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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).

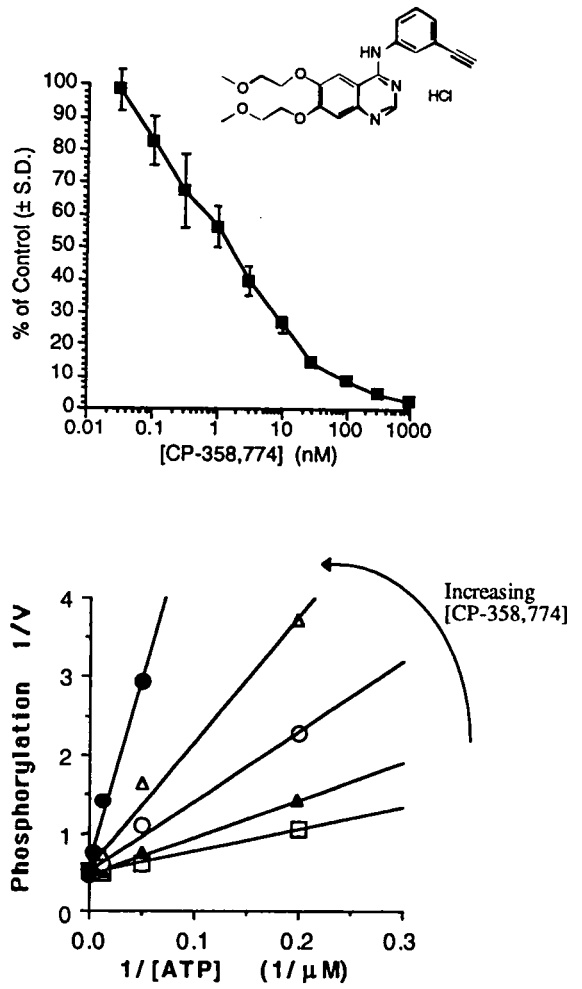

Fig. 1. CP-358,774 inhibits purified EGFR kinase and is competitive with ATP. Phosphorylation of PGT by purified EGFR was measured by immunoassay with antiphosphotyrosine antibodies as described in "Materials and Methods." *A*, *data points*, means of six determinations; *bars*, SD. This result is representative 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

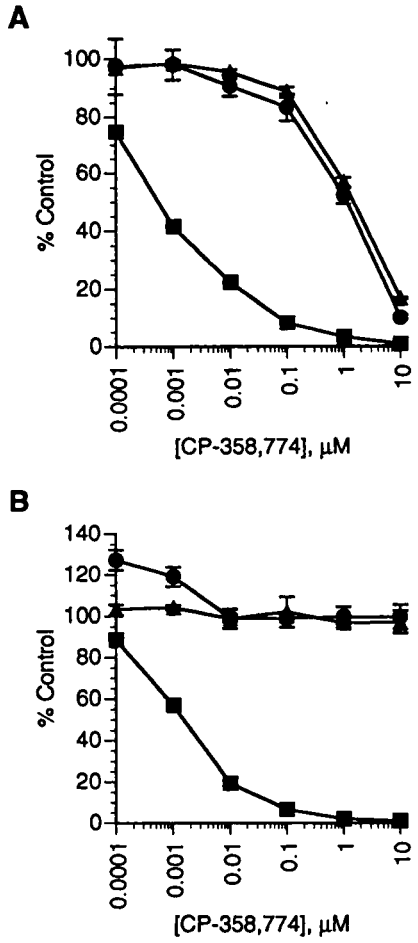

Fig. 2. Selectivity of CP-358,774 for EGFR kinase. Inhibition of purified kinases was measured as described in "Materials and Methods." *Data points*, means of triplicate determinations; *bars*, SE. Similar results were observed in two experiments. *A*, selectivity for native human EGFR *versus* c-src 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

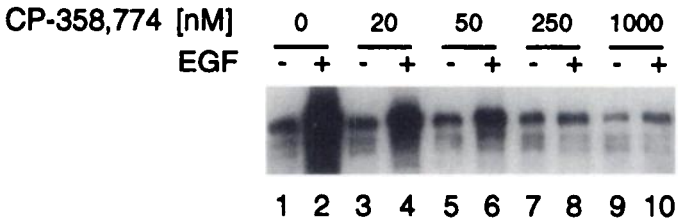

Fig. 3. Effect of CP-358,774 on tyrosine phosphorylation in HN5 cells, as evaluated with Western blots. HN5 cells (10^5 cells/well in 24-well cell culture plates) were exposed to the indicated concentrations of CP-358,774-01 for 1 h and then stimulated with 50 ng/ml EGF (*Lanes 2, 4, 6, 8,* and *10*) 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*).

