Discovery and Pharmacologic Characterization of CP-724,714, a Selective ErbB2 Tyrosine Kinase Inhibitor


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Abstract

Amplification and overexpression of erbB2 (Her-2/neu) proto-oncogene has been linked to human malignancies including tumors of the breast, ovary, and stomach. It has been implicated in tumor growth, sensitivity to standard chemotherapy, prognosis of patients, and disease-free survival. Although the clinical use of trastuzumab (Herceptin) has prolonged the survival of breast cancer patients with erbB2-overexpressing tumors, there is an urgent need for more potent and orally bioavailable small-molecule inhibitors. CP-724,714 is a potent inhibitor of erbB2 receptor autophosphorylation in intact cells and is currently undergoing phase I clinical trials. Here, we describe the effects of CP-724,714 in vitro and in vivo in human breast cancer models. CP-724,714 is selective for inhibiting growth of HER2-driven cell lines. In addition, we show that it induces G1 cell cycle block in erbB2-overexpressing BT-474 human breast carcinoma cells and inhibits erbB2 autophosphorylation in xenografts when administered p.o. to athymic mice. It induces a marked reduction of extracellular signal-regulated kinase and Akt phosphorylation, tumor cell apoptosis, and release of caspase-3. P.o. administration (q.d. or b.i.d.) of CP-724,714 inhibits the growth of erbB2-overexpressing tumors in athymic mice without overt adverse effects.

Introduction

The human epidermal growth factor 2 (erbB2/Her-2/neu) is a member of the human epidermal growth factor (EGF) receptor (EGFR) family that also includes erbB1 (EGFR, Her-1), erbB3 (Her-3), and erbB4 (Her-4). Signaling of this type I family of receptor tyrosine kinases (RTK), erbB1 and erbB2 in particular, is crucial for mediating the survival, proliferation, and differentiation of normal cells (1–5). Amplification and overexpression of erbB2 have frequently been observed in human malignancies, and it has been implicated in oncogenic transformation and tumorigenesis. These observations have triggered extensive efforts to find compounds that can prevent/disrupt erbB2 receptor–mediated growth signaling and prevent tumor growth. Several strategies have been developed to prevent erbB2 activation, such as humanized antibody, which can bind to p185erbB2 and prevent receptor-mediated signal transduction and small-molecule inhibitors that block the ATP-binding sites of the cytosolic domain of the erbB2 receptor.

Trastuzumab (Herceptin), a humanized monoclonal antibody directed against p185erbB2 (6–9), was the first agent targeting these receptors approved for clinical use, and was shown to significantly improve the quality and overall life expectancy of erbB2-overexpressing breast cancer patients (10, 11). Unfortunately, a significant number of patients with tumors overexpressing erbB2 do not respond to trastuzumab (12), and most who respond eventually develop resistance to therapy (13).

An alternative strategy is to discover low molecular weight agents that can be administered p.o. In the EGFR family receptors, gefitinib (Iressa) and erlotinib (Tarceva), which target the erbB1 receptor, have already been approved by the Food and Drug Administration (FDA; refs. 14–16). In addition, several reversible small-molecule inhibitors of EGFR (15–18), a dual inhibitor of EGFR and erbB2 (GW2016, lapatanib; see refs. 18–20), and irreversible pan-erbB receptor inhibitors like canertinib (CI-1033; ref. 21) are in various phases of clinical trials.

Inhibition of EGFR is frequently associated with dose-limiting side effects. For example, acne-like skin rash on the face and upper torso was observed in 86% of patients treated with cetuximab (22). Similarly, adverse acniform skin rash has also been observed during clinical trials with the small-molecule EGFR inhibitors gefitinib (23, 24) and erlotinib (25), the pan-erbB inhibitor canertinib (26), and the dual kinase inhibitor lapatinib. These observations indicate that a potent and erbB2-selective agent might not cause this adverse reaction, and this logic led us to start a program seeking a small-molecule erbB2-selective inhibitor, which led to the discovery of CP-724,714.

ErbB2-selective agents represent a unique class of inhibitors potentially devoid of clinical dose-limiting side effects associated with EGFR inhibition such as skin toxicity. We recently reported the biological and biochemical attributes including the antiproliferative effect of a prototype erbB2-selective agent, CP-654,577 (27). We describe herein the pharmacology and biological effects of CP-724,714, a potent erbB2-selective inhibitor.

