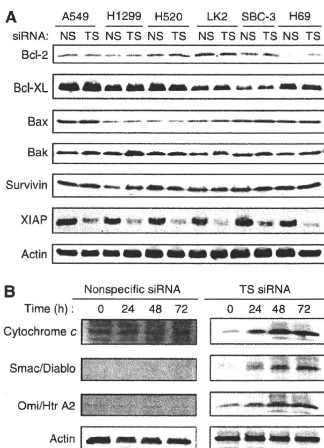

Figure 5 Effects of TS depletion on the expression of Bcl-2 and IAP family members and on the release of mitochondrial proteins into the cytosol in lung cancer cells. (**A**) The indicated cell lines were transfected with nonspecific (NS) or TS-1 siRNAs for 72 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. (**B**) SBC-3 cells were transfected with NS or TS-1 siRNAs for 24, 48, or 72 h, after which a cytosolic fraction was prepared and subjected to immunoblot analysis with antibodies to cytochrome c, Smac/Diablo, Omi/HtrA2, and β-actin. Transfection with the NS siRNA had no substantial effects on the abundance of Bcl-2 or IAP family proteins or on the release of mitochondrial proteins into the cytosol, compared with untreated cells.

S-phase arrest induced by DNA damage or inhibition of DNA synthesis in tumour cells (Mazumder *et al*, 2000; Leonce *et al*, 2001; Lu *et al*, 2009; Sankar *et al*, 2009). The effects of TS depletion on the abundance of cyclin E and c-Myc therefore likely contribute to the associated S-phase arrest and caspase-dependent apoptosis in lung cancer cells. Our present data thus suggest that the antiproliferative effect of TS depletion is attributable to S-phase arrest and the induction of caspase-dependent apoptosis in these cancer cells.

Our investigation of the mechanism by which TS depletion led to caspase-dependent apoptosis revealed that elimination of TS resulted in downregulation of XIAP, a member of the IAP family of proteins. Activation of the mitochondrial signalling pathway for apoptosis results in inhibition of IAP proteins and consequent promotion of caspase-dependent apoptosis (Hengartner, 2000; Takasawa *et al*, 2005; Yu *et al*, 2007). We also found that TS depletion resulted in the release of mitochondrial proteins, including cytochrome *c*, Smac/Diablo, and Omi/HtrA2, into the cytosol, suggestive of a link between activation of the mitochondrial pathway and downregulation of XIAP in lung cancer cells depleted of TS. Activation of the mitochondrial pathway is induced by a variety of stimuli including DNA damage (Hengartner, 2000). Given that TS depletion induced DNA double-strand breakage, our data suggest that loss of TS may contribute to activation of the mitochondrial pathway of apoptosis. We found that TS depletion did not affect the expression level of the IAP protein survivin. Further study will thus be needed to elucidate the precise mechanism by which XIAP is downregulated specifically in TS-depleted cells.

In conclusion, we have shown that the almost complete elimination of TS activity with an RNAi-based approach resulted in an apparently universal antiproliferative effect in lung cancer cells that was attributable to S-phase arrest and the induction of apoptosis. High levels of TS expression have been suggested to predict resistance to TS-targeted agents such as 5-fluorouracil (Johnston *et al*, 2003; Showalter *et al*, 2008). The new TS-targeted agent pemetrexed was found to have low activity in the treatment of SCLC (Ceppi *et al*, 2006; Socinski *et al*, 2009), possibly as a result of a high level of TS expression in such tumours. Our results now suggest that TS depletion inhibits the growth of lung cancer cells including SCLC cells with a high original activity of TS. This apparent discrepancy may be explained by the fact that 5-fluorouracil and pemetrexed inhibit TS activity by only $\sim 60\%$ (van Triest *et al*, 1997, 1999; Codacci-Pisanelli *et al*, 2002; Giovannetti *et al*, 2008), whereas our siRNA-based method inhibit TS activity almost completely. Our present results thus suggest that novel TS-targeted agents with an increased inhibitory efficacy might prove beneficial for the treatment of lung cancer regardless of histotype. They further provide a rationale for future clinical investigation of the therapeutic efficacy of TS-targeted agents for lung cancer patients.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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Research Article

Molecular Cancer Therapeutics

Enhanced Anticancer Effect of the Combination of BIBW2992 and Thymidylate Synthase–Targeted Agents in Non–Small Cell Lung Cancer with the T790M Mutation of Epidermal Growth Factor Receptor

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Abstract

Most non–small cell lung cancer (NSCLC) tumors with activating mutations of the epidermal growth factor receptor (EGFR) are initially responsive to first-generation, reversible EGFR tyrosine kinase inhibitors (TKI) such as gefitinib, but they subsequently develop resistance to these drugs through either acquisition of an additional T790M mutation of EGFR or amplification of the proto-oncogene *MET.* We have now investigated the effects of combination treatment with thymidylate synthase (TS)–targeting drugs and the second-generation, irreversible EGFR-TKI BIBW2992 on the growth of NSCLC cells with the T790M mutation. The effects of BIBW2992 on EGFR signaling and TS expression in gefitinib-resistant NSCLC cells were examined by immunoblot analysis. The effects of BIBW2992 and the TS-targeting agents S-1 (or 5-fluorouracil) or pemetrexed on the growth of gefitinib-resistant NSCLC cells were examined both *in vitro* and *in vivo*. The combination of BIBW2992 with 5-fluorouracil or pemetrexed synergistically inhibited the proliferation of NSCLC cells with the T790M mutation *in vitro*, whereas an antagonistic interaction was apparent in this regard between gefitinib and either of these TS-targeting agents. BIBW2992 induced downregulation of TS in the gefitinib-resistant NSCLC cells, implicating depletion of TS in the enhanced antitumor effect of the combination therapy. The combination of BIBW2992 and either the oral fluoropyrimidine S-1 or pemetrexed also inhibited the growth of NSCLC xenografts with the T790M mutation to an extent greater than that apparent with either agent alone. The addition of TS-targeting drugs to BIBW2992 is a promising strategy to overcome EGFR-TKI resistance in NSCLC with the T790M mutation of EGFR. *Mol Cancer Ther; 9(6); 1647–56. ©2010 AACR.*

Introduction

Somatic mutations of the *epidermal growth factor receptor* (*EGFR*) gene are associated with a therapeutic response to EGFR tyrosine kinase inhibitors (TKI) in individuals with non–small cell lung cancer (NSCLC; 1–3). However, most such patients ultimately develop resistance to these drugs. Among patients with NSCLC who develop resistance to the first-generation EGFR-TKIs gefitinib or erlotinib, ~50% have tumors with a secondary T790M mutation in exon 20 of *EGFR* and ~20% have tumors that manifest amplification of the proto-oncogene *MET* (4–6). The identification of strategies or agents capable of overcoming acquired resistance to EGFR-TKIs is thus an important clinical goal.

Gefitinib and erlotinib act as ATP mimetics and reversible inhibitors at the tyrosine kinase domain of EGFR. In contrast, second-generation, irreversible EGFR-TKIs not only act as ATP mimetics but also covalently bind to Cys^{797} of EGFR, which allows them to inhibit EGFR phosphorylation even in the presence of a T790M secondary mutation. Irreversible EGFR-TKIs including BIBW2992 have been found to be effective in inhibiting the growth of NSCLC cells with the T790M mutation of EGFR both *in vitro* and *in vivo* (7–9). On the basis of these preclinical evaluations, various clinical trials are currently under way to determine the efficacy of these drugs in NSCLC patients.

S-1 is an oral fluoropyrimidine derivative that is also currently under evaluation for the treatment of NSCLC as a thymidylate synthase (TS)–targeted agent (10–12). A new antifolate drug, pemetrexed, has also been shown to inhibit tumor growth by targeting TS and is widely used in clinical settings (13, 14). We have previously shown that gefitinib-induced downregulation of TS and E2F1, a transcription factor that regulates expression of

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Table 1. IC_{50} values of BIBW2992 and gefitinib for inhibition of the growth of NSCLC cells *in vitro*

Cell line	IC_{50} (μmol/L)	
	BIBW2992	Gefitinib
	T790M (–)	
PC9	<0.001	0.031
HCC827	<0.001	0.011
	T790M (+)	
PC9/ZD	0.41	>5
H1975	0.22	>5

NOTE: Data are means of triplicates from representative experiments repeated a total of three times.

the TS gene, is responsible for the enhanced antitumor effect of combined treatment with S-1 (15, 16). However, an enhanced antitumor effect of this combination therapy on the growth of NSCLC cells harboring the T790M mutation of EGFR was not apparent as a result of continuous activation of EGFR and sustained expression of TS during gefitinib exposure. We have now investigated the potential efficacy of combined therapy with an irreversible EGFR-TKI and TS-targeted agents for the treatment of NSCLC with the T790M mutation of EGFR.

Materials and Methods

Cell culture and reagents

The human NSCLC cell lines PC9, PC9/ZD, HCC827, and NCI-H1975 (H1975) were obtained as described previously (16–18). All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. BIBW2992 was kindly provided by Boehringer Ingelheim Pharma; gefitinib was obtained from AstraZeneca; and 5-fluorouracil (5-FU), S-1, and pemetrexed were from Wako. U0126 and LY294002 were obtained from Cell Signaling Technology.

