Phase II Trial of Amrubicin for Second-Line Treatment of Advanced Non-small Cell Lung Cancer

Results of the West Japan Thoracic Oncology Group Trial (WJTOG0401)

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Background: Amrubicin is a synthetic anthracycline drug that is a potent inhibitor of topoisomerase II. We have performed a multicenter phase II trial to evaluate the efficacy and safety of amrubicin for patients with previously treated non-small cell lung cancer (NSCLC).

Methods: Patients with advanced NSCLC who experienced disease recurrence after one platinum-based chemotherapy regimen were eligible for enrollment in the study. Amrubicin was administered by intravenous injection at a dose of 40 mg/m^2 on 3 consecutive days every 3 weeks.

Results: Sixty-one enrolled patients received a total of 192 treatment cycles (median, 2; range, 1–15). Response was as follows: complete response, 0; partial response, seven (11.5%); stable disease, 20 (32.8%); and progressive disease, 34 (55.7%). Median progression-free survival was 1.8 months, whereas median overall survival was 8.5 months, and the 1-year survival rate was 32%. Hematologic toxicities of grade 3 or 4 included neutropenia (82.0%), leukopenia (73.8%), thrombocytopenia (24.6%), and anemia (27.9%). Febrile neutropenia occurred in 18 patients (29.5%). One treatment-related death due to infection was observed. Nonhematologic toxicities were mild.

Conclusions: Amrubicin is a possible alternative for second-line treatment of advanced NSCLC, although a relevant hematological toxicity is significant, especially with a febrile neutropenia.

Key Words: Amrubicin, Non-small cell lung cancer (NSCLC), Platinum refractory, Second-line chemotherapy.

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Non-small cell lung cancer (NSCLC) is the leading cause of death related to cancer worldwide.[1] The first-line platinum-based chemotherapy confers a moderate improvement in survival and quality of life in individuals with advanced NSCLC.[2,3] It has recently become generally accepted that the second-line chemotherapy also has beneficial effects on survival and quality of life in such patients.[3–5] Despite the availability of several options for the second-line treatment of NSCLC,[6] however, the life expectancy of patients with advanced disease remains short, highlighting the urgent need for new treatments.

Amrubicin is a fully synthetic anthracycline anticancer drug with a similar structure to doxorubicin and is a potent inhibitor of topoisomerase II.[7–9] Two phase II trials of amrubicin administered as a single agent yielded response rates of 18.7 to 27.9% with acceptable toxicities in chemotherapy-naive patients with advanced NSCLC,[10,11] suggestive of promising activity for such patients. However, the activity and safety of amrubicin for patients with NSCLC whose

disease progresses after first-line chemotherapy have not been previously described.

Therefore, we conducted a multicenter phase II trial of amrubicin in patients with NSCLC previously treated with platinum-based chemotherapy. This trial was designed to determine the antitumor activity and toxicity of amrubicin in the second-line setting.

PATIENTS AND METHODS

Patient Selection

The eligibility criteria for participation of subjects in the trial included histologic or cytologic evidence of NSCLC; stage IV or stage IIIB disease (including only patients with no indications for curative thoracic radiotherapy) at study entry; recurrent or refractory disease after one previous platinum-containing chemotherapy regimen; measurable disease; no chemotherapy or radiotherapy within the 4 weeks before study entry; an age of 20 to 74 years; an Eastern Cooperative Oncology Group performance status of 0 or 1; adequate bone marrow function (leukocyte count of $\geq$4000 and $\leq$12,000/mm^3, neutrophil count of $\geq$2000/mm^3, platelet count of $\geq$100,000/mm^3, and hemoglobin content of $\geq$9.5 g/dl); adequate other organ function (serum total bilirubin concentration of $\leq$1.5 mg/dl, serum aspartate aminotransferase and alanine aminotransferase levels of $\leq$2.5 times the upper normal limit, and normal serum creatinine concentration); partial pressure of arterial oxygen of $\geq$60 torr; no abnormality on the electrocardiogram requiring treatment; and a left ventricular ejection fraction of $\geq$60% on echocardiography. Patients were ineligible for participation in the study if they had undergone previous amrubicin therapy, a history of a cumulative doxorubicin dose >500 mg/m^2 (epirubicin >900 mg/m^2, pirarubicin >950 mg/m^2, and daunorubicin >25 mg/kg), symptomatic brain metastasis, third-space fluid collection requiring drainage, active concomitant malignancy, radiographic signs of interstitial pneumonia or pulmonary fibrosis, a serious or uncontrolled concomitant systemic disorder (active infection, active gastric or duodenal ulcer, heart disease, diabetes mellitus, or a condition requiring chronic systemic administration of corticosteroids), or a history of drug allergy, or if they were lactating or pregnant. This study was performed in accordance with the principles of the Declaration of Helsinki and the good clinical practice guidelines. Written informed consent was obtained from all patients before study entry. Trial document approval was obtained from the institutional review board of each participating institution.

Study Design and Sample Size

The study was a multicenter, open-label, single-arm, phase II study. The primary end point was the response rate for amrubicin in patients with recurrent or refractory NSCLC who experienced treatment failure with platinum-based chemotherapy, which determined the sample size based on an optimal two-stage design.[12] On the basis of the results of previous studies, the proposed regimen was to be considered worthy or not worthy for additional investigation in the selected patient population if a true response rate was obtained of $\geq$18 or $\leq$5%, respectively, with a power of 0.9 and an α error of 0.05. A total of 55 assessable patients was necessary for the study; 23 in the first stage and 32 in the second stage. Assuming a drop-out rate of 10%, we planned on enrolling 60 patients in the study.

Treatment

Amrubicin was reconstituted in 20 ml of physiological saline or 5% glucose solution and was administered intravenously for more than 5 minutes at a dose of 40 mg/m^2 per day on days 1 to 3 every 3 weeks. Patients with evidence of disease progression or who experienced unacceptable adverse events were withdrawn from the study. Other criteria for treatment discontinuation included treatment refusal by the patient, inadvertent enrollment in the study, use of excluded concomitant therapy, or a decision by the physician to stop treatment. Subsequent courses of treatment were withheld until the following criteria were satisfied: the leukocyte count was $\geq$3000/mm^3, the neutrophil count was $\geq$1500/mm^3, the platelet count was $\geq$100,000/mm^3, and the grade of any nonhematologic toxicity was $\leq$2. If these criteria were not satisfied within 43 days after the onset of the last treatment, the patient was removed from the study. The dose of amrubicin was reduced to 35 mg/m^2 per day if leukopenia or neutropenia of grade 4 for more than 4 days, febrile neutropenia, thrombocytopenia of grade 4, or nonhematologic toxicity of grade $\geq$3 (or of grade 4 for anorexia, nausea, body weight loss, or hyponatremia) occurred during the previous course. If these toxicities occurred after reduction of the amrubicin dose to 35 mg/m^2 per day, the dose was reduced further to 30 mg/m^2 per day. The third reduction of amrubicin dose was not allowed.

Evaluation

Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors.[13] Tumors were measured by computed tomography within 4 weeks before the first cycle of treatment. The same measurement was performed every 4 weeks from the onset of treatment. A central radiologic review was performed to determine the eligibility of patients and the response to treatment. Response was confirmed at least 4 (for a complete or partial response) or 6 weeks (for stable disease) after it was first documented. Progression-free survival was defined as the time from registration until objective tumor progression or death. Patients whose disease had not progressed at the time of discontinuation of the study treatment were assessed until progression was documented. If a patient died without documentation of disease progression, the patient was considered to have had tumor progression at the time of death, unless there was sufficient documented evidence to conclude otherwise. Overall survival was defined as the time from registration until death from any cause. Progression-free and overall survival and the 1-year survival rate were estimated by the Kaplan-Meier method. Adverse events were graded according to National Cancer Institute Common Toxicity Criteria (version 3). All patients who received one dose of chemotherapy were assessable for toxicity. Clinical and laboratory assessment was performed at least once a week.

RESULTS

Patient Characteristics

Between February 2005 and March 2006, 61 patients were enrolled in the study at 12 participating institutions. All patients were eligible for the study and assessable both for the efficacy and safety of treatment and for survival. The characteristics of the study subjects are summarized in Table 1. Thirty-nine patients were men and 22 were women, and their median age was 63 years, with a range of 51 to 74 years. Histologic analysis revealed that 40 patients (65.6%) had adenocarcinoma, and 14 patients (23.0%) had squamous cell carcinoma. Forty-eight patients (78.7%) had stage IV disease, and the other 13 patients had stage IIIB disease at the time of enrollment in the study. All 61 patients had been previously treated with platinum-based chemotherapy, with eight and 22 patients having also undergone surgery or radiation therapy, respectively, before enrollment in the study.

Treatment Administered

Patients received a median of two cycles of treatment (range, 1–15), with 16 patients (26.2%) receiving at least four cycles. A total of 192 cycles of treatment was delivered overall. The mean relative dose intensity of amrubicin was 87.3%. Dose reduction of amrubicin was necessary according to the study protocol in 22 cycles (11.5% of total cycles). The major reasons for dose reduction were neutropenia or leukopenia of grade 4 (13 cycles of all cycles) and febrile neutropenia (nine cycles of all cycles). Treatment was discontinued in 14 patients after the first cycle and in 17 patients after the second cycle; the reasons for discontinuation included progressive disease (25 patients), toxicity (four patients), and patient refusal (two patients). Poststudy, 71% of patients eventually received subsequent therapies. Twenty-eight patients (46%) received docetaxel-containing chemotherapy, 18 (26%) received gefitinib or erlotinib, and 30 (49%) received other chemotherapy.

TABLE 1. Characteristics of the 61 Eligible Patients

Characteristic	No. of Patients (%)
Median age (yr)	
<70	48 (78.7)
≥70	13 (21.3)
Sex	
Male	39 (63.9)
Female	22 (36.1)
Performance status (ECOG)	
0	15 (24.6)
1	46 (75.4)
Disease stage	
III B	13 (21.3)
IV	48 (78.7)
Tumor histology	
Adenocarcinoma	40 (65.6%)
Squamous cell carcinoma	14 (23.0%)
Large cell carcinoma	3 (4.9)
NSCLC, not specified	4 (6.6)
Prior therapy	
Chemotherapy	61 (100)
Radiotherapy	22 (36.1)
Surgery	8 (13.1)
Time since last chemotherapy	
<3 mo	28 (46.0)
3–6 mo	16 (26.0)
≥6 mo	17 (28.0)
Response to prior chemotherapy	
Complete response	1 (1.6)
Partial response	36 (59.0)
Stable or progressive disease	19 (31.1)
Not evaluable	5 (8.2)

ECOG, Eastern Cooperative Oncology Group; NSCLC, non-small cell lung cancer.

