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

Ken Takezawa[1], Isamu Okamoto[1], Junko Tanizaki[1], Kiyoko Kuwata[1], Haruka Yamaguchi[1], Masahiro Fukuoka[3], Kazuto Nishio[2], and Kazuhiko Nakagawa[1]

Abstract

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

Introduction

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

The identification of strategies or agents capable of overcoming acquired resistance to EGFR-TKIs is thus an important clinical goal.

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

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

Authors' Affiliations: Departments of ¹Medical Oncology and ²Genome Biology, Kinki University School of Medicine; ³Department of Medical Oncology, Kinki University School of Medicine, Sakai Hospital, Osaka, Japan

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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

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

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

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

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

Materials and Methods

Cell culture and reagents

The human NSCLC cell lines PC9, PC9/ZD, HCC827, and NCI-H1975 (H1975) were obtained as described previously (16–18). All cells were cultured under a humidified atmosphere of 5% CO$_2$ at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. BIBW2992 was kindly provided by Boehringer Ingelheim Pharma; gefitinib was obtained from AstraZeneca; and 5-fluorouracil (5-FU), S-1, and pemetrexed were from Wako. U0126 and LY294002 were obtained from Cell Signaling Technology.

Growth inhibition assay *in vitro*

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

a well-established index of the interaction between two drugs (19). CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively.

Immunoblot analysis

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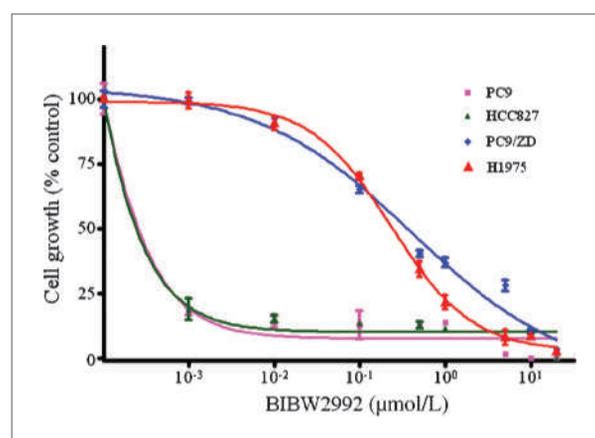

