Inhibition of Growth Factor-Mediated Tyrosine Phosphorylation in Vascular Smooth Muscle by PD 089828, a New Synthetic Protein Tyrosine Kinase Inhibitor

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ABSTRACT

PD 089828, a novel protein tyrosine kinase inhibitor of a new structural class, the 6-aryl-pyrido-[2,3-d]pyrimidines, was identified by screening a compound library with assays that measured protein tyrosine kinase activity. PD 089828 was found to inhibit human full-length fibroblast growth factor (FGF) receptor-1 (FGFR-1), platelet-derived growth factor (PDGF) receptor β subunit (PDGFR-β), Src nonreceptor tyrosine kinase (c-Src) and epidermal growth factor (EGF) receptor (EGFR) tyrosine kinases with half-maximal inhibitory potencies (IC₅₀ values) of 0.15 ± 0.02 (n = 4), 0.18 ± 0.04 (n = 3), 1.76 ± 0.28 (n = 4) and 5.47 ± 0.78 (n = 6) μM, respectively. PD 089828 was further characterized as an ATP competitive inhibitor of the growth factor receptor tyrosine kinases (FGFR-1, PDGFR-β and EGFR) but a noncompetitive inhibitor of c-Src tyrosine kinase with respect to ATP. In addition, PD 089828 inhibited PDGF- and EGF-stimulated receptor autophosphorylation in vascular SMC (VSMC) and basic FGF-mediated tyrosine phosphorylation in A121 cells with IC₅₀ values similar to the potencies observed for inhibition of receptor tyrosine kinase activity. The inhibition of PDGF receptor autophosphorylation in VSMC by PD 089828 occurred rapidly, with maximal effects reached within 5 min of drug exposure. Inhibition after single exposure was long lasting but also rapidly reversible, occurring within 5 min after drug removal. The PDGF-induced association of downstream signaling proteins, including phosphoinositide-3-kinase (PI-3K), growth factor receptor binding protein-2 (GRB2), SH-2 domain and collagen like (Shc) and phospholipase Cγ (PLCγ), with VSMC PDGFR receptors was also blocked as a result of the inhibition of PDGF-stimulated receptor autophosphorylation by PD 089828. PD 089828 also inhibited the PDGF-induced tyrosine phosphorylation of the 44- and 42-kDa mitogen-activated protein kinase isoforms. Moreover, the effects of PD 089828 were demonstrated in functional assays in which PDGF-stimulated DNA synthesis, PDGF-directed migration and serum-stimulated growth of VSMC were all inhibited to the same extent as PDGF receptor autophosphorylation (IC₅₀ = 0.8, 4.5 and 1.8 μM, respectively). These results highlight the biological characteristics of PD 089828 as a novel, broadly active protein tyrosine kinase inhibitor with long-lasting but reversible cellular effects. The potential therapeutic use of these broadly acting, nonselective inhibitors as antiproliferative and antimigratory agents could extend to such diseases as cancer, atherosclerosis and restenosis in which redundancies in growth-signaling pathways are known to exist.

Protein tyrosine kinases comprise a group of enzymes that catalyze the phosphorylation of certain proteins on specific tyrosine residues. The growth factor receptor tyrosine kinases are a subfamily whose kinases are activated on high affinity binding of growth factors to their cognate receptors. Members of this subfamily include PDGFR α and β isoforms (Claesson-Welsh, 1994), FGFR [FGFR-1 (flg), FGFR-2 (bek), FGFR-3 and FGFR-4] (Friesel and Maciag, 1995) and EGFR [EGFR, p185erbB2, erbB3 and erbB4] (Hynes and Tern, 1994). The initial activation of the kinase results in autophosphorylation, followed by subsequent tyrosine phosphorylation of various protein substrates: PLCγ, PI-3-kinase, GTPase-activating protein, GRB2, Shc, p21 ras, MAPKs (Cadena and Gill, 1992; Jaye et al., 1992) and c-Src (Alonso et al., 1995). c-Src is itself a nonreceptor membrane-associated tyrosine kinase that binds via its Src homology-2 (SH2) domain and becomes phosphorylated by the PDGF (Alonso et al., 1995; Kypka et al., 1990). The recruitment of c-Src to the PDGFR is thought

ABBREVIATIONS: FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGFR-1, fibroblast growth factor receptor-1; PDGFR, platelet-derived growth factor receptor β subunit; EGFR, epidermal growth factor receptor; c-Src, Src nonreceptor tyrosine kinase; PI-3-kinase, phosphoinositide-3-kinase; GRB2, growth factor receptor binding protein-2; Shc, SH-2 domain and collagen like; PLCγ, phospholipase Cγ; MAPK, mitogen-activated protein kinase; SMC, smooth muscle cell(s); VSMC, vascular smooth muscle cell(s); aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; MEK, MAP kinase/extracellular signal-regulated protein kinase; EGTA, ethylene glycol bis(α-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
to be important for the mitogenic effects of PDGF. Thus, the process of linking extracellular signals present at the cell membrane, such as growth factor receptor binding and activation of phosphorylation cascades, with changes in gene expression at the nucleus has been found to be a common mechanism for transducing cellular signaling events such as mitogenesis, differentiation, migration and cell survival (Ulrich and Schlessinger, 1990).

