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Biochemical and Antiproliferative Properties of 4-[Ar(alk)ylamino]pyridopyrimidines, a New Chemical Class of Potent and Specific Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor

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ABSTRACT. The tyrosine kinase inhibitors PD 69896, 153717, and 158780, which belong to the chemical class 4-[ar(alk)ylamino]pyridopyrimidines, have been characterized with respect to enzymology, target specificity, and antiproliferative effects in tumor cells. These compounds were competitive inhibitors with respect to ATP against purified epidermal growth factor (EGF) receptor tyrosine kinase and inhibited EGF receptor autophosphorylation in A431 human epidermoid carcinoma with IC_{50} values of 2085, 110, and 13 nM, respectively. Onset of inhibition was immediate once cells were exposed to these compounds, whereas recovery of receptor autophosphorylation activity after the cells were washed free of the compound was dependent on inhibitory potency. Thus, full activity returned immediately after removal of PD 69896 but required 8 hr after exposure to PD 158780. PD 158780 was highly specific for the EGF receptor in Swiss 3T3 fibroblasts, inhibiting EGF-dependent receptor autophosphorylation and thymidine incorporation at low nanomolar concentrations while requiring micromolar levels for platelet-derived growth factor- and basic fibroblast growth factor-dependent processes. PD 158780 inhibited heregulin-stimulated phosphorylation in the SK-BR-3 and MDA-MB-453 breast carcinomas with IC_{50} values of 49 and 52 nM, respectively, suggesting that the compound was active against other members of the EGF receptor family. The antiproliferative effects of this series of compounds against A431 cells correlated precisely with the inhibitory potency against EGF receptor autophosphorylation. PD 158780 reduced clone formation in soft agar of fibroblasts transformed by EGF, EGF receptor, or the *neu* oncogene but not *ras* or *raf*, further demonstrating its high degree of specificity. Finally, this compound was active against clone formation in several breast tumors having different expression patterns of the erbB family, indicating an anticancer utility in tumors expressing these receptors. BIOCHEM PHARMACOL **54**;8:877–887, 1997.

KEY WORDS. tyrosine kinase; EGF receptor; inhibitor; tumor; anticancer; pyridopyrimidine

PTKs[‖] are enzymes that phosphorylate specific tyrosine residues within the sequence of a wide variety of functional proteins and have been found to be a common mechanism for transmitting mitogenic signals and regulating numerous cellular processes [1–4]. A potential role for certain PTKs in tumorigenesis is evident by their ability to transform normal cells to a neoplastic phenotype when expressed in a mutated, unregulated form or to an abnormally high level. Indeed, half of the protooncogenes identified to date encode for proteins having PTK activity [5, 6]. This potential to transform normal cells is compatible with existing data implicating tyrosine phosphorylation and dephosphorylation as events intimately involved in growth regulation and mitogenesis [3, 7–10]. For these reasons, tyrosine kinases have been considered attractive targets for cancer therapies [11–15] as well as for other proliferative diseases including psoriasis [16, 17], atherosclerosis [18], and restenosis [19, 20].

Considerable evidence has emerged, both preclinically and clinically, over the last decade to implicate the epidermal growth factor receptor and, more recently, its family members, erbB2, erbB3 and erbB4, in the development, progression, and severity of certain human cancers. These receptors have been shown to be protooncogenes, and overexpression of one or more of these receptors occurs

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‖*Abbreviations:* bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; and PTK, protein tyrosine kinase.

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with very high frequency in human cancer, especially breast and ovarian [21–24]. Since expression levels often correlate with a poorer patient prognosis and shorter survival time, the EGF receptor family members have been viewed as potential targets for cancer chemotherapy [11–15].

One of the major strategies to disrupt signalling through these receptors has been to suppress their tyrosine kinase activity, which has been shown to be essential for mitogenic and transforming properties [25]. Over the past decade, drug discovery efforts have produced a wide variety of chemical structures, generated either by synthetic means or as fermentation products that reportedly inhibit purified or partially purified preparations of various tyrosine kinases. The results of this work have been summarized in a number of previous review articles [11, 13, 14, 26–29]. Many of these earlier kinase inhibitors suffered from a lack of potency and specificity that perhaps compromised attempts to demonstrate antitumor activity based solely on the original kinase target. Within the past 2 years, new structural classes of tyrosine kinase inhibitors that exhibit enormous improvements in potency and specificity over prior compounds have begun to emerge. Most of these newer compounds are directed against either the EGF or PDGF receptor tyrosine kinases. Inhibitors against the EGF receptor are currently dominated by three series of compounds that include 4-anilinoquinazolines [30–37], 4-[ar(alk)ylamino]pyridopyrimidines [38, 39] and 4-phenylaminopyrrolo-pyrimidines [40]. These compounds have shown remarkable potency and specificity for the EGF receptor, and reports of preclinical antitumor activity in animal tumor models have appeared recently [40, 41].

In this report, we have selected three members of the pyridopyrimidine class of EGF receptor tyrosine kinase inhibitors based on a spectrum of affinities for the enzyme. The enzymology and biochemical pharmacology of this new series of compounds have been characterized in terms of type of enzyme inhibition, specificity in cells against receptor autophosphorylation and growth factor-mediated mitogenesis, as well as antiproliferative effects against tumor cells.

MATERIALS AND METHODS

Chemicals and Reagents

PD 69896, 153717, and 158780 were synthesized as described previously [38, 39]. [^{3}H]Thymidine was from Amersham (Arlington Heights, IL). Antiphosphotyrosine antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). EGF was obtained from the Chiron Corp., Emeryville, CA. PDGF and bFGF were from the Intergen Co., Purchase, NY.