Figure 1. Effect of BIBW2992 on the growth of NSCLC cell lines *in vitro*. The indicated NSCLC cell lines were cultured for 72 h in complete medium containing various concentrations of BIBW2992, after which cell viability was assessed as described in Materials and Methods. Points, mean of triplicates from experiments that were repeated a total of three times with similar results; bars, SD.
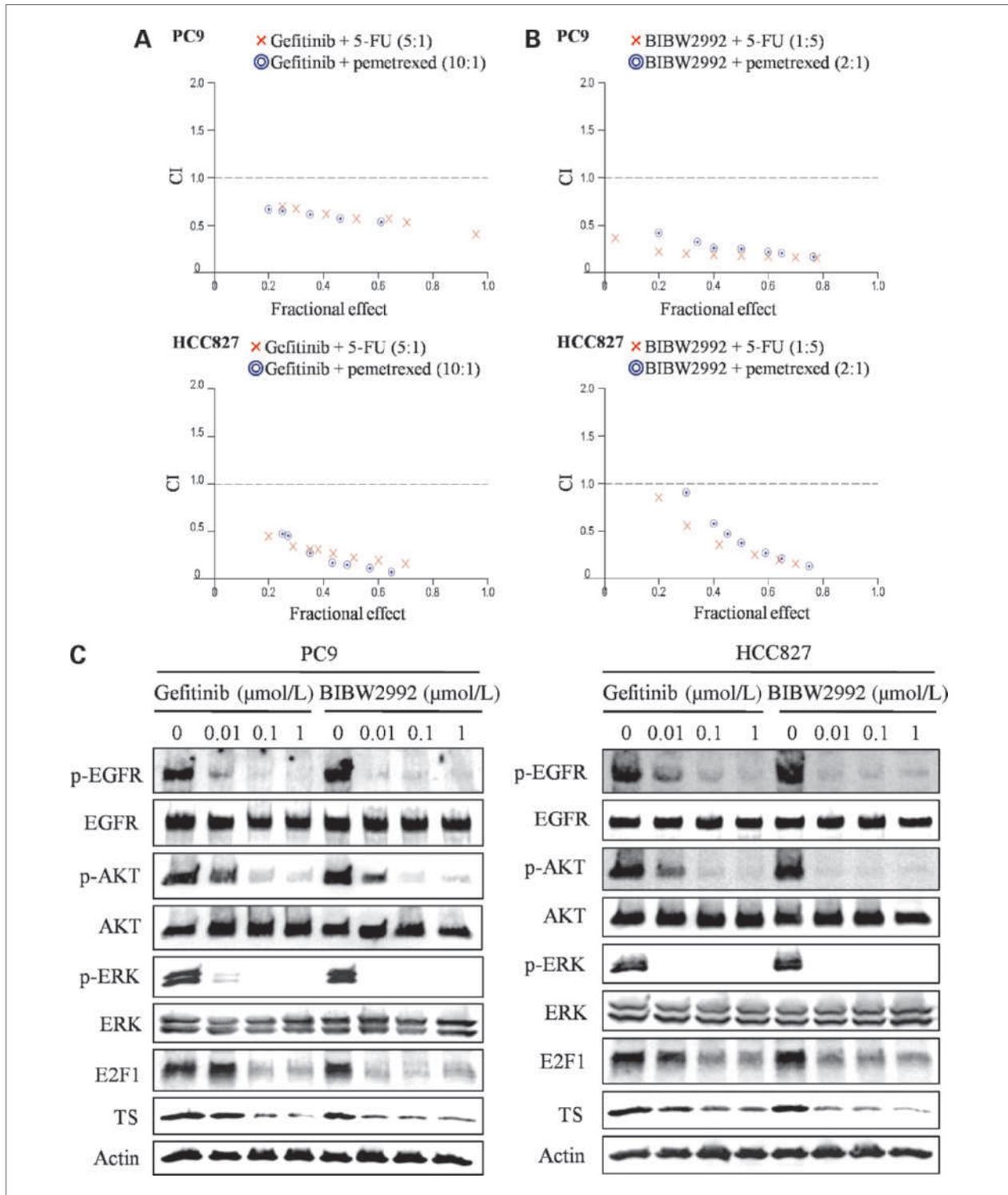
Figure 2. Effects of the combination of TS inhibitors (5-FU or pemetrexed) with EGFR-TKIs (gefitinib or BIBW2992) on the growth of gefitinib-sensitive NSCLC cell lines *in vitro*. A and B, sensitizing *EGFR* mutation-positive NSCLC (PC9 and HCC827) cells were incubated for 72 h with gefitinib (A) or BIBW2992 (B) together with 5-FU or pemetrexed at the indicated molar concentration ratios, after which cell viability was measured. The interaction between the two drugs in each combination was evaluated on the basis of the CI, which is plotted against fractional growth inhibition. Data are means of triplicates from experiments that were