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Induction of Apoptosis and Cell Cycle Arrest by CP-358,774, an Inhibitor of Epidermal Growth Factor Receptor Tyrosine Kinase

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ABSTRACT

The epidermal growth factor receptor (EGFR) is overexpressed in a significant percentage of carcinomas and contributes to the malignant phenotype. CP-358,774 is a directly acting inhibitor of human EGFR tyrosine kinase with an IC_{50} of 2 nM and reduces EGFR autophosphorylation in intact tumor cells with an IC_{50} of 20 nM. This inhibition is selective for EGFR tyrosine kinase relative to other tyrosine kinases we have examined, both in assays of isolated kinases and whole cells. At doses of 100 mg/kg, CP-358,774 completely prevents EGF-induced autophosphorylation of EGFR in human HN5 tumors growing as xenografts in athymic mice and of the hepatic EGFR of the treated mice. CP-358,774 inhibits the proliferation of DiFi human colon tumor cells at submicromolar concentrations in cell culture and blocks cell cycle progression at the G_1 phase. This inhibitor produces a marked accumulation of retinoblastoma protein in its underphosphorylated form and accumulation of $p27^{KIP1}$ in DiFi cells, which may contribute to the cell cycle block. Inhibition of the EGFR also triggers apoptosis in these cells as determined by formation of DNA fragments and other criteria. These results indicate that CP-358,774 has potential for the treatment of tumors that are dependent on the EGFR pathway for proliferation or survival.

INTRODUCTION

EGFR[2] is a transmembrane glycoprotein with an external domain that binds activating ligands, such as EGF and tumor growth factor α, and an intracellular tyrosine kinase domain that, upon activation, phosphorylates both the receptor itself and a variety of "effector" proteins such as SHC and phospholipase Cγ (1). Activation of this signaling cascade triggers DNA synthesis in cells that express the EGFR.

Many human tumors, especially squamous carcinomas of the lung or head and neck, express high levels of EGFR or tumor growth factor α relative to the corresponding normal tissue (2–4). Although overexpression of EGFR is the most prevalent alteration of this pathway in human tumors, constitutively active mutant EGFR has also been reported in gliomas (5), breast tumors (6) and lung tumors (7). This suggests that activation of the EGFR may drive the proliferation of these tumors and that inhibitors of EGFR may be of use as antitumor agents (2, 8–10).

Blockade of the EGFR pathway by several methods inhibits the proliferation of a variety of tumor cell lines. For example, down-regulation of EGFR by antisense expression reduces the proliferation and invasive properties of a human colon tumor cell line (11) and blocks proliferation of human rhabdomyosarcoma cells (12). Moreover, the transformation of cells by overexpression of EGFR in the presence of EGF is reversed by expression of a dominant negative mutant EGFR (13). Inhibition of EGFR as an antitumor approach has been further substantiated by studies that show that antibodies that block EGF binding to the EGFR inhibit tumor cell proliferation in cell cultures and tumor xenografts in athymic mice (2, 8, 14–16). Importantly, anti-EGFR antibodies have been recently shown to produce complete regressions of established human tumor xenografts in athymic mice (17, 18). Clinical trials of a humanized monoclonal antibody against the EGFR for the treatment of patients with tumors overexpressing EGFR are in progress (19). Finally, selective low molecular weight inhibitors of the EGFR kinase have been shown to inhibit EGF-dependent cell proliferation (20–22) and exhibit antitumor activity in a human tumor xenograft model (23). Thus, a subset of tumors are dependent, at least in part, on the EGFR for proliferation.

Although the evidence above suggests that inhibition of EGFR may block proliferation of some tumor cells, EGFR inhibitors must also be well tolerated in patients to provide a useful therapeutic index. Although an inhibitor of EGFR would not be expected to be cytotoxic in the manner of the most current chemotherapy, pharmacological inhibition of the EGFR could interfere with the physiological functions of EGF and other EGFR ligands. However, targeted "knockout" of the mouse EGFR permitted embryonic development and birth of mice that survived as long as 18 days (24, 25). This indicates that EGFR is dispensable for the proliferation of all critical cell types and essential physiological functions. However, various defects such as thin epidermis, distorted colonic and hepatic epithelium, and low body weight were observed in these animals. Mice with the *waved-2* phenotype express mutant EGFR with markedly impaired kinase activity *in vivo* (26) and, thus, may represent a model of partial inhibition of EGFR. These mice are viable and fertile but have hair, skin, and eye abnormalities. Furthermore, early results from clinical trials with the anti-EGFR antibody have not revealed any toxicities that would prevent further development (19). These results suggest that a partial inhibition of EGFR may be reasonably well tolerated in adults or present a much different pattern of toxicity than does standard chemotherapy. However, many normal cells express EGFR, for example, skin, liver, and gastrointestinal epithelium, and may be affected by such an inhibitor.

