Antitumor activity of erlotinib (OSI-774, Tarceva) alone or in combination in human non-small cell lung cancer tumor xenograft models

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Our objective was the preclinical assessment of the pharmacokinetics, monotherapy and combined antitumor activity of the epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor erlotinib in athymic nude mice bearing non-small cell lung cancer (NSCLC) xenograft models. Immunohistochemistry determined the HER1/EGFR status of the NSCLC tumor models. Pharmacokinetic studies assessed plasma drug concentrations of erlotinib in tumor- and non-tumor-bearing athymic nude mice. These were followed by maximum tolerated dose (MTD) studies for erlotinib and each chemotherapy. Erlotinib was then assessed alone and in combination with these chemotherapies in the NSCLC xenograft models. Complete necropsies were performed on most of the animals in each study to further assess antitumor or toxic effects. Erlotinib monotherapy dose-dependently inhibited tumor growth in the H460a tumor model, correlating with circulating levels of drug. There was antitumor activity at the MTD with each agent tested in both the H460a and A549 tumor models (erlotinib 100 mg/kg: 71 and 93% tumor growth inhibition; gemcitabine 120 mg/kg: 93 and 75% tumor growth inhibition; cisplatin 6 mg/kg: 81 and 88% tumor growth inhibition). When each compound was given at a fraction of the MTD, tumor growth inhibition was suboptimal. Combinations of gemcitabine or cisplatin with erlotinib were assessed at 25% of the MTD to determine efficacy. In both NSCLC models, doses of gemcitabine (30 mg/kg) or cisplatin (1.5 mg/kg) with erlotinib (25 mg/kg) at 25% of the MTD were well tolerated. For the slow growing A549 tumor, there was significant tumor growth inhibition in the gemcitabine/erlotinib and cisplatin/erlotinib combinations (above 100 and 98%, respectively), with partial regressions. For the faster growing H460a tumor, there was significant but less remarkable tumor growth inhibition in these same combinations (86 and 53% respectively). These results show that in NSCLC xenograft tumors with similar levels of EGFR expression, the antitumor activity of erlotinib is robust both as monotherapy and in combination with chemotherapies. *Anti-Cancer Drugs* 15:503–512

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Introduction

The epidermal growth factor receptor (HER1/EGFR) is a valuable molecular target in cancer therapy [1]. Many cancers overexpress HER1/EGFR: head and neck squamous cell carcinoma (70–100%), non-small cell lung cancer (NSCLC) (50–90%), prostate cancer (40–70%), glioma (10–50%), gastric cancer (30–60%), breast cancer (35–70%), colorectal cancer (45–80%), pancreatic cancer (30–50%) and ovarian cancer (35–60%) [1,2]. Salomon *et al.* also highlighted the link between overexpressed HER1/EGFR and patients with advanced disease, metastases and poor prognosis [2].

NSCLC is the most common lung cancer. According to the extent of the disease, the treatment approach will differ. For early-stage disease, surgery is the only cure and a multimodal approach with chemotherapy can be associated with improved outcome. In advanced disease, chemotherapy is the main option, which offers small improvements in overall survival. Thus, the medical need remains high in NSCLC with the search for more effective and better-tolerated regimens. Many traditional cytotoxics have been used as monotherapy in NSCLC, including vindesine, carboplatin, etoposide, ifosfamide, cyclophosphamide, vincristine, mitomycin and cisplatin

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[3]. Monotherapy with these drugs produces only small improvement, but combination therapy with cisplatin has lessened patients' illness and improved their quality of life in randomized trials [4].

Gemcitabine was developed in the 1990s and inhibits ribonuclease reductase. Gemcitabine monotherapy has a greater probability of tumor response and improved patient quality of life (in terms of reduced hair loss, nausea and vomiting, and appetite loss) than standard cisplatin/etoposide chemotherapy [5].

Combination trials by the European Organization for Research and Treatment of Cancer (EORTC) compared cisplatin and teniposide to cisplatin and paclitaxel [6]. As the latter combination gave better palliation for advanced NSCLC (even though a clear survival benefit was not met), it has been recommended as one of the standards of care for advanced NSCLC patients. In addition, a combination of gemcitabine and cisplatin has been shown to act synergistically *in vitro* and at least additively *in vivo* [7]. In phase II trials, the response rate for gemcitabine and cisplatin was 47% and median survival 57 weeks, with a 1-year survival rate of 48% [4].

New treatments for cancer take a cancer-cell specific approach and promise less toxicity than the older cytotoxic drugs. As cancer cell-specific targets are only part of the disease etiology, treatments combining targeted and conventional drugs may have a synergistic effect. Optimal treatment of NSCLC is likely to consist of EGFR inhibitors in combination with traditional chemotherapy.

Erlotinib (Tarceva, OSI-774) is a selective, orally available small-molecule inhibitor of the HER1/EGFR tyrosine kinase domain. It has potent antitumor activity in preclinical animal models of head and neck and vulval carcinoma [8]. Erlotinib induces apoptosis *in vitro* and is active against various EGFR-expressing human tumor xenografts *in vivo* [9]. In an open-label, phase II study of NSCLC patients who had failed platinum-based chemotherapy [10], erlotinib had encouraging anticancer activity.

In this study we investigated whether combining erlotinib with cisplatin or gemcitabine in athymic nude mice bearing NSCLC xenograft models acts synergistically or antagonistically in inhibiting tumor growth. The H460a and A549 NSCLC tumor models were chosen because they clearly express EGFR, with around 70 000–80 000 binding sites per cell [11,12]. A549 is slow growing, and H460a is more aggressive and faster growing.

Methods

Animals

Female, athymic, *nu*/*nu*-nuBR nude mice (Charles River, Wilmington, DE) of around 10–12 weeks and weighing 23–25 g were used. The health of the mice was assessed daily by observation and analysis of blood samples taken from sentinel animals on shared shelf racks. All animals were allowed to acclimatize and recover from shipping-related stress for 1 week.

