

Enhanced anti-angiogenic effect of E7820 in combination with erlotinib in epidermal growth factor receptor–tyrosine kinase inhibitor-resistant non-small-cell lung cancer xenograft models

Ken Ito,¹ Taro Semba,¹ Toshimitsu Uenaka,^{1,2} Toshiaki Wakabayashi,¹ Makoto Asada³ and Yasuhiro Funahashi^{1,4}

¹Tsukuba Research Laboratory, Eisai Co., Ltd., Tsukuba, Japan; ²Morphotek, Exton, Pennsylvania, USA; ³Eisai Co., Ltd., Tokyo, Japan; ⁴Eisai Inc., Andover, Massachusetts, USA

Key words

Anti-angiogenesis, E7820, erlotinib, integrin, non-small-cell lung cancer

Correspondence

Yasuhiro Funahashi, Eisai Inc., Andover, MA 01810, U.S.A.
Tel: +1-987-837-4807; Fax: +1-978-689-0543;
E-mail: yasuhirou_funahashi@eisai.com

Funding Information

Eisai Co., Ltd.

Received January 13, 2014; Revised May 1, 2014; Accepted May 11, 2014

Cancer Sci 105 (2014) 1023–1031

doi: 10.1111/cas.12450

Most non-small-cell lung cancers (NSCLCs) harboring activating mutations in the epidermal growth factor receptor (EGFR) are initially responsive to EGFR tyrosine kinase inhibitors (EGFR-TKIs); however, they invariably develop resistance to these drugs. E7820 is an angiogenesis inhibitor that decreases integrin- α 2 expression and is currently undergoing clinical trials. We investigated whether E7820 in combination with erlotinib, an EGFR-TKI, could overcome EGFR-TKI-resistance in the NSCLC cell lines A549 (KRAS; G12S), H1975 (EGFR; L858R/T790M), and H1650 (PTEN; loss, EGFR; exon 19 deletion), which are resistant to erlotinib. Immunohistochemical analysis was carried out in xenografted tumors to investigate anti-angiogenesis activity and endothelial cell apoptosis levels by endothelial cell marker CD31 and TUNEL staining, respectively. Treatment with E7820 (50 mg/kg) with erlotinib (60 mg/kg) showed a synergistic antitumor effect in three xenograft models. Immunohistochemical analysis indicated that combined treatment with E7820 and erlotinib significantly decreased microvessel density and increased apoptosis of tumor-associated endothelial cells compared with use of only one of the agents. This combination increased apoptosis in HUVECs through activation of both intrinsic and extrinsic apoptosis pathways *in vitro*. The combination of E7820 with erlotinib is an alternative strategy to overcome erlotinib resistance in NSCLC by enhancement of the anti-angiogenic activity of E7820.

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death both in the USA and Japan.^(1,2) The disease has served as a model for genotype-directed targeted cancer therapy. Patients with NSCLC harboring activating somatic mutations in the kinase domain of epidermal growth factor receptor (EGFR) often initially respond to treatment with EGFR-targeted tyrosine kinase inhibitors (TKIs) such as erlotinib.⁽³⁾ The most frequently observed mutations in NSCLCs are deletions in exon 19 of the *EGFR* gene or the replacement of leucine with arginine at codon 858 (L858R), which together account for ~90% of mutations; various other mutations such as the exon 20 insertions G719X and L861Q have also been reported.⁽⁴⁾ Lung adenocarcinomas that have these ‘driver’ *EGFR* mutations are considered to be ‘oncogene-addicted’ to the EGFR pathway; hence, they are initially sensitive to EGFR tyrosine kinase inhibition.^(5,6)

Despite the benefits of TKIs in the treatment of NSCLCs, many cases, if not all, ultimately develop resistance to these agents.⁽⁴⁾ In ~50% of TKI-resistant NSCLCs, acquired resistance is associated with a secondary mutation, T790M, in *EGFR*.^(7,8) As other features of EGFR-TKI resistance in NSCLC, mutant KRAS and PTEN loss are important factors. In a study of NSCLC patients, KRAS mutations were identi-

fied in more than 25% of NSCLCs, and tumors with oncogenic KRAS responded poorly to cytotoxic agents and molecular-targeted agents such as EGFR-TKIs.^(9,10) Approximately 2–10% of NSCLC patients are considered to have PTEN loss,⁽¹¹⁾ which contributes to erlotinib resistance by activation of Akt and EGFR.⁽¹²⁾ Currently, there is no standard cancer therapy for NSCLCs with acquired resistance to EGFR-TKIs.⁽¹³⁾ Therefore, the identification of strategies or agents that are capable of overcoming acquired resistance to EGFR-TKIs in NSCLC is an important clinical goal.

Erlotinib hydrochloride is a selective and competitive inhibitor of ATP binding to the EGFR tyrosine kinase domain that functions as an antiproliferative agent and is an inducer of apoptosis and reversible cell cycle arrest.^(14–16) *In vivo* preclinical studies have shown that erlotinib markedly reduces levels of phosphorylated mitogen-activated protein kinase (MAPK), which acts downstream of EGFR, and inhibits the growth of tumor cells in a human NSCLC xenograft model.⁽¹⁷⁾ Erlotinib received US Food and Drug Administration approval in 2004 for use in the treatment of advanced NSCLC in patients.⁽¹⁸⁾ However, its efficacy for prolonging the survival of patients is limited.

E7820, an orally active aromatic sulfonamide derivative, is an angiogenesis inhibitor that modulates α -1, α -2, α -3, and α -5

integrin mRNA expression⁽¹⁹⁾ and is currently undergoing phase II clinical trials.⁽²⁰⁾ In preclinical studies, E7820 significantly inhibited basic fibroblast growth factor (bFGF)-induced angiogenesis in several mice models, as well as the growth of seven s.c. implanted tumors.⁽²¹⁾ This inhibition appears to be mediated through suppression of endothelial integrin α -2, α -3, α -5, and β -1 mRNA expression.

Several preclinical studies on EGFR inhibitors in combination with anti-angiogenesis agents have been carried out.^(22,23) Also, several clinical studies evaluating the combination of erlotinib and bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, are ongoing in some cancer types including NSCLC.^(22,24,25) Encouraging data from these preclinical and clinical studies have led to the initiation of preclinical studies of the combination of E7820 and erlotinib in NSCLC models to investigate whether combination therapy of an angiogenesis inhibitor with erlotinib is able to overcome EGFR-TKI resistance.

In the present study, we showed that E7820 given in combination with erlotinib shows a synergistic antitumor activity against EGFR-TKI-resistant tumors, A549 (KRAS; G12S), H1975 (EGFR; L858R/T790M), and H1650 (PTEN; loss, EGFR; exon 19 deletion) cell lines, by enhancing anti-angiogenesis activity through enhanced apoptosis of tumor-associated endothelial cells.

Materials and Methods

Cell lines and culture conditions. Non-small-cell lung cancer cell lines A549, H1975, and H1650 were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μ g/mL). Human umbilical vein endothelial cells were isolated and cultured from human umbilical vein by using a method described previously.⁽¹⁹⁾

Reagents. Pan-caspase inhibitor zVAD-fmk, caspase-9 inhibitor Ac-LEHD-CHO, caspase-3 inhibitor Ac-DEVD-CHO, and caspase-8 inhibitor Ac-IETD-CHO (all from Peptide Institute Inc., Osaka, Japan) were used to inhibit caspase activities.

