4-anilinoquinazoline base structure, but differ in the substituents attached to the quinazoline and anilino rings. Minor differences in the chemical structures of these compounds may thus influence hepatotoxicity. In conclusion, erlotinib is an effective and well-tolerated treatment option for patients for whom gefitinib has been discontinued because of severe hepatotoxicity. Clinical trials to evaluate the administration of erlotinib after severe hepatotoxicity induced by daily administration of gefitinib are warranted.

Masayuki Takeda and Isamu Okamoto
Department of Medical Oncology, Kinki University School of Medicine, Osaka, Japan

Masahiro Fukuoka
Department of Medical Oncology, Kinki University School of Medicine, Sakai Hospital, Osaka, Japan

Kazuhiko Nakagawa
Department of Medical Oncology, Kinki University School of Medicine, Osaka, Japan

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST
The author(s) indicated no potential conflicts of interest.

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Research Article

Molecular Cancer Therapeutics

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

Junko Tanizaki[1], Isamu Okamoto[1], Ken Takezawa[1], Sayaka Tsukioka[2], Junji Uchida[2], Mamoru Kiniwa[2], Masahiro Fukuoka[3], and Kazuhiko Nakagawa[1]

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

Authors' Affiliations: [1]Department of Medical Oncology, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan; [2]Tokushima Research Center, Taiho Pharmaceutical Co. Ltd., Kawauchi, Tokushima, Japan; and [3]Department of Medical Oncology, Kinki University School of Medicine, Sakai Hospital, Sakai, Osaka, Japan

Corresponding Author: Isamu Okamoto, Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone: 81-72-366-0221; Fax: 81-72-360-5000. E-mail: chi-okamoto@dotd.med.kindai.ac.jp

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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),

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

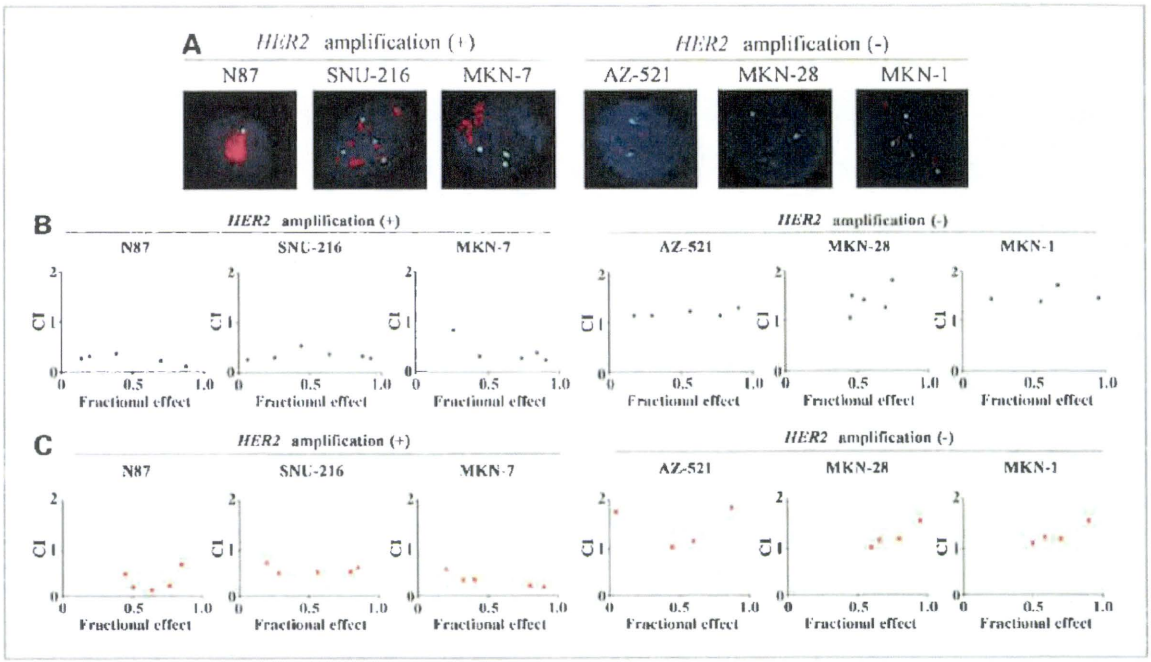

Figure 1. Effect of the combination of 5FU and HER2-targeting agents on the growth *in vitro* of gastric cancer cells positive or negative for *HER2* amplification. A, fluorescence *in situ* hybridization analysis of gastric cancer cell lines. The indicated cell lines were subjected to hybridization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

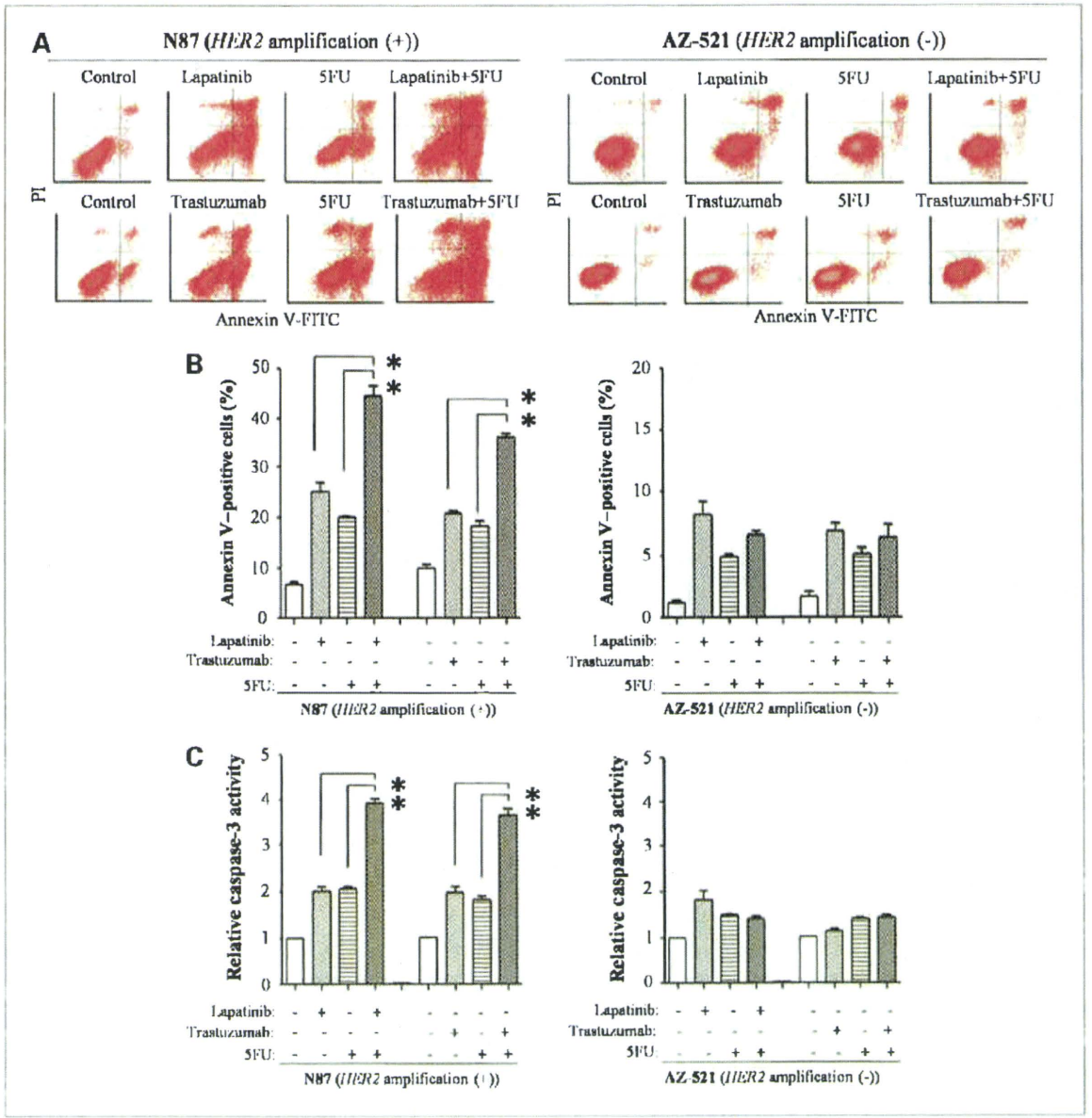

Figure 2. Effect of the combination of 5FU and HER2-targeting agents on apoptosis in gastric cancer cells positive or negative for *HER2* amplification. A, cells were incubated for 72 hours with lapatinib, trastuzumab, or 5FU at their IC_{50} concentrations unless indicated otherwise: 0.02 μmol/L, 1.5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