Autoclaved water and irradiated food [5058-ms Pico Lab (mouse) breed chow; Purina Mills, Richmond, IN] were provided *ad libitum*, and the animals were kept in a 12-h light and dark cycle. Cages, bedding and water bottles were autoclaved before use and changed weekly. All animal experiments were in accordance with protocols approved by the Roche Animal Care and Use Committee, and in accordance with local regulations.

Cell culture and animal studies

H460a cells (provided by Dr Jack Roth, MD Anderson Cancer Center) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). A549 cells (ATCC, Manassas, VA) were grown in RPMI 1640 + 10% FBS. The cell concentrations for implant were 1×10^7 cells/0.2 ml for H460a and 7.5×10^6 cells/0.2 ml for A549.

Cells were suspended in phosphate-buffered saline and implanted s.c. in the right flank of each mouse. Once palpable tumors were established, animals were randomized so that all groups had similar starting mean tumor volumes of 100–150 mm^3. Tumor measurements and mouse weights were taken 3 times per week. Animals were individually monitored throughout the experiment.

Test agents and drug treatment

Erlotinib (OSI Pharmaceuticals, Melville, NY) was formulated as a fine suspension with sodium carboxymethylcellulose and Tween 80 in water for injection. Erlotinib (0.2 ml/animal) was given orally using a 1-ml syringe and an 18-gauge gavage needle. All groups were treated daily for 3 weeks.

Lyophilized gemcitabine (Gemzar; Lilly Research Center, Indianapolis, IN) was formulated in the prepackaged vial with sterile saline according to the label instructions, giving a solution containing 38 mg/ml active compound. An aliquot of the stock vial solutions was taken for each dose group, consisting of the drug needed for the entire study and diluted further with sterile saline, to give a solution of 0.5 ml dosing volume for each animal. Gemcitabine was given i.p. using a 3-ml syringe and a 26-gauge needle. All groups were treated every 3 days for 3 weeks (a total of six injections).

Cisplatin (Platinol-AQTM; Bristol-Myers Squibb, New York, NY) was provided in a stock sterile saline solution of 1 mg/ml. An aliquot of the stock vial solutions was taken for each dose group, consisting of the drug needed for the entire study and diluted further with sterile saline, to give a solution of 0.5 ml dosing volume for each animal. Cisplatin was given i.p. using a 3 ml-syringe and a 26-gauge needle. All groups were treated every 6 days for 3 weeks (a total of three injections).

Calculations and statistical analysis

Weight loss was calculated as percent change in mean group body weight, using the formula:

$$[(W - W_0)/W_0] \times 100$$

where W represents mean body weight of the treated group at a particular day and W_0 represents mean body weight of the same group at start of treatment. Maximum weight loss was also calculated using the above formula, giving the maximum percentage of body weight lost at any time in the entire experiment for a particular group. Treatment efficacy was assessed by tumor growth inhibition. Tumor volumes of treated groups were given as percentages of tumor volumes of the control groups (%T/C), using the formula:

$$100 \times [(T - T_0)/(C - C_0)]$$

where T represents mean tumor volume of a treated group on a specific day during the experiment, T_0 represents mean tumor volume of the same group on the first day of treatment, C represents mean tumor volume of a control group on a particular day of the experiment and C_0 represents mean tumor volume of the same group on the first day of treatment.

Tumor growth inhibition was calculated using the formula:

$$100 - \%\mathrm{T/C}$$

Tumor volume (mm^3) was calculated using the ellipsoid formula:

$$[D \times (d^2)]/2$$

where D represents the large diameter of the tumor and d represents the small diameter. In some cases, tumor regression and/or percentage change in tumor volume was calculated using the formula:

$$[(T - T_0)/T_0] \times 100$$

where T represents mean tumor volume of the treated group at a particular day and T_0 represents mean tumor volume of the same treated group at the start of treatment.

Statistical analysis was by the rank sum test and one-way analysis of variance (ANOVA) and a post-hoc Bonferroni *t*-test (SigmaStat, version 2.03; Jandel Scientific, San Francisco, CA). The significance level was set at $p \leq 0.05$.

Pharmacokinetics (PK) analysis

For single-dose PK, blood samples from three mice per time point were collected by cardiac puncture at 5, 15, 30 and 60 min, and 2, 4, 8, 16 and 24 h post-dose. For chronically treated animals, blood samples from two or three mice per time point were collected via the retro-orbital sinus at 1 and 6 h. Collection tubes contained EDTA as anticoagulant. Samples were stored at −70°C. Plasma concentrations of erlotinib were determined using specific and validated liquid chromatography and tandem mass spectrometry (LC-MS/MS) with quantification limits of 1 ng/ml. PK parameters were estimated by non-compartmental analysis of the composite data, using the PK evaluation programme WinNonlin PRO version 3.1 (Pharsight, Mountain View, CA). In one study, erlotinib tumor (H460a) concentrations were determined using a selective LC-MS/MS method with a quantification limit of 1 ng/g tissue.

Pathology/necropsy

Five mice per treatment from all remaining groups were given a full necropsy at the end of the study. Whole blood was also collected from these mice for hematology and clinical chemistry.

Tumor samples were fixed by immersion in 10% zinc formalin then processed in a Tissue-Tek VIP (Sakura, Torrance, CA) and embedded in paraffin. Sections for immunohistochemistry were cut at 5 µm. Pre-immune rabbit or goat serum (Dako, Carpenteria, CA) was used as the negative control. Sections were immersed in Target Retrieval Solution (Dako) and heated to 94°C in a steamer (Black & Decker, Towson, MD) for 20 min. Endogenous peroxidase activity was quenched with 6% H_2O_2 in methanol for 15 min.

To block non-specific tissue-binding sites, sections were blocked by 10% normal serum from the species in which the secondary antibody was raised. Sections were incubated for 20 min at room temperature in serum prepared in Ultra-V (Lab Vision, Fremont, CA).