Drugs. E7820 was synthesized at Eisai Co., Ltd. (Tsukuba-Shi, Japan). E7820 stock solution was prepared as follows: E7820 (25 mg/mL) was dissolved in DMSO (35% [v/v]), followed by addition of Tween 80 (65% [v/v]). E7820 stock solution was diluted with 5% glucose prior to use. Erlotinib was purchased from OSI Pharmaceuticals (Melville, NY, USA). Erlotinib was suspended in DMSO and diluted 10-fold with saline containing 0.1% pluronic P105 prior to use.

Xenograft models. All animal studies were approved by Eisai Institutional Animal Care and Use Committee. A549, H1975, and H1650 cells were s.c. inoculated into 9-week-old female nude mice (KSN-slc mice; Japan SLC, Inc., Shizuoka, Japan). Approximately 10 days after inoculation, E7820 (25 and 50 mg/kg) was given orally twice daily and erlotinib (60 mg/kg) was given orally once a day for 3 weeks. Tumor volume (TV) and relative TV (RTV) were calculated by using a formula described previously.⁽¹⁶⁾ The combination effect on RTV was analyzed by using two-way ANOVA.

Trypan blue exclusion assay. The HUVECs and A549, H1975, and H1650 cells were placed into 6-well plates (1×10^5 cells/well) and incubated for 48 or 96 h at 37°C. The cell viability was then assayed by Trypan blue staining.

Immunohistochemical staining of CD31. Frozen samples of xenografted-tumor were used for identification of CD31. Frozen cross-sections were fixed in cold acetone and washed with PBS.

Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol. After PBS rinses, the sections were incubated overnight at 4°C with the primary antibody (BD Pharmingen, San Diego, CA, USA), and then incubated for 30 min at room temperature with a secondary antibody kit (Vectastain ABC-AP Rat-IgG Kit; Vector Laboratories, Burlingame, CA, USA). A brown color was developed by incubation in diaminobenzidine solution for 1 min at room temperature. In sections stained for CD31, vessel density was quantified in five random fields.

Immunohistochemical staining of proliferating cell nuclear antigen. Paraffin-embedded samples of xenografted-tumor were used for proliferating cell nuclear antigen (PCNA) staining. Sections were deparaffinized, rehydrated in PBS, incubated overnight at 4°C with the primary antibody (Dako, Copenhagen, Denmark), and then incubated for 1 h at room temperature with a secondary antibody (EnVision system; Dako). A brown color was developed with incubation in diaminobenzidine solution for 1 min at room temperature. To quantify PCNA expression, the number of positively stained cells was counted in five random fields.

Double immunofluorescence staining for CD31/TUNEL. Cross-sections of frozen xenografted tumors were mounted on slides and fixed in cold 1% paraformaldehyde in PBS, stained with biotin-conjugated anti-mouse CD31 (BD Pharmingen) as the primary antibody, and then stained with Cy3-conjugated secondary antibody (Arcturus Bioscience, Mountain View, CA, USA). Apoptotic cells were quantified by using a commercially available ApoDIRECT DNA Fragmentation Kit (BioVision, Mountain View, CA, USA). Quantification of apoptotic endothelial cells is expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in five random fields.

Analysis of apoptosis *in vitro* and *in vivo*. To quantify the number of apoptotic cells, 1×10^5 cells were stained with annexin V-FITC and propidium iodide (PI) at room temperature for 15 min by using an annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen) and analyzed by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) described previously.⁽²⁶⁾

The extent of apoptosis in xenografted-tumors was assessed by means of TUNEL assay with a TdT-Fragel DNA-Fragmentation Kit (Chemicon, Temecula, CA, USA), and then TUNEL-stained cells were quantified in five randomly selected microscope fields. The apoptotic index was calculated as the number of positive cells/0.318-mm² field. Five tumors were analyzed per treatment group.

Caspase activity. Caspase-3-like activity was measured according to the manufacturer's instructions (caspase-3-like activity) (Chemicon) by using the FL2 channel of a FACScan flow cytometer.

Determination of cytochrome c release. To assess the release of cytochrome c from mitochondria into the cytoplasm, HUVECs were washed once with PBS, lysed in ice-cold Sodium chloride Tris-EDTA (STE) buffer described previously,⁽²⁶⁾ and immediately centrifuged at 15 000 g for 15 min. The supernatant was then used for Western blot analysis.

Analysis of mitochondrial membrane potential loss. Changes in mitochondrial membrane potential (MMP) following induction of apoptosis were assessed using a Mito Capture apoptosis detection kit (BioVision) according to the manufacturer's instructions. The status of the MMP was analyzed by using the FL1 channel of a FACScan flow cytometer.

Statistical analysis. All data are expressed as the mean \pm SD. Tukey's multiple comparison test was used to evaluate statistical significance between control and treatment groups with

relation to CD31-positive microvessel density (MVD), PCNA-positive cells, annexin V-positive apoptosis cells, and TUNEL positivity. All *P*-values represent two-sided tests of statistical significance. The combination effect was evaluated using two-way ANOVA.

Results

Synergistic antitumor effect of E7820 and erlotinib in EGFR-TKI-resistant human NSCLC xenograft models in nude mice. We investigated the *in vivo* antitumor activities of E7820, erlotinib, and E7820 in combination with erlotinib in nude mice bearing erlotinib-resistant NSCLC A549, H1975, or H1650 cells. Approximately 10 days after tumor transplantation, E7820 (25 or 50 mg/kg) and/or erlotinib (60 mg/kg) were given to the mice. The combination of E7820 at either dose with erlotinib showed a superior antitumor effect compared with E7820 or erlotinib alone in three xenograft models (Fig. 1a, Table 1; $P < 0.05$). The combination of E7820 at a dose of 50 mg/kg with erlotinib at a dose of 60 mg/kg was found to have a significantly synergistic antitumor effect in three xenograft models without severe body weight loss ($P < 0.05$; Fig. 1b, Table 1). These data show that the combination of E7820 with erlotinib is effective against NSCLC xenograft models resistant to EGFR-TKIs.

Combination treatment with E7820 and erlotinib decreased MVD and enhanced apoptosis in tumor-associated endothelial cells in human NSCLC xenograft models. Because E7820 shows anti-angiogenic activity in xenografted human tumors in

mice,^(19,21) we examined MVD in human NSCLC A549, H1975, and H1650 xenograft models after treatment. Nude mice bearing A549, H1975, or H1650 xenografts were treated with E7820 (50 mg/kg), and/or erlotinib (60 mg/kg) for 7 days. Microvessel density in the tumor xenografts was determined by immunohistochemical staining against CD31. Treatment with E7820 or erlotinib alone did not decrease MVD in the A549 xenograft model (Fig. 2a,b). However, E7820 in combination with erlotinib did significantly decrease the number of CD31-positive cells in the A549 xenografted tumor ($P < 0.05$; Fig. 2a,b). We obtained similar results in the H1975 and H1650 xenograft model ($P < 0.05$; Fig. 2a,b).