Evidence has accumulated that overexpression of receptor protein tyrosine kinases or autocrine production of mitogenic growth factors, leading to constitutive mitogenic signaling, is implicated in a growing number of proliferative diseases, including tumors of epithelial and mesenchymal origin (Antoniades et al., 1992; Perez et al., 1987; Sitarska et al., 1988), psoriasis (Elder et al., 1989), atherosclerosis (Hajjar and Pomerantz, 1992; Ross, 1989) and restenosis (Libby et al., 1992; Schwartz et al., 1992).

The proliferation of VSMC of the arterial wall has been associated with the formation and progression of lesions of atherosclerosis and in restenosis after angioplasty (Clowes and Reidy, 1991; Jackson and Schwartz, 1992). In vivo studies of balloon catheter injury to arteries demonstrate intimal SMC hyperplasia caused by both migration of cells from the media and increased proliferation (Schwartz et al., 1995), and these processes are thought to be mediated by PDGF and FGF. PDGF is both a mitogen and chemotactant for VSMC (Ferns et al., 1991; Jackson et al., 1993; Ross, 1990) and may be synthesized and released by platelets, SMC and endothelial cells (Vlodavsky et al., 1987). In animal models, vascular injury induces expression of mRNA for PDGF-A chain and the PDGRF α and β isoforms (Lindner et al., 1995; Majesky et al., 1990). Moreover, neointima formation induced by injury can be inhibited by a neutralizing antibody against PDGF (Ferns et al., 1991). VSMC can also synthesize and respond mitogenically to aFGF and bFGF via autocrine/paracrine mechanisms evoked after arterial wall injury. Thus, local FGF production could be involved in the VSMC replication that occurs after injury to the vessel wall (Gospodarowicz et al., 1988; Weich et al., 1990). Direct evidence in support of this possibility has been reported by Lindner and Reidi (1991), who demonstrated that systemic injection of a neutralizing antibody against bFGF before balloon injury of the rat carotid artery inhibited injury-induced VSMC proliferation. In addition, balloon injury has been shown to increase expression of mRNA for both bFGF and FGFR-1 by VSMC (Lindner and Reidi, 1993).

It has been suggested that restenosis may, in part, be the result of a cascade of early events involving mechanical strain and acute thrombosis that may trigger the early expression of cytokines and growth factors by VSMC and macrophages (Libby et al., 1992). These mediators could stimulate multiple cell types via paracrine and autocrine mechanisms to regulate their own expression. Redundant signaling pathways could be invoked, thereby circumventing any therapeutic agent aimed at blocking a specific growth factor or cytokine. Broadly acting, nonselective small-molecule inhibitors of protein tyrosine kinases may be required to overcome these redundancies in growth signaling to prevent the accelerated proliferation and migration of cells, which are thought to contribute to the formation of a restenotic lesion.

The importance of protein tyrosine kinases in signal transduction and the association of aberrant protein tyrosine kinase receptor and ligand expression with proliferative disorders makes agents that modulate the activity of protein tyrosine kinases attractive therapeutic targets. Over the past several years, a number of different low-molecular-weight inhibitors of protein tyrosine kinases have been synthesized. Examples of first-generation compounds include the flavonoids, typified by Quercetin (Ogawa et al., 1988), typhostins (Bilder et al., 1991; Gazit et al., 1989; Lyall et al., 1989) and Lavendustin, erstatin and genistein (Burke, 1992; Fry et al., 1994a), agents that have mostly been directed against members of the EGFR or PDGRF tyrosine kinases. Recent reports have highlighted more potent and selective inhibitors of PDGFR tyrosine kinases including the substituted quinolines (Dolle et al., 1994), biarylhydrazones (Sawutz et al., 1996) and phenylamino-pyrimidine analogs (Buchdunger et al., 1995; Zimmermann et al., 1996).

In the present study, we report on PD 089828, a novel pyrido[2,3-d]pyrimidine protein tyrosine kinase inhibitor that is distinguished from previously reported protein tyrosine kinase inhibitors by possessing a novel pyrido[2,3-d]pyrimidine cyclic structure; is ATP competitive for PDGFR, EGFR and FGFR tyrosine kinases; is uniquely noncompetitive for c-Src tyrosine kinase and demonstrates prolonged inhibition of a variety of growth factor-mediated cellular functions whose effects are reversible.

Methods

Chemicals and reagents. Human recombinant PDGF-BB, EGFR and bFGF growth factors, anti-phosphotyrosine monoclonal (clone 4G10), anti-human PDGR-β polyclonal and anti-human EGFR polyclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal antibodies raised to the human FGFR-1 (fg2) were a kind gift from Dr. Wendy Fantl (Chiron, Inc., San Francisco, CA). Radionuclide [125I]protein A was purchased from ICN Biomedicals (Irvine, CA). DMEM, RPMI and PBS were obtained from Life Technologies (Grand Island, NY), and FBS was purchased from HyClone (Provo, UT). The A12(i) human ovarian carcinoma cell line was isolated and generously provided by Dr. Kent Crickard (Department of Gynecology and Obstetrics, State University of New York at Buffalo, Buffalo, NY).

