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In Vitro Characterization of a Novel Series of Platelet-Derived Growth Factor Receptor Tyrosine Kinase Inhibitors

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ABSTRACT. In this report, we describe the discovery and characterization of a novel biarylhydrazone series of platelet-derived growth factor (PDGF) receptor tyrosine kinase inhibitors typified by the prototype WIN 41662 (3-phenyl-N^1-[1-(4-pyridyl)pyrimidine]hydrazone). WIN 41662 inhibited PDGF-stimulated autophosphorylation of PDGF receptors from human vascular smooth muscle cells (hVSMC) with an IC_{50} value of 60 nM. The inhibitor appeared to be competitive with respect to substrate (Mn^{2+}-ATP), having a calculated K_i of 15 ± 5 nM. WIN 41662 was approximately 500-fold more potent in inhibiting the PDGF receptor tyrosine kinase than the $p56^{lck}$ tyrosine kinase. It was inactive against other serine/threonine and tyrosine kinases tested. WIN 41662 produced concentration-dependent inhibition of PDGF-stimulated receptor autophosphorylation in intact hVSMC with an IC_{50} < 100 nM. Intracellular Ca^{2+} mobilization and cell proliferation were events that occurred in hVSMC subsequent to PDGF receptor activation. WIN 41662 inhibited PDGF-stimulated Ca^{2+} mobilization and cell proliferation ([^{3}H]TdR incorporation) with IC_{50} values of 430 nM and 2.3 μM, respectively. These effects appeared to be specifically related to PDGF receptor tyrosine kinase inhibition since WIN 41662 was not cytotoxic (*in vitro*) and since WIN 72039, a close structural analog that does not inhibit PDGF receptor tyrosine kinase, also did not inhibit PDGF-stimulated receptor autophosphorylation, Ca^{2+} mobilization, or hVSMC proliferation. Thus, WIN 41662 is representative of a novel class of selective PDGF receptor tyrosine kinase inhibitors that inhibit PDGF-regulated secondary events in intact cells. BIOCHEM PHARMACOL 51;12:1631–1638, 1996.

KEY WORDS. platelet-derived growth factor; platelet-derived growth factor receptor; tyrosine kinase; platelet-derived growth factor receptor tyrosine kinase inhibitors; vascular smooth muscle cells

Human PDGF,¶ released from platelets during clotting, is a major plasma growth factor. The molecular mass of PDGF ranges from 28 to 35 kDa, and it exists in three biologically active forms: the heterodimer (PDGF-AB) and homodimers of the PDGF A and B chains [1]. PDGF binds to a specific cell surface receptor with a molecular mass of approximately 180 kDa [2]. There are two major PDGF receptor isoforms, α and β, which can form functional heterodimeric and homodimeric complexes [3]. These receptors contain several domains including an external PDGF-binding domain, a single transmembrane region, and a cytoplasmic portion containing a catalytic domain with tyrosine kinase activity [4].

PDGF homo- and heterodimers bind to the PDGF receptor isoforms with high affinity (K_D < 1 nM), producing a variety of physiological responses. Subsequent to PDGF binding, the PDGF receptor tyrosine kinase is activated, resulting in autophosphorylation of the cytoplasmic domain [5] as well as the phosphorylation and activation of several key enzymes including PLC-γ, phosphatidylinositol-3-kinase, and Raf-1 [6–8]. PLC-γ activation results in the mobilization of intracellular Ca^{2+} and the activation of additional second messenger systems [9]. Key physiological activities resulting from PDGF receptor activation include cell mitogenesis and proliferation, cell migration, expression of extracellular matrix proteins, and vasoconstriction.

