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Irreversible Inhibition of Epidermal Growth Factor Receptor Tyrosine Kinase with *In Vivo* Activity by *N*-[4-[(3-Bromophenyl)amino]-6-quinazolinyl]-2-butynamide (CL-387,785)

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ABSTRACT. It has been shown previously that 4-anilino quinazolines compete with the ability of ATP to bind the epidermal growth factor receptor (EGF-R), inhibit EGF-stimulated autophosphorylation of tyrosine residues in EGF-R, and block EGF-mediated growth. Since millimolar concentrations of ATP in cells could reduce the efficacy of 4-anilino quinazolines in cells and the activity of these compounds would not be sustained once they were removed from the body, we reasoned that irreversible inhibitors of EGF-R might improve the activity of this series of compounds in animals. Molecular modeling of the EGF-R kinase domain was used to design irreversible inhibitors. We herein describe one such inhibitor: *N*-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide, known as CL-387,785. This compound covalently bound to EGF-R. It also specifically inhibited kinase activity of the protein (IC_{50} = 370 ± 120 pM), blocked EGF-stimulated autophosphorylation of the receptor in cells ($IC_{50} \cong 5$ nM), inhibited cell proliferation (IC_{50} = 31–125 nM) primarily in a cytostatic manner in cell lines that overexpress EGF-R or c-erbB-2, and profoundly blocked the growth of a tumor that overexpresses EGF-R in nude mice (when given orally at 80 mg/kg/day for 10 days, daily). We conclude that CL-387,785 is useful for studying the interaction of small molecules with EGF-R and may have clinical utility. BIOCHEM PHARMACOL **57**;8:917–925, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. epidermal growth factor receptor; tyrosine kinase; c-*erb*B-2; covalent inhibitor; tumor; anticancer

The EGF-R** is a 170-kDa protein that contains an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. Upon binding ligands (e.g. EGF, transforming growth factor alpha, amphiregulin), the receptor dimerizes, tyrosine kinase activity increases, and the protein autophosphorylates tyrosine residues in the C-terminal region of the receptor [1]. The autophosphorylated receptor recruits a variety of scaffold, anchoring, and adapter proteins that lead to activation of (among other pathways) the ras/raf and phosphoinositol 3-kinase pathways [2]. Ultimately, cell proliferation occurs.

EGF-R is hyperactivated in numerous tumors including those derived from the brain, lung, bladder, prostate, head, and neck [3]. Receptor hyperactivation can occur by overexpression of the protein, mutation of the receptor, or abnormal proximity of the receptor and ligand(s). In many cases, overexpression of EGF-R has been correlated with poor prognosis. Beyond this, another family member of this type I growth factor receptor, c-*erb*B-2, is amplified in approximately 25% of all breast cancer, and its overexpression has been correlated with poor prognosis [4].

Two approaches to specifically inhibit EGF-R and/or c-erbB-2 activation involve the use of neutralizing antibodies directed to the extracellular region of the receptor [5] or small molecules that inhibit the tyrosine kinase activity in these receptors [6–10]. Molecules in the pyrimidine [6], tyrphostin [7], and substituted quinazoline series [8–10], exemplified by CGP 59326 [6], RG13022 [7], and PD

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** *Abbreviations*: EGF-R, epidermal growth factor-receptor; and EGF, epidermal growth factor.

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153035 [8], respectively, are potent inhibitors of EGF-R. The inhibitory action of these compounds is mediated, in part, by competition of ATP with the kinase domain of EGF-R [7, 11]. While these molecules have good activity in tissue culture, only CGP 59326 inhibited the growth of tumors that overexpress EGR-R in multiple *in vivo* tumor models [6]. PD 153035 did not inhibit tumor growth *in vivo*, although short-term inhibition of EGF-R phosphorylation was demonstrated in the same *in vivo* model [12]. RG13022 has demonstrated activity in one *in vivo* model [13] and no activity in another model [14]. The minimal or inconsistent *in vivo* activity of PD 153035 or RG13022 is associated with rapid clearance of these molecules from plasma [13, 14], and, therefore, may require sustained delivery. We have independently discovered a similar phenomenon. Therefore, we have attempted to develop irreversible inhibitors in which the action of the drug would be sustained after the drug was cleared from plasma or tumor. In this report, molecular modeling of the putative kinase domain of EGF-R was used to help identify irreversible low molecular weight inhibitors of EGF-R. One novel irreversible inhibitor of EGF-R with *in vivo* activity, known as *N*-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide and designated CL-387,785, is described below.

MATERIALS AND METHODS

Chemicals

Preparation of CL-387,785 began with 5-nitroanthranilonitrile. Formamidination using *N*,*N*-dimethylformamide dimethyl acetal (98% yield) followed by reduction of the nitro group with palladium on carbon and cyclohexene in refluxing methanol furnished *N′*-(4-amino-2-cyanophenyl)-*N*,*N*-dimethylformamidine (95% yield). Acylation with the mixed anhydride of tetrolic acid and isobutyl chloroformate gave *N*-[3-cyano-4-[[(dimethylamino)methylene]amino]phenyl]-2-butynamide (89% yield). In the final step, cyclization using 3-bromoaniline in refluxing acetic acid gave CL-387,785 (66% yield), which was purified by recrystallization from ethanol. Full experimental details are provided elsewhere [15].