Response and Survival

Among the 61 assessable patients, there were seven partial responses and no complete responses, for an overall response rate of 11.5% (95% confidence interval [CI], 4.7–22.2) (Table 2). Twenty patients (32.8%) had stable disease, yielding an overall disease control rate (complete response + partial response + stable disease) of 44.3% (95% CI, 31.5–57.6). Thirty-four patients had progressive disease as the best response. No correlation was apparent between the response rate and sex, age, tumor histology, disease stage, or smoking status.

Of the 61 subjects, 11 patients were still alive as of October 2008. The progression-free survival curve is shown in Figure 1; the median progression-free survival was 1.8 months (95% CI, 1.4–2.3). The curve for overall survival is shown in Figure 2; the median overall survival time was 8.5 months (95% CI, 7.7–10.4), and the 1-year survival rate was 32% (95% CI, 20.7–44.0).

Safety

The adverse events observed for all 61 treated patients are summarized in Table 3. The most frequent toxicity was myelosuppression, which mostly affected leukocytes. Neutropenia or leukopenia of grade ≥3 occurred in 82.0% and 73.8% of patients, respectively. Anemia and thrombocytopenia of grade ≥3 were relatively infrequent, occurring in 27.9% and 24.6% of patients, respectively. Eighteen patients (29.5%) developed febrile neutropenia. The most common

TABLE 2. Overall Response Rate for Amrubicin (Response Evaluation Criteria in Solid Tumors) as Determined by Independent Radiological Assessment

Response	No. of Patients
Complete response	0
Partial response	7 (11.5%; 95% CI, 4.7–22.2)
Overall response	7 (11.5%)
Stable disease	20 (32.8%)
Disease control	27 (44.3%; 95% CI, 31.5–57.6)
Progressive disease	34 (55.7%)

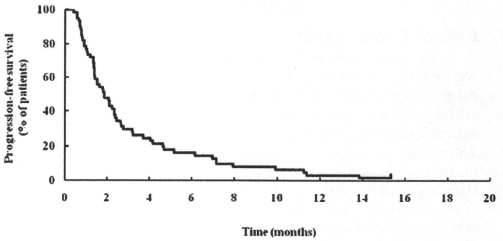

FIGURE 1. Kaplan-Meier analysis of progression-free survival for all 61 treated patients.

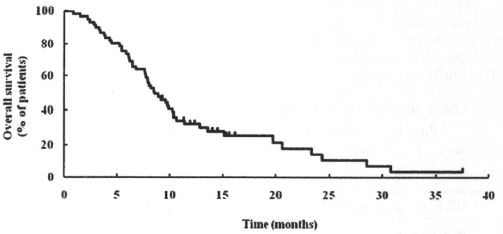

FIGURE 2. Kaplan-Meier analysis of overall survival for all 61 treated patients.

TABLE 3. Toxicity for all 61 Patients During Amrubicin Monotherapy According to the National Cancer Institute Common Toxicity Criteria (Version 3)

	Grade				Grade ≥3	
Toxicity	**1**	**2**	**3**	**4**	**No.**	**Percentage**
Leukopenia	5	8	24	21	45	73.8
Neutropenia	0	5	8	42	50	82.0
Anemia	16	27	13	4	17	27.9
Thrombocytopenia	25	7	7	8	15	24.6
Febrile neutropenia	0	0	18	0	18	29.5
Anorexia	19	9	5	1	6	9.8
Nausea	20	5	2	0	2	3.3
Vomiting	7	3	0	0	0	0
Asthenia	18	13	2	2	4	6.6
Infection	0	1	2	1	4[a]	6.6
Fever	10	6	1	0	1	1.6
Elevation of AST or ALT	15	3	1	3	4	6.6
Pneumonitis	1	0	1	0	1	1.6

[a] Includes one treatment-related death (grade 5).
AST, aspartate aminotransferase; ALT, alanine aminotransferase.

nonhematologic toxicities of grade 3 or 4 were anorexia (9.8%), asthenia (6.6%), an increase in serum alanine aminotransferase and aspartate aminotransferase levels (6.6%), and infection (6.6%), but most nonhematologic toxicities were mild. No cardiac toxicity was observed during the study. Pneumonitis of grade 3 occurred in one patient. One treatment-related death due to sepsis after febrile neutropenia occurred.

DISCUSSION

Amrubicin is a novel, fully synthetic anthracycline agent that is active against both NSCLC and small cell lung cancer (SCLC).[10,11,14–16] No prospective study evaluating the efficacy and safety of amrubicin for previously treated NSCLC has been reported. We have now demonstrated the efficacy of amrubicin monotherapy for patients with NSCLC previously treated with platinum-based chemotherapy, as shown by a response rate of 11.5%, median overall survival of 8.5 months, and 1-year survival rate of 32% in 61 patients. Previous phase III trials for second- or third-line treatment of NSCLC have shown response rates of 7.6 to 9.1%, median overall survival times of 6.7 to 8.3 months, and 1-year survival rates of 29.7 to 34%.[4,5,17–19] Amrubicin is a potent inhibitor of topoisomerase II, with its mechanism of action differing from those of currently available active agents for advanced NSCLC.[7–9] Given the encouraging results from our trial and the unique mode of action of amrubicin, this drug is a good candidate for the development of a new second-line treatment for NSCLC.

Treatment was discontinued in 14 patients after the first cycle and 17 patients after the second cycle. Of these 31 patients, 25 patients were withdrawn because of progressive disease. The study protocol required assessment of antitumor effect by computed tomography every 4 weeks. Such assessment, performed to avoid ineffective therapy, resulted in early discontinuation of treatment due to progressive disease and thereby yielded a median progression-free survival that was slightly shorter than otherwise might have been obtained.

Two recent phase II trials of amrubicin for previously treated SCLC, in which the drug was administered at the

same dose and according to the same schedule as in the present study, found that treatment was associated with a high incidence of bone marrow suppression, although drug toxicity was manageable.[20,21] Consistent with these results, the major adverse events in this study were hematologic toxicities of grade 3 or 4 including neutropenia (82.0%), leukopenia (73.8%), anemia (27.9%), and thrombocytopenia (24.6%). The incidence of these toxicities in this study was similar to that observed previously in the phase II trials for previously treated SCLC. However, the incidence of febrile neutropenia of grade 3 was higher in our study (29.5%) than in these previous trials (5–14%). One possible explanation for this difference is the frequent use of granulocyte colony-stimulating factor for treatment of SCLC, when compared with treatment for NSCLC. The incidents of significant neutropenia and febrile neutropenia were seen primarily in the first cycle. In this study, patients who experienced severe hematologic toxicities were not allowed to receive prophylactically granulocyte colony-stimulating factor in subsequent cycles. One treatment-related death due to sepsis after febrile neutropenia occurred in our study. Therefore, it is important to monitor closely leukocyte and neutrophil counts during amrubicin therapy in patients with previously treated NSCLC. Nonhematologic toxicity was manageable in this study. Another adverse event of particular concern for amrubicin is cardiac toxicity, given that the chemical structure of the drug is similar to that of doxorubicin, whose cardiac toxicity has been experimentally and clinically established. Indeed, cardiac toxicity was detected in previous trials of amrubicin, although its frequency (3.2%) was relatively low.[10,11] For safety reasons, this study allowed the enrollment only of patients with a left ventricular ejection fraction of $\geq$60% as determined by echocardiography. No cardiac toxicity was observed during our trial, even in the three patients who received more than eight cycles of amrubicin therapy.

In conclusion, in this first reported phase II study of the efficacy and safety of amrubicin monotherapy as a second-line treatment for advanced NSCLC previously treated with platinum-based chemotherapy, we obtained a response rate, overall survival, and 1-year survival rate comparable with those of other second-line treatment regimens. This activity despite a relevant hematological toxicity of amrubicin monotherapy is a possible alternative for second-line treatment of advanced NSCLC. Further evaluation of amrubicin for refractory or relapsed NSCLC in randomized phase III trials is warranted.

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De Novo Resistance to Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors in *EGFR* Mutation-Positive Patients with Non-small Cell Lung Cancer

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Background: Somatic mutations in the epidermal growth factor receptor (EGFR) gene are a predictor of response to treatment with EGFR tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung cancer (NSCLC). However, mechanisms of de novo resistance to these drugs in patients harboring *EGFR* mutations have remained unclear. We examined whether the mutational status of *KRAS* might be associated with primary resistance to EGFR-TKIs in *EGFR* mutation-positive patients with NSCLC.

Methods: Forty patients with NSCLC with *EGFR* mutations who were treated with gefitinib or erlotinib and had archival tissue specimens available were enrolled in the study. *KRAS* mutations were analyzed by direct sequencing.

Results: Three (7.5%) of the 40 patients had progressive disease, and two (67%) of these three individuals had both *KRAS* and *EGFR* mutations.

Conclusions: Our results suggest that *KRAS* mutation is a negative predictor of response to EGFR-TKIs in *EGFR* mutation-positive patients with NSCLC.

Key Words: Drug resistance, Epidermal growth factor receptor, KRAS, Non-small cell lung cancer, EGFR-TKI.

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A total of 40 patients with non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (*EGFR*) mutations were treated with gefitinib (n = 36) or erlotinib (n = 4) between September 2002 and January 2009, and three patients exhibited resistance to EGFR-tyrosine kinase inhibitor (TKIs). (i) Case 1 was a 63-year-old man who had never smoked and was diagnosed with lung adenocarcinoma of stage IV. Molecular screening identified a deletion mutation in exon 19 of *EGFR*, and he had received gefitinib as the second-line therapy. Although he tolerated gefitinib well, the primary lung lesion showed slow but steady growth, and he was removed from therapy because of his progressive disease (PD) at day 58 (Figure 1*A*). (ii) Case 2 was a 69-year-old man who had never smoked, had adenocarcinoma of stage IIIB, harbored a deletion in exon 19 of *EGFR*, and was treated with erlotinib as the third-line therapy. A chest computed tomography scan on day 28 revealed enlargement of the primary lung lesion, and the case was subsequently classified as PD (Figure 1*B*). (iii) Case 3 was a 52-year-old man who was a current smoker, had lung adenocarcinoma of stage IV with left adrenal metastasis, harbored a deletion in exon 19 of *EGFR*, and received erlotinib as the fourth-line therapy. Chest computed tomography on day 32 showed enlargement of the left adrenal metastasis, resulting in a classification of PD (Figure 1*C*). Thus, these clinical data demonstrated the existence of de novo resistance to EGFR-TKIs in *EGFR* mutation-positive patients. We examined the mutational status of *KRAS* in the three patients who showed PD as their best response. An amino acid substitution at codon 12 (G12D) of *KRAS* was identified in two of these three patients (Figure 1*D–F*).