Materials and Methods

**CP-724,714 synthesis.** E-2-Methoxy-N-(3-[4-[(3-methyl-4-[(6-methylpyridin-3-yl)oxy]-phenylamino]-quinazolin-6-yl]-allyl)-acetamide (CP-724,714) was prepared according to published procedures (28). The chemical structure of CP-724,714 is shown in Fig. 1A. The mesylate salt of this compound was used for all studies.
Cells and cell proliferation assays. NIH 3T3 fibroblasts transfected with chimeric EGFR/erbB2 or human EGFR have previously been reported (27, 29). FRE-erbB2 cells were generated by transfection of Fischer rat embryo fibroblasts with human erbB2 containing an activating mutation in the transmembrane domain as previously reported (27). FRE-erbB2 cells were grown in DMEM with high glucose, 1.5 mmol/L L-glutamine, and 10% fetal bovine serum (FBS; ref. 27). The effects of CP-724,714 on cell proliferation were studied in human breast cancer cell lines growing in vitro. The cell lines ZR-75-30, HCC-1419, MDA-MB-175, BT-474, SKBR3, MDA-MB-361, UACC-812, T-47D, MDA-MB-453, MDA-MB-468, CAMA-1, MDA-MB-157, MCF-7, MDA-MB-435, ZR-75-1, BT-20, and MDA-MB-231 were obtained from American Type Culture Collection. The cell lines EFM-192A, KPL-1, EFM-19, and CAL-51 were obtained from the German Tissue Repository Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and the cell lines SUM-190 and SUM-225 were obtained from the University of Michigan, MDA-MB-175, UACC-893, UACC-812, and MDA-MB-157 cells were cultured in L15 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, and 1% penicillin G-streptomycin-fungizone solution (PSF; Irvine Scientific). CAL-51 and KPL-1 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and PSF, as above. SUM-190 and SUM-225 cells were cultured in Ham’s F-12 supplemented with 5% heat-inactivated FBS, PSF, 5 mg/mL insulin, and 1 mg/mL hydrocortisone. The remaining cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, and PSF.

Cells were seeded in duplicate at 5,000 to 10,000 per well in 24-well plates. The day after plating, CP-724,714 was added by titrating over six or more dilutions from 10 μmol/L down. Control wells without drug were seeded as well. Cells were grown for 6 to 7 days, at which time surviving cells were counted. After trypsinization, cells were placed in isotope solution and counted immediately using a Coulter Z2 particle counter (Beckman Coulter, Inc.). Growth inhibition was calculated by 1 - (experimental value / control value) x 100 for each concentration. Dose-response curves were repeated at least twice and averaged. IC₅₀ values were calculated using CalcuSyn Software (Biosoft).

Animals. Athymic female mice (CD-1 nu/nu, 20 g) obtained from Charles River Laboratories were used for all of the in vivo studies. Mice were housed in specific pathogen-free conditions according to the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care (30) and all of the in vivo studies were carried out under approved institutional experimental animal care and use protocols. Animal handling was done in a laminar flow hood. Animals were provided pelleted food and water ad libitum and kept in a room conditioned at 70°F to 75°F with 50% to 60% relative humidity. Sentinel mice were monitored at regular intervals by serologic assays and were found free of murine pathogens (murine hepatitis virus, Sendai virus, pneumonia virus of mice, minute virus of mice, mouse poliovirus type 3 reovirus, Mycoplasma pulmonis, mouse parvovirus, epizootic diarrhea of infant mice, lymphocytic choriomeningitis virus, mouse adenovirus, ectromelia, mouse pneumonitis, and polyomavirus). For all of the studies, mice were allowed to acclimate a minimum of 3 days after receipt of shipment and randomized before commencement of experiments.

Kinase assays. The erbB2 and EGFR kinase reactions were done as previously reported (27).

Cell selectivity assay. NIH 3T3 cells transfected with chimeric EGFR/erbB2 or EGF were used to determine the erbB2 selectivity of CP-724,714. Cells were treated with CP-724,714 or erlotinib (EGFR inhibitor) for 2 h and then stimulated with EGF for 10 min. Cell lysates were used for Western blot analysis (27).

Cell cycle analysis. Exponentially growing BT-474 cells were treated with CP-724,714 (1 μmol/L, 24 h) and cell cycle distribution was determined by flow cytometric analysis as described earlier (27).