For platelet endothelial cell adhesion molecule (PECAM-1, CD31) antigen and EGFR antigen, the sections were incubated overnight at room temperature with a polyclonal goat anti-PECAM-1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:800 in Antibody Diluent (Dako) or with a polyclonal rabbit anti-EGFR IgG (BioGenex, San Ramon, CA) diluted 1:50 in Antibody Diluent (Dako). Sections were incubated with Vectastain Elite ABC-peroxidase (Vector, Burlingame, CA) for 45 min at room temperature.

For the Ki-67 antigen, sections were incubated for 1 h at room temperature with a polyclonal anti Ki-67 IgG (NeoMarkers, Fremont, CA) diluted 1:2000 in

Antibody Diluent (Dako), followed by the addition of horseradish peroxidase-labeled streptavidin complex for 30 min.

To detect apoptosis, the TUNEL TdT-FragEL DNA fragmentation detection kit (Oncogene Research Products, San Diego, CA) was used according to the manufacturer's recommendations. For all four antigens, Vector Nova Red (Vector) was the final chromogen and hematoxylin the nuclear counterstain.

Results

EGFR immunohistochemical staining in NSCLC xenografts

The EGFR expression pattern in the H460a and A549 tumors was examined by immunohistochemistry. Both cell lines had a similar membranous pattern of staining for EGFR (data not shown). This confirms previous results showing equivalent expression of EGFR in these two tumor lines [11,12].

Single and chronic-dose PK assessment of erlotinib in athymic nude mice

In non-tumor-bearing mice

Erlotinib 20 and 100 mg/kg was given by gavage to female *nu/nu* athymic mice. The doses refer to the hydrochloride salt with an active drug (free base) content of 91.5%. The formulations were sodium carboxymethylcellulose suspensions containing 2.5 and 12.5 mg/ml of erlotinib, respectively. Three animals per time point were evaluated for PK data (Table 1).

The mice given 100 mg/kg had high systemic exposures to erlotinib, with an AUC_{last} value of approximately 196 000 h·ng/ml. The AUC_{last} following 20 mg/kg was 33 500 h·ng/ml. The exposure (AUC) was dose-proportional. Mean maximum plasma concentrations were approximately 24 000 ng/ml after 100 mg/kg and 9100 ng/ml after 20 mg/kg. Maximum plasma concentration was 0.5–1.0 h post-dose. Mean apparent terminal half-life was about 4 h and the average mean residence time approximately 7 h.

Table 1 Single-dose pharmacokinetics of erlotinib 20 and 100 mg/kg in non-tumor bearing *female nu/nu* athymic mice

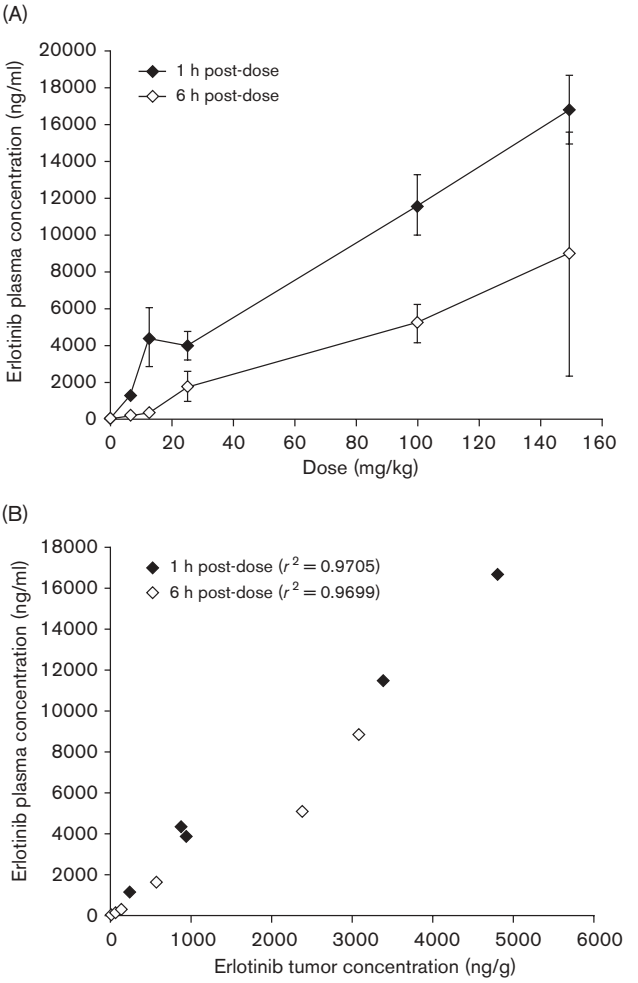
	20 mg/kg	100 mg/kg
C_{max} (ng/ml)	9100	24000
C_{max}/dose ([ng/ml]/[mg/kg])	455	240
T_{max} (h)	0.5–1	0.5–1
T_{last} (h)	8	24
AUC_{last} (h·ng/ml)	33500	196000
AUC_{last}/dose ([h·ng/ml]/[mg/kg])	1680	1960
CL/F (ml/min/kg)	7.6	8.0
λ_z (1/h)	0.17	0.19
$T_{1/2}$ (h)	4.1	4.0
MRT (h)	5.6	8.2
V_z/F (l/kg)	2.7	2.8

C_{max} = peak plasma concentration; T_{max} = time to peak plasma concentration; T_{last} = time of last measurable concentration; AUC_{last} = area under the plasma concentration–time curve from time zero to time of last measurable concentration; CL/F = apparent clearance; λ_z = elimination rate constant; $T_{1/2}$ = plasma terminal half-life; MRT = mean residence time; V_z/F = apparent volume of distribution.

In tumor-bearing mice

After erlotinib 6.3, 12.5, 25.0, 100.0 and 150.0 mg/kg was given orally to *nu/nu* athymic mice, plasma concentration was up to 16 700 and 8870 ng/ml at 1 and 6 h post-dose, respectively (Fig. 1A). The respective mean tumor concentrations following oral doses of 150 mg/kg, sampled at the same time points as the plasma samples, were 4800 and 3090 ng/g tissue.