DISCUSSION

Somatic mutations of the *EGFR* gene are associated with an increased response to EGFR-TKI in patients with NSCLC. Several prospective clinical trials of EGFR-TKI treatment for patients with NSCLC with *EGFR* mutations have subsequently revealed radiographic response rates of 55 to 91%. It remains of clinical concern, however, that a small proportion of patients with NSCLC with *EGFR* mutations show de novo resistance to EGFR-TKIs and that molecular markers to predict a lack of response to these drugs remain to be identified. We have now examined *KRAS* mutation status in *EGFR* mutation-positive patients with NSCLC treated with EGFR-TKIs and found a high incidence of concomitant *KRAS* mutation in individuals who did not respond to these drugs. Our results indicate that *KRAS* mutation may be clinically useful as a negative predictive marker of sensitivity to EGFR-TKIs in patients with NSCLC with *EGFR* mutations. Previous studies have also shown that *KRAS* mutations are associated with resistance to EGFR-TKIs in patients with NSCLC and that *EGFR* and *KRAS* mutations

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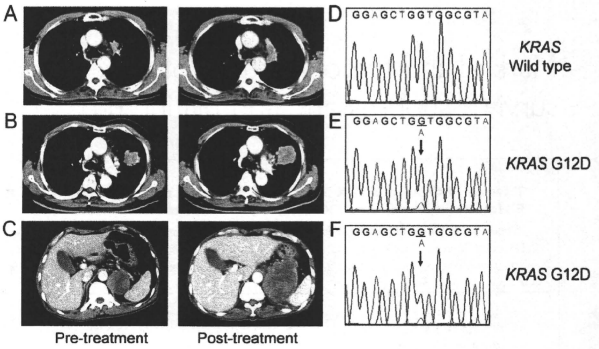

FIGURE 1. Clinical and molecular characteristics of patients with non-small cell lung cancer (NSCLC) who showed de novo resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs). *A–C*, Computed tomography (CT) images obtained before and after EGFR-TKI treatment for cases 1 to 3, respectively. *D–F*, Sequence chromatographs of *KRAS* mutation status determined with tumor tissue isolated before EGFR-TKI treatment for cases 1 to 3, respectively. Arrows indicate the mutated nucleotide (G→A) in codon 12 for cases 2 and 3.

appear to be mutually exclusive in such patients,[1–3] suggesting that *KRAS* mutations are predictors of unresponsiveness to EGFR-TKIs in patients with NSCLC with wild-type *EGFR*. The mutual exclusivity of *EGFR* and *KRAS* mutations combined with their prevalence patterns in lung adenocarcinoma, with *KRAS* mutations being preferentially found in smokers and *EGFR* mutations in nonsmokers, suggests that the mutations in these two genes might arise through different pathogenic pathways. Conversely, some studies have shown that *KRAS* mutations do sometimes coexist with *EGFR* mutations in patients with NSCLC.[4,5] The extent of coexistence of *EGFR* and *KRAS* mutations in NSCLC thus remains unclear, in large part as a result of the low frequency of *KRAS* mutations, and the clinical relevance of *KRAS* mutations in *EGFR* mutation-positive patients has remained unknown. We have now shown that patients with NSCLC harboring *EGFR* mutations who exhibit de novo resistance to EGFR-TKIs have a high incidence of *KRAS* mutation, suggesting that the presence of *KRAS* mutations might provide a basis for the identification of *EGFR* mutation-positive patients who are unlikely to benefit from EGFR-TKI treatment. Our clinical findings are consistent with preclinical data showing that forced expression of mutant KRAS in PC-9 human NSCLC cells, which harbor an activating mutation of *EGFR*, resulted in a reduction in the sensitivity of these cells to gefitinib.[6] Gefitinib shuts down both PI3K-AKT and RAS-RAF-MEK-ERK signaling pathways in PC-9 cells; however, expression of the KRAS mutant resulted in constitutive activation of these signaling pathways in a manner independent of EGFR activation, leading to continued cell growth and survival.

In July 2009, gefitinib received a license from the European Medicines Agency for all lines of therapy in patients with locally advanced or metastatic NSCLC positive for activating mutations of *EGFR*. More patients with *EGFR* mutation-positive tumors will thus now receive EGFR-TKIs. Our present results suggest that EGFR-TKIs should not be given routinely to patients harboring concomitant *KRAS* and *EGFR* mutations. In the event that such patients do receive treatment with EGFR-TKIs, they should be followed up after a short interval to obtain early evidence of possible tumor progression.

KRAS mutations cannot account for all cases of de novo resistance to EGFR-TKIs in *EGFR* mutation-positive patients with NSCLC. A recent study showed that loss of *PTEN* contributes to erlotinib resistance in an *EGFR* mutation-positive NSCLC cell line.[7] Loss of *PTEN* resulted in partial uncoupling of the mutant EGFR from downstream signaling and further activated the receptor, leading to erlotinib resistance. Both homozygous deletion of *PTEN* and *EGFR* mutation were detected in one of 24 clinical specimens of NSCLC with *EGFR* mutations, although the efficacy of EGFR-TKIs was not evaluated in the corresponding patient.

In conclusion, our results suggest that *KRAS* mutation status should be assessed before initiation of EGFR-TKI treatment in *EGFR* mutation-positive patients with NSCLC, allowing enrichment of the population of such patients who are likely to prove responsive to the treatment.

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Marked anti-tumour activity of the combination of YM155, a novel survivin suppressant, and platinum-based drugs

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BACKGROUND: Survivin, a member of the inhibitor of apoptosis protein family, is an attractive target for cancer therapy. We have now investigated the effects of the combination of YM155, a novel small-molecule inhibitor of survivin expression, and platinum compounds (cisplatin and carboplatin) on human non-small cell lung cancer (NSCLC) cell lines.

METHODS: The anti-cancer efficacy of YM155 in combination with platinum compounds was evaluated on the basis of cell death and progression of tumour xenografts. Platinum compound-induced DNA damage was evaluated by immunofluorescence analysis of histone γ-H2AX.

RESULTS: Immunofluorescence analysis of histone γ-H2AX showed that YM155 delayed the repair of double-strand breaks induced in nuclear DNA by platinum compounds. The combination of YM155 and platinum compounds also induced synergistic increases both in the number of apoptotic cells and in the activity of caspase-3. Finally, combination therapy with YM155 and platinum compounds delayed the growth of NSCLC tumour xenografts in nude mice to an extent greater than that apparent with either treatment modality alone.

CONCLUSION: These results suggest that YM155 sensitises tumour cells to platinum compounds both *in vitro* and *in vivo*, and that this effect is likely attributable to the inhibition of DNA repair and consequent enhancement of apoptosis.

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Survivin is a 16.5-kDa member of the inhibitor of apoptosis protein (IAP) family and blocks the mitochondrial pathway of apoptosis by inhibiting caspases (Altieri, 2003a, b). Survivin also regulates cell division through interaction with the proteins INCENP and Aurora B (Wheatley *et al*, 2001). It is abundant in many types of cancer cells but not in the corresponding normal cells (Ambrosini *et al*, 1997; Marusawa *et al*, 2003; Altieri, 2004). High levels of survivin expression in cancer cells are associated with poor patient prognosis and survival, as well as with resistance to therapy and an increased rate of cancer recurrence (Monzo *et al*, 1999; Altieri, 2001; Rodel *et al*, 2005). Survivin has therefore become a therapeutic target and potentially important prognostic marker for many tumour types including non-small cell lung cancer (NSCLC) (Adida *et al*, 1998; Monzo *et al*, 1999). Reflecting the many mechanisms that seem to regulate survivin expression, diverse approaches have been evaluated for targeting survivin in experimental models (Li *et al*, 1999; Olie *et al*, 2000; Grossman *et al*, 2001).

YM155, a small imidazolium-based compound, was identified by high-throughput screening of chemical libraries for inhibitors of the activity of the survivin gene promoter in a reporter assay (Nakahara *et al*, 2007). This compound specifically inhibits the expression of survivin at both the mRNA and protein levels and exhibits pronounced anti-cancer activity in pre-clinical models (Nakahara *et al*, 2007). An advantage of YM155 compared with previously investigated suppressors of survivin expression is that it is active in the subnanomolar range (Carter *et al*, 2001; Milella *et al*, 2001; O'Connor *et al*, 2002; De Schepper *et al*, 2003; Wall *et al*, 2003; Sah *et al*, 2006). Our previous pharmacokinetics analysis also revealed that YM155 becomes highly distributed to tumour tissue in tumour xenograft models *in vivo* (Nakahara *et al*, 2007). In addition, continuous YM155 infusion in mice did not result in systemic toxicity such as body weight loss or decreased blood cell count (Nakahara *et al*, 2007). Furthermore, we have recently shown that YM155 sensitised NSCLC cells to radiation both *in vitro* and *in vivo*, and that this effect of YM155 was likely attributable to the inhibition of DNA repair and enhancement of apoptosis that result from downregulation of survivin expression (Iwasa *et al*, 2008). YM155 is thus an attractive candidate drug for cancer therapy.

Despite its demonstrated efficacy in targeting tumour cells, the effects of YM155 in combination with DNA-damaging drugs have remained largely unknown. We have now examined the effects of the combination of YM155 and platinum compounds on human NSCLC cell lines both *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Cell culture and reagents

The human NSCLC cell lines NCI-H460 (H460), Calu6, NCI-H358 (H358), and PC14 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured under an atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% foetal bovine serum. Cisplatin (CDDP) was obtained from Nippon Kayaku (Tokyo, Japan), and carboplatin (CBDCA) was from Bristol-Myers Squibb (New York, NY, USA). YM155 (Astellas Pharma Inc, Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO).

Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in a solution containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (1 $\mu g\,ml^{-1}$). The protein concentration of lysates was determined with the Bradford reagent (Bio-Rad, Hercules, CA, USA), and equal amounts of protein were subjected to SDS polyacrylamide gel electrophoresis on a 15% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% non-fat dried milk in PBS for 1 h at room temperature before overnight incubation at 4°C with rabbit polyclonal antibodies to human survivin (1 : 1000 dilution; R&D Systems, Wiesbaden, Germany), to human c-IAP1 (1:1000 dilution; MBL International, Woburn, MA, USA), or to human XIAP (1:1000 dilution; Cell Signaling, Beverly, MA). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Sigma). Immune complexes were finally detected with chemiluminescence reagents (PerkinElmer Life Science, Boston, MA, USA).

Detection of apoptotic cells

Cells were fixed with 4% paraformaldehyde for 1 h at room temperature, after which a minimum of 1000 cells per sample was evaluated for apoptosis with the use of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) technique (*In situ* Cell Death Detection Kit; Boehringer Mannheim, Mannheim, Germany).

Assay of caspase-3 activity

The activity of caspase-3 in cell lysates was measured with a CCP32/caspase-3 Fluometric Protease Assay Kit (MBL). Fluorescence attributable to cleavage of the DEVD-AFC substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

Immunofluorescence staining of γ-H2AX

Cells were grown to 50% confluence in two-well Lab-Tec Chamber Slides (Nunc, Naperville, IL, USA) and then cultured for 48 h in the presence of 50 nM YM155 or vehicle (final DMSO concentration of 0.1%; we confirmed that this DMSO concentration did not affect the proliferation of NSCLC cell lines) before additional exposure to 10 μM CDDP or CBDCA. At various times thereafter, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilised with 0.1% Triton X-100 for 10 min at 4°C, and exposed to 5% non-fat dried milk for 10 min at room temperature. The slides were washed with PBS and then incubated at room temperature first for 2 h with mouse monoclonal antibodies to histone γ-H2AX (Upstate Biotechnology, Lake Placid, NY, USA) at a dilution of 1 : 300 and then for 1 h with Alexa Fluor 488-labeled goat antibodies to mouse immunoglobulin G (Molecular Probes, Eugene, OR, USA) at a dilution of 1 : 700. The slides were mounted in fluorescence mounting medium (Dako Cytomation, Hamburg, Germany), and fluorescence signals were visualised with a confocal laser-scanning microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany) equipped with the LSM5 PASCAL system (Carl Zeiss). Three random fields each containing at least 50 cells were examined at a magnification of × 100. Nuclei containing ⩾10 immunoreactive foci were counted as positive for γ-H2AX, and the percentage of positive cells was calculated (De Schepper *et al*, 2003).

Evaluation of tumour growth *in vivo*

Male nude (BALB/cAnNCrj-nu/nu) mice (5 weeks old) were obtained from Charles River Japan (Kanagawa, Japan). All animal studies were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. The ethical procedures followed met the requirements of the UKCCCR guidelines (1998). Tumour cells (2×10^6) were implanted into the right hind leg of 6-week-old male athymic nude mice. Tumour volume was determined from caliper measurement of tumour length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when tumours in each group of animals achieved an average volume of 100–200 mm^3. Treatment groups (each containing eight mice) consisted of vehicle control (0.1% DMSO in physiological saline), YM155 alone, vehicle plus CDDP or CBDCA, and YM155 plus CDDP or CBDCA. Vehicle or YM155 at a dose of 5 mg per kg of body mass was administered over seven consecutive days (days 0–6) with the use of an implanted micro-osmotic pump (Alzet model 1007D; Durect, Cupertino, CA, USA). CDDP (3 $mg\,kg^{-1}$) or CBDCA (60 $mg\,kg^{-1}$) was administered intravenously on each of days 0–3 and days 7–11, respectively.

Statistical analysis

Data are presented as means ± s.e. and were compared between groups with the unpaired Student's t-test. A P-value of <0.05 was considered statistically significant.

RESULTS

Specific inhibition of survivin expression in NSCLC cells by YM155

We first examined the effect of YM155 on survivin expression in human NSCLC cell lines by immunoblot analysis. Exposure of H460, Calu6, H358, or PC14 cells to YM155 at 10–100 nM for 48 h inhibited survivin expression in a concentration-dependent manner (Figure 1A). In contrast, exposure of these cell lines to YM155 at 50 nM for 48 h did not affect the abundance of other members of the IAP family including XIAP and c-IAP1 (Figure 1B), indicating that YM155 specifically inhibits survivin expression in the NSCLC cell lines.

Enhancement of DNA-damaging agent-induced apoptosis in NSCLC cells by YM155

We next examined the effect of YM155 on DNA-damaging agent-induced apoptosis in H460, Calu6, H358, or PC14 cells with the use of the TUNEL assay. Combined treatment of each cell line with YM155 and either CDDP or CBDCA resulted in an increase in the number of apoptotic cells at 24 and 48 h that was greater than the sum of the increases induced by YM155 or by CDDP or CBDCA alone (Figure 2). To confirm the results of the TUNEL assay, we measured the activity of caspase-3 in cell lysates. Again, combined

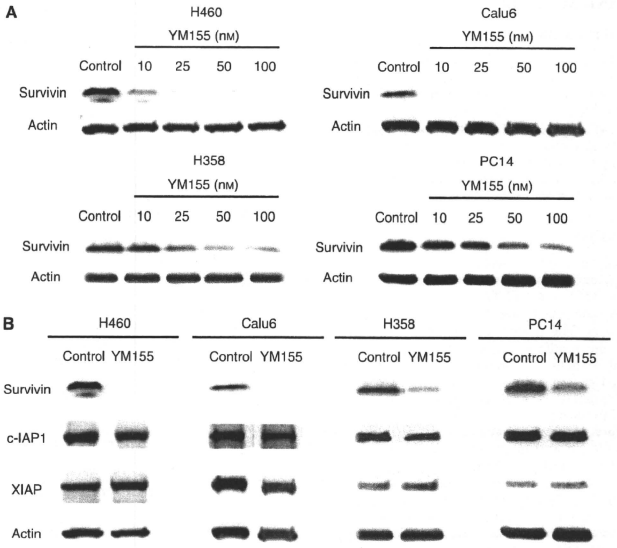


Figure 1 Effect of YM155 on survivin expression in human non-small cell lung cancer (NSCLC) cells. (**A**) H460, Calu6, H358, or PC14 cells were incubated in the absence (control, 0.1% dimethyl sulfoxide (DMSO)) or presence of the indicated concentrations of YM155 for 48 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to survivin or to β-actin (loading control). (**B**) H460, Calu6, H358, or PC14 cells were incubated in the absence or presence of 50 nM YM155 for 48 h, after which cell lysates were subjected to immunoblot analysis with antibodies to survivin, c-IAP1, XIAP, or β-actin.

treatment of H460, Calu6, H358, or PC14 cells with YM155 and either CDDP or CBDCA induced a synergistic increase in caspase-3 activity (Figure 2). These data thus suggested that YM155 promotes the induction of apoptosis by DNA-damaging agents in NSCLC cell lines.

Effect of YM155 in combination with DNA-damaging agents on H2AX phosphorylation

We have previously shown that YM155 sensitises tumour cells to radiation by inhibiting the repair of radiation-induced DNA damage. CDDP and CBDCA are key drugs in NSCLC treatment and are known to induce DNA damage (Diggle *et al*, 2005). We therefore hypothesised that YM155 might inhibit the repair of CDDP- or CBDCA-induced DNA damage and thereby promote CDDP- or CBDCA-induced cell death. To explore this possibility, we determined whether YM155 might affect CDDP- or CBDCA-induced phosphorylation of histone H2AX to yield γ-H2AX, which is a marker of DNA double-strand breaks (DSBs). Exposure of H460 or Calu6 cells to CDDP or CBDCA resulted in the gradual accumulation of γ-H2AX foci, with this effect being maximal at 12 h, after which the number of foci declined (Figure 3). Although YM155 did not affect the extent of CDDP- or CBDCA-induced focus formation, it significantly retarded the loss of foci normally apparent at 24 h after exposure to CDDP or CBDCA (Figure 3). These results thus suggested that downregulation of survivin expression by YM155 results in inhibition of the repair of DSBs induced by DNA-damaging agents in NSCLC cells.

Enhancement of chemotherapy-induced tumour regression by YM155

To determine whether the enhancement of the anti-tumour activity of DNA-damaging agents by YM155 observed *in vitro* might also be apparent *in vivo*, we injected Calu6 cells into nude mice to elicit the formation of solid tumours. After tumour formation, the mice were treated with YM155, CDDP, or both drugs. Combined treatment with CDDP and YM155 inhibited Calu6 tumour growth to a markedly greater extent than did treatment with either drug alone (Figure 4A). No pronounced tissue damage or toxicity such as weight loss (Figure 4A) was observed in mice in any of the four treatment groups.

Finally, we examined the effect of the combination of YM155 and CBDCA on tumour growth. Treatment with YM155 or CBDCA alone delayed tumour growth, whereas combined treatment inhibited tumour growth to a significantly greater extent (Figure 4B). Again, there was no evidence of toxicity on the basis of body weight loss (Figure 4B) and there were no animal deaths in any of the four groups. These data suggested that YM155 enhances the tumour response to platinum-based chemotherapy *in vivo*.

DISCUSSION

The success of anti-cancer therapies is often limited by the development of resistance to apoptosis, which may result from defects in common apoptotic pathways (Hanahan and Weinberg, 2000). In this context, approaches to counteract the action of

survivin in tumour cells have been proposed with the dual aims of inhibiting tumour growth through promotion of spontaneous apoptosis and of enhancing the tumour cell response to apoptosis-inducing agents (Altieri, 2003b). In this study, we found that the combination of YM155 and platinum compounds induced NSCLC cell apoptosis as well as the activation of caspase-3 to an extent greater than that apparent with either type of agent alone. Our findings thus suggest that YM155 acts in a synergistic manner to promote the induction of apoptosis by platinum compounds.