CL-387,785 radiolabeled with carbon-14 at the 4-position in the quinazoline ring was prepared by the reaction of 2-bromo-4-nitroaniline with [^{14}C]KCN (57 mCi/mmol) in the presence of copper(I) iodide, according to the procedure of Carr *et al.* [16], yielding the desired 5-nitroanthranilo[^{14}C]nitrile in 37.8% yield. Following formamidination with *N*,*N*-dimethylformamide dimethyl acetal (93.2% yield), cyclization to the 6-nitro-4-(3-bromoanilino) quinazoline-[4-^{14}C] using 3-bromoaniline in refluxing acetic acid proceeded in 81.5% yield. After iron powder reduction (82.1% yield), the corresponding 6-aminoquinazoline-[4-^{14}C] was acylated with the mixed anhydride of tetrolic acid and isobutyl chloroformate to afford the free base of [^{14}C]CL-387,785 (51.4% yield) after semi-preparative high pressure liquid chromatography, which was then converted to the methane sulfonate salt (83.9% yield). The final material had a specific activity of 139.4 μCi/mg and was 100% radiopure.

Molecular Modeling

A three-dimensional homology model of the catalytic domain of EGF-R, based on a crystallographic structure of the catalytic subunit of cyclic AMP-dependent protein kinase [17], was constructed and was similar to other kinase models for EGF-R [11, 18]. The protein, the bound ATP, and a tyrosine-containing substrate peptide were surrounded by an 8 Å sphere of water, and a 100-psec molecular dynamics simulation was done, allowing everything within a radius of 12 Å to move around ATP. Subsequently, a model of CL-387,785 was docked into the active site using Quanta/CHARMm (Version 97.0711; Molecular Simulation Inc.). A sphere of explicit water molecules (radius 7.5 Å) was placed around the ligand, and the ligand–protein complex was minimized, allowing everything to move within a radius of 12 Å.

Kinase Assay

Kinase assays were performed with a recombinant cytoplasmic domain of EGF-R (HcEGF-R). To obtain recombinant enzyme, cDNA encoding amino acids 645–1186 of EGF-R based on the published sequence [19] was derived from a polymerase chain reaction using a placental cDNA library (Stratagene). This cDNA was cloned into a baculoviral expression vector (pFASTBacHTc; Gibco BRL) that encoded a Met-Ala-$(His)_6$ sequence immediately upstream of the cDNA encoding the EGF-R sequence. Sf9 cells in 100-mm plates were infected with 10 pfu/cell, and cells were harvested 48 hr post-infection. A cytoplasmic extract was prepared using 1% Triton X-100 and applied to a Ni-nitrolotriacetic acid column. After washing the column with 20 mM imidazole, HcEGF-R was eluted with 250 mM imidazole (in 50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl) and dialyzed against 10 mM HEPES, pH 7.0, 50 mM NaCl, 10% glycerol, 1 μg/mL of antipain, 1 μg/mL of leupeptin, and 0.1 mM Pefabloc SC. The protein was frozen in dry ice/methanol and stored at −70°.

For the enzyme reaction, stock solutions of 500 μM CL-387,785 (prepared in 100% DMSO) were diluted to the desired concentration with 30 mM HEPES, pH 7.4. Ten microliters of CL-387,785 at various concentrations were incubated with 3 μL of recombinant enzyme (1:120 dilution in 100 mM HEPES, pH 7.4) on ice for 10 min. Then, 5 μL peptide (400 μM final concentration of RR-SRC composed of Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (Gibco)), 10 μL of 4× reaction buffer containing 50 mM HEPES, pH 7.4, 80 μM ATP, 40 mM $MnCl_2$, and 200 μM sodium orthovanadate, 0.30 μL [^{33}P]ATP (>2500 Ci/mmol; Amersham), and 12 μL H_2O were added. After incubation for 90 min at room temperature, the entire volume was spotted onto precut P81 filter papers. The filter discs were washed two times with 0.5%

phosphoric acid, and radioactivity was measured using a liquid scintillation counter. Under these conditions, the specific activity of EGF-R kinase was approximately 0.50 pmol/mg/min.

