In Vitro Pharmacological Characterization of PD 166285, a New Nanomolar Potent and Broadly Active Protein Tyrosine Kinase Inhibitor

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ABSTRACT
PD 166285, a novel protein tyrosine kinase inhibitor of a new structural class, the 6-aryl-pyrido[2,3-d]pyrimidines, was synthesized as the most potent and soluble analog of a series of small molecules originally identified by screening a compound library with assays that measured protein tyrosine kinase activity. PD 166285 was found to inhibit Src nonreceptor tyrosine kinase, fibroblast growth factor receptor-1, epidermal growth factor receptor and platelet-derived growth factor receptor beta subunit (PDGFR-beta), tyrosine kinases with half-maximal inhibitory concentrations (IC50 values) of 8.4 ± 2.3 nM (n = 6), 39.3 ± 2.8 nM (n = 16), 87.5 ± 13.7 nM (n = 6) and 98.3 ± 7.9 nM (n = 16), respectively. PD 166285 also demonstrated inhibitory activity against mitogen-activated protein kinase (IC50 = 5 µM) and protein kinase C (IC50 = 22.7 µM). PD 166285 was further characterized as an ATP competitive inhibitor of Src nonreceptor tyrosine kinase, PDGFR-beta, fibroblast growth factor receptor-1 and epidermal growth factor receptor tyrosine kinases. In addition, PD 166285 inhibited PDGF- and EGF-stimulated receptor autophosphorylation in vascular smooth muscle cells (VSMCs) and A431 cells, respectively, and basic fibroblast growth factor-mediated tyrosine phosphorylation in SF9 cells, with IC50 values of 6.5 nM, 1.6 µM and 97.3 nM, respectively, further establishing a tyrosine kinase mechanism of inhibition. The inhibition of PDGF receptor autophosphorylation in VSMCs by PD 166285 was long lasting and persisted for 4 days after a single 1-hr exposure followed by extensive washing. The PDGF-induced tyrosine phosphorylation of the 44- and 42-kDa mitogen-activated protein kinase isoforms was also blocked as a result of the inhibition of PDGF-stimulated receptor autophosphorylation by PD 166285 in VSMCs. The effects of PD 166285 were also demonstrated in functional assays of cell attachment, migration and proliferation, in which vascular cell adhesion to vitronectin, PDGF-directed chemotaxis and serum-stimulated cell growth were all potently inhibited with IC50 values of 80 to 120 nM. Finally, PD 166285 uniquely demonstrated potent inhibition of phorbol ester-induced production of 92-kDa gelatinase A (MMP-9) in VSMC without affecting 72-kDa gelatinase B (MMP-2) as measured by gelatin zymography. These results highlight the biological characteristics of PD 166285 as a broadly active protein tyrosine kinase capable of potently inhibiting a number of kinase mediated cellular functions, including cell attachment, movement and replication. The potential therapeutic utility of this broadly acting inhibitor as an antiproliferative and antimigratory agent could extend to such diseases as cancer, atherosclerosis and restenosis, in which redundancies in protein kinase 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, alpha and beta isoforms (Claesson-Welsh, 1994), FGFRs [FGFR-1 (flg), FGFR-2 (bek), FGFR-3 and FGFR-4; Friesel and Maciag, 1995] and the EGFR family (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, including c-Src (Alonso et al., 1995) and MAPK (Cadena and Gill, 1992; Jaye et al., 1992). Src is itself a nonreceptor kinase inhibitor of growth factor receptor tyrosine kinases.

ABBREVIATIONS: FGFR-1, fibroblast growth factor receptor-1; PDGFR-beta, platelet-derived growth factor receptor beta subunit; EGF, epidermal growth factor receptor; c-Src, Src nonreceptor tyrosine kinase; bFGF, basic fibroblast growth factor; MAPK, mitogen-activated protein kinase; VSMC, vascular smooth muscle cell; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N’-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RBRA, rabbit renal artery smooth muscle cell; AM, acetoxyxymethyl ester; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; CDF4, cyclin-dependent kinase 4; MMP, matrix metalloproteinases; BAEC, bovine aortic endothelial cell; PKC, protein kinase C; ECL, enhanced chemiluminescence; ECM, extracellular matrix remodeling.
membrane-associated tyrosine kinase that binds via its Src homology-2 (SH2) domain and becomes phosphorylated by the PDGFR (Alonso et al., 1995; Kypka et al., 1990). The recruitment of c-Src to the PDGFR is thought 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 (Ullrich and Schlessinger, 1990).

Evidence has accumulated that overexpression of receptor protein tyrosine kinases or autoirradiation 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; Sitaras 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, 1995).

The importance of protein tyrosine kinases in signal transduction and the association of aberrant protein tyrosine kinase receptor and ligand expression with proliferative disorders make agents which 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), tyrophostins (Bilder et al., 1991; Gazit et al., 1989; Lyall et al., 1989) and lavendustin, erbstatin and genistein, (Burke, 1989) and lavendustin, erbstatin and genistein, (Burke, 1989) and lavendustin, erbstatin and genistein, (Burke, 1989) and lavendustin, erbstatin and genistein, (Burke, 1989).

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).

With the potential for multiple growth factor receptors to be simultaneously activated in proliferative diseases, broadly acting, 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 or a solid tumor.

Previous reports from our laboratories have described a new series of potent, broadly active tyrosine kinase inhibitors based on two lead structures: PD 089828 (Connolly et al., 1986; Dahring et al., 1997) and PD 09560 (Blankley et al., 1997; Klohs et al., 1997; Kraker et al., 1997; Showalter et al., 1997), which are derived from compound library screening.