Growth inhibition assay *in vitro*

Cells were plated in 96-well flat-bottomed plates and cultured for 24 hours before exposure to various concentrations of drugs for 72 hours. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku) was then added to each well, and the cells were incubated for 3 hours at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of tested drugs resulting in 50% growth inhibition (IC_{50}) was calculated. Data were analyzed by the median-effect method (CalcuSyn software, Biosoft) to determine the combination index (CI), a well-established index of the interaction between two drugs (19). CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively.

Immunoblot analysis

Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of cell lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 hour at room temperature before incubation overnight at 4°C with rabbit polyclonal antibodies to human phosphorylated EGFR (pY1068, 1:1,000 dilution; Cell Signaling Technology), phosphorylated AKT (1:1,000 dilution; Cell Signaling Technology), AKT (1:1,000 dilution; Cell Signaling Technology), phosphorylated extracellular signal-regulated kinase (ERK; 1:1,000 dilution; Santa Cruz Biotechnology), ERK (1:1,000 dilution; Santa Cruz Biotechnology), E2F1 (1:1,000 dilution; Santa Cruz Biotechnology), TS (1:1,000 dilution; Santa Cruz Biotechnology), or β-actin (1:500 dilution; Sigma) or with mouse monoclonal antibodies to EGFR (1:1,000 dilution; Zymed). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 hour 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 (Perkin-Elmer Life Science).

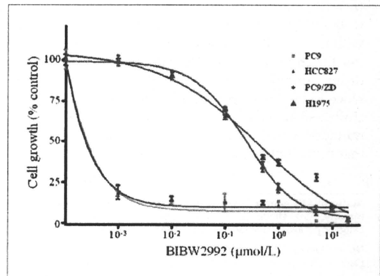

Figure 1. Effect of BIBW2992 on the growth of NSCLC cell lines *in vitro*. The indicated NSCLC cell lines were cultured for 72 h in complete medium containing various concentrations of BIBW2992, after which cell viability was assessed as described in Materials and Methods. Points, mean of triplicates from experiments that were repeated a total of three times with similar results; bars, SD.

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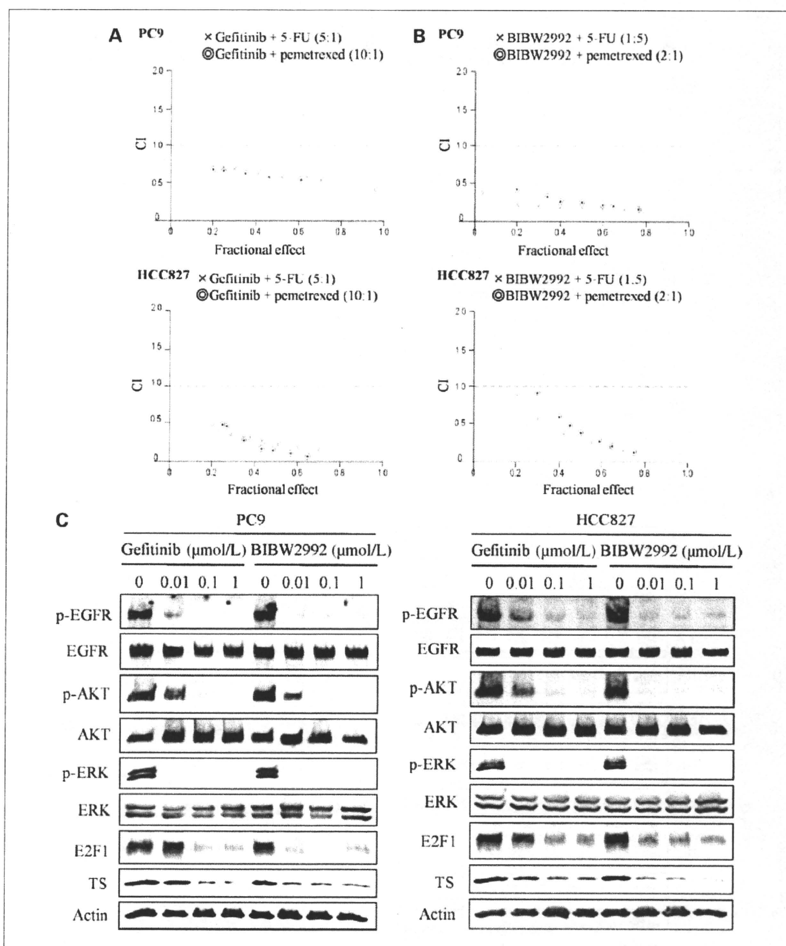

Figure 2. Effects of the combination of TS inhibitors (5-FU or pemetrexed) with EGFR-TKIs (gefitinib or BIBW2992) on the growth of gefitinib-sensitive NSCLC cell lines *in vitro*. A and B, sensitizing *EGFR* mutation-positive NSCLC (PC9 and HCC827) cells were incubated for 72 h with gefitinib (A) or BIBW2992 (B) together with 5-FU or pemetrexed at the indicated molar concentration ratios, after which cell viability was measured. The interaction between the two drugs in each combination was evaluated on the basis of the CI, which is plotted against fractional growth inhibition. Data are means of triplicates from experiments that were repeated a total of three times with similar results. C, cells were incubated for 24 h with gefitinib or BIBW2992 at the indicated concentrations in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, or ERK as well as with those to E2F1, TS, or β-actin (loading control).

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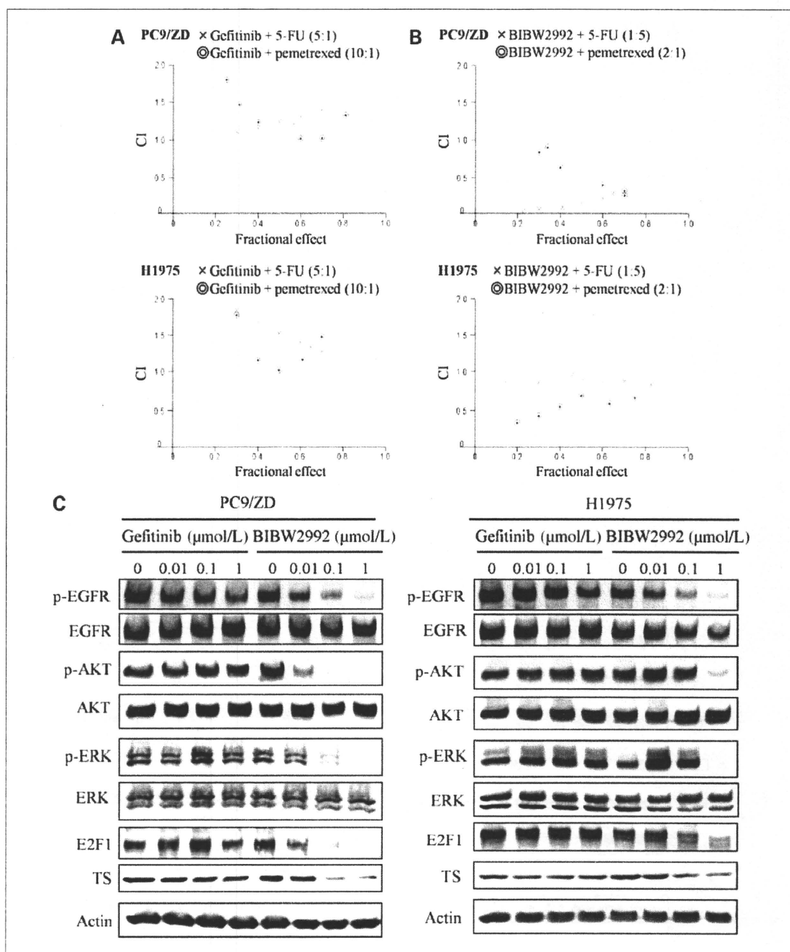

Figure 3. Effects of the combination of TS inhibitors (5-FU or pemetrexed) with EGFR-TKIs (gefitinib or BIBW2992) on the growth of gefitinib-resistant NSCLC cell lines *in vitro*. A and B, cells with a secondary T790M mutation of EGFR (PC9/ZD and H1975) were incubated for 72 h with gefitinib (A) or BIBW2992 (B) together with 5-FU or pemetrexed at the indicated molar concentration ratios, after which cell viability was measured and CI was plotted against fractional growth inhibition. Data are means of triplicates from experiments that were repeated a total of three times with similar results. C, cells were incubated for 24 h with gefitinib or BIBW2992 at the indicated concentrations in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis as in Fig. 2C.

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Annexin V binding assay

Binding of Annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining kit (Roche). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × *g* for 5 minutes. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 minutes at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and CellQuest software (Becton Dickinson).

Animals

Male athymic nude mice were exposed to a 12-hour-light, 12-hour-dark cycle and provided with food and water *ad libitum* in a barrier facility. All animal experiments were done with approval of an international Institutional Animal Care and Use Committee and complied with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care of Japan.