Cellular responses to stress or DNA damage are important for the maintenance of genomic stability and cellular integrity (Bunz *et al*, 1998; Hirao *et al*, 2000). Depending on its extent, cells either repair DNA damage or, when it is too severe for repair, initiate the cell death programme (Zhao *et al*, 2001). Agents that inhibit repair of DNA damage therefore increase the sensitivity of cells to ionising radiation and chemotherapeutic drugs (Banath *et al*, 2004; Taneja *et al*, 2004). The chemotherapeutic effect of platinum compounds results from their interaction with DNA; platinum thus induces the formation of DNA–protein crosslinks, DNA monoadducts, as well as interstrand or intrastrand DNA crosslinks (Chu, 1994; Siddik, 2003). These DNA adducts induce local distortion in the DNA double helix that results in strand unwinding and kinking (Chu, 1994). Survivin was previously shown to enhance tumour cell survival after radiation exposure through regulation of DSB repair (Chakravarti *et al*, 2004). We have previously shown that YM155 inhibited the repair of radiation-induced DSBs in NSCLC cells and that this effect likely accounted for the observed radiosensitising action of YM155 (Iwasa *et al*, 2008). We therefore investigated the effect of YM155 on the repair of platinum compound-induced DSBs by immunofluorescence imaging of γ-H2AX foci. Given that γ-H2AX appears rapidly at DNA DSBs and disappears as repair proceeds (Rogakou *et al*, 1998; Zhou and Elledge, 2000; Khanna *et al*, 2001; Shiloh and Kastan, 2001), it serves as a sensitive and specific marker for unrepaired DNA damage. We found that YM155 inhibited the repair of platinum compound-induced DSBs in NSCLC cells. Overexpression of survivin was previously shown to enhance DSB repair in tumour cells through upregulation of Ku protein (Jiang *et al*, 2009). Although it remains unclear whether YM155 affects Ku protein kinetics, our data suggest that the observed chemosensitisation by YM155 is attributable to inhibition of the repair of DNA damage induced by CDDP or CBDCA. Further investigations will be required to determine the mechanism underlying the effect of YM155 on DNA repair.

In addition to the initially identified isoform, four splice variants of human survivin have been described: survivin-2α, survivin-2β, survivin-δ-Ex3, and survivin-3β (Mahotka *et al*, 1999, 2002; Badran *et al*, 2004; Caldas *et al*, 2005a). However, little is known of

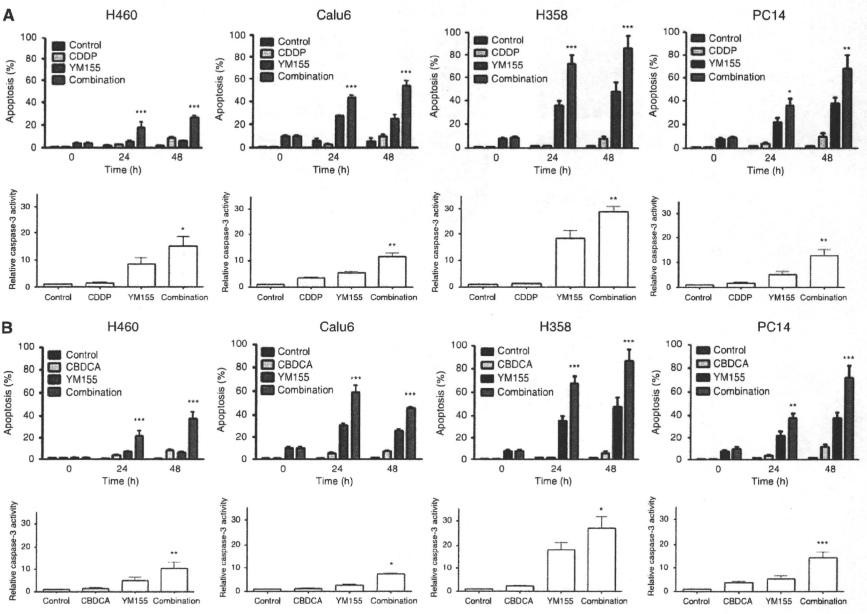
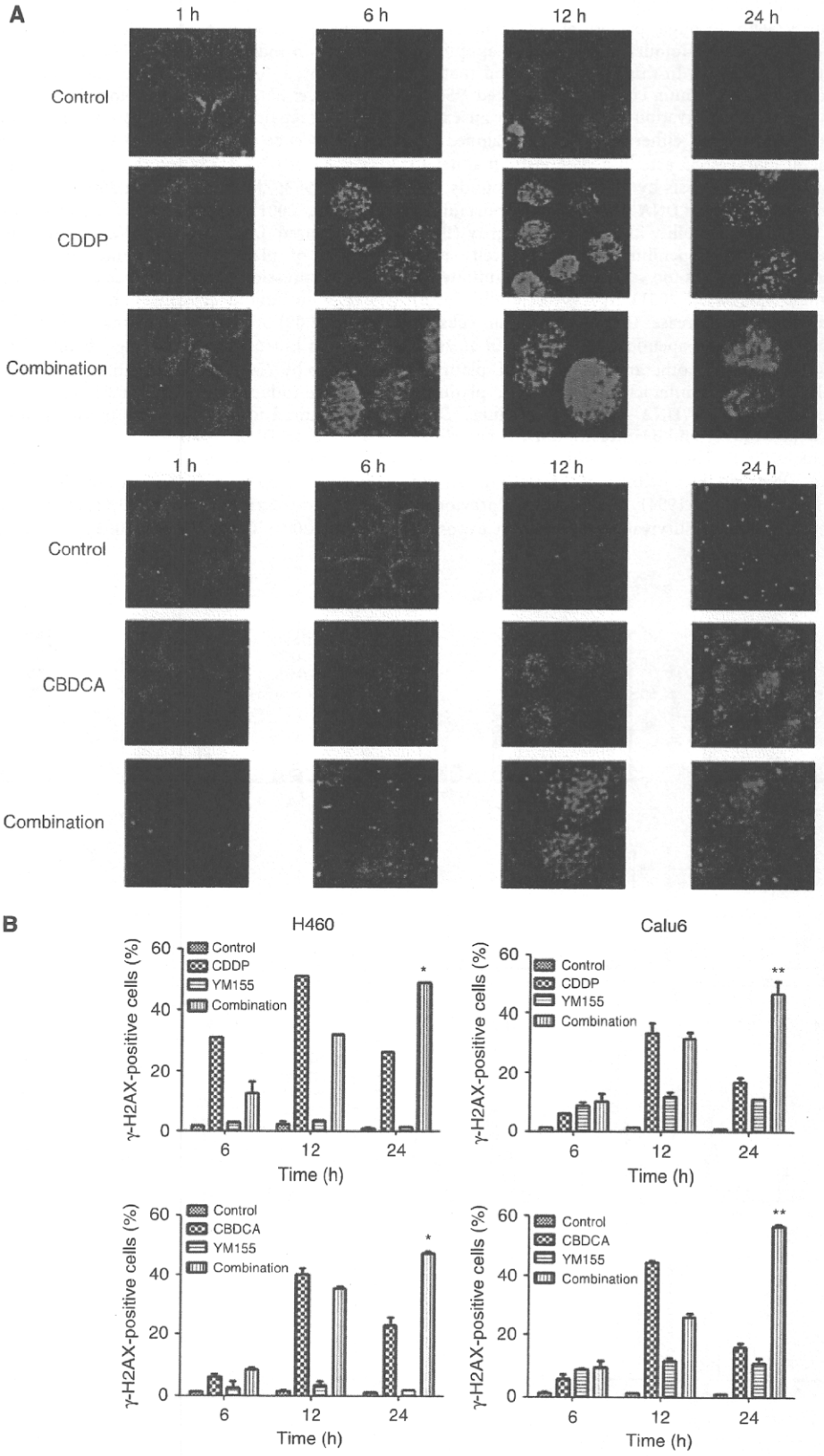


Figure 2 Effects of YM155 on DNA-damaging agent-induced apoptosis and caspase-3 activity in H460, Calu6, H358, or PC14 cells. Cells were incubated with 50 nM YM155 or vehicle (0.1% dimethyl sulfoxide (DMSO)) for 48 h and then for the indicated times (upper panels) or for 24 h (lower panels) in the additional absence or presence of 10 μM CDDP (**A**) or CBDCA (**B**). The percentage of apoptotic cells was then determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (upper panels), and cell lysates were assayed for caspase-3 activity (lower panels). All data are means ± s.e. from three independent experiments; those for caspase-3 activity are expressed relative to the corresponding value for the control condition. $*P<0.05$, $**P<0.001$, $***P<0.0001$ *vs* the corresponding value for CDDP or CBDCA or for YM155 alone.



the differential functions of these alternative splice forms of survivin (Krieg *et al*, 2002; Mahotka *et al*, 2002; Caldas *et al*, 2005b; Noton *et al*, 2006). Given that the suppression of survivin expression by YM155 is mediated through inhibition of the transcriptional activity of the survivin gene promoter (Nakahara *et al*, 2007), it is possible that YM155 also inhibits the expression of these survivin variants.

No serious adverse haematological events related to drug treatment were reported in phase I studies of YM155 in single-agent therapy (Tolcher *et al*, 2008; Satoh *et al*, 2009). A recent phase II study showed that YM155 monotherapy is safe but only moderately effective (objective tumour response rate, 5.4%) in patients with advanced NSCLC (Giaccone *et al*, 2009). Given this limited efficacy of YM155 as a single agent in NSCLC patients, the combination of YM155 with other agents may be beneficial. Platinum-based combination chemotherapy is the standard of care for most individuals with advanced NSCLC (Chu, 1994). We have shown that treatment of NSCLC cells with YM155 results in a marked increase in the anti-tumour effects of CDDP and CBDCA both *in vitro* and *in vivo*, suggesting that the combination of YM155 and platinum compounds may have potential as a novel therapeutic regimen. Clinical studies of YM155 in combination with platinum-based chemotherapy are thus warranted.

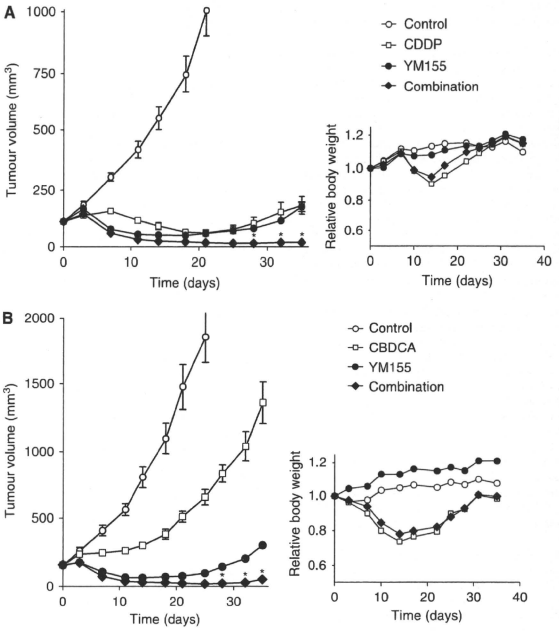


Figure 4 Effects of YM155 on the anti-tumour action of CDDP or CBDCA *in vivo*. Calu6 cells were injected into the right hind limb of nude mice and allowed to form tumours, after which the mice were assigned to one of four treatment groups (control, CDDP (**A**) or CBDCA (**B**) alone, YM155 alone, or the combination of YM155 and either CDDP (**A**) or CBDCA (**B**)) as described in Materials and Methods. Tumour volume was measured at the indicated times after the onset of treatment (left panels); values for mice in the control group are not shown for later time points to highlight differences among the other three groups. Body weight was also measured in each treatment group at the indicated times and is expressed relative to the corresponding value for time 0 (right panels). All data are means ± s.e. from eight mice per group. $^{*}P < 0.0001$ *vs* the corresponding value for treatment with CDDP (**A**) or CBDCA (**B**) alone or with YM155 alone.