repeated a total of three times with similar results. C, cells were incubated for 24 h with gefitinib or BIBW2992 at the indicated concentrations in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, or ERK as well as with those to E2F1, TS, or β-actin (loading control).
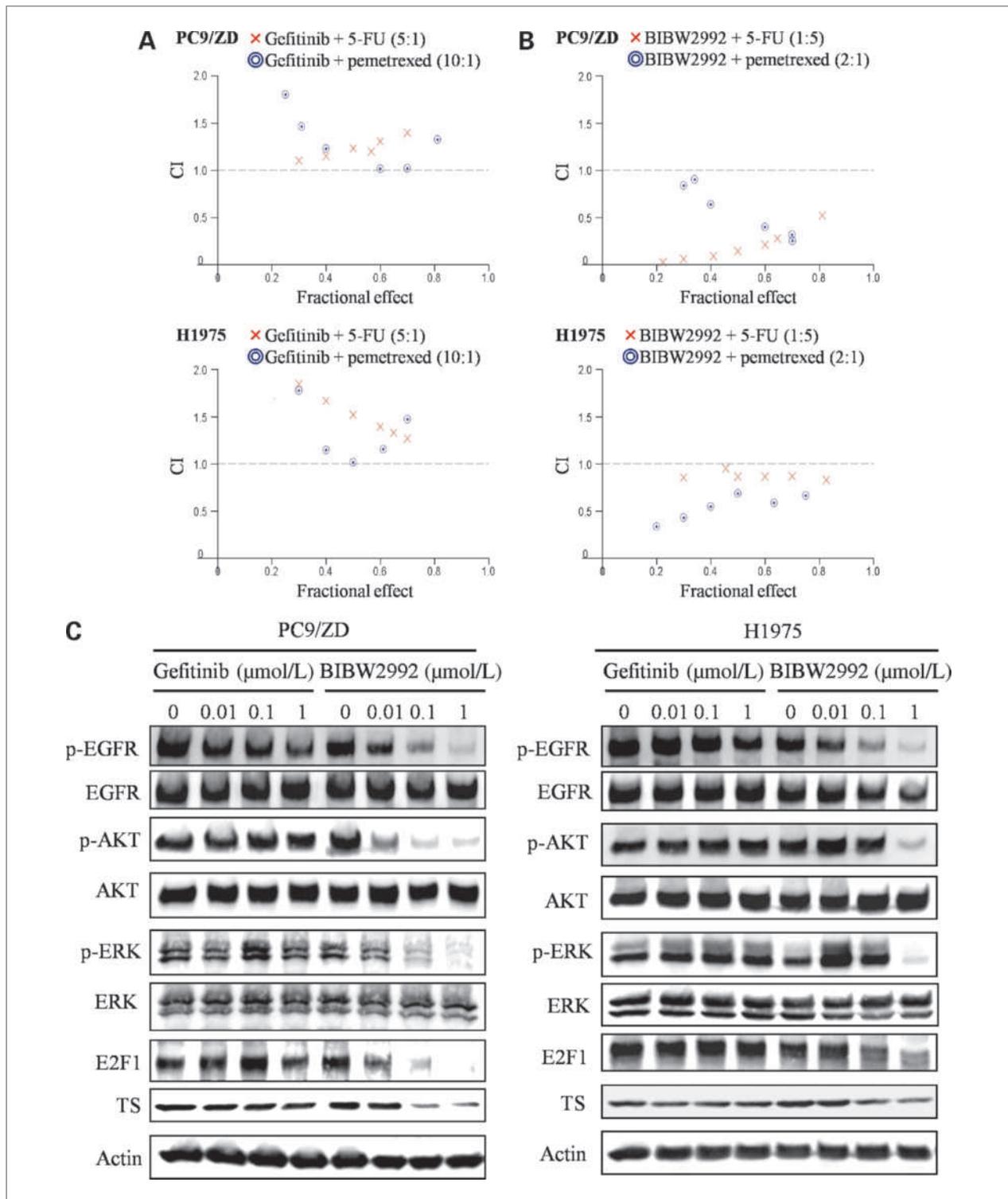
Figure 3. Effects of the combination of TS inhibitors (5-FU or pemetrexed) with EGFR-TKIs (gefitinib or BIBW2992) on the growth of gefitinib-resistant NSCLC cell lines *in vitro*. A and B, cells with a secondary T790M mutation of EGFR (PC9/ZD and H1975) were incubated for 72 h with gefitinib (A) or BIBW2992 (B) together with 5-FU or pemetrexed at the indicated molar concentration ratios, after which cell viability was measured and CI was plotted against fractional growth inhibition. Data are means of triplicates from experiments that were repeated a total of three times with similar results. C, cells were incubated for 24 h with gefitinib or BIBW2992 at the indicated concentrations in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis as in Fig. 2C.