Recombinant kinases. Baculovirus containing sequence for the full-length human PDGRF-β was obtained from Dr. William LaRochelle (National Institutes of Health, Bethesda, MD). Production of PDGFR-β protein in infected Spodoptera frugiperda (Sf9) insect cells was performed as previously described (Jensen et al., 1992). cDNA coding for the full-length human FGFR-1 active tyrosine kinase (three IgG loop form) was kindly provided by Dr. Tom Maciag (American Red Cross, Rockville, MD) and was cloned into the baculovirus transfer vector pBacPAK5 (Clontech, Palo Alto, CA). Recombinant baculovirus bearing the FGFR-1 DNA was prepared, identified and purified using Sf9 insect cells as hosts according to the BaculoGold system (PharMingen, San Diego, CA) (instructions are provided with the kit). Baculovirus-containing sequence for the full-length EGFR and c-Src kinases were prepared in a similar manner and have been previously described (Fry et al., 1994a, 1994b; Thompson et al., 1994). For all of the kinases, Sf9 cells were infected with the individual viruses to overexpress the proteins.

Tyrosine kinase assays. Assays using the full-length PDGFR-β, FGFR-1 and EGFRtyrosine kinases and full-length c-Src kinase were performed in a total volume of 100 μl containing 25 mM HEPES buffer, pH 7.4, 150 mM NaCl, 10 mM MnCl2, 0.2 mM sodium orthovanadate, 750 μM/ml concentration of a random copolymer of glutamic acid and tyrosine (4:1), various concentrations of inhibitor and 60 to 750 ng of enzyme as previously described (Fry et al., 1994a,
lysates were incubated for 2 hr with a 1:100 dilution of either an fluorescent staining with a monoclonal antibody specific for SMC identified as SMC by their “hill-and-valley” growth pattern and by Island, NY) and 1% penicillin/streptomycin (GIBCO). Cells were aorta of adult male Sprague-Dawley rats weighing 350 g and ex-
tensively evaluated with the test compounds. Appropriate DMSO controls were simulta-
tion (Cleland, 1979). A comparison of the $K_i$ (slope) $K_i$ (intercept) was then used to refine the curve fit analysis. Kinetic analyses were performed using GraFit Version 3.0 (Leatherbarrow, 1992).

Cell culture. Smooth muscle cells were isolated from the thoracic aorta of adult male Sprague-Dawley rats weighing 350 g and explanted according to the method of Ross (1971). Cells were grown in DMEM containing 10% FBS, 1% glutamine (GIBCO BRL, Grand Island, NY) and 1% penicillin/streptomycin (GIBCO). Cells were identified as SMC by their “hill-and-valley” growth pattern and by fluorescent staining with a monoclonal antibody specific for SMC $\alpha$-actin (Sigma Chemical Co., St. Louis, MO). Cells were used between passages 8 and 20 for all experiments. Test compounds were used for FGFR studies because FGFR-1 protein levels in VSMC at 37°C to stimulate autophosphorylation of the PDGFR and associa-
tion of SH2 proteins to the phosphorylated receptors. After growth factor treatment, the medium was removed, and cells were washed with cold PBS and immediately lysed with 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM $p$-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin and 10 $\mu$g/ml leupeptin). Lysates were centrifuged at 10,000 $\times$ g for 10 min. Supernatants were incubated for 2 hr with a 1:100 dilution of anti-human PDGFR-β polyclonal antibody, which cross-reacts with rat PDGFRs. After the incubation, protein A-Sepharose beads were added for 2 hr with continuous mixing followed by several 1-ml washes of the immune complexes bound to the beads. Immune complexes were solubilized in 30 $\mu$l of Laemmli’s sample buffer and electrophoresed in 8% to 16% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, separated proteins were transferred to nitrocellulose and immunoblotted with a 1:1000 dilution of anti-phosphotyrosine monoclonal antibody (UBI clone 4G10; $^\circ$5–321). After incubation of blots with $^{125}$I-protein A, protein levels were detected by PhosphorImager analysis, and protein bands were quan-
tified via densitometry. $IC_{50}$ values were generated from the densi-
tometric data.