Colony formation assays. BT-474 and SKBR3 cells were seeded at 3,000 or 1,000 per plate, respectively, in medium supplemented with 10% FBS. After attachment overnight, compound was added and cells were exposed for 6 days followed by replacement of medium without compound. On day 14 after seeding, the medium was removed by aspiration and the plates were rinsed with normal saline and fixed with methanolic crystal violet. Colonies were counted manually.

Pharmacokinetic and pharmacodynamic studies. FRE-erbB2 cells (5 x 10⁶ per mouse) were inoculated s.c. into the flank region of female athymic mice and allowed to grow to 250 mm³. Animals were treated with vehicle (0.5% methylcellulose) or CP-724,714 (25 mg/kg, p.o.) and tumors and plasma were isolated at 0.5, 1, 2, 4, and 8 h after dosing.
**Ex vivo studies.** FRE-erbB2 xenograft–bearing mice were treated with vehicle (0.5% methylcellulose) or CP-724,714 (3.125, 6.25, 12.5, 25, 50, and 100 mg/kg, p.o., in 0.5% methylcellulose) and tumors and plasma were isolated at 1 h after dosing. Tumors were homogenized in ice-cold lysis buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1% Glycerol, 1% Triton X-100, 1.6 mmol/L Na3VO4, 50 mmol/L NaF, Protease Inhibitor Cocktail (Boehringer Mannheim)] at 1 mL buffer/100 mg of tumor wet weight. ErbB2 phosphorylation status was determined using a neu-coated ELISA plate (Oncogene Research Products) to capture the receptor and the plate is probed with a horseradish peroxidase–conjugated anti-phosphotyrosine antibody (PY99). Inhibition of erbB2 phosphorylation was measured as the decrease of ELISA signal relative to the vehicle-treated control tumors (27).

**In vivo efficacy of CP-724,714.** These studies were conducted to establish whether CP-724,714 could inhibit xenograft growth in vivo. Exponentially growing cells were trypsinized and resuspended in sterile PBS and inoculated s.c. [FRE-erbB2 cells at 0.5 × 10^6 per mouse and human tumor cells (BT-474 and MDA-MB-453) at 5 × 10^6 to 10 × 10^6 per mouse in 200 mL] into the right flank of female athymic mice (27). Animals bearing xenografts were randomly divided into groups (7–10 per group) and treated with either vehicle or CP-724,714 formulated in 0.5% methylcellulose. Animal body weight and tumor measurements (in millimeters) were obtained every 2 to 4 days. Tumor volume (in cubic millimeters) was calculated using the formula length (mm) × width (mm) × width (mm) × 0.5, as previously described (27). Tumors were isolated and homogenized in ice-cold lysis buffer. ErbB2 receptor phosphorylation was determined by ELISA as described earlier.

**In vivo induction of apoptosis.** Mice bearing FRE-erbB2 xenografts (~150 mm^3 in size) were treated with vehicle or CP-724,714 (30 and 100 mg/kg, p.o., in 0.5% methylcellulose). Tumors were isolated (0.5, 1, 2, 4, and 8 h after dosing) from five mice per dose at each time point and fixed in formaldehyde. The tumors were processed routinely into paraffin, sectioned at 5 μm, and stained with H&E. Apoptotic cells were counted in 10 randomized 400 fields (Olympus BX51) of each section of the tumor. Apoptotic cells were also evaluated by immunostaining of tumor sections for cleaved caspase-3 using an avidin-biotin-peroxidase technique (Cell Signaling).

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**Figure 2.** A, selective inhibition of erbB2 kinase by CP-724,714. NIH 3T3 cells transfected with chimeric EGFR/erbB2 (top) or human EGFR (bottom) were treated with the indicated concentrations of the compound for 2 h and then stimulated for 10 min with 50 ng/mL EGF. Lysates were prepared and analyzed for tyrosine phosphorylation of the chimeric receptor (top) or EGFR (bottom) as described (27). Triplicate samples were analyzed. The blotting membranes were cut at a midrange molecular weight and the lower molecular weight region was blotted for actin to confirm equal loading. B, receptor autophosphorylation was evaluated as described in A. The Western blot with phosphospecific antibodies was quantitated by use of a Lumi-Imager and were reported as relative levels to the control autophosphorylation in samples treated for 10 min with EGF, normalized to 100%. The values are mean ± SD for n = 3 for cells treated with inhibitor and n = 6 for the controls. C, CP-724,714 induces a G1 cell cycle block. BT-474 cells in culture were treated for 24 h with 1 μmol/L CP-724,714 (B) or vehicle for control (A), and then analyzed for DNA synthesis and DNA content by flow cytometry. D, CP-724,714 reduces colony formation by erbB2-overexpressing cells. Colony formation by BT-474 and SKBR3 cells in the presence of vehicle (control) or the indicated concentration of CP-724,714 was evaluated as described in Materials and Methods. Columns, mean of three independent plates; bars, SD.
and erbB2 phosphorylation status was determined. Blood or plasma, diluted with 50-