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 $200 \times g$ for 5 minutes. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 minutes at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and CellQuest software (Becton Dickinson).

Animals

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

Growth inhibition assay *in vivo*

Cubic fragments of tumor tissue (~2 by 2 by 2 mm) were implanted s.c. into the axilla of 5- to 6-week-old male athymic nude mice. Treatment was initiated when tumors in each group of eight mice achieved an average volume of 150 to 200 mm³. Treatment groups consisted of control, S-1 or pemetrexed alone, gefitinib alone, BIBW2992 alone, the combination of gefitinib and either S-1 or pemetrexed, and the combination of BIBW2992 and either S-1 or pemetrexed. S-1, gefitinib, and BIBW2992 were administered by oral gavage daily for 28 days; control animals received a 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle. Pemetrexed was administered i.p. once a week. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula $LW^2/2$. Both tumor size and body weight were measured twice per week.

Statistical analysis

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

Results

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

We first examined the ability of BIBW2992 to inhibit the proliferation of human NSCLC cells with an *EGFR* mutation. Both PC9 and HCC827 cells harbor an in-frame deletion in exon 19 of *EGFR* and were found to be highly sensitive to BIBW2992, with IC$_{50}$ values of <0.001 μmol/L (Table 1). Gefitinib also potently inhibited the proliferation of these two cell lines (Table 1). PC9/ZD cells are a gefitinib-resistant clone of PC9 and also harbor the T790M mutation of *EGFR*, and H1975 cells possess both L858R and T790M mutations of *EGFR*. Both of these cell lines

manifested resistance to gefitinib, with an IC$_{50}$ for this drug of >5 μmol/L (Table 1). Although BIBW2992 inhibited the growth of PC9/ZD and H1975 cells with IC$_{50}$ values within the range of achievable serum concentrations of the drug, these values were ~1,000 times those apparent for PC9 and HCC827 cells (Fig. 1). These data thus suggested that an additional T790M mutation of *EGFR* reduces the sensitivity of NSCLC cells with a sensitizing *EGFR* mutation (either an exon 19 deletion or L858R in exon 21) to BIBW2992.

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

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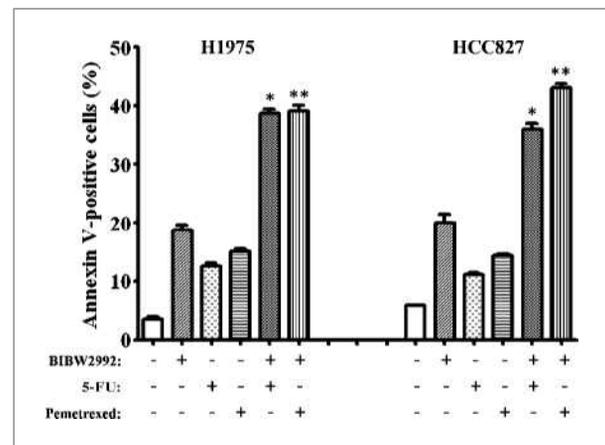