Cell association assays. Rat aortic SMC were grown to conflu-
ency in 100-mm dishes. Growth medium was removed and replaced with serum-free medium, and cells were incubated at 37°C for an additional 24 hr. Test compounds were then added directly to fresh medium, and cells were incubated for an additional 2 hr. After 2 hr, PDGF-BB was added at a final concentration of 30 ng/ml for 5 min at 37°C to stimulate autophosphorylation of the PDGFR and associa-
tion of SH2 proteins to the phosphorylated receptors. After growth factor treatment, the medium was removed, and cells were washed with cold PBS and immediately lysed with 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM $p$-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin and 10 $\mu$g/ml leupeptin). Lysates were centrifuged at 10,000 $\times$ g for 10 min. Supernatants were incubated for 2 hr with a 1:100 dilution of anti-human PDGFR-β polyclonal antibody, which cross-reacts with rat PDGFRs. After the incubation, protein A-Sepharose beads were added for 2 hr with continuous mixing followed by several 1-ml washes of immune complexes bound to the beads. Immune complexes were solubilized in 30 $\mu$l of Laemmli’s sample buffer and electrophoresed in 8% to 16% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, separated proteins were transferred to nitrocellulose and immunoblotted with a 1:1000 dilution of anti-human PDGFR-β polyclonal antibody, which cross-reacts with rat PDGFRs. After the incubation, protein A-Sepharose beads were added for 2 hr with continuous mixing followed by several 1-ml washes of immune complexes bound to the beads. Immune complexes were solubilized in 30 $\mu$l of Laemmli’s sample buffer and electrophoresed in 8% to 16% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, separated proteins were transferred to nitrocellulose and immunoblotted with a 1:1000 dilution of anti-phosphotyrosine monoclonal antibody (UBI clone 4G10; $^\circ$5–321). After incubation of blots with $^{125}$I-protein A, protein levels were detected by PhosphorImager analysis, and protein bands were quan-
tified via densitometry. $IC_{50}$ values were generated from the densi-
tometric data.

DNA synthesis. Rat aortic SMC plated onto 24-well plates were serum-starved for 24 hr and then incubated with PDGF-BB (10 ng/ml), EGF (10 ng/ml) or bFGF (5 ng/ml) and various concentrations of PD 089828. Growth factor and inhibitor incubations continued for an additional 24 hr. During the final 4 hr, cells were supplemented with 0.25 $\mu$Ci/well (37 kBq) of $[^{3}H]$thymidine. Cells were

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1 R. L. Panek, unpublished observations.

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washed with PBS and then fixed with 5% trichloroacetic acid. Cultures were washed several times with water, the trichloroacetic acid-precipitable material was solubilized with 0.25 N NaOH and [3H] was quantified by liquid scintillation counting.

**Cell growth assays.** Rat aortic SMC were plated at 10,000 cells/well onto 24-well plates in 0.5 ml of DMEM containing 10% FBS. After 24 hr, serum-supplemented medium was removed, and cells were washed thoroughly and then maintained in serum-free medium (as described above) for 24 hr to growth arrest the cells. PD 089828 or vehicle (0.5% DMSO, final concentration) were added every day to triplicate cultures of cells together with 10% FBS to stimulate growth. Cell number was measured by Coulter counting on days 1, 3, 6 and 8 after drug exposure. In a second series of experiments, rat aortic SMC were plated at 5000 cells/well as described above. After a 24-hr incubation period in DMEM/10% FBS, cells were growth arrested in serum-free medium for an additional 24 hr. PD 089828 (10 μM) or vehicle was then added only once to the cells together with 10% FBS to stimulate growth. After 8 days of growth in the presence of 10 μM PD 089828, medium containing inhibitor was aspirated, cells washed three times, fresh medium containing vehicle plus 10% FBS was added back and cells growth was allowed to continue. Cell number was measured by Coulter counting on days 1, 3, 6, and 8 and 11 after drug exposure.

**Cell migration assay.** Rat aortic SMC between passages 8 and 20 were used for these studies. Migration of SMC was assayed using Nuclepore polycarbonate 8.0-μm-pore filters (#NFB8) in 48-well chemotaxis chambers (NeuroProbe, Cabin John, MD). The filters were coated with 100 μg/ml concentration of type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) by incubating filters overnight at room temperature followed by drying. SMC were trypsinized and resuspended at a concentration of 500,000 cells/ml in serum-free DMEM containing streptomycin and penicillin. A volume of 50 μl of SMC suspension (25,000 cells) was mixed together with either vehicle or increasing concentrations of PD 089828 and then placed in the upper chamber. DMEM (25 μl) containing 10 ng/ml PDGF-BB was placed in the lower chamber. The chambers were incubated for 4 hr at 37°C in an atmosphere of 95% air/5% CO2. After incubation, the filters were removed, and the SMC on the top of the filter were scraped off. The SMC that had migrated to the lower side of the filter were fixed and stained with Diff-Quick staining solution (Baxter Inc., McGaw Park, IL) and counted under a microscope (200×100 fields).

**Statistics.** Data are expressed as the mean ± S.E.M. except where indicated. Linear regression analysis was used to generate IC₅₀ values. An analysis of variance with Duncan’s multiple range test was used to compare treatment groups. Statistical significance was defined as P < .05.