Tissue Culture

The human breast carcinomas MDA-MB-468, MDA-MB-453, MDA-MB-231, MCF-7, and SK-BR-3, the human epidermoid carcinoma A431, and the Swiss 3T3 mouse fibroblasts were obtained from the American Type Culture Collection, Rockville, MD. NIH 3T3 mouse fibroblasts transfected with the *neu* oncogene and rat1 fibroblasts transfected with the gene for EGF were obtained from David Stern, Yale University, New Haven, CT. NIH 3T3 mouse fibroblasts transfected with the human EGF receptor were obtained from Stewart Decker, Parke-Davis Pharmaceutical Research. NIH 3T3 mouse fibroblasts transfected with mutant *ras* or *raf* were produced in-house. All cell lines were maintained as monolayers in dMEM/F12, 50:50 (Gibco/BRL, Bethesda, MD) containing 10% fetal bovine serum. For growth inhibition assays, dilutions of the designated compound in 10 μL were placed in 24-well Linbro plates (1.7 × 1.6 cm, flat bottom) followed by the addition of cells (2×10^4) in 2 mL of medium. The plates were incubated for 72 hr at 37° in a humidified atmosphere containing 5% CO_2 in air. Cell growth was determined by counting cells with a Coulter model AM electronic cell counter (Coulter Electronics, Inc., Hialeah, FL). For clone formation in soft agar, cells were trypsinized, and 10,000 cells/mL were seeded into DMEM/F12 medium containing 10% fetal bovine serum, 0.4% agarose, and the designated concentration of compound. One milliliter of this solution was placed over a bottom layer of the same medium containing 0.8% agarose in a 35-mm Petri dish and incubated at 37° in a humidified atmosphere containing 5% CO_2 in air. After 3 weeks, colonies were stained with *p*-iodonitrotetrazolium violet (INT) and quantitated with an image analyzer using the software NIH Image version 1.55. Incorporation of radiolabeled thymidine into cellular DNA was monitored by exposing compound-treated or control cells to [*methyl*-^{3}H]thymidine (ICN Biomedicals, Costa Mesa, CA) at a concentration of 1 μM and a specific activity of 1 μCi/nmol. After 2 hr the cells were trypsinized and injected into 2 mL of ice-cold 15% trichloroacetic acid (TCA). The resulting precipitate was collected on glass fiber filters, washed five times with 2-mL aliquots of ice-cold 15% TCA, dried, and placed in scintillation vials plus 10 mL Ready gel (Beckman, Irvine, CA). Radioactivity was determined in a Beckman LS 6800 scintillation counter.

Heregulin

A fragment of heregulin-β1 (Ser_{177}-Glu_{241}), which retains receptor binding activity to cells that express erbB-2, erbB-3, and erbB-4 and induces tyrosine phosphorylation of these receptors, was obtained by recombinant methods similar to those described previously [42]. Briefly, the cDNA for heregulin-β1 was obtained by reverse transcription–polymerase chain reaction (RT-PCR; Stratagene, La Jolla, CA) of mRNA isolated from MDA-MB-231 human breast carcinoma cells. Oligonucleotide primers were used to generate the $heregulin_{177-241}$ fragment and insert a *Nco*I restriction site on the 5′-end and a *Bam*HI site at the 3′-end. The PCR product was cloned into pCR-Script (Stratagene), and the sequence was confirmed by direct

sequencing of double-stranded templates. Heregulin cDNA was excised by *Nco*I and *Bam*HI digests and inserted into a similarly digested pET-26b (Novagen, Madison, WI). The engineered plasmid was transformed and the heregulin fragment expressed in *Escherichia coli* BL21 cells. Since a high percentage of the recombinant protein was secreted into the growth medium, bacterial cells were removed by centrifugation, and proteins in the supernatant were concentrated by ultrafiltration. The heregulin fragment, which was approximately 8 kDa, was partially purified by passing the solution through a molecular weight exclusion membrane with a 30 kDa cutoff.

Purification of EGF Receptor Tyrosine Kinase

Full-length human EGF receptor tyrosine kinase was isolated from A431 human epidermoid carcinoma cells by the method described in Gill and Weber [43]. Briefly, cells were grown in roller bottles in dMEM/F12 medium (Gibco) containing 10% fetal bovine serum. Approximately 10^9 cells were lysed in 2 vol. of buffer containing 20 mM HEPES, pH 7.4, 5 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1 mM sodium orthovanadate, 5 mM sodium fluoride, 4 mM pyrophosphate, 4 mM benzamide, 1 mM dithiothreitol, 80 μg/mL aprotinin, 40 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 25,000 *g* for 10 min, the supernatant was equilibrated for 2 hr at 4° with 10 mL of Sepharose conjugated with anti-EGF receptor antibody. Contaminating proteins were washed from the resin with 1 M NaCl followed by 1 M urea. The enzyme was eluted with 0.1 mg/mL EGF. The receptor was homogeneous as assessed by Coomassie blue stained polyacrylamide gels.

EGF Receptor Tyrosine Kinase Assay

The enzyme assay was performed in 96-well filter plates (Millipore MADVN6550). The total volume was 0.1 mL containing 20 mM HEPES, pH 7.4, 50 μM sodium vanadate, 40 mM magnesium chloride, 10 μM ATP containing 0.5 μCi of [^{32}P]ATP, 20 μg of polyglutamic acid/tyrosine (Sigma Chemical Co., St. Louis, MO), 1 ng of EGF receptor tyrosine kinase, and appropriate dilutions of inhibitor and/or ATP. All components except the ATP were added to the well, and the plate was incubated with shaking for 10 min at 25°. The reaction was started by adding [^{32}P]ATP, and the plate was incubated with shaking at 25° for 10 min. The reaction was terminated by the addition of 0.1 mL of 20% TCA, and the plate was kept at 4° for at least 15 min to allow the substrate to precipitate. The wells were then washed five times with 0.125 mL of 10% TCA, and [^{32}P] incorporation was determined with a Wallac beta plate counter. To assess the type of inhibition, kinetic data were fitted by nonlinear regression computer programs (GraFit, Erithacus Software, Ltd., London, U.K.) to equation 1 where I is the inhibitor concentration and v_0 is the initial reaction rate [44].