Next, we determined whether this combination therapy was associated with apoptosis of endothelial cells by using a CD31/TUNEL fluorescent double-labeling technique. Tumor tissues from mice receiving vehicle treatment showed no apoptosis in the tumor-associated endothelial cells in either xenograft model. Treatment of mice with E7820 in combination with erlotinib significantly increased apoptosis in tumor-associated endothelial cells compared to single agent alone in both the A549 and H1975 xenograft models ($P < 0.05$; Fig. 2c,d). These results suggested that the superior antitumor activity of E7820 in combination with erlotinib is caused by a decrease in MVD through the enhancement of apoptosis in tumor-associated endothelial cells.

Combination treatment with E7820 and erlotinib inhibited tumor cell proliferation and enhanced apoptosis in human NSCLC xenograft models. We next examined whether E7820 in combination with erlotinib resulted in the inhibition of tumor cell

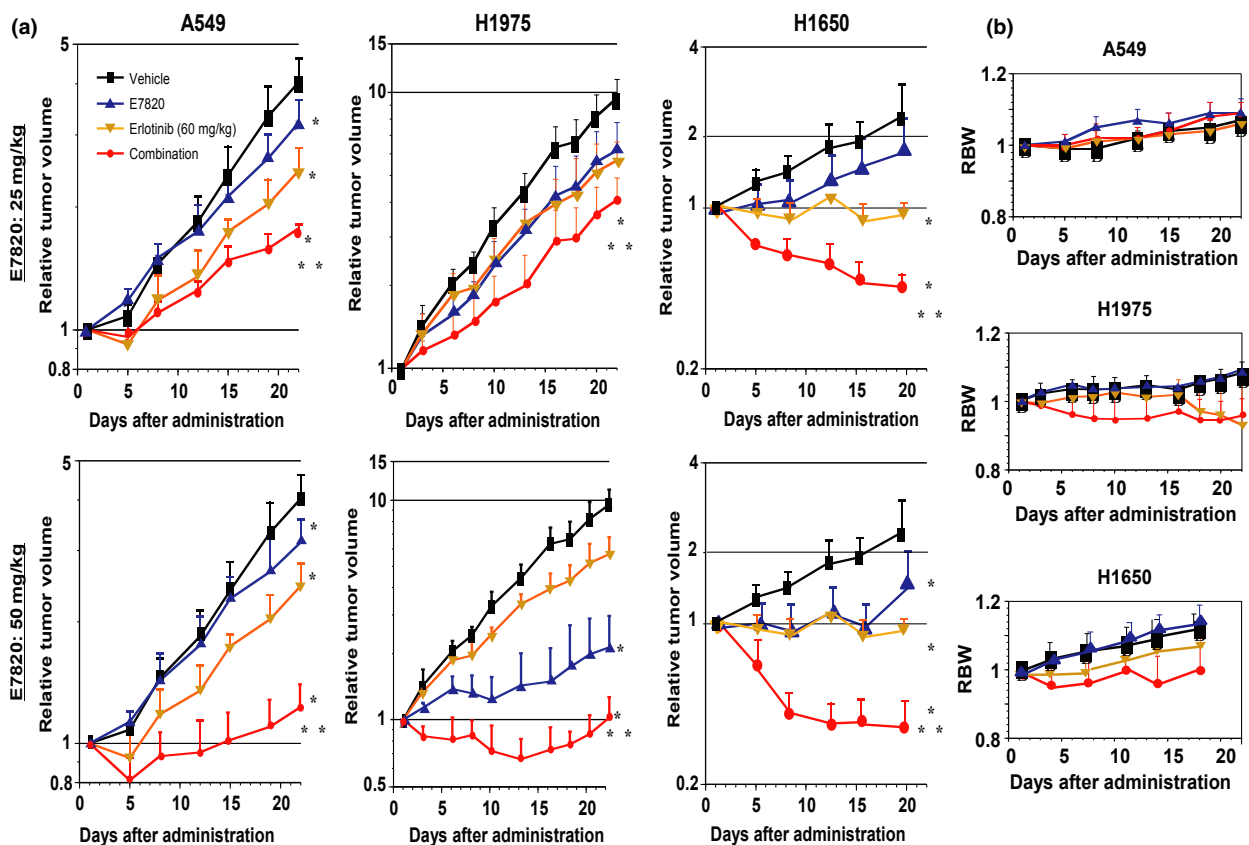


Fig. 1. E7820 in combination with erlotinib enhanced antitumor activity. (a) *In vivo* antitumor activities of E7820 and/or erlotinib in non-small-cell lung cancer xenograft models ($n = 6$, per group). (b) Relative body weight (RBW). Experiment was carried out more than twice. * $P < 0.05$, compared with vehicle. ** $P < 0.05$, compared with erlotinib and E7820.

Table 1. Studies of combined treatment using E7820 and erlotinib in human non-small-cell lung cancer xenograft models

Treatment (mg/kg)	A549		H1975		H1650	
	Tumor volume, mm ³	Δ T/C	Tumor volume, mm ³	Δ T/C	Tumor volume, mm ³	Δ T/C
Vehicle	792 ± 186	100	1362 ± 412	100	333 ± 65	100
Erlotinib (60)	490 ± 95*	48	826 ± 324	55	152 ± 31*	-1
E7820 (25)	638 ± 163*	72	863 ± 202	60	271 ± 93	65
E7820 (25) + erlotinib (60)	347 ± 43***	25	562 ± 122***	36	212 ± 48***	34
E7820 (50)	609 ± 35*	68	304 ± 122*	13	80 ± 18*	-48
E7820 (50) + erlotinib (60)	247 ± 45***	7	145 ± 52***	0	62 ± 16***	-59

Values are means ± SD. Drug efficacy was assessed at day 22 (A549 and H1975) and day 19 (H1650). **P* < 0.05 compared with vehicle group; ***P* < 0.05 compared with erlotinib and E7820 group. ΔT/C (% of control for Δ growth) were calculated from the formula; (ΔT/ΔC) × 100. ΔT and ΔC are changes in tumor volume (Δ growth) for each treated and vehicle control group. The details are described previously.⁽¹⁹⁾

proliferation and enhancement of tumor cell apoptosis in tumor tissues. Nude mice bearing A549, H1975, or H1650 tumors were treated with E7820 (50 mg/kg) and/or erlotinib (60 mg/kg), for 7 days. Staining with PCNA was carried out to assess cell proliferation and TUNEL staining was used to assess apoptosis in the tumor cells. E7820 in combination with erloti-

nib significantly decreased the number of PCNA-positive cells compared with E7820 or erlotinib alone in three xenograft models (*P* < 0.05; Fig. 3a,b). Furthermore, combination treatments of these drugs significantly increased TUNEL-positive tumor cells compared with either E7820 or erlotinib alone in three xenograft models after 7 days of treatment (Fig. 3c,d).