### Results

**Effect of PD 089828 on protein tyrosine kinase activity.** PD 089828, the prototype of a novel structural class of tyrosine kinase inhibitors, the 6-aryl-pyrido-2,3-d’ pyrimidines, was identified by screening a compound library with assays that measured protein tyrosine kinase activity (fig. 1). PD 089828 was found to inhibit human full-length FGFR-1, c-Src, PDGF-β and EGFR tyrosine kinases with half-maximal inhibitory potencies (IC₅₀ values) of 0.15 ± 0.02 (n = 4), 0.18 ± 0.04 (n = 3), 1.76 ± 0.28 (n = 4) and 5.47 ± 0.78 (n = 6) μM, respectively. Additional biochemical characterization of kinase inhibition was accomplished through analysis of reaction kinetics as a function of inhibitor concentration effects on ATP utilization by the enzyme. Table 1 shows representative inhibitory constants (Kᵢ) and IC₅₀ determinations for PD 089828 against the various protein kinases. The Kᵢ values obtained via nonlinear regression analysis for FGFR-1, c-Src, PDGF-β and EGFR tyrosine kinases were similar to their respective IC₅₀ values. PD 089828 was also found to inhibit MAPK with an IC₅₀ of 7.1 μM but had no effect on insulin receptor tyrosine kinase, protein kinase C or cyclin-dependent kinase 4 at concentrations as high as 50 μM (table 1). In figure 2, Lineweaver-Burke plots for inhibition of FGFR-1, PDGFβ and EGFR tyrosine kinases by PD 089828 with respect to ATP concentration showed all curves intersecting the y-intercept at zero, which is indicative of a competitive mechanism of inhibition. In contrast, Lineweaver-Burke analysis of c-Src kinase inhibition by PD 089828 with respect to ATP concentration showed all curves intersecting the y-intercept at different points and converging at the
x-intercept, suggesting a noncompetitive mechanism of inhibition.

**Effect of PD 089828 on growth factor-mediated tyrosine phosphorylation in intact cells.** The inhibitory effects of PD 089828 on PDGF-, EGF- and bFGF-mediated tyrosine kinase receptor autophosphorylation were apparent in viable cells. VSMC were pretreated with varying concentrations of PD 089828 for 2 hr and then exposed to either PDGF-BB or EGF. A121(p) ovarian carcinoma cells were pretreated in the same fashion as VSMC and then exposed to bFGF. Figure 3 shows the effect of PD 089828 on PDGFR autophosphorylation (fig. 3A), EGFR autophosphorylation (fig. 3B) or FGFR-1 tyrosine phosphorylation (fig. 3C). In VSMC, both PDGF-BB and EGF elicited a robust stimulation of tyrosine kinase receptor autophosphorylation as identified by anti-phosphotyrosine immunoblotting of immunoprecipitated PDGFRs and EGFRs, respectively. PD 089828 inhibited PDGFR autophosphorylation by 50% at a concentration of 0.82 ± 0.21 μM (n = 3), whereas EGFR autophosphorylation was inhibited with an IC_{50} value of 10.9 ± 0.45 μM (n = 3). In A121(p) cells, FGFR-1 tyrosine phosphorylation was ligand independent because exposure of cells to bFGF did not lead to a further increase in level of receptor phosphorylation. PD 089828 potently inhibited the phosphorylation of this 130-kDa protein with an IC_{50} value of 0.63 ± 0.55 μM (n = 3).

The inhibition of PDGFR autophosphorylation was further studied by examining the kinetics of inhibition by PD 089828. VSMC were incubated with inhibitor for varying times, and an assessment was made of the exposure time required to obtain maximal inhibition of PDGF-induced autophosphorylation as well as the reversibility of the inhibition once PD 089828 was removed. Figure 4 shows a time course for the inhibition of PDGFR-stimulated autophosphorylation in VSMC exposed to 3 μM PD 089828. Inhibition occurred rapidly and was maximal within 5 min of drug exposure. Figure 4 also shows that when PD 089828 was added to the culture medium one time without changing
medium, complete inhibition could be demonstrated every day for 4 consecutive days, indicating that the compound was stable over this time period and did not induce receptor desensitization. In figure 4, VSMC were exposed to 3 \(\mu\)M PD 089828 for 2 hr, after which the drug was removed by washing the cells twice with drug-free medium. PDGF was then added at various times after drug removal to stimulate PDGFR autophosphorylation. Within 10 min of drug wash-out, PDGFR autophosphorylation was returned to maximal stimulation levels.

