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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[1]

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ABSTRACT

Phosphorylation of tyrosine residues on the epidermal growth factor (EGF) receptor (EGFr) is an important early event in signal transduction, leading to cell replication for major human carcinomas. CP-358,774 is a potent and selective inhibitor of the EGFr tyrosine kinase and produces selective inhibition of EGF-mediated tumor cell mitogenesis. To assess the pharmacodynamic aspects of EGFr inhibition, we devised an ex vivo enzyme-linked immunosorbent assay for quantification of EGFr-specific tyrosine phosphorylation in human tumor tissue specimens obtained from xenografts growing s.c. in athymic mice. When coupled with pharmacokinetic analyses, this measurement can be used to describe the extent and duration of kinase inhibition in vivo. CP-358,774 is an effective, orally active inhibitor of EGFr-specific tyrosine phosphorylation (ED_{50} = 10 mg/kg, single dose). It has a significant duration of action, producing, on average, a 70% reduction in EGFr-associated phosphotyrosine over a 24-h period after a single 100 mg/kg dose. Inhibition of EGFr phosphotyrosine in an ex vivo assay format effectively estimates the potency and degree of inhibition of EGFr-dependent human LICR-LON-HN5 head and neck carcinoma tumor growth. Substantial growth inhibition of human tumor xenografts was achieved with p.o. doses of the compound (ED_{50} = 10 mg/kg q.d. for 20 days). Combination chemotherapy with cisplatin produced a significant response above that of cisplatin alone with no detectable effects on body weight or lethal toxicity. Taken together, these observations suggest that CP-358,774 may be useful for the treatment of EGFr-driven human carcinomas.

For the majority of human carcinomas, growth factor receptors play an important role in tumorigenesis and progression to terminal disease states. The epidermal growth factor (EGF) receptor (EGFr) has been implicated in many human squamous cell carcinomas (Ozanne et al., 1986), such as non-small cell lung carcinoma and brain, bladder, breast, and ovarian carcinomas (Gullick, 1991). EGF at picomolar concentrations is mitogenic for cells overexpressing the receptor, and antibodies to EGFr abolish EGF-stimulated mitogenesis in LICR-LON-HN5 head and neck carcinoma (HN5; Modjtahedi et al., 1993b,c) and other tumor cells (Aboud-Pirak et al., 1988; Yoneda et al., 1991a). As an early event in the signal transduction process, the ligand transforming growth factor-α or EGF binds to EGFr on the surface of tumor cells and stimulates: 1) heterodimerization and homodimerization of EGFr molecules; 2) intermolecular cross-phosphorylation of intracytoplasmic tyrosine residues (EGFr autophosphorylation; Honegger et al., 1989); and 3) activation of the tyrosine kinase activity of EGFr. Apart from binding to the cognate ligand, all known functions of EGFr depend on tyrosine kinase activity. Point mutations in the kinase domain that abrogate ATP binding also abolish ligand-dependent kinase activity and abrogate EGF/transforming growth factor-α-induced mitogenesis (Moolenaar et al., 1988). An intact kinase domain is essential for activation of numerous downstream effectors, including phospholipase C-γ (Margolis et al., 1990; Nishibe et al., 1990; Wahl et al., 1990) phosphatidylinositol 3-kinase (Bjorge et al., 1990), and mitogen-activated protein

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ABBREVIATIONS: EGF, epidermal growth factor; EGFr, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks' balanced salt solution; HN5, LICR-LON-HN5 head and neck carcinoma.

kinase (Ahn et al., 1990), with the ultimate cellular response being DNA synthesis and cell division (Honegger et al., 1987). Transfection experiments have shown that EGFr overexpression alone may lead to constitutive activation of signal transduction, leading to uncontrolled mitosis (Di Fiore et al., 1987; Velu et al., 1987). The degree of EGFr overexpression has been shown to be related to tumorigenicity in some tumor systems (Santon et al., 1986; Velu, 1990). Recent studies of biopsy specimens suggest that overexpression of EGFr is associated with a poor prognosis in bladder (Neal et al., 1985) and breast (Sainsbury et al., 1985) carcinomas.