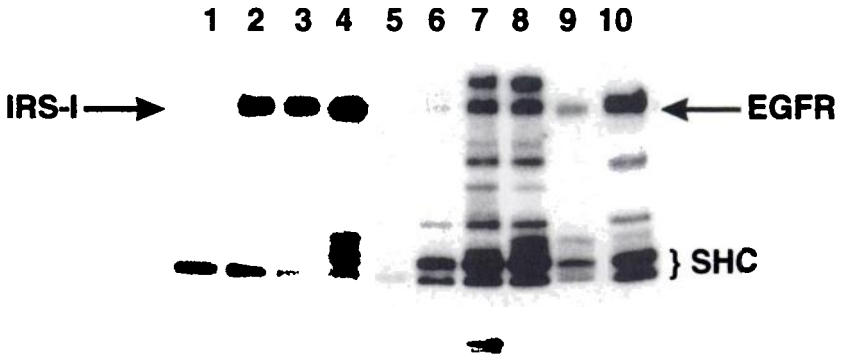

Fig. 4. CP-358,774 selectively inhibits EGFR signaling in HN5 cells. Following serum starvation for 2 h, HN5 cells were incubated for an additional hour in the presence of 0.125% DMSO (*Lanes 1–3* and *6–8*), 1 μM CP-358,774–01 (*Lanes 4* and *9*), or 10 μM staurosporine (*Lanes 5* and *10*). Cells were 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-

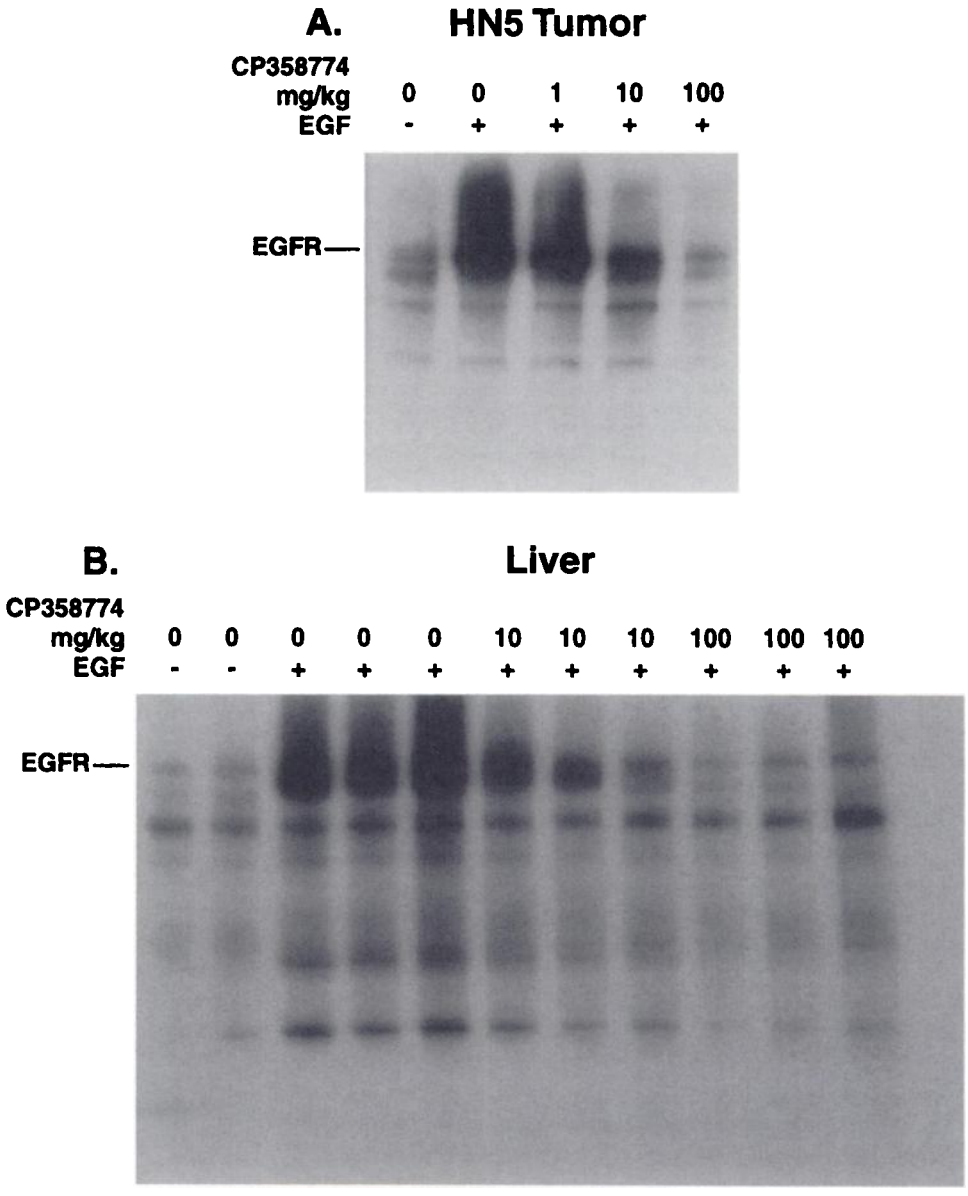

Fig. 5. CP-358,774 pretreatment inhibits EGF-induced EGFR phosphorylation in HN5 tumor (*A*) and murine liver (*B*) *in vivo*. Athymic *nu/nu* mice bearing s.c. HN5 tumors were treated with the indicated doses of CP-358,774 or vehicle (0.1% P105 in saline containing 10% DMSO) by i.p. injection. The mice 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.

