. S-1 is only available in 20-mg or 25-mg capsules. Therefore, it is easier to plan the dose escalation procedure or a dosage adjustment of docetaxl than S-1. The initial starting dose of docetaxel was 40 mg/m^2 (dose level 1), and step-wise dose increases to 50 (dose level 2) and 60 mg/m^2 (dose level 3) were planned for successive patient cohorts. DLT was determined during the first treatment cycle. At least three patients were enrolled at each dose level: (i) the dose was defined as MTD when two or more of three patients developed DLT; (ii) when one of three patients developed DLT, three other patients were enrolled; (iii) when three or more of six patients developed DLT, the dose was defined as MTD; (iv) when one or two of six patients developed DLT, the dose was increased to the next level.

DLT was defined as follows: grade 4 neutropenia; grade 3 or 4 neutropenia associated with a fever $\geq$38°C; grade 4 thrombocytopenia; or grade 3 or 4 nonhematological toxicities. A DLT was also reported if 7 days or more omission of S-1, or if the second cycle was delayed until after day 29 because the dosing requirements were not satisfied.

S-1 80 mg/m^2 per day was given orally in 2 divided dose after a meal for 2 weeks, after a drug-free interval of 1 week (one cycle). Three doses of S-1 were selected according to body surface area (BSA). So that they would be approximately equivalent to 80 mg/m^2: BSA<1.25 m^2, 40 mg b.i.d.; BSA 1.25, but <1.5 m^2, 50 mg b.i.d.; and BSA $\geq$1.5 m^2, 60 mg b.i.d. Docetaxel 40 mg/m^2 was diluted in 500 ml of 0.9% salaine and administered as a 1-hour infusion on the morning of day 1 of each cycle (i.e., every 3 weeks). Dexamethasone 8 mg was infused 1 hour before docetaxel administration. Granulocyte colony-stimulating factor was permitted if a patient developed grade 4 neutropenia; primary prophylaxis was not allowed. Antiemetic (ondansetron) treatment was allowed at the discretion of the treatment physician.

In the phase II part of this study, patients received the RD of docetaxel on day 1 and oral S-1 80 mg/m^2 in accordance with the treatment schedule described above. The treatment was repeated every 21 days for at least two cycles unless there was disease progression, unacceptable toxicity, patient refusal, or the physician's decision to stop treatment.

S-1 was stopped if there was a leukocyte count of $<2000/\mu l$, neutrophil count of $<1000/\mu l$, platelet count of $<50,000/\mu l$, and a grade 3 or 4 nonhematological toxicity.

The next course of treatment was initiated only when the neutrophil count recovered to $\geq 2000/\mu l$, platelet count to $\geq 100,000/\mu l$, creatinine within the ULN, total bilirubin ≤ 1.5 mg/dl, and the level of aspartate aminotransferase/alanine aminotransferase became <2.5 times the ULN. If patients did not recover from these toxicities within 2 weeks of the last administration of S-1, they were withdrawn from this study. If patients experienced grade 4 neutropenia, fever $\geq 38.0°C$ with grade 3 to 4 neutropenia, grade 3 or more thrombocytopenia, the dose of docetaxel was reduced by 10 mg/m^2 in the subsequent cycle. The dose of S-1 was to be reduced by 20 or 30 mg per day if any grade 3 or 4 nonhematological toxicity was recognized including nausea/vomiting, anorexia, and general fatigue.

Statistical Analysis

Based on the assumption that a response rate of higher than 20% would warrant a further investigation of this combination chemotherapy, and a rate of below 5% would make such an investigation unnecessary, a sample size of 27 patients was required with an alpha error of 0.05 and a beta error of 0.2. Therefore, the accrual of 30 patients was planned for a 2-year period since we considered that several ineligible patients might be identified in the course of the study. PFS was defined as the interval from the start of the treatment to the diagnosis of progression or death from any cause. Overall survival was defined as the interval between when treatment was started and death or the final follow-up visit. Median overall survival and median PFS were estimated by the Kaplan-Meier method.[18] Survival time was recorded at the last confirmation date if the patients were alive.

RESULTS

Between January 2005 and May 2006, 33 patients were enrolled on this study. Nine patients (6 in level 1 and 3 in level 2) were enrolled into the phase I part. Of 30 patients enrolled into the phase II part of the study, one patient did not receive either docetaxel or S-1 because his disease had progressed rapidly. This patient was excluded from all analyses. Twenty-nine patients who were given the RD were evaluated for efficacy and detailed safety profile: these patients consisted of 6 and 23 patients who entered into the study at phase I and II, respectively.

Phase I

The first cohort of 6 patients received docetaxel 40 mg/m^2 plus S-1 80 mg/m^2 (dose level 1). Among these patients, one experienced cerebral infarction (grade 4 CNS cerebrovascular ischemia). No other DLT was observed at dose level 1. At dose level 2 (docetaxel 50 mg/m^2), 2 of the 3 patients developed grade 4 neutropenia which was considered DLT. From these results, the MTD and RD were determined to be level 2 and level 1, respectively.

Phase II

Baseline characteristics of the 29 patients treated at the RD are shown in Table 1. Ages ranged from 48 to 79 years, with a median of 67 years. There were 23 men and 6 women. Nine patients had Eastern Cooperative Oncology Group PS 0, 20 patients had PS 1. Seven patients had clinical stage IIIB disease and 22 had stage IV disease. Histology consisted of adenocarcinoma in 16 patients, squamous cell carcinoma in 10, large-cell carcinoma in 2, and other in one. A single prior chemotherapy regimen had been given in 23 patients, 2 regimen in 4 patients and 3 in 2 patients. Twenty-eight (96.5%) patients had received a platinum-based chemotherapy.

Response and Survival

Of 29 patients assessable for response, none of the patients achieved a CR; 7 (24.1%) achieved a PR with an overall response rate of 24.1% [95% confidence interval (95% CI), 10.3–44.8%]. Thirteen (44.8%) had SD and 7 patients (24.1%) had PD as best response. Two were unevaluable. The tumor control rate (CR + PR+SD) was 68.9% (95% CI, 49.2–84.7%). Among all 29 patients, the median PFS was 3.9 months. As shown in Figure 1, the MST of all patients was 11.8 months, and the 1-year survival rate was 41.8% (95% CI, 21.8–61.8%).