Despite homology with other tyrosine kinases, selective inhibitors have been identified (for a review, see Traxler, 1998). The EGFr tyrosine kinase therefore represents an attractive molecular target for pharmacological intervention. To monitor the effects of kinase inhibition, the degree of EGFr autophosphorylation was examined, because: 1) autophosphorylation of effector-specific tyrosine residues increases the velocity of the kinase reaction (Bertics and Gill, 1985); 2) autophosphorylation increases the affinity of the EGFr for its substrates, such as phospholipase C-γ (Magni et al., 1991), allowing these substrates to bind the activated receptor (docking site) and thereby become tyrosine phosphorylated; and 3) EGFr phosphotyrosine represents the last known biochemical event before committed steps toward cellular division are mediated by downstream effector mechanisms. For these reasons, we believe quantification of EGFr autophosphorylation is related to, and characterizes, inhibition of the kinase functionality.

CP-358,774 is a potent inhibitor of the EGFr tyrosine kinase with an IC_{50} value of 2 nM; CP-358,774 and its analogs have been shown to be direct-acting, reversible, ATP-competitive inhibitors of EGFr tyrosine phosphorylation (Moyer et al., 1997; Pustilnik et al., 1997). Specificity analysis has indicated >1000-fold selectivity against other tyrosine kinases, such as $pp60^{v\text{-}src}$, $pp145^{c\text{-}abl}$, the tyrosine kinase activities of the insulin and the insulin-like growth factor-1 receptors; selectivity has been shown against isolated kinases as well as in intact cells (Moyer et al., 1997). CP-358,774 inhibits autophosphorylation of the EGFr in a variety of EGFr-overexpressing tumor cells (IC_{50} = 20 nM) and produces inhibition of mitogenesis, inhibition of tumor cell division, and cell cycle arrest. In some cell types, such as DiFi, CP-358,774 induces concentration-dependent apoptosis in vitro.

Here, we report that CP-358,774 is an effective, orally active inhibitor of EGFr tyrosine autophosphorylation. CP-358,774 can effectively inhibit EGFr tyrosine phosphorylation in human tumors growing s.c. in athymic mice with an ED_{50} value of 10 mg/kg p.o. It has significant duration of action and produces substantial inhibition of human EGFr-overexpressing tumors growing s.c. in athymic mice. Moreover, the degree of inhibition of EGFr phosphotyrosine shows good agreement with the degree of tumor growth inhibition in treated animals. The results of these experiments were previously reported at the American Association for Cancer Research annual meeting (Pollack et al., 1997). The data suggest that CP-358,774 may be a useful new compound for therapy of human neoplastic diseases.

Materials and Methods

Mice. Three- to 4-week-old female athymic mice (CD-1 *nu/nu*) were used for human tumor xenografts. Mice were obtained from Charles River Laboratories (Wilmington, MA) and were housed in specific pathogen-free conditions, according to the guidelines of the American Association for Laboratory Animal Care; all studies were carried out with approved institutional experimental animal care and use protocols. During these studies, animals were provided pelleted food and water ad libitum and kept in a room conditioned at 70–75°C and 50 to 60% relative humidity with >15 fresh air changes per hour. Sentinel heterozygous littermates of the athymic animals were monitored routinely (3-week intervals) by serological assays and were found to be free of exposure to the following agents: murine hepatitis virus, ectromelia virus, and Sendai virus. For all studies, the mice were allowed to acclimate for 1 to 3 days after receipt of shipment; test animals were randomized before commencement of treatments and examined twice daily thereafter for compound-induced or tumor-related deaths. Moribund animals were sacrificed to reduce suffering.

Tumor Cell Lines. The HN5 cells were obtained from Dr. M. J. O'Hare (Haddow Labs., Institute of Cancer Research, Sutton, Surrey, UK). All other cells were purchased from the American Type Culture Collection (Rockland, MD). All cell lines were free of reovirus type 3, pneumonia virus of mice, K-virus, Theiler's virus, Sendai virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Bethesda, MD).