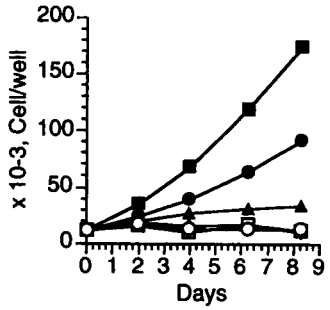

Fig. 6. CP-358,774 inhibits DiFi cell population growth. DiFi cells were seeded in 12-well plates (1.5×10^4 cells/well) in DMEM:Ham's F-12 plus 10% FBS and grown at 37°C. The next day (day 0), the medium was replaced with medium containing 0.5% FBS and the indicated concentrations of CP-358,774. Cell number was determined for each condition in duplicate using a Coulter counter on days 0, 2, 4, 6, and 8. *Data points*, means of duplicate determinations that agreed within 10%. Similar results were obtained in four experiments. ■, control; ●, 0.1 μM; ▲, 0.2 μM; ♦, 0.4 μM; □, 0.6 μM; ○, 1.2 μM.

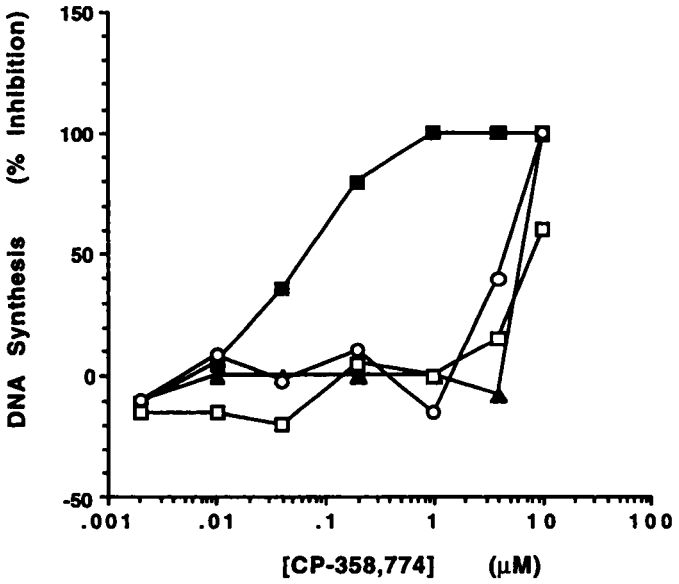

Fig. 7. CP-358,774 selectively inhibits EGF-induced mitogenesis. FRE cells were rendered quiescent by incubation in serum-free medium for 12–20 h and then incubated with the indicated growth factors and the indicated concentrations of CP-358,774 overnight. ■, 0.5 ng/ml EGF; ▲, 15 ng/ml bFGF; □, 50 ng/ml IGF-I; ○, 50 ng/ml PDGF. DNA synthesis was estimated as described in "Materials and Methods." This result is representative of two independent experiments.

creased from 14 ± 0.5% (mean ± SE; n = 4) to 9 ± 1% and 0.7 ± 0.1%, and the percentage of cells in G_1 increased from 82 ± 0.9% to 85 ± 1.4% and 95 ± 1.3% upon 24 h exposure to 0.1 μM and 1 μM CP-358,774, respectively, as evaluated by this method (data not shown).

The accumulation of cells in G_1 shown in Fig. 8 presumably ensues from decreased signaling through the EGFR and the resulting loss of downstream signals required for cell cycle progression. The phosphorylation of pRB is a key permissive step in the progression of cells into S phase and is regulated by growth factors (45). Disruption of the EGFR pathway by anti-EGFR antibodies was shown to result in loss of the permissive (hyperphosphorylated) ppRB in DiFi human colon tumor cells (28). Similarly, treatment of DiFi cells with CP-358,774 leads to a loss of hyperphosphorylated ppRB (Fig. 9*A*) in a concentration-dependent manner. Studies with the HN5 cell line also revealed loss of the hyperphosphorylated ppRB. This loss is not observed at 2 h of treatment but is obvious (>75%) by 12 h of exposure to this compound (data not shown). The phosphorylation of pRB is mediated by a complex of cyclins and cyclin-dependent kinases and is negatively regulated by the expression of endogenous inhibitors of those kinases such as $p27^{KIP1}$. Treatment of DiFi cells (Fig. 9*B*) or HN5 cells (data not shown) with CP-358,774 resulted in a marked induction of $p27^{KIP1}$ in a concentration-dependent manner. These actions of CP-358,774 are consistent with the compound acting as a negative regulator of cell proliferation by blockade of the EGFR signal transduction pathway.