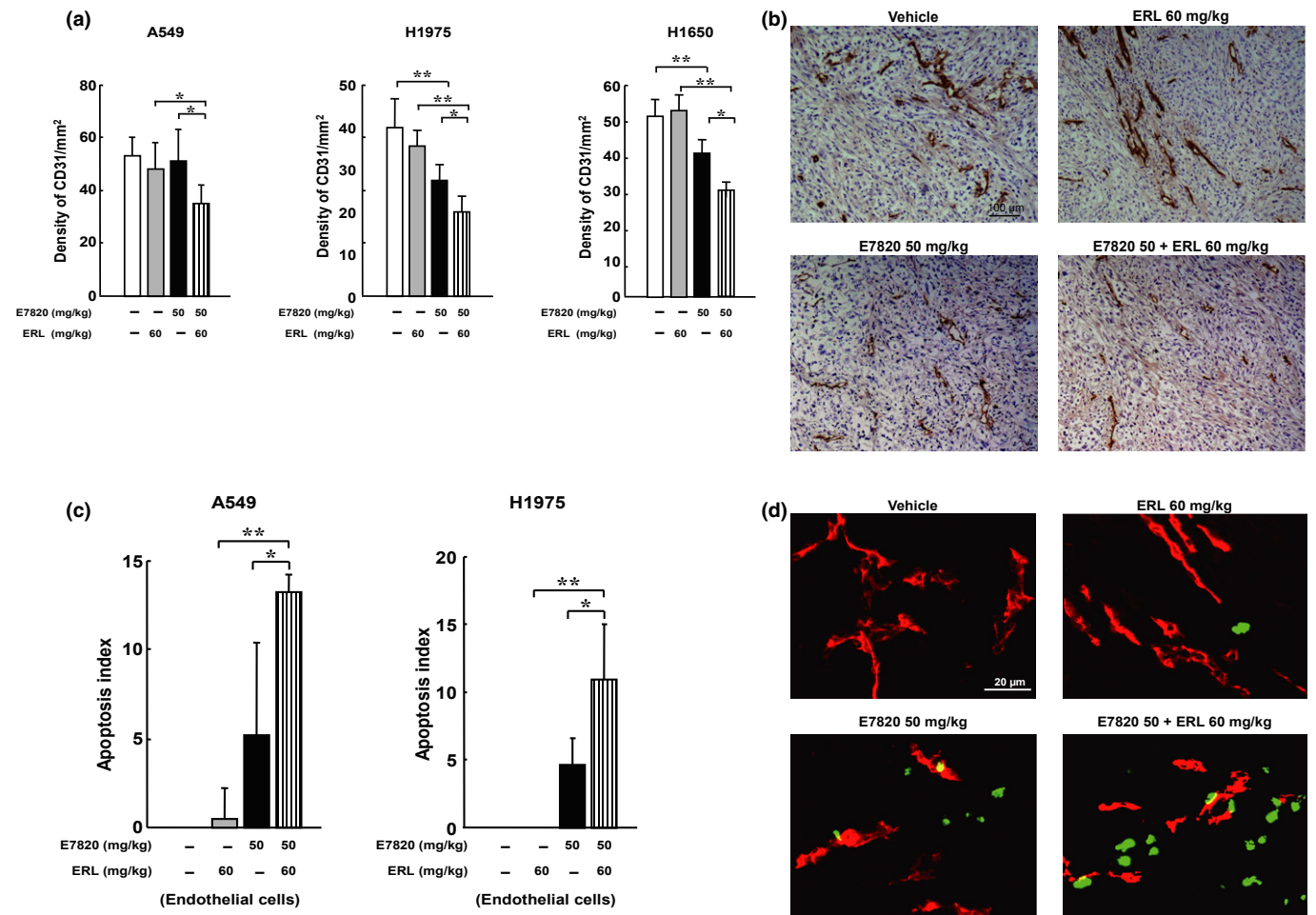


Fig. 2. Combination treatment with E7820 and erlotinib (ERL) preferentially decreased microvessel density and enhanced apoptosis of tumor-associated endothelial cells in non-small-cell lung cancer xenograft models. Mice (*n* = 5, per group) were treated with vehicle, E7820 (50 mg/kg), and/or erlotinib (60 mg/kg) for 7 days. The tumors were resected and processed for immunohistochemical evaluation of CD31. (a) Columns indicate means of density of CD31-positive count. **P* < 0.05 (b) Representative results are shown with CD31 staining in H1975. (c) Double immunofluorescence staining of CD31/TUNEL. Columns indicate means of apoptosis index in endothelial cells. **P* < 0.05; ***P* < 0.01. (d) Representative results are shown with CD31/TUNEL staining in H1975. Endothelial cells (CD31+) stained with red fluorescence and apoptotic cells (TUNEL+) stained with green fluorescence. Colocalization of endothelial cells undergoing apoptosis yielded yellow fluorescence.