Cell Culture. Cell lines were passaged by monolayer culture in 175-cm^2 tissue culture flasks (Nunclon; Marsh Biomedical Products, Rochester, NY) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS (Hazelton Research Products, Inc., Lenexa, KS), 300 μg/ml glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10 μg/ml gentamycin at 37°C in a humidified 95% air/5% CO_2 atmosphere. Routine periodic samples of cell culture broths tested negative for *Mycoplasma* contamination (Microbiological Associates). For implantation in vivo, the tumor cells were harvested from exponentially growing cultures (60–80% confluence), detached by light trypsinization (0.25% trypsin and 0.02% EDTA, 1 min), washed in Hanks' balanced salt solution (HBSS), resuspended in HBSS, mixed with the basement membrane preparation Matrigel (40234; Collaborative Biomedical Products, Bedford, MA), and held in an ice bath <1 h before injection.

Chemotherapeutants. CP-358,774 [6,7-bis(2-methoxy-ethoxy)quinazoline-4-yl]-(3-ethynylphenyl)amine; MF = $C_{22}H_{23}N_3O_4$), a colorless, crystalline, anhydrous compound, was synthesized in our laboratories (Arnold and Schnur, 1998). In these studies, the hydrochloride salt (molecular weight = 429.9) was used in all cases, except for that represented in Fig. 7, which used the free base (molecular weight = 393.4), and the dosage levels shown represent the quantity of free base administered, excluding the contribution of the salt. The compound was formulated for i.p. or p.o. administration by dissolution of the dry powder in a small amount (10% of final volume) of dimethyl sulfoxide (DMSO), mixed by vortexing until dissolved; during vortexing, sufficient sterile, pyrogen-free physiological saline (0.15 N NaCl), containing 0.10% (w/v) Pluronic P105 (BASF Wyandotte, Parsippany, NJ), was added to produce a homogeneous fine suspension. The prepared dosage forms did not produce microbial colonies after incubation on brain-heart infusion agar and did not contain endotoxin detectable by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA). Doxorubicin (Adriamycin; Rapid Dissolution Formula) was purchased from Adria Labs. (Columbus, OH). Cisplatin was obtained as a powder from Sigma Chemical Co. (St. Louis, MO). All dosage forms were freshly prepared for each day's treatment. CP-358,774 and the reference agents were dosed according to the optimum formulation, route, and regimens, as empirically derived in previous studies; aggressive dosing parameters (single bolus treatments at maximum tolerated dosage levels) were used for maximum antitumor efficacy of the cytoreductive agents. Test animals were treated between 7 and 9 AM, immediately after a 12-h dark photoperiod (active phase), to control for variability introduced by

circadian physiological cycles, according to the methods of Halberg et al. (1973).

EGFr Phosphotyrosine Determinations by Enzyme-Linked Immunosorbent Assay (ELISA). To determine compound-induced inhibition of EGFr-associated tyrosine phosphorylation in human tumor explants from athymic mice, an ELISA specific for EGFr phosphotyrosine was developed. Tumor tissue was harvested at various times after dosing (usually 1 h) by careful dissection, immediately flash frozen in liquid nitrogen, and then homogenized in buffer formulated to prevent further tyrosine phosphorylation as well as enzymatic phosphatase activity. A double-antibody ELISA provided quantitative determinations of the degree of EGFr tyrosine phosphorylation after specific capture of EGFr protein.

Briefly, athymic mice with s.c. tumors (5–10 mm in diameter) were euthanized humanely, and tumors were excised with the use of small dissecting scissors and mosquito forceps, after which the tumor tissue was immediately flash frozen in liquid nitrogen and stored at −70°C before homogenization and immunoassay. Tumors were weighed, and for each 100 mg of tumor tissue, 1 ml of ice-cold, sterile lysis buffer was added. Lysis buffer contained (per liter) 50 ml of 1 M HEPES, pH 7.4, buffer, 37.5 ml of 4 M sodium chloride, 0.75 ml of 2 M magnesium chloride, 10 ml of 100 mM EDTA, 10 ml of glycerol, 10 ml of Triton X-100, 8 ml of 200 mM sodium orthovanadate, 4.2 g of sodium fluoride, 50 μg/ml phenylmethylsulfonyl fluoride, 25 mg of soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Tumors were homogenized with a Thomas Teflon pestle homogenizer attached to a power drill (or equivalent) and then clarified by centrifugation; the resulting supernatant liquid (800 μl) was transferred to microtiter plates in 200-μl aliquots and maintained at −70°C before assay.