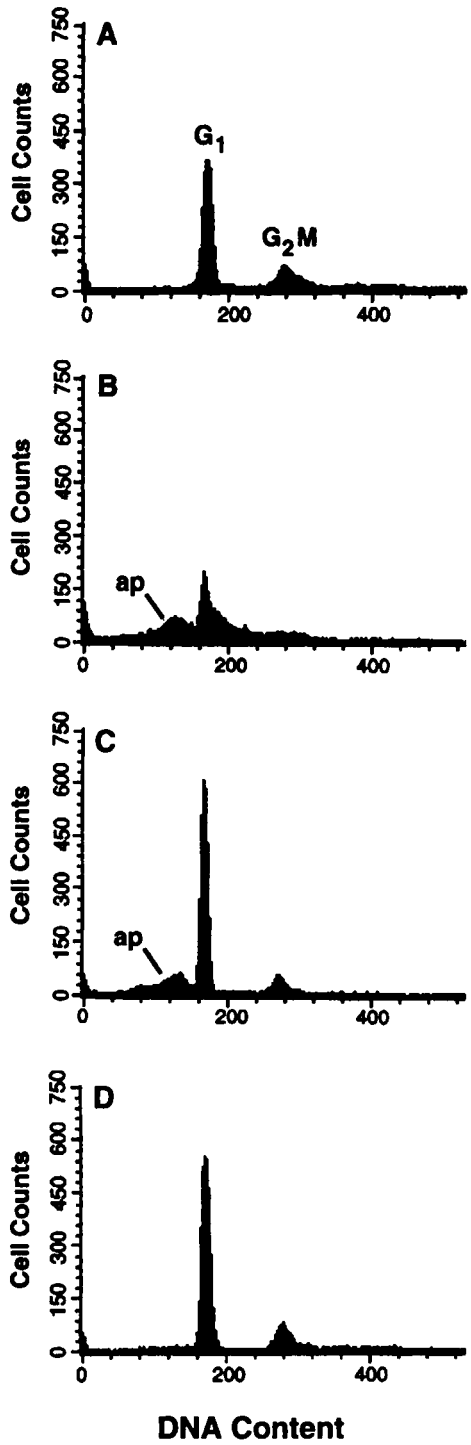

Fig. 8. Effects of CP-358,774 on DiFi cell cycle. DiFi cells were exposed to 0.125% DMSO (*A*), 8 μg/ml cisplatin (*B*), or 1 μM CP-358,774 in the absence (*C*) or presence (*D*) of 20 nM IGF-I for 24 h and then analyzed for DNA content (PI uptake) as described in "Materials and Methods." Ten thousand cells were analyzed per determination. Cell population distribution analysis was done using the CellQuest software. See Table 1 for quantitation of the G_1, S, and G_2-M peaks. *ap*, apoptotic peak.

Table 1 *Cell cycle arrest and induction of apoptosis by CP-358,774*

Quantitation of cell cycle arrest, DNA fragmentation, and annexin V binding induced by CP-358,774 in DiFi cells. These values are quantitation of the flow cytometry experiments shown as histograms or dot plots in Fig. 8, 11, and 12. All values are the average of duplicate determinations that agreed within 3%. All experiments were repeated at least twice with similar results. For the experimental details, see the legends of those figures.

Treatment	Cell cycle distribution (% of cycling cells)			% apoptotic by	
	G_1	S	G_2	TUNEL	Annexin binding
Control	64	8	28	2.3	6
Cisplatin (8 μg/ml)	56	32	12	ND[a]	26
CP-358,774 (1 μM)	77	5	18	17	21
CP-358,774 (1 μM) + 20 nM IGF-1	77	2	21	7.4	6

[a] ND, not done.

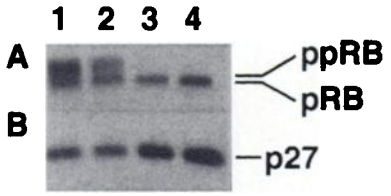

Fig. 9. CP-358,774 reduces pRB phosphorylation and increases $p27^{KIP1}$ expression in DiFi cells. DiFi cells were incubated at 37°C in 1:1 DMEM:Ham's F-12 with 10% FBS, supplemented with CP-358,774 at 50 nM (*Lane 2*), 250 nM (*Lane 3*), or 1000 nM (*Lane 4*) or without inhibitor (*Lane 1*) for 24 h. Cell extracts were prepared, and pRB (*A*) and $p27^{KIP1}$ (*B*) were measured as described in "Materials and Methods." The positions of the hyperphosphorylated (*ppRB*) and hypophosphorylated (*pRB*) retinoblastoma protein and of $p27^{KIP1}$ (*p27*) are indicated.

Induction of Apoptosis by CP-358,774. Although inhibition of EGFR signaling may produce only tumor stasis if cells are retained in G_1, other evidence suggests that inhibition of EGFR triggers cell death in some tumors. We, therefore, examined induction of apoptosis by CP-358,774 in DiFi human colon tumor cells because these cells were reported to undergo apoptosis in response to anti-EGFR antibodies (28).

Apoptosis (programmed cell death) is associated with the activation of Ca^{2+}-dependent endonucleases and cleavage of genomic DNA at the linker sites between nucleosomes. The low molecular weight DNA fragments generated in this process leak out of cells when cells are treated with detergent to make them permeable to PI. Thus, apoptotic cells have a lower DNA content than nonapoptotic, cycling cells indicated by a "sub-G_1" peak in DNA histograms (46). In the analysis of DNA content of CP-358,774- and cisplatin-treated cells by flow cytometry, there is a prominent sub-G_1 peak (Fig. 8, *B* and *C*). Also in HN5 cells, a population of cells with DNA content less than that of G_1 cells appeared upon treatment with CP-358,774, but this population was less prominent than that seen in Fig. 8 for DiFi cells. The emergence of this peak cannot be considered definitive evidence for apoptosis because several other conditions, *e.g.*, mechanically damaged cells, can generate cells with sub-G_1 DNA content. However, IGF-I prevented the formation of the sub-G_1 population in the CP-358,774-treated cells (Fig. 8*D*), and IGF-I is known to protect DiFi cells from apoptosis that is induced by anti-EGFR antibodies (28). To confirm the induction of apoptosis by CP-358,774, we used additional techniques to more directly assess this process.