Growth inhibition assay *in vivo*

Cubic fragments of tumor tissue (~2 by 2 by 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 of eight mice achieved an average volume of 150 to 200 mm^3. Treatment groups consisted of control, S-1 or pemetrexed alone, gefitinib alone, BIBW2992 alone, the combination of gefitinib and either S-1 or pemetrexed, and the combination of BIBW2992 and either S-1 or pemetrexed. S-1, gefitinib, and BIBW2992 were administered by oral gavage daily for 28 days; control animals received a 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle. Pemetrexed was administered i.p. once a week. 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 twice per week.

Statistical analysis

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

Results

An additional T790M mutation reduces the sensitivity of sensitizing EGFR mutation-positive NSCLC cells to BIBW2992

We first examined the ability of BIBW2992 to inhibit the proliferation of human NSCLC cells with an *EGFR* mutation. Both PC9 and HCC827 cells harbor an in-frame deletion in exon 19 of *EGFR* and were found to be highly sensitive to BIBW2992, with IC_{50} values of <0.001 μmol/L (Table 1). Gefitinib also potently inhibited the proliferation of these two cell lines (Table 1). PC9/ZD cells are a gefitinib-resistant clone of PC9 and also harbor the T790M mutation of *EGFR*, and H1975 cells possess both L858R and T790M mutations of *EGFR*. Both of these cell lines manifested resistance to gefitinib, with an IC_{50} for this drug of >5 μmol/L (Table 1). Although BIBW2992 inhibited the growth of PC9/ZD and H1975 cells with IC_{50} values within the range of achievable serum concentrations of the drug, these values were ~1,000 times those apparent for PC9 and HCC827 cells (Fig. 1). These data thus suggested that an additional T790M mutation of *EGFR* reduces the sensitivity of NSCLC cells with a sensitizing *EGFR* mutation (either an exon 19 deletion or L858R in exon 21) to BIBW2992.

Synergistic effects of EGFR-TKIs and 5-FU or pemetrexed in sensitizing EGFR mutation-positive NSCLC cells

We have previously shown that the combination of gefitinib and S-1 has a synergistic antiproliferative effect on sensitizing *EGFR* mutation-positive NSCLC cells, with downregulation of TS by gefitinib underlying its synergistic interaction with S-1 (16). We used 5-FU instead of S-1 for *in vitro* experiments because tegafur, which is a component of S-1, is metabolized to 5-FU primarily in the liver. The combined effect of the two drugs was evaluated on the basis of the CI. The combination of 5-FU and gefitinib manifested a synergistic inhibitory effect (CI of <1.0) on the growth of both PC9 and HCC827 cells (Fig. 2A; Supplementary Fig. S1; Supplementary Table S1), consistent with the results of our previous study (15). Given that pemetrexed also inhibits tumor growth by targeting TS, we examined the effect of the combination of this drug with gefitinib on the proliferation of PC9 and HCC827 cells. We again obtained a CI value of <1.0 for both cell lines (Fig. 2A; Supplementary Fig. S1; Supplementary

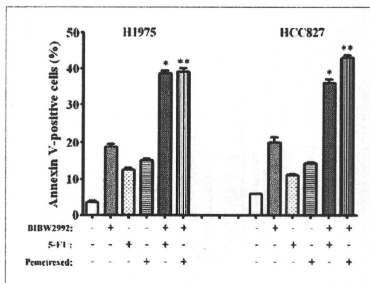

Figure 4. Effects of the combination of BIBW2992 and either 5-FU or pemetrexed on apoptosis in NSCLC cells. H1975 or HCC827 cells were incubated for 72 h with BIBW2992, 5-FU, or pemetrexed at their IC_{50} values, after which the proportion of apoptotic cells was assessed by staining with FITC-conjugated Annexin V and propidium iodide followed by flow cytometry. Columns, mean of triplicates from an experiment that was repeated a total of three times with similar results; bars, SD. *, P < 0.05, for the combination of BIBW2992 plus 5-FU versus 5-FU alone; **, P < 0.05, for the combination of BIBW2992 plus pemetrexed versus pemetrexed alone.

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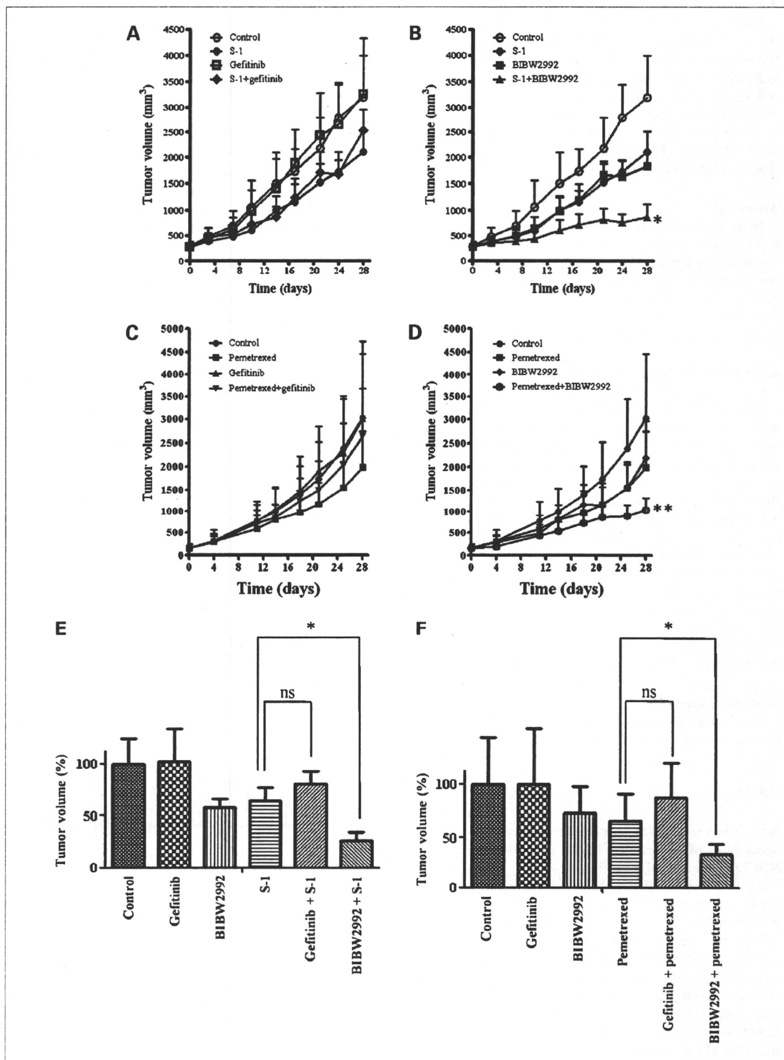

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Table S1). We further examined the effects of the combination of the irreversible EGFR-TKI BIBW2992 with either 5-FU or pemetrexed, finding that each drug combination showed a synergistic antiproliferative effect in sensitizing *EGFR* mutation-positive NSCLC cells (Fig. 2B; Supplementary Fig. S1; Supplementary Table S1).

To investigate the underlying mechanism of the synergistic growth-inhibitory effects of these various drug combinations, we examined the effects of gefitinib and BIBW2992 on the expression of the transcription factor E2F1 and TS as well as on the phosphorylation of EGFR and downstream signaling molecules in sensitizing *EGFR* mutation-positive NSCLC cell lines. Immunoblot analysis revealed that the phosphorylation of EGFR, the protein kinase AKT, and the mitogen-activated protein kinase (MAPK) ERK as well as the expression of E2F1 and TS were markedly inhibited by gefitinib or BIBW2992 in a concentration-dependent manner (Fig. 2C). These data thus suggested that not only reversible EGFR-TKIs (gefitinib) but also irreversible EGFR-TKIs (BIBW2992) downregulate the expression of TS, resulting in a synergistic antiproliferative interaction with 5-FU or pemetrexed in sensitizing *EGFR* mutation-positive NSCLC cells.

Synergistic effects of BIBW2992 and 5-FU or pemetrexed in NSCLC cells with the T790M mutation

We examined the combined effects of gefitinib and either 5-FU or pemetrexed on the growth of NSCLC cell lines with a secondary T790M mutation of *EGFR*. We found that gefitinib and either 5-FU or pemetrexed manifested an antagonistic interaction (CI of >1.0) in their effects on the growth of both PC9/ZD and H1975 cells (Fig. 3A; Supplementary Fig. S2; Supplementary Table S1). In contrast to gefitinib, the combination of BIBW2992 and either 5-FU or pemetrexed exhibited a synergistic growth-inhibitory effect (CI of <1.0) in these cells (Fig. 3B; Supplementary Fig. S2; Supplementary Table S1). We next examined the effects of gefitinib and BIBW2992 on the expression of E2F1 and TS as well as on the phosphorylation of EGFR, AKT, and ERK in NSCLC cell lines with a secondary T790M mutation of *EGFR*. Immunoblot analysis revealed that BIBW2992, but not gefitinib, markedly inhibited the expression of E2F1 and TS as well as the phosphorylation of EGFR, AKT, and ERK in a concentration-dependent manner in PC9/ZD or H1975 cells (Fig. 3C). The combination of BIBW2992 with either 5-FU or pemetrexed thus had a synergistic antiproliferative effect even in gefitinib-resistant NSCLC cells harboring the T790M mutation, and this effect correlated with downregulation of TS expression.