In the present study, we report on PD 166285, the most potent identified analog of the pyrido-[2,3-d]pyrimidine class of protein tyrosine kinase inhibitors. PD 166285 is distinguished from previously reported protein tyrosine kinase inhibitors by possessing a number of unique structural and biological features, including (1) a novel pyrido[2,3-d]pyrimidine bicyclic structure; (2) ATP competitiveness for PDGFR, EGFR and FGFR tyrosine kinases and c-Src kinase; (3) a highly potent inhibitor of tyrosine kinase-mediated cellular growth, adhesion, migration and MMP production and (4) a molecule that demonstrates long-lasting inhibition of growth factor-mediated cellular functions.

Methods

Chemicals and reagents. Human recombinant PDGF-BB, EGF and bFGF growth factors; anti-phosphotyrosine monoclonal (clone 4G10); and anti-human PDGFR-β polyclonal and anti-human EGF receptor polyclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies raised to the human PDGFR-1 (flg) were a kind gift from Dr. Wendy Fautl (Chiron, San Francisco, CA). p44/p42 MAPK antibodies were purchased from New England Biolabs (Beverly, MA). ECL reagents were purchased from Amersham Life Science (Arlington Heights, IL). Calcine-AM was obtained from Molecular Probes (Eugene, OR). Collagen Type I was obtained from Collagen Corp. (Palo Alto, CA). Fibronectin was purchased from Collaborative Biomedical Products (Bedford, MA). Vi- tronecin, soybean trypsin inhibitor, BSA (fraction V), selenium, transferrin and hydrocortisone were purchased from Sigma Chemical (St. Louis, MO). DMEM, DMEM/Ham’s F-12, RPMI, Dulbecco’s PBS, 1% glucose and 1% penicillin/streptomycin were obtained from Gibco Life Technologies (Grand Island, NY). FBS was purchased from Hyclone (Provo, UT).

Recombinant kinases. Bacterial-covirus-containing sequence for the full-length human PDGFR-β was obtained from Dr. William LaRochele (National Institutes of Health, Bethesda, MD). Production of PDGFR-β protein in infected Spodoptera frugiperda (SB) insect cells was performed as previously described (Jensen et al., 1992). cDNA coding for the full-length human PDGFR-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 bacu- lovirus transfer vector pBacPAK8 (Clontech, Palo Alto, CA). Recom- binant baculovirus bearing the FGFR-1 DNA was prepared, identi- fied and purified using SB9 insect cells as hosts according to the BaculoGold system (PharMingen, San Diego, CA) (instructions provided with the kit). Baculovirus-containing sequences for the full- length EGF receptor and c-Src kinases were prepared in a similar manner and have been previously described (Fry et al., 1994a, 1994b; Thomp- son et al., 1994). For all of the kinases, SB9 cells were infected with the individual viruses to overexpress the proteins.

Tyrosine kinase assays. Assays using the full-length PDGFR-β, FGFR-1 and EGFR tyrosine 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 μg/ml concentration of a random copolymer of glutamic acid and tyrosine (4:1), various concentrations of inhibitor and 60 to 75 ng of enzyme as previously described (Fry et al., 1994a, 1994b). The reaction was initiated by the addition of [γ-32P]ATP (50 μM ATP containing 0.4 μCi of [γ-32P]ATP per incubation), and samples were incubated at 25°C for 10 min. The reaction was terminated by the addition of 30% trichloroacetic acid and the precipitation of material onto glass-fiber filter mats. Filters were washed three times with 15% trichloroacetic acid, and the incorporation of [32P] into the glutamate tyrosine polymer substrate was determined by counting the radioactivity retained on the filters in a Wallac (Gaithersburg, MD) 1250 betaplate reader. Nonspecific activity was defined as ra- diocactivity retained on the filters following incubation of samples without enzyme. Specific activity was determined as total activity (enzyme plus buffer) minus nonspecific activity. The concentration of compound that inhibited specific enzymatic activity by 50% (IC50) was determined graphically. For determination of ATP kinetics, assay conditions were the same as above except that varying con- centrations of ATP were added in the absence or presence of a single concentration of PD 166285 to generate ATP concentration curves. K1 determinations for PD 166285 were obtained by a nonlinear regres- sion analysis to fit the inhibition data to equations that describe different types of inhibition (Cleland, 1979). A comparison of the K1 (slope) vs. K1 (intercept) was then used to refine the curve-fit analy-
sisk. Kinetic analyses were performed using GraFit v 3.0 (Leatherbarrow, 1992).

Cell culture. Smooth muscle cells were isolated from the thoracic aorta of adult male Sprague-Dawley rats (300–350 g; Charles River, Portage, MI) or male New Zealand White rabbits (2.0–2.5 kg; Covance, Denver, PA) and explanted according to the method of Ross (1971). Cells were grown in DMEM containing 10% FBS, 1% glutamine and 1% penicillin/streptomycin. Cells were identified as smooth muscle cells by their “hill-and-valley” growth pattern and by fluorescent staining with a monoclonal antibody specific for smooth muscle cell α-actin. Cells were used between passages 8 and 20 for all experiments. A431 human epidermal carcinoma cells were obtained from Dr. David Fry (Parke-Davis, Cancer Research) and have previously been shown to express high levels of EGFRs (Fry et al., 1994b). Test compounds were prepared in DMSO to achieve consistency in the vehicle and ensure compound solubility. Appropriate DMSO controls were simultaneously evaluated with the test compounds.