In endonuclease-mediated DNA fragmentation, the ultimate DNA fragments are multimers of ~180-bp nucleosomes that resolve into a "DNA ladder" when electrophoresed on standard agarose gels. This ladder represents the low molecular weight DNA that leaks out of apoptotic cells during detergent permeabilization, resulting in the sub-G_1 peak discussed above. DiFi cells treated for 24 h with 1 μM CP-358,774 reproducibly produced such ladders (Fig. 10) to a degree similar to that produced by a positive control compound, cisplatin, which has been reported to induce internucleosomal DNA fragmentation in DiFi cells (28). Simultaneous treatment with 20 nM IGF-I rescued cells from CP-358,774-induced DNA fragmentation (Fig. 10, *Lanes 7* and *8*) but did not protect from cisplatin-induced DNA fragmentation (data not shown).

We also confirmed the induction of DNA fragmentation by CP-358,774 using TUNEL. TUNEL is a more sensitive and quantitative method for the detection of 3'-OH ends generated by endonuclease-mediated DNA strand breaks (47). Exposure to 1 μM CP-358,774 for 24 h caused a ~7-fold increase in the percent apoptotic cells (Fig. 11 and Table 1). Coincubation with 20 nM IGF-I reduced the induction of DNA strand breaks by CP-358,774 to less than 3-fold (Fig. 11*C* and Table 1) or even completely during the 24-h exposure in similar experiments not shown.

Another biochemical change that takes place in cells undergoing apoptosis is exposure of PS on the outer leaflet of the plasma membrane due to activation of a membrane translocase. This is a general feature of apoptosis, having been observed in a wide variety of cells in response to many different stimuli (48). Annexin V binds with high affinity to PS and can be used as a specific probe to detect PS externalization. Because annexin V also binds to necrotic cells, it is necessary to counterstain with PI to distinguish between apoptotic and necrotic cells. Thus, by simultaneous detection of PI and annexin V-FITC fluorescence, one can discriminate between normal (PI−/FITC−), apoptotic (PI−/FITC+), and necrotic (PI+/FITC+) cells.

We examined annexin V-FITC binding to DiFi cells that had been treated with 8 μg/ml cisplatin or with 1 μM CP-358,774 for 24 h (Fig. 12 and Table 1). CP-358,774 induced a 3.5-fold increase in apoptotic

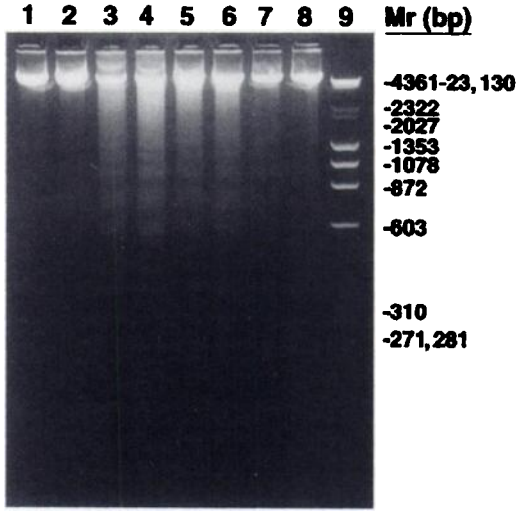

Fig. 10. CP-358,774 induces internucleosomal DNA cleavage in DiFi cells. DiFi cells were incubated for 24 h in DMEM:Ham's F-12 plus 0.5% FBS containing DMSO (duplicates, *Lanes 1* and *2*), 12 μg/ml cisplatin (duplicates, *Lanes 3* and *4*), 1 μM CP-358,774 (duplicates, *Lanes 5* and *6*), or 1 μM CP-358,774 plus 20 nM IGF-I (duplicates, *Lanes 7* and *8*). The cells were harvested and the DNA extracted and electrophoresed as described in "Materials and Methods." This result was reproduced in three experiments. Molecular size markers (*Lane 9*) are indicated in bp.