$$v_0 = \frac{V_{max}[ATP]}{K_m\left[1 + \frac{I}{K_{is}}\right] + [ATP]\left[1 + \frac{I}{K_{ii}}\right]} \quad (1)$$

Western Blotting Procedure

Inhibition of receptor autophosphorylation in viable cells was assessed by antiphosphotyrosine western blot. Cells were grown to 90% confluency in 6-well plates, made serum-free for 18 hr, and then treated with compound as described in the specific figure legends. Then the cells were stimulated for 5 min with 100 ng/mL of EGF or 10 ng/mL of heregulin, and extracts were made by lysing the monolayers in 0.2 mL of boiling Laemmli buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 50 mM Tris, pH 6.8). After heating to 100° for 5 min, 30 μL of the lysate was loaded and separated on a polyacrylamide gel (4–20%), and the proteins were electrophoretically transferred to nitrocellulose. The membrane was washed once in 10 mM Tris, pH 7.2, 150 mM NaCl, 0.01% azide (TNA) and blocked overnight in TNA containing 5% bovine serum albumin and 1% ovalbumin. The membrane was blotted for 2 hr with antiphosphotyrosine antibody (UBI, 1 μg/mL in blocking buffer) and then washed twice in TNA, once in TNA containing 0.05% Tween-20 and 0.05% Nonidet P-40, and twice in TNA. Then the membranes were incubated for 2 hr in blocking buffer containing 0.1 μCi/mL of [^{125}I]protein A and washed again as above. After the blots were dry, they were loaded into a film cassette and exposed to X-AR X-ray film for 1–7 days. Band intensities were determined with a Molecular Dynamics laser densitometer.

RESULTS

Biochemical Effects of PD 69896, 153717 and 158780

Figure 1 shows the chemical structures for the three pyridopyrimidines used in this study. They are part of a large series of analogs [38, 39] and were selected for the purpose of investigating how compounds with widely different affinities for the enzyme affect recovery of kinase activity in cells. Previously published data indicate that these compounds have IC_{50} values for inhibition of purified EGF receptor tyrosine kinase of 578, 9.6, and 0.008 nM, respectively [38, 39]. Figure 2 shows double-reciprocal plots from a steady-state kinetic analysis for inhibition of EGF receptor tyrosine kinase by PD 69896. When ATP was made the variable substrate, the lines converged on the Y-axis (Fig. 2A), indicating that the inhibition was simple competitive with respect to ATP. Analysis of the data by nonlinear regression (see Materials and Methods) confirmed this conclusion. In contrast, when glu-tyr was made the variable substrate, the lines converged on the X-axis (Fig. 2B), indicating that the inhibition was noncompetitive with respect to peptide polymer. PD 153717 showed a similar pattern of kinetics (data not shown). Because PD 158780 inhibits the enzyme at 10^{-11} to 10^{-12} M, it can be

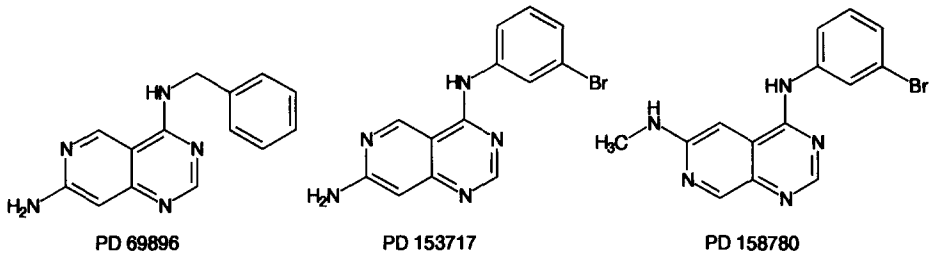

FIG. 1. Chemical structures for PD 69896, 153717, and 158780.

considered a tight binding inhibitor, and a conventional steady-state analysis was not possible; however, because it is very similar structurally to PD 153717, it too is most likely competitive with ATP. Figure 3A–C is an antiphosphotyrosine western blot showing the effects of these three compounds on EGF-stimulated receptor autophosphorylation in A431 cells. All three produced a concentration-dependent inhibition of this activity and exhibited IC_{50} values of 2085, 110, and 13 nM for PD 69896, 153717, and 158780, respectively. The onset of inhibition was extremely rapid, and time–course experiments showed complete suppression of receptor autophosphorylation activity by these compounds at the shortest possible time period between exposing the cells to inhibitor and stimulating with EGF (about 12 sec, data not shown). The time needed for recovery of enzyme activity in cells that were treated with these inhibitors and then washed free of the agent, however, appeared to be dependent on their potency. Figure