Enhanced induction of apoptosis by the combination of BIBW2992 and either 5-FU or pemetrexed in NSCLC cells with EGFR mutations

To investigate the mechanism of the synergistic growth inhibition induced by the combination of BIBW2992 and either 5-FU or pemetrexed in NSCLC cells with a secondary T790M mutation of *EGFR*, we examined the effects of each agent alone and in combination on apoptosis. An assay based on the binding of Annexin V to the cell surface revealed that the frequency of apoptosis was markedly greater for H1975 cells (harboring both L858R and T790M mutations) treated with the combination of BIBW2992 and either 5-FU or pemetrexed than for those treated with either agent alone (Fig. 4). Such combination therapy also induced apoptosis to a significantly greater extent in HCC827 cells (harboring only an exon 19 deletion) compared with either monotherapy (Fig. 4). These data thus suggested that the combination of BIBW2992 and either 5-FU or pemetrexed exhibits an enhanced proapoptotic effect in sensitizing *EGFR* mutation-positive NSCLC cells with or without the T790M mutation.

Effects of combined treatment with BIBW2992 and either S-1 or pemetrexed on the growth of gefitinib-resistant NSCLC cells with the T790M mutation *in vivo*

We next investigated whether combined treatment with BIBW2992 and either S-1 or pemetrexed might exhibit an enhanced effect on the growth of gefitinib-resistant NSCLC (H1975) cells with the T790M mutation of *EGFR in vivo*. When their tumors became palpable, mice were divided into nine groups and treated with vehicle, S-1 or pemetrexed alone, gefitinib alone, BIBW2992 alone, both gefitinib and either S-1 or pemetrexed, or both BIBW2992 and either S-1 or pemetrexed for 4 weeks. Combination therapy with gefitinib and S-1 did not exhibit an enhanced effect on the growth of tumors formed by H1975 cells (Fig. 5A and E), consistent with our previous findings (16). Combination therapy with gefitinib and pemetrexed also did not exhibit an enhanced antitumor effect (Fig. 5C and F). In contrast, combination therapy with BIBW2992 and either S-1 or pemetrexed inhibited the growth of H1975 tumors to a significantly greater extent than did treatment with either drug alone (Fig. 5B, D, E, and F). All of the treatments were well tolerated by the mice, with no signs of toxicity or weight loss during therapy (data

Figure 5. Effects of combination therapy with S-1 or pemetrexed and either gefitinib or BIBW2992 on the growth of NSCLC cells harboring the T790M mutation of EGFR *in vivo*. Nude mice with tumor xenografts established by s.c. implantation of tumor fragments formed by H1975 cells were treated daily for 4 wk by oral gavage with vehicle (control), S-1 (10 mg/kg), or either gefitinib (50 mg/kg; A) or BIBW2992 (10 mg/kg; B) alone or together with S-1 (10 mg/kg). Alternatively, the mice were treated with vehicle (control), pemetrexed (100 mg/kg, i.p., on days 1, 8, 15, and 22), or either gefitinib (50 mg/kg; C) or BIBW2992 (10 mg/kg; D) alone or together with pemetrexed (100 mg/kg). Tumor volume was determined at the indicated times after the onset of treatment. Points, mean of values from eight mice per group; bars, SE. *, $P < 0.05$, for the combination of S-1 plus BIBW2992 versus either S-1 or BIBW2992 alone; **, $P < 0.05$, for the combination of pemetrexed plus BIBW2992 versus either pemetrexed or BIBW2992 alone. Final tumor volume relative to that in the vehicle-treated (control) group for combined therapy with S-1 and either gefitinib or BIBW2992 (E) or with pemetrexed and either gefitinib or BIBW2992 (F) was also calculated. *, $P < 0.05$. ns, not significant.

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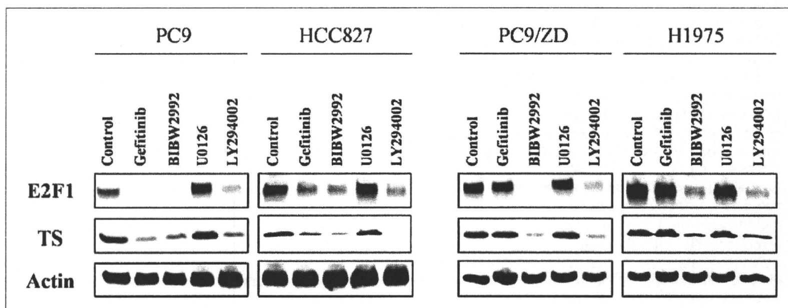

Figure 6. Effects of MEK or PI3K inhibitors on E2F1 and TS expression in NSCLC cells. Cells were incubated for 24 h with vehicle (DMSO, control), gefitinib (1 μmol/L), BIBW2992 (1 μmol/L), U0126 (10 μmol/L), or LY294002 (20 μmol/L for HCC827 and H1975; 50 μmol/L for PC9 and PC9/ZD) in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to E2F1, TS, or β-actin.

not shown). These findings thus suggested that combination therapy with BIBW2992 and either S-1 or pemetrexed exhibited an enhanced antitumor effect *in vivo* with gefitinib-resistant xenografts harboring the T790M mutation of *EGFR*, consistent with the results obtained *in vitro*.

Role of phosphoinositide 3-kinase in the effects of EGFR-TKIs on E2F1 and TS expression in NSCLC cells

To investigate the mechanism underlying the downregulation of E2F1 and TS by EGFR-TKIs, we examined the effects of specific inhibitors of the ERK kinase MAPK/ERK kinase (MEK) or phosphoinositide 3-kinase (PI3K) on E2F1 and TS expression. The amounts of both E2F1 and TS in *EGFR* mutation–positive NSCLC cells were reduced by treatment with the PI3K inhibitor LY294002 but were not affected by that with the MEK inhibitor U0126 (Fig. 6). These results thus suggested that EGFR-TKIs regulate E2F1 and TS expression primarily through inhibition of PI3K signaling rather than through that of MAPK signaling.

Discussion

We have shown that the sensitivity of sensitizing *EGFR* mutation–positive NSCLC cells to the antiproliferative effect of the irreversible EGFR-TKI BIBW2992 is reduced by the acquisition of a secondary T790M mutation of *EGFR*. Consistent with previous observations (9), we found that BIBW2992 inhibited the proliferation of NSCLC cells harboring the T790M mutation with IC_{50} values within the clinically achievable range of serum concentrations (20), whereas gefitinib exhibited no such activity against these cells. However, the IC_{50} value for the antiproliferative effect of BIBW2992 in PC9/ZD cells (which harbor the T790M mutation) was ~1,000 times that in the parental PC9 cells (which do not harbor the T790M mutation). Indeed, NSCLC cells harboring the T790M mutation were previously found to be more resistant to other irreversible EGFR-TKIs (CL-387,785 and PF00299804) compared with those without the mutation (8, 21). These observations thus indicate that an additional T790M mutation reduces the sensitivity of sensitizing *EGFR* mutation–positive NSCLC cells to irreversible EGFR-TKIs, suggesting that the antitumor effects of these drugs may be limited for the treatment of NSCLC patients. We therefore propose that combination therapy with BIBW2992 and cytotoxic agents is likely to be more effective than treatment with BIBW2992 alone.

We previously showed that the combination of S-1 and gefitinib has an enhanced antitumor effect on NSCLC cells regardless of the presence or absence of sensitizing *EGFR* mutations, and that downregulation of TS by gefitinib contributes to its enhanced interaction with S-1 (15). However, gefitinib failed to inhibit the phosphorylation of EGFR as well as the expression of E2F1 and TS in NSCLC cells harboring the T790M mutation of *EGFR*, resulting in the lack of an enhanced interaction with S-1 (16). These findings suggest that gefitinib-induced downregulation of E2F1 and TS is mediated by modulation of EGFR signaling. In the present study, we found that BIBW2992 inhibited EGFR phosphorylation and induced downregulation of E2F1 and TS even in NSCLC cells with an additional T790M mutation of *EGFR*. These data thus provide further support for the link between EGFR signaling and the expression of E2F1 and TS. Furthermore, we have now shown that combination therapy with BIBW2992 and S-1, as well as that with BIBW2992 and the new TS-targeted agent pemetrexed, had an enhanced antitumor effect on NSCLC cells with the T790M mutation. A low level of TS expression in human solid tumors is thought to predict a better response to 5-FU (22–25).

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Pemetrexed sensitivity has also been suggested to correlate inversely with TS expression in human cancer (26, 27). Several preclinical studies also support such an inverse relation between TS expression and sensitivity to TS-targeted agents, likely reflecting the role of TS as a target for these drugs (28–31). These observations support the notion that BIBW2992-induced downregulation of TS underlies, at least in part, the enhanced antitumor effect of combination therapy with either S-1 or pemetrexed. Our present data thus provide a rationale for combination therapy with BIBW2992 and either S-1 or pemetrexed for NSCLC with a secondary T790M mutation of *EGFR*.