Autophosphorylation assay. Rat aortic smooth muscle cells were grown to confluency in 100-mm dishes with DMEM containing 10% FBS, and A431 human epidermal carcinoma cells were grown to confluency in 100-mm dishes with DMEM/F12 containing 10% FBS. Growth medium was removed and replaced with serum-free medium consisting of DMEM/F12 (1:1), 30 mM sodium, 50 μg/ml transferrin, 10 mM hydrocortisone and 5 μg/ml insulin, and cells were incubated for an additional 24 hr. PD 166285 was then added directly to fresh medium and cells incubated for an additional 2 hr. PDGF-BB was added at a final concentration of 30 ng/ml for 5 min at 37°C to stimulate autophosphorylation of PDGF-Rs in rat aortic smooth muscle cells. EGF was added at a final concentration of 20 ng/ml for 10 min at 37°C to stimulate autophosphorylation of EGFRs in A431 cells. After growth factor treatment of cells, the medium was removed and cells were washed with cold phosphate-buffered saline 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 μg/ml aprotinin and 10 μg/ml leupeptin). Lysates were centrifuged at 10,000 × g for 10 min. Supernatants from rat aortic smooth muscle cell lysates were incubated for 2 hr with a 1:100 dilution of anti-human PDGF-R β-polyclonal antibody (UBI; No. 06–498), and supernatants from A431 cell lysates were incubated with anti-human EGFR polyclonal antibody (UBI; No. 06–498). This immunoprecipitate was precipitated with 10 μg/ml of Protein A-Sepharose beads and washed with 1 ml of cold wash buffer for each wash step. After the final wash, lysates were run on a 5–10% SDS-polyacrylamide gel electrophoresis, separated proteins were transferred to nitrocellulose and immunoblotted with a 1:1000 dilution of anti-phosphotyrosine monoclonal antibody (UBI clone 4G10; No. 05–321) or anti-human PDGF-R β polyclonal antibody (UBI; No. 06–498), respectively. The remaining 200-μl samples were analyzed for PD166285 lysate concentrations using high-performance liquid chromatography and UV spectrophotometric detection.

PD 166285 high-performance liquid chromatography assay. PD 166285 was extracted by protein precipitation with acetonitrile. Liquid chromatographic separation was achieved on a Varian Bondel 5-μm, 4.6 × 100-mm C8 reverse-phase column. The mobile phase consisted of 30% acetonitrile/70% ammonium phosphate buffer (0.1 M, pH 3.5). Column effluent was monitored by a UV detector at a wavelength of 364 nm. Assay quantification ranged from 25 to 5000 ng/ml for a 200-μl sample aliquot.

DNA synthesis. Rat aortic smooth muscle cells plated onto 24-well plates were serum-starved for 24 hr and then incubated with PDGF-BB (10 ng/ml) or bFGF (25 ng/ml) and various concentrations of PD 166285. Growth factor and inhibitor incubations continued for an additional 24 hr. During the final 4 hr, cells were supplemented with 0.25 μCi/well (37kBq) of methyl-[3H] thymidine. Cells were washed with phosphate-buffered saline and then fixed with 5% trichloroacetic acid. Cultures were washed several times with water, and the trichloroacetic-acid-precipitable material was solubilized with 0.25N NaOH, and [3H] was quantified by liquid scintillation counting.

Cell growth assays. Rat aortic smooth muscle cells were plated at 10,000 cells/well in 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 166285 or vehicle (0.5% DMSO, final concentration) was 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.

Cell adhesion assay. RBBs between passages 8 and 16 were used in these experiments. The wells of uncharged polystyrene 96-
well microtiter plates (Corning Glass Works, Corning, NY) were precoated with either collagen type I (10 μg/ml), fibronectin (10 μg/ml) or vitronectin (10 μg/ml), in sterile PBS overnight at 4°C. After the coating period, wells were rinsed with PBS, and nonspecific binding sites were blocked with 2% BSA at 37°C for 2 hr. Confluent cells were detached from culture dishes with minimal trypsinization (2 min), placed immediately into an equal volume of 0.2% soybean trypsin inhibitor and centrifuged. Cells were resuspended in DMEM containing 1 mg/ml BSA. Approximately 60,000 cells were placed into each well and allowed to adhere at 37°C for 1 hr. Various concentrations of PD 166285 were added to the wells just before the addition of the cells. Nonadherent cells were rinsed off with PBS, and the remaining cells were incubated for 45 min at 37°C with calcine-AM (15 μg/ml), a fluorescent dye that is taken up into the cytosol of cells, where intracellular esterases cleave the AM group, allowing free calcein to emit fluorescent light. The cellular fluorescence was quantified with a Cytofluor 2350 fluorescence scanner (Millipore, Bedford, MA) with excitation/emission filter wavelengths set at 485/530 nm. Under these conditions, fluorescence emission is proportional to cell number.

Cell migration assay. RBRAs between passages 8 and 16 were used for these studies. Migration of RBRAs was assayed using Costar 24-well Transwell cell culture chambers with 8.0-μm pore polycarbonate filter inserts (No. 3422; Costar, Cambridge, MA). The filters were coated with 100 μg/ml collagen type I (Vitrogen 100; Collagen Corp., Palo Alto, CA.), fibronectin, vitronectin (Sigma Chemical) or Matrigel (Becton Dickinson, Bedford, MA) basement membrane matrix (300 μg/ml) by incubating filter inserts overnight at room temperature under a laminar flow hood. Coated inserts were rehydrated the next day with 0.5 ml of serum-free DMEM, which was removed just before the addition of cells. RBRAs were trypsinized, washed two times with 0.2% soybean trypsin inhibitor and then resuspended at a concentration of 300,000 cells/ml in serum-free DMEM containing streptomycin and penicillin. A volume of 100 μl of RBA suspension (30,000 cells) was mixed together with either vehicle or increasing concentrations of PD 166285 and then placed in the coated filter insert. Next, 500 μl of DMEM containing 10 ng/ml of PDGF-BB was placed in the lower chamber. The inserts were placed into the transwell chambers, and cells were incubated for 22 hr at 37°C in an atmosphere of 95% air/5% CO2. After incubation, the filter inserts were removed, medium was decanted off and the RBRAs on the top side of the filter were scraped off. The RBRAs that had migrated to the lower side of the filter were incubated for 45 min at 37°C with calcine-AM (15 μg/ml) as described above. Quantification of the fluorescent-labeled RBRAs was achieved with a Nikon Diaphot inverted microscope equipped with epifluorescence and attached to an Apple power PC 8100 using image analysis (National Institutes of Health Image software version 1.56). Migration was quantified by converting the fluorescent light emitted by the RBRAs into pixels and measuring the mean number of pixels per three microscopic fields imaged per individual filter. Data were expressed as a percentage of total pixels measured after stimulation with PDGF-BB, which was taken to represent the maximal amount of migration (100%).