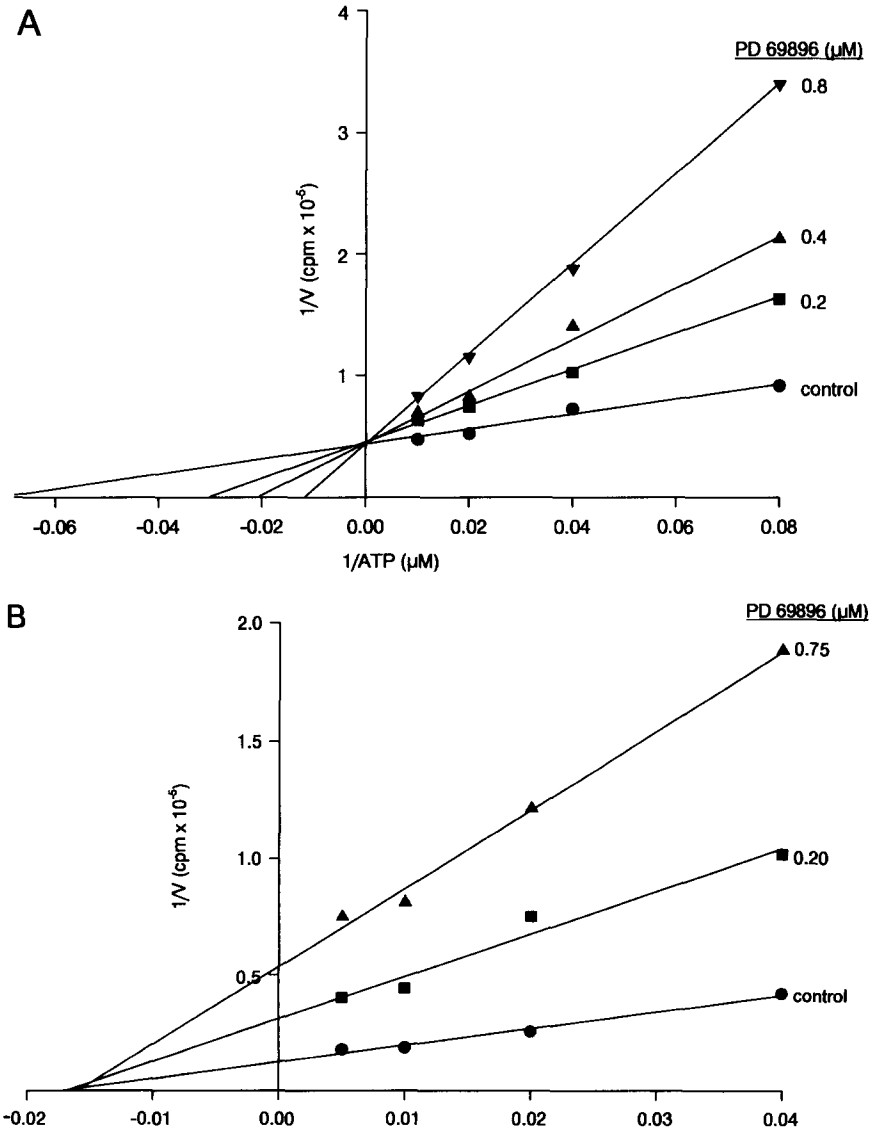

FIG. 2. Double-reciprocal plots for the inhibition of EGF receptor tyrosine kinase where the variable substrate is either ATP (A) or the peptide polymer glu-tyr (B).

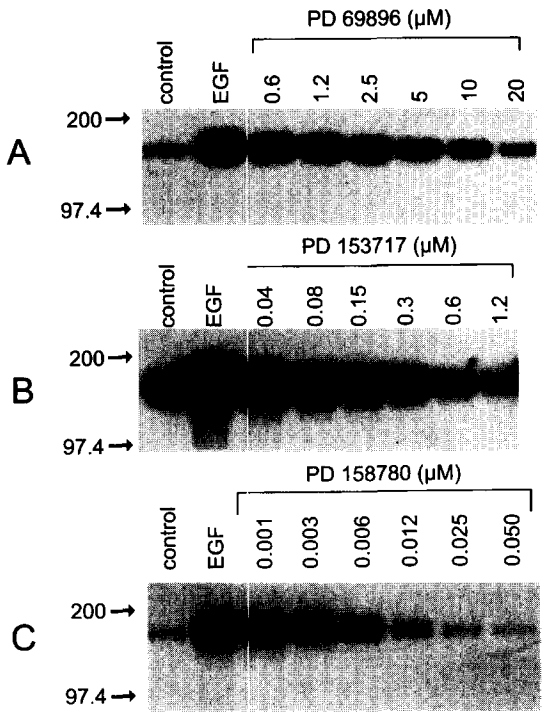

FIG. 3. Effects of PD 69896 (A), 153717 (B), and 158780 (C) on EGF-dependent tyrosine phosphorylation in A431 human carcinoma cells. Cells were treated for 2 hr with various concentrations of compound and then were stimulated with 100 ng/mL of EGF for 5 min. Extracts and antiphosphotyrosine western blots were completed as described in Materials and Methods. Numbers at the left of the gel indicate the molecular weight standards in kilodaltons.

4A–C represents an experiment in which cells were treated with either 20 μM PD 69896, 1 μM PD 153717, or 0.2 μM PD 158780 for 2 hr to completely suppress EGF receptor autophosphorylation activity. Then the cells were washed twice to remove the compounds and were stimulated at varying times after washing to assess the return of activity. Figure 4A shows that autophosphorylation activity in the cells treated with PD 69896 returned to 100% of control immediately after the inhibitor was removed. Cells treated with PD 153717 recovered about 50% of their autophosphorylation activity immediately and then regained the rest of the activity over about 2 hr (Fig. 4B). In contrast, cells treated with PD 158780 were very slow to recover, requiring approximately 8 hr for the return of full autophosphorylation activity.

