Research Article

Molecular Cancer Therapeutics

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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doi: 10.1158/1535-7163.MCT-10-0045

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),

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

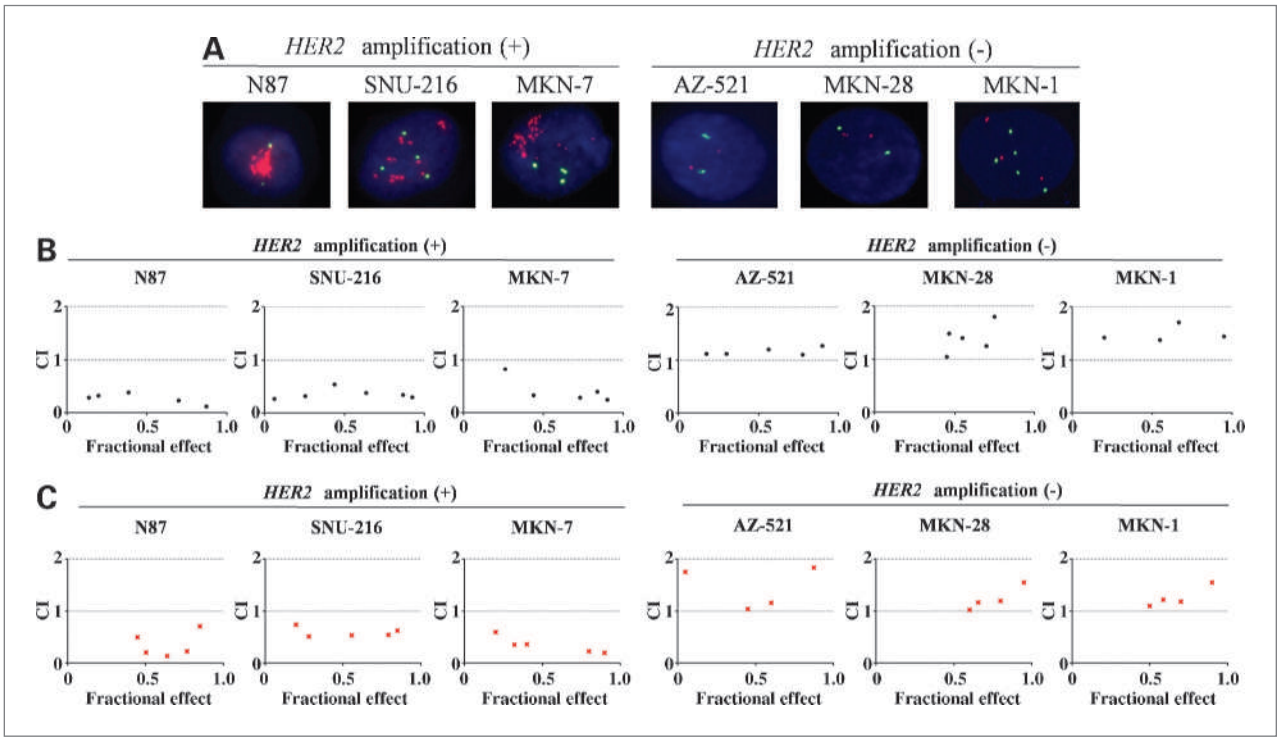

Figure 1. Effect of the combination of 5FU and HER2-targeting agents on the growth *in vitro* of gastric cancer cells positive or negative for *HER2* amplification. A, fluorescence *in situ* hybridization analysis of gastric cancer cell lines. The indicated cell lines were subjected to hybridization 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.