Toxicity of Treatment

Hematological toxicity and nonhematological toxicity were analyzed during treatment and the follow-up period. The major toxicities during the study period are shown in Tables 2 and 3. The grade 3 to 4 hematological toxicities were neutropenia (34.5%), leukopenia (20.6%), and anemia (10.3%). None of the patients developed grade 2 or more thrombocytopenia. The grade 3 to 4 nonhematological toxicities included fever 2 (6.9%), diarrhea 1 (3.4%), stomatitis 1 (3.4%), cerebral infarction 1 (3.4%), and pneumonitis 1 (3.4%). There was one treatment-related death. The patient died 54 days after the first cycle of chemotherapy due to relapse of drug induced pneumonitis.

Treatment Delivery

The median number of cycles administered was 3 (range, 1–8 cycles).

TABLE 1. Patients' Characteristics

No. patients	30
Eligible	29
Male/Female	23/6
Median age, in yr (range)	67 (48–79)
PS 0/1	9/20
ad/sq/la/other	16/10/2/1
Stage IIIb/IV	7/22
No. previous chemo regimens	
1/2/3	23/4/2
RT	13
Operation	3

PS, performance status; Chemo, chemotherapy; RT, radiotherapy.

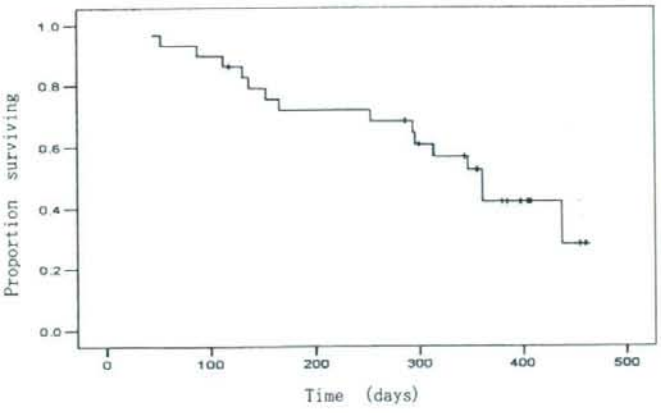

FIGURE 1. Overall survival curve.

TABLE 2. Hematological Toxicity

Grade				
Toxicity	**1**	**2**	**3**	**4**
Leukopenia	3	8	6	0
Neutropenia	1	5	7	3
Thrombocytopenia	2	0	0	0
Hemoglobin	7	7	3	0

TABLE 3. Nonhematological Toxicity

Grade				
Toxicity	**1**	**2**	**3**	**4**
Nausea	5	0	0	0
Vomiting	1	2	0	0
Fatigue	1	5	0	0
Infection	0	0	0	0
Fever	3	0	2	0
Diarrhea	4	0	1	0
Ulcer	0	1	0	0
Cerebrovascular ischemia	0	0	0	1
Skin	2	3	0	0
Stomatitis	3	0	1	0
Pneumonitis	1	0	0	1*

*One patient died from relapse of drug induced pneumonitis.

In all, 20 (62.5%) patients received at least 2 cycles of treatment. The reasons for terminating the chemotherapy before the second treatment cycle were disease progression in seven patients and adverse events in two patients. Five patients each required dose reductions of docetaxel or S-1, respectively.

Poststudy Therapy

Eighteen patients received at least one form of antitumoral treatment after disease progression. Thirteen patients received chemotherapy alone, the most frequently prescribed treatment was carboplatin plus gemcitabine. Ten patients received gefitinib.

DISCUSSION

The benefit of second-line chemotherapy has been substantiated by randomized trials using docetaxel, pemetrexed, topotecan, and erlotinib.[10,11,19–21] The response rate was reported to be 6.7 to 10.8% for docetaxel, 9.1% for pemetrexed, 5% for topotecan, and 8.9% for erlotinib. The 1-year survival rate of these reports ranges from 25 to 37%. It is clear that there is an urgent need for more active treatment regimens to patients with relapsed or refractory NSCLC. On the other hand, second-line chemotherapy is a palliative treatment. Therefore, pretreated patients have poorer tolerance to second-line chemotherapy, lower toxicity, and efficacy, which is important when considering the second-line chemotherapy.

To improve the efficacy of second-line chemotherapy, a number of studies have conducted two-drug second-line therapy combinations.[22–25] Georgoulias et al. reported a randomized phase II study that compared single agent irinotecan with a combination of irinotecan plus gemcitabine.[24] Their results failed to demonstrate a statistically significant survival advantage of the combination of irinotecan and gemcitabine over irinotecan alone, although the combination regimen was better in terms of response rate and QOL. A phase III study by Takeda et al. comparing docetaxel alone versus docetaxel plus gemcitabine was terminated early with unexpected incidence of interstitial lung disease and treatment-related deaths due to interstitial lung disease, only in the combination chemotherapy group.[25] Indeed, a comparison of combination chemotherapy versus monotherapy in patients with previously treated NSCLC failed to demonstrate any difference in terms of overall survival. For the moment, single-agent therapy remains the standard option for patients with relapsed or refractory NSCLC.

In the present study, we administered S-1 plus docetaxel to previously treated patients with NSCLC. Seven of the 29 patients (24.1%) achieved a PR as a result. The MST of this regimen was 11.8 months and the 1-year survival rate was 41.8% (Figure 1). The results of the present study are promising, suggesting that the survival of patients treated

with combination therapy could be improved compared with the survival of those treated with docetaxel alone as a second-line treatment. However, we can not exclude the possibility that the poststudy treatment such as gefitinib or selection bias might also have played a role in prolonging the survival times. Various combination chemotherapy regimens including oral fluoropyrimidine, such as UFUR and capecitabine, have been investigated in NSCLC.[26–28] Kindwall-Keller et al. reported a phase II study of docetaxel and capecitabine in previously treated patients with NSCLC.[27] The response rate was 26% with the MST and 1-year survival rate of 9.1 month and 37%. Chen et al. used UFUR with gemcitabine for 45 patients who failed previous platinum-based chemotherapy.[28] Their patients were treated with 1000 mg/m^2 gemcitabine on days 1 and 8, plus oral UFUR 200 mg/m^2/d from days 1 to 14 of every 3 weeks. They reported that 7 patients (15.6%) had a PR. The MST was 13.2 months.