EGF-R Tyrosyl Phosphorylation Assays in Cells

A431 cells (8×10^5) were seeded in 100-mm dishes overnight in complete medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium). Then the monolayer of cells was exposed to various concentrations of CL-387,785 at 37° for 3 hr, followed by stimulation with recombinant human EGF (100 ng/mL, R & D Systems) for 20 min. Cells were harvested by washing twice with cold PBS, and lysed in 1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.6, 0.2 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM Na_3VO_4, 5 μg/mL of aprotinin, and 5 μg/mL of leupeptin on ice for 20 min. After 10 min of 300 *g* centrifugation, protein in the supernatant was determined by the Bradford method (Bio-Rad). Equal amounts of protein were then either subjected to immunoprecipitation or boiled in 2× Laemmli sample buffer for 5 min and resolved on SDS-polyacrylamide gels. After electrophoresis, protein was electrotransferred to PVDF membrane. Membrane was blocked in 10 mM Tris, pH 7.4, containing 150 mM NaCl, 0.05% Tween (TBST), and 1% BSA for 1 hr and then incubated with a 1:2000 dilution of anti-phosphotyrosine antibody conjugated with horseradish peroxidase (Transduction Laboratories) for 1 hr. After washing, membrane was developed by enhanced chemiluminescence (ECL; Amersham).The amount of EGF-R protein in the samples was analyzed by stripping the membrane, quenching the blot in 5% nonfat dry milk in TBST, reprobing with 1:2000 anti-EGF-R antibody (Transduction Laboratories), washing, incubating with 1:2000 anti-mouse IgG conjugated to horseradish peroxidase (Transduction Laboratories), and developing by the ECL method. Alternatively, immunoprecipitation of EGF-R was done by incubating 500 μg of lysate with 1.25 μg anti-EGF-R antibody (Transduction Laboratories) at 4° for 3 hr. Then 60 μL of protein A slurry (UBI) was added. After 2 hr, protein A beads were washed with Dulbecco's PBS three times to remove unbound proteins, 2× Laemmli sample buffer was added, and the eluate was analyzed by immunoblot analysis as described above.

Covalent Binding Analysis

Membranes from A431 cells were prepared by sucrose density gradient centrifugation according to previously described methods [20] except that 2 mM sodium orthovanadate and 100 mM sodium fluoride were added to the protease inhibitor mixture and maintained throughout the procedure. Protein determination was done by the Bradford method, and the material was frozen as aliquots at −70°.

For covalent labeling experiments, 100 μg of membrane protein was incubated with or without 50 μM unlabeled CL-387,785. This material was mixed immediately with 20–5000 nM [^{14}C]CL-387,785. Ten millimolar Tris, pH 7.4 was added to bring the final reaction volume to 37 μL. The sample was placed in the dark. After 30 min, the maximal reaction occurred. The reaction was terminated by the addition of 5× Laemmli sample buffer to achieve the final concentrations of 2% SDS, 5 mM β-mercaptoethanol, 62.5 mM Tris, pH 6.8. Samples were boiled for 3 min. The material was resolved in 7% SDS gels, and protein was stained with Coomassie Brilliant Blue R to ensure equivalent loading. Radioactivity in the gel was determined by incubating 1-mm slices of each gel lane in 200 μL of 90% Beckman BTS for 16 hr, adding 6 mL of Ecolume scintillation fluid, and performing liquid scintillation counting. In some cases, membranes were incubated with [^{14}C]CL-387,785, lysed, and subjected to immunoprecipitation with an EGF-R directed antibody as described above. The immunoprecipitated material was resolved in gels, and radioactivity in dried gels was observed using a PhosphoImager (Molecule Dynamics).

Cell Survival Assay

The following cell lines were used in this assay: A431, SK-BR-3, MDA-MB-435, MCF-7, SW-620, 3T3, and 3T3 transfected with c-*erb*B-2. All lines were obtained from the American Type Culture Collection, with the exception of the 3T3, 3T3/c-*erb*B-2 pair, which were provided by Dr. M. Disis (University of Washington). Cells were grown in RPMI medium supplemented with 5% fetal bovine serum. Cell survival assays were done by plating cells in 96-well dishes at the following density per well: A431 and SW-620: 30,000; SK-BR-3: 50,000; and MCF-7, MDA-MB-435, 3T3, and 3T3/c-*erb*B-2: 25,000. On the next day, drug was prepared at 10-fold dilutions in medium and incubated with the cells for 2 days. Cell survival was determined by the sulforhodamine B assay as previously described [21]. The IC_{50} values were obtained from the growth curves.

Estimation of Levels of EGF-R and c-erbB-2 in Cells

The relative levels of EGF-R and c-erbB-2 were determined by flow cytometry or immunoblot analysis. For flow cytometry, cells were briefly trypsinized and washed in PBS. Then cells were sequentially incubated in a 1:10 dilution of monoclonal antibody 528 to EGF-R (Santa Cruz Biotechnology) or monoclonal antibody 9G6 to c-erbB-2 for 15 min, washed in PBS, incubated with a 1:10 dilution of goat-anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Dako) for 20 min, washed, and analyzed by flow cytometry (FACSort; Becton Dickinson). All procedures except trypsinization were carried out at 4°. For immunoblot analysis, EGF-R and c-erbB-2 levels in cell lysates were determined under conditions described above with polyclonal antibodies to EGF-R (Santa Cruz antibody No. 1005; 1:500 dilution) or to c-erbB-2 (Oncogene Re-

