Combination Treatment with Erlotinib and Pertuzumab against Human Tumor Xenografts Is Superior to Monotherapy

Thomas Friess, Werner Scheuer, and Max Hasmann

Abstract

In many solid tumors, overexpression of human epidermal growth factor receptors (e.g., HER1/EGFR and HER2) correlates with poor prognosis. Erlotinib (Tarceva) is a potent HER1/EGFR tyrosine kinase inhibitor. Pertuzumab (Omnitarg), a novel HER2-specific, recombinant, humanized monoclonal antibody, prevents heterodimerization of HER2 with other HERs. Both mechanisms disrupt signaling pathways, resulting in tumor growth inhibition. We evaluated whether inhibition of both mechanisms is superior to monotherapy in tumor cell lines expressing different HER levels. Human non–small cell lung cancer (NSCLC) cells (Calu-3: HER1/EGFR 0+, HER2 3+; QG56: HER1/EGFR 2-3+, HER2 0+) and breast cancer cells (KPL-4: HER1/EGFR 2-3+, HER2 3+) were implanted into BALB/c nu/nu mice and severe combined immunodeficient mice, respectively. Drug monotherapy had antitumor activity in all models. Tumor volume treatment-to-control ratios (TCR) with erlotinib were 0.36 (Calu-3), 0.79 (QG56), and 0.51 (KPL-4). Pertuzumab TCR values were 0.42, 0.51, and 0.64 in Calu-3, QG56, and KPL-4 models, respectively. Combination treatment resulted in greater than additive (QG56: TCR 0.39; KPL-4: TCR 0.38) or additive (Calu-3: TCR 0.12) antitumor activity. Serum tumor markers for NSCLC (Cyfra 21.1) and breast cancer (soluble HER2) were markedly inhibited by combination treatment (80-97% in Calu-3 and QG56; 92% in KPL-4), correlating with decreased tumor volume. Overall, erlotinib and pertuzumab are active against various human xenograft models, independently of HER1/EGFR or HER2 expression. A combination of these HER-targeted agents resulted in additive or greater than additive antitumor activity.

Lung and breast cancer are among the most prevalent types of cancer and account for up to 30% of all cancer-related deaths (1–3). Unfortunately, advanced disease in either cancer type cannot be cured. With current treatment options, median survival times are low for non–small cell lung cancer (NSCLC; e.g., 12–15 months; refs. 4, 5) and moderate for breast cancer (2–4 years; ref. 6). Thus, there is an urgent need for new therapies to survival and quality of life in these cancers.

Tyrosine kinase receptors, like human epidermal growth factor receptors (HER, EGFR), are pivotal in regulating cell proliferation and differentiation (reviewed in ref. 7). The HER group consists of four transmembrane receptors (HER1/EGFR, HER2, HER3, and HER4) with homodimerization and heterodimerization providing signaling diversity (8). Many cancer types have abnormal or enhanced expression of HER1/EGFR or HER2, and possibly HER3 and HER4, suggesting a potential role in tumorigenesis. For example, in NSCLC and breast cancer, HER2 can be overexpressed (9, 10). Overexpression of HER1/EGFR and HER2 in cancer can be associated with advanced disease, metastasis, and poor survival (11–15). Thus, these receptors are potential targets for the development of novel anticancer therapies (16, 17).

Erlotinib (Tarceva) is a highly potent, orally available, reversible inhibitor of HER1/EGFR tyrosine kinase. This enzyme activity is activated by extracellular binding of specific ligands like epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) and is potentiated by dimerization of activated receptors (Fig. 1A). In in vitro and in vivo studies, erlotinib inhibited several key events downstream of the receptor, resulting in growth inhibition in a variety of human tumors (18, 19). Single-agent erlotinib had greater antitumor activity against slow-growing versus fast-growing human NSCLC xenografts, independently of HER1/EGFR and HER2 expression (20, 21). In phase I and II clinical trials, erlotinib has shown activity in a wide range of tumor types, including NSCLC, head and neck, colorectal, pancreatic, and metastatic breast cancers (22–26). A phase III trial showed that erlotinib monotherapy in a second/third-line setting significantly prolonged survival, delayed disease progression, and delayed worsening of lung cancer–related symptoms in patients with advanced, relapsed NSCLC (27). Erlotinib, in combination...
with gemcitabine, significantly improved survival in advanced pancreatic cancer compared with gemcitabine alone in a recent phase III trial (28).