The degree of specificity for PD 158780 among different tyrosine kinases in cells was assessed in Swiss 3T3 murine fibroblasts that express multiple receptors including those for EGF, PDGF, and FGF. Figure 5 is an antiphosphotyrosine western blot showing the inhibitory effects of PD 158780 on EGF, PDGF, or bFGF-stimulated tyrosine phosphorylation. Figure 5A shows that in response to EGF, phosphorylation of the EGF receptor was suppressed at low nanomolar concentrations of PD 158780, whereas phosphorylation of the PDGF receptor in response to PDGF (Fig. 5B) or the FGF receptor in response to bFGF (Fig. 5C) required micromolar concentrations to have a significant effect. The specificity against receptor tyrosine kinases by PD 158780 translated into a selective inhibition of EGF-mediated mitogenesis as shown in Fig. 6, which demonstrates the effect of this compound on EGF-, PDGF-, bFGF- and serum-dependent thymidine incorporation into DNA. PD 158780 inhibited EGF-mediated mitogenesis with an IC_{50} value of 0.1 μM, whereas concentrations from 23- to over 100-fold higher were required for inhibition of PDGF, bFGF, and serum-stimulated effects, exhibiting IC_{50} values of 2.3, 5.5, and 11.6 μM, respectively.

Inhibitory Activity of PD 158780 against Heregulin-Stimulated Tyrosine Phosphorylation

Heregulins are a family of polypeptides that are considered to be ligands for erbB4 and erbB3 as well as heterodimers between erbB2 and erbB3 or erbB2 and erbB4 [45–48]. These polypeptides activate either autophosphorylation of erbB4 or transphosphorylations of erbB2, erbB3, and erbB4 through heterodimerization [49–54]. PD 158780 was evaluated against heregulin-dependent tyrosine phosphorylation in two human breast carcinomas that overexpress erbB2: the SK-BR-3, which, in addition to erbB2 [55, 56], expresses EGF receptor [55] and erbB3 [57, 58], and the MDA-MB-453, which expresses erbB2 [55, 56], erbB3 [57,

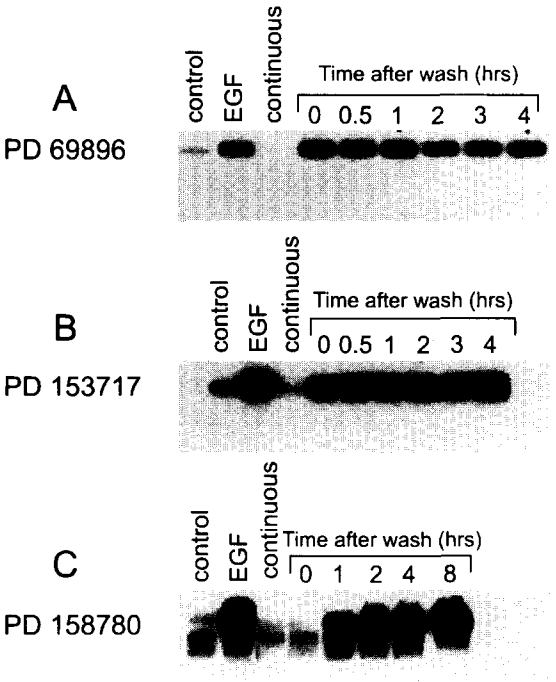

FIG. 4. Time–course for recovery of EGF receptor autophosphorylation in A431 cells after exposure to PD 69896 (A), 153717 (B), or 158780 (C). Cells were exposed to either 20 μM PD 69896, 1 μM PD 153717, or 0.2 μM PD 158780 for 2 hr to completely suppress EGF-dependent tyrosine phosphorylation (lane 3), washed twice to remove the compound, and then incubated for various lengths of time before stimulating with 100 ng/mL. Extracts and antiphosphotyrosine western blots were completed as described in Materials and Methods.

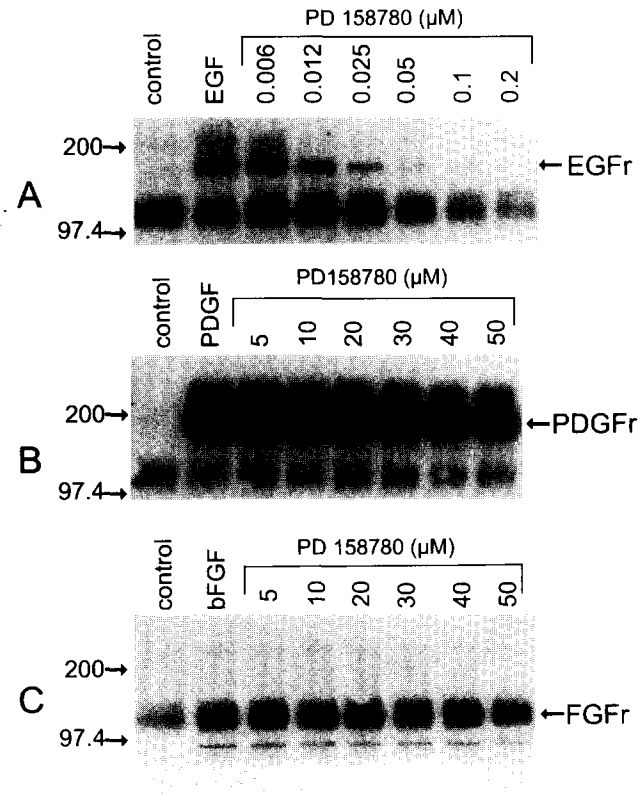

FIG. 5. Effect of PD 158780 on EGF, PDGF, or bFGF-dependent tyrosine phosphorylation in Swiss 3T3 fibroblasts. Cells were treated for 2 hr with the indicated concentrations of PD 158780 and then exposed to 20 ng/mL of EGF (A), PDGF (B), or bFGF (C) for 5 min. Extracts and antiphosphotyrosine western blots were completed as described in Materials and Methods. Numbers at the left of the gel indicate the molecular weight standards in kilodaltons.