Although treatment with anti-EGFR antibodies has produced regressions of established human tumors in some xenograft tumor models (17, 18, 27), inhibition of growth factor pathways may only produce a cytostatic effect if tumor cells survive in a G_0 state when the EGFR pathway is blocked. However, an important recent report by Wu *et al.* (28) showed that an anti-EGFR antibody could trigger apoptosis in a human colorectal cell line, DiFi, that overexpresses EGFR. Addition of IGF-I was able to protect the cells from the apoptotic effect of the antibody. These results, together with recent studies of inhibition of IGF-IR function (29, 30), indicate that, in some tumors, inhibition of growth factor pathways may kill tumor cells

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; FBS, fetal bovine serum; FRE cells, contact-inhibited Fischer rat embryo cells; PGT, poly(glutamic acid:tyrosine) 4:1; HRP, horseradish peroxidase; BrdUrd, bromodeoxyuridine; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; pRB, retinoblastoma gene protein; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; IRS-1, insulin receptor substrate-1; PS, phosphatidylserine.

rather than only produce cell cycle arrest. Here, we extend these findings with a novel EGFR tyrosine kinase inhibitor and demonstrate that this inhibitor blocks DiFi cell proliferation by a combination of cell cycle arrest and induction of apoptosis.

MATERIALS AND METHODS

CP-358,774. [6,7-Bis(2-methoxy-ethoxy)-quinazolin-4-yl]-(3-ethynylphenyl)amine, molecular formula $C_{22}H_{23}N_3O_4$, was prepared as described (31). The structure is shown in Fig. 1*A*. The monohydrochloride salt form of this compound was used in the studies reported here.

Cell Lines and Culture Conditions. DiFi is a human colorectal carcinoma cell line derived from a familial adenomatous polyposis patient, as described previously, that expresses 5×10^6 EGFRs/cell (16, 32). DiFi cells were maintained in 1:1 DMEM:Ham's F-12 with 10% FBS. HN5 human head and neck tumor cells that express 1.4×10^7 EGFRs/cell (15, 33) were obtained from Dr. M. J. O'Hare of Haddow Laboratories, The Institute of Cancer Research, Sutton (Surry, United Kingdom), and grown in DMEM with 10% FBS. MDA-MB-468 human breast cancer cells that express 1.5×10^6 EGFRs/cell (15) were obtained from the American Type Culture Collection (Bethesda, MD) and grown in DMEM with 5% FBS. FRE cells (34) were maintained in DMEM with 10% FBS. All cells were cultured at 37°C in 5% carbon dioxide/95% air in the presence of 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. DiFi, MDA-MB-468, and HN5 cells were tested and found to be mycoplasma free using the Gen-Probe kit (Fisher Scientific, Pittsburgh, PA).

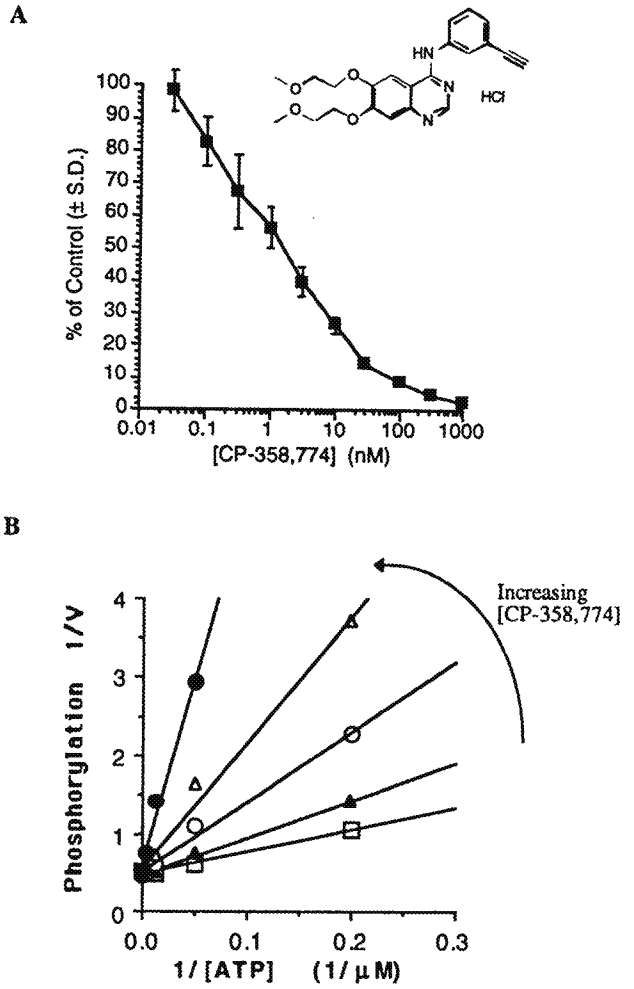

Fig. 1. CP-358,774 inhibits purified EGFR kinase and is competitive with ATP. Phosphorylation of PGT by purified EGFR was measured by immunoassay with antiphosphotyrosine antibodies as described in "Materials and Methods." *A*, *data points*, means of six determinations; *bars*, SD. This result is representative of four independent experiments. *B*, ATP competition experiments. CP-358,774 concentrations were: 10 nM (●); 3 nM (△); 1 nM (○); 0.3 nM (▲); or 0 nM (□). The ATP concentrations tested were 5 μM, 20 μM, 80 μM, 320 μM, and 3 mM. The K_m for ATP under these conditions was 7 μM. Phosphorylation is measured in arbitrary units, and the data were fit by a linear least squares method. This experiment was repeated with identical results.