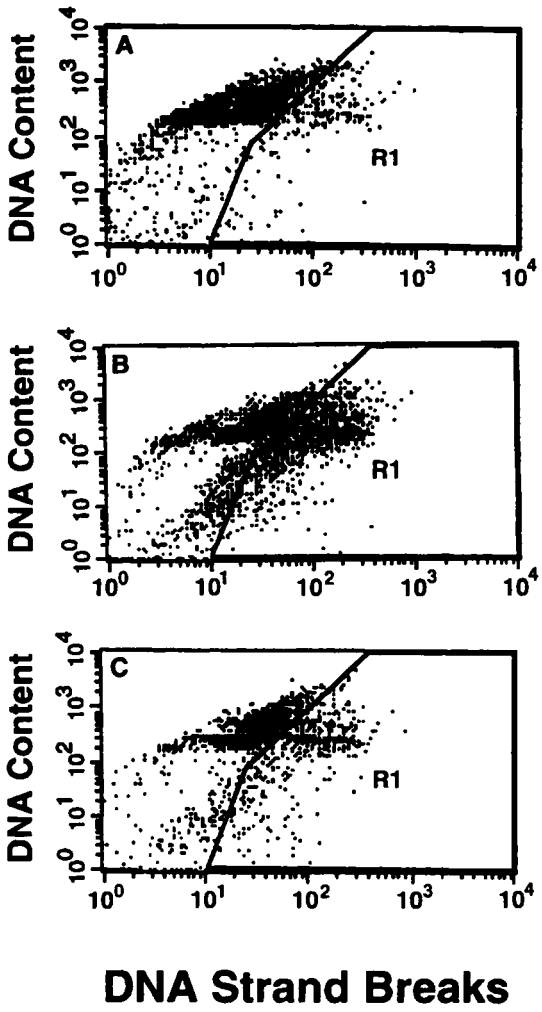

Fig. 11. CP-358,774 causes 3'-OH DNA strand breaks in DiFi Cells. DiFi human colon carcinoma cells were incubated for 24 h in DMEM:Ham's F-12 plus 0.5% FBS containing diluent (*A*), 1 μM CP-358,774 (*B*), or 1 μM CP-358,774 plus 20 nM IGF-I (*C*). Following harvest, fixation, and permeabilization, cells were labeled using the TUNEL technique, counterstained with PI, and analyzed by flow cytometry. Ten thousand cells were analyzed per determination. Dot plots show DNA strand breaks (TUNEL labeling) on the *X* axis and DNA content (PI staining) on the *Y* axis. *R1*, region of apoptotic cells. In other similar experiments, CP-358,774 treatment increased TUNEL labeling by 4- to >7-fold.

cells and a corresponding decrease in the number of normal cells but did not increase the number of necrotic cells. IGF-I completely protected from the increase in annexin V-FITC binding induced by CP-358,774 (Fig. 12*D*). Cisplatin caused a ~4-fold increase in apoptosis but also increased the number of necrotic cells by 2.7-fold (Fig. 12*B*). In other experiments, the fold increase in annexin V binding induced by CP-358,774 ranged from 2- to 5-fold. A 6-fold induction of annexin binding by CP-358,774 was also observed upon treatment of HN5 cells (data not shown).

Cleavage of the nuclear enzyme PARP has been reported in a variety of cell types undergoing chemotherapy-induced programmed cell death, including the EGFR-overexpressing breast carcinoma cell line, MDA-MB-468 (49). PARP has been shown to be a substrate for several of the ICE-like proteases thought to be the universal effectors of apoptosis (50). Therefore, we examined DiFi cells for proteolysis of PARP upon treatment with CP-358,774. Examination of control cells revealed that the immunolabeled protein was in the M_r 116,000 form (Fig. 13, *Lanes 1* and *2*). Treatment with either cisplatin (Fig. 13, *Lane 3*) or CP-358,774 (Fig. 13, *Lanes 4* and *5*) for 24 h resulted in a decrease in the M_r 116,000 protein and a corresponding increase in the M_r 85,000 fragment. Coculture with IGF-I protected cells from induction of PARP cleavage by CP-358,774 (Fig. 13, *Lanes 6* and *7*).

Thus, we have evidence for induction of apoptosis in DiFi cells by the EGFR kinase inhibitor CP-358,774 and protection by IGF-I using