In vascular tissue, PDGF mediates smooth muscle cell

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¶ *Abbreviations:* ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate); FGF, fibroblast growth factor; $hVSMC_i$, immortalized human vascular smooth muscle cells; $hVSMC_p$, primary hVSMC; INDO-1 AM, INDO-1 acetoxymethyl ester; LDH, lactate dehydrogenase; PDGF, platelet-derived growth factor; PK-A, cyclic AMP-dependent protein kinase; PK-C, protein kinase C; PLC-γ, phospholipase C-γ; SmBM, smooth muscle cell basal medium; SmGM, smooth muscle cell growth medium; [^{3}H]TdR, [^{3}H]thymidine; and TGF, transforming growth factor.

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proliferation and chemotaxis. One of the characteristics of atherosclerosis is the proliferation and migration of smooth muscle cells from the medial layer to the intimal layer of the vessel [10, 11]. PDGF, basic FGF, and TGF-β all produce a pattern of arterial wall thickening and smooth muscle cell migration consistent with the development of atherosclerosis and may be critical in the pathogenesis of vascular wall diseases [12]. Inhibition of PDGF receptor tyrosine kinase may attenuate the proliferative effects of the growth factor under pathological conditions [13].

Initial inhibitors of PDGF receptor tyrosine kinase, the tyrphostins, are neither extremely potent nor selective for PDGF relative to other tyrosine kinases. Accordingly, we sought to identify novel, potent (IC_{50} < 1 μM), and selective inhibitors of PDGF receptor tyrosine kinase activity. A series of pyridinyl quinolines meeting these criteria was identified from our initial screening efforts [14]. In this report, we present biological data on a second, novel biarylhydrazone class of selective PDGF receptor tyrosine kinase inhibitors, typified by WIN 41662 (3-phenyl-N^1-[1-(4-pyridyl)pyrimidine]hydrazone).

MATERIALS AND METHODS

Materials

$hVSMC_i$, isolated from the aorta of a 51-year-old man [15], were obtained from the Fred G. Hutchinson Cancer Institute (Seattle, WA). $hVSMC_p$, isolated from the aorta of a 21-year-old man (strain AOSMC-2176), SmGM and SmBM were obtained from the Clonetics Corp. (San Diego, CA). PDGF-BB (recombinant human homodimer), polyclonal anti-PDGF receptor antibody (No. 06-131), and biotinylated monoclonal antiphosphotyrosine antibody (No. 05-321) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Streptavidin-horseradish peroxidase was obtained from the Pierce Chemical Co. (Rockford, IL). An ABTS peroxidase substrate detection system was obtained from Kirkegaard & Perry, Inc. (Gaithersburg, MD). Dipotassium ATP (No. 1190) was obtained from Calbiochem, Inc. (La Jolla, CA). INDO-1 AM was purchased from Molecular Probes, Inc. (Eugene, OR). [^{3}H]TdR (20 Ci/mmol) was obtained from Dupont/NEN (Boston, MA; Catalog No. NET-027X). Protease-free BSA and LDH assay reagents (Catalog No. DG1340-K) were obtained from the Sigma Chemical Co. (St. Louis, MO). Geneticin (G418 sulfate) was from Gibco BRL Life Technologies, Inc. (Grand Island, NY), All other chemicals and reagents were obtained as indicated in Materials and Methods. The biarylhydrazones reported here were synthesized at Sterling Winthrop Pharmaceuticals Research Division, Collegeville, PA, according to the method of Coppola *et al.* [16].

Cell Culture

$hVSMC_i$ and $hVSMC_p$ were cultured in Falcon Primaria tissue culture dishes in SmGM, which consists of MCDB 131 medium supplemented with fetal bovine serum (5%), human epidermal growth factor (10 ng/mL), human fibroblast growth factor-B (2 ng/mL), dexamethasone (0.4 μg/mL), gentamicin (50 μg/mL), and amphotericin-B (50 ng/mL). The growth medium for $hVSMC_i$ was supplemented additionally with G418 (0.6 mg/mL) to prevent loss of the immortalized phenotype. $hVSMC_i$ were subcultured once a week at a 1:20 dilution, with changes of medium every second or third day. $hVSMC_p$ were received as cryopreserved tertiary cultures and used for experiments in the fifth passage.