Kinase Assays. The EGFR kinase assay is similar to one described previously (35). Nunc MaxiSorp 96-well plates were coated by incubation overnight at 37°C with 100 μl per well of 0.25 mg/ml PGT (Sigma Chemical Co., St. Louis, MO) in PBS. Excess PGT was removed by aspiration, and the plate was washed 3 times with wash buffer (0.1% Tween 20 in PBS). The kinase reaction was performed in 50 μl of 50 mM HEPES (pH 7.3), containing 125 mM sodium chloride, 24 mM magnesium chloride, 0.1 mM sodium orthovanadate, 20 μM ATP, 1.6 μg/ml EGF, and 15 ng of EGFR, affinity purified from A431 cell membranes as described (36). The compound in DMSO was added to give a final DMSO concentration of 2.5%. Phosphorylation was initiated by addition of ATP and proceeded for 8 min at room temperature, with constant shaking. The kinase reaction was terminated by aspiration of the reaction mixture and was washed 4 times with wash buffer. Phosphorylated PGT was measured by 25 min of incubation with 50 μl per well HRP-conjugated PY54 (Oncogene Science Inc., Uniondale, NY) antiphosphotyrosine antibody, diluted to 0.2 μg/ml in blocking buffer (3% BSA and 0.05% Tween 20 in PBS). Antibody was removed by aspiration, and the plate was washed 4 times with wash buffer. The colorimetric signal was developed by addition of TMB Microwell Peroxidase Substrate (Kirkegaard and Perry, Gaithersburg, MD), 50 μl per well, and stopped by the addition of 0.09 M sulfuric acid, 50 μl per well. Phosphotyrosine is estimated by measurement of absorbance at 450 nm. The signal for controls was typically 0.6–1.2 absorbance units, with essentially no background in wells without ATP, EGFR, or PGT and was proportional to the time of incubation for 10 min.

Conditions for selectivity assays were the same as those for the EGFR kinase assay, except for the addition of 1 mM manganese chloride to the assay buffer and a final ATP concentration of 100 μM. The reaction was terminated by the addition of 50 μl of 250 mM EDTA prior to aspiration. For experiments comparing inhibition of EGFR to v-abl or c-src kinase, recombinant bacterially expressed v-abl (3.4 ng/well) or purified human platelet c-src (1.2 units/well, Oncogene Science Inc.) was substituted for the EGFR. For experiments comparing inhibition of EGFR to insulin receptor or IGF-IR, purified recombinant proteins were substituted for native EGFR. Baculovirus-expressed cytoplasmic domain of the insulin receptor β subunit (10 units/well) was from Stratagene (La Jolla, CA). Recombinant EGFR kinase domain (2 ng), IGF-IR kinase domain (3 ng), and v-*abl* kinase were prepared as described below.

Preparation of Recombinant Kinases. The complete intracellular domain of human EGFR (amino acids 644-1186) was PCR amplified and subcloned into pAcG2T to generate a glutathione *S*-transferase fusion protein with a thrombin cleavage site. Plaque-purified recombinant baculovirus was used to infect Sf9 insect cells for 60 h. The complete intracellular domain of human IGF-IR β subunit (amino acids 711-1377) was PCR amplified and subcloned into pAcG2T to generate a glutathione *S*-transferase fusion protein with a thrombin cleavage site. Plaque-purified recombinant baculovirus was used to infect High V insect cells for 36 h. For both preparations, active kinase was purified using glutathione Sepharose followed by elution with free glutathione. The v-abl was expressed as a His6-tagged protein and purified by affinity chromatography with the QIAexpress system (Qiagen).

FRE Mitogenesis. FRE cells were plated in 96-well plates at 1.5×10^4 cells/well in 100 μl of DMEM with 10% FBS. The next day, the medium was replaced with 100 μl of serum-free medium (RPMI 1640). After 12–20 h, growth factors and BrdUrd were added in the presence or absence of CP-358,774. The growth factors and their concentrations were as follows: murine EGF (Collaborative Biomedical Products), 0.5 ng/ml; PDGF (Genzyme, Cambridge, MA), 15 ng/ml; IGF-I (Genzyme), 50 ng/ml; and bFGF (Genzyme), 50 ng/ml. After overnight incubation, BrdUrd incorporation was measured using the Cell Proliferation Assay Kit (Amersham, Arlington Heights, IL) according to the supplier's instructions, except for substitution of *o*-phenylenediamine (Pierce Chemical Co., Rockford, IL) as the peroxidase substrate. The color reaction was stopped with 2 N sulfuric acid, and absorbance was read at 490 nm.