Although the mechanism responsible for the downregulation of E2F1 and TS by EGFR-TKIs remains unclear, our results suggest that these effects are mediated in part through inhibition of PI3K as a consequence of EGFR inactivation. Consistent with this notion, EGF-induced activation of PI3K-AKT signaling has previously been shown to result in E2F1 accumulation and inhibition of apoptosis, whereas activation of the MAPK pathway had no such effects (32–35). In the present study, we further found that the combination of the PI3K inhibitor LY294002 and either 5-FU or pemetrexed manifested a synergistic inhibitory effect (CI of <1.0) on the growth of *EGFR* mutation–positive NSCLC cells (Supplementary Table S2), likely as a result of the observed downregulation of TS by LY294002. Although the precise mechanism by which inhibition of PI3K signaling results in downregulation of E2F1 and TS expression remains to be elucidated, our present findings suggest that the antitumor effects of TS-targeted agents might be affected by the activity of the PI3K signaling pathway.

In conclusion, we have shown that the combination of BIBW2992 and either S-1 or pemetrexed has an enhanced antitumor effect in both gefitinib-sensitive NSCLC cells and gefitinib-resistant cells with the T790M mutation of *EGFR*. BIBW2992-induced downregulation of TS expression was associated with increased sensitivity to S-1 or pemetrexed, and the enhanced antitumor effect of such combination therapy was reflected in an increased proapoptotic effect *in vitro*. Further development and assessment of such combination therapy is warranted as a means of overcoming gefitinib resistance in NSCLC patients with the T790M mutation of *EGFR*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Research Article

Synergistic Antitumor Effect of S-1 and HER2-Targeting Agents in Gastric Cancer with *HER2* Amplification

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Abstract

Amplification of *human epidermal growth factor receptor 2* (*HER2*) has been detected in 20% to 30% of gastric cancers and is associated with a poor outcome. Combination therapies with HER2-targeting agents and cytotoxic agents are considered a potential therapeutic option for gastric cancer with *HER2* amplification. We have now investigated the effects of combination treatment with the oral fluoropyrimidine S-1 and the HER2-targeting agents lapatinib or trastuzumab in gastric cancer cells with or without *HER2* amplification. We used 5-fluorouracil (5FU) instead of S-1 for *in vitro* experiments, given that tegafur, a component of S-1, is metabolized to 5FU in the liver. The combination of 5FU and HER2-targeting agents synergistically inhibited cell proliferation and exhibited an enhanced proapoptotic effect in gastric cancer cells with *HER2* amplification, but not in those without it. Lapatinib or trastuzumab also induced downregulation of thymidylate synthase (TS) expression and activity only in cells with *HER2* amplification. The combination of 5FU and TS depletion by RNA interference also exhibited an enhanced proapoptotic effect in cells with *HER2* amplification. These observations thus suggest that lapatinib-induced or trastuzumab-induced downregulation of TS is responsible, at least in part, for the synergistic antitumor effect of combined treatment with 5FU and HER2-targeting agents. The antitumor effect of the combination of S-1 and HER2-targeting agents *in vivo* was also greater than that of either drug alone. Our preclinical findings thus indicate that the combination of S-1 and HER2-targeting agents is a promising treatment option for gastric cancer with *HER2* amplification. *Mol Cancer Ther; 9(5); 1198–207. ©2010 AACR.*

Introduction

Gastric cancer is the second leading cause of cancer mortality worldwide, with 700,000 confirmed deaths annually (1, 2). Advanced gastric cancer is treated predominantly by combination chemotherapy that includes fluoropyrimidine derivatives, but overall survival time remains <1 year (3, 4). Further improvement in such therapy is therefore warranted. S-1 is a novel oral anticancer drug that combines tegafur, a prodrug of 5-fluorouracil (5FU), with 5-chloro-2,4-dihydropyrimidine and potassium oxonate. 5-Chloro-2,4-dihydropyrimidine increases the plasma concentration of 5FU through competitive inhibition of dihydropyrimidine dehydrogenase, which catalyzes 5FU catabolism (5), whereas potassium oxonate reduces the gastrointestinal toxicity of 5FU (6). Clinical trials have revealed response rates of ~30% to 50% for S-1 in advanced gastric cancer (6–9), and S-1 is now recognized as one of the standard chemotherapeutic drugs for this condition, especially in East Asia (9–11).

Recent years have seen substantial advances in the development of molecularly targeted therapy for various types of cancer. Amplification of *human epidermal growth factor receptor 2* (*HER2*) has been detected in 20% to 30% of gastric cancers and is associated with a poor outcome and aggressiveness of the disease (12, 13). Targeting of HER2 is therefore thought to be beneficial for those gastric cancer patients with *HER2* amplification. Clinical trials to evaluate the efficacy of HER2-targeting agents—including lapatinib, a dual tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR) and HER2, and trastuzumab, a humanized monoclonal antibody to HER2—in individuals with gastric cancer positive for *HER2* amplification are under way. However, the development of HER2-targeted therapy for gastric cancer lags behind that for breast cancer, for which trastuzumab is now recognized as a standard therapy for HER2-positive patients. Preclinical studies of HER2-targeting agents with gastric cancer cells positive for *HER2* amplification are still limited (14–17), with further investigations to clarify the efficacy and mechanism of action of HER2-targeting agents alone or in combination with cytotoxic drugs being required. We have now investigated the effects of combination treatment

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with S-1 (or 5FU) and the HER2-targeting agents lapatinib or trastuzumab in gastric cancer cells with or without *HER2* amplification, and we have further examined the mechanism of such effects.

Materials and Methods

Cell culture and reagents. Human gastric cancer cell lines were obtained from the following sources: NCI-N87 from American Type Culture Collection; MKN-1, MKN-7, and AZ-521 from Health Science Research Resources Bank; MKN-28 from Immuno-Biological Laboratories; and SNU-216 from Korean Cell Line Bank. All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. The human gastric cancer line 4-1ST was obtained from Central Institute for Experimental Animals and was maintained in BALB/c-nu/nu mice by s.c. injection of tumor pieces. Lapatinib was obtained from Sequoia Research Products, trastuzumab was from Hoffmann-La Roche, and 5FU and S-1 were from Wako. Tegafur, gimeracil, and oteracil, all of which are components of S-1, were synthesized by Taiho Pharmaceutical.

Fluorescence* in situ *hybridization analysis. The gene copy number per cell for *HER2* was determined by fluorescence *in situ* hybridization with the use of *HER2/neu* (17q11.2-q12) Spectrum Orange and CEP17 (chromosome 17 centromere) Spectrum Green probes (Vysis; Abbott). Cells were centrifuged onto glass slides with a Shandon cytocentrifuge (Thermo Electron) and were fixed by consecutive incubations with ice-cold 70% ethanol for 10 minutes, 85% ethanol for 5 minutes, and 100% ethanol for 5 minutes. The slides were stored at −20°C until analysis. Cells were subsequently subjected to digestion with pepsin for 10 minutes at 37°C, washed with water, dehydrated with a graded series of ethanol solutions, denatured with 70% formamide in 2× SSC for 5 minutes at 72°C, and dehydrated again with a graded series of ethanol solutions before incubation with a hybridization mixture consisting of 50% formamide, Cot-1 DNA, and labeled DNA in 2× SSC. The slides were washed for 5 minutes at 73°C with 3× SSC, for 5 minutes at 37°C with 4× SSC containing 0.1% Triton X-100, and for 5 minutes at room temperature with 2× SSC before counter-staining with antifade solution containing 4′,6-diamidino-2-phenylindole. Hybridization signals were scored in 40 nuclei with the use of a 100× immersion objective lens. Nuclei with a disrupted boundary were excluded from the analysis. Gene amplification was defined as a mean *HER2*/chromosome 17 copy number ratio of >2.0 (18).

***Growth inhibition assay* in vitro.** Cells were plated in 96-well flat-bottomed plates and cultured for 24 hours before exposure to various concentrations of drugs for 72 hours. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku) was then added to each well, and the cells were incubated for 3 hours at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of tested drugs resulting in 50% growth inhibition (IC_{50}) was calculated. Data were analyzed by the median-effect method (CalcuSyn software; Biosoft) to determine the combination index (CI), a well-established index of the interaction between two drugs (19). CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively.

Annexin V binding assay. Binding of Annexin V to cells was measured with the use of an Annexin V-FLUOS Staining kit (Roche). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × *g* for 5 minutes. The cell pellets were resuspended in 100 μL of Annexin V-FLUOS labeling solution, incubated for 10 to 15 minutes at 15°C to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson).

Assay of caspase-3 activity. The activity of caspase-3 in cell lysates was measured with the use of a CCP32/Caspase-3 Fluometric Protease Assay kit (MBL). 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.