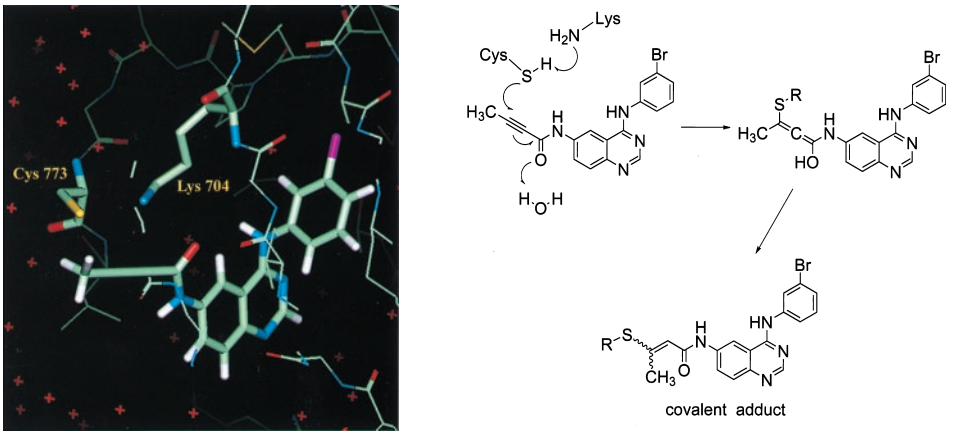

FIG. 1. Model of CL-387,785 binding to the ATP site of EGF-R and the proposed alkylation mechanism.

search Products antibody Ab-1; 1:50 dilution). Immunoreactivity was detected with 1:1000 donkey anti-rabbit IgG (Amersham) followed by development with ECL.

Cell Cycle Analysis

T75 flasks of A431 or SK-BR-3 cells were grown to approximately 50% confluency and then treated with increasing concentrations of CL-387,785 or paclitaxel. After 3 days, the medium was collected, and cells were washed with PBS and trypsinized. The medium and trypsinized cells were centrifuged (300 *g* for 10 min). To the resultant pellet, 1 mL of 80% ethanol at −20° was added per 10^6 cells. The ethanol-treated cells were vortexed lightly and centrifuged (300 *g* for 5 min). The resultant pellet was washed twice in PBS, incubated with 1 mL of propidium iodide (50 μg/mL; Calbiochem) and 200,000 units of RNase (Sigma) for 1 hr at room temperature, and analyzed by flow cytometry using a 488 nm emission wavelength and a 650 nm excitation filter.

Growth of Tumors in Nude Mice

Athymic *nu/nu* female mice (Charles River Laboratories) were injected s.c. with either 5×10^6 A431 cells or 6×10^6 MDA-MB-435 cells. When tumors attained a mass of 80–120 mg (day 0), animals were randomized into treatment groups. Depending upon the experiment, there were 5 or 10 animals per experimental group. Mice were treated either i.p. or p.o. with a suspension of CL-387,785 prepared in 0.2% Klucel (hydroxypropylcellulose) or with vincristine (Lilly) prepared in saline. Tumor mass ([length × width^2] /2) was determined every 7 days for up to 49 days, and the percent treated/control (%T/C) was calculated. The %T/C is defined as the mean relative tumor growth of the treated group divided by the mean relative tumor growth of the vehicle control group multiplied by 100.

RESULTS

Using the homology model of EGF-R as a starting point and the observations by us and others [22] that the 6- and 7-positions of the quinazoline ring can tolerate considerable bulk, we proposed that CL-387,785 bound to EGF-R at the ATP-binding region with an orientation and conformation similar to that shown in Fig. 1. This orientation placed the bromoaniline side chain of CL-387,785 in a hydrophobic pocket surrounded by Pro^{770}, Met^{769}, Gln^{767}, Ala^{719}, Val^{702}, and Leu^{820}. The N1 nitrogen of the quinazoline was in contact with Arg^{817}. The N3 nitrogen of the quinazoline was close to Thr^{830} and a few water molecules. Most significantly, the β-carbon atom of the Michael acceptor functional group of CL-387,785 was potentially within bonding distance to the sulfhydryl group of Cys^{773}. (A second cysteine, Cys^{751}, was also located in the binding pocket, but it was not as close to the Michael acceptor functional group as Cys^{773}.) In addition, this model suggested that the amino group of Lys^{704} was located close enough to this sulfhydryl group to serve as a basic catalyst for the Michael addition reaction. Our proposed mechanism for the alkylation of the enzyme by CL-387,785 is shown in Fig. 1.