For calculation of inhibition, all wells were corrected for the background signal obtained from cells incubated without BrdUrd. Percent inhibition was calculated as follows: 100 − 100[(treated − basal)/(control − basal)], where basal is signal from cells not stimulated with growth factor, control is signal

from growth factor-stimulated cells, and treated is signal from CP-358,774-treated, growth factor-stimulated cells.

Measurement of Phosphotyrosine, pRB Phosphorylation, $p27^{KIP1}$ Expression, and PARP Cleavage by Western Blotting. Cells (DiFi or HN5) were incubated in the presence or absence of CP-358,774 or cisplatin. After 24 h, cells were washed with 50 mM Tris-HCl, 140 mM sodium chloride, 3.3 mM potassium chloride, and 500 μM sodium orthovanadate (pH 7.4) and lysed by boiling in 2× Laemmli sample buffer (37) with 2 mM sodium orthovanadate for 10 min. Cellular protein was determined using the BCA protein assay (Pierce Chemicals, Rockford, IL). Equal amounts of total protein (10–20 μg) were loaded onto 4–20% Tris-glycine minigels (Integrated Separated Systems, Natick, MA) for phosphotyrosine determinations, 7.5% Daiichi Tris-glycine minigels for pRB, 12.5% Daiichi Tris-glycine minigels for $p27^{KIP1}$, or 4–12% Bis-Tris NuPage minigels (Novex, San Diego, CA) for PARP. After electrophoresis, proteins were transferred to an Immobilon-*P* membrane (Millipore, Bedford, MA) for 2 h at 250 mA. After transfer, membranes were blocked for 1 h or overnight with 5% BSA in TBST [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20] for antiphosphotyrosine blotting or with 4–5% nonfat dry milk in TBST. For phosphotyrosine determinations, blots were probed with HRP-conjugated antiphosphotyrosine PY20 (ICN, Costa Mesa, CA) or HRP-PY54 (Oncogene Science). For pRB determinations, blots were probed with 1 μg/ml monoclonal antibody G3-245 (PharMingen, San Diego, CA), followed by HRP-conjugated goat antimouse (PharMingen; 1:1000). The identification of the lower band as underphosphorylated pRB was confirmed by use of an antibody specific for this form (PharMingen). For $p27^{KIP1}$, blots were probed with 0.1 μg/ml anti-$p27^{KIP1}$ Clone 57 (Transduction Labs, Lexington, KY) followed by HRP-goat antimouse. For PARP, blots were probed with anti-PARP, Clone C-2-10 (Biomol, Plymouth Meeting, PA), followed by HRP-conjugated rabbit antimouse IgG (Pierce Chemicals). The M_r 116,000 PARP and its M_r 85,000 proteolytic fragment were identified by comparison with lysates of untreated HL-60 cells or HL-60 cells induced to undergo apoptosis with etoposide (Biomol). All HRP-labeled antibodies were detected using enhanced chemiluminescence (Amersham) according to the supplier's directions and quantitated by densitometry.

Cell Cycle Analysis of DiFi Cells. Semiconfluent DiFi cells growing in six-well plates were incubated for 24 h in DMEM:Ham's F12 and 0.5% FBS containing diluent (0.125% DMSO), cisplatin, or CP-358,774 in the absence or presence of IGF-I. Cells were harvested and incubated in medium containing 0.2% Triton X-100 and 50 μg/ml PI for 10 min, according to published methods (38). PI uptake was analyzed by flow cytometry using a FACSort (Becton Dickinson, San Jose, CA). Data were acquired using linear amplification of FL2 and analyzed using CellQuest software (Becton Dickinson). For measurement of BrdUrd incorporation, DiFi cells were treated for 24 h with diluent or 1 μM CP-358,774, pulse labeled with 10 μM BrdUrd, incubated with FITC-conjugated anti-BrdUrd monoclonal antibody (Becton Dickinson), and counterstained with PI.

Agarose Gel Analysis of DNA Fragmentation. DiFi cells were treated to induce apoptosis as described for cell cycle analysis. After trypsinization of the cells, DNA was extracted using a TACS Apoptotic DNA Laddering Kit according to the supplier's instructions (Trevigen, Gaithersburg, MD). Isolated DNA was quantitated by absorbance at 260 nm. Samples (6 μg/lane) were electrophoresed on a 1.5% agarose gel containing 0.5 μg/ml PI for 2 h at 110 V in TAE buffer (40 mM Tris-acetate, 10 mM EDTA, and 20 mM glacial acetic acid, pH 8.4). Gels were visualized on a UV transilluminator.

TUNEL Analysis. DiFi cells were treated to induce apoptosis as described for cell cycle analysis. Cells were harvested, and aliquots of 2×10^6 cells were fixed in 1% paraformaldehyde, permeabilized in 70% ethanol, and stored at −20°C. 3′-OH DNA strand breaks were detected by the TUNEL technique using the ApopTag Plus Fluorescein (FITC) kit (Oncor, Gaithersburg, MD) according to the supplier's instructions. FITC-labeled cells were counterstained with PI and analyzed by flow cytometry using a FACSort. Excitation was at 488 nm with emission read in the FL1 (BP 530/30) and FL2 (BP 585/42) channels. Data were acquired using logarithmic amplification of both FL1 and FL2 (the FITC and PI signals, respectively). Data were analyzed using CellQuest software. Regions of FL1 *versus* FL2 dot plots were set using control (cells treated with diluent alone) samples (39).