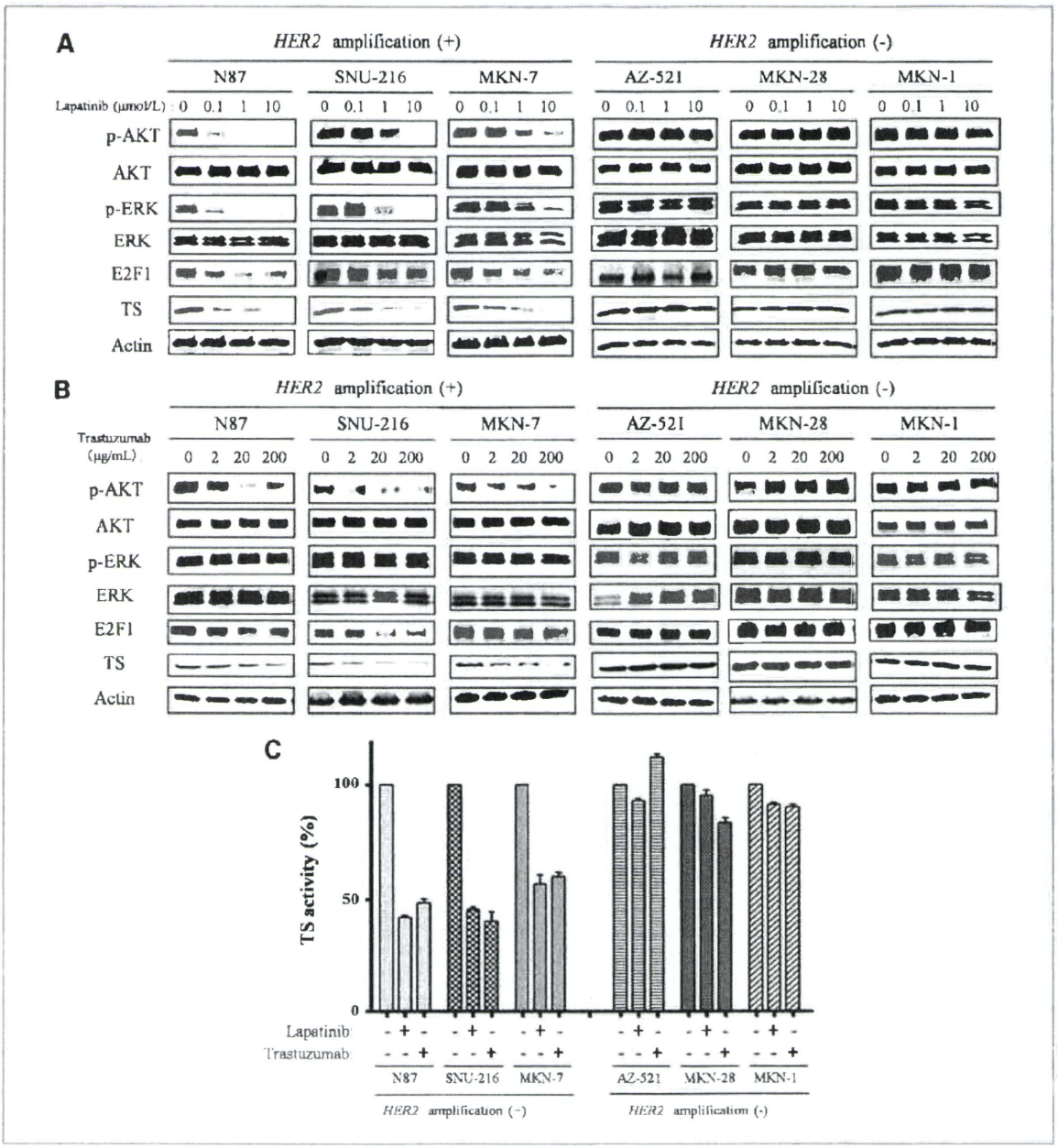

Figure 3. Effect of HER2-targeting agents on E2F1 and TS expression or activity in gastric cancer cells positive or negative for *HER2* amplification. A and B, cells were incubated with the indicated concentrations of lapatinib for 24 hours (A) or trastuzumab for 48 hours (B), after which cell lysates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

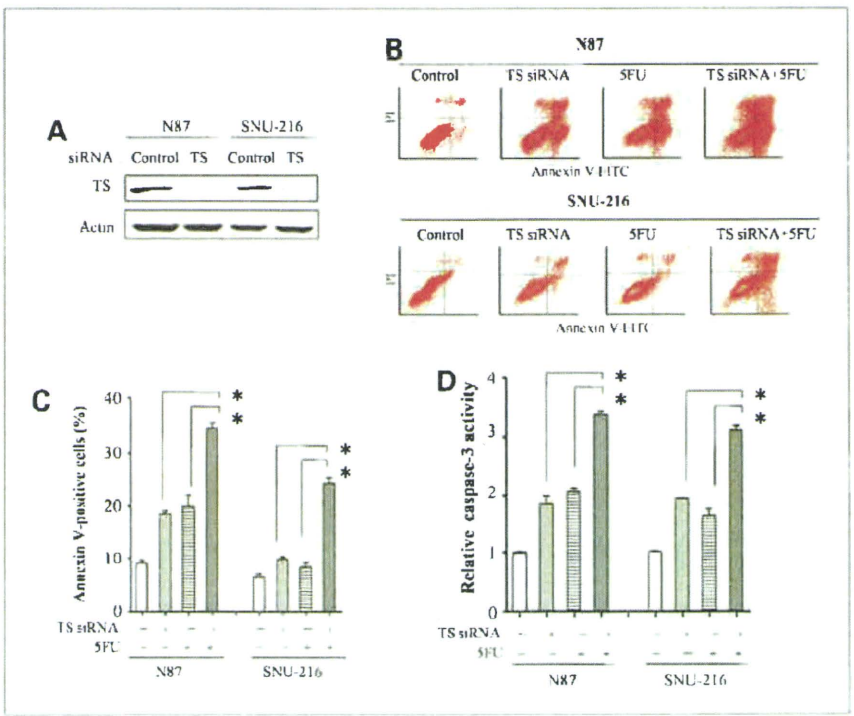

Figure 4. Effect of RNA interference–mediated depletion of TS on the proapoptotic action of 5FU in gastric cancer cells positive for *HER2* amplification. A, cells were transfected with nonspecific (control) or TS siRNAs for 48 hours, after which cell lysates were prepared and subjected to immunoblot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