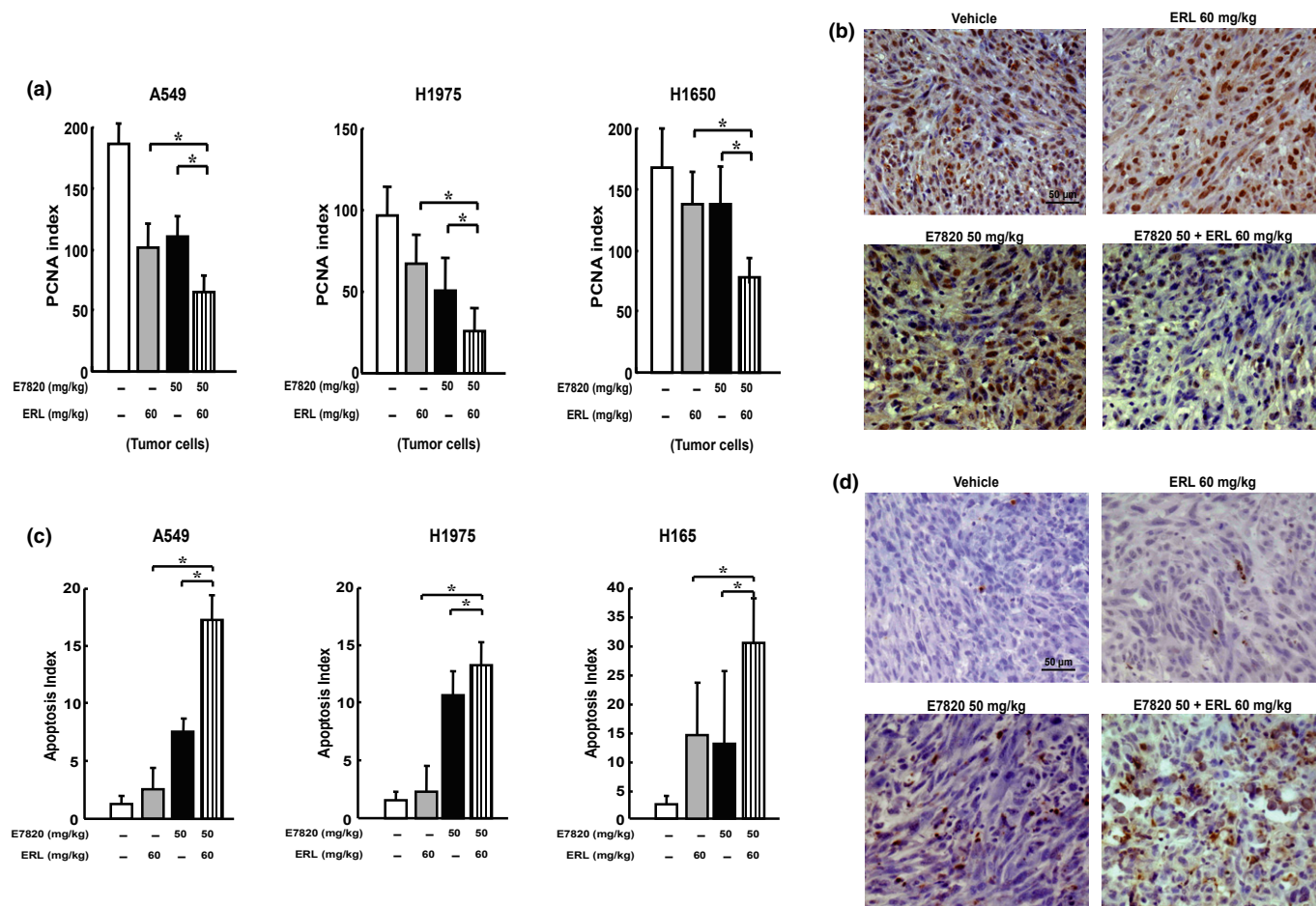


Fig. 3. Combination treatment with E7820 and erlotinib (ERL) inhibited tumor cell proliferation and enhanced apoptosis in human non-small-cell lung carcinoma xenograft models. Mice ($n = 5$, per group) were treated with vehicle, E7820 (50 mg/kg) and/or erlotinib (60 mg/kg) for 7 days. The tumors were resected and processed for immunohistochemical evaluation. (a) Columns indicate means of percentages of proliferating cell nuclear antigen (PCNA)-positive cells/mm². * $P < 0.05$. (b) Representative results are shown with PCNA staining in H1975 xenograft tumor. (c) Columns indicate means of apoptosis-index in tumor cells. * $P < 0.05$. (d) Representative results are shown with TUNEL staining in H1975 xenograft tumor.

These results indicated that the superior antitumor activity of E7820 in combination with erlotinib was caused by decreased tumor cell proliferation and increased tumor cell apoptosis based on enhanced anti-angiogenesis activity.

Combination treatment with E7820 and erlotinib enhanced inhibition of cell proliferation and apoptosis through activation of both intrinsic and extrinsic apoptosis pathways in cultured HUVECs. To confirm the efficacy of E7820 in combination with erlotinib in terms of decreased MVD and enhanced apoptosis of tumor-derived endothelial cells in the NSCLC A549, H1975, and H1650 xenograft models, we next examined the growth-inhibiting and apoptosis-inducing effects of E7820 in combination with erlotinib on cultured HUVECs and also tumor cells (A549, H1975, and H1650). The HUVECs and A549, H1975, and H1650 cells were treated with E7820 and/or erlotinib at the indicated doses for 48 or 96 h. E7820 in combination with erlotinib significantly inhibited HUVEC growth ($P < 0.05$; Fig. 4). Annexin V-PI two-color flow cytometry revealed significantly enhanced apoptosis in HUVECs treated with E7820 in combination with erlotinib compared with either single agent alone or control cells ($P < 0.05$; Fig. 5a,b). The enhancement of apoptosis in HUVECs after combination treatment was confirmed by signifi-

cantly increased MMP loss ($P < 0.05$) and cytochrome c release, which are markers of apoptosis (Fig. 5c,d). Furthermore, the results of annexin V-PI two-color flow cytometry showed that the pan-caspase inhibitor zVAD-fmk significantly inhibited apoptosis in HUVECs that is induced by combination treatment with E7820 and erlotinib ($P < 0.05$; Fig. 5e). Caspase-3 activity was also significantly increased in HUVECs treated with E7820 in combination with erlotinib compared to single agent or control ($P < 0.05$), indicating that E7820 in combination with erlotinib induced apoptosis in a caspase-dependent manner (Fig. 5f). Blockade of pro-apoptosis signals by the caspase-3 inhibitor Ac-DEVD-CHO at a point where the intrinsic and extrinsic apoptosis pathways meet, by the caspase-9 inhibitor Ac-LEHD-CHO at a point downstream of the mitochondria in the intrinsic pathway, or by the caspase-8 inhibitor Ac-IETD-CHO at a point in the extrinsic pathway, resulted in inhibition of the apoptosis induced by combination treatment (Fig. 5e). In terms of tumor cells, E7820 in combination with erlotinib significantly inhibited H1650 cell growth ($P < 0.05$; Fig. 4) and enhanced H1650 cell apoptosis (Figs. 5a,S1). We observed neither inhibition of cell proliferation nor induction of apoptosis in both cell lines (A549 and H1975) after combination treatment (Figs. 4,5a,

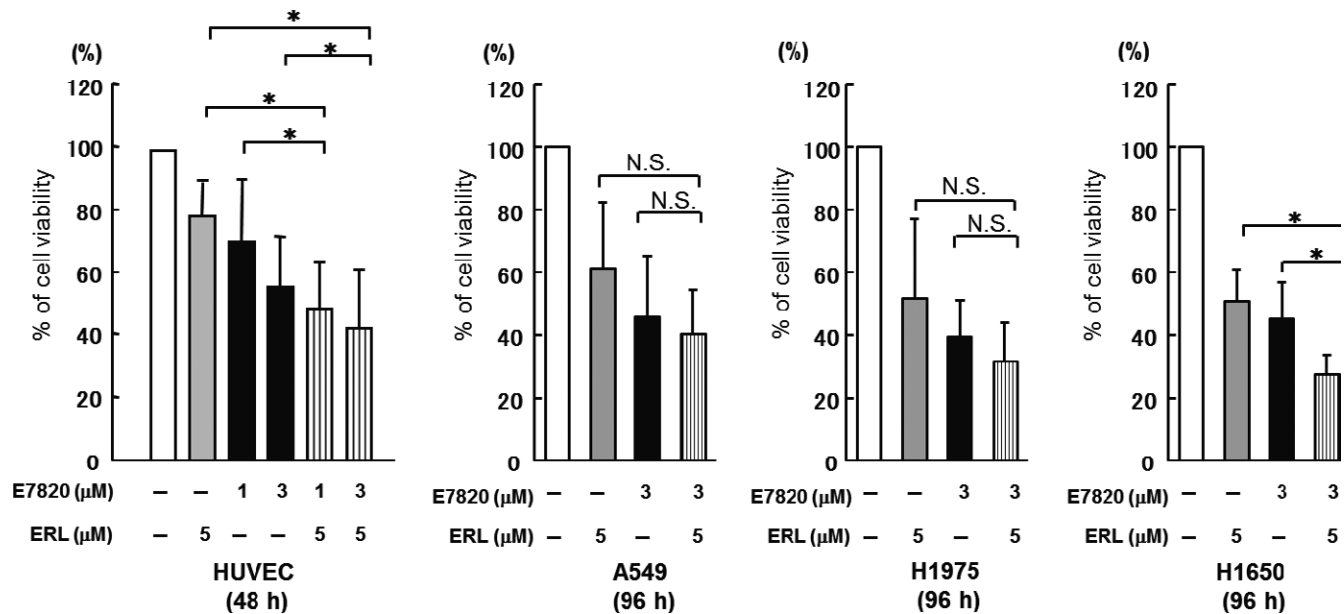


Fig. 4. E7820 in combination with erlotinib (ERL) enhanced inhibition of cell proliferation in HUVECs and H1650 but not A549 and H1975 non-small-cell lung cancer cell lines *in vitro*. HUVECs, A549, H1975, and H1650 cells were treated with E7820 and/or erlotinib for 48 or 96 h then analyzed for cell proliferation. Experiments were carried out three times independently. * $P < 0.05$

S1). These data showed that E7820 in combination with erlotinib inhibited cell proliferation and enhanced apoptosis through activation of both the intrinsic and extrinsic apoptosis pathways in cultured HUVECs.