Annexin V Binding. DiFi cells were treated to induce apoptosis as described for cell cycle. Cells were harvested, and aliquots of 2×10^5 cells were treated with annexin V-FITC and PI using the Apoptosis Detection Kit (R&D Systems, Minneapolis, MN) and the supplier's protocol. Annexin V-FITC and PI binding were analyzed by flow cytometry using a FACSort. Data were collected using logarithmic amplification of both the FL1 (FITC) and FL2 (PI) channels. Quadrant analysis of coordinated dot plots was done using CellQuest software. Unstained cells were used to control for autofluorescence. Singly stained cells were used to adjust the photomultiplier voltages and compensation settings to eliminate spectral overlap between the FL1 and FL2 signals.

Preparation of Tissue Extracts for Western Blot Analysis. Frozen tumor or liver samples were pulverized, extracted with boiling 2× Laemmli sample buffer (37) with 2 mM sodium orthovanadate, placed in a boiling water bath for 10 min, and stored at −80°C until analysis. After the tissue extracts of insoluble material were cleared by centrifugation, the protein content of the extracts was determined, and the samples were analyzed by Western blotting as described above.

RESULTS

Inhibition of EGFR Tyrosine Kinase by CP-358,774. CP-358,774 inhibits purified EGFR kinase with an IC_{50} of 2 nM (Fig. 1*A*). Kinetic analysis indicates that the inhibition is competitive with ATP. A Lineweaver-Burk plot of phosphorylation in the presence of varied ATP and inhibitor indicates that the inhibition is reduced at higher concentrations of ATP and can be restored to the uninhibited rate at high ATP concentrations (Fig. 1*B*). The extent of inhibition may, therefore, be influenced by the intracellular ATP concentration. A replot of the $K_{m,app}$ *versus* concentration of CP-358,774 from the data of Fig. 2*B* indicated a K_i for CP-358,774 of 2.7 nM. EGFR is more sensitive to inhibition by CP-358,774 than are the other tyrosine kinases we have examined, and it is >1000-fold more sensitive than human c-*src* or v-*abl* when compared under identical conditions (Fig. 2*A*). CP-358,774 is also a potent inhibitor of the recombinant intracellular (kinase) domain of the EGFR, with an IC_{50} of 1 nM (Fig 2*B*), essentially identical to that observed with full-size EGFR (Fig. 1*A*), which indicates that the inhibitor binding site is in the kinase domain. The kinase domains of the human insulin receptor and IGF-IR are much less sensitive to this inhibitor and are essentially unaffected at concentrations as high as 10 μM (Fig. 2*B*). Thus, CP-358,774 is a potent, selective, and directly acting inhibitor of the EGFR tyrosine kinase.

Inhibition of Tyrosine Phosphorylation in Intact Cells. The addition of EGF to cells that express EGFR leads to a rapid autophosphorylation of the EGFR on tyrosine residues in the COOH terminus, thus providing a facile assay for inhibition of the EGFR tyrosine kinase activity in intact cells. CP-358,774 potently inhibits EGFR autophosphorylation in HN5 human head and neck tumor cells (Fig. 3), a cell line that expresses high levels of EGFR (15). Evaluation of these Western blots by densitometry indicates an IC_{50} for inhibition of EGFR phosphorylation of 20 nM. At the higher concentrations of the compound, the extent of autophosphorylation after EGF stimulation is lower than that in the controls without EGF, indicating reduction of basal EGFR activity, which may arise from autocrine stimulation. Additional studies indicated that the inhibition of kinase is rapidly obtained (<10 min) on addition of CP-358,774 to cell medium and is rapidly reversed after the inhibitor is washed out and the cells are incubated in inhibitor-free medium (data not shown). Similarly potent inhibition of EGF-induced EGFR autophosphorylation by CP-358,774 was seen with DiFi human colon cancer cells and MDA-MB-468 human breast cancer cells (data not shown).

The selectivity of CP-358,774 as an inhibitor of the phosphorylation of endogenous intracellular substrates by EGFR tyrosine kinase can also be demonstrated in intact cells. The phosphorylation of the adaptor protein SHC (40, 41) upon addition of EGF serves as a convenient physiological marker for EGFR activity. The EGF-induced tyrosine phosphorylation of SHC proteins is completely