### Effects of PD 089828 on SH2 protein associations.

PDGFR activation induces receptor autophosphorylation on tyrosine residues in its intracellular domain resulting in the recruitment of SH2-containing proteins, which are thought to serve as downstream regulators of cell proliferation and migration. SH2-containing proteins such as PI-3-kinase, PLC\(\gamma\), GRB2 and Shc have been implicated in these cellular events. PD 089828 was tested for its ability to inhibit the cellular binding association of these SH2 proteins with tyrosine-phosphorylated PDGFR in VSMC. Quiescent cells were exposed for 2 hr to increasing concentrations of PD 089828 followed by a 5-min exposure with PDGF-BB to stimulate receptor autophosphorylation and SH2 protein association. Cells were lysed, and PDGFR receptor/SH2 protein complexes were immunoprecipitated with PDGFR antibodies. SH2 proteins were visualized by immunoblotting with specific antibodies. Figure 5 shows that PD 089828 inhibited the PDGFR-\(\beta\)/SH2 protein associations with potencies that appeared to be roughly similar to its potency for inhibition of PDGFR autophosphorylation (0.82 \(\mu\)M): (fig. 5A) the 85-kDa...
subunit of PI-3-kinase, IC$_{50}$ = 6.85 μM; (fig. 5B) GRB2, IC$_{50}$ = 1.08 μM; (fig. 5C) Shc (66-, 52- and 46-kDa isoforms), IC$_{50}$ = 1.36, 1.24 and 1.06 μM, respectively; and (fig. 5D) PLCγ, IC$_{50}$ > 5.0 μM. To determine whether inhibition of growth factor receptor tyrosine kinase activity by PD 089828 would lead to inhibition of signal transduction events downstream of the receptor/SH2 protein interactions, VSMC were exposed for 2 hr to PD 089828 followed by treatment with PDGF-BB for 5 min to stimulate phosphorylation of MAPKs. Figure 6 shows a Western blot of the phosphorylated 44- and 42-kDa MAPK isoforms after incubation of VSMC with PD 089828. The PDGF-induced phosphorylation of the 44- and 42-kDa MAPK isoforms was inhibited by PD 089828 with IC$_{50}$ values of 1.15 and 1.71 μM, respectively, which were also similar to the potency of PD 089828 for inhibition of PDGFR autophosphorylation.

**Effects of PD 089828 on growth factor-mediated functional responses.** VSMC are mitogenically responsive to growth factors such as PDGF, EGF and bFGF via activation of phosphorylation cascades that link extracellular signal events present at the cell membrane with changes in gene expression in the nucleus. To determine whether inhibition of growth factor receptor tyrosine kinase activity by PD 089828 would lead to interruption of mitogenesis, VSMC were treated for 18 hr with PD 089828 and then stimulated with PDGF-BB, EGF or bFGF to induce DNA synthesis. Mitogenesis was measured as an increase in the incorporation of [³H]thymidine into DNA as an index of DNA synthesis. Figure 7 shows that PDGF, EGF and bFGF stimulated DNA synthesis to different maximal levels, with PDGF producing the greatest increases (~30-fold) and bFGF eliciting only an ~7-fold increase in the incorporation of [³H]thymidine into DNA. However, PD 089828 inhibited increases in DNA synthesis stimulated by all three growth factors, with IC$_{50}$ values of 0.8 ± 0.13 for PDGF-, 1.7 ± 0.29 for EGF- and 0.48 ± 0.09 μM for bFGF-induced mitogenesis.

To determine whether the inhibition of growth factor-stimulated DNA synthesis by PD 089828 would lead to inhibition of cell proliferation, PD 089828 was administered for 8 consecutive days to VSMC stimulated to grow in 10% serum. Figure 8 shows that PD 089828 produced a concentration-related inhibition of serum-stimulated cell growth with an IC$_{50}$ value of 1.8 ± 0.19 μM (n = 3) on day 8 of growth. A concentration of 10 μM PD 089828 inhibited growth completely over the entire 8-day growth period. Removal of 10 μM PD 089828 from the cell media on day 3 after growth restored the growth response to within 60% of the control growth curve by day 8.

VSMC growth was also monitored after a single addition of...
10 μM PD 089828 to cells to examine the efficacy and stability of the inhibitor under longer-term growth conditions. VSMC were exposed to a single 10 μM concentration of PD 089828 on day 1 of growth, and cell growth allowed to continue for 8 consecutive days without a medium change. After 8 days of growth in the presence of 10 μM PD 089828, medium containing inhibitor was aspirated, fresh medium without inhibitor added back and cell growth was allowed to continue for 3 additional days. Cell number was measured by Coulter counting on days 1, 3, 6, 8 and 11 after initial drug exposure. Values represent the mean ± S.E. of three separate experiments performed in triplicate.

PD 089828 resulted in complete inhibition of serum-stimulated cell growth for 8 consecutive days. In addition, removal of the inhibitor from the growth medium restored cell growth to ~70% of the control growth values at day 11.

The ability of PDGF to stimulate chemotaxis in cells is thought to occur via stimulation of PDGFR autophosphorylation with subsequent association of PI-3-kinase and possibly PLCγ. The inhibition of PDGFR-induced autophosphorylation and association with PI-3-kinase and PLCγ by PD 089828 was also associated with an inhibition of PDGF-BB-stimulated migration of VSMC. Figure 10 shows that PDGF-BB stimulated a robust migratory response of VSMC,
which was inhibited by PD 089828 in a concentration-related manner with an IC\textsubscript{50} value of 4.5 ± 0.37 μM (n = 3).