58], and erbB4 [59] but not the EGF receptor [55]. This pattern of receptor expression was confirmed by western blot analysis in the cell lines used in this study. Cells were preincubated with various concentrations of PD 158780 for 2 hr and then exposed to 10 ng/mL of the active fragment of heregulin for 5 min. Antiphosphotyrosine western blots were performed on whole cell extracts to visualize total tyrosine phosphorylation. Panels A and B of Fig. 7 show that when exposed to the heregulin fragment, both the SK-BR-3 and the MDA-MB-453 cells exhibited prominent phosphorylation of a protein(s) in the vicinity of 185 kDa. This phosphorylation was inhibited in a concentration-dependent manner by PD 158780, which exhibited IC_{50} values of 49 and 52 nM, respectively, and indicates that this compound has excellent inhibitory activity against those receptor kinases activated by heregulin with potency comparable to that shown against the EGF receptor.

In Vitro *Antiproliferative Activity of PD* 158780

The next series of experiments were designed to determine whether the antiproliferative properties of these compounds are related to their inhibitory potency against the EGF receptor tyrosine kinase. Table 1 shows the concentration of PD 69896, 153717, or 158780 necessary to produce a 50% inhibition of either EGF receptor autophosphorylation in A431 human epidermoid carcinoma cells or proliferation in these same cells. PD 153240 is an analog from the quinazoline series of kinase inhibitors [30, 33], which had no activity against the EGF receptor tyrosine kinase and was included as an inactive control. There was a direct correlation between the concentration of compound necessary to inhibit the enzyme and that necessary to prevent proliferation of A431 cells.

Clone formation in soft agar is considered to be an identifying characteristic of the transformed phenotype. PD 158780 was examined for its effect on clone formation of a series of fibroblasts that had been transformed by specific oncogenes or protooncogenes. Preliminary experiments (not shown) indicated that the effects of these inhibitors on clone formation were reversible and, therefore, a continuous exposure protocol was maintained. A 1 μM concentration was chosen because earlier experiments indicated that complete suppression and specificity was retained against the EGF receptor tyrosine kinase family with no effects on other receptor tyrosine kinases. Figure 8 shows a visual display of colony formation and indicates that at 1 μM, PD 158780 had significant inhibitory effects on those cells transfected with the EGF receptor or the *neu* oncogene but little effect on those fibroblasts transfected with *ras* or *raf*. Table 2 is a quantitation of these and other data including an EGF transformed fibroblast and further illustrates the specific effect that this compound has on cells transformed by members of the EGF receptor family but not other oncogenes.

PD 158780 was evaluated further for its effect on clone formation against a panel of established human breast carcinoma cell lines. Table 3 lists the percent inhibition of clone formation in the presence of 1 μM PD 158780. MDA-MB-468, SK-BR-3, and MCF-7 cells were sensitive to this treatment, showing a decrease in clone formation of 73, 68, and 93%, respectively. Although the MDA-MB-453 cells were clearly less sensitive with regard to clone formation, PD 158780 did produce a distinct morphological change. Whereas the untreated cells developed clones that appeared as amorphous aggregates of refractile globular shaped cells, treated clones contained flatter cells that were packed in a very orderly fashion with a uniform spherical symmetry. It is interesting that this compound had no effect on clone formation against the MDA-MB-231 cells. The reason for the refractory nature of these cells is unknown. They express the EGF receptor as well as erbB2, and previous studies have shown that PD 158780 inhibits autophosphorylation of the EGF receptor with a potency similar to that seen in the A431 and MDA-MB-468 cells (data not shown). These cells are known to secrete substantial amounts of heregulin into the extracellular medium, however, which may have some down-regulating effect on this receptor family. Alternately, they may simply be driven by oncogenes other than the EGF receptor family.

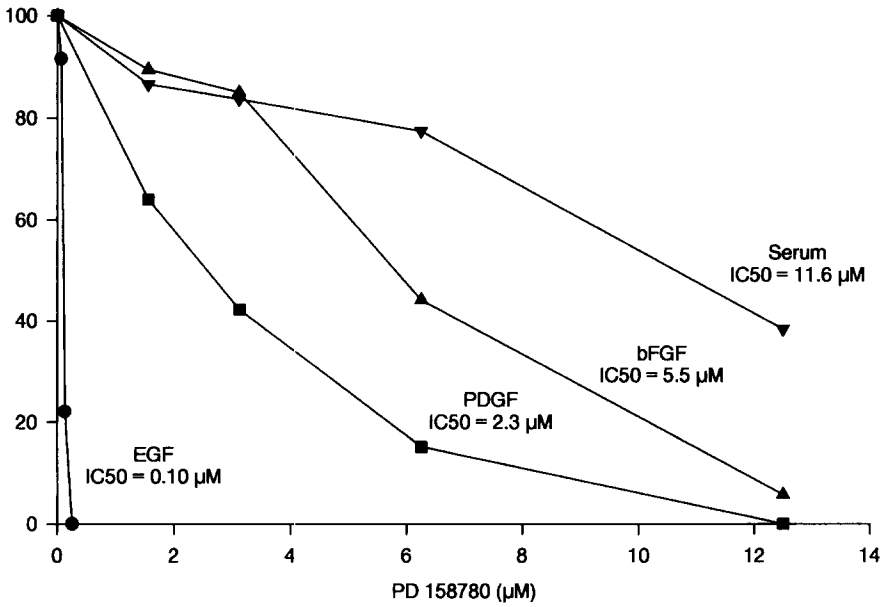

FIG. 6. Effect of PD 158780 on growth factor-dependent thymidine incorporation in Swiss 3T3 fibroblasts. Cell were treated with the indicated concentrations of PD 158780 for 2 hr and then exposed to 20 ng/mL of either EGF, PDGF, bFGF, or 10% fetal bovine serum for 24 hr. Thymidine incorporation was measured as described in Materials and Methods. Incorporation values of [^{3}H]thymidine for the controls were 400, 830, 800, and 2690 cpm for EGF, PDGF, bFGF, and serum, respectively.