Discussion

In the present study, the combination of E7820 with erlotinib showed superior antitumor activity compared to treatment with each single agent in mouse xenograft models of human NSCLC, A549 (KRAS; G12S), H1975 (EGFR; L858R/T790M), and H1650 (PTEN; loss, EGFR; exon 19 deletion) cell lines, which are resistant to erlotinib. Combination treatment with these agents showed a synergistic antitumor effect accompanied by a decrease in MVD and an increase in apoptosis in tumor-associated endothelial cells *in vivo*. Furthermore, immunohistochemical analyses showed a reduction of PCNA-positive cells and increased TUNEL-positive tumor cells after treatment with E7820 in combination with erlotinib.

Many studies of combination therapy using bevacizumab, which is an anti-VEGF antibody, with not only cytotoxic agents such as cisplatin or vinflunine,⁽²⁷⁾ but also molecular-targeted agents such as erlotinib, have shown enhanced antitumor effects compared with each single agent.^(22,28) Thus, the strategy of combining bevacizumab with chemotherapeutic drugs shows promise for finding more effective anticancer therapies. However, there remains an unmet medical need for those patients with NSCLC who are ineligible to receive bevacizumab because of squamous histology or brain metastases, or because they are receiving anticoagulant therapy.⁽²⁹⁾

Developments of next-generation angiogenesis inhibitors, which do not target the VEGF signal, are expected to improve the efficacy of anti-angiogenesis therapies. Currently, integrin-targeted agents are being investigated in both preclinical and clinical studies as potential next-generation angiogenesis inhibitors.⁽³⁰⁾ Indeed, in preclinical models, blocking integrin-mediated

signaling suppresses angiogenesis and tumor growth.⁽³¹⁾ Two integrin-targeted agents, cilengitide, a synthetic peptide that inhibits the binding of integrins $\alpha v \beta 3$ and $\alpha v \beta 5$ to the extra cellular matrix (ECM), and volociximab, a chimeric mAb that blocks fibronectin binding to $\alpha 5 \beta 1$, are currently undergoing phase II clinical trials in patients with NSCLC.^(32,33) The targeting of integrin $\alpha 2$ with E7820 may produce a different antitumor spectrum compared with bevacizumab.

In clinical practice, most NSCLCs develop EGFR-TKI resistance through a second-site mutation in EGFR (T790M).⁽⁸⁾ This mutation is assumed to inhibit the ability of gefitinib or erlotinib to form a critical hydrogen bond within the ATP-binding pocket of the catalytic region of EGFR.⁽⁷⁾ To overcome EGFR-TKI resistance in NSCLC, several preclinical and clinical studies are being carried out with the aim of developing dual inhibitors against EGFR and other receptor tyrosine kinases: MP-412, HKI-272, and BIBW-2992 are novel dual TKIs for EGFR and ErbB2; XL-647 is a novel dual TKI for EGFR and VEGFR; and BMS-690514 is a novel pan-EGFR/VEGFR inhibitor.^(34–38) KRAS mutation and PTEN loss are other mechanisms of resistance to EGFR inhibitors that develops in NSCLC.^(8,12) Ganetespib, which is a heat shock protein (HSP)-90 inhibitor, is effective against the KRAS gain-of-function mutation that predominantly arises as single amino acid substitutions at residues G12, G13, and/or Q61.⁽⁹⁾ Some kinase inhibitors are currently undergoing clinical trials by targeting the PI3K/AKT/mTOR pathway, which may be effective to PTEN-loss tumors.⁽³⁹⁾ These approaches may acquire other types of resistance, because they mainly target the tumor cell itself.

E7820 in combination with erlotinib enhanced anti-angiogenesis activity through increased apoptosis in endothelial cells from erlotinib-resistant tumors, and this combination approach may be useful to overcome EGFR-TKI-resistant tumors, regardless of EGFR, KRAS mutation, or PTEN status. Our results suggest that E7820 causes apoptosis triggered by