Gel zymography. Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in 10% polyacrylamide gels containing 1 mg/ml gelatin (Novex, San Diego, CA). Rabbit renal artery smooth muscle cells were grown to 80% confluency in 100-mm dishes with DMEM containing 10% FBS. Growth medium was removed and replaced with serum-free medium consisting of DMEM/F12 (1:1), 30 nM selenium, 50 μg/ml transferrin, 10 nM hydrocortisone and 5 μg/ml insulin, and cells were incubated for an additional 24 hr. Cells were stimulated to induce expression of gelatinases as the addition directly to fresh medium of PMA (Sigma) in the absence or presence of PD 166285 and incubated for an additional 24 hr. After a 24-hr incubation, culture medium was mixed 1:1 with Laemmelli’s sample buffer in the absence of β-mercaptoethanol, and 40 μl was loaded onto gels and electrophoresed at 120 V for 2 hr. After electrophoresis, gels were renatured by exchanging SDS with Triton X-100 (two 30-min incubations in 25% Triton X-100 at 25°C). Gels were subsequently incubated overnight at 25°C in 50 mM Tris-HCl, pH 7.4, containing 10 mM CaCl2 and 0.05% Brij 35 developing buffer (Novex). To verify the MMP nature of lytic activity detectable by zymography, identical gels were incubated in the above buffer containing either 10 mM EDTA, a catalytic-site MMP inhibitor, or 1 mM phenylmethylsulfonyl fluoride, an inhibitor of serine proteases (data not shown). At the end of the incubation, gels were stained with 0.5% Coomassie blue stain for 1 hr and then destained. Gels were scanned into Adobe Photoshop (version 4.0), and bands of gelatinolytic activity were quantified with Image version 1.66.

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 a Duncan multiple range test was used to compare treatment groups. Statistical significance was defined as P < .05.

Results

Effect of PD 166285 on protein tyrosine kinase activity. PD 166285 (fig. 1) was identified as a potent inhibitor of c-Src, FGFR-1, EGFR, and PDGFR-β tyrosine kinases with half-maximal inhibitory potencies (IC50 values) of 8.4 ± 2.3 (n = 6), 39.3 ± 2.8 (n = 16), 87.5 ± 13.7 (n = 6), and 98.3 ± 7.9 (n = 16) nM, respectively. Additional biochemical characterization of kinase inhibition was accomplished by analysis of reaction kinetics as a function of inhibitor concentration effects on ATP utilization by the enzyme. Table 1 shows representative inhibitory constants (K) and IC50 determinations for PD 166285 against the various protein kinases. The K and IC50 values obtained via nonlinear regression analysis for c-Src, FGFR-1, EGFR and PDGFR-β tyrosine kinases were similar to their respective IC50 values. PD 166285 was also found to inhibit MAPK and PKC with IC50 values of 5.0 and 22.7 μM, respectively, but had no effect on insulin receptor tyrosine kinase or CDK4 at concentrations as high as 50 μM (table 1). In figure 2, Lineweaver-Burke plots for inhibition of FGFR-1, PDGFR-β and EGFR tyrosine kinases by PD 166285 with respect to ATP concentration showed all curves intersecting the y intercept at zero, which indicative of a competitive mechanism of inhibition.

Effect of PD 166285 on growth factor-mediated tyrosine phosphorylation in intact cells. The inhibitory effects of PD 166285 on PDGF-α, EGFR- and bFGF-mediated...
tyrosine kinase receptor autophosphorylation were apparent in viable cells. Rat aortic smooth muscle, A431 and Sf9 cells were pretreated with varying concentrations of PD 166285 for 2 hr and then exposed to PDGF-BB, EGF or bFGF respectively. Figure 3 shows the effect of PD 166285 on PDGFR autophosphorylation in rat aortic smooth muscle cells (fig. 3A), EGFR autophosphorylation in A431 cells (fig. 3B) and FGFR-1 tyrosine phosphorylation in Sf9 cells (fig. 3C). In cells, 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 166285 inhibited PDGFR autophosphorylation by 50% at a concentration of 6.5 ± 6.2.5 nM (n = 4), whereas EGFR autophosphorylation in A431 cells was inhibited with an IC50 value of 1.6 ± 0.5 µM (n = 3). In Sf9 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 166285 potently inhibited the phosphorylation of this 130-kDa protein with an IC50 value of 97.3 ± 12.9 nM (n = 3).

To determine whether inhibition of growth factor receptor tyrosine kinase activity by PD 166285 would lead to inhibition of signal transduction events downstream of receptor activation, rat aortic smooth muscle cells were exposed for 2 hr to PDGF-BB, EGF or bFGF respectively. Figure 3 shows the effect of PD 166285 on PDGFR autophosphorylation in rat aortic smooth muscle cells (fig. 3A), EGFR autophosphorylation in A431 cells (fig. 3B) and FGFR-1 tyrosine phosphorylation in Sf9 cells (fig. 3C). In cells, 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 166285 inhibited PDGFR autophosphorylation by 50% at a concentration of 6.5 ± 2.5 nM (n = 4), whereas EGFR autophosphorylation in A431 cells was inhibited with an IC50 value of 1.6 ± 0.5 µM (n = 3). In Sf9 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 166285 potently inhibited the phosphorylation of this 130-kDa protein with an IC50 value of 97.3 ± 12.9 nM (n = 3).