PDGF Receptor Tyrosine Kinase Assay

PDGF receptor tyrosine kinase activity was determined in an ELISA format measuring PDGF-stimulated receptor autophosphorylation as previously described [17]. PDGF receptors were isolated by wheat germ lectin purification from Triton X-100 extracts of immortalized $hVSMC_i$. Partially purified receptor preparations were incubated overnight at 4° in 96-well plates (Beckman No. 267001) with PDGF-BB (150 ng/mL) in the presence of 4 mM $MnCl_2$, 50 mM HEPES (pH 7.4), 10% glycerol, 0.2% Triton X-100, 0.1 mM sodium vanadate, and 0.5 mM dithiothreitol (total volume = 65 μL) to fully activate the receptor. Test compounds (10-μL aliquots) were incubated with the PDGF–PDGF receptor complex for 30 min at 4° prior to the addition of ATP. All test compounds were dissolved in DMSO. Autophosphorylation of the receptor was initiated at 4° by the addition of 10 μM ATP, and the assay was terminated after 10 min by the addition of 20 mM EDTA. Aliquots of the reaction mixture were transferred to a second 96-well plate (Nunc Maxisorb No. 4-68-667) previously coated with a polyclonal antibody directed against the cytoplasmic domain of the PDGF receptor. Following a 1-hr incubation at room temperature, the wells were washed three times with DuPont plate wash solution to remove unbound receptor. Phosphorylated tyrosine residues on the captured receptors were detected by incubation with biotinylated antiphosphotyrosine monoclonal antibodies followed by incubation with strepavidin-horseradish peroxidase. The bound antiphosphotyrosine antibody-strepavidin complexes in each well were detected at 25° in a spectrophotometric plate reader (OD = 405 nm) using an ABTS detection system.

PDGF Receptor Autophosphorylation in Intact Cells

$hVSMC_i$ were grown to confluence in 24-well dishes with SmGM. The cells were washed twice with serum-free medium and incubated overnight in that medium to arrest growth. On the day of the experiment, the medium was removed and replaced with 150 μL of fresh medium in the absence or presence of test compound. After 3 min, medium with or without PDGF-BB (100 ng/mL final concentration) was added to each well for an additional 3 min. The reaction mixture was removed by aspiration and replaced with 100 μL of lysis buffer containing 10% glycerol,

10 mM HEPES (pH 7.5), 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 mM sodium vanadate. After 15 min, each 100-μL aliquot was diluted in half with 2× SDS–PAGE sample buffer and to each sample was added 10 μL of 2-mercaptoethanol. The samples were heated to 95° for 3 min and then separated on 4 to 15% SDS–PAGE gradient gels (Bio-Rad Laboratories, Richmond, CA). The proteins were transferred to nitrocellulose (Millipore, Inc., Bedford, MA) and probed with anti-phosphotyrosine antibodies using a Promega Protoblot western blot system.

PDGF-Stimulated Ca^{2+} Mobilization

Confluent $hVSMC_i$ in 100 mm dishes were rinsed twice with serum-free medium (SmBM plus 1.4 mg/mL protease-free BSA) and incubated in that medium for 24 hr prior to determining intracellular calcium concentrations. The cells were loaded with INDO-1 by replacement of the medium with (mM): NaCl (145), KCl (5), $NaHCO_3$ (2.3), $MgSO_4$ (1.12), $CaCl_2$ (1.2), HEPES (10), dextrose (10), INDO-1 AM (0.002), sulfinpyrazone (0.1), BSA (0.1 mg/mL), pH 7.55, at 37° for 30 min. The cells were removed from the plate by gentle trypsinization and resuspended in the same solution without INDO-1. Cells (1×10^6 in 1 mL) were transferred to a cuvette maintained at 37° in a Perkin-Elmer LS-50 luminescence spectrometer. Cells were preincubated with test compounds for 5 min prior to the addition of 30 ng/mL PDGF-BB; responses were monitored for 10 min thereafter. The fluorescence intensities at the emission wavelengths of 390 and 485 nm were recorded with an excitation wavelength of 345 nm. The emission ratio (390 nm/485 nm) was calculated and converted to calcium concentration by comparison with the emission ratios of a standard solution containing a known concentration of free calcium [18]. For each cuvette, ionomycin (10 μM) and EGTA (100 μM) were added to determine R_{max} and R_{min}, respectively, thereby allowing a correction to be made for variations in cell concentrations between samples.