Figure 4. Effects of the combination of BIBW2992 and either 5-FU or pemetrexed on apoptosis in NSCLC cells. H1975 or HCC827 cells were incubated for 72 h with BIBW2992, 5-FU, or pemetrexed at their IC$_{50}$ values, after which the proportion of apoptotic cells was assessed by staining with FITC-conjugated Annexin V and propidium iodide followed by flow cytometry. Columns, mean of triplicates from an experiment that was repeated a total of three times with similar results; bars, SD. *, $P < 0.05$, for the combination of BIBW2992 plus 5-FU versus 5-FU alone; **, $P < 0.05$, for the combination of BIBW2992 plus pemetrexed versus pemetrexed alone.
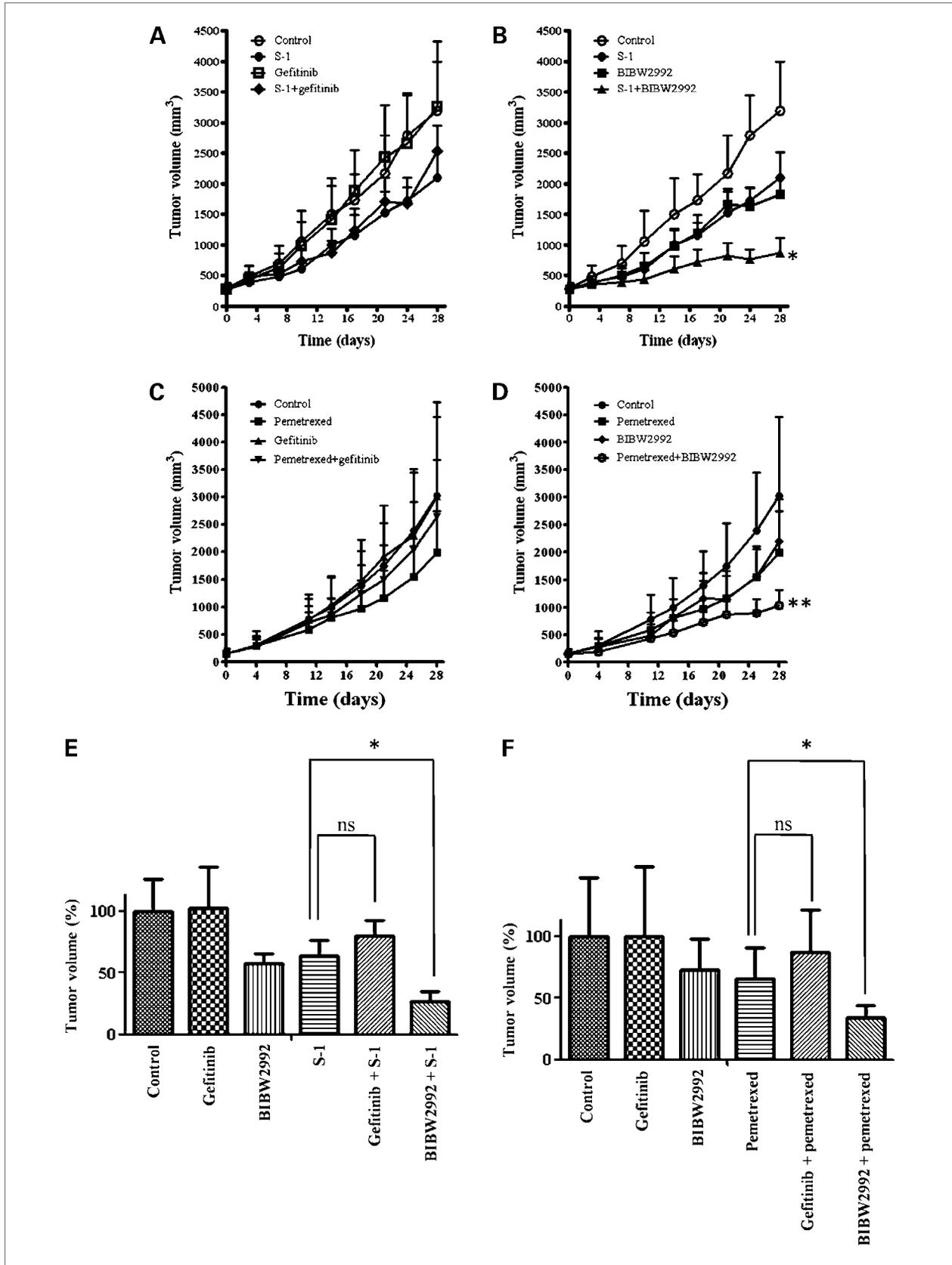
Table S1). We further examined the effects of the combination of the irreversible EGFR-TKI BIBW2992 with either 5-FU or pemetrexed, finding that each drug combination showed a synergistic antiproliferative effect in sensitizing *EGFR* mutation-positive NSCLC cells (Fig. 2B; Supplementary Fig. S1; Supplementary Table S1).

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

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

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

mutation, and this effect correlated with downregulation of TS expression.