To determine whether inhibition of growth factor receptor tyrosine kinase activity by PD 166285 would lead to inhibition of signal transduction events downstream of receptor activation, rat aortic smooth muscle cells were exposed for 2 hr to PD 166285 followed by treatment with PDGF-BB for 5 min to stimulate phosphorylation of MAPKs. Figure 3 shows an immunoblot of the phosphorylated 44- and 42-kDa MAPK isoforms after incubation of cells with PD 166285. The PDGF-induced phosphorylations of the 44- and 42-kDa MAPK isoforms were inhibited by PD 166285 with IC50 values of 12.1 and 24.3 nM, respectively. The inhibitory potencies for the MAPKs were similar to the potency of PD 166285 for inhibition of PDGFR autophosphorylation (table 2); however, a somewhat steeper dose response was observed for inhibition of MAPK phosphorylation compared with PDGFR autophosphorylation (fig. 3).

The inhibition of PDGFR autophosphorylation was further studied by examining the kinetics of inhibition by PD

### Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC50a</th>
<th>Ki</th>
<th>Inhibitor type</th>
</tr>
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<tbody>
<tr>
<td>c-Src</td>
<td>8.4 ± 2.3 nM</td>
<td>4.9</td>
<td>Competitive</td>
</tr>
<tr>
<td>FGFR-1</td>
<td>39.3 ± 2.8 nM</td>
<td>54.4</td>
<td>Competitive</td>
</tr>
<tr>
<td>EGFR</td>
<td>87.5 ± 13.7 nM</td>
<td>104.8</td>
<td>Competitive</td>
</tr>
<tr>
<td>PDGFR</td>
<td>98.3 ± 7.9 nM</td>
<td>138.8</td>
<td>Competitive</td>
</tr>
<tr>
<td>MAPKc</td>
<td>5.0 µM</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PKC5</td>
<td>22.7 µM</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Insulinc</td>
<td>&gt;50 µM</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CDK4</td>
<td>&gt;50 µM</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Values are mean ± S.E.M. of three to six separate experiments performed in triplicate and are expressed as the concentration of inhibitor producing 50% inhibition of kinase activity.

* * Values are the mean of a single experiment performed in triplicate with varying concentrations of ATP. Ki is defined as the dissociation constant of the enzyme inhibitor complex.

* The assay combines cyclin D1 with CDK4 and measures the phosphorylation of the substrate, retinoblastoma protein. Single observation performed in triplicate.

* P < .05 compared with FGFR-1, EGFR, PDGFR, MAPK and PKC.

* P < .05 compared with c-Src, EGFR, PDGFR, MAPK and PKC.

N.D. = not determined.
Rat aortic smooth muscle cells were maintained in DMEM containing 10% FBS to mimic the plasma milieu the cells might be exposed to after vessel wall injury (i.e., endothelial denudation). Cells were incubated with inhibitor at concentrations varying from 10 nM to 10 μM for 1 hr, followed by removal of drug from the culture dishes through extensive washing with PBS. Immediately after the 1-hr incubation, a group of cell cultures representing the maximal drug exposure time point (0 hr) was incubated with 30 ng/ml PDGF-BB for 5 min to stimulate PDGFR autophosphorylation. The remaining groups of cells were rinsed with PBS and refreshed with DMEM containing 10% serum. Sets of cultures maintained in DMEM/10% serum were then harvested at 24, 48, 72 and 96 hr immediately after the 5-min PDGF stimulation. As cells from each time point were harvested, the remaining groups of cells were rinsed with PBS and maintained in DMEM/10% serum until the time of harvest. An assessment was made of the duration of inhibition of PDGF-induced receptor autophosphorylation (measured by anti-phosphotyrosine blotting), status of PDGFR levels (measured by anti-PDGFR blotting) and intracellular concentrations of PD 166285 retained in cells over the 96-hr period. Figure 4 and table 3 show inhibition of PDGF-induced receptor autophosphorylation was concentration related, with complete inhibition achieved within 60 min of drug exposure. Figure 4 also shows that despite removal of PD 166285 from the culture medium immediately after the 1-hr incubation, maximal inhibition of PDGFR autophosphorylation could be maintained but was both concentration and time dependent.
TABLE 3

<table>
<thead>
<tr>
<th>Concentration of PD 166285 exposed to cells in culture</th>
<th>Concentration of PD 166285 in cell lysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of lysis</td>
<td>ng/ml</td>
</tr>
<tr>
<td>0 hr</td>
<td>&lt;25 a</td>
</tr>
<tr>
<td>24 hr</td>
<td>&lt;25 a</td>
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<td>48 hr</td>
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</tr>
<tr>
<td>96 hr</td>
<td>&lt;25</td>
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</tbody>
</table>

*The limit of detection for PD 166285 via high-performance liquid chromatography/UV detection was 25 ng/ml.

Maximal inhibition was maintained for ≥24 hr for all concentrations (10 nM to 10 μM) and persisted over the next 4 days after 1-hr exposure of 10 μM of PD 166285, which yielded intracellular concentrations of PD 166285 of 460 ng/ml or ~1 μM based on the molecular weight of PD 166285 (512) (table 3). In addition, there was a parallel increase in PDGF expression over time except for the cells exposed to 10 μM PD 166285, in which the levels of PDGFR appeared to remain constant throughout the 96-hr washout time period (fig. 4, right).