PDGF-Stimulated hVSMC Proliferation

PDGF-stimulated VSMC proliferation was assessed by measuring [^{3}H]TdR incorporation as an index of DNA synthesis. $hVSMC_p$ were grown in 24-well plates for 3 days from an initial density of 10,000 cells/well. The subconfluent cells were rinsed twice with serum-free medium and incubated in that medium for 48 hr. After changing to fresh medium, cells were incubated with or without inhibitor for 2 hr, followed by addition of PDGF-BB (10 ng/mL) for 24 hr and [^{3}H]TdR (0.5 μCi/well) for an additional 24 hr. Extremely low levels of [^{3}H]TdR uptake were observed in the absence of PDGF. Following the 48-hr incubation period, the medium was aspirated and the cells were washed twice gently with 0.5 mL of ice-cold 5% trichloroacetic acid. The cells were extracted with 0.1 N aqueous NaOH (0.25 mL, 30 min, 25°), which was neutralized with 1.0 N aqueous HCl and counted in a liquid scintillation counter. The percentage inhibition for each concentration of inhibitor was calculated by comparison to vehicle-treated (<0.5% DMSO) cells.

LDH Release Assay

The release of LDH into the extracellular medium following exposure of cells to test compound was measured as an index of cytotoxicity. Primary hVSMCs were incubated with PDGF and increasing concentrations of test compound for 48 hr, identical to the conditions used for the cell proliferation assay except that [^{3}H]TdR was omitted. At the end of the incubation period, a 10-μL aliquot of the cell culture medium was transferred to a 96-well microtiter assay plate to measure extracellular LDH activity. The remaining medium was aspirated and replaced with Hanks' balanced salt solution containing 1% Triton X-100 for 30 min to lyse the cells. A second aliquot was assayed for intracellular LDH activity. The assay, which measures the reduction of NAD^+, was performed in a kinetic plate reader according to manufacturer's directions.

Data Analysis

The IC_{50} values from concentration–response curves, representing data normalized to control were calculated using a four-parameter logistic equation [19] and represent the means ± SEM of 3–4 experiments. K_i values were determined using a competitive fit for the Michaelis–Menten equation in NLIN on a Vax computer as previously described by Faltynek *et al.* [20].

RESULTS

PDGF Receptor Tyrosine Kinase ELISA

An ELISA was developed to measure PDGF receptor tyrosine kinase activity for identification of inhibitors from synthetic chemical and natural products libraries. Autophosphorylation of partially purified PDGF receptors from $hVSMC_i$, detected using an anti-phosphotyrosine monoclonal antibody, was linear for up to 10 min (Fig. 1). Receptor autophosphorylation was not detected in this assay in the absence of either ATP or PDGF. The K_m for Mn^{2+}-ATP in this assay was 4.2 ± 0.7 μM (N = 7), while the K_m for Mg^{2+}-ATP was approximately 25-fold higher (K_m = 112 μM; N = 2). ATPγS demonstrated competitive inhibition of tyrosine kinase activity with a calculated K_i of 6.7 μM (data not shown).