**Discussion**

The growth factor receptor families along with their array of ligands represent a complex network of receptor tyrosine kinases involved in growth, mitogenesis, migration and differentiation (Fantl et al., 1993). Consequently, interruption of protein tyrosine kinase signaling has been considered a potential strategy for inhibition of angiogenesis, tumor growth and restenosis. A number of inhibitors of protein tyrosine kinases have been previously reported (Burke, 1992; Fry et al., 1994a; Traxler and Lydon, 1995); however, suppression of intracellular tyrosine phosphorylation by most of the existing compounds has been demonstrated mainly against EGFR tyrosine kinase activity and include such structures as tyrphostins (Lyall et al., 1989), lavindustin (Onoda et al., 1990), diaminonaphthalimidines (Trinks et al., 1994) and phenylamino-quinazolines (Ward et al., 1994; Fry et al., 1994b). In addition, there have been more recent reports describing the selective inhibition of PDGFR tyrosine kinase activity by tyrphostins (Bilder et al., 1991), substituted quinolines (Dolle et al., 1994), phenylaminopyrimidines (Buchdunger et al., 1995; Zimmermann et al., 1996) and biarylhydrazones (Sawutz et al., 1996).

An argument can be made for development of selective inhibitors for specific kinases reputed to play a key role in a particular proliferative disease. In theory, selective tyrosine kinase inhibitors should be less likely to affect healthy cells, producing fewer unwanted side effects. On the other hand, however, broadly acting, nonselective inhibitors may be required to overcome redundancies in growth signaling pathways to arrest aggressively proliferating cells. This is further complicated by the fact that >200 protein kinases are known and many more remain to be discovered, making it impossible to evaluate inhibitors against a complete panel of isolated enzymes. Thus, given the complex nature of signal transduction (i.e., redundancies and cross-talk between signal transduction pathways), absolute selectivity may not be achievable or necessarily desirable when the need may arise to simultaneously inhibit multiple growth signals.

In this report, we describe the biological characteristics of PD 089828, a novel, broadly active protein tyrosine kinase inhibitor that is the prototype of a newly discovered structural class of small molecules known as the pyrido-[2,3-d] pyrimidines (Connolly et al., 1996). PD 089828 exhibits characteristics that are distinctly different from previously reported protein tyrosine kinase inhibitors, including possession of a novel pyrido-[2,3-d]pyrimidine bicyclic ring structure; ATP competitive for PDGFR, EGFR and FGFR tyrosine kinases, unique noncompetitiveness for c-Src tyrosine kinase and demonstration of prolonged inhibition of growth factor-mediated cellular functions whose effects are reversible.

The results show that PD 089828 is a nonselective inhibitor of FGFR-1, PDGFR-β and EGFR tyrosine kinases and the nonreceptor c-Src tyrosine kinase. Inhibition data showed PD 089828 to be 10- to 15-fold more potent at inhibiting FGFR-1 (150 nM) and c-Src (180 nM) kinase activity that PDGFR-β (1.8 μM) or EGFR (5.5 μM), respectively. The inhibitory potency of PD 089828 was also determined against several other recombinant protein kinases. PD 089828 had little effect on the insulin receptor tyrosine kinase, protein kinase C or cyclin-dependent kinase 4 at concentrations as high as 50 μM. The only other kinase that was appreciably inhibited by PD 089828 was MEK/MAPK (IC\textsubscript{50} = 7.1 μM). This assay, however, did not distinguish between MEK and MAPK inhibitory activity produced by PD 089828. To further investigate the potency differences between the kinases, additional biochemical characterization of kinase inhibition was accomplished by performing kinetic experiments to determine the effects of PD 089828 concentration on ATP utilization by the enzyme. Through the use of conventional Michaelis-Menten kinetic analyses, PD 089828 was found to be a competitive inhibitor of all three growth factor receptor tyrosine kinases with respect to ATP. This is not, however, the first description of ATP competitive inhibition for protein tyrosine kinases. Protein tyrosine kinases constitute a large family of proteins with highly conserved topology for the ATP binding site (Hanks et al., 1988). Indeed, ATP competitive inhibitors represent one of the largest mechanistic categories of tyrosine kinase inhibitors. Moreover, selective protein tyrosine kinase inhibitors that are ATP competitive have previously been reported for Src family members (Faltynek et al., 1995), EGFR (Bridges et al., 1996; Traxler et al., 1996) and PDGFR tyrosine kinase (Dolle et al., 1994; Sawutz et al., 1996; Zimmermann et al., 1996). Unlike these inhibitors, PD 089828 is a nonselective protein tyrosine kinase. A surprising yet intriguing finding was that PD 089828 demonstrated noncompetitive inhibition of c-Src kinase with respect to ATP. The disparity in ATP kinetics along with the 10- to 15-fold differences in potencies for PD 089828 between FGFR-1/c-Src and PDGFR-β/EGFR tyrosine kinases is at present unclear but may conceivably be related to differences in the accessibility of the ATP binding pocket between the protein tyrosine kinases. Furthermore, the different inhibition kinetics between c-Src kinase and FGFR-1, PDGFR-β and EGFR tyrosine kinases have also been observed for several other unsubstituted pyrido-pyrimidines of this structural class, suggesting a class effect of the molecule as an alternative explanation.