Finally, the potency of PD 158780 for the inhibition of clone formation was assessed in the sensitive MDA-MB-468 cells. Figure 9 shows the effects on clone formation of exposing these cells to various concentrations of the compound, which resulted in an IC_{50} value of 0.7 μM.

DISCUSSION

Inhibition of the EGF receptor tyrosine kinase has been viewed as a valid approach to cancer chemotherapy for over a decade. During this period, an enormous number of synthetic compounds and fermentation products have been reported to have at least some inhibitory activity against this kinase [11, 13, 14, 26–29]. Initially, most compounds suffered from a lack of potency and specificity that compromised attempts to demonstrate definitive biochemical effects *in vitro* and antitumor activity *in vivo*. Within the last 3 years, a series of anilinoquinazolines [30–37] and 4-[ar(alk)ylamino]pyridopyrimidines [38, 39] have been disclosed that circumvent these limitations. In this study, several of the pyridopyrimidines have been explored in terms of detailed biochemical pharmacology and effects on cellular proliferation and clonogenicity in soft agar. Some of the salient features that are brought out in this analysis, which distinguish this structural class of inhibitor from many of the previously reported inhibitors, are picomolar potency against the purified enzyme, competitive inhibition with respect to ATP, and extremely rapid kinetics in terms of inhibitory effects on the EGF receptor autophosphorylation in treated cells. Although these inhibitors appear to be reversible, the time needed for recovery of receptor autophosphorylation activity in treated cells is related to the potency of the compound where the most potent compound, PD 158780, exhibited a pseudo irreversible character for several hours. Previously, these compounds were shown to be highly specific for the EGF receptor tyrosine kinase with respect to other purified

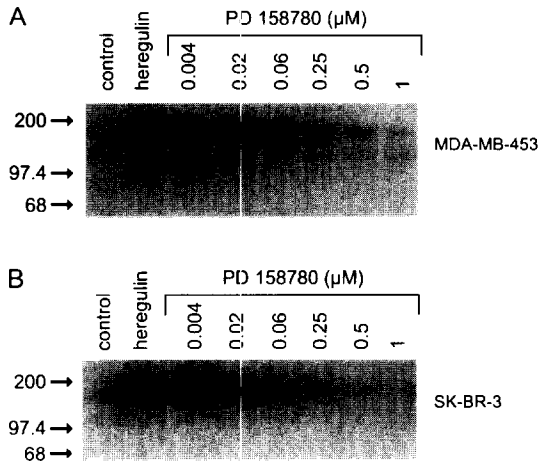

FIG. 7. Effect of PD 158780 on heregulin-stimulated tyrosine phosphorylation in MDA-MB-453 (A) or SK-BR-3 (B) human breast carcinomas. Cells were treated for 2 hr with various concentrations of compound and then were stimulated with 100 ng/mL of heregulin for 5 min. Extracts and antiphosphotyrosine western blots were completed as described in Materials and Methods. Numbers at the left of the gel indicate the molecular weight standards in kilodaltons.

TABLE 1. Correlation between the inhibition of EGF receptor autophosphorylation and proliferation in A431*

PD No.	IC_{50} (nM)†	
	Autophosphorylation	Proliferation
153240	100,000	40,000
69896	2,085	6,100
153717	110	450
158780	13	207

* Experimental details are described in Materials and Methods.
† Values represent the averages of two separate experiments.

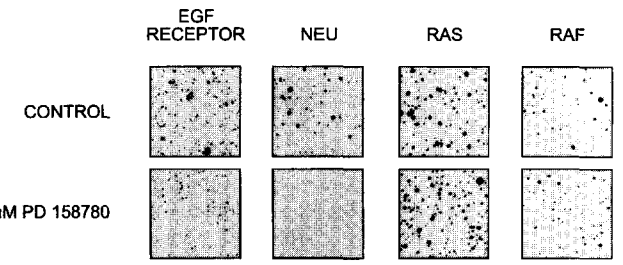

FIG. 8. Effect of 1 μM PD 158780 on clone formation in soft agar of fibroblasts transformed with different oncogenes or proto-oncogenes. Cells were exposed to the compound continuously throughout the cloning period. Experimental details are described in Materials and Methods.

enzymes [38, 39]. The highly selective nature of these compounds has been further demonstrated in this study in which PD 158780 inhibited EGF receptor autophosphorylation in Swiss 3T3 fibroblasts at low nanomolar concentrations but required micromolar levels to inhibit PDGF- or bFGF-mediated tyrosine phosphorylation. Furthermore, the specific activity toward EGF-dependent events was also evident against growth factor-mediated mitogenesis where thymidine incorporation was exquisitely sensitive to this compound when stimulated by EGF but not PDGF, bFGF, or serum.

The results in this study indicate that a significantly higher concentration of PD 158780 is required to inhibit EGF-dependent receptor autophosphorylation in whole viable cells than the purified enzyme [39] and still greater concentrations to inhibit EGF-dependent mitogenesis. The reason for these differences is probably multifold and perhaps is a function of the type of assay and the physical environment encountered by the compound. Possibilities include differences in enzyme concentration between the isolated enzyme assay and whole cell studies, unequal distribution of the compound across the plasma membrane, binding to intracellular proteins or the cell surface, or differences in ATP concentration between cell-free and cellular experiments. The differences in IC_{50} values between the cell autophosphorylation assay and the mitogenic assay might be an indication that 50% suppression of receptor autophosphorylation activity may not be enough to inhibit mitogenesis since it is not known how many functional receptors are necessary to elicit a mitogenic response. It

TABLE 2. Effect of PD 158780 on clone formation of oncogene-transformed fibroblasts in soft agar*

Cell line	Transforming gene	% Reduction in clones at 1 μM†
FR-3T3	EGF	78
NIH/EGFR	EGF receptor	43
B104.1.1	*neu*	96
NIH/RAF	*raf*	0
ME-12	*ras*	0

* Experimental details are described in Materials and Methods.
† Values represent the averages of two separate experiments.