The chemical structures and tyrosine kinase inhibitory activities for related biarylhydrazones are presented in Table 1. The most active analog in this series, WIN 41662, inhibited PDGF receptor autophosphorylation in the ELISA with an IC_{50} of 60 nM (Fig. 2, top) at a substrate (Mn^{2+}-ATP) concentration of 10 μM ([S]/K_m = 2). The parent hydrazine, WIN 45838, was much less active (IC_{50} = 7.4 μM) and the hydrazide analog, WIN 72039, was com-

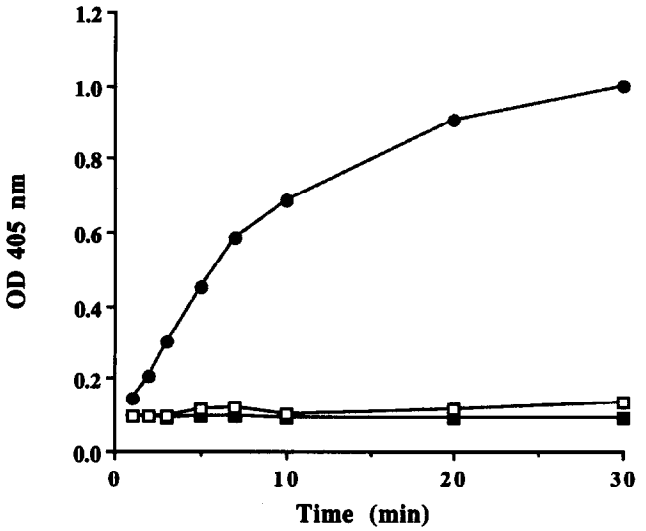


FIG. 1. Kinetics of PDGF receptor tyrosine kinase activity in an ELISA format. The PDGF receptor was incubated for the indicated periods of time with 7 μM ATP under the following conditions: -ATP/+PDGF (□); +ATP/-PDGF (■); and +ATP/+PDGF (●).

pletely inactive. At higher ATP concentrations, the potency of WIN 41662 was decreased. An IC_{50} of 840 nM was obtained in the ELISA at an ATP concentration of 200 μM ([S]/K_m = 50). A linear relationship between the IC_{50} for WIN 41662 inhibition of kinase activity and the concentration of ATP in the assay was observed consistent with a mechanism of competitive inhibition with respect to substrate. Michaelis–Menten analysis of WIN 41662 inhibition of kinase activity as a function of ATP indicated that the data best fit a competitive model of inhibition (Fig. 2, bottom). The calculated K_i value for WIN 41662 was 15 ± 5 nM (N = 4).

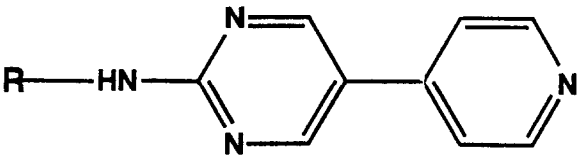
PDGF Receptor Autophosphorylation in Intact Cells

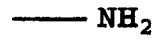
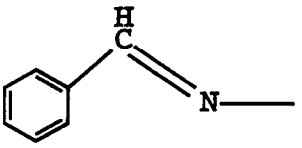
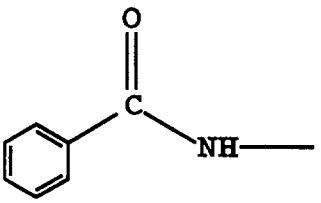
PDGF stimulated the phosphorylation of a 185 kDa protein at PDGF concentrations between 10 and 100 ng/mL in $hVSMC_i$ (Fig. 3A). WIN 41662 produced a concentration-dependent inhibition of this effect with an estimated IC_{50} between 30 and 100 nM (Fig. 3B). WIN 72039, an inactive biarylhydrazone, did not inhibit PDGF receptor autophosphorylation at concentrations up to 100 μM (Fig. 3C).

PDGF-Stimulated Ca^{2+} Mobilization

Exposure of INDO-1-loaded cells to PDGF resulted in a transient increase in intracellular calcium ($Ca^{2+}{}_i$). The peak increase was related to the concentration of PDGF; an EC_{50} of 7 ng/mL was observed for PDGF-BB in this assay (Fig. 4A). WIN 41662 produced concentration-dependent inhibition of Ca^{2+} mobilization with an IC_{50} of 430 nM in cells stimulated with 30 ng/mL PDGF-BB (Fig. 4B). In contrast, WIN 72039 was ineffective at concentrations up to 300 μM (data not presented).