Appropriate dilutions of tumor homogenates (1:20–1:40 dilutions) were made in blocking buffer containing (per liter) 50 g of bovine serum albumin, 10 g of ovalbumin, 0.90% NaCl, and 10 mM Tris · HCl buffer, pH 7.4. After dilution, 100-μl aliquots were transferred to microtiter wells containing adsorbed monoclonal antibody to EGFr protein (Q1A08; Oncogene Science, Uniondale, NY). The plates were then incubated for 30 min at 30°C (or 3 h at room temperature) to allow efficient capture of the EGFr protein from the tumor homogenates. Microtiter wells were washed six times in a 1:10 dilution of Plate Wash Concentrate (PN 77 0550; DuPont NEN, Boston, MA). To detect phosphotyrosine residues, 100 μl of horseradish peroxidase-conjugated monoclonal antibody specific for phosphotyrosine (diluted 1:1000 in blocking buffer) was added to each well (PY54 conjugate, PT03; Oncogene Science), and plates were incubated for 1 h at 30°C. Microtiter wells were then washed six times in a 1:10 dilution of Plate Wash Concentrate, after which 100 μl/well of 3,3′,5,5′-tetramethylbenzidine substrate was added (50-76-04; Kirkegaard and Perry Laboratories, Gaithersburg, MD); color development was monitored over 30 min, after which all reactions were stopped with 100 μl/well of 0.09 M sulfuric acid. For quantification, absorbance was determined at 450 nm with a Bio-Rad (Hercules, CA) model 3550 microplate reader. EGFr phosphotyrosine content was calculated after normalization of each sample for total protein with a commercial kit (BCA Protein; Pierce, Rockford, IL).

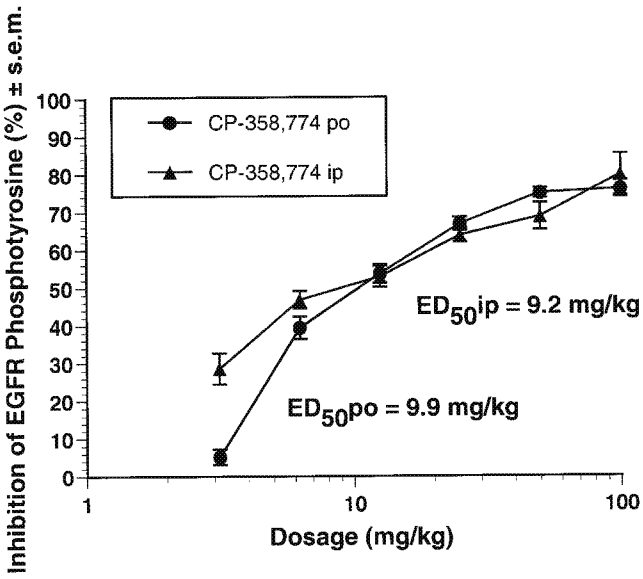
The absorbance values for samples from each of the tumor-bearing animals (sample size, four mice/treatment group) were entered into a custom Microsoft Excel spreadsheet, where the endpoints (i.e., protein concentrations and phosphotyrosine levels) were calculated. In all cases, the EGFr-associated tyrosine phosphorylation was expressed as absorbance units/mg total protein. For statistical inferences, the relationships between groups (i.e., test versus control group) were identified using a computer program for the one-way ANOVA, where the α significance level was assigned at 0.05. P values were determined using Dunnett's t statistic. A set of internal laboratory standards (i.e., aliquots from previously frozen tissue for both treated and control groups) was used to assess the quality and reproducibility of the immunoassay; in the course of 5 years' routine testing, the results were highly reproducible (i.e., the coefficient of variation was <6.0%).