Effects of PD 166285 on growth factor-mediated mitogenic responses. VSMCs are mitogenically responsive to growth factors such as PDGF, EGF and bFGF via activation of phosphorylation cascades, which 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 166285 would lead to interruption of mitogenesis, rat aortic smooth muscle cells were treated for 18 hr with PD 166285 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 5 shows that PDGF, EGF and bFGF stimulated DNA synthesis to different maximal levels, with PDGF and EGF producing the greatest increases (~12-fold) and bFGF elicited only a 3- to 4-fold increase in the incorporation of [3H]thymidine into DNA. However, PD 166285 inhibited increases in DNA synthesis stimulated by all three growth factors with IC50 values of 18.9 ± 0.3 nM (n = 3) for PDGF, 43.3 ± 0.6 nM (n = 3) for bFGF and 321.1 ± 29.6 nM for EGF (n = 3).

To determine whether the inhibition of growth factor-stimulated DNA synthesis by PD 166285 would lead to inhibition of cell proliferation, PD 166285 was administered for 8 consecutive days to rat aortic smooth muscle cells stimulated to grow in 10% serum. Figure 6 shows that PD 166285 produced a concentration-related inhibition of serum-stimulated cell growth with an IC50 value of 92.8 ± 12.5 nM (n = 3) by the eighth day. Removal of 300 nM PD 166285 from the cell media on day 3 after growth completely restored the growth response by day 8.

Effects of PD 166285 on VSMC matrix interactions; matrix degradation, attachment and migration. VSMC migration, proliferation, and ECM are regulated by various growth factors and cytokines and thought to depend on matrix-degrading enzymes such as the MMPs. To further test this hypothesis, we examined the effects of PD 166285 on the ability of VSMCs in culture to synthesize and secrete two matrix-degrading MMPs: the 72-kDa gelatinase (MMP-2 or gelatinase A) and the 92-kDa gelatinase (MMP-9 or gelatinase B). We used PMA (phorbol ester), a direct activator of PKC and a mitogen for rabbit VSMCs, to increase 92-kDa gelatinase activity in RBRAs because it has previously been reported as a potent inducer of 92-kDa gelatinase expression in VSMCs from rabbit aorta (Fabunmi et al., 1996). In figure...
7, zymography revealed that exposure of RBRAs to increasing concentrations of PMA alone for 24 hr stimulated a concentration-related production of 92 kDa gelatinase, whereas production of 72-kDa gelatinase was constitutive and unaltered (fig. 7A). To test whether PD 166285 could act as an inhibitor of the catalytic site of the 72- and 92-kDa gelatinases, RBRAs were first incubated for 24 hr with PMA (10 ng/ml) to induce gelatinase secretion. Aliquots of cell culture media were then electrophoresed onto gelatin gels, and individual gel slices, each representing a separate lane, were incubated overnight with 1 or 10 μM PD 166285 or 0.1 μM BB 94, a nonselective catalytic-site MMP inhibitor, as a reference control. Figure 7B shows that PD 166285 had no effect on either 72- or 92-kDa gelatinolytic activities secreted from RBRAs, whereas BB 94 was a potent inhibitor of both. In contrast, RBRAs incubated simultaneously with PMA and PD 166285 (0.1–3 μM) for 24 hr followed by gel zymography of the media demonstrated a concentration-related inhibition of 92-kDa gelatinase production (IC_{50} = 160 nM), with no effect on 72-kDa gelatinase production secreted by these cells. A similar incubation of RBRAs with PMA and BB 94 (1 μM), on the other hand, had no effect on production of either 72- or 92-kDa gelatinolytic activities. Similar results were obtained with interleukin-1β as the stimulant (data not shown).

The ECM proteins, collagen, fibronectin and vitronectin appear to play an important role for both cell adhesion and migration; therefore, we examined the effects of PD 166285 on cell adhesion to ECM proteins using vascular smooth muscle cells (RBRAs) and BAECs, which served as a positive control. BAECs have been previously been reported to express the vitronectin receptor alpha-v beta-3 and attach to vitronectin (Liaw et al., 1995). Figure 8 shows that all three ECM proteins (collagen type I, fibronectin and vitronectin) promoted maximal adhesion of both RBRAs and BAECs during a 60-min incubation. The addition of increasing concentrations of PD 166285 together with cells to 96-well culture plates precoated with ECM proteins resulted in a specific, concentration-related inhibition of vitronectin-mediated attachment of both RBRAs and BAECs, with IC_{50} values of 121 ± 13.9 (n = 3) and 245 ± 9.4 (n = 3) nM, respectively. The specific alpha-v beta-3 integrin receptor antagonist Gly-Pen-Gly-Arg-Gly-Asp-Pro-Cys-Ala cyclical RGD peptide, used as a positive control, inhibited adhesion of RBRAs to vitronectin with an IC_{50} value of 4.3 μM, which was >10-fold less potent than PD 166285. Adhesion of cells to either collagen type I or fibronectin was relatively unaffected by PD 166285 or the cyclic PenRGD peptide.

The ability of PDGF to stimulate cell migration is not well understood; however, a number of signaling pathways have been implicated, including PDGF-induced receptor autophosphorylation and the subsequent activation of downstream proteins, such as MAPK, as well as signaling pathways activated by cell surface integrin receptors. Therefore, we examined the ability of PD 166285 to inhibit PDGF-stimulated migration of VSMC. Figure 9 shows that PDGF-BB stimulated a robust migration of RBRAs through matrices of either Matrigel, collagen type I or vitronectin, which was inhibited by PD 166285 in a concentration-related manner, with an IC\textsubscript{50} value of 89 ± 11.4 nM (n = 3) for Matrigel, 107 ± 1.7 nM (n = 3) for collagen type I and 95 ± 16.1 nM (n = 3) for vitronectin (table 2). In contrast, PDGF-BB produced only a weak stimulation of RBRB migration through matrices of fibronectin.