TABLE 1. Structure–activity relationships for inhibition of PDGF receptor autophosphorylation by biarylhydrazone-based tyrosine kinase inhibitors



WIN no.	R	IC_{50} (μM)
45838		7.4 ± 0.1
41662		0.06 ± 0.002
72039		Inactive at 100 μM

The IC_{50} values for inhibition of PDGF receptor autophosphorylation in an ELISA were determined as described in Materials and Methods. The data for each compound are means ± SEM of at least 3 independent determinations.

$hVSMC_p$ Proliferation

[^{3}H]TdR incorporation, an index of DNA synthesis, was stimulated in a concentration-dependent manner by PDGF-BB with an EC_{50} of 2.0 ng/mL (Fig. 5A). This effect of PDGF was inhibited by WIN 41662 with an IC_{50} of 2.0 ± 0.4 μM. WIN 72039 was essentially inactive, demonstrating only 5% inhibition at 46 μM (Fig. 5B). Experiments in which cells were prelabeled with [^{3}H]TdR prior to exposure to test compounds produced similar results, thereby ruling out any possible effects on [^{3}H]TdR uptake systems. Treatment of cells with WIN 41662 or WIN 72039 (up to 30 μM) did not result in a detectable increase in extracellular LDH activity, a marker of cytotoxicity. The nonselective tyrosine kinase inhibitor staurosporine, under identical assay conditions, produced a 4-fold increase in extracellular LDH activity at 1μM.

Tyrosine and Serine/Threonine Kinase Selectivity of WIN 41662

To determine the selectivity of WIN 41662 for the PDGF receptor tyrosine kinase, the compound was tested against