Immunoblot analysis. Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of cell lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque) for 20 minutes at room temperature before incubation overnight at 4°C with primary antibodies including those to phosphorylated AKT (1:1,000 dilution; Cell Signaling Technology), to AKT (1:1,000 dilution, Cell Signaling Technology), to phosphorylated extracellular signal-regulated kinase (ERK; 1:1,000 dilution; Santa Cruz Biotechnology), to ERK (1:1,000 dilution, Santa Cruz Biotechnology), to E2F1 (1:1,000 dilution, Santa Cruz Biotechnology), to thymidylate synthase (TS; 1:1,000 dilution, Santa Cruz Biotechnology), or to β-actin (1:500 dilution, Sigma). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 hour at room temperature with horseradish peroxidase–conjugated antibodies to rabbit IgG (Sigma). Immune complexes were finally detected with ECL Western Blotting Detection Reagents (GE Healthcare).

TS activity assay. TS activity was quantified with the use of a tritiated fluoro-dUMP binding assay (20). Cells were harvested and disrupted by ultrasonic treatment in a solution containing 0.2 mol/L Tris-HCl (pH 7.4),

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20 mmol/L 2-mercaptoethanol, 15 mmol/L CMP, and 100 mmol/L NaF. The cell lysate was centrifuged at 1,630 × *g* for 15 minutes at 4°C, and the resulting supernatant was centrifuged at 105,000 × *g* for 1 hour at 4°C. A portion (50 μL) of the final supernatant was mixed with 50 μL of a solution containing 600 mmol/L NH_4HCO_3 buffer (pH 8.0), 100 mmol/L 2-mercaptoethanol, 100 mmol/L NaF, and 15 mmol/L CMP. After the addition of 50 μL of [6-^{3}H]fluoro-dUMP (7.8 pmol, 0.12 μCi) plus 25 μL of cofactor solution containing 50 mmol/L potassium phosphate buffer (pH 7.4), 20 mmol/L 2-mercaptoethanol, 100 mmol/L NaF, 15 mmol/L CMP, 2% bovine serum albumin, 2 mmol/L tetrahydrofolic acid, 16 mmol/L sodium ascorbate, and 9 mmol/L formaldehyde, the mixture was incubated for 20 minutes at 30°C. The reaction was terminated by the addition of 100 μL of 2% bovine serum albumin and 275 μL of 1 mol/L $HClO_4$ followed by centrifugation at 1,630 × *g* for 15 minutes at 4°C. The resulting pellet was resuspended in 2 mL of 0.5 mol/L $HClO_4$, and the suspension was subjected to ultrasonic treatment followed by centrifugation at 1,630× *g* for 15 minutes at 4°C. The final precipitate was solubilized in 0.5 mL of 98% formic acid, mixed with 10 mL of ACS II scintillation fluid (GE Healthcare), and assayed for radioactivity.

Gene silencing. Cells were plated at 50% to 60% confluence in six-well plates or 25-cm^2 flasks and then incubated for 24 hours before transient transfection for 48 hours with small interfering RNAs (siRNA) mixed with the Lipofectamine reagent (Invitrogen). An siRNA specific for TS mRNA (5′-CAAUCCGCAUCCAACUAUUTT-3′) and a nonspecific siRNA (control) were obtained from Nippon EGT.

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 animal experiments were done in compliance with the regulations of the Animal Experimentation Committee of Taiho Pharmaceutical Co. Ltd.

***Growth inhibition assay* in vivo.** Cubic fragments of tumor tissue (~2 × 2 × 2 mm) were implanted s.c. into the axilla of 5-week-old to 6-week-old male athymic nude mice. Treatment was initiated when tumors in each group achieved an average volume of 50 to 200 mm^3. Treatment groups consisted of control, S-1 alone, lapatinib alone, trastuzumab alone, and the combination of S-1 and either lapatinib or trastuzumab. Each treatment group contained seven mice. S-1 and lapatinib were given by oral gavage daily for 28 days; control animals received a 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle. Trastuzumab was given i.p. weekly. 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 twice per week.

Statistical analysis. Unless indicated otherwise, data are presented as means ± SEM from three independent experiments or for seven animals per group. The unpaired two-tailed Student's t test was used to evaluate the significance of differences in the percentage of Annexin V–positive cells, relative caspase-3 activity, or tumor volume. A P value of <0.05 was considered statistically significant.

Results

Synergistic antiproliferative effect of 5FU and either lapatinib or trastuzumab in gastric cancer cells positive for* HER2 *amplification. We first examined the effect of the combination of 5FU and either lapatinib or trastuzumab on the growth *in vitro* of gastric cancer cells positive or negative for *HER2* amplification. We used 5FU instead of S-1 for *in vitro* experiments, given that tegafur, a component of S-1, is metabolized to 5FU in the liver. The combined effect of each pair of drugs was evaluated on the basis of the CI. The combination of 5FU and lapatinib exhibited a synergistic inhibitory effect (CI < 1.0) on the growth of cells with *HER2* amplification, including NCI-N87, SNU-216, and MKN-7 cells, but not on that of cells without *HER2* amplification, including AZ-521, MKN-28, and MKN-1 cells (Fig. 1A and B). A synergistic interaction between 5FU and trastuzumab was also apparent in cells with *HER2* amplification but not in those without it (Fig. 1C). The combination of 5FU with either lapatinib or trastuzumab thus exerted a synergistic antiproliferative effect in gastric cancer cells positive for *HER2* amplification but not in those negative for *HER2* amplification.

Enhanced induction of apoptosis by the combination of 5FU and either lapatinib or trastuzumab in gastric cancer cells positive for* HER2 *amplification. To investigate the mechanism of the synergistic growth inhibition induced by the combination of 5FU and either lapatinib or trastuzumab, we examined the effects of each agent alone and in combination on apoptosis in gastric cancer cells. An assay based on the binding of Annexin V to the cell surface revealed that the frequency of apoptosis was markedly greater for *HER2* amplification–positive cells treated with the combination of 5FU and either lapatinib or trastuzumab than for those treated with either agent alone (Fig. 2A and B). Such an effect was not apparent in cells negative for *HER2* amplification. To confirm the results of the Annexin V binding assay, we measured the activity of caspase-3. Again, the combination of 5FU and either lapatinib or trastuzumab induced an increase in caspase-3 activity greater than that apparent with either agent alone in cells with *HER2* amplification but not in those without it (Fig. 2C). Together, these data thus indicated that the combination of 5FU and either lapatinib or trastuzumab exhibits an enhanced proapoptotic effect in gastric cancer cells positive for *HER2* amplification but not in those negative for this genetic change.

Downregulation by lapatinib or trastuzumab of the expression and activity of TS in gastric cancer cells positive for* HER2 *amplification. To investigate further the molecular mechanism of the synergistic antiproliferative effect of the combination of 5FU and HER2-targeting

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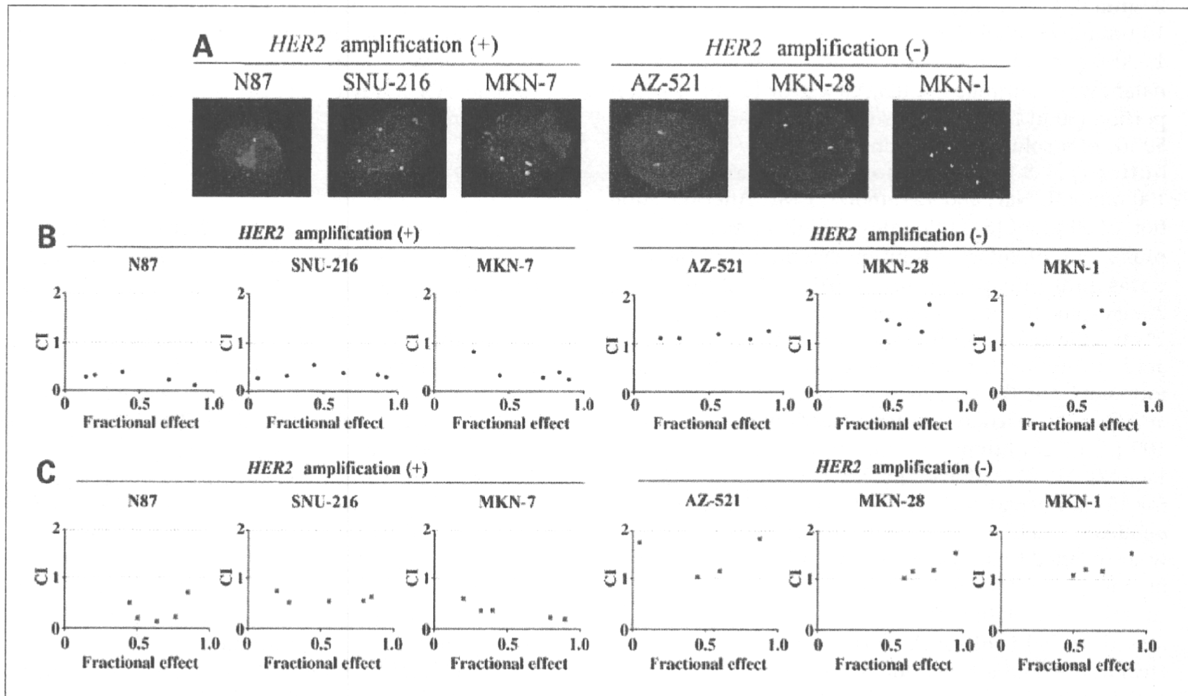