The rank order inhibitory potency was also apparent in its effects on viable cells. PD 089828 was ~2- to 3-fold more potent at inhibiting FGFR-1 phosphorylation in A121(p) cells compared with inhibition of PDGFR autophosphorylation and ~10-fold more potent compared with inhibition of EGFR autophosphorylation in VSMC. It is important to point out that the relative differences in kinase vs. cellular potentials could be due a number of processes occurring in cells that are not present in the kinase assay. The in vitro kinase assays were optimized for enzyme, substrate (ATP, Glu-Tyr) and cation concentrations, pH and time. These components may not mimic the intracellular milieu in which subcellular localization and/or compartmentalization is likely to occur. For example, the concentration of ATP found to elicit optimal kinase activity for the recombinant enzymes used in these assays was 50 μM, whereas the intracellular ATP concentrations are ~5 mM. In addition, FGFR-1 phosphorylation in A121(p) cells is constitutive, probably due to the fact that these cells also express the FGF ligands and therefore require higher concentrations of PD 089828 to overcome the

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2 R. L. Panek, unpublished observations.
elevated FGFR tyrosine kinase activity. In contrast, basal PDGFR and EGFR tyrosine kinase activity was low in unstimulated VSMC, and therefore, lower concentrations of PD 089828 were needed inhibit growth factor-induced receptor autophosphorylation.

To further examine the kinetics of inhibition of intracellular tyrosine phosphorylation and interruption of other receptor tyrosine kinase-associated proteins by PD 089828, we performed additional experiments in VSMC with PDGFR-β autophosphorylation measurements taken as representative of growth factor receptor tyrosine kinase activity. The PDGF receptors were immunoprecipitated from VSMC that were treated with various concentrations of PD 089828 and then stimulated with PDGF-BB. The inhibition of autophosphorylation was rapid and occurred virtually as soon as the cells were exposed to PD 089828. The compound also appeared to be very stable, as demonstrated by its ability to inhibit PDGFR autophosphorylation for ≥4 days after a single exposure. The reasons for the rapid reversal of inhibition of PDGFR autophosphorylation are under further investigation.

PDGFR stimulates mitogenesis of vascular SMC and may be critical for cell proliferation and migration at sites of vascular injury. PDGFR activation induces receptor autophosphorylation on tyrosine residues and recruitment of SH2 domain-containing proteins such as PI-3 kinase and PLCγ, both of which have been implicated in PDGFR-induced mitogenesis and chemotaxis (Bornfeldt et al., 1994; Kundra et al., 1994; Valius and Kazlauskas, 1993; Wennström et al., 1994). A third protein, Src, has been implicated in signaling through the p21 ras pathway in VSMC responding to PDGF (Benjamin et al., 1994). PDGFR also stimulates tyrosine phosphorylation on Shc and subsequent complex formation between Shc and GRB2 (Benjamin et al., 1994; Pelicci et al., 1992). Therefore, PD 089828 was examined for its ability to inhibit the association of SH2-containing cytosolic proteins with the phosphorylated PDGFR in VSMC. Immunoblot analysis for the 85-kDa subunit of PI-3 kinase in PDGFR immunoprecipitated from VSMC treated with PD 089828 and then stimulated with PDGF-BB showed a concentration-related inhibition of the association of PI-3 kinase with phosphorylated PDGFR. A similar inhibition of PDGFR with PLCγ, Shc and GRB2 was also observed. The inhibition of the association of these SH2-domain containing signaling proteins with VSMC PDGFR by PD 089828 indicates that the suppression of PDGFR tyrosine kinase activity results in reduced phosphorylation of key tyrosine residues on the kinase intracellular domain and, consequently, reduces the binding of the SH2 proteins.

PD 089828 inhibited both PDGFR-stimulated migration and DNA synthesis of VSMC, presumably via its effects on suppression of PDGFR-induced receptor tyrosine kinase phosphorylation in these cells. Stimulation of DNA synthesis in VSMC by bFGF and EGF was also inhibited by PD 089828. Because of its broad activity as a protein tyrosine kinase inhibitor, the effects of PD 089828 on growth factor-induced functional responses may be due to inhibition of other protein kinases associated with growth factor receptor signaling. As previously mentioned, PD 089828 inhibited MAPK activity with an IC50 value of 7.1 μM and was a potent inhibitor of PDGFR-stimulated tyrosine phosphorylation of MAPK in VSMC, suggesting that part of the inhibition by PD 089828 on growth factor-mediated functional responses such as DNA synthesis, cell migration and proliferation may be through direct inhibition of the MAPK cascade.

In summary, a novel compound, PD 089828, was discovered to be a nonselective inhibitor of protein tyrosine kinases with long-lasting cellular activity. The compound displayed the unique characteristics of an ATP competitive inhibitor of PDGFR-β, FGFR-1 and EGFR tyrosine kinases but a non-competitive inhibitor of c-Src kinase. The profile of PD 089828 as a broadly active inhibitor of protein tyrosine kinases makes this small molecule attractive for use in a number of diseases characterized by excessive cell proliferation and migration, including cancer, atherosclerosis and restenosis, in which multiple growth factor and cytokine signal transduction pathways are likely to be activated.

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