Our study used 40 mg/m^2 of docetaxel every 3 weeks is lower than that commonly using docetaxel alone at the dose of 75 mg/m^2 as second-line setting in the United States and Europe. By combining docetaxel at 40 mg/m^2 on day 1 with S-1 at 80 mg/m^2/d on days 1 to 14 every 3 weeks, we expected less toxicity, with preserved efficacy. In Japan, docetaxel 60 mg/m^2 every 3 weeks is the commonly used dose. In a phase I study of docetaxel plus S-1, the RD of docetaxel was determined to be 40 mg/m^2 in combination with S-1 80 mg/m^2/d on days 1 to 14. This combination chemotherapy has been evaluated in gastric cancer in Japan.[29–31] The RD of docetaxel was 40 mg/m^2 in combination with S-1 80 mg/m^2/d in the gastric cancer which was the same is our study as a second-line setting. Yamaguchi et al. speculate that the reason for the lower dose of docetaxel may be that the pharmacokinetic parameters (AUC and C_{max}) of 5-FU increase according to the dose of docetaxel.[31]

In our study, the main toxicity was myelosuppression. The most common hematological toxicities were neutropenia and leukopenia. Grade 3 or 4 neutropenia occurred in 34.5% and grade 3 or 4 anemia occurred in 10.3%. In phase III studies of docetaxel 75 mg/m^2 given as a single agent, grade 3 or 4 neutropenia occurred in 40.2 to 67.3% and grade 3 or 4 anemia occurred in 4.3 to 10%.[10,19,20] It seemed that the incidence of grade 3 or 4 neutropenia were lower in our study than in those phase III studies. The majority of nonhematological toxicities were relatively mild. However, grade 4 cerebral infarction and pneumonitis were observed. It is unclear whether this adverse CNS event was related to this combination chemotherapy. This may be due to the hypercoagulability associated with lung cancer. Clotting activation and thromboembolic manifestations are common features in patients with cancer. Therefore, this CNS event might have occurred by chance.[32]

In conclusion, our study indicates that the combination of docetaxel pulse S-1 is an effective and well-tolerated regimen for the treatment of patients with previously treated NSCLC. This regimen seems suitable as a second-line treatment for patients with NSCLC. The response rate and median survival are encouraging and warrant additional investigation.

ACKNOWLEDGMENTS

The authors are indebted to Professor J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

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Synergistic antitumor effect of S-1 and the epidermal growth factor receptor inhibitor gefitinib in non-small cell lung cancer cell lines: role of gefitinib-induced down-regulation of thymidylate synthase

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Abstract

Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the therapeutic response to EGFR tyrosine kinase inhibitors (TKI) in patients with advanced non-small cell lung cancer (NSCLC). The response rate to these drugs remains low, however, in NSCLC patients with wild-type *EGFR* alleles. Combination therapies with EGFR-TKIs and cytotoxic agents are considered a therapeutic option for patients with NSCLC expressing wild-type *EGFR*. We investigated the antiproliferative effect of the combination of the oral fluorouracil S-1 and the EGFR-TKI gefitinib in NSCLC cells of differing *EGFR* status. The combination of 5-fluorouracil and gefitinib showed a synergistic antiproliferative effect *in vitro* in all NSCLC cell lines tested. Combination chemotherapy with S-1 and gefitinib *in vivo* also had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of *EGFR* mutations. Gefitinib inhibited the expression of the transcription factor E2F-1, resulting in the down-regulation of thymidylate synthase at the mRNA and protein levels. These observations suggest that gefitinib-induced down-regulation of thymidylate synthase is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib and provide a basis for clinical evaluation of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors. [Mol Cancer Ther 2008;7(3):599–606]

Introduction

Targeted therapy in the treatment of cancer has made substantial progress over the last few years. The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR; ErbB1), ErbB2 (HER2/*neu*), ErbB3, and ErbB4 and is important for normal development as a result of its roles in cell proliferation and differentiation (1–3). Aberrant expression of EGFR has been detected in a wide range of human epithelial malignancies, including non-small cell lung cancer (NSCLC), and is correlated with poor prognosis and reduced survival time (4, 5). Agents that specifically target EGFR are therefore under development as anticancer drugs. Indeed, two inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the catalytic pocket of the receptor, have been extensively studied in individuals with NSCLC (6–9). Somatic mutations in the region of *EGFR* that encodes the tyrosine kinase domain have been associated with tumor responsiveness to EGFR-TKIs in a subset of NSCLC patients (10–17). In contrast, achievement of a clinical benefit of these drugs in NSCLC patients who express wild-type *EGFR* has been problematic.

S-1 (Taiho Pharmaceutical) is an oral anticancer agent composed of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (18). Tegafur is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome *P*450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase (DPD), which catalyzes 5-FU catabolism (19). Oxonate reduces the gastrointestinal toxicity of 5-FU (20). A response rate of 22% and a median survival time of 10.2 months were obtained in a clinical trial of S-1 in patients with advanced NSCLC not subjected previously to chemotherapy (21). Few severe gastrointestinal or hematologic adverse events were reported. Moreover, a phase II trial of S-1 plus cisplatin in NSCLC patients revealed a 47% response rate and an acceptable safety profile (22).

Based on this background, we examined the anticancer effect of the combination of S-1 and gefitinib in NSCLC cell lines of differing *EGFR* status. We found that the combination of S-1 (or 5-FU) and gefitinib exhibited a marked and synergistic antiproliferative effect both *in vivo*

Received 8/16/07; revised 10/24/07; accepted 1/25/08.

