Erlotinib Antitumor Activity in Non-small Cell Lung Cancer Models is Independent of HER1 and HER2 Overexpression

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Abstract. Background: The human epidermal growth factor receptors HER1/EGFR and HER2 offer potential targets for treating non-small cell lung cancer (NSCLC). The antitumor efficacy of erlotinib (Tarceva, F. Hoffmann-La Roche, Ltd., Basel, Switzerland), a HER1/EGFR tyrosine-kinase inhibitor, was investigated in relation to HER1/EGFR and HER2 expression in five NSCLC xenograft models. Materials and Methods: Tumor-bearing mice were randomized to daily oral erlotinib, 50 mg/kg, or vehicle (controls) for 20-50 days. The antitumor efficacy of erlotinib was measured through tumor volume, serum tumor markers and tumor biomarkers. Tumor HER1/EGFR and HER2 expression were analyzed immunohistochemically. Results: Erlotinib reduced tumor volume in three NSCLC models. It also reduced serum tumor marker levels and the extent of inhibition correlated with tumor growth inhibition. HER1/EGFR and HER2 expression differed between the five tumor models, suggesting that expression level does not predict response to treatment. Conclusion: Erlotinib showed differing antitumor activity in five NSCLC models, suggesting that its antitumor effect is independent of HER1/EGFR and HER2 overexpression.

Epidermal growth factor receptors (EGFR) regulate cell differentiation and proliferation (1). Four receptors have been identified – human epidermal growth factor receptor 1 (HER1/EGFR), HER2, HER3 and HER4. HER1/EGFR is overexpressed in many human cancers, including non-small cell lung cancer (NSCLC) (2, 3) and this overexpression is linked to advanced disease, metastases and poor prognosis (3). Overexpression of HER2 is also associated with poor survival in patients with NSCLC (4-10). EGFRs have, therefore, been identified as potential targets for novel anticancer therapies (2, 11).

Lung cancer, of which NSCLC is the most common form, is the second most common type of cancer in men and women, and is the leading cause of death from cancer in both sexes (12). The treatment approach for NSCLC differs with the extent of disease. Surgery offers the only cure for early disease, while a combination of surgery and chemotherapy and/or radiotherapy can improve outcomes (13, 14). For advanced disease, chemotherapy is the preferred option. Several new chemotherapeutic agents have been shown to provide a small improvement in overall survival when given in combination with platinum-based compounds (15-22). However, the prognosis for patients with advanced NSCLC remains poor, with 5-year survival rates ranging from 5-10% and median survival times from 12-15 months (19, 21). Furthermore, current chemotherapy regimens are non-specific, non-selective and toxic. Thus, new cancer-specific, non-toxic therapies that prolong progression-free disease and overall survival, and improve quality of life are urgently needed for patients with NSCLC (23).

Erlotinib (Tarceva, F. Hoffmann-La Roche, Ltd., Basel, Switzerland) is a highly potent, orally active inhibitor of HER1/EGFR that less effectively inhibits HER2 tyrosine kinase (TK), thereby preventing phosphorylation of the receptors and the subsequent cascade of signaling events (24). It is active against a variety of tumor cell lines in vitro (25) and in vivo (26, 27), while phase I/II clinical trials have shown that it is well tolerated with antitumor effects against several common solid tumors (28-33). In two phase III trials, erlotinib in combination with chemotherapy produced no overall survival benefit in patients with NSCLC (34, 35), but did increase survival in patients who had never smoked (35, 36). In contrast, in an another phase III trial, erlotinib monotherapy significantly prolonged survival, delayed disease progression and delayed worsening of lung cancer-related symptoms in patients with advanced, relapsed NSCLC (37).

The present study examined the antitumor activity of erlotinib in relation to HER1/EGFR and HER2 expression in...
five different NSCLC xenograft models. Secondary objectives were to evaluate the inhibitory effects of erlotinib on tumor biomarkers and serum tumor markers and to compare the antitumor activity of erlotinib with that of gemcitabine.

Materials and Methods

The experimental study protocol was reviewed and approved by the local government.