Pertuzumab (Omnitarg, formerly designated rhuMAb 2C4) is a humanized monoclonal antibody (mAb) against the dimerization domain of HER2. This agent is the first in a new class of targeted therapeutics known as HER dimerization inhibitors (29). Following binding to HER2, pertuzumab blocks ligand-associated heterodimerization with other HERs (Fig. 1B). As a result, downstream signaling pathways and cellular processes associated with tumor growth and progression are inhibited as shown by in vitro and in vivo models (30). Pertuzumab has undergone phase I trials in patients with advanced solid malignancies (31, 32) and is currently in phase II clinical trials in NSCLC, metastatic breast, ovarian, and prostate cancers.

Tumor growth involves multiple signaling pathways and cellular processes. Thus, the combination of agents that inhibit more than one crucial pathway/process, such as erlotinib and pertuzumab, could be an effective therapeutic strategy for patients with solid tumors. Recently, it has been shown that the combination of gefitinib and the phosphatidylinositol 3’ kinase inhibitor LY294002 is significantly more effective than either drug alone (33). Erlotinib in combination with bevacizumab (Avastin, a recombinant humanized monoclonal anti–vascular endothelial growth factor antibody) is being evaluated in a phase I/II trial in patients with recurrent NSCLC (34).

The primary objective of the current study was to examine the antitumor activity of erlotinib in combination with pertuzumab in human NSCLC and breast cancer xenograft models. Further aims were to examine the effects of single-agent therapy on tumor growth and on levels of serum tumor markers.

Materials and Methods

Test agents and vehicles. Erlotinib hydrochloride was supplied as a fine powder (F. Hoffmann-La Roche, Nutley, NJ) and stored at 4 °C. A frozen (−20°C) stock solution of pertuzumab (25 mg/mL) was obtained from Genentech, Inc. (San Francisco, CA). The formulation vehicles were Captisol (sulfobutyl ether β-cyclodextrin, sodium salt, 6% solution in water; Cydex, Inc., Lenexa, KS), and buffer [10 mmol/L L-histidine, 240 mmol/L sucrose, 0.02% polysorbate 20 (pH 6)] for erlotinib and pertuzumab, respectively. Dosing preparations of both agents were prepared on the day of use.

Animals. Female mice, 6 to 8 weeks old, were purchased from Bomholtgard (Ry, Denmark; BALB/c nu/nu nude) or Charles River (Sulzfeld, Germany; severe combined immunodeficient (SCID) beige). Animals were housed in suitable cages under specified pathogen-free conditions in rooms maintained at 23 ± 3°C and 50% humidity, with a 12-hour light/12-hour dark cycle, according to Committee Guidelines (CV-Solas; Falasas, Tienshch). The mice were quarantined for at least a week. Food (Standard 1320 and 1430; Altromin, Lage, Germany) and acidified water (pH 2.5–3.0) were available ad libitum. Regular health checks were done. The experimental study protocol was reviewed and approved by the local government.
Cell lines and culture conditions. Chugai Pharmaceuticals, Co., Ltd. (Kamakura, Kanagawa, Japan) supplied the NSCLC cell lines, Calu-3 and QG56. KPL-4, human inflammatory breast cancer cells (35), were kindly provided by Prof. J Kurebayashi (Kawasaki Medical School, Kurashiki, Japan).