EC50, FRE-erbB2 xenograft–bearing mice were treated with CP-724,714 (3.125,

CP-724,714 produces a reduction of erbB2 tyrosine phosphorylation

in FRE-erbB2 xenografts. FRE-erbB2 cells (5 × 10⁵ per mouse) were

isolated at 1 h (B). Tumors were homogenized in ice-cold lysis buffer

and erbB2 phosphorylation status was determined.

Blood and plasma analysis of CP-724,714. Aliquots (50 μL) of whole

blood or plasma, diluted with 50-mM acetonitrile (25%) containing an

internal standard (0.5 μg/mL), were subsequently extracted with methyl-
tert-butyl ether and 0.1 mol/L sodium hydroxide by 96-well liquid-liquid

extraction. Concentrations of CP-724,714 and internal standard were

determined by liquid chromatography-tandem mass spectrometry with a

Sciex API 3000 triple quadrupole mass spectrometer. CP-724,714 and

internal standard were separated chromatographically using a reverse-

phase analytical column (50 × 2.1 mm; 5-μm particle, Waters X Terra MS C18

5 μm) at a flow rate of 250 μL/min at ambient temperature. The mobile

phase was delivered as 90% 10 mmol/L ammonium acetate with 0.1%

formic acid and 10% acetonitrile for the first minute followed by a linear

gradient from 10% to 90% acetonitrile over 1 min. Then the mobile phase

was delivered isocratically at 90% acetonitrile for 1 min before a subsequent

gradient back to 10% acetonitrile over 0.1 min. The column was allowed to

reequilibrate at 90% 10 mmol/L ammonium acetate with 0.1% formic acid

and 10% acetonitrile for 1 min before the next sample injection.

CP-724,714 and internal standard were analyzed by a turbo ion-spray

interface operating in positive ion mode by multiple reactions monitoring

with m/z transitions being 470.3→381.2 and 454.2→383.1 atomic mass

units, respectively. The retention times of CP-724,714 and internal standard

were 2.54 and 2.62 min, respectively. Data collection and integration were

accomplished using MacQuan (version 1.6). The ratio of peak area

responses of the drug relative to internal standard was used to construct

a standard curve using a linear least squares regression with a 1/x

weighting. The dynamic range of the assay was 1.0 to 1,000 ng/mL. The

performance of the assay was monitored by inclusion of quality control

samples prepared in mouse whole blood from a separate weighing.

Results

In vitro inhibition of erbB2 kinase and selectivity assays. CP-724,714 (Fig. 1A)

was evaluated as an inhibitor of erbB2 and EGFR kinases in an assay of poly-GluTyr phosphorylation by

recombinant intracellular domains of the respective kinases as previously described (27). It was determined to be a potent

inhibitor of erbB2 kinase (IC₅₀ 10 ± 3 nmol/L) with a marked selectivity against EGFR kinase (IC₅₀ 6,400 ± 2,100 nmol/L; Fig. 1B).

Furthermore, CP-724,714 was >1,000-fold less potent for insulin

receptor, insulin-like growth factor-1 receptor, platelet-derived

growth factor β, vascular endothelial growth factor 2, Abl, Src,
c-Met, c-jun NH₂-terminal kinase (JNK)-2, JNK-3, ZAP-70, cyclin-
dependent kinase (Cdk)-2, and Cdk-5 in in vitro kinase assays (data

not shown).