Fig. 1



Erlotinib plasma concentrations over time. (A) Dose-dependent plasma concentrations. (B) Correlation between tumor drug concentrations and plasma drug concentrations. Tumor-bearing mice were given daily oral doses of erlotinib at 0, 6.3, 12.5, 25.0, 100.0 or 150.0 mg/kg for 21 days. On day 28 post-tumor implant, blood (from the retro-orbital sinus) and tumor samples were collected at 1 and 6 h post-dosing. Concentrations of erlotinib were determined using LC-MS/MS. Values are means ± SD, $n = 3$.

Inter-individual variability of the plasma concentrations was moderate, with a relative standard deviation (RSD) of about 35–40% (range 5.2–120%). The exposure was dose-dependent and more than dose-proportional with ascending doses. Tumor concentrations also correlated well with plasma concentrations in this study (Fig. 1B).

Determination of maximum tolerated doses (MTD) in athymic nude mice

Erlotinib MTD

The MTD for erlotinib was 100 mg/kg (Table 2). Mice showing signs of toxicity all had similar lesions. Gross toxicity was found in the skin and gastrointestinal tract. One mouse in the 400 mg/kg group died. The rest of the animals in this group were euthanized because of morbidity. Mice given 200 mg/kg had marked weight loss and all were euthanized. Our previous efficacy studies have shown, however, that erlotinib 150 mg/kg in this formulation is also well tolerated for 3 weeks (unpublished observation).

Gemcitabine MTD

In a 2-week MTD study in nude mice given gemcitabine, there were no signs of overt toxicity (weight loss or gross clinical signs) in any of the treated groups. Gemcitabine's main toxicity is myelosuppression [13–15]. Terminal blood samples for complete blood counts were not taken; therefore, it is not known if there was myelosuppression in any of the dose groups.

Based on these findings and data found in the literature [3–5], we decided to use a dose of 120 mg/kg every 3 days in later efficacy studies as the maximum dose. We were being cautious in using higher doses as different sensitivities have been shown for tumor-bearing animals and the level of toleration can even be tumor-line-specific [16].

Table 2 MTD assessment in non-tumor-bearing athymic nude mice treated for 14 days ($n=5$)

Compound	Dose (mg/kg)	Change in body weight at end of study (%)	Mortality
Vehicle control	0	0	0
Erlotinib in CMC/Tween	400	N/A	5
Erlotinib in CMC/Tween	200	N/A	5
Erlotinib in CMC/Tween	100	−1	0
Erlotinib in CMC/Tween	50	−1	0
Vehicle control	0	−1	0
Gemcitabine	150	−3	0
Gemcitabine	120	−1	0
Gemcitabine	90	−2	0
Gemcitabine	60	4	0
Gemcitabine	30	−3	0
Vehicle control	0	2	0
Cisplatin	12	N/A	5
Cisplatin	9	−15	2
Cisplatin	6	3	0
Cisplatin	3	4	0

N/A = not available; animals died before the end of the study.

Cisplatin MTD

The MTD in this study was 6 mg/kg every 6 days × 3 i.p. (Table 2). There are reports of various MTDs in mice for cisplatin, using either the i.p. or i.v. route, including 4 mg/kg every 6 days × 2 i.v. in nude mice [10], 6 mg/kg every 6 days × 3 i.p. in C57/Bl6 mice [17] and 4 mg/kg every 4 days × 3 i.v. in nude mice [18].

Mice were given cisplatin i.p. in increasing doses from 3 to 12 mg/kg. The classic toxic side-effects of cisplatin therapy are renal, gastrointestinal and neurological [10]. Mice in both groups (to a lesser extent in the 9 mg/kg group) had clear signs of gastrointestinal toxicity. A complete necropsy was not done, therefore it is not known if there were histological lesions in the kidneys, central nervous system or gastrointestinal tract in these mice, or in those in lower dose groups. However, there were no gross signs of nephrotoxicity, or behavioral or postural signs of neurotoxicity in any dose group.

Effects of erlotinib on established NSCLC xenografts

Dose–response study in H460a

At the end of the study in the H460a NSCLC xenograft (day 28 post-tumor implantation), erlotinib, as a monotherapy, had significant dose-dependent efficacy. In the 100 mg/kg group there was growth inhibition of 61% ($p \leq 0.001$ versus vehicle control).

The other groups had the following growth inhibition: 25 mg/kg: 46% ($p \leq 0.001$ versus vehicle control); 12.5 mg/kg: 36% ($p = 0.003$ versus vehicle control); 6.25 mg/kg: 28% ($p = 0.014$ versus vehicle control) (Fig. 2). There were no partial or complete regressions.

Combination activity of erlotinib and gemcitabine in H460a

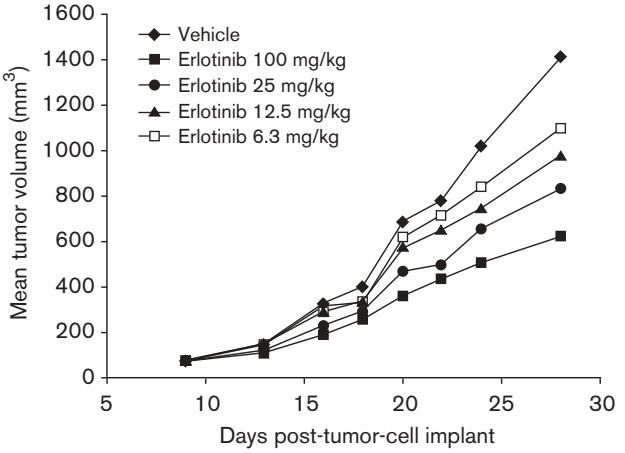
At the 28-day endpoint, erlotinib 100 mg/kg had significantly inhibited tumor growth by 71% ($p = 0.002$) (Fig. 3). Erlotinib 25 mg/kg had a suboptimal efficacy of 30%.

Gemcitabine monotherapy was tested at the MTD of 120 mg/kg every 3 days and at a quarter of the MTD, 30 mg/kg, every 3 days. Gemcitabine 120 mg/kg every 3 days significantly inhibited tumor growth (93%, $p \leq 0.001$). At the fraction of the MTD, tumor growth inhibition was 64% ($p \leq 0.001$).