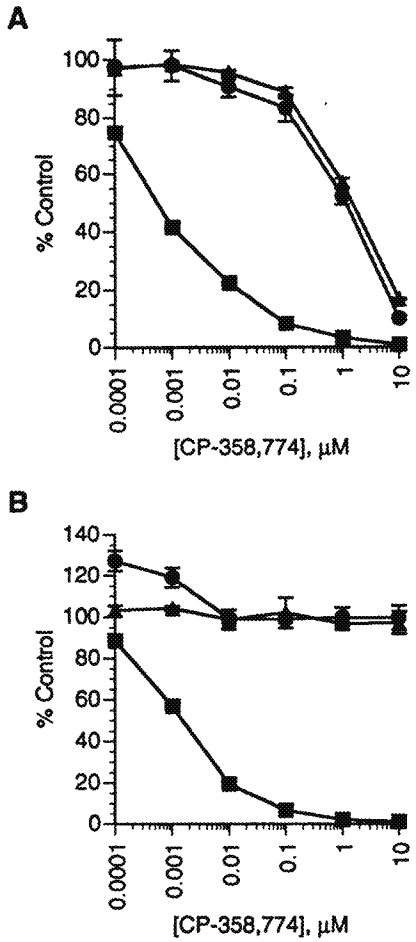

Fig. 2. Selectivity of CP-358,774 for EGFR kinase. Inhibition of purified kinases was measured as described in "Materials and Methods." *Data points*, means of triplicate determinations; *bars*, SE. Similar results were observed in two experiments. *A*, selectivity for native human EGFR *versus* c-src and recombinant v-abl. ■, EFGR; ●, c-src; ▲, v-abl. *B*, selectivity for recombinant EGFR kinase domain (■) *versus* insulin receptor kinase domain (▲) and IGF-IR kinase domain (●). As a positive control, tyrphostin A25 (Calbiochem, San Diego, CA) produced 50% inhibition of IGF-IR kinase in this assay at 20 μM.

blocked by CP-358,774 (Fig. 4, *Lane 9*), whereas the insulin-induced phosphorylation of IRS-1, a prominent insulin receptor tyrosine kinase substrate, is unaffected (Fig. 4., *Lane 4*). Staurosporine, a relatively nonselective kinase inhibitor (42), completely blocked IRS-1 phosphorylation in response to insulin (Fig. 4, *Lane 5*) and therefore served as a positive control. This experiment demonstrates that CP-358,774 markedly inhibits *in situ* phosphorylation of an endogenous substrate of activated EGFR at concentrations that have no effect on phosphorylation of the major physiological substrate of another transmembrane tyrosine kinase, the insulin receptor.

Inhibition of Murine and Human EGFR *in Vivo* by CP-358,774. Intravenous administration of EGF to mice produces a marked autophosphorylation of EGFR in liver and other tissues (43), thus providing a dynamic assay for EGFR inhibition *in vivo*. We examined the ability of CP-358,774 to block EGFR autophosphorylation in liver and HN5 tumors in athymic mice, as shown in Fig. 5. As reported, EGFR in mouse liver is hypophosphorylated but is rapidly tyrosine phosphorylated when mice receive EGF (Fig. 5*B*). This phosphorylation is inhibited 54% by pretreatment with 10 mg/kg CP-358,774 and is nearly completely inhibited (93%) at 100 mg/kg. Similarly, EGFR in human HN5 tumors is rapidly autophosphorylated in response to EGF (Fig. 5*A*). Analysis of a larger set of samples prepared as in Fig. 5 indicates that EGF-induced autophosphorylation of tumor EGFR is completely inhibited by pretreatment with 100 mg/kg CP-358,774 and is reduced by 89 ± 11% (mean ± SE; $n = 4$) and 61 ± 7% at doses of 25 and 10 mg/kg, respectively, at 1 h after an i.p. treatment. The doses of CP-358,774 used here (10, 25, and 100 mg/kg) can be administered daily for at least 5 consecutive days without lethality in mice. These results indicate that CP-358,774 treatment of mice effectively inhibits both murine EGFR kinase in liver and human EGFR in transplanted tumors.

Inhibition of Tumor Cell Proliferation. The DiFi human colon tumor cell line expresses high levels of EGFR and is inhibited by the anti-EGFR antibody (16, 28, 44). The proliferation of DiFi cells is strongly inhibited by CP-358,774 with an IC_{50} of 100 nM for an 8-day proliferation assay (Fig. 6). CP-358,774 did not produce a rapid loss of viability: the percentage of viable cells was 98 ± 0.8% (mean ± SD) in untreated cell cultures and 97 ± 1.7% in cultures exposed to 1 μM CP-358,774 for 24 h. The HN5 human head and neck tumor cell line (15, 28, 33), which also expresses a very high level of the EGFR, is markedly inhibited by CP-358,774 at concentrations as low as 50 nM and is completely blocked at 250 nM (data not shown). In contrast, the proliferation of *raf*-transformed NIH-3T3 cells or *ras*-transformed FRE cells is much less sensitive, with IC_{50}s for proliferation of 7 and 3 μM, respectively (data not shown).