The ability of CL-387,785 to inhibit the kinase activity of EGF-R was evaluated in an enzyme assay. In this assay, the recombinant cytoplasmic domain of EGF-R transferred phosphate onto the tyrosine residue of a peptide substrate (that contained no serine or threonine). The IC_{50} of CL-387,785 was 0.370 ± 0.120 nM (mean ± SEM; N = 12). Consistent with these results, the IC_{50} of CL-387,785 was 0.42 ± 0.19 nM (N = 10) when the full length enzyme was enriched from a membrane preparation of A431 cells. (A431 cells were chosen since they express a large amount of EGF-R [19].) While the IC_{50} value for CL-387,785 was considerably higher than previously reported quinazoline-based inhibitors (e.g. PD 153035, IC_{50} = 5 pM; see Ref. 6), in our recombinant enzyme assay system the IC_{50} for PD 153035 was 0.158 ± 0.03 nM (N = 3). Similar discrepancies have been reported by others [11]. Therefore, PD 153035 and CL-387,785 had approximately the same activity when compared in the same enzyme assay. The activity of CL-387,785 was specific, since it did not inhibit receptor tyrosine kinase activity of VEGF-R or the cyto-

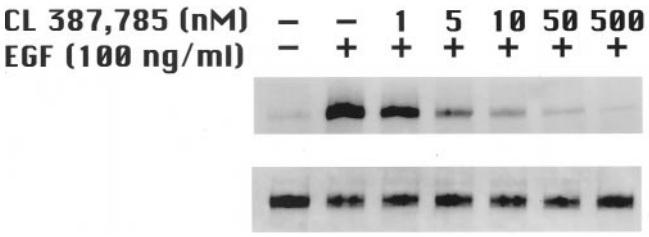

FIG. 2. Inhibition of tyrosine phosphorylation of EGF-R by CL-387,785. A431 cells were preincubated with or without CL-387,785 for 3 hr prior to the addition of 100 ng/mL of EGF. After 20 min, the cells were lysed, and protein was resolved on gels and transferred to PVDF membrane. The phosphorylation status of tyrosine-containing proteins (upper panel) and the protein expression of EGF-R (lower panel) were determined by immunoblot analysis of the samples with anti-phosphotyrosine and anti-EGF-R antibodies, respectively.

plasmic tyrosine kinase activity of src, fyn, lyn, met, and lck when tested up to 10 μM.

The ability of CL-387,785 to inhibit phosphorylation of EGF-R was assessed in A431 cells. To do this, cells were pretreated with CL-387,785 and then phosphorylation of EGF-R was stimulated with EGF. This effect was compared with the same procedure where no pretreatment with CL-387,785 was done. Based on immunoblot analysis of cell lysates, it was found that EGF markedly stimulated tyrosine phosphorylation of a 170-kDa species. This effect was inhibited by CL-387,785 with an $IC_{50} \approx 5$ nM (Fig. 2, upper panel). EGF-R comigrated with the 170-kDa phosphotyrosine-containing species (Fig. 2, lower panel). The drug did not alter EGF-R expression (Fig. 2, lower panel). Similar results were obtained if the analysis was repeated with immunoprecipitated EGF-R (data not shown). The effects of CL-387,785 were also examined on c-erbB-2. Since the ligand for c-erbB-2 is unknown [23], the effect of CL-387,785 on constitutive phosphorylation of c-erbB-2 in SK-BR-3 cells (grown in medium that contained 10% serum) was examined. One hundred nanomolar CL-387,785 also inhibited the constitutive phosphorylation of c-erbB-2 when the drug was incubated with SK-BR-3 cells (data not shown).

The ability of CL-387,785 to bind covalently to EGF-R was examined. To do this, 2.5 μM [^{14}C]CL-387,785 (approximately 40,000 dpm) was incubated with membranes prepared from A431 cells. The reaction was terminated by boiling the sample in a solution containing 2% SDS and 5% β-mercaptoethanol. Proteins were resolved in SDS-containing gels, and gel slices were counted. A radiolabeled 170-kDa species was detected, and such labeling was competed by unlabeled CL-387,785 (Fig. 3). No other radiolabeled species was reproducibly detected in this analysis, despite the presence of numerous other proteins in the membrane preparation. The 170-kDa radiolabeled species was immunoprecipitated with an antibody against EGF-R (Fig. 3; inset). No radiolabel was found in a membrane preparation derived from a cell line that did not overexpress EGF-R. Interestingly, the amount of radiolabel incorpo-