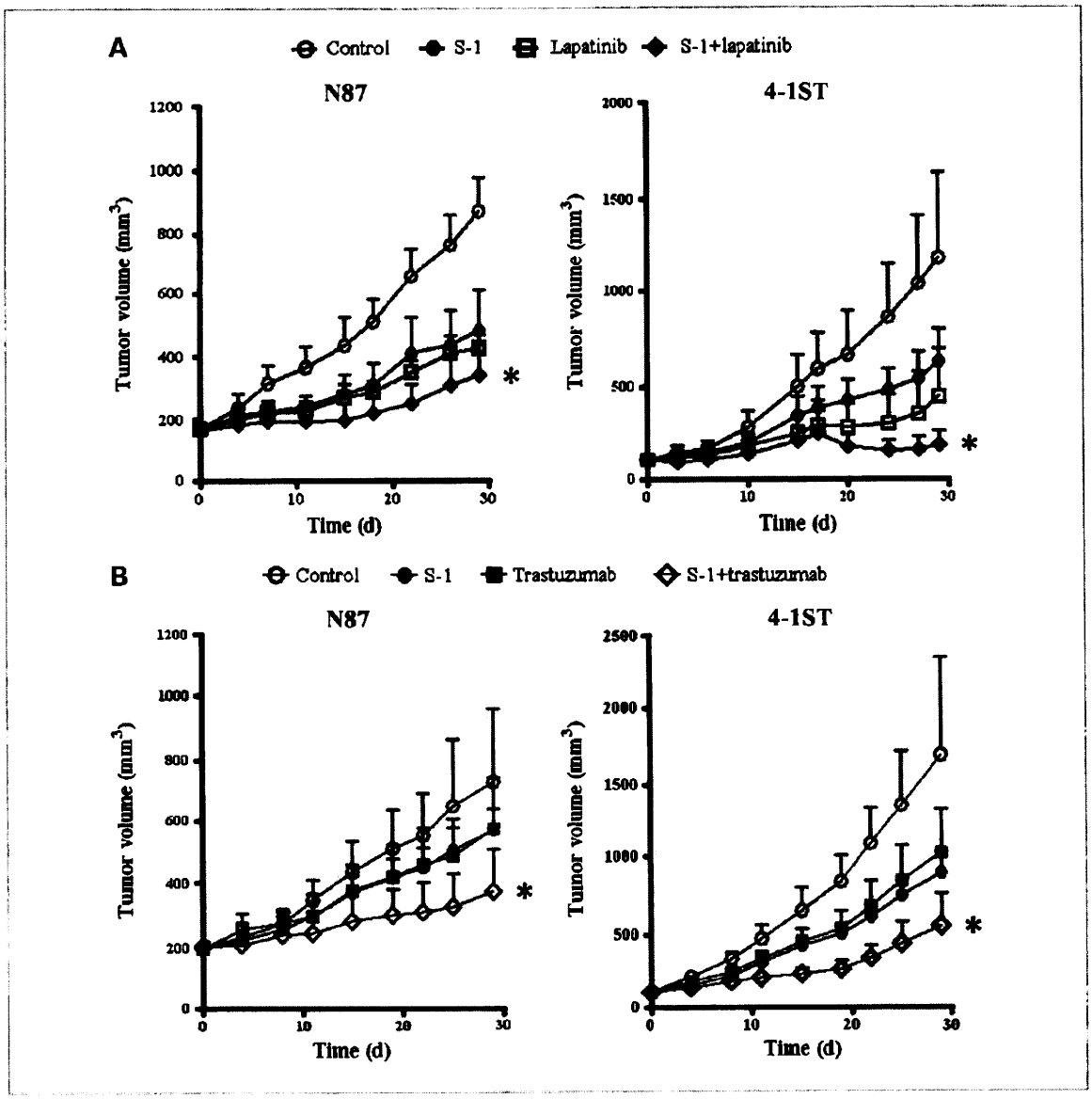

Figure 5. Effect of the combination of S-1 and HER2-targeting agents on the growth *in vivo* of gastric cancer cells with *HER2* amplification. Nude mice with tumor xenografts established by s.c. implantation of NCI-N87 cells were treated for 4 weeks by daily oral gavage with vehicle (control), S-1 (10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

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ORIGINAL REPORT

Phase III Study Comparing Second- and Third-Generation Regimens With Concurrent Thoracic Radiotherapy in Patients With Unresectable Stage III Non–Small-Cell Lung Cancer: West Japan Thoracic Oncology Group WJTOG0105

Nobuyuki Yamamoto, Kazuhiko Nakagawa, Yasumasa Nishimura, Kayoko Tsujino, Miyako Satouchi, Shinzoh Kudo, Toyoaki Hida, Masaaki Kawahara, Koji Takeda, Nobuyuki Katakami, Toshiyuki Sawa, Soichiro Yokota, Takashi Seto, Fumio Imamura, Hideo Saka, Yasuo Iwamoto, Hiroshi Semba, Yasutaka Chiba, Hisao Uejima, and Masahiro Fukuoka

From the Kinki University Hospital, Osaka-Sayama, Shizuoka Cancer Center, Naga-izumi, Hyogo Cancer Center, Akashi; Osaka City University Hospital; Osaka City General Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Aichi Cancer Center Hospital, Nagoya Medical Center, Nagoya, Kinki-chuo Chest Medical Centre, Sakai, Kobe City Medical Center General Hospital, Kobe, Gifu Municipal Hospital, Gifu; Toneyama National Hospital, Toyonaka, Tokai University Hospital, Isehara, Hiroshima City Hospital, Hiroshima; and Kumamoto Regional Medical Center, Kumamoto, Japan.

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Corresponding author: Nobuyuki Yamamoto, MD, Thoracic Oncology Division, Shizuoka Cancer Center, 1007 Naga-izumicho Shimonagakubo, Sunto-gun, Shizuoka 411-8777, Japan; e-mail: n.yamamoto@scchr.jp.

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ABSTRACT

Purpose
This phase III trial of concurrent thoracic radiotherapy (TRT) was conducted to compare third-generation chemotherapy with second-generation chemotherapy in patients with unresectable stage III non–small-cell lung cancer (NSCLC).

Patients and Methods
Eligible patients received the following treatments: A (control), four cycles of mitomycin (8 mg/m^2 on day 1)/vindesine (3 mg/m^2 on days 1, 8)/cisplatin (80 mg/m^2 on day 1) plus TRT 60 Gy (treatment break for 1 week); B, weekly irinotecan (20 mg/m^2)/carboplatin (area under the plasma concentration-time curve [AUC] 2) for 6 weeks plus TRT 60 Gy, followed by two courses of irinotecan (50 mg/m^2 on days1, 8)/carboplatin (AUC 5 on day1); C, weekly paclitaxel (40 mg/m^2)/carboplatin (AUC 2) for 6 weeks plus TRT 60 Gy, followed by two courses of paclitaxel (200 mg/m^2 on day1)/carboplatin (AUC 5 on day 1).

Results
The median survival time and 5-year survival rates were 20.5, 19.8, and 22.0 months and 17.5%, 17.8%, and 19.8% in arms A, B, and C, respectively. Although no significant differences in overall survival were apparent among the treatment arms, noninferiority of the experimental arms was not achieved. The incidences of grade 3 to 4 neutropenia, febrile neutropenia, and gastrointestinal disorder were significantly higher in arm A than in arm B or C ($P < .001$). Chemotherapy interruptions were more common in arm B than in arm A or C.

Conclusion
Arm C was equally efficacious and exhibited a more favorable toxicity profile among three arms. Arm C should be considered a standard regimen in the management of locally advanced unresectable NSCLC.