The combination of gemcitabine 120 mg/kg every 3 days and erlotinib oral 100 mg/kg was lethal, with signs of toxicity at day 5 post-tumor implantation. All mice were dead by day 25 post-tumor implantation (treatment day 15).

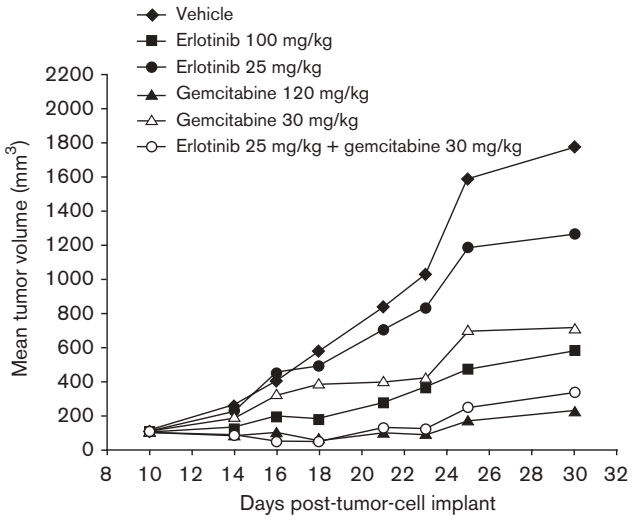
The combination of gemcitabine at 30 mg/kg every 3 days and erlotinib 25 mg/kg inhibited tumor growth by 86%

Fig. 2



Effect of erlotinib on mean tumor volume in the H460a NSCLC xenograft model. Mice were implanted with H460a NSCLC cells. When palpable tumors were established, animals were randomized such that each group had a mean starting tumor volume of 100–150 mm^3. Mice were given daily oral doses of erlotinib at 0, 6.3, 12.5, 25 or 100 mg/kg for 21 days. Tumor size was measured 3 times per week. Values are means, $n=10$.

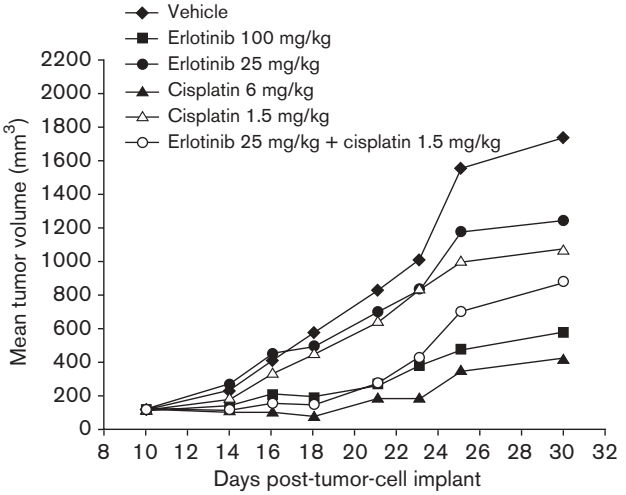
Fig. 3



Effect of erlotinib and gemcitabine alone and in combination on mean tumor volume in the H460a NSCLC xenograft model. Mice were implanted with H460a NSCLC cells. When palpable tumors were established, animals were randomized such that each group had a mean starting tumor volume of 100–150 mm^3. Mice were treated for 21 days with vehicle, oral erlotinib alone at 25 or 100 mg/kg/day, i.p. gemcitabine alone at 30 or 120 mg/kg every 3 days or erlotinib at 25 mg/kg/day with gemcitabine at 30 mg/kg every 3 days. Tumor size was measured 3 times per week. Values are means, $n=10$.

($p \leq 0.001$ versus vehicle control). There were no partial or complete regressions. This inhibition was not additive as it was not significantly better than either gemcitabine or erlotinib administered at 25% of the MTD. This combination was also not significantly better than erlotinib 100 mg/kg or gemcitabine 120 mg/kg.

Fig. 4



Effect of erlotinib and cisplatin alone and in combination on mean tumor volume in the H460a NSCLC xenograft model. Mice were implanted with H460a NSCLC cells. When palpable tumors were established, animals were randomized such that each group had a mean starting tumor volume of 100–150 mm^3. Mice were treated with vehicle, oral erlotinib alone at 25 or 100 mg/kg/day for 3 weeks, i.p. cisplatin alone at 1.5 or 6 mg/kg every 6 days for 3 weeks or erlotinib at 25 mg/kg/day with cisplatin at 1.5 mg/kg every 6 days. Tumor size was measured 3 times per week. Values are means, $n=10$.

Combination activity of erlotinib and cisplatin in H460a

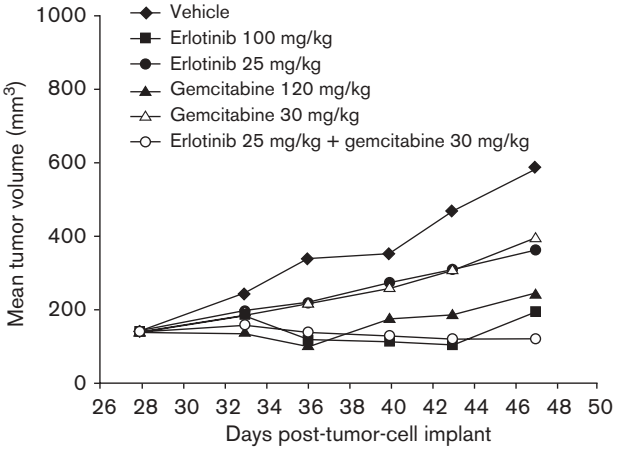
Cisplatin 6 mg/kg significantly inhibited tumor growth by 81% ($p \leq 0.001$) (Fig. 4). Cisplatin 1.5 mg/kg inhibited tumor growth by 42% ($p = 0.014$). Combined cisplatin 6 mg/kg and erlotinib 100 mg/kg was lethal, with signs of toxicity at day 5 post-tumor implantation. All mice were dead by day 23 post-tumor implantation (treatment day 13).