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doi:10.1158/1535-7163.MCT-07-0567

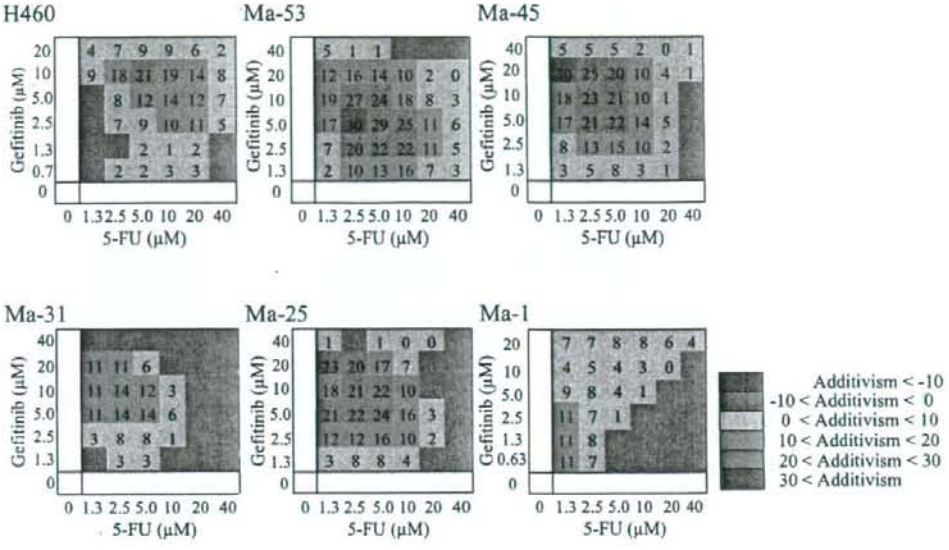

Figure 1. Inhibition of NSCLC cell growth by the combination of 5-FU and gefitinib *in vitro*. Cells with wild-type (H460, Ma-53, Ma-45, Ma-31, and Ma-25) or mutant (Ma-1) EGFR alleles were exposed for 72 h to 5-FU and gefitinib at the indicated concentrations, after which cell viability was measured with a colorimetric assay. The observed excess inhibition (%) relative to that predicted by the Bliss additivism model is shown color-coded in a drug concentration matrix for each cell line. Yellow, orange, pink, and red, synergy; light and dark blue, antagonism. Mean of triplicates from a representative experiment.

and *in vitro* in cells regardless of the absence or presence of *EGFR* mutations. Furthermore, we assessed the effects of gefitinib on the expression of enzymes that function in 5-FU metabolism, including thymidylate synthase (TS), DPD, and orotate phosphoribosyltransferase (OPRT), to gain insight into the mechanism underlying the synergistic effect of combination therapy with S-1 and gefitinib.

Materials and Methods

Cell Lines and Reagents

The human NSCLC cell lines NCI-H460 (H460), Ma-1, Ma-25, Ma-31, Ma-45, and Ma-53 were obtained as described previously (23). MiaPaca-2 cells were obtained from Japan Health Sciences Foundation. These cell lines were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Gefitinib was provided by AstraZeneca. S-1 and CDHP were provided by Taiho Pharmaceutical. 5-FU was obtained from Wako.

Growth Inhibition Assay *In vitro*

Cells (2.0×10^3) were plated in 96-well flat-bottomed plates and cultured for 24 h before the addition of various concentrations of 5-FU and gefitinib and incubation for an additional 72 h. Cell Counting Kit-8 solution (Dojindo) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 450 nm. Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of 5-FU or gefitinib resulting in 50% growth inhibition (IC_{50}) was calculated. The effect of combining 5-FU and gefitinib was classified as additive, synergistic, or antagonistic with the Bliss additivism model (24–26). A theoretical curve was calculated for combined inhibition with the equation: $E_{bliss} = E_A + E_B - (E_A \times E_B)$, where E_A and E_B are the fractional inhibitory effects of drug A alone and drug B alone at specific concentrations. E_{bliss} is then the fractional inhibition that would be expected if the effect of the combination of the two drugs was exactly additive. In this study, the Bliss variable is expressed as percentage decrease in cell growth above what would be expected for the combination. Bliss = 0 indicates that the effect of the combination is additive; Bliss > 0 is indicative of synergy; and Bliss < 0 indicates antagonism.

Animals

Male athymic nude mice were exposed to a 12-h light, 12-h dark cycle and provided with food and water *ad libitum* in a barrier facility. All experiments were done in compliance with the regulations of the Animal Experimentation Committee of Taiho Pharmaceutical.

Growth Inhibition Assay *In vivo*

Cubic fragments of tumor tissue (~2 × 2 × 2 mm) were implanted s.c. into the axilla of 5- to 6-week-old male athymic nude mice. Treatment was initiated when tumors in each group achieved an average volume of 100 to 150 mm^3. Treatment groups consisted of control, S-1 alone, gefitinib alone, and the combination of S-1 and gefitinib. Each treatment group contained seven mice. S-1 (10 mg/kg body mass) and gefitinib (50 or 3 mg/kg) were administered by oral gavage once a day for 14 days; control animals

received 0.5% (w/v) hydroxypropylmethylcellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula $LW^2 / 2$. Both tumor size and body weight were measured two or three times per week.

Immunoblot Analysis

Cell lysates were fractionated by SDS-PAGE on 12% gels (NuPAGE Bis-Tris Gels; Invitrogen), and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites with 5% skim milk, the membrane was incubated overnight at room temperature with primary antibodies. Antibodies to DPD, OPRT, and TS were obtained from Taiho Pharmaceutical; those to E2F-1 were from Santa Cruz Biotechnology; and those to β-actin (loading control) were from Sigma. Immune complexes were detected by incubation of the membrane for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to mouse or rabbit immunoglobulin and by subsequent exposure to enhanced chemiluminescence reagents (Pierce).

Immunoprecipitation Analysis

Immunoprecipitation of EGFR was done according to standard procedures. Whole-cell lysates (800 μg protein) were incubated overnight at 4°C with antibodies to EGFR (Santa Cruz Biotechnology), after which Protein G Plus/Protein A-Agarose Suspension (Calbiochem) was added and the mixtures were incubated for an additional 1 h at 4°C. Immunoprecipitates were isolated, washed, resolved by SDS-PAGE on a 7.5% gel (Bio-Rad), and subjected to immunoblot analysis with antibodies to phosphotyrosine (PY20) and EGFR (Zymed).

Reverse Transcription and Real-time PCR Analysis

Total RNA (1 μg) extracted from cells with the use of an RNeasy Mini Kit (Qiagen) was subjected to reverse transcription with the use of a SuperScript Preamplification System (Invitrogen Life Technologies). The resulting cDNA was then subjected to real-time PCR analysis with the use of a TaqMan PCR Reagent Kit and a Gene Amp 5700 Sequence Detection System (Applied Biosystems). The forward and reverse primers and TaqMan probe for TS cDNA were 5-GCCTCGGTGTGCCTTTCA-3 and 5-CCCGTGATGTGCGCAAT-3 and 6-FAM-5′-TCGCCA-GCTACGCCCTGCTCA-3′-TAMRA, respectively. Glyceraldehyde-3-phosphate dehydrogenase mRNA were used as an internal standard.