The selectivity and potency of CP-724,714 for inhibition of

erbB2 and EGFR kinases were further evaluated in studies with

NIH 3T3 cells transfected either with human EGFR or with a

chimera consisting of EGFR extracellular domain and erbB2

intracellular domain as previously described (27). CP-724,714

potently reduced the EGF-induced autophosphorylation of the

chimera containing the erbB2 kinase domain at a concentration

as low as 50 nmol/L (IC₅₀ 32 nmol/L) but was markedly less

potent against EGFR (Fig. 2A and B). In contrast, as previously

reported, the EGFR-selective inhibitor erlotinib was more potent

against the EGFR than the chimera (27). The onset of inhibition

of erbB2 autophosphorylation was very rapid, reached maximal

within 10 min, and was rapidly reversed on washout (data not

shown).

Antiproliferative effects. The antiproliferative effects of CP-

724,714 in vitro across a panel of human breast cancer cell lines

with variable levels of erbB2 and EGFR are shown in Fig. 1C. There is a significant relationship between sensitivity to CP-724,714 and

erbB2 amplification (P = 0.00056; Pearson χ²). BT-474 and SKBR3

were selected for further study as representative cell lines that are

sensitive to CP-724,714 and are erbB2 amplified.

In vitro cell cycle inhibition by CP-724,714. Treatment of the

Her2-amplified BT-474 breast cancer cells, which overexpress erbB2, with 1 μmol/L CP-724,714 for 24 h resulted in accumulation of cells

in G1 phase and a marked reduction in S-phase cells (Fig. 2C) as

measured by flow cytometry. The S-phase fraction decreased from a

control level (untreated) of 26 ± 3% (mean ± SD) to 3.2 ± 0.2% and the

G1 fraction increased from 59 ± 5% to 83 ± 0.2%. Thus, the

inhibition of erbB2 in these cells resulted in a G1 block of

BT-474 cell cycle progression. Exposure of BT-474 or SKBR3 cells

to CP-724,714 also led to cell death as indicated by a decrease in

colony formation (Fig. 2D). Colony formation was reduced by

>80% at 1 μmol/L (BT-474) and 3 μmol/L (SKBR3). CP-724,714 at

1 μmol/L also reduced the level of phospho-erbB2 in these cells

by >90% at 24 h of exposure as measured by Western blotting with

a specific anti–phospho-erbB2 antibody (data not shown).

In vivo reduction of erbB2 receptor phosphorylation by CP-

724,714. We next examined the effect of CP-724,714 on erbB2

receptor phosphorylation in athymic mice bearing FRE-erbB2

xenografts as described in Materials and Methods. CP-724,714

(25 mg/kg) was rapidly absorbed after p.o. administration and

causd ~75% reduction of tumor erbB2 receptor phosphorylation

at 30 min after dosing with a plasma concentration of ~12.5 μg/mL.

Figure 3. CP-724,714 produces a reduction of erbB2 tyrosine phosphorylation

in FRE-erbB2 xenografts. FRE-erbB2 cells (5 × 10⁵ per mouse) were

inoculated s.c. and mice bearing FRE-erbB2 xenografts (~250 mm³ in size)

were used for all studies. Tumors were isolated at 0.5, 1, 2, 4, and 8 h after

CP-724,714 administration (25 mg/kg, p.o.; A). ○, plasma concentration of

CP-724,714; ●, reduction of erbB2 receptor phosphorylation. To determine

EC₅₀, FRE-erbB2 xenograft–bearing mice were treated with CP-724,714 (3.125,

6.25, 12.5, 25, 50, and 100 mg/kg, p.o., in 0.5% methylcellulose) and tumors

were isolated at 1 h (B).
(Fig. 3A). The reduction of erbB2 receptor phosphorylation was correlated with the plasma concentration of CP-724,714 and a complete recovery of this reduction was achieved within 4 h. Studies were also conducted to establish a relationship between the concentration of CP-724,714 and the reduction of erbB2 phosphorylation in the FRE-erbB2 model (Fig. 3B). P.o. administration of CP-724,714 resulted in a concentration-dependent reduction of erbB2 receptor phosphorylation (EC$_{50}$ 1 μg/mL). Similarly, CP-724,714 treatments (p.o.) in the BT-474 model showed marked reduction of erbB2 receptor phosphorylation with a comparable EC$_{50}$ value (0.77 μg/mL; data not shown).