Combined cisplatin 1.5 mg/kg and erlotinib 25 mg/kg was well tolerated and inhibited tumor growth by 53% ($p = 0.003$ versus vehicle). There were no partial or complete regressions. This tumor growth inhibition was not additive as it was not significantly better than either cisplatin or erlotinib administered at 25% of the MTD. This combination was also not significantly better than erlotinib 100 mg/kg or cisplatin 6 mg/kg.

Combination activity erlotinib and gemcitabine in A549

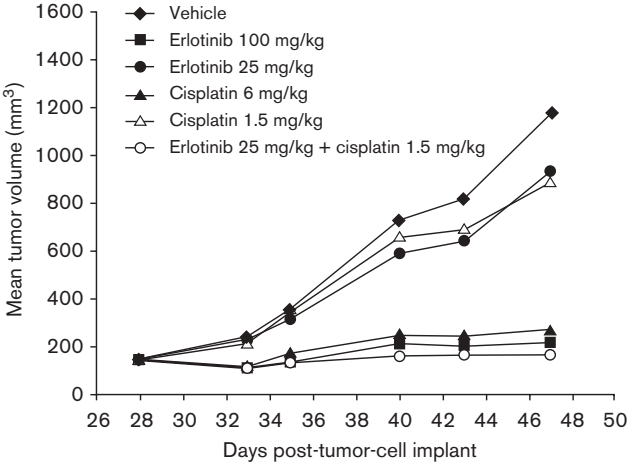
At the end of this study (day 47 post-tumor implantation, treatment day 19), erlotinib 100 mg/kg significantly inhibited tumor growth by 87% ($p \leq 0.001$) (Fig. 5).

Fig. 5



Effect of erlotinib and gemcitabine alone and in combination on mean tumor volume in the A549 NSCLC xenograft model. Mice were implanted with A549 NSCLC cells. When palpable tumors were established, animals were randomized such that each group had a mean starting tumor volume of 100–150 mm^3. Mice were treated for 21 days with vehicle, oral erlotinib alone at 25 or 100 mg/kg/day, i.p. gemcitabine alone at 30 or 120 mg/kg every 3 days or erlotinib at 25 mg/kg/day with gemcitabine at 30 mg/kg every 3 days. Tumor size was measured 3 times per week. Values are means, $n=10$.

Fig. 6



Effect of erlotinib and cisplatin alone and in combination on mean tumor volume in the A549 NSCLC xenograft model. Mice were implanted with A549 NSCLC cells. When palpable tumors were established, animals were randomized such that each group had a mean starting tumor volume of 100–150 mm^3. Mice were treated with vehicle, oral erlotinib alone at 25 or 100 mg/kg/day for 3 weeks, i.p. cisplatin alone at 1.5 or 6 mg/kg every 6 days for 3 weeks or erlotinib at 25 mg/kg/day with cisplatin at 1.5 mg/kg every 6 days. Tumor size was measured 3 times per week. Values are means, $n=10$.

There were two partial regressions (16 and 7%). As in the previous studies, erlotinib 25 mg/kg had suboptimal efficacy of 48% tumor growth inhibition ($p = 0.004$).

Gemcitabine 120 mg/kg significantly inhibited tumor growth by 75% ($p \leq 0.001$) with one partial regression (5%). Gemcitabine 30 mg/kg inhibited tumor growth by 42% ($p = 0.001$). Because of toxicities in previous studies, gemcitabine and erlotinib were not combined at the high doses. Gemcitabine 30 mg/kg and erlotinib 25 mg/kg combined were well tolerated by all mice, with no significant weight loss or overall signs of toxicity. The combination significantly inhibited tumor growth by 103% ($p \leq 0.001$ versus vehicle control), with six partial regressions (range 5–67%). This tumor growth inhibition was additive, as it was significantly better than either gemcitabine or erlotinib administered at a quarter of the MTD ($p \leq 0.05$). The combination was not significantly better than erlotinib 100 mg/kg or gemcitabine 120 mg/kg.

Combination activity erlotinib and cisplatin in A549

At the end of this study (day 47 post-tumor implantation, treatment day 19), erlotinib 100 mg/kg significantly inhibited tumor growth by 93% ($p \leq 0.001$) (Fig. 6). There was one partial regression (2%). Erlotinib 25 mg/kg inhibited tumor growth by 25%. Cisplatin 6 mg/kg significantly inhibited tumor growth by 88% ($p \leq 0.001$) with one partial regression (2%). Cisplatin 1.5 mg/kg inhibited tumor growth by 30%, although this was non-significant.

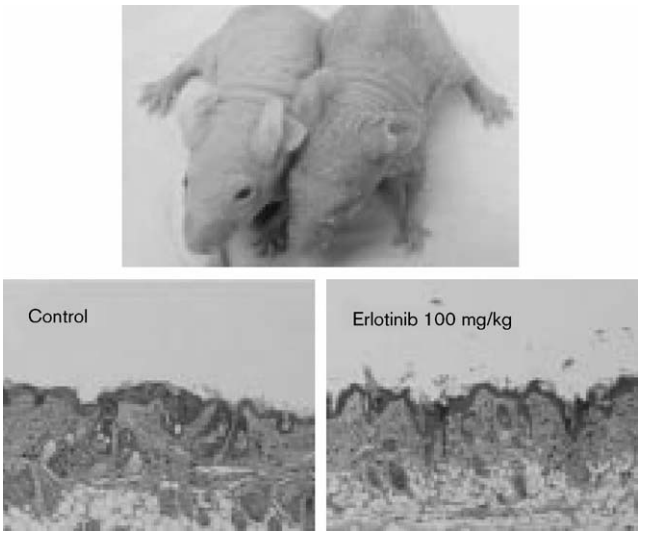
Because of toxicities in previous studies, cisplatin and erlotinib were not combined at the high doses. Combined cisplatin 1.5 mg/kg and erlotinib 25 mg/kg were well tolerated by all mice, with no significant weight loss or overall signs of toxicity. This combination significantly inhibited tumor growth by 98% ($p \leq 0.001$ versus vehicle control), with five partial regressions (range 2–28%). This tumor growth inhibition was synergistic, as it was significantly better than either cisplatin ($p \leq 0.05$) or erlotinib ($p \leq 0.05$) given at 25% of the MTD. This combination was not significantly better than erlotinib 100 mg/kg and cisplatin 6 mg/kg.