HPLC Determinations of CP-358,774 in Plasma and Tumor Tissue. Determination of drug concentration was made by organic extraction (acetonitrile) of plasma and tumor samples, followed by HPLC. CP-358,774 in plasma and tumor tissues was extracted from 200-μl samples spiked with 100 μl of internal standard (CP-292,597; 0.8 ng/μl in acetonitrile) with 5 ml of methyl t-butyl ether using an Oberbach reciprocating shaker for 10 min. Before extraction, tumor tissue was homogenized in 4 parts deionized water to 1 part tumor specimen (v/m) using an Omni 2000 (Omni International, Gainesville, VA) tissue homogenizer. Samples were centrifuged at 3000 rpm for 5 min at 22°C using a Jouan centrifuge. The organic layer of each sample was transferred to a clean tube, and the methyl t-butyl ether was evaporated to dryness in a Zymark Turbo-Vap at 60°C. All samples were reconstituted in 200 μl of mobile phase consisting of 70% water and 30% acetonitrile (v/v) brought to pH 2.4 with trifluoroacetic anhydride (Acros Organics). A 2-liter volume of mobile phase consisted of 1400 ml of Milli-Q deionized water, 600 ml of acetonitrile, and 550 μl of trifluoroacetic anhydride. The analytical column was a YMC Basic C-18 (4.6 mm × 150 mm, 3 μm). A pump (Thermo Separation Products Constametric 4100) was used to establish a 1.5 ml/min flow rate through the column. CP-358,774 was detected at 345 nm (AUFS 0.001) using an ultraviolet detector (Milton Roy Spectro Monitor 3100 variable wavelength detector). The retention time for CP-358,774 was 6.5. The lower limit of quantification of the assay was 10 ng/ml for plasma and 50 ng/g for tumor tissue.

Tumor Growth Inhibition Studies In Vivo. The tumor growth inhibitory effects of CP-358,774 were measured in young athymic mice bearing established, palpable (2–4-mm diameter) human HN5 or A431 tumors. Tumors were induced in the left flank of 3- to 4-week old athymic mice by s.c. injection of 1×10^6 cultured, log phase HN5 or A431 cells in 0.20 ml of HBSS containing 50% Matrigel. Tumor size was measured in millimeters with Vernier calipers across two diameters three times/week, and the tumor volume (mm^3) was calculated using the formula: tumor volume = (length × [width]^2)/2, according to standard methods (Geran et al., 1972); results are expressed as tumor volume (TuV) in mm^3. To calculate tumor growth inhibition, the following formula was used: inhibition (%) = $(TuG_{control} - TuG_{test})/TuG_{control} \times 100\%$, where tumor growth (TuG) equals the final tumor size minus the pretreatment tumor size for individual treatment groups. This method of tumor implantation provided reproducible growth in athymic mice, enabling the determination of dose-response effects for a variety of chemotherapeutic agents. For each experiment, athymic mice were randomized on receipt of shipment and again after tumor implantation (i.e., before commencement of treatment). Data collected from the antitumor studies (e.g., tumor volume) were evaluated for statistical significance using one-way ANOVA (for significant antitumor activity, $P < .05$).

Results

Inhibition of EGFr Phosphotyrosine in HN5 Xenografts. HN5 possesses many of the characteristics of EGFr-dependent squamous cell carcinomas both in vitro (Modjtahedi et al., 1993b,c) and in vivo (Modjtahedi et al., 1993a,b). In particular, monoclonal antibodies directed at the EGFr can completely block cellular proliferation in vitro, and for this reason, the tumor cell line was selected to evaluate a large series of EGFr tyrosine kinase inhibitors. When administered orally (by gavage) or parenterally (i.p.), CP-358,774 consistently produced significant, dose-related inhibition of HN5 EGFr tyrosine phosphorylation 1 h after dosing (Fig. 1). Compared with vehicle-treated controls, a maximum of 80% reduction in phosphotyrosine was observed after dosing by p.o. or i.p. routes. In several preliminary experiments, the



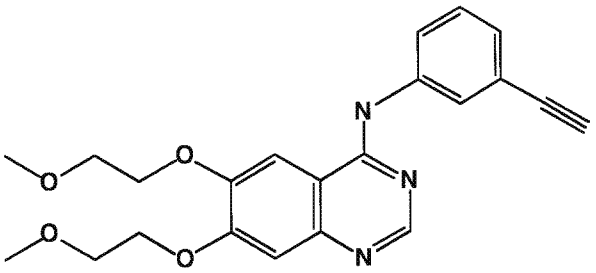


Fig. 1. a, CP-358,774-mediated inhibition of EGFr-associated phosphotyrosine of HN5 tumor xenografts. Human head and neck carcinomas growing s.c. in athymic mice were excised 1 h after dosing either p.o. or i.p. with CP-358,774 formulated in sterile, pyrogen-free 10% DMSO, 0.85% sodium chloride, and 0.1% Pluronic P105. The tumor EGFr-associated phosphotyrosine was measured by ELISA; the data are a summation of 22 (i.p.) and 28 (p.o.) independent experiments. b, CP-358,774 is [6,7-*bis*(2-methoxy-ethoxy)-quinazoline-4-yl]-(3-ethynylphenyl)amine (MF = $C_{22}H_{23}N_3O_4$, MW = 393.4).