CP-358,774 is selective for the EGFR kinase pathway, as evaluated in cellular proliferation assays. Unlike tumor cell lines, FRE fibroblasts can be rendered quiescent by incubation in serum-free medium and then triggered to proliferate by addition of defined growth factors such as EGF, PDGF, IGF-I, or bFGF. Each of these growth factors acts through a cognate transmembrane receptor with tyrosine kinase activity (1); thus, FRE cells are a well-defined model, unambiguously dependent on the added factors, that can be used to evaluate selectivity of tyrosine kinase inhibitors. CP-358,774 inhibits EGF-stimulated mitogenesis with an IC_{50} of 70 nM but only inhibits mitogenesis stimulated by the other factors at concentrations of >1 μM (Fig. 7). Thus, CP-358,774 is not simply indiscriminately cytotoxic because the FRE cells continue to undertake DNA synthesis when stimulated with mitogens other than EGF. This indicates that CP-358,774 is selective for EGFR kinase relative to other tyrosine kinase-linked receptors and that, at concentrations that inhibit EGF-induced proliferation, it does not effectively inhibit any of the many kinases or other enzymes that are necessary for mitogenesis in response to other mitogens.

Additional studies were done to further characterize the effects of CP-358,774 on DiFi cell proliferation. Analysis of the cell cycle distribution of these cells indicated that the cells were partially blocked in the G_1 phase of the cell cycle by this EGFR inhibitor (Fig. 8 and Table 1). Although the changes were somewhat masked by the appearance of an apoptotic cell population discussed below, marked

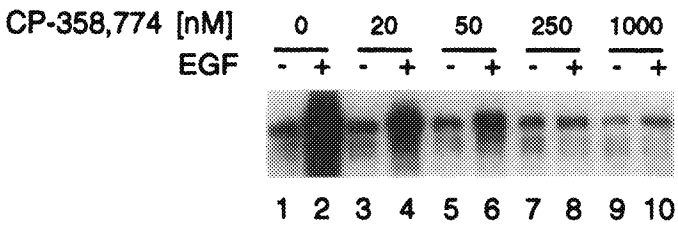

Fig. 3. Effect of CP-358,774 on tyrosine phosphorylation in HN5 cells, as evaluated with Western blots. HN5 cells (10^5 cells/well in 24-well cell culture plates) were exposed to the indicated concentrations of CP-358,774-01 for 1 h and then stimulated with 50 ng/ml EGF (*Lanes 2, 4, 6, 8,* and *10*) or not (*Lanes 1, 3, 5, 7,* and *9*). Cell lysates were prepared after a 5-min exposure to EGF, and EGFR-associated phosphotyrosine was measured by Western blotting as described in "Materials and Methods." The CP-358,774 was present at 0 nM (*Lanes 1* and *2*); 20 nM (*Lanes 3* and *4*); 50 nM (*Lanes 5* and *6*); 250 nM (*Lanes 7* and *8*); or 1000 nM (*Lanes 9* and *10*).

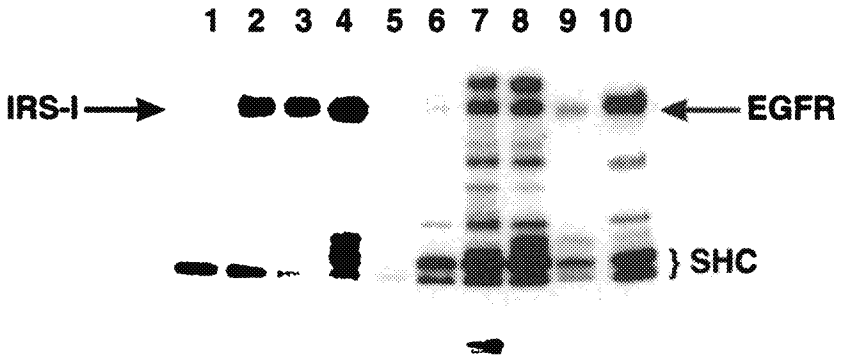

Fig. 4. CP-358,774 selectively inhibits EGFR signaling in HN5 cells. Following serum starvation for 2 h, HN5 cells were incubated for an additional hour in the presence of 0.125% DMSO (*Lanes 1–3* and *6–8*), 1 μM CP-358,774–01 (*Lanes 4* and *9*), or 10 μM staurosporine (*Lanes 5* and *10*). Cells were then stimulated for 5 min with 100 nM insulin (*Lanes 2–5*) or 100 ng/ml EGF (*Lanes 7–10*) and lysed in 1% Triton X-100 buffer. *Lanes 1–5*, lysates were subjected to immunoprecipitation with antibody to IRS-1. *Lanes 6–10*, lysates were subjected to immunoprecipitation with antibody to SHC. Immunoprecipitates were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with antiphosphotyrosine antibodies as described in "Materials and Methods." *Lanes 2* and *3*, duplicates of the insulin-treated controls (no inhibitor); *Lanes 7* and *8*, duplicates of the EGF-treated controls (no inhibitor). Other conditions are single lanes.

decreases in the percentage of cycling cells in S phase and G_2-M and an increase in the percentage of cells in G_1 were observed, indicating a G_1 block. Similar experiments were performed with the HN5 cells: 48 h of exposure to 1 μM CP-358,774 reduced the S-phase cells from 55% of the total to 24% and increased the percentage of cells in G_1 from 24 to 56% (data not shown). Thus, for both DiFi and HN5 cells, inhibition of the EGFR kinase by CP-358,774 leads to a partial G_1 arrest.