Statistical Analysis

Data are presented as mean ± SE and were analyzed by the Aspin-Welch t test. $P < 0.05$ was considered statistically significant.

Results

Effect of the Combination of 5-FU and Gefitinib on NSCLC Cell Growth *In vitro*

Tegafur, which is a component of S-1, is metabolized to 5-FU in the liver and exerts antitumor effects. We first examined the antiproliferative activity of the combination of 5-FU and gefitinib in six NSCLC cell lines. Five of the cell lines (H460, Ma-53, Ma-45, Ma-31, and Ma-25) possess wild-type *EGFR* alleles, whereas Ma-1 cells harbor an *EGFR* mutation (E746_A750del) that is associated with a high response rate to the EGFR-TKIs gefitinib and erlotinib in individuals with advanced NSCLC. We assessed

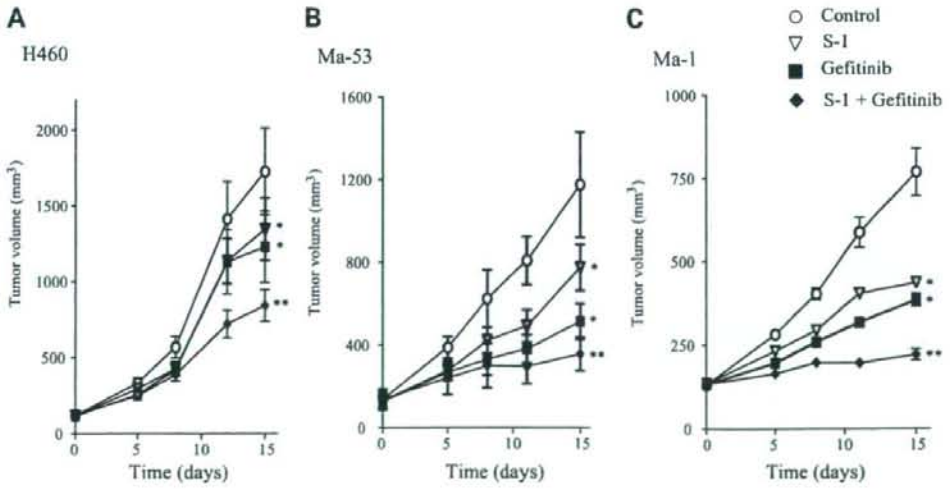

Figure 2. Antitumor activity of the combination of S-1 and gefitinib *in vivo*. **A** and **B**, nude mice with tumor xenografts established by s.c. implantation of NSCLC cells (H460 and Ma-53) possessing wild-type EGFR were treated daily for 2 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage. **C**, nude mice with tumor xenografts derived from NSCLC cells (Ma-1) expressing mutant EGFR were treated daily for 2 weeks with vehicle (control), S-1 (10 mg/kg), gefitinib (3 mg/kg), or both drugs by oral gavage. Tumor volume in all animals was determined at the indicated times after the onset of treatment. Mean ± SE of values from seven mice per group. *, $P < 0.05$ versus control; **, $P < 0.05$ versus S-1 or gefitinib alone for values 15 d after treatment onset (Aspin-Welch t test).

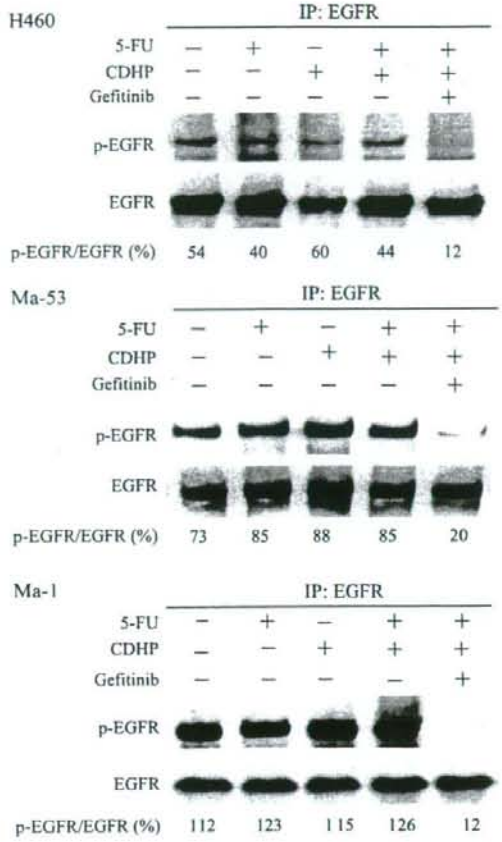

Figure 3. Lack of effect of 5-FU and CDHP on EGFR phosphorylation in NSCLC cell lines. NSCLC cells (H460, Ma-53, and Ma-1) were incubated for 24 h in medium supplemented with 2% fetal bovine serum and with 5-FU (10 μmol/L), CDHP (3 μmol/L), or gefitinib (5 μmol/L). Cell lysates were then prepared and subjected to immunoprecipitation (*IP*) with antibodies to EGFR, and the resulting precipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (for detection of phosphorylated EGFR) and with antibodies to EGFR. The intensity of the phosphorylated EGFR band relative to that of the EGFR band was determined by densitometry and is expressed as a percentage below each lane.

whether 5-FU and gefitinib showed additivity, synergy, or antagonism based on the Bliss additivism model (24–26). We chose this model rather than isobologram or combination index analysis because it would allow us to evaluate the nature of drug interactions even in instances in which the maximal inhibition by 5-FU or gefitinib alone was too low to obtain a reliable IC_{50} value. The six test concentrations for each agent were chosen after first determining the corresponding IC_{50} values. The IC_{50} values for 5-FU chemosensitivity were not associated with *EGFR* status and ranged from 7 to 11 μmol/L. The effect of combined treatment with 5-FU and gefitinib on the proliferation of the six NSCLC cell lines was tested in triplicate in a 6 × 6 concentration matrix. Calculation of the percentage inhibition in excess of that predicted by the Bliss additivism model revealed synergistic effects of Bliss > 0 for 5-FU and gefitinib in all of the six cell lines tested (Fig. 1). These results suggested that 5-FU and gefitinib act synergistically to inhibit cell growth in NSCLC cells.