Treatment-related effects on normal and tumor tissue

Necropsy in animals given monotherapy

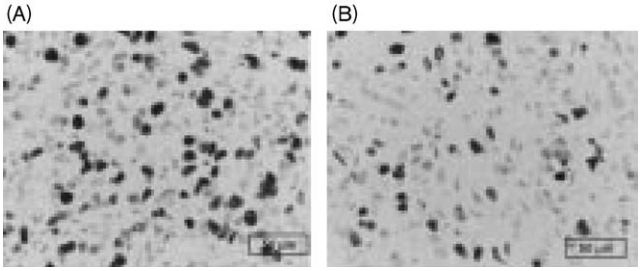
In animals given erlotinib monotherapy, there were no changes in hematology parameters or clinical chemistry parameters (data not shown). There were treatment-related macroscopic changes in the skin. The mice had substantial reddening and crusting of the skin of the muzzle (Fig. 7) that might have been due to the high level of expression of EGFR in the skin. These lesions were transient and dissipated with continued treatment. Treatment-related anti-tumor effects consisted of a mild decrease in Ki-67 proliferative index in the erlotinib 100 mg/kg in both NSCLC xenograft tumor models

Fig. 7



Skin lesions in mice administered erlotinib. At necropsy, skin samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin & eosin. In mice given erlotinib at 100 mg/kg/day for 21 days, skin lesions were grossly characterized as reddened and flaky. Histologically the lesions consisted of diffuse, mild to moderate epidermal acanthosis, epidermal hyperkeratosis, focal escharosis and infiltration of mostly acute inflammatory cells in the dermis. The lesions were transient and dissipated with continued treatment.

Fig. 8



Photomicrographs of immunohistochemical staining of NSCLC in xenograft models. Sections of tumors from nude mice were stained for the antigen Ki-67 to detect cell proliferation in control mice (A) and mice treated with erlotinib at 100 mg/kg/day for 21 days (B) Dark areas represent Ki-67 staining indicative of proliferative activity.

(Fig. 8). There was no significant difference in the frequency of apoptosis in tumor cells in the treated xenografts and no clear effect on angiogenesis as measured by microvascular density (MVD) via immunohistochemical staining for the endothelial cell marker, CD31.

Necropsy in animals given the erlotinib/gemcitabine combination

For mice given erlotinib and gemcitabine at a quarter of the MTD, there were no significant findings in the major organ systems assessed histologically. Treatment-related effects on hematology and serum chemistry parameters were minimal. There was little evidence of treatment-related toxicity under the conditions of this study. Therefore, although the combination of erlotinib 25 mg/kg plus gemcitabine 30 mg/kg had clear antineoplastic effects, it did not appear to increase toxicity. Effects on proliferation in the combination group (assessed by Ki67 staining) were similar to those in erlotinib monotherapy-treated mice (Fig. 8B).

Necropsy in animals given the erlotinib/cisplatin combination

Severity of microscopic lesions was dose dependent in the kidneys of groups given cisplatin alone and in combination. The primary lesions were tubular necrosis and tubular basophilia (data not shown). Treatment-related effects on hematology and serum chemistry parameters were minimal. Cisplatin is a nephrotoxin, producing proximal and distal tubular cell injury [19]. Based on the pathology data, the combination of erlotinib 25 mg/kg and cisplatin 1.5 mg/kg also had clear antineoplastic effects, and, again, toxicity did not appear to be increased. Effects on tumor cell proliferation in the combination group were similar to those of high-dose erlotinib monotherapy (Fig. 8B).

Discussion

These results show that erlotinib, a potent, orally available and selective small-molecule inhibitor of HER1/EGFR, has strong antitumor activity in human NSCLC xenograft models expressing similar numbers of HER1/EGFR, as monotherapy and in combination with conventional chemotherapeutics. In the xenograft model H460a, it had an excellent dose–response relationship and tumor concentration correlated well with plasma concentration.

The two human NSCLC cell lines, when grown as s.c. tumors in athymic mice, had different tumor growth kinetics, with a doubling time of 5 days for H460a and 10 days for A549. Erlotinib monotherapy at 100 mg/kg significantly inhibited tumor growth in the H460a xenograft model.

There was significant growth inhibition and partial regression with the gemcitabine/erlotinib and cisplatin/erlotinib combinations, administered at 25% of the MTD, in the slow-growing A549 tumor (more than 100 and 90%, respectively). Tumor growth inhibition with erlotinib in combination with either agent was significantly increased compared with erlotinib monotherapy ($p \leq 0.05$).

In the faster-growing H460a tumor, there was substantial tumor growth inhibition with the gemcitabine/erlotinib combination (86%) and the cisplatin/erlotinib combination (53%) using a quarter of the MTD of either of the compounds. However, tumor growth inhibition with these combinations was not significantly different from that with monotherapy. A549 is slow growing and therefore assumed to be more dependent on angiogenesis. Erlotinib is thought to be an indirect anti-angiogenic [20], so it is not surprising that it has greater efficacy against A549. Erlotinib inhibits the binding of adenosine triphosphate (ATP) to the intracellular tyrosine kinase domain of HER1/EGFR, blocking receptor phosphorylation and associated downstream signaling [9]. The result is inhibition of cellular processes associated with tumor growth and progression, such as proliferation, angiogenesis, metastasis and protection from apoptosis [9]. Unfortunately, anti-angiogenic effects were not detected by MVD in the tumors treated with erlotinib, possibly because the assay was not sensitive enough.