The reduction in S-phase cells by CP-358,774 was confirmed by flow cytometric measurements of total DNA and incorporated BrdUrd, a more specific method for identifying cells in S-phase. The percentage of S-phase DiFi cells measured by this procedure de-

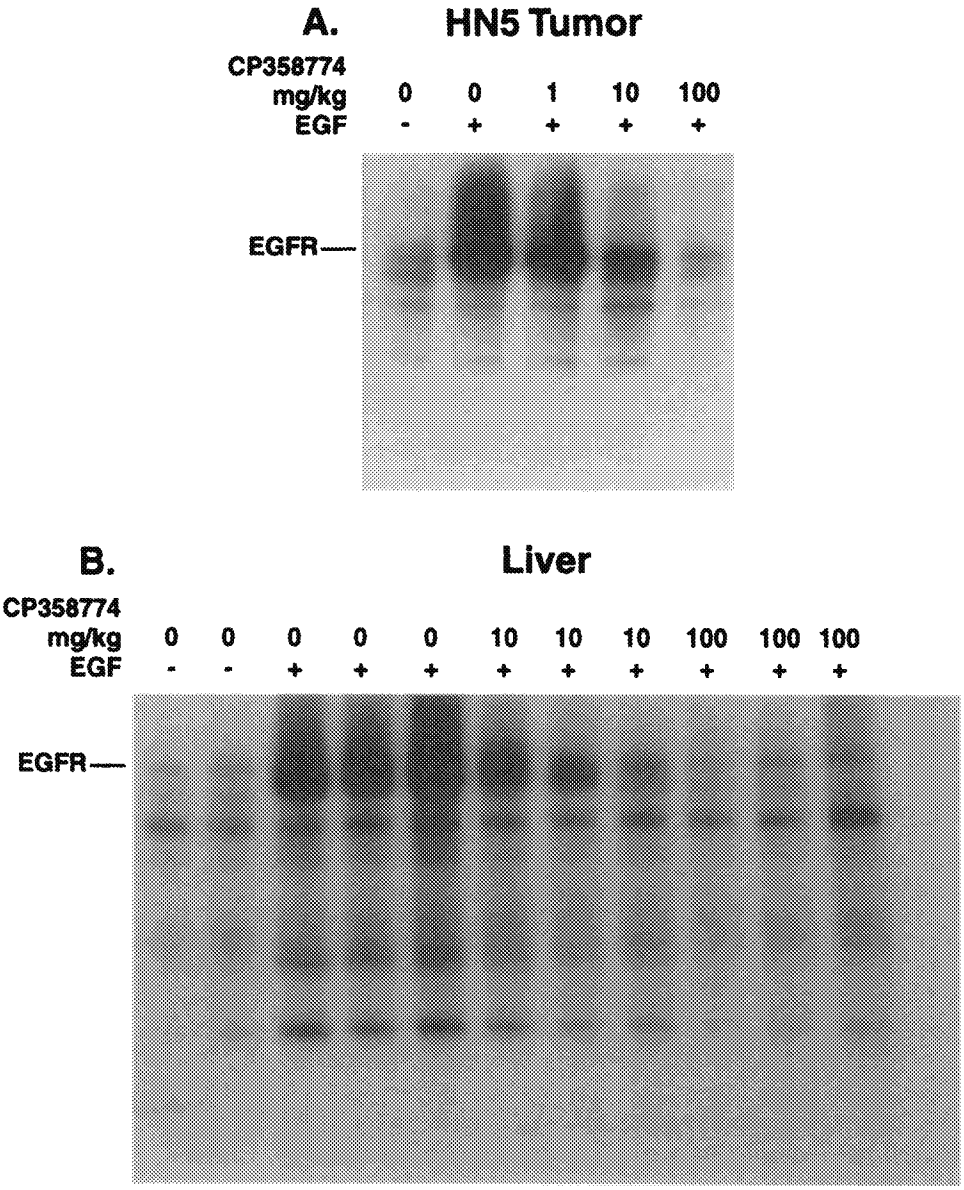

Fig. 5. CP-358,774 pretreatment inhibits EGF-induced EGFR phosphorylation in HN5 tumor (*A*) and murine liver (*B*) *in vivo*. Athymic *nu/nu* mice bearing s.c. HN5 tumors were treated with the indicated doses of CP-358,774 or vehicle (0.1% P105 in saline containing 10% DMSO) by i.p. injection. The mice received 100 μg of murine EGF (Sigma) or vehicle, as indicated, 1 h after administration of the indicated dose of drug by i.p. injection. The mice were killed by cervical dislocation 5 min after administration of EGF. The entire tumor and part of the liver were removed and immediately frozen in liquid nitrogen. EGFR autophosphorylation was measured by Western blotting as described in "Materials and Methods." *B*, triplicate livers are shown for independently treated mice, except for the mice without drug or EGF, for which duplicate liver extracts are shown. This result is representative of three independent experiments.

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