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INTRODUCTION

Lung cancer remains the leading cause of cancer-related deaths worldwide.[1] Non–small-cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases, and approximately 30% of patients with NSCLC present with locally advanced lung cancer.[2]

The standard treatment for stage III locally advanced NSCLC was a combined modality of thoracic radiotherapy (TRT) and chemotherapy.[3,4] Phase III studies have also been conducted to assess the efficacy and toxicity of concurrent chemoradiotherapy in comparison with that of sequential chemoradiotherapy. In two studies (ie, a Japanese report[5] and the RTOG9410[6]) that employed older, second-generation regimens, the survival period was reported to be significantly prolonged by concurrent chemoradiotherapy, although the toxicity was worse. Thus the standard of treatment for stage III locally advanced lung cancer is currently recognized as concurrent chemoradiotherapy.

During the last decade, the usefulness of several new agents, such as paclitaxel, gemcitabine, vinorelbine, and docetaxel, have been studied, usually administered in combination with the platinum compounds. These newer-agent/platinum combinations, the so-called third-generation regimens, have been proven to be more effective than

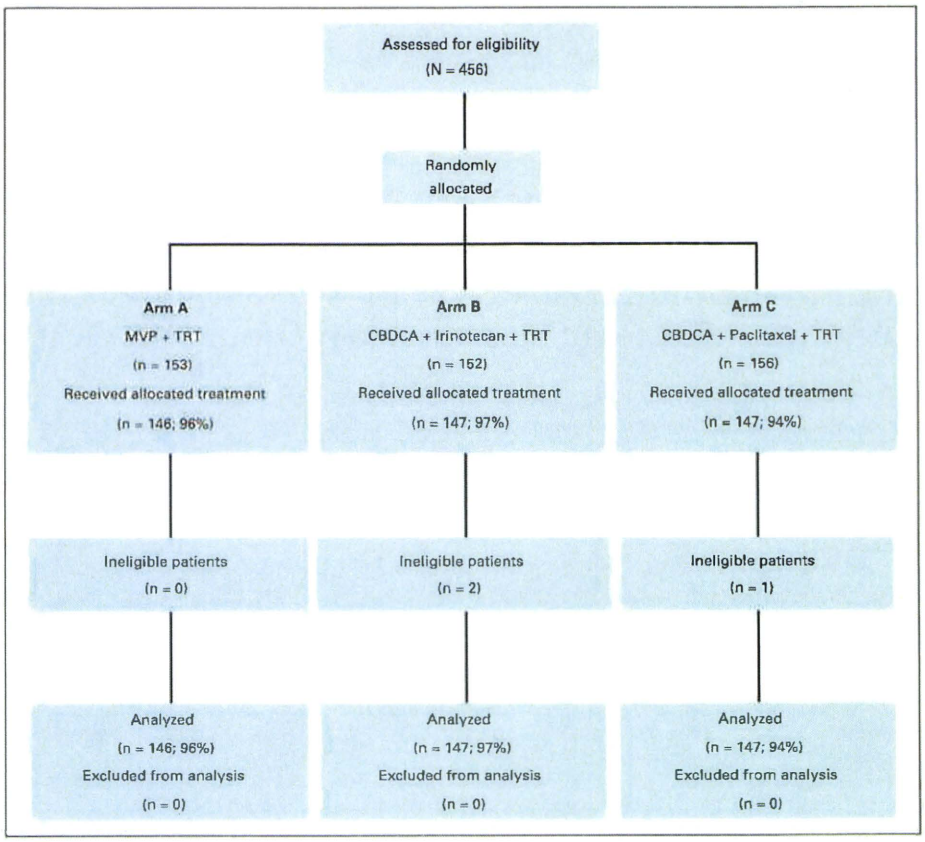

Fig 1. CONSORT diagram. MVP, mitomycin, vindesine, and cisplatin; TRT, thoracic radiotherapy; CBDCA, carboplatin

second-generation regimens, as demonstrated by the increased survival of patients with metastatic NSCLC treated with these regimens.[7-9]

Because the chemotherapy regimens used in the above-described two reports were second-generation regimens, the benefit of the introduction of third-generation regimens for chemoradiotherapy has begun to be assessed. Although concurrent administration of full-dose chemotherapy and thoracic radiotherapy has been reported to be possible by some investigators, it is considered difficult for many regimens[10,11]; third-generation agents can hardly be used at their full doses for concurrent chemoradiotherapy because of the high incidence of toxicity associated with these agents. Therefore, for concurrent chemotherapy with TRT, these chemotherapeutic agents have been used at reduced doses in several reported clinical studies.[12-14] However, some reports have suggested that the marked efficacy of concurrent chemoradiotherapy using third-generation chemotherapeutic agents can hardly be achieved using these agents at reduced doses.[15]

However, it remains to be clearly established regarding which would be superior in terms of both the efficacy and toxicity: concurrent chemoradiotherapy using the second-generation regimens at full doses or the third-generation regimens at reduced doses. We, the West Japan Thoracic Oncology Group, therefore performed a phase III study to compare these therapeutic strategies. The doses of the chemotherapeutic agents were determined based on the results of Japanese phase I studies.[16,17]

PATIENTS AND METHODS

Patient Selection

Patients with histologically or cytologically confirmed NSCLC with unresectable stage III disease were assessed for eligibility (see CONSORT diagram, Fig 1). Unresectable stage IIIA disease was defined by the presence of multiple and/or bulky N2 mediastinal lymph nodes on computed tomography (CT), which rendered, in the opinion of the treating investigator, the patients unsuitable as candidates for surgical resection. Eligible patients also needed to meet the following criteria: measurable disease of 20 mm or more; no prior history of chemotherapy or TRT; Eastern Cooperative Oncology Group performance status $\leq$ 1; age $\leq$ 75 years; leukocytes $\geq$ 4,000/μL, platelets $\geq$ 100,000/μL, and hemoglobin $\geq$ 9.5 g/dL, serum creatinine $\leq$ institutional upper limit of normal, 24-hour creatinine clearance $\geq$ 60 mL/min, bilirubin $\leq$ 1.5 mg/dL, AST and ALT $\leq$ 2.0$\times$ upper limit of normal, and partial pressure of arterial oxygen $\geq$ 70 mmHg.

Patients were excluded if they had pulmonary fibrosis; other active, invasive malignancies in the 3 years leading up to protocol entry; malignant effusion; pyrexia of 38°C or more at baseline; infections; significant cardiac disease; uncontrolled diabetes mellitus; paresis of the intestine ileus; or regular use of corticosteroids. The institutional ethics committee of each of the participating institutions approved the protocol, and all patients provided written informed consent before the start of the study.

For staging, all patients underwent CT of the thorax, including the upper abdomen, and either a brain CT or brain magnetic resonance imaging. A radioisotopic bone scan was also performed for all patients. Positron emission tomography was not obtained in any of the enrollees at baseline.

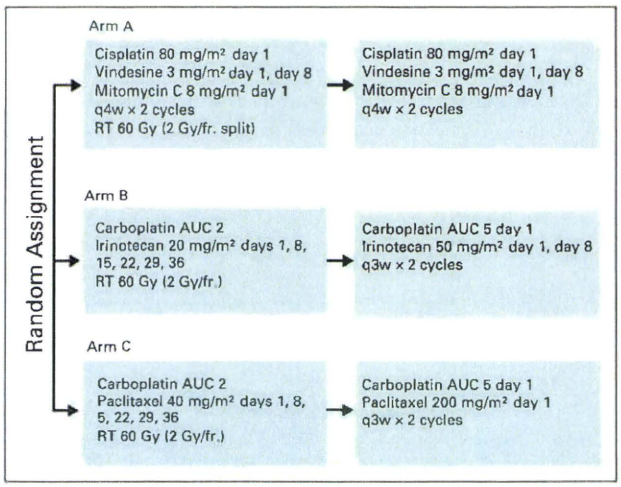

Fig 2. Treatment schema. q4w, every 4 weeks; RT, radiotherapy; fr, fraction; AUC, area under the plasma concentration-time curve.