Monolayers of all cell lines were cultured in flask (Greiner Bio-One GmbH, Frickenhausen, Germany). Calu-3 and QG56 cells were maintained in RPMI 1640 (PAA Laboratories, Linz, Austria) and KPL-4 cells were cultured in DMEM medium (PAA Laboratories). For all cell lines, the medium was supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories) and 2 mmol/L L-glutamine (Gibco Invitrogen, Karlsruhe, Germany). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Cells were passaged using trypsin/EDTA (1:1, Roche Diagnostics, Mannheim, Germany) for cell detachment every 3 days (Calu-3 and QG56) or twice a week (KPL-4).

On the day of tumor cell injection, cells were detached from the flasks with trypsin/EDTA and transferred into 50 mL of the appropriate culture medium, washed once in medium, and resuspended in PBS.

Cell concentration and cell size were determined using a cell counter and analyzer system (Vi-CELL, Beckman Coulter, GmbH, Krefeld, Germany).

Tumor growth inhibition studies in vivo. Mice were anesthetized with isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethylether; CuraMED Pharma, Karlsruhe, Germany). One hundred–microliter suspensions of Calu-3 or QG56 tumor cells (final concentration: 4 x 10^5-5 x 10^6 cells/mL; 4 x 10^5-3 x 10^6 cells/mouse) were transplanted s.c. into the right flank of BALB/c nu/nu nude mice using a 1.0 mL tuberculin syringe (needle 26G x 1 in.; 4.05 x 25 mm; Dispomed Witt oHG, Gelnhausen, Germany). KPL-4 cells (3 x 10^6 cells/mouse in 20 mL of culture medium) were orthotopically transplanted using a Hamilton syringe with a 30G x 1/2 inch needle into the right retro-orbital sinus under anesthesia and serum was prepared (6,800 rpm, 15 min). Mice were anesthetized with isoflurane (1-chloro-2,2,2-trifluoroethyldifluoromethylether; Curamed Pharma, Karlsruhe, Germany). One hundred—microliter suspensions of Calu-3 or QG56 tumor cells (final concentration: 4 x 10^4-5 x 10^5 cells/mL; 4 x 10^5-3 x 10^2 cells/mouse) were transplanted s.c. into the right flank of BALB/c nu/nu nude mice using a 1.0 mL tuberculin syringe (needle 26G x 1 in.; 0.45 x 25 mm; Dispomed Witt oHG, Gelnhausen, Germany). KPL-4 cells (3 x 10^6 cells/mouse in 20 mL of culture medium) were orthotopically transplanted using a Hamilton syringe with a 30G x 1/2 inch needle into the right penultimate inguinal mammary fat pad of SCID beige mice.

Tumor-bearing mice were randomized (n = 12 or 15 per group) when the mean tumor volume was 100 mm^3 (NSCLC xenografts) or 50 mm^3 (KPL-4 xenograft). Each group was closely matched before treatment, which began 2 to 3 weeks after cell transplantation.

Twice a week, each xenograft was measured in two dimensions (a = length; b = width) with a caliper. Tumor volume (V) was determined by the following equation: V = ab^2 / 2. Tumor growth inhibition (TGI) for each group was calculated according to the following formula: (1 - [T - T_{0}]/[C - T_{0}]) x 100. T and T_{0} are the mean tumor volumes on a specific experimental day and on day 1 of treatment, respectively, for the experimental groups. Likewise, for the control groups, C and C_{0} are the mean tumor volumes on a given day and at the start of the study, respectively.

At the end of the studies, all animals were killed humanely by cervical dislocation under isoflurane anesthesia. Samples of explanted tumors were weighed twice a week. All animals were observed daily for clinical signs of toxicity and weighed twice a week.

Explanted tumor investigations. Samples of explanted tumors were fixed in 3.8% buffered formaldehyde, embedded in Paraplast (Sherwood Medical, Norfolk, NE), and 5 mm sections prepared.