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

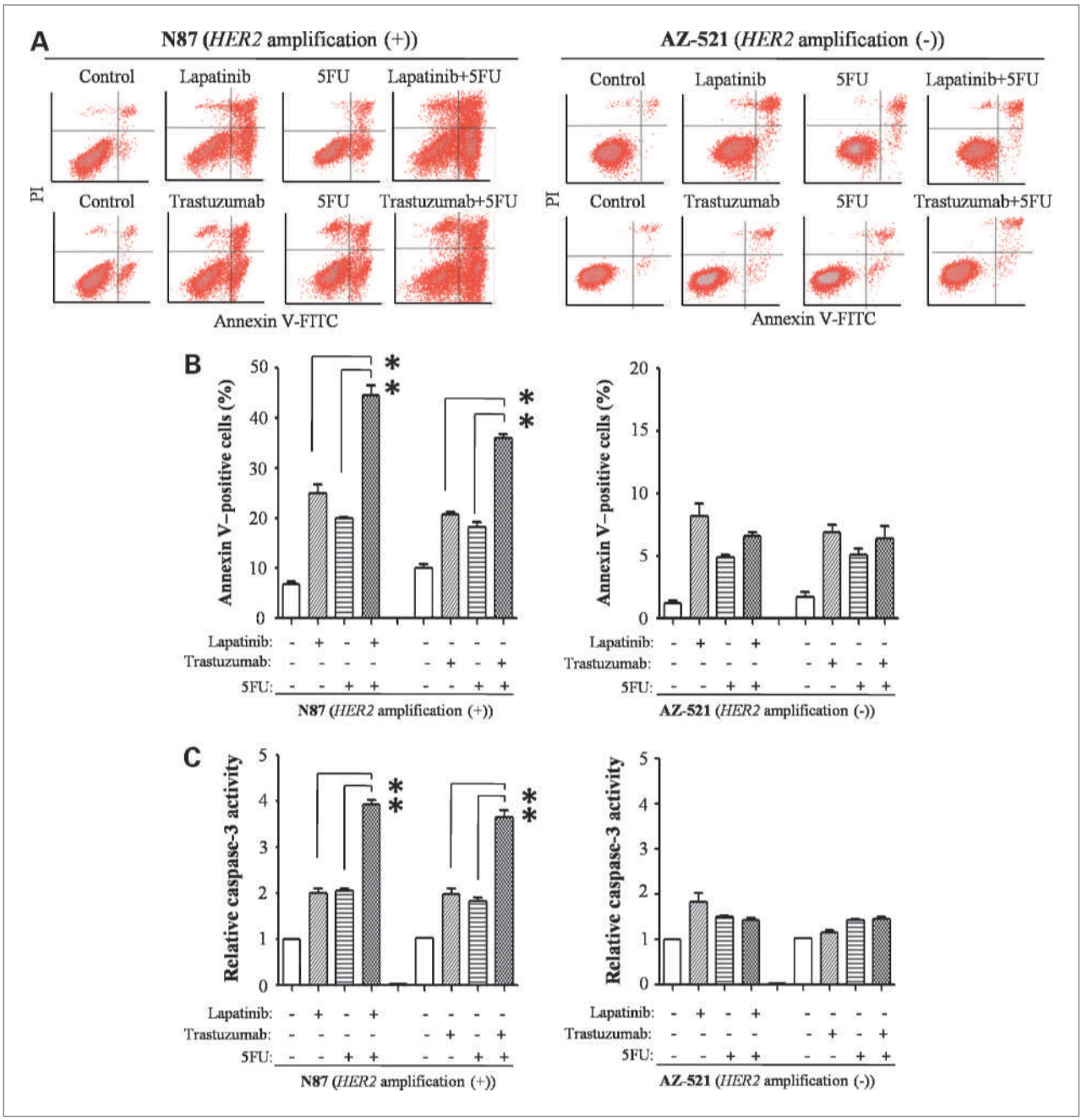

Figure 2. Effect of the combination of 5FU and HER2-targeting agents on apoptosis in gastric cancer cells positive or negative for *HER2* amplification. A, cells were incubated for 72 hours with lapatinib, trastuzumab, or 5FU at their IC_{50} concentrations unless indicated otherwise: 0.02 μmol/L, 1.5 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.