Animals. Female BALB/c nu/nu nude mice (Bomholtgard, Ry, Denmark), aged 6-8 weeks, and female severe combined immunodeficient (SCID)/beige mice (Charles River, Sulzfeld, Germany), aged 6-8 weeks, were quarantined and then maintained under specific pathogen-free conditions according to Committee guidelines (GV-Solas; Felasa; TierSchG). Food (Standard 1320 and 1430; Altromin, Lange, Germany) and acidified water (pH 2.5-3.0) were provided ad libitum.

Cell lines and culture conditions. NCI-H522 and NCI-H322M cell lines (ATCC, Rockville, MD, USA), and Calu-3, QG56 and NCI-H441 cell lines (Chugai Pharmaceuticals Co., Ltd., Kamakura, Kanagawa, Japan) were cultured as monolayers in RPMI 1640 medium (PAA Laboratories, Linz, Austria) supplemented with 10% fetal bovine serum (PAA Laboratories) and 2 mM/l glutamine (PAA Laboratories). Tumor cells were harvested every 3 days using trypsin/ethylenediamine tetra-acetic acid (EDTA; Roche Diagnostics GmbH, Mannheim, Germany) to split the monolayers. The harvested cells were transferred into 50 ml of RPMI 1640 medium, washed with phosphate-buffered saline (PBS) and resuspended in PBS. Cell size and concentration were determined using a cell counter and analyzer system (Vi-CELL, Beckman Coulter GmbH, Krefeld, Germany).

Tumor-growth inhibition studies in vivo. Calu-3, QG56 and NCI-H441 tumor cells were established in BALB/c nude mice through the 100 µl subcutaneous injection of tumor cell suspension (4 or 5x10⁶ tumor cells/ml) into the right-hand flank of anesthetized animals. NCI-H522 and NCI-H322M cells were co-implanted with Matrigel™ (Basement Membrane Matrix, Becton Dickinson Laboratories). Tumor cells were harvested every 3 days using trypsin/ethylenediamine tetra-acetic acid (EDTA; Roche Diagnostics GmbH, Mannheim, Germany) to split the monolayers. The harvested cells were transferred into 50 ml of RPMI 1640 medium, washed with phosphate-buffered saline (PBS) and resuspended in PBS. Cell size and concentration were determined using a cell counter and analyzer system (Vi-CELL, Beckman Coulter GmbH, Krefeld, Germany).

Tumor-bearing mice (10-12 animals in each treatment group) were randomized to once-daily oral erlotinib 50 mg/kg, or vehicle (Captisol®; control group), for 20 (NCI-H522, NCI-H441), 21 (QG56), 42 (NCI-H322M) or 50 days (Calu-3) (note: in a previous preclinical study, 50 mg/kg erlotinib was well tolerated in mice, both as monotherapy and in combination with pertuzumab [39]). Mice with NCI-H322M tumors were also randomized to intraperitoneal gemcitabine at 120 mg/kg, q3dx4. Clinical signs of toxicity were monitored daily, while body weight was measured twice weekly.

Explanted tumor investigations. HER1/EGFR and HER2 expressions were analyzed in all NSCLC xenograft models. Samples of explanted tumor were embedded in paraffin (Paraplast®; Sherwood Medical, Norfolk, NE, USA) and HER1/EGFR and HER2 receptor expression was visualized in 5-µm sections using the HER1/EGFR-specific monoclonal antibody clone 2-18C9 (DAKO Ltd., Hamburg, Germany) and the DAKO HercepTest™ (DAKO Ltd.).

Tumor biomarkers – phosphorylated mitogen-activated protein kinase (pMAPK) and the cell proliferation marker Ki-67 – were examined in mice with advanced Calu-3 tumors (~500 mm³ in volume) that received once-daily oral erlotinib 50 mg/kg, or vehicle, for 4 days. The tumors were explanted 6 h after the last treatment dose and analyzed immunohistochemically (IHC). For pMAPK, endogeneous peroxidase activity was blocked prior to incubation with primary antibody (1:70 dilution; overnight at 4°C). The samples were then incubated with a secondary antibody (goat anti-mouse/rabbit antimouse (K5001; DAKO Ltd.) was added and the samples incubated for 30 min at room temperature. Antigen was visualized with DAB substrate (DAKO Ltd., Hamburg, Germany) and the DAKO HercepTest™ (DAKO Ltd.).