For histopathologic examination, sections were stained with H&E and examined by light microscopy at ×200 magnification by a certified histopathologist (Dr. O. Vogel, TPC, Kiel, Germany). Semiquantitative analysis of the tumor slides included measurement of cell polymorphism, proliferation, necrosis, apoptosis, invasive growth, and intratumoral fibrosis.

The HER1/EGFR and HER2 expression status of the three types of xenografts were evaluated in control animals. Receptors were visualized by immunohistochemistry in the sections using the HER1/EGFR-specific mAb clone 2-18C9 (DAKO, Ltd., Hamburg, Germany) and DAKO HercepTest (DAKO) according to the manufacturer’s protocol. Receptor expression was scored qualitatively.

Serum tumor markers. Cyfra 21.1 is a soluble fragment of the protein cytokeratin 19 cleaved by caspase. Serum Cyfra 21.1 is a prognostic factor in patients with lung cancer and is now evaluated as an early response prediction marker (38–40) and as a prognostic marker after surgical intervention (41). For breast cancer, serum levels of soluble HER2 (sHER2) correlate with tumor growth and aggressiveness (42, 43). Recent studies indicate that sHER2 may have predictive value in the clinic (44–46).

At the end of the study, blood was collected from all mice via the retro-orbital sinus under anesthesia and serum was prepared (6,800 x g, 10 minutes). Cyfra 21.1 and sHER2 were analyzed by ELISA according to the manufacturer’s protocol (Cytokertatin-19 ELISA, Roche Diagnostics; sHER2, Bender MedSystems, Vienna, Austria).

Statistical analysis. Antitumor efficacy was determined by the treatment-to-control ratio (TCR) for tumor volume, percent TGI, and percent tumor marker inhibition (TMI). Statistical analyses were done using SAS software version 8.1 (47) and the TUMGRO module according to Fieller (48) and Munzel and Hothorn (49). A parametric approach (area under the curve) was used. Due to tumor regression, the ratio to baseline was used. Two-sided 95% upper confidence intervals (95% CI) were calculated. 95% CI values below 1.0 were used to indicate statistical significance.

Results

HER1/EGFR and HER2 expression in different human tumor xenografts. Table 1 and Fig. 2 show the expression patterns of HER1/EGFR and HER2 in the Calu-3, QG56, and KPL-4 human xenograft models. Calu-3 cells had high levels of HER2 and no detectable levels of HER1/EGFR (Fig. 2A). In contrast, QG56 cells stained intensively for HER1/EGFR, but certain exclusion size or show surface ulceration. The tumor growth rate differed for each tumor type, hence the differences in treatment periods. All animals were observed daily for clinical signs of toxicity and weighed twice a week.

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Origin</th>
<th>Type</th>
<th>HER1/EGFR</th>
<th>HER2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calu-3</td>
<td>NSCLC</td>
<td>AD</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>QG56</td>
<td>NSCLC</td>
<td>AD</td>
<td>2-3</td>
<td>0</td>
</tr>
<tr>
<td>KPL-4</td>
<td>MBC</td>
<td>AD</td>
<td>2-3</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1. Expression levels of HER1/EGFR and HER2 in different human xenograft models

Abbreviation: IHC, immunohistochemistry; AD, adenocarcinoma; MBC, metastatic breast cancer.
HER2 was undetectable (Fig. 2B). The KPL-4 xenografts had high levels of both receptors (Fig. 2C), confirming published findings (35). Thus, the three human xenograft models selected for the present study expressed different levels of HER1/EGFR and HER2.

Antitumor activity of monotherapy treatment in xenograft models. Erlotinib, 50 mg/kg/d, was well tolerated with no significant effects on body weight or clinical observations in any of the tumor models (data not shown).