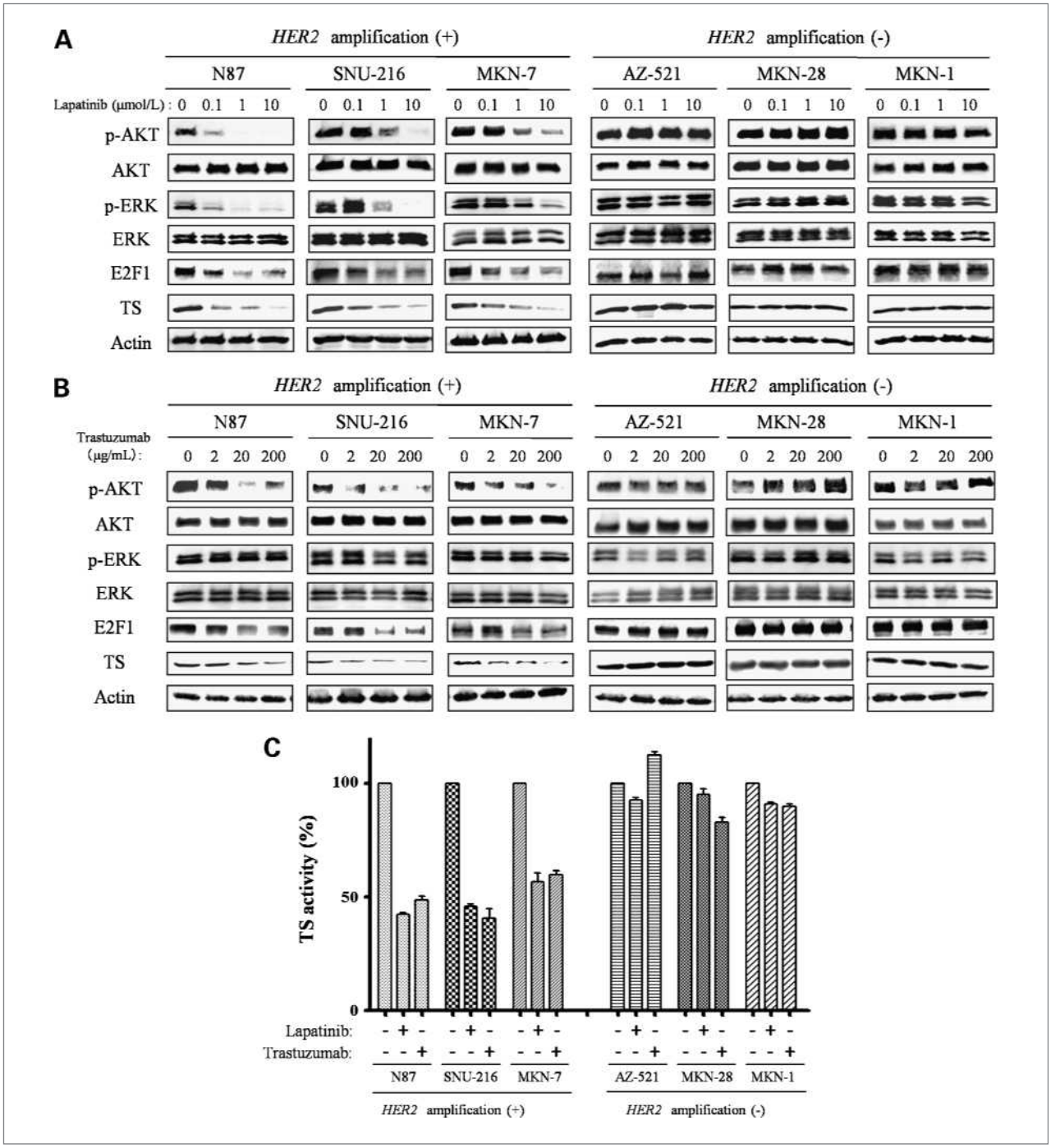

Figure 3. Effect of HER2-targeting agents on E2F1 and TS expression or activity in gastric cancer cells positive or negative for *HER2* amplification. A and B, cells were incubated with the indicated concentrations of lapatinib for 24 hours (A) or trastuzumab for 48 hours (B), after which cell lysates 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