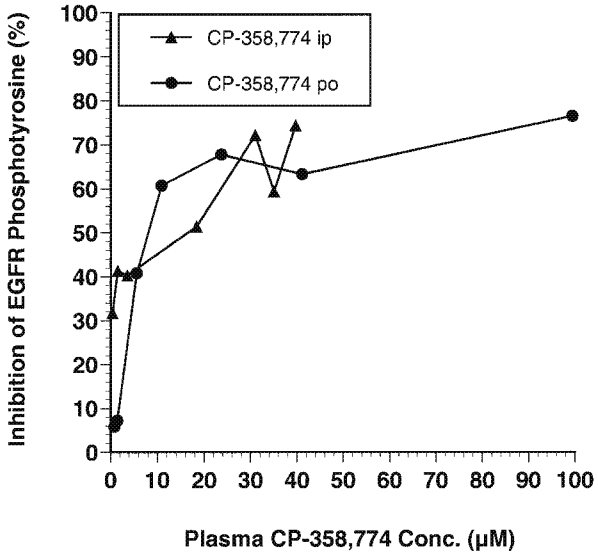


Fig. 2. The relationship of plasma CP-358,774 concentration to reduction in tumor-associated EGFr phosphotyrosine. At 1 h post-treatment with graded doses of 2.9 to 92 mg/kg by either the p.o. or i.p. route, plasma CP-358,774 concentrations were determined by HPLC and HN5 tumor-associated EGFr phosphotyrosine inhibition by ELISA. The effective plasma concentration for 50% inhibition of the target receptor was estimated at 8 μM (3.1 μg/ml) and ~12 μM (4.7 μg/ml) for p.o. and i.p. dosing, respectively. These data are representative of three independent experiments.

vehicle (10% DMSO, 0.85% NaCl, and 0.10% Pluronic P105) produced no inhibition of EGFr phosphotyrosine compared with water or saline treatments.

The data in Fig. 1 are a compilation of 28 (p.o.) and 22 (i.p.) independent experiments, attesting to the reproducible inhibition by this agent. The effective dose for 50% inhibition of the target receptor (ED_{50}) was similar for p.o. and i.p. administration: 9.9 mg/kg p.o. and 9.2 mg/kg i.p. The minimal effective single dose eliciting statistically significant ($P < .05$) reduction in EGFr-associated phosphotyrosine was 5.5 mg/kg (39% reduction) and 2.8 mg/kg (47% reduction) for the p.o. and i.p. routes, respectively. These extrapolated ED_{50} values are within one order of magnitude of the IC_{50} value (20 nM) for the inhibition of EGFr phosphotyrosine by CP-358,774 in homogeneous populations of HN5 cells growing in vitro.

Relationship of Plasma Concentration to EGFr Phosphotyrosine Inhibition. Figure 2 illustrates the relationship of plasma concentration of CP-358,774 to inhibition of EGFr-associated phosphotyrosine of HN5 xenografts. At 1 h post-treatment with a single dose, plasma concentrations of 2 to 10 μM CP-358,774 (0.79–3.9 μg/ml) were associated with a significant reduction in EGFr phosphotyrosine (~40% reduction relative to vehicle-treated controls). At higher plasma concentrations (i.e., 10–100 μM, 3.9–39 μg/ml), the reduction in EGFr phosphotyrosine ranged from 65 to 75%. By interpolation, the effective plasma concentration for 50% inhibition of the target receptor was estimated at 8 μM (3.1 μg/ml) and ~12 μM (4.7 μg/ml) for p.o. and i.p. dosing, respectively. In mouse plasma, 95% of CP-358,774 is bound to plasma proteins. Taking these data into account, at 1 h after the dose, 50% inhibition of EGFr-associated phosphotyrosine of HN5 tumors occurred at free plasma concentrations of 400 nM (160 ng/ml) for p.o. and 600 nM (240 ng/ml) for i.p. doses of CP-358,774.