In control animals bearing Calu-3 tumors, the tumor volume was 1,770 ± 168 mm³ (mean ± SE, n = 12) on day 49. After daily erlotinib treatment, the tumor volume only reached 540 ± 143 mm³ (95% CI, 0.15-0.60; ref. Fig. 3). The overall response (TCR) was 0.36 and the percentage TGI was 74%. Mean percent TMI (Cyfra 21.1) was 84%; 12.1 ± 10.2 ng/mL (±SD; 95% CI, 0.06-0.27).

Erlotinib monotherapy significantly inhibited tumor growth in mice with established QG56 xenografts (Fig. 4). After 20 days, the tumor volume was 463 ± 58 mm³ (mean ± SE, n = 12), less than the control (597 ± 95 mm³, 95% CI, 0.59-1.05). The overall TCR was 0.79 and the TGI was 24%. Mean percent TMI (Cyfra 21.1) was 23%; 64.4 ± 33.4 ng/mL (±SD; 95% CI, 0.40-1.58).

Breast cancer xenografts were also sensitive to growth inhibition by erlotinib (Fig. 5). In mice with KPL-4 tumors, the overall TCR after 27 days of treatment was 0.51 and the TGI was 73%. The mean tumor volumes on day 27 were 314 ± 74 and 109 ± 24 mm³ (mean ± SE, n = 15; 95% CI, 0.32-0.71) for control and treated groups, respectively. Mean percent TMI (sHER2) was 65%; median, 37 ng/mL (95% CI, 0.19-1.02).

The dosing schedule of pertuzumab as monotherapy was also well tolerated and had no significant effects on body weight or
clinical observations (data not shown). The effects of pertuzumab monotherapy on mean tumor volume in the three xenograft models are shown in Figs. 3 to 5. The mean tumor volumes after exposure to pertuzumab were 466 ± 263 mm³ (Calu-3; 95% CI, 0.21-0.67), 232 ± 43 mm³ (QG56; 95% CI, 0.23-0.72), and 204 ± 47 mm³ (KPL-4; 95% CI, 0.44-0.87), all statistically significantly different from vehicle controls. At the end of the dosing period, the TCR values were 0.42, 0.51, and 0.64 for the Calu-3, QG56, and KPL-4 models, respectively. The overall TGI was 78% (Calu-3), 72% (QG56), and 29% (KPL-4). In addition, the mean percent TMI values for pertuzumab monotherapy for the Calu-3, QG56 (Cyfra 21.1), and KPL-4 (sHER2) models, respectively, were as follows: 79%, 15.6 ± 16.1 ng/mL (±SD; 95% CI, 0.02-0.40); 44%, 22.6 ± 23.9 ng/mL (±SD; 95% CI, 0.04-0.55), and 61%, median 41 ng/mL (95% CI, 0.18-0.78).

Antitumor activity of combination treatment in xenograft models. Combination treatment with erlotinib and pertuzumab had no adverse effects on clinical observations and body weights (data not shown). Table 2 shows the enhanced antitumor activity of erlotinib/pertuzumab combination therapy.

Erlotinib (50 mg/kg) with pertuzumab (6 mg/kg) in the Calu-3 model resulted in a marked reduction in tumor volume (106 ± 86 mm³) compared with vehicle control (95% CI, 0.00-0.31; Fig. 3). The TCR was 0.12 and the TGI was 100%. The inhibitory effect of this combination therapy was greater than additive compared with either treatment alone. In 4 of 12 animals (33%), the Calu-3 tumors had completely regressed. The serum tumor marker, Cyfra 21.1, was also substantially reduced by combination treatment with erlotinib and pertuzumab. The mean (±SD) values were 75.2 (±23.2) and 2.2 (±3.9) ng/mL for the control and combination treatment groups, respectively, and the TMI was 97% (95% CI, 0.01-0.03).