Figure 3 Effect of YM155 on the formation of γ-H2AX foci induced by DNA-damaging agents in non-small cell lung cancer (NSCLC) cells. (**A**) H460 cells were incubated with vehicle (0.1% dimethyl sulfoxide (DMSO)) or 50 nM YM155 for 48 h and then for the indicated times in the additional absence or presence of 10 μM CDDP or CBDCA . The cells were then fixed and subjected to immunofluorescence staining for γ-H2AX (green fluorescence). (**B**) H460 or Calu6 cells were incubated with vehicle or 50 nM YM155 for 48 h and then for the indicated times in the additional absence or presence of 10 μM CDDP or CBDCA . They were then fixed and subjected to immunofluorescence staining for γ-H2AX, and the percentage of cells containing γ-H2AX foci was determined. Data are means ± s.e. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ *vs* the corresponding value for CDDP or CBDCA alone.

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Identification of thymidylate synthase as a potential therapeutic target for lung cancer

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BACKGROUND: Thymidylate synthase (TS), a key enzyme in the *de novo* synthesis of thymidine, is an important chemotherapeutic target for malignant tumours including lung cancer. Although inhibition of TS has an antiproliferative effect in cancer cells, the precise mechanism of this effect has remained unclear.

METHODS: We examined the effects of TS inhibition with an RNA interference-based approach. The effect of TS depletion on the growth of lung cancer cells was examined using colorimetric assay and flow cytometry.

RESULTS: Measurement of the enzymatic activity of TS in 30 human lung cancer cell lines revealed that such activity differs among tumour histotypes. Almost complete elimination of TS activity by RNA interference resulted in inhibition of cell proliferation in all tested cell lines, suggestive of a pivotal role for TS in cell proliferation independent of the original level of enzyme activity. The antiproliferative effect of TS depletion was accompanied by arrest of cells in S phase of the cell cycle and the induction of caspase-dependent apoptosis as well as by changes in the expression levels of cyclin E and c-Myc. Moreover, TS depletion induced downregulation of the antiapoptotic protein X-linked inhibitor of apoptosis (XIAP), and it seemed to activate the mitochondrial pathway of apoptosis.

CONCLUSION: Our data provide insight into the biological relevance of TS as well as a basis for clinical development of TS-targeted therapy for lung cancer.

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Thymidylate synthase (TS) is an essential enzyme that catalyses the transfer of a methyl group from methylenetetrahydrofolate to dUMP to generate dTMP (Carreras and Santi, 1995). The subsequent phosphorylation of dTMP to dTTP provides a direct precursor for DNA synthesis. The level of TS expression is increased in highly proliferative cells, and an increased abundance of TS in a broad range of tumours is associated with a poor treatment response and prognosis (Costi *et al*, 2005). Transfection of nontransformed cells with an expression vector for TS has also been found to confer transformed-like behaviour, suggesting that TS itself is a potential oncoprotein (Rahman *et al*, 2004). These findings have led to TS being considered as a molecular target for cancer therapy. To date, the antiproliferative effect of TS inhibition has been examined mostly with the use of drugs such as 5-fluorouracil and its active metabolite 5-fluoro-dUMP, the former of which is used in cancer chemotherapy. Although studies with antisense oligodeoxynucleotides have also shown that depletion of TS results in growth inhibition in human tumour cells (Ferguson *et al*, 1999; Lin *et al*, 2001; Flynn *et al*, 2006), the underlying mechanism of the antiproliferative effect of specific TS inhibition has remained largely unknown.

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Lung cancer is one of the most common forms of cancer worldwide. Advanced lung cancer is treated by combination chemotherapy, although further improvement in such therapy is warranted. High levels of TS in tumours have been associated with a poor response to TS-targeting agents in individuals with advanced lung cancer (Oguri *et al*, 2005; Kubota *et al*, 2009; Ozasa *et al*, 2009), although the biological relevance of TS in lung cancer has remained to be well established. We have now abrogated both the expression and activity of TS in lung cancer cells by the application of RNA interference (RNAi). With this approach, we investigated the precise mechanism of the antiproliferative effect of TS depletion in lung cancer cells and further examined the potential role of TS as a target for chemotherapeutic agents in these cells. Our results provide a basis for the further development of TS-targeted therapy in lung cancer patients.

MATERIALS AND METHODS

Cell culture and reagents

The human lung cancer cell lines A549, H1975, H1650, H358, SW1573, H460, H1299, H520, Calu-1, H226, H82, H526, and H69 were obtained from American Type Culture Collection (Manassas, VA, USA); PC9 and PC9/ZD were obtained as described previously (Koizumi *et al*, 2005); PC3, LK2, PC1, EBC-1, PC10, HARA, SBC-3,

MS-1, COR-L47, STC-1, SBC-1, and RERF-LC-MA were obtained from Human Science Research Resources Bank (Osaka, Japan); Lu135 and Lu134B were from Riken Cell Bank (Tokyo, Japan); and QG56 was obtained from Immuno-Biological Laboratories (Gunma, Japan). All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum. The pan-caspase inhibitor ZVAD-FMK was from Wako (Osaka, Japan).

Assay of TS activity

Thymidylate synthase activity was quantified using tritiated 5-fluoro-dUMP binding assay (Spears *et al*, 1984). Cells were harvested, diluted in 0.2 M Tris-HCl (pH 7.4) containing 20 mM 2-mercaptoethanol, 15 mM CMP, and 100 mM NaF, and disrupted by ultrasonic treatment. The cell lysate was centrifuged at 1600 **g** for 15 min at 4°C, and the resulting supernatant was centrifuged at 105 000 **g** for 1 h at 4°C. A portion (50 μl) of the final supernatant was mixed consecutively with 50 μl of Buffer A (600 mM NH_4HCO_3 buffer (pH 8.0), 100 mM 2-mercaptoethanol, 100 mM NaF, and 15 mM CMP) and with 50 μl of [6-^{3}H]5-fluoro-dUMP (7.8 pmol) plus 25 μl of cofactor solution (50 mM potassium phosphate buffer (pH 7.4), 20 mM 2-mercaptoethanol, 100 mM NaF, 15 mM CMP, 2% bovine serum albumin, 2 mM tetrahydrofolic acid, 16 mM sodium ascorbate, and 9 mM formaldehyde). The resulting mixture was incubated at 30°C for 20 min, after which the reaction was terminated by the addition of 100 μl of 2% bovine serum albumin and 275 μl of 1 M $HClO_4$ and by centrifugation at 1630 **g** for 15 min at 4°C. The resulting precipitate was suspended in 2 ml of 0.5 M $HClO_4$, and the mixture was subjected to ultrasonic treatment followed by centrifugation at 1600 **g** for 15 min at 4°C. The final precipitate was solubilised with 0.5 ml of 98% formic acid, mixed with 10 ml of ACS II scintillation fluid, and assayed for radioactivity.

RNAi

Cells were plated at 50–60% confluence in six-well plates or 25 cm^2 flasks and then incubated for 24 h before transient transfection for the indicated times with small interfering RNAs (siRNAs) mixed with the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). The siRNAs specific for TS mRNA (TS-1, 5′-CAAUCCGCAUCCA ACUAUU-3′; TS-2, 5′-GCUCAGGAUUCUUCGAAAA-3′; and TS-3, 5′-AGCUCAGGAUUCUUCGAAA-3′) and a nonspecific siRNA (5′-GUUGAGAGAUAUUAGAGUU-3′) were obtained from Nippon EGT (Toyama, Japan). The cells were then subjected to immunoblot analysis, flow cytometry, or assay of TS or caspase-3 activity.

Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (1 μg ml^{-1}). The protein concentration of cell lysates was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA), and equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis on a 7.5 or 12% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C either with rabbit polyclonal antibodies to TS (1 : 1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1 : 500 dilution, Sigma), survivin (1 : 1000 dilution; R&D Systems, Minneapolis, MN, USA), or c-Myc, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-x_L, Bax, Bak, X-linked inhibitor of apoptosis (XIAP), or Omi/HtrA2 (all in a 1 : 1000 dilution and from Cell Signaling Technology, Danvers, MA, USA) or with mouse monoclonal antibodies to cyclin E (1 : 1000 dilution, Santa Cruz Biotechnology), cytochrome *c* (1 : 1000 dilution, Cell Signaling Technology), to Smac/Diablo (1 : 1000 dilution, Cell Signaling Technology). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat antibodies to rabbit (Sigma) or mouse (Santa Cruz Biotechnology) immunoglobulin G. Immune complexes were finally detected with chemiluminescence reagents (GE Healthcare, Little Chalfont, UK).

Growth inhibition assay *in vitro* (MTT assay)

Cells were plated at 50–60% confluence in 25 cm^2 flasks and then incubated for 24 h before transient transfection with an siRNA specific for TS mRNA or a control siRNA as described above. The cells were then isolated by exposure to trypsin, transferred to 96-well flat-bottom plates, and cultured for 72 h before the addition of TetraColor One (5 mM tetrazolium monosodium salt and 0.2 mM 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku, Tokyo, Japan) to each well and incubation for an additional 3 h at 37°C. The absorbance at 490 nm of each well was measured using Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA, USA), and absorbance values were expressed as a percentage of that for nontransfected control cells.

Cell cycle analysis

Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg ml^{-1}) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A (0.05 mg ml^{-1}). The stained cells ($\sim 1 \times 10^6$) were then analysed for DNA content using flow cytometer (FACS Caliber; Becton Dickinson, Franklin Lakes, NJ, USA) and Modfit software (Verity Software House, Topsham, ME, USA).