![Fig. 7. Effects of PD 166285 on MMP production in RBRAs stimulated by PMA. A, Cells were stimulated with increasing concentrations of PMA for 24 hr, and aliquots of media were electrophoresed via gelatin gel zymography. B, RBRAs were stimulated with PMA (10 ng/ml) for 24 hr, and medium samples were electrophoresed on zymogram gels as above. The gelatin gels were sliced into sections and incubated with PD 166285 in developing buffer overnight. Far right lane shows BB94 run as a positive control. C, Cells were stimulated with PMA (50 ng/ml) ± PD 166285 or BB94 (as negative control) for 24 hr and aliquots of media were electrophoresed on gelatin gels. D, RBRAs were stimulated with PMA (10 ng/ml) ± increasing concentrations of PD 166285 for 24 hr, and aliquots of media were electrophoresed on gelatin gels. The bands at 92 kDa identify gelatinase A or MMP-9 gelatinolytic activity, and bands at 72 kDa identify gelatinase B or MMP-2 gelatinolytic activity.]

**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; Panayotou and Waterfield, 1993). Consequently, interruption of protein tyrosine kinase signaling has been considered a potential strategy for inhibiting angiogenesis, tumor growth and restenosis. A number of inhibitors of protein tyrosine kinases have been 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), lavendustin (Onoda et al., 1990), diphenilaminonaphthamides (Trinks et al., 1994) and phenylamino quinazolines (Fry et al., 1994b; Ward et al., 1994) or PDGFR tyrosine kinase activity, including inhibition by tyrophostins (Bilder et al., 1991), substituted quinolines (Dolle et al., 1989), phenylamino pyrimidines (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, broadly acting, nonselective inhibitors may be required to overcome redundancies in growth signaling pathways to arrest aggressively proliferating cells. The downside may be a higher potential for nonselective side effects and toxicities associated with systemic administration of a broad acting inhibitor, although local delivery approaches would avoid this. 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 arises to simultaneously inhibit multiple growth signals.

In this report, we describe the biological characteristics of PD 166285, a nanomolarly potent, broadly active protein kinase inhibitor of a novel structural class of small molecules known as the pyrido[2,3-d]pyrimidines (Connolly et al., 1997). PD 166285 exhibits several characteristics that are distinct from previously reported protein tyrosine kinase inhibitors, including (1) a novel pyrido[2,3-d]pyrimidine bicyclic structure; (2) being ATP competitive for PDGFR, EGFR and FGFR and c-Src tyrosine kinases; (3) being highly potent inhibitor of tyrosine kinase-mediated cellular functions, including growth, adhesion, migration and MMP activity and (4) demonstrating long-lasting inhibition of growth factor-mediated cellular functions.

Inhibition data showed PD 166285 to be a broadly active nanomolar inhibitor of c-Src, FGFR-1, EGFR and PDGFR-β tyrosine kinases. In addition, the inhibitory effects of PD 166285 were demonstrated against MAPK and PKC, albeit with lower potencies. PD 166285 had little effect on the insulin receptor tyrosine kinase and CDK4 at concentrations as high as 50 μM. Additional biochemical characterization of kinase inhibition was accomplished by performing kinetic experiments to determine the effects of PD 166285 concentration on ATP utilization by the enzyme. Using conventional Michaelis-Menten kinetic analyses, PD 166285 was found to be a competitive inhibitor of c-Src, FGFR-1, EGFR and PDGFR-β tyrosine kinases with respect to ATP. 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 the PDGFR tyrosine kinase (Dolle et al., 1994; Sawutz et al., 1996; Zimmermann et al., 1996). PD 166285, unlike these aforementioned inhibitors, is a nonselective protein tyrosine kinase. The differences in potencies for PD 166285 between the various protein 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.

The inhibitory potency of PD 166285 was also apparent in its effects on viable cells. PD 166285 inhibited PDGF- and EGF-stimulated receptor autophosphorylation and bFGF re-
receptor-mediated tyrosine kinase phosphorylation in cells in culture, confirming a tyrosine kinase mechanism of inhibition. Interestingly, the potency of PD 166285 for inhibition of PDGFR autophosphorylation in cells was >10-fold greater than its potency for inhibition of the recombinant PDGFR tyrosine kinase activity. The reasons for this disparity are unclear but could involve cellular processes that are not present in the in vitro recombinant kinase assays, such as inhibitor localization, subcellular compartmentalization and/or availability and accessibility of enzyme, substrates, cations and pH. Alternatively, the potent inhibition of PDGFR autophosphorylation in cells by PD 166285 could be due to the simultaneous inhibition of intracellular PDGFR-β and c-Src tyrosine kinase activities. We have demonstrated that PD 166285 is a potent inhibitor of both c-Src and PDGFR-β recombinant protein tyrosine kinases. In addition, it was recently shown that Tyr934 in the kinase domain of the PDGFR-β is phosphorylated by c-Src in cells (Hansen et al., 1996). Thus, by inhibiting the ability of Src kinase to phosphorylate the PDGFR-β and by directly inhibiting PDGFR-β autophosphorylation in the same cells, PD 166285 could achieve enhanced cellular inhibitory efficacy.

To examine the kinetics of inhibition of intracellular tyrosine phosphorylation by PD 166285, we performed additional experiments in VSMCs with PDGFR-β autophosphorylation measurements taken as representative of growth factor receptor tyrosine kinase activity. VSMCs were exposed to PD 166285, and an assessment was made of the duration of inhibition of PDGF-induced receptor autophosphorylation and the intracellular concentrations of PD 166285 retained in cells over the next 96 hr. Despite the removal of PD 166285 from the culture medium immediately after the 1-hr incubation, maximal inhibition of PDGFR autophosphorylation could be maintained but was both concentration and time dependent. These data indicate that when exposed to cells for a short time, PD 166285 can achieve intracellular concentrations of sufficient quantity to maintain a long-lasting inhibition of PDGFR tyrosine phosphorylation. In addition, the failure of cells to express more PDGFRs after the 10 μM exposure of PD 166285 suggests that PD 166285 also sustained VSMC growth because these experiments were performed with cells maintained continuously in serum. Thus, PD 166285 may be a suitable candidate for use in local drug delivery applications where sustaining sufficient intracellular drug concentrations for prolonged periods of time may be necessary to achieve the desired effect.