Treatment Schedules

Patients were randomly assigned to one of the three following treatment arms (Fig 2). Treatment was composed of concurrent chemoradiotherapy and subsequent consolidation chemotherapy.

In arm A, chemotherapy consisted of vindesine 3 mg/m^2 on days 1 and 8, cisplatin 80 mg/m^2 on day 1, and mitomycin 8 mg/m^2 on day 1. This chemotherapy was repeated every 4 weeks, and four courses were administered. On day 2 of chemotherapy, TRT was begun at the dose of 2 Gy/fraction given in 15 fractions over 3 weeks, followed by a rest period of 1 week. Subsequently, radiation was again resumed at the dose of 2 Gy/fraction given in 15 fractions over 3 weeks. The total dose of radiation administered was 60 Gy.

In arms B and C, concurrent chemoradiotherapy was undertaken with the agents administered at reduced doses weekly for 6 weeks, followed by full-dose chemotherapy during the consolidation phase. The consolidation phase chemotherapy, initiated 3 to 4 weeks after the concurrent chemoradiotherapy, was administered in two cycles. TRT was initiated on day 1 at the dose of 2.0 Gy daily, five times per week. The total dose of 60 Gy was given in 30 fractions over a 6-week period.

The concurrent-phase chemotherapy consisted of irinotecan 20 mg/m^2 followed by carboplatin area under the plasma concentration time curve (AUC) 2 mg/mL/min in arm B and paclitaxel 40 mg/m^2 followed by carboplatin AUC 2 mg/mL/min in arm C. The consolidation chemotherapy consisted of 3-week cycles of irinotecan (50 mg/m^2 on days 1 and 8)/carboplatin (AUC 5 mg/mL/min on day 1) in arm B and paclitaxel (200 mg/m^2 administered over 3 hours) followed by carboplatin (AUC 5 mg/mL/min on day 1) in arm C.

Radiation Therapy

All patients were treated with a linear accelerator photon beam of 4 MV or more. The primary tumor and involved nodal disease received 60 Gy in 2-Gy fractions over 6 weeks in arms B and C and 7 weeks in arm A.

At the start of this multi-institutional study, three-dimensional (3D) treatment planning system using CT was not available at all institutions. Therefore, two-dimensional (2D) treatment planning techniques were allowed, and 3D dose constraints for both planning target volume and normal-risk organs were not determined in the protocol. Radiation doses were specified at the center of the target volume. In 2D treatment planning, doses were calculated assuming tissue homogeneity without correction for lung tissues, whereas lung inhomogeneity correction was performed in 3D treatment planning. Among 412 patients who received ≥ 54 Gy (arm A, n = 139; arm B, n = 137; and arm C, n = 136), 2D and 3D treatment planning was performed for 200 and 212 patients, respectively.

The initial 40 Gy was delivered to clinical target volume 1 (CTV1), and the final 20 Gy was delivered to a reduced volume defined as clinical target volume 2 (CTV2). CTV1 included the primary tumor, ipsilateral hilum, and mediastinal nodal areas from the paratracheal (no. 2) to subcarinal lymph nodes (no. 7). The contralateral hilum was not included in CTV1. The supraclavicular areas were not to be treated routinely, but could be treated when supraclavicular nodes were involved. For the primary tumors and the involved lymph nodes of 1 cm in the shortest diameter, a margin of 1.5 to 2 cm was added. CTV2 included only the primary tumor and the involved lymph nodes with a margin of 0.5 to 1 cm. The spinal cord was excluded from the fields for CTV2 by appropriate methods, such as the oblique opposing method. Appropriate planning target volume margin and leaf margin were added for CTV1 and CTV2. When grade 4 hematologic toxicity, grade 3 to 4 esophagitis or dermatitis, pyrexia of ≥ 38°C, or a partial pressure of arterial oxygen of less than 60 mmHg occurred, the TRT was interrupted.

Evaluation of Response and Toxicity

All eligible patients who received any treatment at all were considered as assessable for response and toxicity. Chest x-rays, CBCs, and blood chemistry studies were repeated once a week during the treatment period. Thoracic CT was performed once a month during the treatment period. After the treatment, thoracic CT was obtained every 3 months, and other imaging examinations were obtained when recurrence was suspected. The response was evaluated in accordance with Response Evaluation Criteria in Solid Tumors (RECIST). In the evaluation of the antitumor effects, extramural review was conducted. Overall survival (OS) was defined as the time from registration until death from any cause. Progression-free survival (PFS) was defined as the time between random assignment and disease progression, death, or last known follow-up. OS and PFS were estimated by the Kaplan-Meier method.

Statistical Analysis

The primary end point of this study was comparison of the OS between the control group (arm A) and each of the treatment groups (arm B or C). It was projected that the control group would achieve a median OS time of 16.5 months,[5] whereas the treatment group would show an increase in the median OS to 20.5 months, on the basis of previously published data.[14] When the upper limit of the adjusted CI of the hazard ratio of the control group to each treatment group was low 1.176 (1/0.85), the results were recognized as demonstrating noninferiority of the experimental treatment to the control treatment. The sample size was calculated assuming a 2.5% one-sided type I error and 80% power. The patient accumulation period was 4.5 years, and the follow-up period was 3 years. In view of the possibility of variance inflation owing to censoring, the sample size was set at 450 patients.

Baseline characteristics were compared among the treatment groups using the Kruskal-Wallis test for continuous variables and Fisher's exact test for discrete variables. Rates of occurrence of specific toxicities and treatment delivery were compared among the groups using Fisher's exact test.

RESULTS

Patient Characteristics

From September 2001 to September 2005, a total of 456 patients were registered for the study, and 153, 152, and 151 patients were allocated to arms A, B, and C, respectively. Of the total, 16 patients (arm A, n = 7; arm B, n = 5; arm C, n = 4) did not receive the protocol treatment because they were deemed ineligible for the study before the start of treatment after registration in five patients (large irradiation area, n = 2; stage IIB, n = 1; stage IV, n = 2), worsening of the underlying disease in four patients, worsening of complications in five patients, patient refusal in one patient, and unknown reason in one patient. The safety and antitumor effects of the treatments were eventually assessed on the basis of the data of 440 patients after exclusion of these 16 patients from the total of 456 patients enrolled. After the start of the treatment, three patients were found to be ineligible because of stage IV disease, but the data of these patients were included in all the analyses.

Table 1. Patient Characteristics

Characteristic	Arm A No.	Arm A %	Arm B No.	Arm B %	Arm C No.	Arm C %	P
Sex							.879
Female	18	12.3	21	14.3	19	12.9	
Male	128	87.7	126	85.7	128	87.1	
Age, years							.378
Median	63.0		62.0		63.0		
Range	31-74		30-74		38-74		
≥ 70	27	18.5	36	24.5	31	21.1	
Smoking history							.240
Absence	17	11.6	15	10.2	9	6.1	
Presence	129	88.4	132	89.8	138	93.9	
Performance status							.447
0	56	38.4	66	44.9	65	44.2	
1	90	61.6	81	55.1	81	55.1	
Unknown	0	0.0	0	0.0	1	0.7	
Weight loss during the previous 6-month period							.680
< 5%	92	63.0	100	68.0	95	64.6	
≥ 5%	28	19.2	24	16.3	29	19.7	
Unknown	26	17.8	23	15.6	23	15.6	
Staging							.901
IIIA	49	33.6	46	31.3	49	33.3	
IIIB	97	66.4	101	68.7	98	66.7	
N status							—
N2	94	64.4	86	58.5	99	67.3	
N3	33	22.6	43	29.3	32	21.8	
Histology							—
Adenocarcinoma	58	39.7	69	46.9	62	42.2	
Squamous cell carcinoma	70	47.9	62	42.2	71	48.3	

There were no statistically significant differences among the three arms in terms of patient characteristics (Table 1).