In the other NSCLC xenograft model, QG56, combination treatment with erlotinib and pertuzumab also reduced tumor volume (151 ± 41 mm³) compared with vehicle control.
The TCR and TGI values were 0.39 and 89%, respectively. This tumor inhibition was additive compared with either agent alone. Tumors were still present in all animals at necropsy. Tumor growth inhibition was confirmed by the dramatic reduction in mean serum levels of Cyfra 21.1. The mean (±SD) values were 83.7 (42.9) and 16.4 (16.9) ng/mL for the control and combination treatment groups, respectively, and the TMI was 80% (95% CI, 0.05-0.41).

Erlotinib plus pertuzumab had an additive effect on tumor inhibition compared with either single agent in the breast cancer model (KPL-4; Fig. 5). The mean tumor volume at study termination was 82 ± 25 mm³ (95% CI, 0.20-0.59). The TCR was 0.38 and the TGI was 85%. In 3 of 15 animals (20%), the KPL-4 tumors had completely regressed. The serum tumor marker, sHER2, was also substantially reduced by single-agent and combination treatment (Fig. 6). The median serum values were 106 and 9 ng/mL for vehicle control and combination therapy, respectively. For erlotinib combined with pertuzumab, the overall TMI was 92% (95% CI, 0.06-0.35).

Antitumor activity was confirmed by histology as shown in Figs. 7 (Calu-3), 8 (QG56), and 9 (KPL-4). Histopathologic analysis of xenograft samples showed that combination treatment with erlotinib and pertuzumab decreased the rate of cell proliferation, decreased apoptosis (for Calu-3 and KPL-4 models only), and increased capsular and intratumoral fibrosis compared with single-agent administration. Due to the marked reduction in tumor mass in response to combination treatment, it was not possible to obtain a meaningful statistical analysis of Ki67 staining (data not shown).

Discussion

Increasing evidence indicates that the HER family are involved in tumorigenesis (12, 50) and new antitumor agents targeting these receptors are being developed (16, 51, 52). Erlotinib and pertuzumab have independent mechanisms of action. Erlotinib, a 4-anilinoquinazoline small molecule tyrosine kinase inhibitor (53), specifically targets the HER1/EGFR receptor (18, 19), with some activity against HER2 (54). In contrast, pertuzumab is a mAb that, by binding to the dimerization domain of HER2, prevents heterodimerization with other HERs (29). Receptor inhibition by either mechanism blocks downstream signaling pathways, reducing cell growth and proliferation (16). The concept of blocking HER1/EGFR or HER2 as an effective anticancer strategy is supported by preclinical studies (33) and by phase III clinical trial data with erlotinib (27, 28) and trastuzumab (Herceptin), respectively. Trastuzumab, a humanized anti-HER2 mAb, combined with chemotherapy has significant clinical benefit (e.g., antitumor activity, improved survival) in patients with HER2-positive metastatic breast cancer (10, 55, 56).

In the present study, cell lines with different expression levels of HER1/EGFR and HER2 were selected and confirmed using immunohistochemistry. The Calu-3 xenografts had no detectable HER1/EGFR and expressed high HER2 levels. In contrast,

Table 2. Antitumor activity of erlotinib/pertuzumab combination therapy and reduction of serum tumor marker concentrations

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Response (TCR)</th>
<th>95% CI</th>
<th>TGI (%)</th>
<th>Tumor free</th>
<th>Tumor marker</th>
<th>TMI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calu-3</td>
<td>0.12</td>
<td>0.00-0.317</td>
<td>100</td>
<td>4/12</td>
<td>Cyfra 21.1</td>
<td>97</td>
</tr>
<tr>
<td>QG56</td>
<td>0.39</td>
<td>0.21-0.59</td>
<td>89</td>
<td>0/12</td>
<td>Cyfra 21.1</td>
<td>80</td>
</tr>
<tr>
<td>KPL-4</td>
<td>0.38</td>
<td>0.20-0.59</td>
<td>85</td>
<td>3/15</td>
<td>sHER2</td>
<td>92</td>
</tr>
</tbody>
</table>
the other NSCLC tumor, QG56, had high levels of HER1/EGFR and virtually no HER2 expression. Both receptors were highly expressed in the breast cancer model KPL-4.