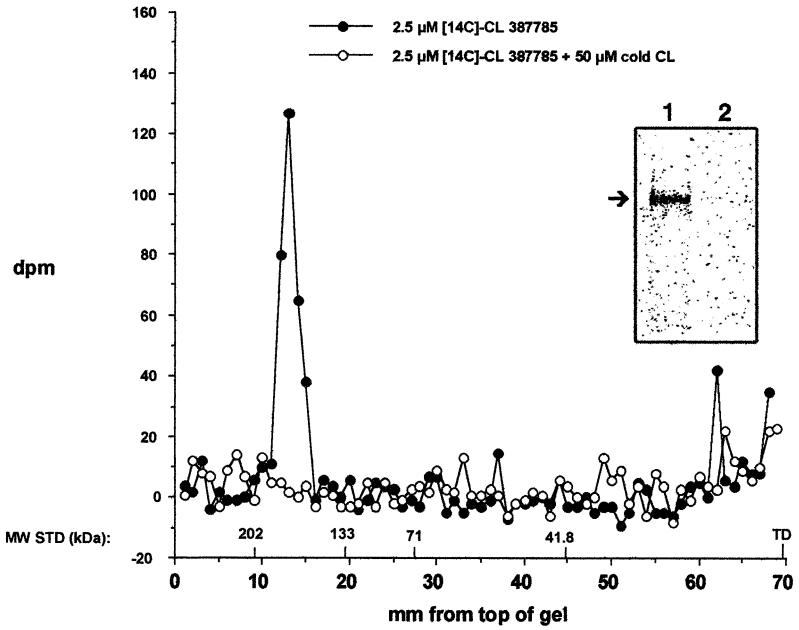

FIG. 3. Covalent labeling of EGF-R by CL-387,785. Fifty micrograms of membrane protein from A431 cells was incubated in 2.5 μM [^{14}C]CL-387,785 alone (●) or in the presence of 50 μM unlabeled (cold) CL-387,785 (○) for 1 hr at room temperature. The reaction was terminated by the addition of Laemmli sample buffer, the mixture was boiled for 3 min, and protein in the sample was resolved by SDS–PAGE. Gel lanes were sliced, and the radioactivity per slice was determined. Molecular weight standards (MW STD) were used to calibrate the gel (TD = tracking dye). Inset: 1000 μg of membrane protein was labeled as above in the absence (lane 1) or presence (lane 2) of unlabeled CL-387,785. After lysing the membranes, the supernatant was subjected to immunoprecipitation, resolved on gels, and radioactivity in the dried gel was determined by PhosphoImage analysis (7-day exposure). The arrow indicates radiolabeled 170-kDa species.

TABLE 1. Effect of CL 387,785 on cell proliferation

Cell line	IC_{50}(nM)*	EGF-R expression†	c-erbB-2 expression†
A431	67 ± 7.6	++++	+
SK-BR-3	26 ± 1.7	++	++++
MDA-MB-435	600 ± 37	+	+
MCF-7	232 ± 15	+	+
SW-620	731 ± 45	+	+
3T3	400 ± 32	+	+
3T3/c-*erb*B-2	31 ± 9	+	++++

*Cell growth in 96-well dishes over a 72-hr period was determined by the sulforhodamine B assay. CL-387,785 was added 24 hr after plating cells. Values are means ± SD; N = 19 independent determinations.

†EGF-R and c-erbB-2 levels were determined by immunological methods using flow cytometry and confirmed by immunoblot assay as described in Materials and Methods. Range was + to ++++.

rated into the 170-kDa species was low, approximately 74–400 dpm, even though 148–32,100 dpm (equivalent to 20–5000 nM, respectively) were present in the reaction. Upon further investigation, it was found that the binding of [^{14}C]CL-387,785 to EGF-R was linear up to 100 nM and then saturated with higher amounts of CL-387,785. This indicated that the reaction was efficient (up to 50% bound) and was highly specific because only one species was labeled when the radiolabeled molecule was present at approximately a 50-fold excess.

The ability of CL-387,785 to inhibit the growth of tumor cell lines that overexpressed EGF-R, c-erbB-2, or neither protein was assessed (Table 1). Consistent with previous reports [24], we determined that A431 cells markedly overexpressed EGF-R, SK-BR-3 cells markedly overexpressed c-erbB-2, while SW-620, MDA-MB-435, and MCF-7 cells had much lower levels of expression of both proteins. The ability of CL-387,785 to inhibit the growth of a mouse fibroblast cell line transfected with c-*erb*B-2, designated 3T3/c-*erb*B-2, was also compared with the nontransfected parental line [25]. It was found that the concentration of CL-387,785 needed to inhibit cell proliferation was 4- to 12-fold lower in cells that overexpressed EGF- or c-erbB-2 compared with cells that had low-level expression of both proteins. This was particularly obvious in the isogenic cell pair (3T3 vs 3T3/c-*erb*B-2).

Before determining if CL-387,785 would have activity in tumor-bearing animals, it was important to distinguish whether CL-387,785 was cytostatic or cytotoxic and whether it would inhibit growth transiently or irreversibly, respectively. These results would help guide dose schedules in animals. We suspected that even though the agent bound irreversibly to EGF-R, the drug would be cytostatic, since inhibition of this signal transduction pathway might not be lethal, and new, functionally active EGF-R would be made when cells were grown in the absence of the drug. Therefore, we determined the time course of growth inhibition, the ability of the cell to recover after removal from CL-387,785, and the cell cycle status when inhibition of proliferation occurred. Comparisons were made with paclitaxel, a cytotoxic agent.

When A431 cells were grown continuously for 7 days in drug, the IC_{50} of CL-387,785 was 133 nM. However, if cells were pulsed for 1 day in drug and then grown for 6 days in drug-free medium, the IC_{50} of CL-387,785 was 1067 nM. In contrast, the IC_{50} of paclitaxel (1.5 nM) did not change when the cells were grown under both conditions. Furthermore, A431 cell number increased within days after they were removed from CL-387,785. Similar results were obtained with SK-BR-3 cells. These findings suggested that the action of CL-387,785 was slow, reversible, and indicative of cell cycle arrest.