Treatment Administered

Table 2 shows the status of implementation of chemotherapy. During the concurrent phase, 40.8% of patients in arm B and 58.5% of patients in arm C received six weekly cycles of chemotherapy ($P = .003$); 67.3% of patients in arm B and 87.8% patients in arm C completed at least five cycles ($P < .001$). In regard to the consolidation phase, 41.1%, 29.3%, and 49.7% in arms A, B, and C, respectively, received the two scheduled courses of therapy ($P = .002$). Chemotherapy interruptions were more common in arm B than in arms A and C in both the concurrent and consolidation phases.

Table 2. Chemotherapy Administered

Chemotherapy Cycles	No. of Patients Arm A	Arm B	Arm C	P
Concurrent chemotherapy cycles				
1	18.5	0.7	2.0	
2	81.5	2.0	2.0	
3		5.4	1.4	
4		24.5	6.8	
5		26.5	29.3	B v C: .003
6		40.8	58.5	B v C: < .001
Consolidation chemotherapy				
0	46.6	34.0	30.6	
1	12.3	36.7	19.7	
2	41.1	29.3	49.7	A v B v C: .002

In most of the patients, TRT at 60 Gy was completed, and 6.8%, 8.2%, and 8.8% of patients in arms A, B, and C, respectively, received a radiation dose of less than 60 Gy. The reason for the reduced radiation dose was toxicity in two thirds of the patients (three patients from arm A; six patients from arm B, including two cases of esophagitis and two cases of pneumonitis; and seven patients from arm C, including one case of esophagitis and two cases of pneumonitis).

Toxicity

Table 3 lists the grade 3 or worse severe toxicities. There were a total of 11 treatment-related deaths. The cause of death was radiation pneumonitis in one patient and sepsis in one of the two patients in arm A; meningitis in one patient, pneumonia in one patient, radiation pneumonitis in two patients, and mycosis in one of the five patients in arm B; and radiation pneumonitis in three patients and death from other cause in one of the four patients in arm C. The clinical course of the patients who died of radiation pneumonitis are presented next. One patient from arm A developed pneumonitis on day 2 of the fourth course of treatment. In this patient, the pneumonitis subsided temporarily in response to corticosteroid therapy, but it aggravated again subsequently, resulting in death. In arm B, one patient developed pneumonitis after 54 Gy of TRT and died despite mechanical ventilation, and another patient developed pneumonitis at the end of the concurrent phase. In the latter patient, the pneumonitis subsided temporarily in response to pulsed corticosteroid therapy, but it aggravated again, resulting in death. In arm C, two patients developed pneumonitis at the end of the concurrent phase. Another patient from arm C developed pneumonitis on day 16 of the concurrent phase.

The incidences of grade 3 or worse severe hematologic toxicity, infection, febrile neutropenia, and gastrointestinal toxicity were significantly higher in arm A than in arm B or C. The incidence of grade

Table 3. Grade 3 or Worse Toxicity

Toxicity	All Treatment: Arm A	Arm B	Arm C	P	Concurrent Phase: Arm A	Arm B	Arm C	P
Neutropenia	95.9	60.5	61.9	< .001	93.8	53.7	23.1	< .001
Leukopenia	96.6	75.5	66.0	< .001	95.9	72.1	46.9	< .001
Anemia	25.3	17.7	8.8	< .001	15.8	8.8	6.1	0.019
Thrombocytopenia	28.8	28.6	7.5	< .001	21.9	11.6	5.4	< .001
Febrile neutropenia	37.0	8.8	10.2	< .001	30.8	6.1	3.4	< .001
Nausea	21.9	4.8	4.8	< .001	21.9	3.4	3.4	< .001
Vomiting	6.8	2.7	0.7	.012	6.2	1.4	0.0	.001
Fatigue	13.0	6.1	4.8	.019	9.6	2.0	1.4	< .001
Constipation	11.6	6.1	2.7	.009	8.9	6.1	1.4	.015
Diarrhea	0.7	2.0	1.4	.606	0.7	0.7	0.7	.999
Neurogenic (sensory)	0.7	0.7	4.8	.017	0.0	0.0	0.0	—
Esophagitis	5.5	2.7	8.2	.121	4.1	2.0	7.5	.077
Infection	26.0	16.3	17.0	.066	22.6	12.2	10.2	.006
Dyspnea	6.2	5.4	6.1	.957	2.7	0.7	2.0	.406
Pneumonitis	1.4	4.1	4.1	.312	0.0	0.0	0.7	.368

Table 4. Objective Response

Response	Arm A (n = 146) No.	Arm A (n = 146) %	Arm B (n = 147) No.	Arm B (n = 147) %	Arm C (n = 147) No.	Arm C (n = 147) %
CR	3	2.1	4	2.7	5	3.4
PR	94	64.4	79	53.7	88	59.9
SD	16	11.0	32	21.8	32	21.8
PD	19	13.0	19	12.9	16	10.9
NE	14	9.6	13	8.8	6	4.1
Response rate, CR + PR*	97	66.4	83	56.5	92	63.0

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated.
*P = .198.

3 or worse severe neurogenic toxicity was significantly higher in arm C as compared with that in the other two arms. There were no statistically significant differences in the incidences of esophagitis, dyspnea, or pneumonitis, which are manifestations of radiation-related toxicity, among the three groups. The incidence of grade 2 or worse severe esophagitis was significantly higher in arm C (20.5%, 23.1%, and 33.3% from arms A, B and C, respectively; P = .003).

Efficacy

The objective response rates were 66.4%, 56.5%, and 63.3% in arms A, B, and C, respectively (Table 4). The response rates in arms B and C were not statistically significantly different from the rate in arm A.

The OS and PFS are shown in Figure 3. Most of the patients had been observed for more than 3 years, and 343 patients had died. The median survival time and 3- and 5-year survival rates in arm A were 20.5 months, 35.3%, and 17.5%, respectively. The corresponding values were 19.8 months, 24.2%, and 17.8% in arm B, and 22.0 months, 26.4%, and 19.5% in arm C. There was no statistically significant difference in the OS between arm B or C and arm A (arm A v B, P = .392; arm A v C, P = .876). The upper limits of the adjusted CI of the hazard ratio between arm A and B (1.402) or C (1.204) exceeded 1.176. Thus the results did not show noninferiority of the three experimental regimens (arm B and C) as compared with the reference treatment (arm A).

The OS was not significantly different according to sex (male, female), stage (IIIA, IIIB), and weight loss (< 5%, ≥ 5%) among the three arms. The causes of death after the third year are disease progression (n = 15, 6, and 9 in arms A, B, and C, respectively) and other disease (n = 3, 1, and 0 in arms A, B, and C, respectively).

The median PFS was 8.2, 8.0, and 9.5 months in arms A, B, and C, respectively. There was also no statistically significant difference of the PFS between arm B or C and A (arm A v B, P = .466; arm A v C, P = .621).