In both NSCLC models, gemcitabine (30 mg/kg) or cisplatin (1.5 mg/kg) with erlotinib (25 mg/kg), administered at a quarter of the MTD, were well tolerated, with no or insignificant weight loss, suggesting potential significant quality of life benefits for patients, by maintaining efficacy with less risk of side effects. In contrast, the high-dose combination of erlotinib and conventional agents at their individual maximum tolerated doses was not tolerated. This may be related to the fact that supportive care cannot be used preclinically.

Phase III trials of erlotinib in combination with gemcitabine and cisplatin, or with carboplatin and paclitaxel in humans with NSCLC have been disappointing since a conclusive survival benefit was not demonstrated. Nevertheless, the preclinical studies reported here have clearly shown that erlotinib in combination with chemotherapy has an additive effect on inhibiting tumor growth. A confounding factor here is that the phase III combination studies used erlotinib and chemotherapy at the effective MTD. This was not possible in the preclinical studies reported here. Therefore, it remains possible that additive effects of erlotinib and chemotherapy would not be seen if the drugs were given at their combined murine MTD. These findings support the need for further examination of the effects of erlotinib in various clinical settings such as its sequential use with other chemotherapy agents and in selected patient populations. In addition, HER1/EGFR is overexpressed in numerous cancers, including head and neck, prostate, glioma, gastric, breast, cervical, pancreatic and ovarian cancer [1,2]. Therefore, erlotinib in combination with gemcitabine or cisplatin may have efficacy benefits in other cancers with HER1/EGFR-expressing solid-cell tumors.

In conclusion, in NSCLC, the antitumor activity of erlotinib in xenograft tumors with similar levels of EGFR expression is robust both as monotherapy and in combination with chemotherapies. Further research is needed to fully evaluate this promising new avenue in cancer treatment.

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References

1 Ciardiello TG. Anti-epidermal growth factor receptor drugs in cancer therapy. *Expert Opin Invest Drugs* 2002; **11**:755–768.
2 Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995; **19**:183–232.
3 Rajkumar SV, Adjei AA. A review of the pharmacology and clinical activity of new chemotherapeutic agents in lung cancer. *Cancer Treat Rev* 1998; **24**:35–53.
4 Bunn Jr PA, Kelly K. New chemotherapeutic agents prolong survival and improve quality of life in non-small cell lung cancer: a review of the literature and future directions. *Clin Cancer Res* 1998; **4**:1087–1100.
5 ten Bokkel Huinink WW, Bergman B, Chemaissani A, Dornoff W, Drings P, Kellokumpu-Lehtinen PL, *et al.* Single-agent gemcitabine: an active and better-tolerated alternative to standard cisplatin-based chemotherapy in locally advanced or metastatic non-small cell lung cancer. *Lung Cancer* 1999; **26**:85–94.
6 Giaccone G, Splinter TA, Debruyne C, Kho GS, Lianes P, van Zandwijk N, *et al.* Randomized study of paclitaxel–cisplatin versus cisplatin–teniposide in patients with advanced non-small-cell lung cancer. The European Organization for Research and Treatment of Cancer Lung Cancer Cooperative Group. *J Clin Oncol* 1998; **16**:2133–2141.
7 Peters GJ, Bergman AM, Ruiz van Haperen VW, Veerman G, Kuiper CM, Braakhuis BJ. Interaction between cisplatin and gemcitabine *in vitro* and *in vivo*. *Semin Oncol* 1995; **22**(**4 suppl 11**):72–79.
8 Pollack VA, Savage DM, Baker DA, Tsaparikos KE, Sloan DE, Moyer JD, *et al.* Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition *in situ* and antitumor effects in athymic mice. *J Pharmacol Exp Ther* 1999; **291**:739–748.
9 Moyer JD, Barbacci EG, Iwata KK, Arnold L, Boman B, Cunningham A, *et al.* Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 1997; **57**:4838–4848.
10 Perez-Soler R, Chachoua A, Huberman M, Karp D, Rigas J, Hammond L, *et al.* A Phase II trial of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor OSI-774, following platinum-based chemotherapy, in patients with advanced, EGFR-expressing, non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* 2001; **20**:310a (abstr 1235).
11 Bianco C, Tortora G, Bianco R, Caputo R, Veneziani BM, Caputo R, *et al.* Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). *Clin Cancer Res* 2002; **8**: 3250–3258.
12 Lee M, Draoui M, Zia F, Gazdar A, Oie H, Bepler G, *et al.* Epidermal growth factor receptor monoclonal antibodies inhibit the growth of lung cancer cell lines. *J Natl Cancer Inst Monogr* 1992; **13**: 117–123.

13 Hoang T, Kim K, Jaslowski A, Koch P, Beatty P, McGovern J, *et al.* Phase II study of second-line gemcitabine in sensitive or refractory small cell lung cancer. *Lung Cancer* 2003; **42**:97–102.
14 Philip PA. Gemcitabine and platinum combinations in pancreatic cancer. *Cancer* 2002; **95**(**4 suppl**):908–911.
15 Tripathy D. Overview: gemcitabine as single-agent therapy for advanced breast cancer. *Clin Breast Cancer* 2002; **3**(**suppl 1**):8–11.
16 Merriman RL, Hertel LW, Schultz RM, Houghton PJ, Houghton JA, Rutherford PG, *et al.* Comparison of the antitumor activity of gemcitabine and ara-C in a panel of human breast, colon, lung and pancreatic xenograft models. *Invest New Drugs* 1996; **14**:243–247.
17 van Moorsel CJ, Pinedo HM, Veerman G, Vermorken JB, Postmus PE, Peters GJ. Scheduling of gemcitabine and cisplatin in Lewis lung tumor bearing mice. *Eur J Cancer* 1999; **35**:808–814.
18 Riccardi A, Meco D, Ferlini C, Servidei T, Carelli G, Segni G, *et al. In vitro* and *in vivo* antitumor activity of the novel trinuclear platinum complex BBR 3464 in neuroblastoma. *Cancer Chemother Pharmacol* 2001; **47**:498–504.
19 Klaassen CD (editor). *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn. New York: McGraw-Hill; 2001, pp. 399–401, 496, 497, 500, 503, 506, 511, 695, 852, 853.
20 Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002; **2**:727–739.