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

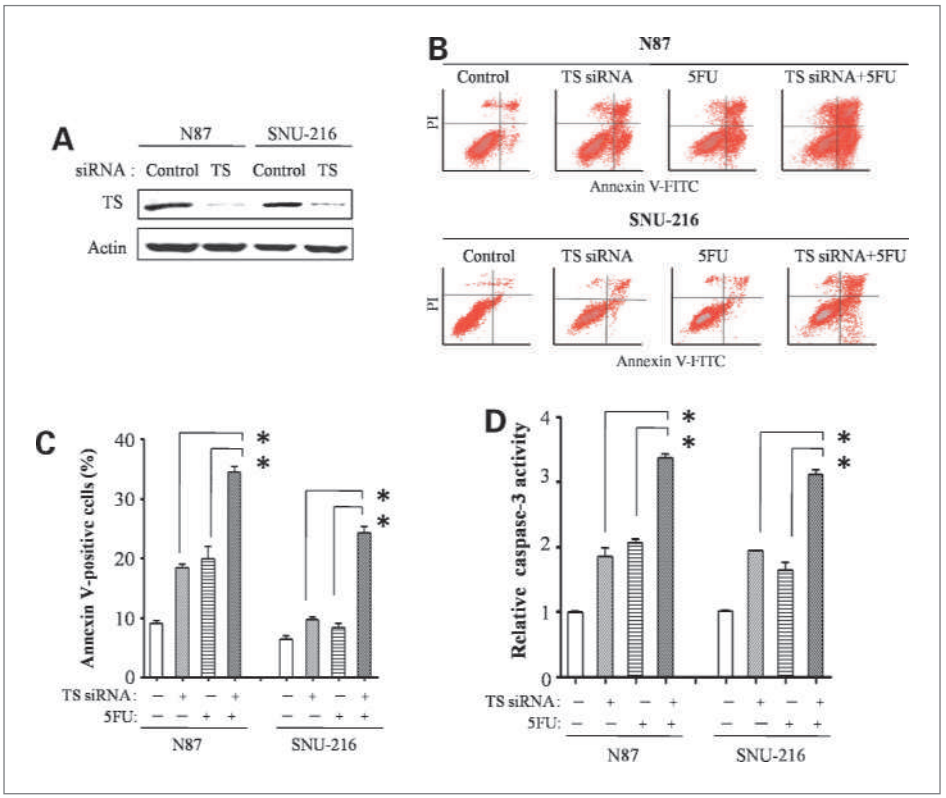

Figure 4. Effect of RNA interference–mediated depletion of TS on the proapoptotic action of 5FU in gastric cancer cells positive for *HER2* amplification. A, cells were transfected with nonspecific (control) or TS siRNAs for 48 hours, after which cell lysates were prepared and subjected to immunoblot 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.