DISCUSSION

To our knowledge, this is the first phase III trial designed for direct comparison between second-generation and third-generation regimens applied in combination with concurrent TRT in patients with locally advanced lung carcinoma. This study was additionally aimed at comparing a cisplatin-based regimen with a carboplatin-based regimen and also more frequent radiosensitizing doses during TRT with systemic doses of chemotherapy during radiotherapy. In regard to chemotherapy for advanced lung cancer, a previous meta-analysis demonstrated that a cisplatin-based regimen is superior to a carboplatin-based regimen in terms of OS. In the present study, however, the OS in arm A (cisplatin-based regimen) was not significantly longer than that in arm B or C (carboplatin-based regimen). The observed intergroup differences possibly reflect the differences between the second- and third-generation regimens or between more frequent radiosensitizing doses and systemic doses of chemotherapy. In any event, the results of this study suggest that the third-generation

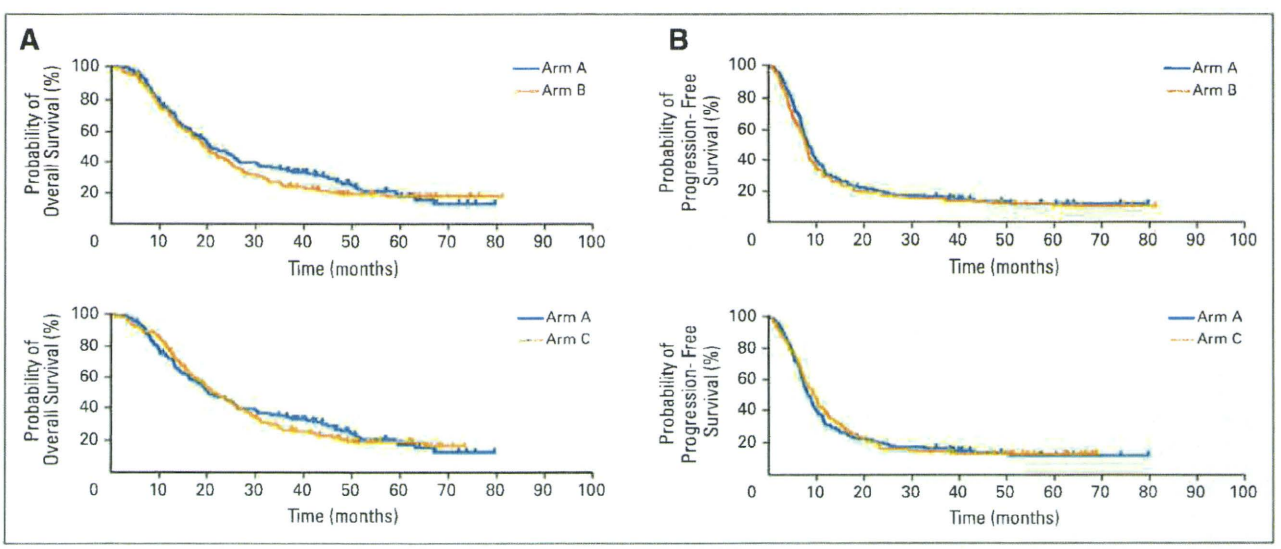

Fig 3. (A) Comparison of overall survival among the three randomly assigned arms. (B) Comparison of progression-free survival among the three randomly assigned arms.

carboplatin regimen (particularly carboplatin plus paclitaxel) was at least comparable to the second-generation cisplatin regimen, which is the conventionally used therapeutic regimen, in terms of the survival-prolonging effect when applied in combination with concurrent thoracic radiotherapy.

Unfortunately, noninferiority of OS was not demonstrated in the present study, probably because the number of the patients in this study resulted in a deficiency of power, because the therapeutic outcome in the reference arm was more favorable than that in conventional reports. The therapeutic outcome in the reference arm in recent phase III studies of chemoradiotherapy was more favorable than the estimated numerical data.[18] The favorable data may be attributable to bias as a result of the patient inclusion criteria or the development of radiotherapy, but no distinct cause could be identified.

Although noninferiority in terms of OS was not demonstrated in this study, the survival curves themselves mostly coincided among the three groups, as shown in Figure 2. The hematologic and gastrointestinal toxicities noted in arm A were significantly serious as compared with those in the experimental arms. Although the incidence of grade 3 or worse severe neurotoxicity was significantly higher, most of the other toxicities were the mildest in arm C among the three groups. Between the experimental arms, the rate of implementation of chemotherapy tended to be lower for arm C than for arm B. It was considered, from the viewpoint of feasibility, that arm C may be superior to arm B.

From these data on the efficacy and toxicity, we judged that concurrent chemoradiotherapy involving the combined use of carboplatin plus paclitaxel and TRT yielded the best results among the three groups, and we, the West Japan Thoracic Oncology Group, will select this treatment method as the reference arm for phase III studies in the future.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

Conception and design: Nobuyuki Yamamoto, Kazuhiko Nakagawa, Yasumasa Nishimura, Hisao Uejima, Masahiro Fukuoka
Administrative support: Kayoko Tsujino, Yasutaka Chiba
Provision of study materials or patients: Nobuyuki Yamamoto, Kazuhiko Nakagawa, Yasumasa Nishimura, Kayoko Tsujino, Miyako Satouchi, Shinzoh Kudo, Toyoaki Hida, Masaaki Kawahara, Koji Takeda, Nobuyuki Katakami, Toshiyuki Sawa, Soichiro Yokota, Takashi Seto, Fumio Imamura, Hideo Saka, Yasuo Iwamoto, Hiroshi Semba, Hisao Uejima, Masahiro Fukuoka
Collection and assembly of data: Nobuyuki Yamamoto, Kazuhiko Nakagawa, Yasumasa Nishimura, Miyako Satouchi, Shinzoh Kudo, Toyoaki Hida, Masaaki Kawahara, Koji Takeda, Nobuyuki Katakami, Toshiyuki Sawa, Soichiro Yokota, Takashi Seto, Fumio Imamura, Hideo Saka, Yasuo Iwamoto, Hiroshi Semba, Hisao Uejima, Masahiro Fukuoka
Data analysis and interpretation: Nobuyuki Yamamoto, Kazuhiko Nakagawa, Yasumasa Nishimura, Yasutaka Chiba, Masahiro Fukuoka
Manuscript writing: Nobuyuki Yamamoto, Yasumasa Nishimura, Yasutaka Chiba
Final approval of manuscript: Nobuyuki Yamamoto, Kazuhiko Nakagawa, Yasumasa Nishimura, Kayoko Tsujino, Miyako Satouchi, Shinzoh Kudo, Toyoaki Hida, Masaaki Kawahara, Koji Takeda, Nobuyuki Katakami, Toshiyuki Sawa, Soichiro Yokota, Takashi Seto, Fumio Imamura, Hideo Saka, Yasuo Iwamoto, Hiroshi Semba, Yasutaka Chiba, Hisao Uejima, and Masahiro Fukuoka

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Phase I Safety, Pharmacokinetic, and Biomarker Study of BIBF 1120, an Oral Triple Tyrosine Kinase Inhibitor in Patients with Advanced Solid Tumors

Isamu Okamoto[1], Hiroyasu Kaneda[1,2], Taroh Satoh[1], Wataru Okamoto[1], Masaki Miyazaki[1], Ryotaro Morinaga[1], Shinya Ueda[1], Masaaki Terashima[1], Asuka Tsuya[1], Akiko Sarashina[3], Koichi Konishi[3], Tokuzo Arao[2], Kazuto Nishio[2], Rolf Kaiser[4], and Kazuhiko Nakagawa[1]

Abstract

BIBF 1120 is an oral multitargeted tyrosine kinase inhibitor that blocks the activity of vascular endothelial growth factor (VEGF) and other growth factor receptors. We have done a phase I study to evaluate the safety, pharmacokinetics, and pharmacodynamic biomarkers of BIBF 1120. Patients with advanced refractory solid tumors were treated with BIBF 1120 at oral doses of 150 to 250 mg twice daily. Drug safety and pharmacokinetics were evaluated, as were baseline and post-treatment levels of circulating CD117-positive bone marrow–derived progenitor cells and plasma soluble VEGF receptor 2 as potential biomarkers for BIBF 1120. Twenty-one patients were treated at BIBF 1120 doses of 150 (n = 3), 200 (n = 12), or 250 mg twice daily (n = 6). Dose-limiting toxicities of reversible grade 3 or 4 elevations of liver enzymes occurred in 3 of 12 patients at 200 mg twice daily and 3 of 6 patients at 250 mg twice daily. Stable disease was achieved in 16 (76.2%) patients, and median progression-free survival was 113 days (95% confidence interval, 77-119 d). Pharmacokinetic analysis indicated that the maximum plasma concentration and area under the curve for BIBF 1120 increased with the dose within the dose range tested. Levels of CD117-positive bone marrow–derived progenitors and soluble VEGF receptor 2 decreased significantly during treatment over all BIBF 1120 dose cohorts. In conclusion, the maximum tolerated dose of BIBF 1120 in the current study was determined to be 200 mg twice daily, and our biomarker analysis indicated that this angiokinase inhibitor is biologically active. *Mol Cancer Ther; 9(10), 2825–33. ©2010 AACR.*