Effect of Combined Treatment with S-1 and Gefitinib on NSCLC Cell Growth *In vivo*

We therefore next investigated whether combined treatment with S-1 and gefitinib might also exert a synergistic effect on NSCLC cell growth *in vivo*. Doses of both agents were selected so that their independent effects on tumor growth would be moderate. Nude mice were implanted s.c. with H460, Ma-53, or Ma-1 tumor fragments to establish tumor xenografts. When the H460 or Ma-53 tumors, which harbor wild-type *EGFR*, became palpable (100-150 mm^3), the mice were divided into four groups for daily treatment with vehicle, S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage over 2 weeks. For xenografts formed by H460 or Ma-53 cells, combination therapy with S-1 and gefitinib resulted in a significant reduction in tumor size compared with that apparent in animals treated with S-1 or gefitinib alone (Fig. 2*A* and *B*). Mice bearing Ma-1 tumors, which express mutant *EGFR*, were treated with vehicle, S-1 (10 mg/kg), gefitinib (3 mg/kg), or both agents daily over 2 weeks. Combination treatment with S-1 and gefitinib significantly inhibited the growth of Ma-1 xenografts relative to that apparent in mice treated with either agent alone (Fig. 2C). None of the drug treatments induced a weight loss of >20% during the 2-week period, and no signs of overt drug toxicity were apparent (data not shown). These results thus suggested that combination chemotherapy with S-1 and gefitinib *in vivo* had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of *EGFR* mutations, consistent with our results *in vitro*.

Effects of 5-FU and CDHP on EGFR Phosphorylation in NSCLC Cell Lines

To investigate the mechanism responsible for the observed interaction between S-1 and gefitinib, we examined the effect of 5-FU on EGFR signal transduction in NSCLC cells expressing wild-type (H460 and Ma-53) or mutant (Ma-1) *EGFR*. Immunoprecipitation analysis revealed that exposure of H460 or Ma-53 cells to 5-FU (10 μmol/L) for 24 h had no effect on the basal level of EGFR phosphorylation (Fig. 3). We have shown previously that EGFR is constitutively phosphorylated in Ma-1 cells maintained in serum-free medium (23). Exposure of Ma-1 cells to 5-FU for 24 h did not affect this constitutive level of EGFR phosphorylation (Fig. 3). We next examined the effects of both CDHP, which is a component of S-1, and the combination of CDHP and 5-FU on EGFR phosphorylation in H460, Ma-53, and Ma-1 cells. Neither CDHP alone nor the combination of CDHP and 5-FU affected the level of EGFR phosphorylation in any of these three cell lines (Fig. 3). These results thus indicated that 5-FU and CDHP have no effect on EGFR signal transduction.

Effects of Gefitinib on the Expression of DPD, OPRT, and TS in NSCLC Cell Lines

We next investigated whether gefitinib might affect the expression of DPD, OPRT, or TS, enzymes that are major determinants of the sensitivity of cells to 5-FU. We first examined the abundance of these enzymes in the NSCLC cell lines H460, Ma-53, and Ma-1 by immunoblot analysis. The expression of DPD was detected in MiaPaca-2 cells (positive control) but not in H460, Ma-53, or Ma-1 cells (Fig. 4*A*). In contrast, OPRT and TS were detected in all three NSCLC cell lines and their abundance did not appear related to *EGFR* status (Fig. 4*A*). Treatment of H460, Ma-53, or Ma-1 cells with gefitinib (5 μmol/L) for up to 48 h resulted in a time-dependent decrease in the amount of TS, whereas that of OPRT or DPD remained unaffected (Fig. 4*B*). A reduced level of TS expression in tumors has been associated previously with a higher response rate to 5-FU-based chemotherapy (27, 28). Our data thus suggested that the suppression of TS expression by gefitinib might increase the sensitivity of NSCLC cells to 5-FU.

The transcription factor E2F-1 regulates expression of the TS gene (29–31). We therefore examined the possible effect of gefitinib on E2F-1 expression in NSCLC cell lines. Incubation of H460, Ma-53, or Ma-1 cells with gefitinib for up to 48 h also induced a time-dependent decrease in the amount of E2F-1 (Fig. 4*B*), suggesting that this effect might contribute to the down-regulation of TS expression by gefitinib in these cell lines.

Effect of Gefitinib on TS mRNA Abundance in NSCLC Cell Lines

The abundance of TS mRNA would be expected to be decreased if the down-regulation of E2F-1 expression by gefitinib was responsible for the reduced level of TS. We determined the amount of TS mRNA in H460, Ma-53, or Ma-1 cells at various times after exposure to gefitinib with the use of reverse transcription and real-time PCR analysis. Gefitinib indeed induced a time-dependent decrease in the amount of TS mRNA in all three NSCLC cell lines (Fig. 5), suggesting that the down-regulation of TS expression by gefitinib occurs at the transcriptional level and may be due to suppression of E2F-1 expression.

Discussion

The recent identification of activating somatic mutations of *EGFR* in NSCLC and their relevance to prediction of the therapeutic response to EGFR-TKIs such as gefitinib and erlotinib have had a major effect on NSCLC treatment (10–17). The response rate to these drugs remains low, however, in NSCLC patients with wild-tye *EGFR* alleles. Combination therapy with EGFR-TKIs and cytotoxic agents is a potential alternative strategy for NSCLC expressing wild-type *EGFR*. In the present study, we have evaluated the potential cooperative antiproliferative effect of combined treatment with the EGFR-TKI gefitinib and the new oral fluorouracil S-1 in NSCLC cell lines of differing *EGFR* status. We found that S-1 (or 5-FU) and gefitinib exert a synergistic antiproliferative effect on NSCLC cells both *in vivo* and *in vitro* regardless of the absence or presence of *EGFR* mutation. We chose a gefitinib dose of 50 mg/kg for treatment of mice bearing H460 or Ma-53 tumors. The median effective dose of gefitinib was shown previously to be ~50 mg/kg in athymic nude mice bearing A431 cell-derived xenografts (32). A gefitinib dose of 50 mg/kg has therefore subsequently been widely used in tumor xenograft studies (33–36). The U.S. Food and Drug Administration recommends that drug doses in animals be converted to those in humans based on body surface area (37). According to this guideline, a gefitinib dose of 50 mg/kg in mouse xenograft models is approximately equivalent to the therapeutic dose (250 mg/d) of the drug in humans. In addition, the tumor concentrations of gefitinib in NSCLC xenografts of mice treated with this drug (50 mg/kg) ranged from 9.7 to 13.3 μg/g, values that were similar to the