Duration of Action of CP-358,774. The duration of reduction in EGFr phosphotyrosine after a single 92 mg/kg dose of CP-358,774 was evaluated in the HN5 model (Fig. 3). After p.o. dosing, significant and substantial inhibition of EGFr phosphotyrosine (75–85%) was observed for 12 h; reduction was still measurable (25–40%), and statistically significant, after 24 h. To a similar degree of efficacy, parenterally (i.p.) dosed mice showed substantial inhibition of EGFr phosphotyrosine for 12 h; however, no reduction was observed at 24 h (data not shown). Calculation of the area under the curve for reduction in EGFr phosphotyrosine provides an estimation of the overall degree of inhibition over a 24-h period. Based on the assumption that complete inhibition (100%) of EGFr autophosphorylation over a 24-h period would produce inhibition of 2400%-h (100% "coverage"), p.o. dosing elicited inhibition of 1690%-h (70.4% coverage), whereas parenteral dosing (i.p.) showed an area under the curve of 1420%-h (59.0% coverage).

Within 1 h after p.o. dosing, peak plasma and tumor CP-358,774 concentrations were reached (124.6 μM and 54.8 μmol/kg, respectively). Plasma and tumor concentrations then declined rapidly until 6 h, after which concentrations

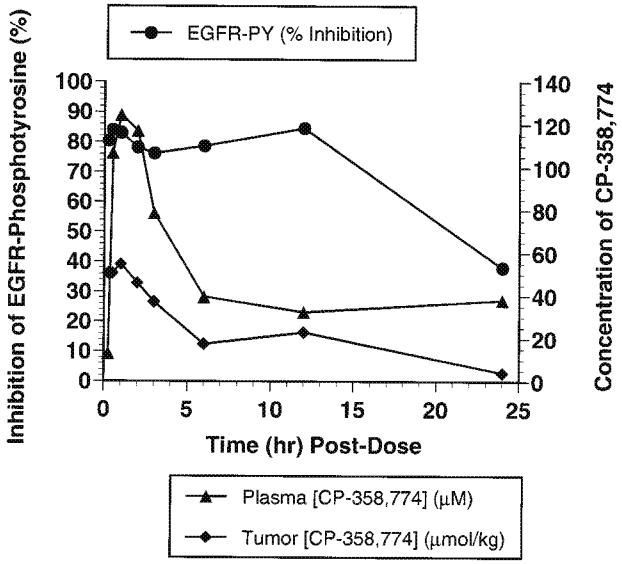


Fig. 3. Duration of action of CP-358,774 for inhibition of EGFr phosphotyrosine in HN5 tumors. Athymic mice bearing s.c. bilateral HN5 received a single dose of CP-358,774 of 92 mg/kg p.o., and at various times after the dose, mice were sacrificed and the tumors were harvested and assayed for EGFr phosphotyrosine by ELISA or for CP-358,774 by extraction and HPLC analysis. These data are representative of three independent experiments.

remained low, although detectable, for several hours. The terminal elimination half-life in plasma after p.o. administration could not be determined because plasma concentrations from 6 to 24 h did not significantly decline; the mean tumor half-life after p.o. administration was estimated at 2.9 h. At 24 h after the dose, plasma and tumor concentrations were 38 μM and 4.0 μmol/kg, respectively. It is clear from Fig. 3 that although plasma and tumor CP-358,774 concentrations follow similar time courses, the EGFr-associated phosphotyrosine reduction does not decline with declining plasma and tumor levels and remains at a high level (80% inhibition) at 12 h after the dose. The reason for this is unclear but seems to be a consistent observation for this compound and related analogs.