Introduction

Angiogenesis, defined as the formation of new blood vessels from a preexisting vasculature, is essential for tumor growth and the spread of metastases (1, 2). Tyrosine kinase receptors, including vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors, and fibroblast growth factor receptors, together with their corresponding ligands, play key roles in angiogenesis (1). Antiangiogenic therapy that targets signaling by these receptor-ligand systems represents an important advance in clinical oncology (3). Given that most angiogenesis inhibitors are cytostatic, however, it has been difficult to assess their biological effects in early clinical trials. Validated biomarkers that allow monitoring of the biological activity of these agents are thus urgently needed (4, 5). The most intuitive approach to measurement of the biological activity of such targeted agents is evaluation of their effects on tumor cells or the vasculature. However, this invasive approach raises practical and ethical concerns (6, 7). Noninvasive, blood-based biomarkers that allow repetitive sampling throughout treatment and follow-up are therefore preferred.

BIBF 1120 is an orally available triple tyrosine kinase inhibitor that predominantly blocks VEGFR1 to 3, fibroblast growth factor receptors 1 to 3, as well as platelet-derived growth factor receptors α and β tyrosine kinases at nanomolar concentrations (Fig. 1; refs. 8–10). In preclinical studies, BIBF 1120 has been shown to inhibit the growth of and to reduce vessel density in s.c. implanted human tumor xenografts in nude mice (8, 11). A previous phase I BIBF 1120 monotherapy study in patients with advanced and heavily pretreated malignancies showed encouraging antitumor activity and a tolerable safety profile. The maximum tolerated dose (MTD) was determined as 250 mg twice daily (12). A further phase I combination study showed that BIBF 1120 at 200 mg twice daily can be combined with standard doses

Authors' Affiliations: Departments of [1]Medical Oncology and [2]Genome Biology, Kinki University School of Medicine, Osaka-Sayama, Japan; [3]Nippon Boehringer Ingelheim Co. Ltd., Tokyo, Japan; and [4]Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany

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Corresponding Author: Isamu Okamoto, Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone: 81-72-366-0221; Fax: 81-72-360-5000, E-mail: chi-okamoto@dotd.med.kindai.ac.jp

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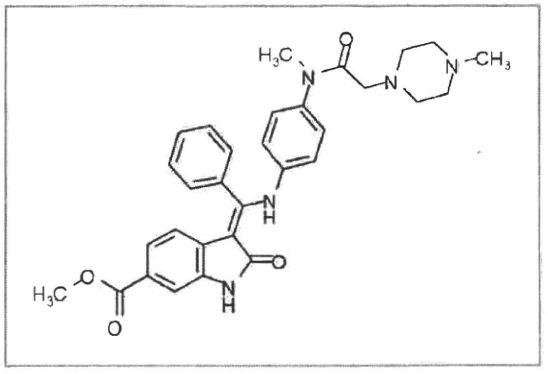

Figure 1. Structure of BIBF 1120.

of paclitaxel and carboplatin (13). Several phase II monotherapy trials have gone on to show promising signs of efficacy in patients with advanced non–small cell lung cancer and ovarian cancer (14, 15).

We have done a phase I dose-escalation study to determine the MTD, tolerability, basic pharmacokinetics, and antitumor effect of BIBF 1120 given p.o. on a twice daily schedule in Japanese patients with advanced refractory solid tumors. To identify biomarkers that reflect the pharmacodynamics and dose-response relation of BIBF 1120, we further evaluated baseline (before BIBF 1120 treatment) and post-treatment levels of circulating CD117 (c-KIT)-positive bone marrow–derived (BMD) progenitor cell subsets as well as of plasma soluble VEGFR2 (sVEGFR2). We show that a subset of $CD117^+$ BMD progenitors, immunophenotypically defined as $CD45^{dim}CD34^+CD117^+$ cells, is a potential biomarker for guidance of optimal therapy with BIBF 1120.

Patients and Methods

Patient eligibility

Eligible patients were 20 years of age or older with a confirmed diagnosis of advanced solid tumors who had not responded to conventional treatment or for whom no therapy of proven efficacy was available. They were required to have an Eastern Cooperative Oncology Group performance status of <2 and adequate organ function. Individuals were excluded if they had a brain tumor or brain metastases requiring therapy, gastrointestinal disorders that might interfere with absorption of the study drug, or serious illness or concomitant nononcologic disease that was difficult to control by medication. Patients were also excluded if they had a history of obvious pulmonary fibrosis or interstitial pneumonitis, autoimmune disease, serious drug hypersensitivity, cardiac infarction, or congestive heart failure. All subjects received information about the nature and purpose of the study, and they provided written informed consent in accordance with institutional guidelines.

Study design

This study was designed as a single-center, open-label, dose-escalation phase I trial. The primary objectives of this dose-escalation trial were to determine if BIBF 1120 doses from 150 to 250 mg given twice daily on a continuous daily schedule could be confirmed as safe and tolerable treatment, and to collect overall safety data. The secondary objectives included the determination of the MTD, pharmacokinetic variables, pharmacodynamics, and preliminary information about the antitumor activity and the efficacy on angiogenic peripheral blood biomarkers in this treatment population. The study was reviewed and approved by the Institutional Review Board.

Dose levels of BIBF 1120 were 150, 200, and 250 mg twice daily. Intrapatient dose escalation was not permitted. Each treatment course comprised 28 days of continuous daily treatment with BIBF 1120. If a patient experienced a drug-related dose-limiting toxicity (DLT), the treatment with BIBF 1120 had to be discontinued. If all DLTs were recovered to baseline or below grade 1 according to the Common Toxicity Criteria for Adverse Events version 3.0 within 14 days of stopping treatment with BIBF 1120, treatment could be resumed at one-dose lower level.

The dose escalation/reduction scheme was based on the occurrence of drug-related DLTs within the first treatment course. If a DLT was not observed in any of the first three patients, the dose was escalated to the next level. If a DLT was observed in one of the first three patients, three additional patients were recruited to that dose level. If a DLT occurred in only one of six patients, dose escalation was permitted. If two or more of six patients experienced a DLT, additional patients were recruited at one-dose lower level for a total of at least six patients. In addition to this dose escalation/reduction scheme, if the investigators and independent data monitoring committee agreed that additional patients were necessary to confirm the dose escalation/reduction decision in cases in which two or more patients experienced DLTs, which were not life-threatening, and were reversible and manageable with or without medication, entering additional patients at that dose level was allowed. The MTD was defined as the highest dose level at which <33% of the patients would experience a DLT during the first treatment course. Once the MTD had been determined, that cohort was expanded to at least 12 patients in total to more completely assess the safety and tolerability of the dose level.

Safety and efficacy assessments

The safety and tolerability of BIBF 1120 were assessed according to Common Toxicity Criteria for Adverse Events version 3.0. The following adverse events were defined as DLTs: drug-related adverse events involving hematologic or nonhematologic toxicity of Common Toxicity Criteria for Adverse Events grade 3 or 4 within the first treatment course with BIBF 1120. Objective