Assay of caspase-3 activity

The activity of caspase-3 in cell lysates was measured using CCP32/Caspase-3 Fluometric Protease Assay kit (MBL, Woburn, MA, USA). Fluorescence attributable to cleavage of the Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

Subcellular fractionation

A cytosolic fraction was isolated from cells by centrifugation. In brief, cells were washed, resuspended in homogenisation buffer (0.25 M sucrose, 10 mM HEPES-NaOH (pH 7.4), and 1 mM EGTA), and homogenised by 50 strokes in a Dounce homogeniser. The homogenate was centrifuged at 1000 **g** for 15 min at 4°C to remove nuclei and intact cells, and the resulting supernatant was centrifuged at 10 000 **g** for 15 min at 4°C. The final supernatant (cytosolic fraction) was subjected to immunoblot analysis.

Statistical analysis

Data were analysed using Student's two-tailed *t*-test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

TS activity varies among histotypes of lung cancer cells

We first examined the enzymatic activity of TS in 30 human lung cancer cell lines (Table 1). The median TS activity in small cell lung cancer (SCLC) lines was significantly higher than that in non-SCLC (NSCLC) lines. Among NSCLC cell lines, the values for squamous cell carcinoma were higher than those for non-squamous cell

Table 1 TS activity of lung cancer cell lines classified according to histology

Cell line	Histology	TS activity (pmol per mg protein)
A549	Adeno	1.003 ± 0.142
H1975	Adeno	1.005 ± 0.276
H1650	Adeno	0.705 ± 0.177
PC9	Adeno	0.370 ± 0.042
PC9/ZD	Adeno	0.635 ± 0.148
H358	Adeno	1.140 ± 0.127
PC3	Adeno	0.591 ± 0.325
SW1573	Adeno	1.695 ± 0.544
H460	Large cell	0.420 ± 0.184
H1299	Large cell	1.121 ± 0.594
H520	Squamous	1.755 ± 0.813
Calu-1	Squamous	4.805 ± 3.061
H226	Squamous	1.930 ± 0.820
LK2	Squamous	1.121 ± 0.042
PC1	Squamous	3.055 ± 0.997
EBC-1	Squamous	1.055 ± 0.078
PC10	Squamous	1.204 ± 0.052
QG56	Squamous	0.870 ± 0.030
HARA	Squamous	2.590 ± 0.340
SBC-3	SCLC	5.795 ± 0.247
H82	SCLC	5.170 ± 0.641
H526	SCLC	1.125 ± 0.092
H69	SCLC	4.005 ± 0.078
MS-1	SCLC	2.555 ± 0.474
COR-L47	SCLC	3.760 ± 0.560
STC-1	SCLC	6.832 ± 0.490
SBC-1	SCLC	0.753 ± 0.023
Lu135	SCLC	3.698 ± 0.190
Lu134B	SCLC	0.310 ± 0.100
RERF-LC-MA	SCLC	1.413 ± 0.183

Abbreviations: SCLC = small cell lung cancer; TS = thymidylate synthase. Data are means ± s.d. from three independent experiments.

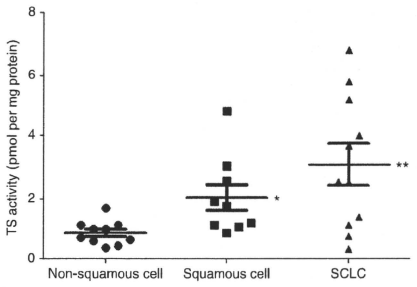

Figure 1 Thymidylate synthase (TS) activity in lung cancer cell lines stratified according to histotype. Central horizontal lines represent median values, with the upper and lower bars representing the 95% confidence interval. *$P<0.05$ for squamous cell carcinoma *vs* non-squamous cell carcinoma; **$P<0.05$ for SCLC *vs* either squamous cell or non-squamous cell carcinoma.

carcinoma (Figure 1). There was no significant correlation between TS activity and cell proliferation rate among these lung cancer cell lines (data not shown). These data thus suggested that TS activity varies according to histotype among lung cancer cell lines.

TS depletion induces growth inhibition regardless of original TS activity level in lung cancer cells

We next examined the effect of TS depletion by RNAi on the growth of lung cancer cell lines. The abundance of TS was markedly decreased as a result of cell transfection with either of three different siRNAs targeted to TS mRNA (Figure 2A). Given that the TS-1 siRNA induced the most pronounced downregulation of TS expression, we selected this siRNA for use in subsequent experiments. In all tested lung cancer cells, transfection with TS-1 resulted in marked depletion of TS, whereas no such effect was observed in cells transfected with a nonspecific siRNA (Figure 2B). Moreover, transfection of cells with TS-1 resulted in a >90% decrease in TS activity compared with that in corresponding cells transfected with a nonspecific siRNA or in untreated cells (Figure 2C), regardless of the original levels of TS expression and activity. The antiproliferative effect of TS depletion was evaluated using the MTT assay. Depletion of TS resulted in pronounced inhibition of proliferation in all tested cells compared with the corresponding cells transfected with a nonspecific siRNA or untreated cells (Figure 2D), and this antiproliferative effect was found to be time dependent (Figure 2E). These data thus suggested that the almost complete elimination of TS activity resulted in marked inhibition of the proliferation of lung cancer cells regardless of the original level of such activity.

TS depletion induces S-phase arrest in lung cancer cells

To investigate the mechanism by which TS depletion inhibits lung cancer cell growth, we examined the cell cycle profile by flow cytometry. Transfection with TS-1 siRNA increased the proportion of cells in S phase of the cell cycle and reduced that of cells in G_1 or G_2–M phases in all tested cell lines regardless of histotype (Figure 3A). Immunoblot analysis of proteins implicated in regulation of the G_1–S transition revealed that TS depletion increased the abundance of cyclin E in all tested cell lines (Figure 3B) without affecting that of cyclins D or A (data not shown). In addition, TS depletion induced downregulation of c-Myc (Figure 3B), a transcription factor that activates the expression of several cell cycle-related genes. However, expression of c-Myc was not detected in H69 cells, consistent with previous observations (Plummer *et al*, 1993). These results thus suggested that the S-phase arrest induced by TS depletion in lung cancer cells was related to upregulation of cyclin E and downregulation of c-Myc.

TS depletion induces caspase-dependent apoptosis in lung cancer cells

We next examined the effect of TS depletion on apoptosis in lung cancer cells. Flow cytometric analysis revealed that TS depletion induced a time-dependent increase in the size of the sub-G_1 (apoptotic) cell population (Figure 4A). Depletion of TS also induced the cleavage of PARP (Figure 4B), a characteristic of apoptosis, in the cell lines examined. Furthermore, the activity of caspase-3 in cell lysates was increased as a consequence of TS depletion (Figure 4C), and previous exposure of lung cancer cells to the pan-caspase inhibitor ZVAD-FMK significantly inhibited the increase in the size of the sub-G_1 cell population induced by depletion of TS (Figure 4D). These data thus indicated that TS depletion induces caspase-dependent apoptosis in lung cancer cells.

TS depletion activates the mitochondrial pathway of apoptosis and induces downregulation of XIAP

To elucidate further the mechanism of apoptosis induced by TS depletion, we examined the expression of members of the Bcl-2

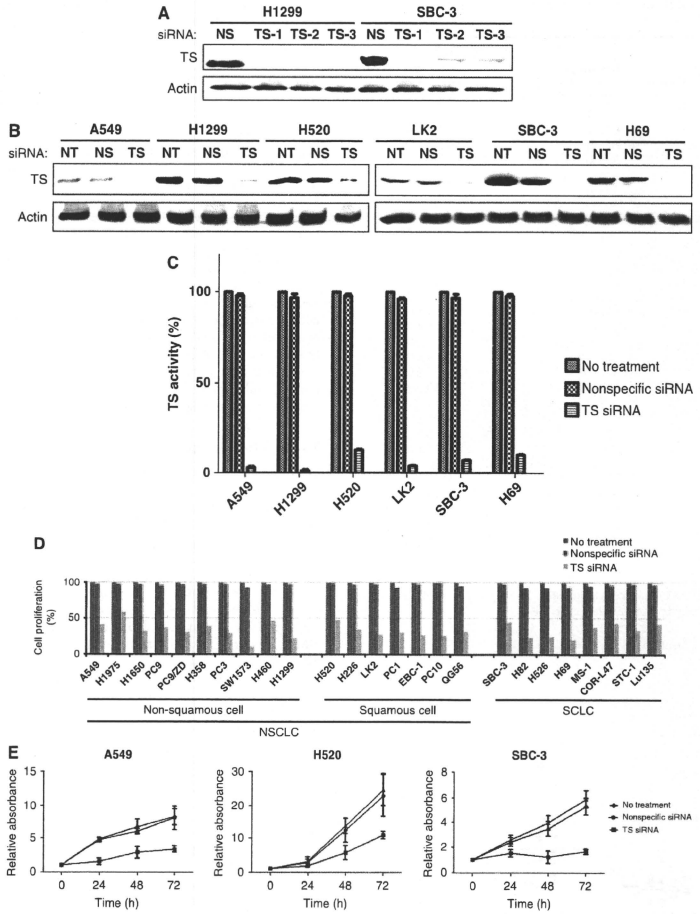

Figure 2 Effects of transient depletion of TS on TS activity and the proliferation of lung cancer cell lines. (**A**) The indicated cell lines were transfected with a nonspecific (NS) siRNA or with either of three different siRNAs specific for TS mRNA (TS-1, TS-2, and TS-3) for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS and β-actin (loading control). (**B**) The indicated cell lines were left untreated (NT) or were transfected with nonspecific or TS-1 siRNAs for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS and β-actin. (**C**) Cells were left untreated or were transfected with NS or TS-1 siRNAs for 72 h, after which cell lysates were prepared and assayed for TS activity. Data are expressed as a percentage of the value for untreated cells and are means ± s.d. of triplicates from experiments that were repeated on at least one additional occasion with similar results. (**D**) Cells were left untreated or were transfected with NS or TS-1 siRNAs for 72 h, after which cell viability was assessed with the MTT assay. Data are expressed as a percentage of the value for untreated cells and are means of triplicates from experiments that were repeated on two additional occasions with similar results. (**E**) Cells were left untreated or were transfected with NS or TS-1 siRNAs for the indicated times, after which cell viability was assessed with the MTT assay. Data are means ± s.d. of triplicates from experiments that were repeated on two additional occasions with similar results.