**Tumor cell apoptosis.** Athymic, female FRE-erbB2 xenograft–bearing mice (~150 mm$^3$ in size, s.c.) treated with a single dose of vehicle or CP-724,714 (30 or 100 mg/kg, p.o.) were sacrificed at the indicated times and tumors were isolated and processed for quantitative microscopic evaluation of apoptosis. Apoptotic cells were counted in 10 random 400× fields of each section of the tumor. CP-724,714 treatments resulted in a time- and dose-dependent induction of apoptosis, which was evident as early as 4 to 8 h after dosing. Approximately 75% more tumor cells exhibited apoptotic changes in the 100 mg/kg treatment group compared with vehicle control group at 8 h after dosing (Fig. 4A). An increased number of cleaved caspase-3 immunostained cells in tumors of this group was also observed by immunohistochemical analysis (Fig. 4B).

**Inhibition of FRE-erbB2 xenograft growth by CP-724,714.** We extended our investigations to determine the antitumor efficacy of CP-724,714 in FRE-erbB2 tumor model. Athymic female mice bearing FRE-erbB2 xenografts (~150 mm$^3$ in size, s.c.) were randomized in groups consisting of nine animals each as described in Materials and Methods. P.o. administration of CP-724,714 (6.25–100 mg/kg, q.d.) resulted in an inhibition of FRE-erbB2 xenografts (Fig. 5A). Approximately 50% tumor growth inhibition was observed in animals receiving daily doses of 50 mg/kg, q.d. (P < 0.001). The plasma C$_{max}$ and C$_{ave}$ 0–4 h values in this group were 4.5 and 1.3 μg/mL, respectively. CP-724,714 was well tolerated and there was no weight loss or mortality.

**Inhibition of human breast cancer xenografts by CP-724,714.** We evaluated the p.o. antitumor efficacy of CP-724,714 in two human breast carcinoma models, BT-474 and MDA-MB-453, which are Her2 amplified and highly overexpress erbB2 (confirmed by flow cytometry; data not shown). CP-724,714 produced a dose-dependent inhibition of BT-474 xenograft growth with antiproliferative efficacy observed within 4 days of dosing at the two higher doses (Fig. 5B). BT-474 xenograft growth at 30 mg/kg ceased after 14 days of dosing and there was no tumor regrowth until day 40 when the study was terminated. Overall, on day 40, the tumor growth inhibition was 41%, 42%, 81%, and 100% (relative to vehicle) in 1, 10, 30, and 100 mg/kg treatment groups, respectively. Tumor regression occurred in the 100 mg/kg treatment group by day 9 (~6%), and it reached 61% regression on day 40 (P < 0.001). On day 40, the reduction of tumor erbB2 receptor phosphorylation was nonlinear (10–30% in 1–30 mg/kg treatment groups); however, 81% reduction of erbB2 receptor phosphorylation was observed in the 100 mg/kg group. The antitumor efficacy of CP-724,714 was also determined in the MDA-MB-453 xenograft model, which also expresses a higher level of erbB2. On day 29, ~19%, 66%, and 83% tumor growth inhibition was achieved in the 25, 50, and 100 mg/kg b.i.d. treatment groups, respectively (Fig. 5C). The reduction of tumor erbB2 phosphorylation in these groups was in the range of 69% to 79%.

The antitumor efficacy of CP-724,714 was also determined in athymic mice bearing xenografts expressing lower levels of erbB2, specifically MDA-MB-231 (breast), LoVo (colon), and Colo-205 (colon). CP-724,714 treatments were efficacious in all these models; however, the growth inhibition obtained in these models...
on 100 mg/kg (p.o.) b.i.d. dosing for 14 to 31 days of treatment was in the range of 46% to 71% (detailed data not shown). Thus, response in these models was less than that seen in the very high erbB2 expressing tumors MDA-MB-453 and BT-474 in which 83% inhibition of growth or regressions were obtained (Fig. 5).

Reduction of extracellular signal–regulated kinase and Akt phosphorylation. The mitogen-activated protein kinases (MAPK) extracellular signal–regulated kinase Erk1 and Erk2 play critical roles in cell growth and survival. CP-724,714 treatments (30 or 100 mg/kg, p.o.) resulted in 75% reduction of these activated (phosphorylated) MAPKs in BT-474 xenografts (Fig. 6A and B). We also determined the effect of CP-724,714 on tumor phospho-Akt levels because it plays an important role in transducing cell survival signaling through erbB2 RTK. Administration of CP-724,714 markedly reduced phospho-Akt within 0.5 h (Fig. 6A and B). Approximately 42% and 70% reductions of phospho-Akt in BT-474 xenograft was observed at 0.5 h with 30 and 100 mg/kg treatments, respectively.