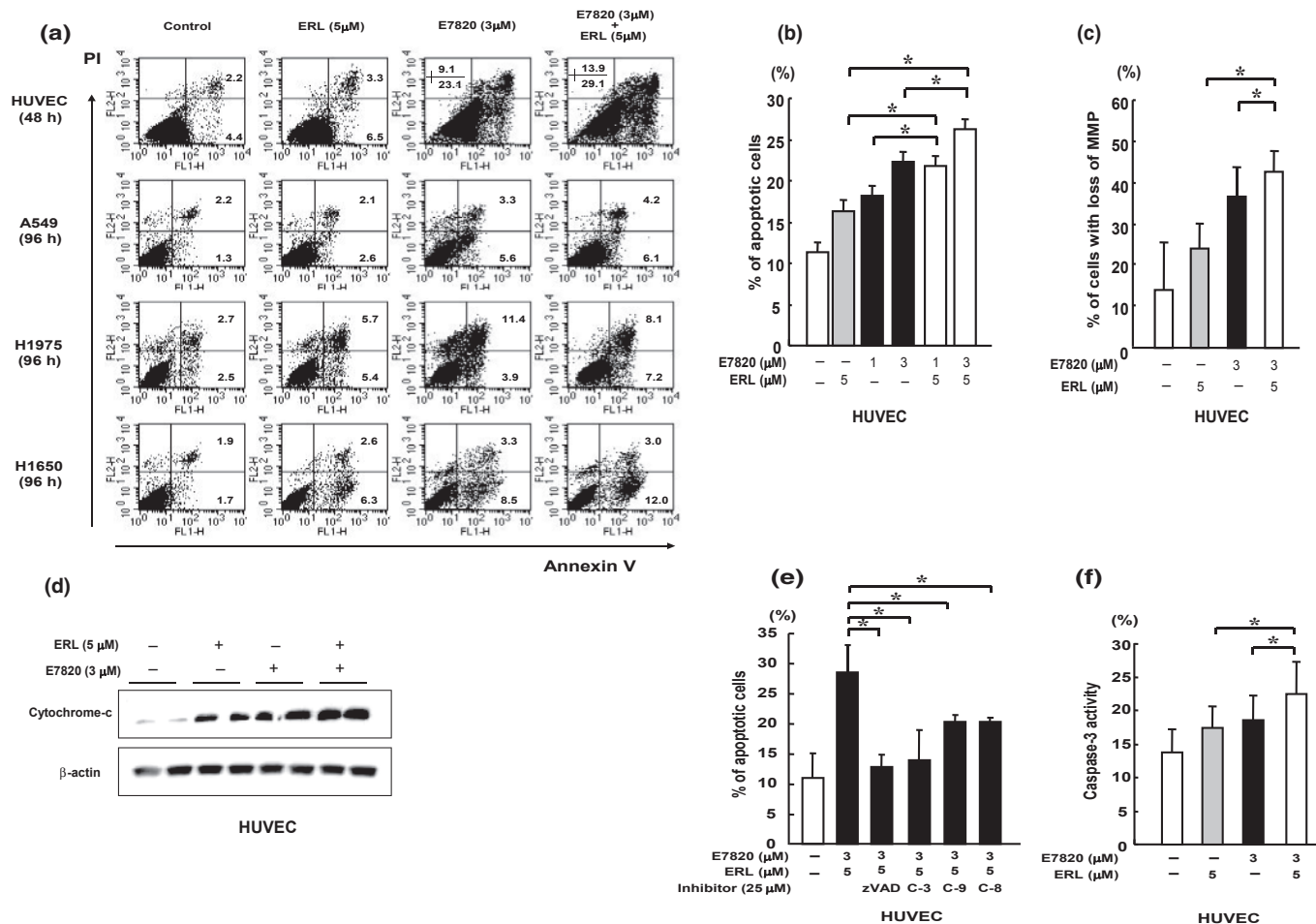


Fig. 5. E7820 in combination with erlotinib (ERL) enhanced apoptosis in cultured HUVECs through activation of both the intrinsic and extrinsic apoptosis pathways. The HUVECs and A549, H1975, and H1650 cells were treated with E7820 and/or erlotinib in the presence or absence of broad caspase inhibitor (zVAD-fmk), caspase-3, caspase-9, and caspase-8 inhibitor for 48 or 96 h. The experiments were carried out three times independently. (a) Cells were analyzed for apoptosis by annexin V–propidium iodide (PI) two-color flow cytometry. Representative results are shown in dot-plots. (b) Columns indicate means of percentage of apoptotic cells in HUVECs. * $P < 0.05$ (c) Loss of mitochondrial membrane potential (MMP). Columns indicate means of percentages of cells with loss of MMP. * $P < 0.05$. (d) Mitochondrial release of cytochrome c in HUVECs. Representative results of Western blot analysis. (e) Columns indicate means of percentage of apoptotic cells in HUVECs. * $P < 0.05$ (f) Caspase-3-like activity. Columns indicate means of percentages of caspase-3 activity. * $P < 0.05$

reactive oxygen species generation, which consequently induces MMP loss in HUVECs (KI and YF, unpublished data). Epidermal growth factor stimulation protects HUVECs against chemically induced pro-apoptotic stimuli by signals transmitted through PI-3K and ERKs.⁽⁴⁰⁾ This evidence suggests that the different mechanism of action of erlotinib compared to that of E7820 might result in greater induction of apoptosis in endothelial cells. Integrin $\alpha 2\beta 1$ plays a key role in angiogenesis development⁽⁴¹⁾ and cross-talk between EGF and integrin $\alpha 2\beta 1$ through MAPK activation has been reported in many types of cancers.^(42,43) E7820 in combination with erlotinib reduced p-Akt levels in HUVECs and H1975 and H1650 cells (Fig. S2). This has led to the hypothesis that treatment with E7820 in combination with EGFR-TKIs may be a promising strategy for treating NSCLC that merits further investigation in a clinical setting.

Erlotinib has been shown to cause more tolerable adverse effects than conventional chemotherapy.⁽⁴⁴⁾ Rash and diarrhea are two well-known side-effects of erlotinib treatment.⁽¹⁰⁾ The adverse effects of E7820 at the clinical recommended dose (100 mg/day) are nausea, fatigue, diarrhea, and anemia, which

are all grade 1 or 2.⁽¹⁷⁾ In the present study, we showed a synergistic antitumor activity with 60 mg/kg (3/5 the maximum tolerated dose [MTD]) of erlotinib combined with 25 mg/kg (1/8 MTD) or 50 mg/kg (1/4 MTD) of E7820 in NSCLC xenograft models without severe body weight loss. These results show that combination therapy with these two drugs may not enhance the adverse effects seen with the single agents.

In conclusion, we showed that treatment with E7820 in combination with erlotinib showed synergistic antitumor activity through enhancement of apoptosis in tumor-associated endothelial cells, decreased MVD, and inhibition of tumor growth, along with induction of tumor cell apoptosis in three EGFR-TKI-resistant NSCLC tumor cell lines, A549, H1975, and H1650. These cumulative results suggest that E7820 combined with erlotinib merits further investigation in a clinical setting as an alternative strategy to overcome EGFR-TKI-resistant NSCLC based on angiogenesis inhibition.

Acknowledgments

We thank Dr. Toru Haneda for drug production of E7820.

Disclosure Statement

All authors are employees of Eisai Co., Ltd.