Figure 1. Effect of the combination of 5FU and HER2-targeting agents on the growth *in vitro* of gastric cancer cells positive or negative for *HER2* amplification. A, fluorescence *in situ* hybridization analysis of gastric cancer cell lines. The indicated cell lines were subjected to hybridization with a *HER2/neu* probe (orange) and a chromosome 17 centromere probe (green). B and C, gastric cancer cells with or without *HER2* amplification were incubated for 72 hours with lapatinib (B) or trastuzumab (C) together with 5FU at a fixed lapatinib/5FU molar ratio of 1:10 or a fixed trastuzumab/5FU molar ratio of 15:1, after which cell viability was measured. The interaction between the two drugs in each combination was evaluated on the basis of the CI. CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively. Data are means of triplicates from a representative experiment.

agents, we next examined the effects of lapatinib and trastuzumab on TS expression and activity in gastric cancer cells, given that a reduced level of TS expression has been associated with a higher response rate to 5FU-based chemotherapy (21, 22). Exposure of *HER2* amplification–positive cells to either lapatinib or trastuzumab resulted in downregulation of TS expression in a concentration-dependent manner, whereas TS expression was not affected by these agents in cells without *HER2* amplification (Fig. 3A and B). Consistent with these results, lapatinib or trastuzumab reduced TS activity in cells with *HER2* amplification but not in those without it (Fig. 3C). Furthermore, lapatinib or trastuzumab downregulated the expression of E2F1, a transcription factor that promotes expression of the TS gene (23), in cells positive for *HER2* amplification but not in those negative for this genetic change (Fig. 3A and B).

To explore the mechanism of TS downregulation by HER2-targeting agents, we examined the effects of these agents on the phosphoinositide 3-kinase (PI3K)–AKT signaling pathway as well as on signaling by the mitogen-activated protein kinase ERK. Immunoblot analysis showed that phosphorylation of AKT in *HER2* amplification–positive cells was inhibited by lapatinib or trastuzumab, whereas phosphorylation of ERK in these cells was inhibited only by lapatinib (Fig. 3A and B). Phosphorylation of AKT or ERK was not affected by either HER2-targeting agent in cells without *HER2* amplification. These data thus suggested that lapatinib and trastuzumab each induce downregulation of TS expression and activity in *HER2* amplification–positive gastric cancer cells and that this effect is attributable to downregulation of E2F1, possibly mediated by inhibition of the PI3K-AKT signaling pathway.

Enhancement of 5FU-induced apoptosis by depletion of TS in gastric cancer cells positive for* HER2 *amplification. To investigate whether the downregulation of TS by lapatinib or trastuzumab indeed contributes to the synergistic antiproliferative effect of these drugs with 5FU in gastric cancer cells positive for *HER2* amplification, we depleted such cells of TS by transfection with an siRNA specific for TS mRNA (Fig. 4A). Similar to the action of lapatinib or trastuzumab, RNA interference–mediated depletion of TS enhanced the effects of 5FU treatment on the number of apoptotic cells and the activity of caspase-3 compared with those apparent in cells transfected with a control siRNA (Fig. 4B–D). These data thus indicated that downregulation of TS by lapatinib or trastuzumab contributes, at least in part, to the observed synergistic antiproliferative and proapoptotic interaction of these drugs with 5FU.

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Enhanced inhibition of the growth of* HER2 *amplification–positive gastric cancer cells* in vivo *by combined treatment with S-1 and either lapatinib or trastuzumab. Finally, we investigated the effect of combined treatment with S-1 and either lapatinib or trastuzumab on the growth *in vivo* of gastric cancer cells positive for *HER2* amplification. Mice with palpable tumors formed by NCI-N87 or 4-1ST cells were divided into groups for treatment with vehicle, S-1, lapatinib, trastuzumab, or the combination of S-1 and either lapatinib or trastuzumab

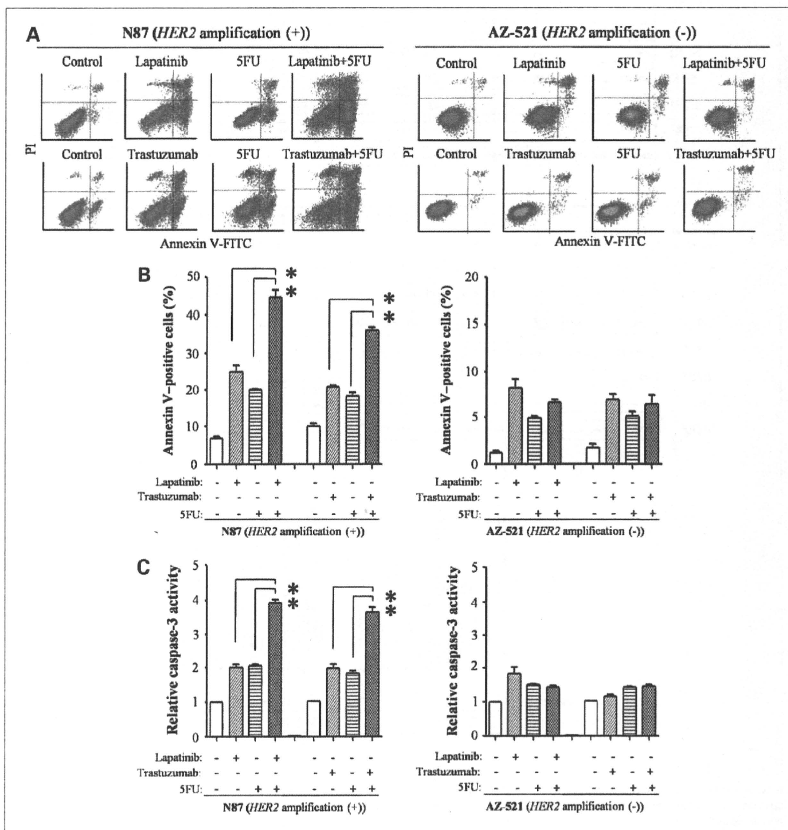

Figure 2. Effect of the combination of 5FU and HER2-targeting agents on apoptosis in gastric cancer cells positive or negative for *HER2* amplification. A, cells were incubated for 72 hours with lapatinib, trastuzumab, or 5FU at their IC_{50} concentrations unless indicated otherwise: 0.02 μmol/L, 1.5 μg/mL, and 2.5 μmol/L, respectively, for NCI-N87 cells and 2.0 μmol/L, 200 μg/mL (IC_{50} not determined), and 4.5 μmol/L, respectively, for AZ-521 cells. The proportion of apoptotic cells was then assessed by staining with FITC-conjugated Annexin V and propidium iodide (PI) followed by flow cytometry. B, the proportion of apoptotic cells in experiments similar to that shown in A was determined. Data are means ± SEM from three independent experiments. C, lysates prepared from cells exposed to drugs as in A for 48 hours were assayed for caspase-3 activity. Data are expressed relative to the corresponding value for the control condition and are means ± SEM from three independent experiments. *, $P < 0.05$, for the indicated comparisons.

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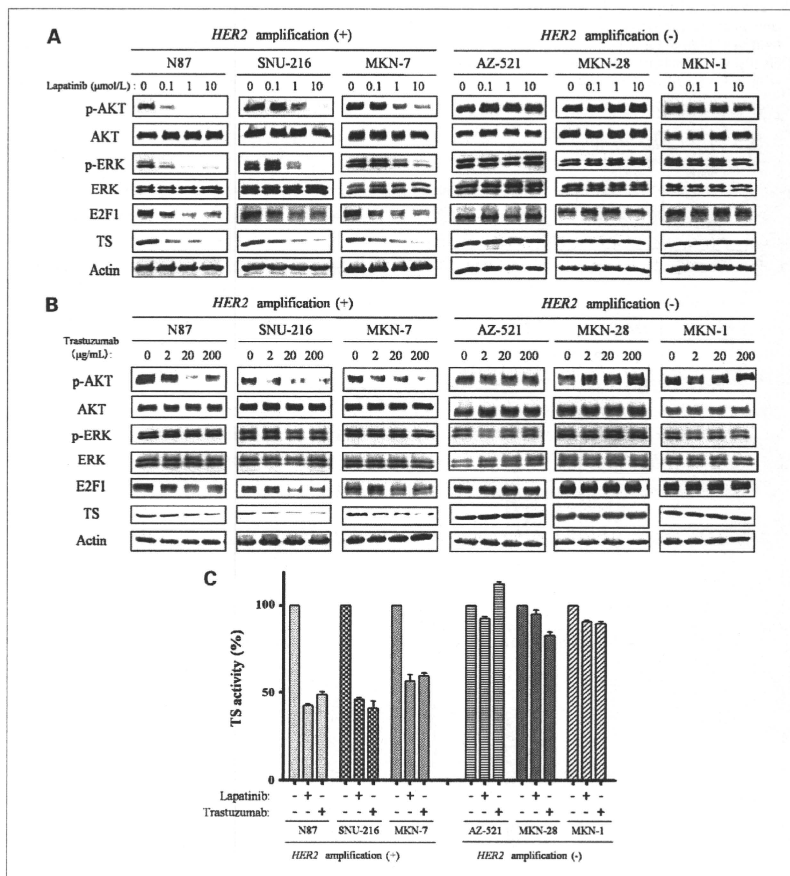

Figure 3. Effect of HER2-targeting agents on E2F1 and TS expression or activity in gastric cancer cells positive or negative for *HER2* amplification. A and B, cells were incubated with the indicated concentrations of lapatinib for 24 hours (A) or trastuzumab for 48 hours (B), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of AKT or ERK as well as with those to E2F1, TS, and β-actin (loading control). C, cells were treated with lapatinib (1 μmol/L) for 24 hours or with trastuzumab (200 μg/mL) for 48 hours, after which cell lysates were prepared and assayed for TS activity. Data are expressed as a percentage of the corresponding value for control cells and are means ± SEM from three independent experiments.

for 4 weeks. Combination therapy with S-1 and lapatinib (Fig. 5A) or with S-1 and trastuzumab (Fig. 5B) inhibited the growth of tumors formed by NCI-N87 or 4-1ST cells to a significantly greater extent than did treatment with either drug alone. All treatments were well tolerated by the mice, with no signs of toxicity or weight loss during therapy (data not shown). These findings thus suggested that combination therapy with S-1 and either lapatinib or

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trastuzumab exhibits an enhanced antitumor effect in gastric cancer xenografts positive for *HER2* amplification, consistent with the results obtained *in vitro*.