Discussion

In summary, overexpression or amplification of erbB RTK in human cancers has provided a rationale for targeting these receptors for the discovery of novel target-based therapeutics. Recent FDA approvals of the EGFR-targeted small-molecule tyrosine kinase inhibitors gefitinib and erlotinib, the dual EGFR/erbB2 tyrosine kinase inhibitor lapatinib, and the monoclonal antibody cetuximab have significantly improved the quality of life and overall survival of cancer patients with EGFR and

![Graph A](image1.png)

**Figure 5.** Effect of CP-724,714 on the growth of tumor xenografts. Exponentially growing cancer cells were harvested and inoculated s.c. into the flank of female athymic mice as described in Materials and Methods. Animals bearing xenografts (85–150 mm³ in size) were treated with vehicle or CP-724,714 in 0.5% methylcellulose [p.o., q.d. (FRE-erbB2, A) or b.i.d. (BT-474, B and MDA-MB-453, C)] for 8 to 40 d. The tumors were measured with a caliper and tumor volume (mm³) was calculated. Points, mean; bars, SE. P.o. administration of CP-724,714 resulted in a dose-dependent inhibition of tumor growth. No weight loss or mortality of the animals was noted in any group.

![Graph B](image2.png)

**Figure 6.** Effect of CP-724,714 on phospho-Erk and phospho-Akt in BT-474 xenografts. Animals bearing BT-474 tumors (~ 85 mm³ in size) were treated with vehicle or CP-724,714 in 0.5% methylcellulose (30 or 100 mg/kg, p.o.). Tumors isolated at 0.5 h after dosing were homogenized and phospho-Erk and phospho-Akt levels were determined by Western blot analysis (A). Quantitation by densitometry of the blots is shown in B. Columns, mean; bars, SE. β-Actin was used as a gel loading control.
HER2-dependent malignancies (14, 31, 32–34). Similarly, the survival benefit of trastuzumab treatments in combination with paclitaxel in breast cancer patients (8, 9) has brought higher expectations and enthusiasm for the discovery of new agents targeting the signal transduction pathway of the EGFR family. Although the successful clinical outcome of trastuzumab treatments validates the benefit of targeting the erbB2 RTK signaling pathway, long-term use of trastuzumab has several limitations. Trastuzumab is administered i.v. on a weekly basis and long-term treatment is not economical. Furthermore, a large number of patients do not respond to trastuzumab treatments due to de novo resistance. In addition, many patients who initially respond have their tumors begin to regrow within 1 to 2 years, leaving them very limited treatment options (12, 13). Hence, there is an urgent need for potent and erbB2 RTK-selective small-molecule inhibitors for long-term p.o. treatment.

We have described the identification and pharmacologic characterization of CP-724,714, a novel erbB2 receptor-selective RTK inhibitor. CP-724,714 is a potent inhibitor of erbB2 receptor kinase (IC_{50} 10 nmol/L) in vitro and also in an intact cell–based assay (IC_{50} 32 nmol/L). It is an erbB2 RTK–selective compound (500–1,000-fold selective against erbB1 and other related RTKs). It induces G1 cell cycle block in vitro in erbB2-overexpressing BT-474 human breast carcinoma cells. In ex vivo studies, CP-724,714 treatments resulted in a concentration-dependent reduction of erbB2 receptor phosphorylation. When administered p.o., CP-724,714 preferentially inhibited tumor growth of erbB2-overexpressing human breast carcinomas in athymic mice. In addition, CP-724,714 treatments induce reduction of downstream erbB2 RTK signaling (phospho-Akt, phospho-Erk1, and phospho-Erk2), tumor cell apoptosis, and release of caspase-3. CP-724,714 induced regression of BT-474 tumors and significant inhibition in a number of other human tumor xenografts. Additionally, CP-724,714 showed a favorable nonclinical toxicity profile with no apparent effects on cardiac tissue. On the basis of these promising preclinical results, CP-724,714 was advanced to phase I clinical trials and is potentially another option for women with Her2-driven breast cancer.

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Discovery and Pharmacologic Characterization of CP-724,714, a Selective ErbB2 Tyrosine Kinase Inhibitor

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