TABLE 3. Effect of PD 158780 on clone formation of different breast tumors*

Tumor	% Reduction in clones at 1 μM†
MDA-MB-468	73
MDA-MB-231	0
SK-BR3	68
MCF-7	93
MDA-MB-453	13

* Experimental details are described in Materials and Methods.
† Values represent the averages of two separate experiments.

may be a very small percentage of the total receptor capacity, and, therefore, enough compound to inhibit by 90% or more might be required to produce effects on thymidine incorporation.

The inhibitory properties of PD 158780 do appear to broaden out at least within the EGF receptor family. Heregulin-stimulated tyrosine phosphorylation in either the SK-BR-3 or MDA-MB-453 cells was inhibited with IC_{50} values of 49 and 52 nM, respectively. Heregulin has been reported to be a specific ligand for erbB4 [49]; however, it also binds and activates heterodimers between erbB2 and erbB4 and erbB2 and erbB3 [46, 48, 50, 54]. The SK-BR-3 breast carcinoma expresses the EGF receptor [55], erbB2 [55, 56], and erbB3 [57, 58]. We have shown that the heregulin preparation used in this study has no activity for stimulating EGF receptor autophosphorylation (data not shown), and since erbB3 has negligible kinase activity [60], the inhibitory activity in these cells is most likely directed against erbB2. Since the MDA-MB-453 cells do not express the EGF receptor [55] but do express erbB2 [55, 56], erbB3 [57, 58], and erbB4 [59], the inhibition could be against erbB2 or erbB4 or both. Nevertheless, the data strongly imply that PD 158780 has potent inhibitory activity against other members of the EGF receptor family, which is

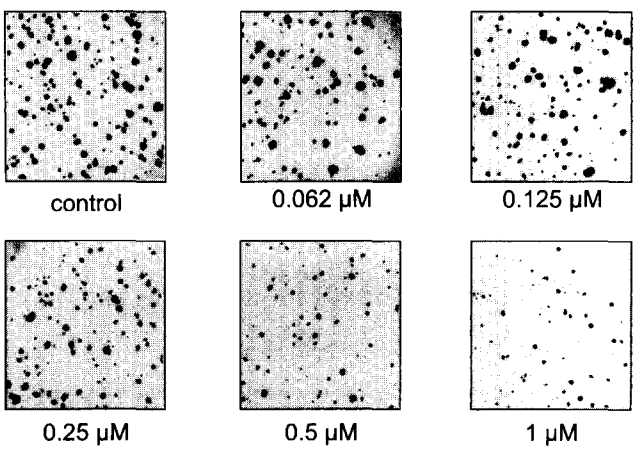

FIG. 9. Effect of PD 158780 on clone formation in soft agar of MDA-MB-468 human carcinoma cells. Cells were exposed to the indicated concentrations of compound continuously throughout the cloning period. Experimental details are described in Materials and Methods.

compatible with the extremely high homology within the kinase domains of these receptors.

The basis for assuming that suppression of the EGF receptor family is a valid approach to cancer chemotherapy stems from a substantial accumulation of data demonstrating the oncogenic capacity of these receptors when overexpressed to levels that are seen frequently in the clinic and a high correlation between these expression levels and poor prognosis in clinical cancers, especially breast and ovarian [21–24]. The question arises as to whether inhibiting the tyrosine kinase activity of one or more of these receptors will result in an antitumor effect. Certainly one well-established function for the EGF receptor is its involvement and contribution to mitogenesis. The effect of PD 158780 on this process is reflected very cleanly through efficient and specific suppression of EGF-dependent thymidine incorporation. Depending on the contribution of the EGF receptor to the ability of a cell to grow on plastic *in vitro*, an antiproliferative effect can also be demonstrated as shown in this study for the A431 human epidermoid carcinoma, where growth inhibition by a series of compounds was directly related to their inhibitory potency against the EGF receptor tyrosine kinase.

The precise role of the EGF receptor family is far from clear and appears to be multifunctional in its contribution to cell growth and differentiation [23]. The major therapeutic effect resulting from eliminating the kinase activity of this receptor family may stem not simply from an inhibition of cell growth or mitogenesis but from a removal of one major component of the transformed phenotype of the tumor cells. In this study, the effects of PD 158780 on clone formation in soft agar illustrate two major points. The first is a further demonstration of the selectivity of this compound. Among the fibroblasts transformed by specific oncogenes or proto-oncogenes, only those transfected with genes related to the EGF receptor family, including EGF, the EGF receptor, or the *neu* oncogene, were sensitive while those cells transformed by *raf* kinase or mutant *ras* were insensitive. The second point to be made is the fact that selectively reducing the kinase activities of the EGF receptor family does indeed impair clonal growth in certain tumors. The IC_{50} for inhibition of clone formation in the MDA-MB-468 human breast carcinoma was 0.7 μM, which, based on previous biochemical data, is well below the threshold where effects other than inhibition of its target kinases would occur. Similar inhibition was seen in MCF-7 and SK-BR-3 human breast carcinomas, indicating that PD 158780 has activity against cells having widely different spectrums of expression for the four erbB receptor members.

Clearly, the next step in the development of these compounds is a firm demonstration of *in vivo* antitumor activity. A few reports have already indicated that inhibitors of the EGF receptor tyrosine kinase [40, 41], as well as antibodies against erbB2 or EGF receptor [61, 62], cause growth delay in animal tumor models. Preliminary data indicate that the pyridopyrimidines do indeed have activity against certain erbB receptor-expressing tumors grown as xenografts in mice, and a detailed study to be published subsequently is underway.

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