References

- 1 Jemal A, Siegel R, Ward E *et al.* Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71–96.
- 2 Katanoda K, Matsuda T, Matsuda A *et al.* An updated report of the trends in cancer incidence and mortality in Japan. *Jpn J Clin Oncol* 2013; **43**: 492–507.
- 3 Lynch TJ, Bell DW, Sordella R *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**: 2129–39.
- 4 Rosell R, Moran T, Queralt C *et al.* Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009; **361**: 958–67.
- 5 Paez JG, Janne PA, Lee JC *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.
- 6 Ji H, Li D, Chen L *et al.* The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell* 2006; **9**: 485–95.
- 7 Kobayashi S, Boggon TJ, Dayaram T *et al.* EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005; **352**: 786–92.
- 8 Pao W, Miller VA, Politi KA *et al.* Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005; **2**: e73.
- 9 Acquaviva J, Smith DL, Sang J *et al.* Targeting KRAS-mutant non-small cell lung cancer with the Hsp90 inhibitor ganetespib. *Mol Cancer Ther* 2012; **11**: 2633–43.
- 10 Riely GJ, Marks J, Pao W. KRAS mutations in non-small cell lung cancer. *Proc Am Thorac Soc* 2009; **6**: 201–5.
- 11 Jin G, Kim MJ, Jeon HS *et al.* PTEN mutations and relationship to EGFR, ERBB2, KRAS, and TP53 mutations in non-small cell lung cancers. *Lung cancer* 2010; **69**: 279–83.
- 12 Sos ML, Koker M, Weir BA *et al.* PTEN loss contributes to erlotinib resistance in EGFR-mutant lung cancer by activation of Akt and EGFR. *Cancer Res* 2009; **69**: 3256–61.
- 13 Nguyen KS, Neal JW. First-line treatment of EGFR-mutant non-small-cell lung cancer: the role of erlotinib and other tyrosine kinase inhibitors. *Biologics* 2012; **6**: 337–45.
- 14 Kim TE, Murren JR. Erlotinib OSI/Roche/Genentech. *Curr Opin Investig Drugs* 2002; **3**: 1385–95.
- 15 Huether A, Hopfner M, Sutter AP, Schuppan D, Scherubl H. Erlotinib induces cell cycle arrest and apoptosis in hepatocellular cancer cells and enhances chemosensitivity towards cytostatics. *J Hepatol* 2005; **43**: 661–9.
- 16 Sutter AP, Hopfner M, Huether A, Maaser K, Scherubl H. Targeting the epidermal growth factor receptor by erlotinib (Tarceva) for the treatment of esophageal cancer. *Int J Cancer* 2006; **118**: 1814–22.
- 17 Friess T, Scheuer W, Hasmann M. Erlotinib antitumor activity in non-small cell lung cancer models is independent of HER1 and HER2 overexpression. *Anticancer Res* 2006; **26**: 3505–12.
- 18 Gridelli C, Bareschino MA, Schettino C, Rossi A, Maione P, Ciardiello F. Erlotinib in non-small cell lung cancer treatment: current status and future development. *Oncologist* 2007; **12**: 840–9.
- 19 Funahashi Y, Sugi NH, Semba T *et al.* Sulfonamide derivative, E7820, is a unique angiogenesis inhibitor suppressing an expression of integrin alpha2 subunit on endothelium. *Cancer Res* 2002; **62**: 6116–23.
- 20 Mita M, Kelly KR, Mita A *et al.* Phase I study of E7820, an oral inhibitor of integrin alpha-2 expression with antiangiogenic properties, in patients with advanced malignancies. *Clin Cancer Res* 2011; **17**: 193–200.
- 21 Semba T, Funahashi Y, Ono N *et al.* An angiogenesis inhibitor E7820 shows broad-spectrum tumor growth inhibition in a xenograft model: possible value of integrin alpha2 on platelets as a biological marker. *Clin Cancer Res* 2004; **10**: 1430–8.
- 22 Zappa F, Droege C, Betticher D *et al.* Bevacizumab and erlotinib (BE) first-line therapy in advanced non-squamous non-small-cell lung cancer (NSCLC) (stage IIIB/IV) followed by platinum-based chemotherapy (CT) at disease progression: a multicenter phase II trial (SAKK 19/05). *Lung Cancer* 2012; **78**: 239–44.
- 23 Schicher N, Paulitschke V, Swoboda A *et al.* Erlotinib and bevacizumab have synergistic activity against melanoma. *Clin Cancer Res* 2009; **15**: 3495–502.
- 24 Montagna E, Canello G, Bagnardi V *et al.* Metronomic chemotherapy combined with bevacizumab and erlotinib in patients with metastatic HER2-negative breast cancer: clinical and biological activity. *Clin breast Cancer* 2012; **12**: 207–14.
- 25 Yoo DS, Kirkpatrick JP, Craciunescu O *et al.* Prospective trial of synchronous bevacizumab, erlotinib, and concurrent chemoradiation in locally advanced head and neck cancer. *Clin Cancer Res* 2012; **18**: 1404–14.
- 26 Ito K, Oji Y, Tatsumi N *et al.* Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. *Oncogene* 2006; **25**: 4217–29.
- 27 Kaira K, Tomizawa Y, Yoshino R *et al.* Phase II study of oral S-1 plus cisplatin with bevacizumab for advanced non-squamous non-small cell lung cancer. *Lung Cancer* 2013; **82**: 449–54.
- 28 Zappa F, Droege C, Betticher D *et al.* Bevacizumab and erlotinib (BE) first-line therapy in advanced non-squamous non-small-cell lung cancer (NSCLC) (stage IIIB/IV) followed by platinum-based chemotherapy (CT) at disease progression: a multicenter phase II trial (SAKK 19/05). *Lung Cancer* 2012; **78**: 239–44.
- 29 Perez-Moreno P, Brambilla E, Thomas R, Soria JC. Squamous cell carcinoma of the lung: molecular subtypes and therapeutic opportunities. *Clin Cancer Res* 2012; **18**: 2443–51.
- 30 Rogosin S, Sandler AB. Beyond bevacizumab: antiangiogenic agents. *Clin Lung Cancer* 2012; **13**: 326–33.
- 31 Ramakrishnan V, Bhaskar V, Law DA *et al.* Preclinical evaluation of an anti-alpha5beta1 integrin antibody as a novel anti-angiogenic agent. *J Exp Ther Oncol* 2006; **5**: 273–86.
- 32 Hariharan S, Gustafson D, Holden S *et al.* Assessment of the biological and pharmacological effects of the alpha nu beta3 and alpha nu beta5 integrin receptor antagonist, cilengitide (EMD 121974), in patients with advanced solid tumors. *Ann Oncol* 2007; **18**: 1400–7.
- 33 Besse B, Tsao LC, Chao DT *et al.* Phase Ib safety and pharmacokinetic study of volociximab, an anti-alpha5beta1 integrin antibody, in combination with carboplatin and paclitaxel in advanced non-small-cell lung cancer. *Ann Oncol* 2013; **24**: 90–6.
- 34 Suzuki T, Fujii A, Ohya J *et al.* Antitumor activity of a dual epidermal growth factor receptor and ErbB2 kinase inhibitor MP-412 (AV-412) in mouse xenograft models. *Cancer Sci* 2009; **100**: 1526–31.
- 35 Awada A, Dirix L, Manso Sanchez L *et al.* Safety and efficacy of neratinib (HKI-272) plus vinorelbine in the treatment of patients with ErbB2-positive metastatic breast cancer pretreated with anti-HER2 therapy. *Ann Oncol* 2013; **24**: 109–16.
- 36 Solca F, Dahl G, Zoephel A *et al.* Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker. *J Pharmacol Exp Ther* 2012; **343**: 342–50.
- 37 Pietanza MC, Gadgeel SM, Dowlati A *et al.* Phase II study of the multitargeted tyrosine kinase inhibitor XL647 in patients with non-small-cell lung cancer. *J Thorac Oncol* 2012; **7**: 856–65.
- 38 de La Motte Rouge T, Galluzzi L, Olaussen KA *et al.* A novel epidermal growth factor receptor inhibitor promotes apoptosis in non-small cell lung cancer cells resistant to erlotinib. *Cancer Res* 2007; **67**: 6253–62.
- 39 Dillon LM, Miller TW. Therapeutic targeting of cancers with loss of PTEN function. *Curr Drug Targets* 2014; **15**: 65–79.
- 40 Vinci MC, Visentin B, Cusinato F, Nardelli GB, Trevisi L, Luciani S. Effect of vascular endothelial growth factor and epidermal growth factor on iatrogenic apoptosis in human endothelial cells. *Biochem Pharmacol* 2004; **67**: 277–84.
- 41 San Antonio JD, Zoeller JJ, Habursky K *et al.* A key role for the integrin alpha2beta1 in experimental and developmental angiogenesis. *Am J Pathol* 2009; **175**: 1338–47.
- 42 Kawahara E, Nakada N, Hikichi T, Kobayashi J, Nakanishi I. EGF and beta1 integrin convergently regulate migration of A431 carcinoma cell through MAP kinase activation. *Exp Cell Res* 2002; **272**: 84–91.
- 43 Krensel K, Lichtner RB. Selective increase of alpha2-integrin sub-unit expression on human carcinoma cells upon EGF-receptor activation. *Int J Cancer* 1999; **80**: 546–52.
- 44 Rosell R, Carcereny E, Gervais R *et al.* Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EORTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 2012; **13**: 239–46.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. E7820 in combination with erlotinib enhanced apoptosis in H1650 cells but not A549 and H1975 cells. Cells were treated with E7820 and/or erlotinib for 96 hours. The columns means of percent of apoptotic cells. $*P < 0.05$.

Fig. S2. E7820 in combination with erlotinib reduced p-Akt in HUVEC, H1975, and H1650 cells but not A549 cells. Cells were incubated with erlotinib and/or E7820 for 24 hours (HUVEC) and 96 hours (A549, H1975, and H1650).