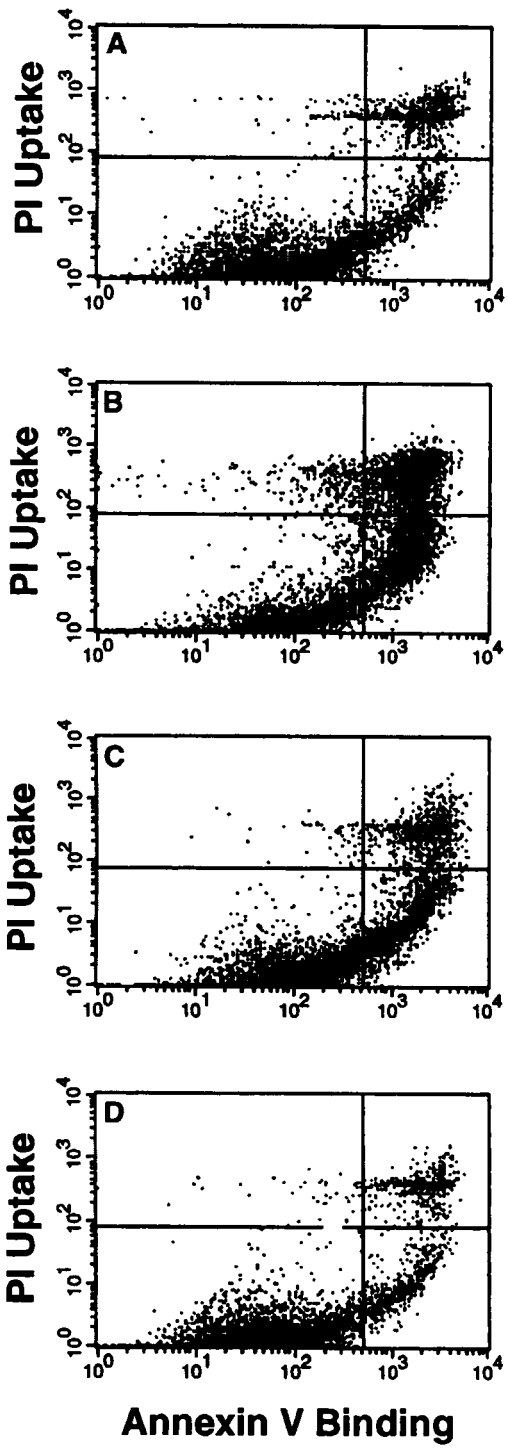

Fig. 12. CP-358,774 induces annexin V binding in DiFi cells and IGF-I protects. DiFi cells were incubated in DMEM:Ham's F-12 plus 0.5% FBS containing diluent (*A*), 8 μg/ml cisplatin (*B*), 1 μM CP-358,774 (*C*), or 1 μM CP-358,774 plus 20 nM IGF-I (*D*). After 24 h, the cells were harvested and incubated with annexin V-FITC and PI as described in "Materials and Methods." Annexin V-FITC and PI binding were quantitated by flow cytometry. Ten thousand cells were analyzed per determination. Dot plots show Annexin V-FITC binding on the *X* axis and PI staining on the *Y* axis. Dots represent cells as follows: *lower left quadrant*, normal cells (FITC−/PI−); *lower right quadrant*, apoptotic cells (FITC+/PI−); upper right quadrant, necrotic cells (FITC+/PI+). The results shown here are representative of three experiments.

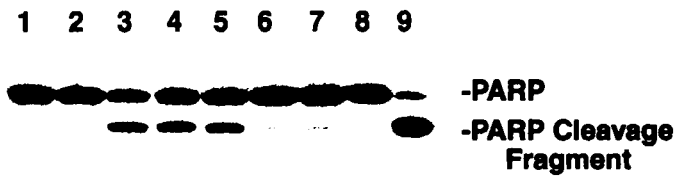

Fig. 13. CP-358,774 induces PARP cleavage in DiFi cells, and IGF-I protects. DiFi cells were incubated in DMEM:Ham's F-12 plus 0.5% FBS containing diluent (*Lanes 1* and *2*), 12 μg/ml cisplatin (*Lane 3*), 1 μM CP-358,774 (*Lanes 4* and *5*), or 1 μM CP-358,774 and 20 nM IGF-I (*Lanes 6* and *7*). After 24 h, detached and adherent cells were harvested, lysed, and analyzed for PARP cleavage by Western blotting as described in "Materials and Methods." HL60 cells, either uninduced (*Lane 8*) or induced to undergo apoptosis with etoposide (*Lane 9*), were used as negative and positive controls, respectively. The M_r 116,000 PARP and its M_r 85,000 PARP cleavage fragment are indicated. The result shown is representative of two experiments.

five independent criteria: accumulation of cells with sub-G_1 DNA content, DNA ladder formation, DNA fragments identified by TUNEL, PS externalization shown by annexin V binding, and PARP cleavage.

DISCUSSION

The EGFR represents an attractive target for antitumor therapy because it offers the prospect of disrupting a pathway that is essential for proliferation of some tumor cells without producing a general block of cell proliferation in the host. Evaluation of this hypothesis in the clinic will require a specific inhibitor of EGFR signaling, such as the monoclonal antibodies currently in clinical trials (19) or a highly selective inhibitor of the EGFR kinase activity. CP-358,774 is shown here to have many of the properties desired for such an EGFR tyrosine kinase inhibitor. It is a potent, directly acting inhibitor of the EGFR kinase (Fig. 1) with excellent potency in cellular assays for both EGFR autophosphorylation (Fig. 3) and inhibition of cell proliferation (Figs. 6 and 7).

Studies of the specificity of CP-358,774 indicated that it is a selective EGFR kinase inhibitor relative to four other distinct tyrosine kinases (Fig. 2). However, because the conditions used for kinase assays are arbitrary, these assays may not accurately reflect the selectivity of this inhibitor in intact cells. Therefore, the selectivity of CP-358,774 for EGFR is more convincingly shown by cellular assays as in Figs. 4 and 7. Fig. 4 shows that CP-358,774 blocks downstream signaling by SHC initiated by EGFR tyrosine kinase but does not block signaling by IRS-1 initiated by the insulin receptor tyrosine kinase. Fig. 7 indicates that this inhibitor blocks EGF-stimulated mitogenesis at concentrations that do not reduce mitogenesis triggered by distinct tyrosine kinase-linked growth factor receptors (Fig. 7). The continued capacity for cell proliferation in response to PDGF, IGF-I, and bFGF in the presence of CP-358,774 also excludes the possibility of significant inhibition of other kinases essential for cell proliferation. Thus, CP-358,774 has the advantage of being selective for inhibition of the EGFR pathway, without inhibiting other pathways involved in growth regulation.

Multiple proteins are phosphorylated upon activation of the EGFR, and these proteins mediate the cellular mitogenic response. As one example, phosphorylation of the SH2-domain protein SHC mediates the mitogenic effects of EGF (40, 41) in at least some cells, and constitutive phosphorylation of SHC, a common feature of tumors, may contribute to the transformed phenotype (51). Thus, the reduction of SHC phosphorylation in response to CP-358,774 (Fig. 4) may contribute to the antiproliferative action of CP-358,774 and indicates that the extent of inhibition of EGFR kinase is sufficient to block activation of downstream steps of the signaling cascade.