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

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

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

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

Figure 5. Effects of combination therapy with S-1 or pemetrexed and either gefitinib or BIBW2992 on the growth of NSCLC cells harboring the T790M mutation of EGFR *in vivo*. Nude mice with tumor xenografts established by s.c. implantation of tumor fragments formed by H1975 cells were treated daily for 4 wk by oral gavage with vehicle (control), S-1 (10 mg/kg), or either gefitinib (50 mg/kg; A) or BIBW2992 (10 mg/kg; B) alone or together with S-1 (10 mg/kg). Alternatively, the mice were treated with vehicle (control), pemetrexed (100 mg/kg, i.p., on days 1, 8, 15, and 22), or either gefitinib (50 mg/kg; C) or BIBW2992 (10 mg/kg; D) alone or together with pemetrexed (100 mg/kg). Tumor volume was determined at the indicated times after the onset of treatment. Points, mean of values from eight mice per group; bars, SE. *, *P* < 0.05, for the combination of S-1 plus BIBW2992 versus either S-1 or BIBW2992 alone; **, *P* < 0.05, for the combination of pemetrexed plus BIBW2992 versus either pemetrexed or BIBW2992 alone. Final tumor volume relative to that in the vehicle-treated (control) group for combined therapy with S-1 and either gefitinib or BIBW2992 (E) or with pemetrexed and either gefitinib or BIBW2992 (F) was also calculated. *, *P* < 0.05. ns, not significant.
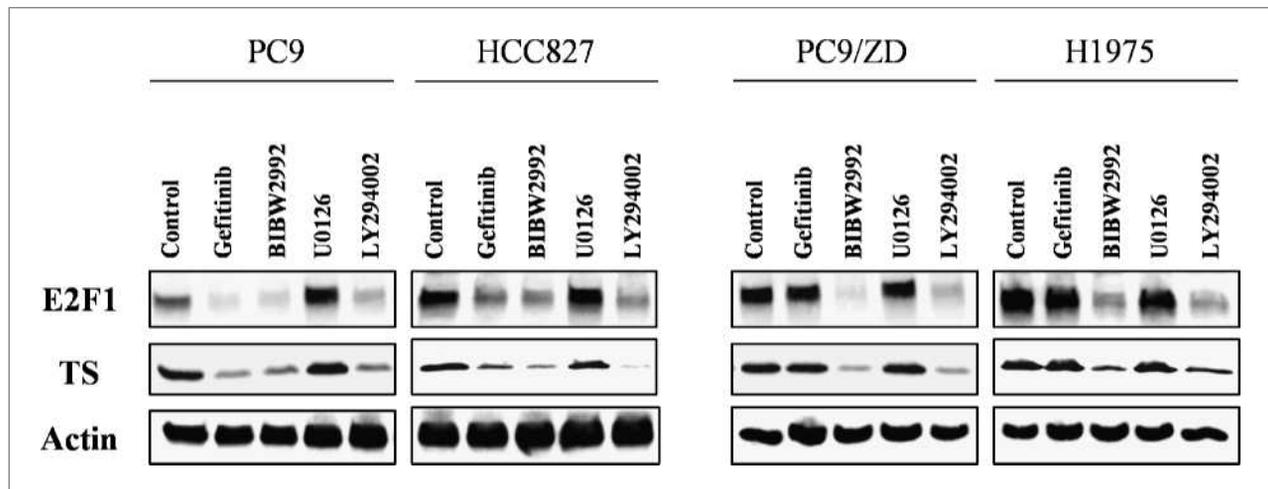
Figure 6. Effects of MEK or PI3K inhibitors on E2F1 and TS expression in NSCLC cells. Cells were incubated for 24 h with vehicle (DMSO, control), gefitinib (1 μmol/L), BIBW2992 (1 μmol/L), U0126 (10 μmol/L), or LY294002 (20 μmol/L for HCC827 and H1975; 50 μmol/L for PC9 and PC9/ZD) in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to E2F1, TS, or β-actin.

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

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

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

Discussion

We have shown that the sensitivity of sensitizing *EGFR* mutation–positive NSCLC cells to the antiproliferative effect of the irreversible EGFR-TKI BIBW2992 is reduced by the acquisition of a secondary T790M mutation of *EGFR*. Consistent with previous observations (9), we found that BIBW2992 inhibited the proliferation of NSCLC cells harboring the T790M mutation with IC_{50} values within the clinically achievable range of serum concentrations (20), whereas gefitinib exhibited no such activity against these cells. However, the IC_{50} value for the antiproliferative effect of BIBW2992 in PC9/ZD cells (which harbor the T790M mutation) was ~1,000 times that in the parental

PC9 cells (which do not harbor the T790M mutation). Indeed, NSCLC cells harboring the T790M mutation were previously found to be more resistant to other irreversible EGFR-TKIs (CL-387,785 and PF00299804) compared with those without the mutation (8, 21). These observations thus indicate that an additional T790M mutation reduces the sensitivity of sensitizing *EGFR* mutation–positive NSCLC cells to irreversible EGFR-TKIs, suggesting that the antitumor effects of these drugs may be limited for the treatment of NSCLC patients. We therefore propose that combination therapy with BIBW2992 and cytotoxic agents is likely to be more effective than treatment with BIBW2992 alone.

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

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

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

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

Disclosure of Potential Conflicts of Interest

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

Ken Takezawa, Isamu Okamoto, Junko Tanizaki, et al.

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