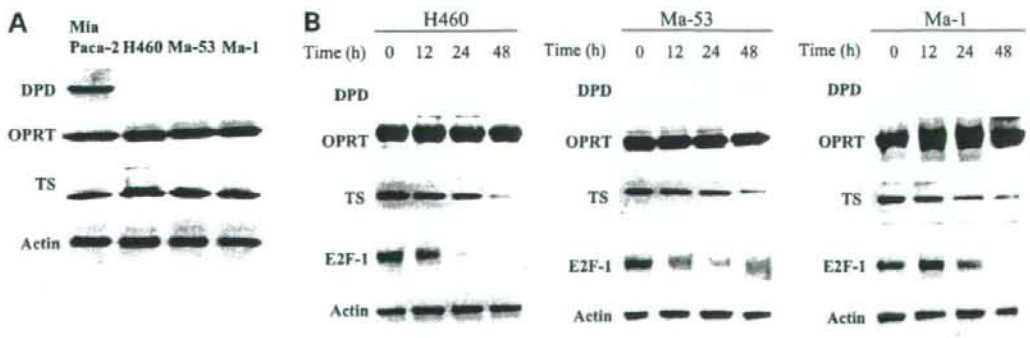

Figure 4. Effects of gefitinib on the expression of E2F-1, DPD, OPRT, and TS in NSCLC cell lines. **A**, lysates of H460, Ma-53, or Ma-1 cells were subjected to immunoblot analysis with antibodies to DPD, OPRT, TS, or β-actin (loading control). MiaPaca-2 cells were also examined as a positive control for DPD expression. **B**, NSCLC cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which cell lysates were subjected to immunoblot analysis as in **A**, with the addition that E2F-1 expression was also examined.

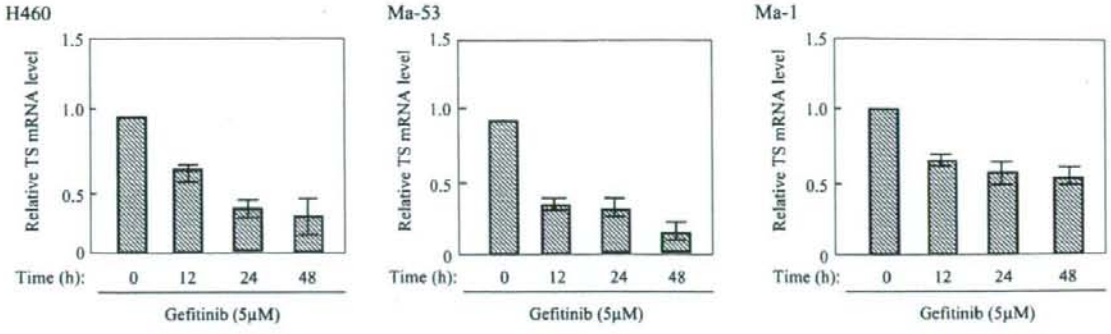

Figure 5. Down-regulation of TS mRNA by gefitinib in NSCLC cell lines. H460, Ma-53, or Ma-1 cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which total RNA was extracted from the cells and subjected to reverse transcription and real-time PCR analysis of TS mRNA. The amount of TS mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Mean ± SE of values from three separate experiments.

achievable concentrations of gefitinib in tumor tissues of treated humans (34). These observations suggest that a gefitinib dose of 50 mg/kg in mouse xenograft models is appropriate for mimicking the therapeutic dose in humans.

EGFR-TKIs have been shown previously to act synergistically with radiation or cytotoxic agents such as cisplatin, paclitaxel, and irinotecan (38–40). These cytotoxic agents and radiation have been shown to increase the phosphorylation level of EGFR, possibly reflecting the activation of prosurvival signaling, and this effect is blocked by EGFR-TKIs, resulting in the synergistic antitumor effects of the combination therapies. Such a synergistic effect of 5-FU and gefitinib was attributed to 5-FU-induced EGFR phosphorylation in colorectal cancer cells (41). In contrast, we found that 5-FU had no effect on the level of EGFR phosphorylation in NSCLC cell lines. Further examination of different concentrations of 5-FU and different exposure times also failed to reveal an effect of 5-FU on EGFR phosphorylation in these cells (data not shown). These findings indicate that NSCLC cell lines respond differently to 5-FU than do colorectal cancer cells and that the synergistic antiproliferative effect of 5-FU and gefitinib in NSCLC cells is not mediated at the level of EGFR phosphorylation.

Our results indicate that the synergistic interaction of 5-FU (or S-1) and gefitinib is attributable, at least in part, to down-regulation of TS expression by gefitinib. The active metabolite of 5-FU, FdUMP, forms a covalent ternary complex with 5,10-methylenetetrahydrofolate and TS, resulting in inhibition of DNA synthesis (42). TS is thus an important therapeutic target of 5-FU. The amount of TS in neoplastic cells has been found to increase after exposure to 5-FU, resulting in the maintenance of free enzyme in excess of that bound to 5-FU (43–47). Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5-FU resistance in cancer cells (48–50). The development of a new therapeutic strategy that reduces TS expression would therefore be of interest. Indeed, preclinical studies have shown that the down-regulation of TS by antisense oligonucleotides or other means enhances the efficacy of 5-FU (51–54). Down-regulation of TS would be expected to enhance the cytotoxicity of 5-FU as a result of the decrease in the amount of its protein target (55). Consistent with these preclinical data, an inverse relation between TS expression and 5-FU sensitivity has been shown in various human solid tumors (27, 28, 56–60). We have now shown that gefitinib alone induced down-regulation of TS expression, suggesting that this effect of gefitinib contributes to its synergistic interaction with 5-FU (or S-1) in NSCLC cell lines.