To determine whether inhibition of EGFr tyrosine phosphorylation could lead to decreased expression or increased turnover of the surface-bound receptor, tumor homogenates were assayed for EGFr protein using a commercial kit (Oncogene Science). In several experiments, EGFr protein concentrations did not change within 24 h of a single dose of CP-358,774 or within 1 h in multiply dosed animals (n = 20 consecutive daily doses; data not shown). Moreover, it appeared from our data that in vivo receptor density remained relatively constant at 9.4 fmol/μg total protein among several experiments. Although we cannot conclude that transient changes in receptor density did not occur in these animals, it is apparent that a prolonged drug-induced reduction in EGFr-associated tyrosine phosphorylation could not be explained by concurrent reductions in receptor density. Similarly, tumor tissue concentrations of CP-358,774 correlated well with plasma concentrations. In Fig. 3, the use of athymic mice bearing bilateral tumors allowed the simultaneous measurements of EGFr phosphotyrosine, tumor tissue drug concentration, and plasma drug concentrations. On average, the peak tumor tissue concentration was 44 and 29% of the peak circulating plasma levels for p.o. and i.p. dosing, respectively, and CP-358,774 was not retained in tumors relative to plasma (Fig. 3). In tissue culture, the effects of CP-358,774 on EGFr phosphotyrosine in HN5 cells is freely reversible. Cellular EGFr phosphotyrosine levels return to levels found in untreated cells within minutes of removal of CP-358,774 from the culture medium. Thus there is no clear pharmacodynamic explanation for a persistent inhibitory effect of CP-358,774 in tumors growing in vivo.

Antitumor Effects of CP-358,774 (HN5). The antitumor effects of CP-358,774 were determined in the EGFr-overexpressing human HN5 and human A431 epidermoid carcinomas. Both tumors have been shown to be inhibited by monospecific anti-EGFr antibodies in cell culture and in xenograft models (Fan et al., 1993; Modjtahedi et al., 1993a). Oral administration of CP-358,774 produced significant dose-related antitumor effects against established HN5 growing s.c. in athymic mice (Fig. 4). When test animals were dosed for 20 consecutive days beginning at 4 days after tumor implantation (tumor diameter, 2–4 mm), the minimal effective dose for significant antitumor effects was 5.7 mg/kg/day p.o., using one-way ANOVA ($P < .05$ with Dunnett's test). Doses of 11 to 92 mg/kg/day p.o. produced substantial antitumor effects (i.e., >50% inhibition). During the course of dosing (days 4–23 after implantation), tumor-bearing mice treated with vehicle alone showed progressive enlargement of tumors; spontaneous regressions in vehicle-treated or untreated animals have not been observed in this model.

In the experiment described above, tumor sizes for CP-358,774-treated animals were significantly reduced, and this inhibitory effect was observed as long as the test animals were being treated. On the cessation of treatment, we have found that although tumors gradually enlarge, tumor growth rates do not generally equal those of the vehicle controls. Using the tumor size measurements taken only during the

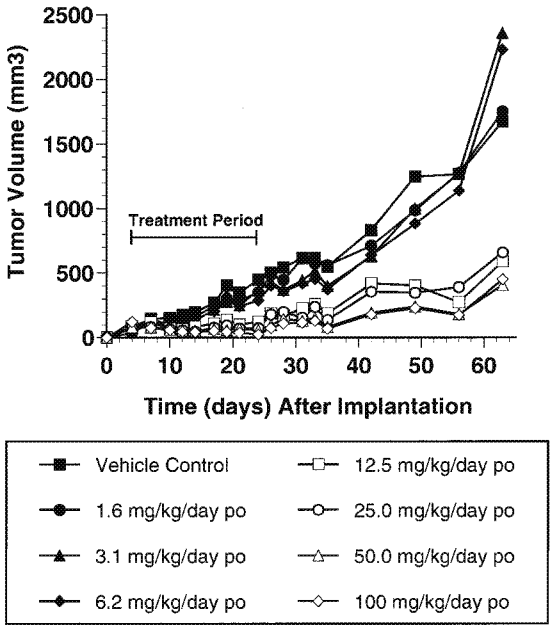


Fig. 4. Antitumor effects of CP-358,774 p.o. on HN5 xenografts in athymic mice. HN5 cells were implanted s.c. in the flank of athymic mice, and after tumors became palpable (2–4-mm diameter, day 4 postimplantation), test animals were treated once daily for 20 consecutive days. Tumors were measured across two diameters according to standard methods. These data are representative of three independent experiments.

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