Discussion

HER2 amplification is a frequent molecular abnormality in gastric cancer as well as in various other cancers. Trastuzumab is widely used as a standard therapy for HER2-positive patients with breast cancer, with the drug showing clinical efficacy both alone and in combination with chemotherapeutic agents (24, 25). HER2 is thus considered to be a potential target for the treatment of gastric cancer positive for *HER2* amplification. A recently reported phase III clinical trial showed a significant gain in overall survival for HER2-positive patients with advanced gastric cancer who received combined treatment with trastuzumab and fluoropyrimidine-cisplatin compared with those treated without trastuzumab (26). However, there has been limited examination of HER2-targeting agents in gastric cancer models, and most such studies have been restricted to cells with *HER2* amplification. Furthermore, the mechanisms of action of HER2-targeting agents in combination with cytotoxic agents have remained unclear.

In the present study, we have shown that the combination of S-1 (or 5FU) and HER2-targeting agents exerts a synergistic antitumor effect in gastric cancer cells with *HER2* amplification but not in those without it. We found that HER2-targeting agents inhibit TS activity as well as TS expression in *HER2* amplification–positive gastric cancer cells, but not in cells without *HER2* amplification. Lapatinib is a dual inhibitor of EGFR and HER2, and so its downregulation of TS might be attributable to inhibition of either of these tyrosine kinases. However, given that trastuzumab downregulated TS expression and activity to an extent similar to that observed with lapatinib, the effects of both lapatanib and trastuzumab on TS are likely mediated by inhibition of HER2. This conclusion is further supported by the observation that transfection of *HER2* amplification–positive gastric cancer cells with an siRNA specific for HER2 mRNA resulted in marked inhibition of TS expression, whereas transfection with an EGFR siRNA had no such effect (data not shown). Downregulation of TS by HER2-targeting agents was accompanied by a reduction in the abundance of E2F1, suggesting that this effect on TS results from attenuation of E2F1-dependent transcription of the TS gene. Although the mechanism responsible for regulation of TS and E2F1 remains unclear, our observations indicate that inhibition of the PI3K-AKT pathway contributes, at least in part, to the downregulation of TS by HER2-targeting agents. Activation of PI3K-AKT signaling has been found to result in E2F1 accumulation (27, 28), supporting the notion that inhibition of such signaling by HER2-targeting agents leads to downregulation of E2F1 and TS. We previously showed that inhibition of EGFR by EGFR–tyrosine kinase inhibitors results in downregulation of TS and E2F1

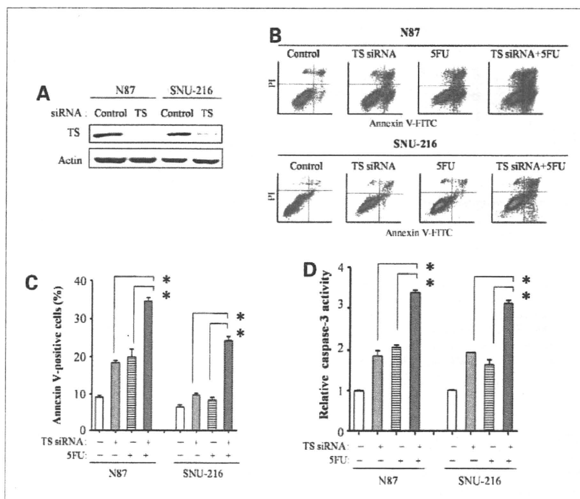

Figure 4. Effect of RNA interference–mediated depletion of TS on the proapoptotic action of 5FU in gastric cancer cells positive for *HER2* amplification. A, cells were transfected with nonspecific (control) or TS siRNAs for 48 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS and to β-actin. B, cells were transfected with nonspecific or TS siRNAs as in A, replated and incubated for 72 hours in complete medium in the absence or presence of 5FU at IC_{50} concentrations (2.5 and 1.5 μmol/L for NCI-N87 and SNU-216 cells, respectively), and then evaluated for apoptosis by staining with Annexin V. C, the proportion of apoptotic cells in experiments similar to that in B was determined. Data are means ± SEM from three independent experiments. D, cells treated as in B were lysed and assayed for caspase-3 activity after exposure to 5FU for 48 hours. Data are means ± SEM from three independent experiments. *, $P < 0.05$ for the indicated comparisons.

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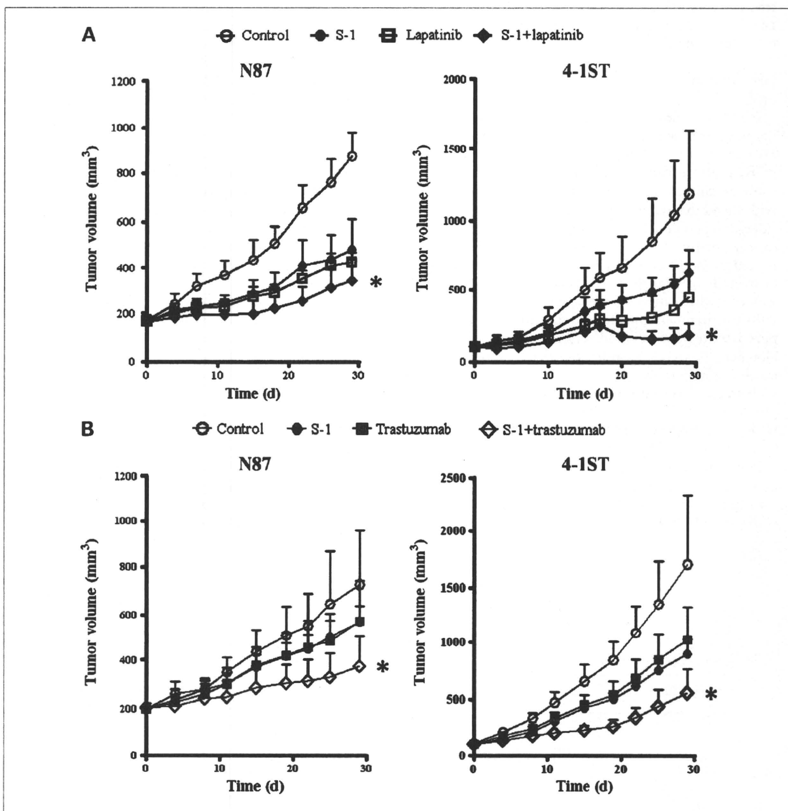

Figure 5. Effect of the combination of S-1 and HER2-targeting agents on the growth *in vivo* of gastric cancer cells with *HER2* amplification. Nude mice with tumor xenografts established by s.c. implantation of NCI-N87 cells were treated for 4 weeks by daily oral gavage with vehicle (control), S-1 (10 mg/kg), or lapatinib (50 × 2 mg/kg, twice a day; A) or by weekly i.p. administration of trastuzumab (20 mg/kg on days 1, 8, 15, and 22; B), as indicated. Nude mice with 4-1ST xenografts were similarly treated with vehicle (control), S-1 (8.3 mg/kg), lapatinib (30 × 2 mg/kg, twice a day; A), or trastuzumab (10 mg/kg on days 1, 8, 15, and 22; B). Tumor volume was determined at the indicated times after the onset of treatment. Data are means ± SEM of values from seven mice per group. *, $P < 0.05$, for the combination of S-1 plus lapatinib or trastuzumab at 28 days versus the corresponding value for S-1, lapatinib, or trastuzumab alone.

expression in non–small cell lung cancer cells (29, 30). Given that downregulation of TS was induced by HER2-targeting agents in gastric cancer cells with *HER2* amplification and by EGFR–tyrosine kinase inhibitors in non–small cell lung cancer cells, the expression of TS is likely dependent on receptor tyrosine kinase signaling, which is essential for cell survival.

Downregulation of TS expression has been found to enhance the efficacy of 5FU, possibly as a direct result of the decrease in the amount of this protein target of

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