We further explored the molecular mechanism by which gefitinib induces down-regulation of TS expression in NSCLC cells. Given that EGFR signal transduction has been shown to be involved in activity of E2F-1 that regulates the expression of several genes including TS (61, 62), which controls the expression of several genes including that for TS, we examined the possible effects of gefitinib on E2F-1 expression and on the abundance of TS mRNA. Gefitinib induced down-regulation of E2F-1 in NSCLC cell lines harboring wild-type *EGFR*, consistent with previous observations (63), as well as in those expressing mutant *EGFR*. In addition, gefitinib reduced the amount of TS mRNA in NSCLC cells, consistent with the notion that the suppression of TS expression by gefitinib is attributable to inhibition of gene transcription as a result of down-regulation of E2F-1. For our experiments examining the effects of gefitinib on TS and E2F-1 expression, we used a drug concentration of 5 μmol/L. The concentration of gefitinib in tumor xenografts was shown previously to be 5 to 14 times that in the plasma concentration of the mouse hosts (34). Daily oral administration of gefitinib (250 mg) in patients also gave rise to a drug concentration in tumor tissue that was substantially higher (mean, 42-fold) than that in plasma concentration (34). We showed previously that the maximal concentration of gefitinib in the plasma of patients with advanced solid tumors had a mean value of 0.76 μmol/L at a daily dose of 225 mg (64). Based on these data, we considered that a gefitinib concentration of 5 μmol/L was appropriate for our

analyses of TS and E2F-1 expression. Together, our present findings suggest that down-regulation of E2F-1 and consequently that of TS by gefitinib is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib.

Somatic mutations of *EGFR* have been associated with sensitivity to EGFR-TKIs in patients with advanced NSCLC (13–16). However, although most NSCLCs with *EGFR* mutations initially respond to EGFR-TKIs, the vast majority of these tumors ultimately develop resistance to the drug. In the present study, the synergistic effect of combination chemotherapy with S-1 and gefitinib was observed even in *EGFR* mutant cells. Our findings thus suggest that the addition of S-1 (or 5-FU) to EGFR-TKIs might overcome chemoresistance to EGFR-TKIs and that exploration of the effect of such combination therapy in cells resistant to EGFR-TKIs is warranted. *EGFR* mutations appear to be largely limited to lung cancer, with few such mutations having been detected in other types of cancer (65–67). 5-FU is widely used as an anticancer agent and is considered a key drug in chemotherapy for solid tumors such as gastrointestinal and cervical cancer (68–70). Our present results show that gefitinib suppressed the expression of TS in NSCLC cell lines regardless of the absence or presence of *EGFR* mutations, suggesting that the addition of EGFR-TKIs to a 5-FU-containing regimen might increase the effectiveness of such treatment for solid cancers without *EGFR* mutations. Oral combined chemotherapy with drugs, such as S-1 and gefitinib, may also prove to be of low toxicity and therefore maintain quality of life. Our preclinical results provide a basis for future clinical investigations of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors.

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Phase II Study of Combination Therapy with S-1 and Irinotecan for Advanced Non–Small Cell Lung Cancer: West Japan Thoracic Oncology Group 3505

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Abstract **Purpose:** To evaluate the efficacy and toxicity of combination therapy with the oral fluoropyrimidine formulation S-1 and irinotecan for patients with advanced NSCLC.

Experimental Design: Chemotherapy-naive patients with advanced NSCLC were treated with i.v. irinotecan (150 mg/m^2) on day 1 and with oral S-1 (80 mg/m^2) on days 1 to 14 every 3 weeks.

Results: Fifty-six patients (median age, 63 years; range, 40-74 years) received a total of 286 treatment cycles (median, 5; range, 1-15). No complete responses and 16 partial responses were observed, giving an overall response rate of 28.6% [95% confidence interval (95% CI), 17.3-42.2%]. Twenty-four patients (42.9%) had stable disease and 12 patients (21.4%) had progressive disease as the best response. The overall disease control rate (complete response + partial response + stable disease) was thus 71.4% (95% CI, 57.8-82.7%). Median progression-free survival was 4.9 months (95% CI, 4.0-6.4 months), whereas median overall survival was 15 months. Hematologic toxicities of grade 3 or 4 included neutropenia (25%), thrombocytopenia (3.6%), and anemia (3.6%), with febrile neutropenia being observed in four patients (7.1%). The most common nonhematologic toxicities of grade 3 or 4 included anorexia (14.3%), fatigue (8.9%), and diarrhea (8.9%). There were no deaths attributed to treatment.

Conclusions: The combination of S-1 and irinotecan is a potential alternative option with a favorable toxicity profile for the treatment of advanced NSCLC. This nonplatinum regimen warrants further evaluation in randomized trials.

Non–small cell lung cancer (NSCLC) is the leading cause of death related to cancer worldwide (1). Platinum-based chemotherapy is the standard first-line treatment for advanced NSCLC based on the moderate improvement in survival and quality of life it confers compared with best supportive care alone (2–4). The poor outlook even for patients with advanced NSCLC who receive such treatment has prompted a search for new chemotherapeutic agents and combination regimens.

S-1 is an oral fluorinated pyrimidine formulation that combines tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (5). Tegafur is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome *P*450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase, which catalyzes 5-FU catabolism (6). CDHP also attenuates the cardiotoxic and neurotoxic effects of 5-FU by reducing the production of fluoro-β-alanine, the main catabolite of 5-FU (7, 8). Oxonate reduces the gastrointestinal toxicity of 5-FU. After its oral administration, oxonate becomes distributed selectively to the small and large intestine, where it inhibits the phosphorylation of 5-FU to fluoropyrimidine monophosphate catalyzed by orotate phosphoribosyltransferase within gastrointestinal mucosal cells, thereby reducing the incidence of diarrhea (9). In a phase II trial of S-1 as a single agent for treatment of advanced NSCLC, a response rate of 22% and a median survival time of 10.2 months were obtained in 59 patients without prior chemotherapy (10). Few severe gastrointestinal or hematologic adverse events were reported (10). Moreover, a phase II trial of S-1 plus cisplatin in advanced NSCLC patients revealed a response rate of 47% and a median survival time of 11 months (11).

Irinotecan is an inhibitor of DNA topoisomerase I. It has shown activity as a single agent in first-line chemotherapy for advanced NSCLC (12). Weekly administration of irinotecan

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Received 2/25/08; revised 3/29/08; accepted 4/14/08.

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doi:10.1158/1078-0432.CCR-08-0511