The regulation of cell adhesion, migration and proliferation is thought to involve specific components of the ECM as well as proteolytic modification of the ECM. The matrix-degrading MMPs are thought to be primarily responsible for the turnover of ECM components. The gelatinases, a subgroup of MMPs that comprise 72- and 92-kDa gelatinases, efficiently degrade type IV collagen (Wilhelm et al., 1989), the major structural component of basement membranes. Thus, gelatinases are implicated in removing the first ECM barrier to migration and proliferation of VSMCs. In the present study, we investigated the effects of PD 166285 on the capacity of VSMCs to express 72- and 92-kDa gelatinases in the basal state or after stimulation. The results showed that PD 166285 was unique in its ability to potently inhibit phorbol ester-mediated production of 92-kDa gelatinase from VSMCs via a mechanism that appears to be unrelated to a direct catalytic site inhibition of the enzyme, such as that produced by BB94. Instead, PD 166285 may be interacting at the level of transcriptional regulation of 92-kDa gelatinase expression in RBRAs. There are binding sites for both activator protein-1 and nuclear factor-κB transcription factors in the conserved regions of the rabbit 92-kDa gelatinase gene promoter (Fini et al., 1994). In addition, several growth factors and cytokine regulatory pathways converge at the activator protein-1 binding site on the 92 kDa gelatinase gene promoter which also constitutes the phorbol ester response element (Angel et al., 1987). Thus, the exact mechanism of PD 166285 inhibition is unclear and may be independent of its effects on tyrosine kinase inhibition. However, the effects of PD 166285 on 92 kDa gelatinase production by phorbol ester appear to be consistent with the proposed regulatory roles of activator protein-1 and nuclear factor-κB.

The adhesive interactions between cells and the ECM are mediated by integrins, a family of cell surface receptors that bind to ECM proteins, including collagen type I, fibronectin and vitronectin (Hynes, 1992). Activation of integrin receptors on binding to specific ECM ligands has been shown to enhance tyrosine phosphorylation of several proteins, including MAPK (Chen et al., 1994) and focal adhesion kinase (pp125 FAK), previously identified as a substrate for c-Src tyrosine kinase (Cobb et al., 1994). Furthermore, recent evidence suggests that coordinated regulation of these signaling molecules is central to the control of cell adhesion, formation of the actin cytoskeleton and activation of intracellular signaling cascades (Parsons, 1996). Because PD 166285 was a potent inhibitor of c-Src kinase, broadly active against several other kinases (i.e., MAPK), and an inhibitor MMP production, we reasoned that PD 166285 might affect adhesion of VSMCs with certain ECM proteins. The ECM proteins collagen type I, fibronectin and vitronectin promoted maximal attachment to both RBRAs and BAECs, and PD 166285 specifically inhibited adhesion of both vascular cell types to vitronectin, whereas adhesion of cells to either collagen type I or fibronectin was relatively unaltered. PD 166285 was >1 log order more potent than the cyclical PenRGD peptide Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala, which is a specific inhibitor of vitronectin binding to the vitronectin receptor alpha-v beta-3. The mode of inhibition by PD 166285 probably is not via a direct interaction with the ligand binding site (Arg-Gly-Asp, or RGD) on the alpha-v beta-3 receptor surface, as is the interaction with the PenRGD peptide. Instead, we speculate that PD 166285 may be inhibiting tyrosine phosphorylation of the beta-3 subunit of the vitronectin receptor alpha-v beta-3. Recent studies by Blystone et al. (1996) have clearly shown that tyrosine phosphorylation of the integrin beta-3 cytoplasmic tail is induced by adhesion to the alpha-v beta-3-specific ligand vitronectin. It has not yet been established whether beta-1 integrins such as alpha-v beta-3, which binds collagen, or alpha-5 beta-1, which binds fibronectin, contain tyrosine residues that can be phosphorylated. The lack of tyrosines on beta-1 subunits could in part explain the lack of effect of PD 166285 on inhibition of collagen or fibronectin adhesion to vascular cells.

In addition to its inhibitory effects on vascular cell adhesion, PD 166285 was a potent inhibitor of PDGF-induced migration of VSMCs through a simple matrix of type I collagen or vitronectin as well as a complex basement membrane, Matrigel, which is made up of several ECM components,
including collagens, laminin and proteoglycans. The ability of PDGF to stimulate cell migration is not well understood; however, a number of signaling pathways have been implicated, including PDGF-induced receptor autophosphorylation and the subsequent activation of downstream proteins, such as MAPK, as well as signaling pathways activated by cell surface integrin receptors.

PD 166285 inhibited growth factor-stimulated DNA synthesis and serum-induced growth of VSMCs, presumably via its effects on suppression of growth factor-induced receptor tyrosine kinase phosphorylation in these cells. Because of its broad activity as a protein tyrosine kinase inhibitor, the effects of PD 166285 on growth factor-induced functional responses could also be due to inhibition of other unknown protein kinases associated with growth factor receptor signaling.

In summary, we have described PD 166285 as a novel, nanomolar potency and broadly active ATP competitive inhibitor of protein tyrosine kinases, a highly potent inhibitor of protein tyrosine-mediated cellular functions including growth, adhesion, migration and MMP production. Furthermore, PD 166285 is a molecule that demonstrates prolonged intracellular retention and persistent inhibition of growth factor-mediated cellular phosphorylation and growth. The profile of PD 166285 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, where multiple growth factor and cytokine signal transduction pathways are likely to be activated.

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