Both erlotinib (orally, 50 mg/kg/d) and pertuzumab (i.p. dose of 6 mg/kg weekly), as single agents, had significant antitumor activity in mice with human NSCLC or breast cancer xenografts. In all models, tumor volume was significantly decreased compared with controls for both compounds. These results were confirmed by histopathology.

Based on the expression levels of HER1/EGFR and HER2 in the three cell lines, it might be expected that erlotinib, which targets HER1/EGFR with limited activity against HER2, would only have antitumor activity in those models with high levels of HER1/EGFR expression (i.e., QG56 and KPL4). Interestingly, erlotinib was also effective against Calu-3 xenografts (no detectable HER1/EGFR), albeit with a longer dosing period (49 days versus 20-27 days for the other models). Similarly, pertuzumab, specific for HER2, inhibited tumor growth in all three xenograft models evaluated, despite the different levels of HER2 expression (KPL-4 and Calu-3 = high; QG56 = not detectable). Collectively, these results suggest that the antitumor activity of these agents is independent of HER1/EGFR and HER2 expression and may reflect the receptor activation status of these cell lines. These findings for erlotinib and pertuzumab are not unique to this study. Erlotinib has differential antitumor activity against the human NSCLC xenografts H460a (fast growing) and A549 (slow growing), which express similar levels of HER1/EGFR receptors (21). In other human NSCLC xenografts (NCI-H322M, NCI-H522, and NCI-H441), erlotinib inhibited tumor growth independently of HER1/EGFR and HER2 overexpression (20). Clinical studies with erlotinib have also shown that response to HER1/EGFR-targeted therapy is not correlated with HER1/EGFR expression (22, 57). Pertuzumab has also been shown to be effective in suppressing the growth of a number of xenografted tumors that do not overexpress HER2 (30).

![Fig. 6. Effect of erlotinib and pertuzumab on serum levels of sHER2 in KPL-4 xenograft model. Mice with established KPL-4 xenografts were treated with vehicle, erlotinib (50 mg/kg/d, orally), pertuzumab (12 mg/kg loading dose, then 6 mg/kg/wk, i.p.), or erlotinib and pertuzumab for 27 days. At termination, blood samples were collected from the retro-orbital sinus and serum levels of sHER2 were evaluated by ELISA. Values are the median (ng/mL), n = 15 per group.](image)

![Fig. 7. Effect of erlotinib and pertuzumab on histologic parameters of explanted Calu-3 tumors. Female BALB/c nu/nu mice with established Calu-3 xenografts were treated with (A) vehicle, (B) erlotinib (50 mg/kg/d), (C) pertuzumab (12 mg/kg loading dose, then 6 mg/kg/wk), or (D) erlotinib in combination with pertuzumab for 49 days. Sections of fixed samples of explanted tumors embedded in Paraplast were stained with H&E (magnification, × 200).](image)
The antitumor activity of erlotinib combined with pertuzumab was also evaluated, and substantial tumor growth inhibition was shown, which was greater than single-agent activity. With regard to decreased tumor volume, the effect of erlotinib in combination with pertuzumab was additive in the QG56 and KPL-4 xenografts, and greater than additive (i.e., the fixed dose combination of erlotinib and pertuzumab was superior to each agent alone) in the Calu-3 model compared with monotherapy. The antitumor results of the combination therapy were particularly striking in the Calu-3 and KPL-4 models, as complete tumor regression was achieved in some of the mice bearing these tumors. Histopathology clearly confirmed the antitumor activity of the combination therapy in all three models. Both erlotinib and pertuzumab have different mechanisms of action. Erlotinib directly inhibits HER1/EGFR tyrosine kinase activity (18, 19), whereas pertuzumab blocks ligand-associated dimerization of HER2 with other HERs (29). Active heterodimers stimulate at least two different signaling pathways; one pathway promotes cell growth (via mitogen-activated protein kinase) and the other pathway enhances cell survival (via Akt; ref. 50). Thus, inhibition of cell proliferation rates or induction of apoptosis may contribute to the additive effect with erlotinib and pertuzumab. Furthermore, combining erlotinib with other targeted agents has also shown greater effects compared with single-agent activity. For example, erlotinib with cetuximab (an anti-HER1/EGFR mAb) significantly enhanced growth inhibition of cancer cell lines in vitro over that observed with either agent alone (58). This observation supports the concept that mAbs targeting the receptor may potentially improve therapeutic efficacy of tyrosine kinase inhibitors.