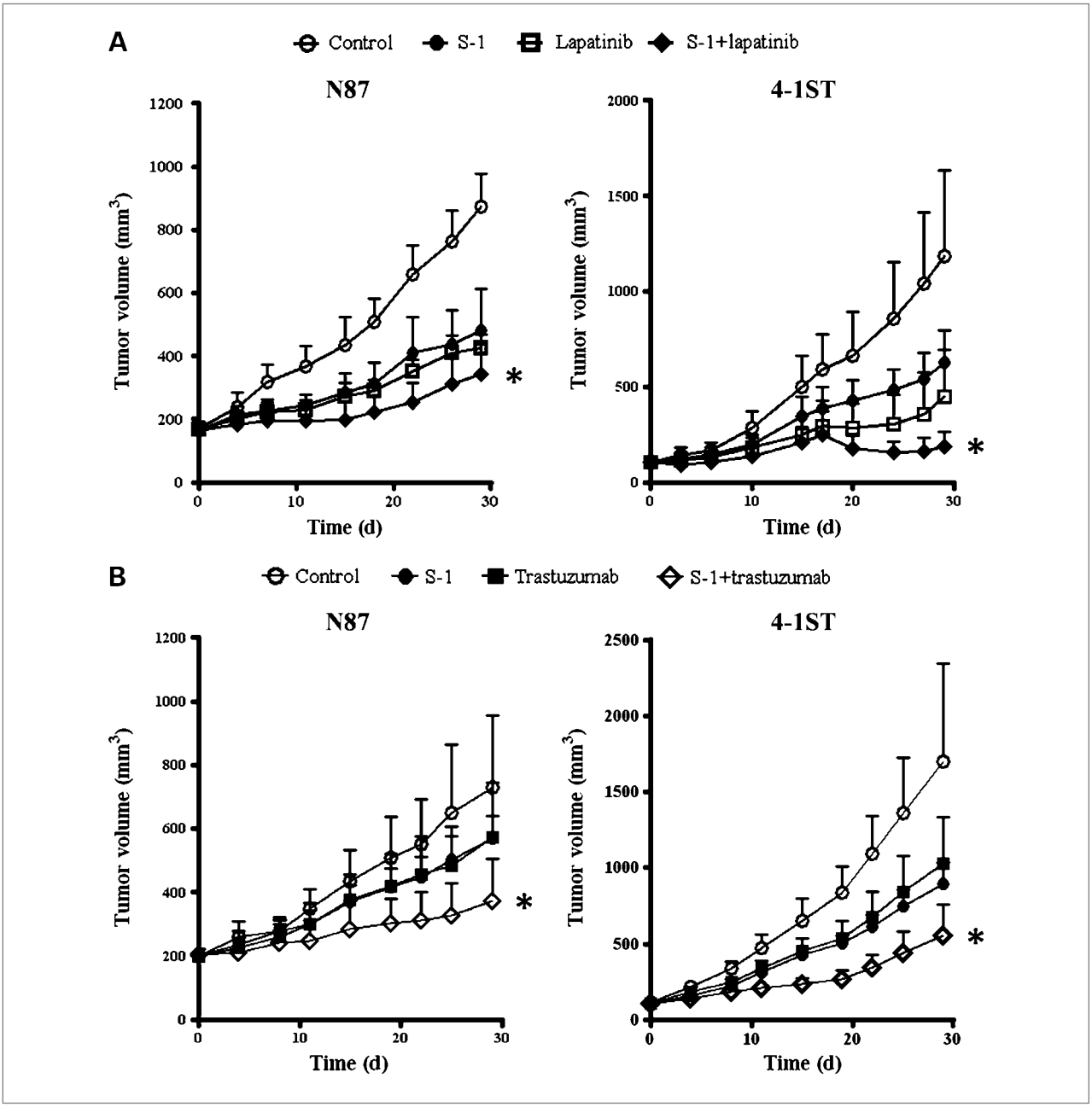

Figure 5. Effect of the combination of S-1 and HER2-targeting agents on the growth *in vivo* of gastric cancer cells with *HER2* amplification. Nude mice with tumor xenografts established by s.c. implantation of NCI-N87 cells were treated for 4 weeks by daily oral gavage with vehicle (control), S-1 (10 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

5FU (31). In the present study, we found that depletion of TS by RNA interference enhanced the induction of apoptosis by 5FU in gastric cancer cells with *HER2* amplification, suggesting that the proapoptotic effect of the combination of 5FU and HER2-targeting agents is attributable to TS inhibition. The abundance of TS in neoplastic cells has been found to increase after exposure to 5FU, resulting in maintenance of the amount of the free enzyme in excess of that of enzyme bound to 5FU (32–34). Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5FU resistance in cancer cells (22, 35–39). Downregulation of TS by HER2-targeting agents might thus contribute to reversal of the 5FU-induced increase in TS expression, resulting in enhancement of 5FU-induced apoptosis. In addition, prolonged inhibition of TS has been shown to trigger apoptosis by inducing an imbalance in the deoxyribonucleoside pool and consequent disruption of DNA synthesis and repair (40–42). Given that the TS siRNA itself induced apoptosis in gastric cancer cells positive for *HER2* amplification in the present study, the depletion of TS by HER2-targeting agents might also contribute directly to the combined proapoptotic action with 5FU.

The *HER2* amplification–positive gastric cancer cell line MKN-7 has been found to be insensitive to trastuzumab. In contrast to their insensitivity to trastuzumab, we found that MKN-7 cells retain sensitivity to lapatinib (IC_{50} values of >200 μg/mL and 0.99 ± 0.055 μmol/L for trastuzumab and lapatinib, respectively; data not shown). Most HER2-positive breast cancer patients who initially respond to trastuzumab ultimately develop resistance to this drug (25). Preclinical studies have indicated several molecular mechanisms that might contribute to the development of trastuzumab resistance, including signaling by a HER2-HER3-PI3K-PTEN pathway (43, 44). One possible explanation for trastuzumab resistance in MKN-7 cells is activation of the EGFR signaling pathway (45, 46). MKN-7 cells might prove to be a good model for the study of trastuzumab-resistant cells positive for *HER2* amplification. We found that lapatinib and trastuzumab each inhibit TS expression and activity in MKN-7 cells, likely accounting for the synergistic antiproliferative effect observed with 5FU. These data suggest that the synergistic antitumor effect of the combination of 5FU and HER2-targeting agents is conserved in trastuzumab-resistant cells with *HER2* amplification.

In conclusion, we have shown that the combination of S-1 and HER2-targeting agents exerts a synergistic antitumor effect mediated by TS inhibition in gastric cancer cells with *HER2* amplification, but not in those negative for *HER2* amplification. Our observations provide a rationale for clinical evaluation of combination chemotherapy with S-1 and HER2-targeting agents according to *HER2* amplification status.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank M. Iki for helpful discussion and E. Hatashita, K. Kuwata, and H. Yamaguchi for technical assistance.

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Received 01/15/2010; revised 03/15/2010; accepted 03/17/2010; published OnlineFirst 04/27/2010.

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