Consistent with results from the cell proliferation experiments, little evidence for cell death was found when cell cycle analysis was done. In particular, it was found that after exposure of A431 or SK-BR-3 cells to 300-1000 nM CL-387,785 for 3 days, an increase in the number of cells in the G_0/G_1 state was seen with a corresponding decrease in the number of cells in the S and G_2M phases of the cell cycle (Table 2). Effects were more dramatic in SK-BR-3 cells and correlated with the higher potency of CL-387,785 on these cells versus A431 cells. A small number of SK-BR-3 cells displayed reduced fluorescence with respect to the G_0/G_1 cell cycle region. Other studies have shown that this region of the analysis contains apoptotic cells [26]. No changes in cell cycle analysis were seen in cell lines that did not overexpress EGF-R (data not shown). In contrast, when A431 or SK-BR-3 cells were exposed to 3–10 nM paclitaxel for 3 days (a concentration and time course where cell growth was inhibited), a large increase in the apoptotic population was observed.

The effects of CL-387,785 on tumor growth were assessed in human tumor xenografts implanted s.c. in the flanks of nude mice (Fig. 4). CL-387,785 inhibited the growth of tumors derived from A431 cells when the drug was administered i.p. or p.o. (Fig. 4A). Inhibitory effects were observed within 7 days after the onset of therapy. In general, efficacy was achieved only after multiple doses (data not

TABLE 2. Effect of CL-387,785 or paclitaxel on the cell cycle in A431 or SK-BR-3 cells*

	A431 cells				SK-BR-3 cells			
	% of Cells in cell stages							
Drug (nM)	A†	G_o/G_1	S	G_2/M	A	G_o/G_1	S	G_2/M
0	1	56	28	15	4	63	18	15
CL-387,785								
30	2	65	23	10	3	63	19	15
300	3	73	16	8	11	82	4	3
1000	7	66	17	11	10	84	4	3
Paclitaxel								
1	13	61	19	8	5	60	18	16
3	38	25	30	7	51	18	22	10
10	52	12	24	13	49	8	24	18

*Cells were treated with no drug or CL-387,785 for 3 days, and cell cycle status was determined by staining with propidium iodide and flow cytometry as described in Materials and Methods.

†A = apoptosis.

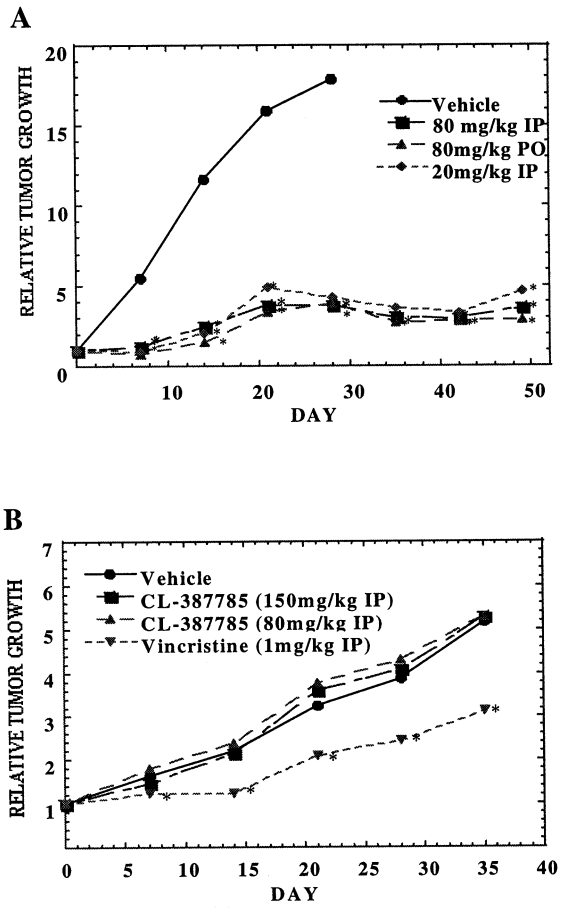

FIG. 4. ***In vivo*** **activity of CL-387,785 on tumors derived from A431 cells in nude mice. Female** ***nu/nu*** **mice were injected s.c. with 5 × 10^6 A431 cells (panel A) or 6 × 10^6 MDA-MB-435 cells (panel B). Mice bearing staged tumors (100–150 mg) from A431 cells were treated with 80 mg/kg of CL-387,785 i.p. on days 1, 5, 9, 21, 25, and 29 or with 20 mg/kg i.p. or 80 mg/kg p.o. on days 1 through 10 and days 21 through 30. For mice bearing staged tumor from MDA-MB-435 cells, animals were treated on days 1, 5, and 9 with CL-387,785 or vincristine given i.p. Relative tumor growth for each animal was determined on post-staging at the time points indicated. Data are the mean fold increase in tumor volume for the five mice in each group. Significant tumor inhibition (*P* < 0.05 by Student's *t*-test) was observed in all drug-treated A-431 tumors, whereas MDA-MB-435 tumors were inhibited significantly only by vincristine.**

shown). While tumors regrew shortly after one course of therapy (i.e. after 10 daily treatments), they responded to a second course of therapy. No efficacy was observed with CL-387,785 in tumors derived from cell lines that did not overexpress EGF-R or c-erbB-2, including the human breast carcinoma MDA-MB-435 (Fig. 4B).