The critical committed step toward cell division, progression of cells into S phase, normally requires phosphorylation of pRB to initiate the steps required for DNA synthesis (40). The accumulation of cells in G_1 (Fig. 8), loss of hyperphosphorylated pRB (Fig. 9*A*) and accumulation of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ (Fig. 9*B*) indicate that inhibition of EGFR in DiFi or HN5 cells reduces cell proliferation in part by reducing pRB phosphorylation. Similar results were obtained on treatment of DiFi cells with anti-EGFR antibodies (28, 52). Although the increased $p27^{KIP1}$ levels may contribute to the reduced ppRB levels, several other signals may also be involved, for example, changes in other inhibitors of the cyclin-dependent kinases such as $p21^{WAF1}$ and $p15^{INK4b}$ or in expression levels of cyclin D may also influence pRB phosphorylation (45). The effect of CP-358,774 on the expression of these proteins remains to be determined. The two cell lines used in these studies, DiFi and HN5, were selected because previous studies indicated that they were highly sensitive to the antiproliferative effects of the anti-EGFR antibodies (15, 16, 28). They are also the most sensitive cell lines we have examined in regard to inhibition of proliferation by CP-358,774. Both cell lines express pRB and respond to CP-358,774 with an accumulation of hypophosphorylated pRB. It would be of interest to determine whether the expression of functional pRB is essential for a robust antiproliferative response to EGFR inhibitors, given that pRB is inactivated in several types of human cancers (45).

The antiproliferative effects of CP-358,774, directly measured by cell counts, could arise primarily by the effects on cell cycle, *i.e.*, by producing a complete G_1/G_0 arrest. However, the cell cycle measurements (Fig. 8) do not indicate complete arrest of cells at G_1, even at concentrations that completely prevent any increase in cell number in DiFi cell cultures (compare Figs. 6 and 8) or HN5 cell cultures (data not shown). This apparent discrepancy may be explained by the induction of apoptosis in these cells documented in Figs. 8–13.

One way that activation of growth factor receptors exert an antiapoptotic effect is by propagating a "survival" signal that prevents apoptosis (30). Indeed, an antiapoptotic action of the EGFR was demonstrated directly by Pierce *et al.* (53) in experiments in which EGFR was transfected into hematopoietic cells; in these cells, apoptosis induced by withdrawal of IL-3 could be blocked by addition of EGF. Additional support for a survival signal propagated by the EGFR is provided by studies with a mutant EGFR frequently expressed by gliomas (54). Glioma cells transfected with kinase competent truncated EGFR but not with a kinase-defective truncated EGFR had lower rates of apoptosis (54). A potential role for EGFR in promoting survival of human keratinocytes is indicated by studies in which monoclonal antibodies to the EGFR induced apoptosis in cells following detachment from the extracellular matrix (55). Finally, a role for EGFR in promoting survival was indicated by the induction of apoptosis in DiFi cells by antibodies that block the EGFR (28).

The current study indicates that inhibition of the EGFR kinase activity by CP-358,774 induces apoptosis in a significant fraction of DiFi cells: 17 *versus* 2.3% apoptotic cells in controls, as measured by the TUNEL method. As had been observed with the anti-EGFR antibody (28), this effect could be prevented by concomitant exposure to IGF-I, possibly by providing an alternative survival signal. These results suggest that therapy directed against the EGFR may induce tumor cell apoptosis but that the effectiveness of EGFR kinase inhibitors may be modified by other endogenous survival factors.

The detailed mechanism by which inhibition of EGFR results in apoptosis in DiFi cells is unclear. Further work is needed to link the inhibition of the EGFR to the multiple downstream processes, such as DNA fragmentation, externalization of PS, and cleavage of PARP, by a coherent sequence of biochemical events. The pathways linking surface receptors to apoptosis are under intense investigation (56).

The relative contributions of cell cycle arrest and apoptosis to the

antitumor activity that results from inhibition of EGFR may vary with different tumor cells. Thus anti-EGFR antibodies trigger G_0/G_1 cell cycle arrest, differentiation, and immune destruction of HN5 tumor xenografts, without evidence of apoptosis (27), and DU-145 prostate cancer cells are also arrested without evidence of apoptosis (57). Although inhibition of EGFR kinase triggers apoptosis of DiFi cells in culture, further studies with tumor xenografts are required to establish whether EGFR blockade induces apoptosis of tumor cells *in vivo*.

The studies described here examined the effect of 1 μM inhibitor CP-358,774, a concentration that completely blocks DiFi cell proliferation, on apoptosis and cell cycle arrest; more detailed studies are needed to determine which effects are critical, especially at lower concentrations. Finally, additional studies are required to establish the utility of inhibitors of EGFR in the treatment of human disease. The results described above, as well as additional antitumor data to be reported separately,[3] indicate that CP-358,774 is sufficiently potent, selective, effective, and safe in animal models to warrant examination in the clinic. Ultimately, clinical studies are required to determine the prevalence of human tumors that are dependent on EGFR for proliferation, as well as the clinical toleration of an inhibitor of the EGFR kinase in patients.

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