and inhibitor of apoptosis (IAP) families of proteins, both of which are important regulators of apoptotic signalling pathways (Hengartner, 2000). Although depletion of TS did not substantially affect the expression levels of Bcl-2, Bcl-x_L, Bax, Bak, and survivin, it resulted in a substantial decrease in the abundance of X-linked inhibitor of apoptosis (XIAP) (Figure 5A). The activity of XIAP is

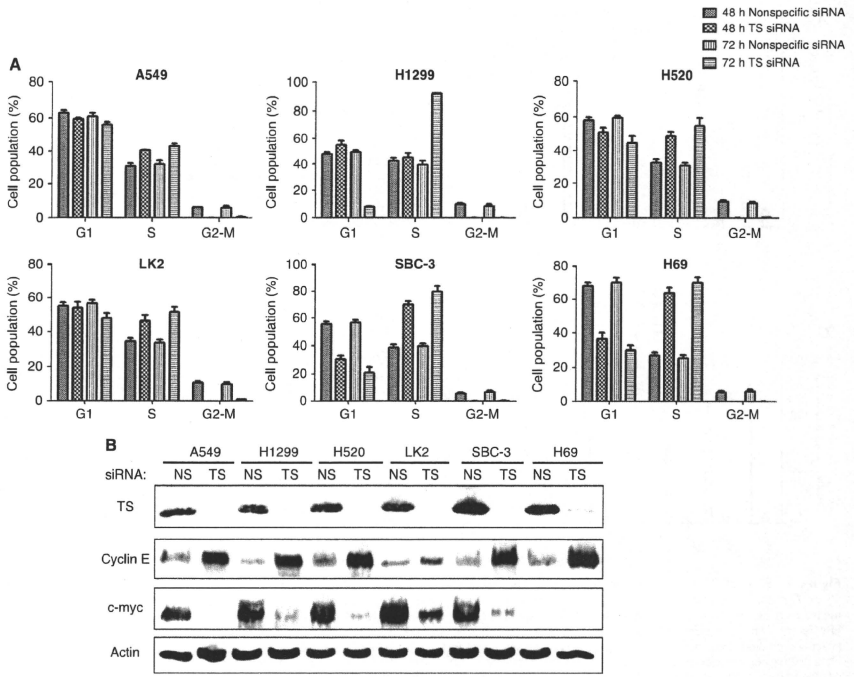

Figure 3 Effects of TS depletion on cell cycle distribution and on cyclin E and c-Myc expression in lung cancer cells. (**A**) The indicated cell lines were transfected with nonspecific (NS) or TS-1 siRNAs for 48 or 72 h and were then fixed, stained with propidium iodide, and analysed for cell cycle distribution by flow cytometry. Data are means ± s.d. of triplicates from experiments that were repeated on two additional occasions with similar results. (**B**) Cells were transfected with NS or TS-1 siRNAs for 72 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS, cyclin E, c-Myc, and β-actin. Transfection with the NS siRNA had no substantial effect on cell cycle distribution or on the expression of cyclin E or c-Myc compared with untreated cells.

modulated by mitochondrial proteins such as cytochrome *c*, Smac (also known as Diablo), and Omi (also known an HtrA2) (Hengartner, 2000; Srinivasula *et al*, 2003; Martinez-Ruiz *et al*, 2008). To investigate the mechanism of the downregulation of XIAP induced by TS depletion, we examined the release of these mitochondrial proteins into the cytosol. Immunoblot analysis revealed that the amounts of these mitochondrial proteins in the cytosol were increased by TS depletion in a time-dependent manner (Figure 5B). These data thus suggested that TS depletion-induced apoptosis is mediated, at least in part, by the mitochondrial signalling pathway.

DISCUSSION

Studies of TS-targeted therapy as well as the role of TS in DNA synthesis have provided the rationale for consideration of this enzyme as a prime therapeutic target. However, the precise mechanism by which inhibition of TS results in inhibition of tumour cell growth has remained incompletely understood. The aim of this study was therefore to investigate the underlying mechanism of the antiproliferative effect of specific TS inhibition in lung cancer cells with the use of an siRNA-based approach.

We first examined TS activity in lung cancer cell lines of different histotypes. Thymidylate synthase activity was determined with the use of the well-established 5-fluoro-dUMP binding assay. We found that TS activity was significantly higher in SCLC lines than in NSCLC lines, and that, among the latter, TS activity was higher in squamous cell carcinoma lines than in non-squamous cell carcinoma lines. A previous microarray analysis showed that mRNAs for proliferation-related proteins including TS were more abundant in SCLC lines than in NSCLC lines (Bhattacharjee *et al*, 2001). In addition, recent studies showed that the amount of TS mRNA was higher in resection specimens from patients with squamous cell carcinoma of the lung than in those from individuals with other histotypes of NSCLC (Ceppi *et al*, 2006; Ishihama *et al*, 2009; Monica *et al*, 2009). Given that TS activity in lung cancer cell lines was proportional to the abundance of TS protein in the present study (data not shown), our data showing a differential profile of TS activity among histotypes of lung cancer

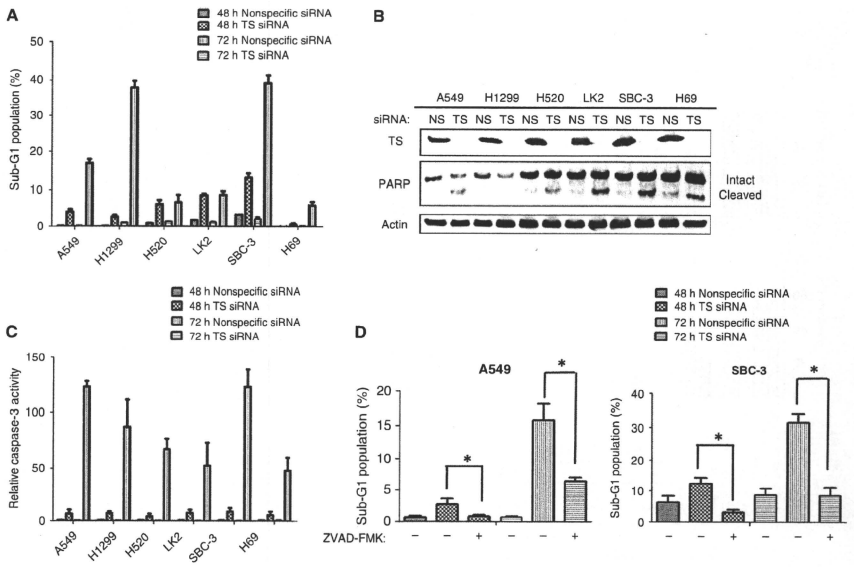

Figure 4 Effect of TS depletion on apoptosis in lung cancer cells. (**A**) The indicated cell lines were transfected with nonspecific (NS) or TS-1 siRNAs for 48 or 72 h and were then fixed, stained with propidium iodide, and subjected to flow cytometry for quantitation of the sub-G_1 population. Data are means ± s.d. of triplicates from experiments that were repeated on two additional occasions with similar results. (**B**) Cells were transfected with NS or TS-1 siRNAs for 72 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS, PARP, and β-actin. The positions of intact and cleaved forms of PARP are indicated. (**C**) Cells were transfected with NS or TS-1 siRNAs for 48 or 72 h, lysed, and assayed for caspase-3 activity. Data are expressed relative to the value for cells transfected with NS siRNA and are means ± s.d. from three independent experiments. (**D**) Cells were incubated for 2 h with or without ZVAD-FMK (50 μM), transfected with NS or TS-1 siRNAs for 48 or 72 h (in the continued absence or presence of ZVAD-FMK), and then evaluated for the size of the sub-G_1 population as in (**A**). Data are means ± s.d. of triplicates from experiments that were repeated on two additional occasions with similar results. *$P < 0.05$ for the indicated comparisons. Transfection with the NS siRNA had no substantial effects on these markers of apoptosis compared with untreated cells.

are consistent with these previous findings. The cell line SCLC differs from NSCLC in terms of its faster growth and earlier spread (Allen and Jahanzeb, 2008), and recent clinical trials in NSCLC patients have revealed a poorer prognosis for squamous cell carcinoma than for adenocarcinoma (Asamura *et al*, 2008). The differential activity of TS among histotypes of lung cancer is thus suggestive of a role for this enzyme in promoting cell proliferation, with TS activity being a potential marker of tumour aggressiveness in lung cancer, although TS activity was not correlated with cell proliferation rate among the lung cancer cell lines examined in this study. We induced downregulation of both TS abundance and enzymatic activity in lung cancer cell lines by RNAi. The almost complete elimination of TS activity was associated with a marked antiproliferative effect in all tested lung cancer cell lines, including those with an original relatively low level of TS activity. These data suggest that TS is important for tumour cell proliferation in a manner independent of the original activity level.

We found that depletion of TS induced S-phase arrest and caspase-dependent apoptosis in lung cancer cells. Previous studies have shown that TS inhibition results in an imbalance between the amounts of dUTP and dTTP and a consequent decrease in the efficiency of DNA synthesis (Curtin *et al*, 1991; Houghton *et al*, 1993). Furthermore, this dUTP–dTTP imbalance results in misincorporation of dUTP into DNA and consequent DNA damage (Curtin *et al*, 1991; Houghton *et al*, 1993). In this study, we examined the effect of TS depletion on DNA damage as determined by immunofluorescence imaging of histone γ-H2AX foci, a sensitive and specific marker of DNA double-strand breaks (Burma *et al*, 2001; Stiff *et al*, 2004). Such foci were detected in ~90% of lung cancer cells transfected with TS siRNA (Supplementary Figure S1). Given that DNA damage or a reduced rate of DNA synthesis triggers the S-phase checkpoint mechanism (Sclafani and Holzen, 2007), the observed S-phase arrest induced by TS depletion likely results from activation of the S-phase checkpoint. Cellular responses to DNA damage are important for maintenance of genomic stability and cellular integrity (Bunz *et al*, 1998; Hirao *et al*, 2000). Cells either repair DNA damage or, if it is too severe for repair, initiate the cell death program (Zhao *et al*, 2001). Our data thus suggest that cells that arrest in S phase after TS depletion subsequently undergo apoptosis as a result of the accumulation of unrepairable DNA damage. We further showed that TS depletion resulted in upregulation of cyclin E and downregulation of c-Myc. Both cyclin E and c-Myc contribute to the transition of cells from G_1 to S phase (Wang *et al*, 2008; Malumbres and Barbacid, 2009) and have recently been implicated in promotion of caspase-dependent apoptosis subsequent to