Serum tumor markers. Blood collected from tumor-bearing mice at the end of the study was analyzed for one of the two tumor markers, Cyfra 21.1 or neuron-specific enolase (NSE), which were selected for each model based on their significant release into serum. Tumor marker levels, which give an indication of the response to treatment, were analyzed using enzyme-linked immunosorbent assay (ELISA; Roche Diagnostics and Research
Diagnostics Inc., New Jersey, USA). The response to treatment was calculated as the percent serum tumor marker inhibition (%TMI).

Statistical analysis. The antitumor efficacy of erlotinib was determined by calculating the treatment-to-control ratio (TCR) for tumor volume, percent tumor-growth inhibition (%TGI) and %TMI. Statistical analysis of the data was performed using SAS software (40) and the TUMGRO module (41, 42). Two-sided 95% upper confidence intervals (CI) of less than 1.0 were taken to indicate statistical significance.

Results

HER1/EGFR and HER2 expression in NSCLC xenografts. The level of HER1/EGFR and HER2 expression differed between the five NSCLC xenograft models: NCI-H441 and NCI-H522 cells stained for both HER1/EGFR and HER2 receptors; Calu-3 cells expressed high levels of HER2, but HER1/EGFR was undetectable; QG56 cells stained highly for HER1/EGFR, but HER2 was undetectable; and NCI-H322M cells expressed high levels of HER1/EGFR and low levels of HER2 (Table I).

Antitumor activity of erlotinib in NSCLC xenograft models. Oral erlotinib 50 mg/kg once daily was well tolerated, with no signs of toxicity and no significant effect on body weight in any of the NSCLC xenograft models (data not shown). Treatment with erlotinib significantly inhibited tumor growth in three NSCLC xenograft models (NCI-H322M, NCI-H522, Calu-3; Figure 1) and the response to treatment was similar in the three models, with TCR values of 0.08-0.21 (Table II). In animals with NCI-H322M tumors that received gemcitabine 120 mg/kg, q3dx4, the reduction in tumor volume was significantly less (mean tumor volume at 42 days 653.4±272 mm³; TCR: 0.53, 95% CI 0.31, 0.80; TGI 57.9%) than that in erlotinib-treated animals (Figure 1).

Erlotinib also inhibited tumor growth in the other two NSCLC xenograft models (QG56, NCI-H441), but the reduction in tumor volume was much less than that in the NCI-H322M, NCI-H522 and Calu-3 models (Table II). Treatment with erlotinib led to a substantial reduction in serum tumor marker concentrations and the extent of serum tumor marker inhibition correlated with the antitumor activity of erlotinib (Table III). Tumor marker levels were reduced by 55-98% in the NSCLC xenograft models sensitive to erlotinib, whereas tumor marker inhibition was less than 50% (32-48%) in the less sensitive models. Cyfra 21.1 levels were reduced to a greater extent than NSE levels.

Effect of erlotinib on biological markers in vivo. Histopathological analysis revealed that long-term treatment (20-50 days) with erlotinib suppressed tumor-cell proliferation and cell growth.
increased intratumoral fibrosis in NCI-H322M, NCI-H441 and Calu-3 xenograft models. In animals with NCI-H322M tumors treated with gemcitabine, only a moderate reduction in cell proliferation and a smaller increase in fibrosis were seen (data not shown).

Immunohistochemical (IHC) analysis showed that short-term treatment with erlotinib (4 days) significantly suppressed pMAPK expression and reduced tumor-cell proliferation by 30%, as measured by Ki-67 staining, in mice with advanced Calu-3 tumors (Figure 2).

Discussion

The results of this study indicate that once-daily, oral erlotinib has significant antitumor activity against three NSCLC xenograft models and minor antitumor effects in two models, as indicated by decreased tumor volumes. In the NCI-H322M model, erlotinib proved more effective than gemcitabine in inhibiting tumor growth. The inhibitory effect of erlotinib on tumor growth was further demonstrated by a significant reduction in the levels of
serum tumor markers, Cyfra 21.1 and NSE, which have been shown to be markers of treatment effect (43, 44). However, the extent of serum tumor marker inhibition with erlotinib was greater in NCI-H322M, NCI-H522 and Calu-3 than in QG56 and NCI-H441 xenograft models. This pattern was also reflected in tumor mass at study termination. Collectively, these data suggest that serum tumor markers provide a reliable tool for monitoring the activity of antitumor agents in preclinical tumor models.