The antitumor activity of erlotinib/pertuzumab combination treatment is confirmed by the reduction of serum tumor markers. Cyfra 21.1, a soluble fragment of the structural protein cytokeratin 19, is released upon tumor cell death (59). Serum levels of Cyfra 21.1 in advanced NSCLC patients can be used to monitor tumor response to treatment (39). In the current study, mice bearing human NSCLC xenografts had measurable levels of serum Cyfra 21.1, which were markedly decreased following treatment with erlotinib and pertuzumab. These reductions correlated well with changes in tumor volume, indicating that this protein fragment could be a reliable marker for antitumor activity in preclinical models. Similar findings with Cyfra 21.1 have been reported for other human NSCLC xenograft models (20). For the breast cancer model, KPL-4, serum levels of sHER2 were measured. This molecule, the soluble, extracellular domain portion of HER2, is shed from breast cancer cells via protease activity. In some clinical settings, monitoring serum levels of sHER2 may assess breast tumor response to therapy (42, 45, 46). In the KPL-4 model, median serum levels of sHER2 were considerably reduced in response to treatment with either erlotinib or pertuzumab and to the combined agents. These results correlated well with the inhibition of tumor growth of KPL-4 xenografts and support the use of sHER2 as a marker for tumor response in preclinical studies.

Erlotinib is currently under investigation in an extensive range of clinical trials as a monotherapy and in combination with chemotherapy or radiotherapy (reviewed in ref. 60). In a phase III trial in patients with advanced, relapsed NSCLC, the receptor may potentially improve therapeutic efficacy of tyrosine kinase inhibitors.
erlotinib monotherapy significantly prolonged survival, delayed disease progression, and delayed worsening of lung cancer–related symptoms (27). Erlotinib in combination with chemotherapy significantly prolonged survival in a phase III trial in patients with advanced pancreatic cancer (28). In contrast, erlotinib combined with conventional chemotherapy did not provide a conclusive survival benefit for NSCLC (61, 62). These latter findings are in contrast to early clinical studies (e.g., ref. 22) and in preclinical investigations (18, 19, 21) with erlotinib. Such discrepancies have also occurred with other tyrosine kinase inhibitors under development (e.g., gefitinib; refs. 63, 64). The schedule and sequence of administration may critically affect the outcome with tyrosine kinase inhibitor/chemotherapy combinations, and is a key area for research to identify the most effective use of EGFR tyrosine kinase inhibitors with standard chemotherapy agents.

Further clinical and preclinical research is required to fully optimize the use of targeted therapies to treat cancer. Multiple molecular abnormalities are likely to influence cell growth and development of cancer (16). Therefore, future therapeutic strategies need to consider combining agents targeting different receptors and pathways. Erlotinib is already in early clinical trials in NSCLC in combination with the anti–vascular endothelial growth factor antibody, bevacizumab (34), which inhibits angiogenesis.

The preclinical results reported are encouraging as combining erlotinib with pertuzumab has enhanced antitumor activity in human NSCLC and breast xenografts. The outcome of this investigation suggests that further investigations of simultaneous HER1/EGFR and HER2 inhibition for cancer treatment are warranted.

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