DISCUSSION

These data show that CL-387,785 is a specific inhibitor of EGF-R. The molecule bound covalently to EGF-R. Such binding inhibited autophosphorylation of EGF-R and blocked proliferation of tumor cells both *in vitro* and *in vivo*. It is likely that CL-387,785 inhibits c-*erb*B-2, since the growth of cells that overexpress the receptor was inhibited and the drug inhibited autophosphorylation of c-erbB-2 in cells. Further studies are underway to determine whether CL-387,785 directly binds to c-erbB-2 or inhibition of the receptor is accomplished by heterodimerization with EGF-R.

The data demonstrate the utility of homology models for the discovery of small molecules that bind to receptor tyrosine kinase domains. The model makes use of the fact that eukaryotic protein kinase domains share homologous catalytic domains. To date, approximately sixty receptor tyrosine kinases have been described from vertebrates [27]. Many of these receptors play important roles in disease processes [28], and irreversible, covalent inhibitors of such kinases may be a useful therapeutic approach for treatment of these diseases. Our homology model of EGF-R predicts that CL-387,785 alkylates Cys^{773}, which is located in the ATP-binding region of the protein. This cysteine residue in the kinase domain, along with the Lys^{704} (located close enough to this sulfhydryl group to serve as a basic catalyst for the Michael addition reaction) is unique to EGF-R and c-erbB-2 and would explain, in part, why CL-387,785 selectively interacts with these two tyrosine kinases. While additional work is needed to prove this prediction, preliminary data indicate that [^{14}C]CL-387,785 specifically binds to the recombinant cytoplasmic portion of EGF-R. Since radiolabeled [^{14}C]CL-387,785 has low specific activity and binding to EGF-R is saturated at low concentrations, it has been difficult to immunoprecipitate EGF-R bound to [^{14}C]CL-387,785 and to perform radioactive peptide mapping. However, the high efficiency of binding indicates that direct mapping, using non-radiolabeled material and mass spectrometry methods, can be used to identify the amino acid residue(s) that binds to the drug. During the preparation of this manuscript, other investigators reported that a closely related analog of CL-387,785 specifically alkylates Cys^{773} of EGF-R with a 1:1 stoichiometry [29]. Consistent with this conclusion, assuming that our membrane preparation contained 0.1 to 1.0% EGF-R, and the labeling was saturated at 100 nM [^{14}C]CL-387,785, we estimate that 7–70% of all EGF-R molecules bound the inhibitor.

While CL-387,785 binds irreversibly to EGF-R, its effect on cell growth is reversible. This apparent paradox suggests that inhibition of EGF-R by CL-387,785 does not lead to cell death. Cell growth may resume after drug exposure is terminated because new EGF-R receptor is made. Consistent with the cytostatic nature of the drug, cell cycle arrest, without marked apoptosis, is the predominant response to CL-387,785. Similar conclusions have been made with another quinazoline-based inhibitor of EGF-R, PD 153035 [30]. However, another EGF-R inhibitor, CP-358,774, causes apoptosis in a colon carcinoma, Difi, that overexpresses EGF-R [31]. In Difi cells, apoptosis was assessed by cell cycle analysis (using flow cytometry) and DNA fragmentation. The amount of apoptosis reported with CP-358,774 is similar to that seen in SK-BR-3 cells exposed to CL-387,785. Therefore, CL-387,785 may be more cytotoxic

in certain cell lines than in others. Further assessment of CL-387,785 on synchronized cell populations and evaluation of the apoptotic populations of tumor cells in animals will help clarify this issue.

Consistent with the cytostatic and slow-acting effects of CL-387,785 *in vitro*, we found that good activity of CL-387,785 was achieved only when the compound was given repeatedly (over multiple days). Furthermore, tumors regrew after drug exposure was terminated and were inhibited again if the drug was re-administered after an initial course of therapy. If this is a general mechanism for EGF-R inhibitors and if drug is cleared rapidly in animals, irreversible inhibitors of EGF-R may be advantageous, since the drug will sustain its inhibitory effect on EGF-R even after the drug is cleared. Further work is in progress to define the pharmacokinetics of CL-387,785 in animals and to determine if CL-387,785 inhibits EGF-R phosphorylation after the drug is cleared. Beyond this, additional work is needed to determine if resistance to therapy with EGFR inhibitors will occur when chronic dosing is used.

In conclusion, CL-387,785 is an inhibitor of EGF-R tyrosine kinase with activity *in vivo*. The drug inhibits proliferation of cells that overexpress EGF-R or c-erbB-2 by covalently binding to EGF-R. The molecule appears suitable for further development and clinical studies.

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