The antitumor effects of erlotinib were also demonstrated by the suppression of tumor biomarker expression after short-term (4-day) treatment, as indicated by a significant decrease in pMAPK expression in Calu-3 tumor cells. These results lend support to the finding from other studies that the inhibitory effect of erlotinib on HER1/EGFR TK led to a reduction in downstream signaling and a consequent reduction in tumorigenesis (25). Furthermore, the suppression of Ki-67 expression in Calu-3 tumor cells suggests that erlotinib inhibits tumor growth by reducing tumor cell proliferation.

IHC revealed that HER1/EGFR and HER2 expression levels differed between the five NSCLC xenograft models. In the two models expressing high levels of HER1/EGFR (QG56 and NCI-H441), IHC also showed that these receptors were phosphorylated (i.e., in an activated state). The relatively small inhibitory effect of erlotinib in these models may indicate that growth regulation of QG56 and NCI-H441 xenografts is not dominated by HER1/EGFR alone, despite its high expression and activation. Other growth regulatory pathways that are not affected by

Figure 2. Effect of short-term (4-day) treatment with erlotinib 50 mg/kg/day on the expression of (A) pMAPK and (B) Ki-67 in Calu-3 tumor cells.
erlotinib may be implicated in these models. Taken together, these limited results (due to the small number of preclinical models and no statistical analysis) suggest that the antitumor effects of erlotinib are independent of HER1/EGFR and HER2 overexpression. This observation is supported by the results of a phase II clinical trial of erlotinib in NSCLC, which showed that tumors staining strongly for HER1/EGFR were not associated with a better response to therapy or with stable disease (30). The lack of an association between antitumor efficacy and HER1/EGFR overexpression has also been reported with other HER1/EGFR inhibitors (45-48).

Cancer research is currently focused on identifying markers that correlate with the response to treatment, enabling patients who are most likely to benefit from treatment to be identified. The value of HER1/EGFR expression as a prognostic indicator in NSCLC is controversial because some studies found an association between HER1/EGFR overexpression and poor survival (3, 7, 49), while others have found no such association (30). The relationship between HER1/EGFR expression and prognosis in cancer patients was examined in a literature review (7). The results showed that HER1/EGFR expression was a strong prognostic indicator in many types of cancer, but not in NSCLC. However, the authors suggested that the value of HER1/EGFR may have been underestimated; total cellular HER1/EGFR levels rather than the activated receptor were assessed and methods were not standardized. In another literature review (50), meta-analysis of the results from retrospective studies in NSCLC showed that HER1/EGFR expression may be a prognostic factor for poor survival if receptor expression is measured using IHC techniques. However, the authors pointed out that the analysis of IHC results is subjective and that different cut-off points for receptor expression were used in the different studies reviewed. Consequently, they suggested that an optimal threshold for HER1/EGFR expression needs to be defined using IHC techniques and that the results obtained with these techniques should be compared with those obtained using molecular biology methodology. Until this has been achieved, HER1/EGFR expression cannot be used as a prognostic indicator in NSCLC.

The situation with respect to HER2 is more conclusive. In a literature review (7), 75% of studies showed an association between HER2 overexpression and poor survival in patients with NSCLC. Furthermore, one of the studies reviewed (51) showed that HER1/EGFR expression alone was not a significant prognostic indicator, whereas analysis of both HER1/EGFR and HER2 expression did identify patients with a poor prognosis. Thus, it is possible that a combination of components of the HER1/EGFR signaling network may be of value as prognostic indicators in patients with NSCLC.

The preclinical results reported here clearly demonstrate that erlotinib as a single agent has antitumor activity against different NSCLC xenografts. Recent positive results in the clinical setting confirm these findings: in a phase III trial in advanced, relapsed NSCLC, erlotinib monotherapy significantly prolonged survival, delayed disease progression and delayed worsening of lung cancer-related symptoms when given as second- or third-line treatment (37).

In conclusion, erlotinib showed substantial antitumor activity in three out of five NSCLC xenograft models and minor activity in two models, as indicated by decreased tumor volumes, reduced tumor marker levels (Cyfra 21.1 and NSE) and the suppression of tumor biomarker (pMAPK and Ki-67) expression. The antitumor effects of erlotinib were independent of tumor HER1/EGFR and HER2 overexpression. Further studies to evaluate the antitumor effects of erlotinib in NSCLC are warranted.

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