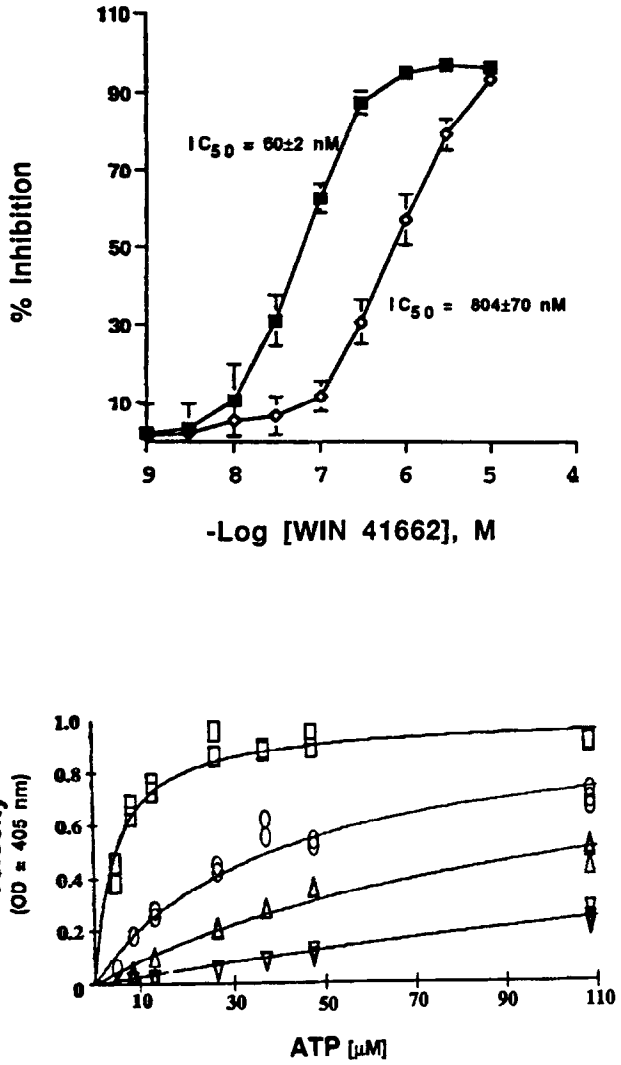


FIG. 2. (Top) Inhibition of PDGF receptor tyrosine kinase activity by WIN 41662. Increasing concentrations of WIN 41662 (as indicated) were incubated with PDGF receptor in the presence of 10 μM (■) or 200 μM (◇) ATP. The data are means ± SD from 3 independent experiments. (Bottom) Inhibition of PDGF receptor tyrosine kinase activity as a function of various ATP concentrations, determined in the absence (■) or presence of 0.1 μM (○), 0.3 μM (△), and 1.0 μM (▽) WIN 41662. The data are the best nonlinear regression fits to a model of competitive inhibition as described in Materials and Methods where the K_m for ATP is 4 μM. The calculated K_i for WIN 41662 from 4 similar experiments was 15 ± 5 nM.

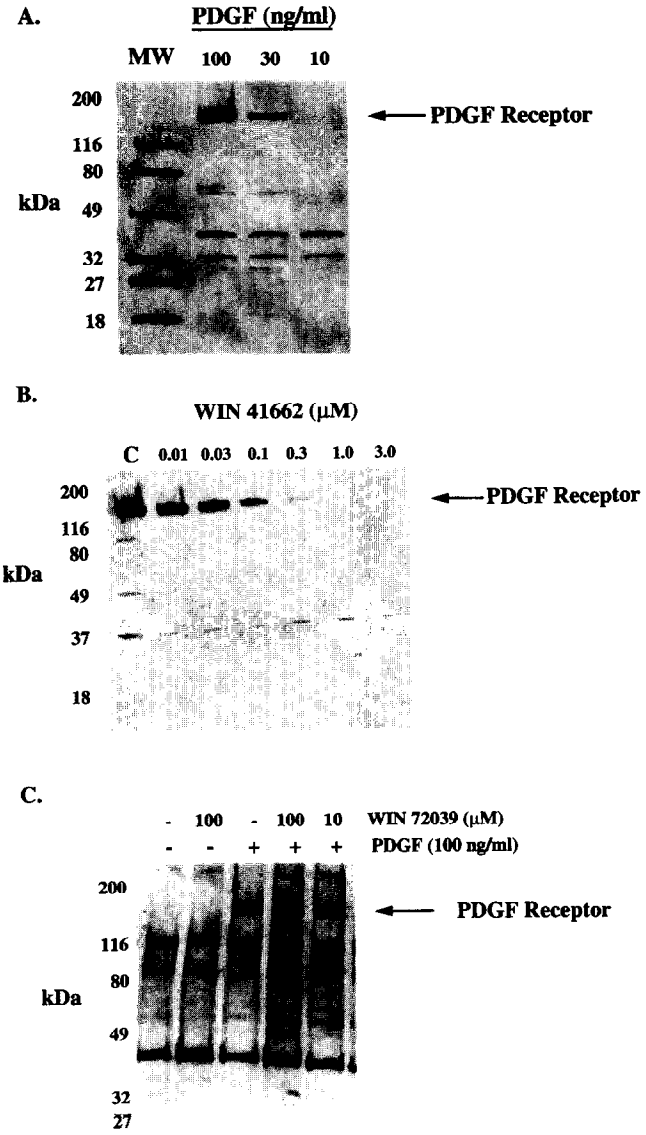


FIG. 3. (A) Western blot of PDGF receptor autophosphorylation in intact $hVSMC_i$. $hVSMC_i$ were incubated with PDGF (as indicated) for 3 min, and phosphotyrosine residues were visualized by western blotting as described in Materials and Methods. (B) Inhibition of PDGF receptor autophosphorylation by WIN 41662 in intact $hVSMC_i$. $hVSMC_i$ were incubated with PDGF-BB (100 ng/mL) in the absence (C = control) or presence of increasing concentrations of WIN 41662 as indicated. Phosphotyrosine residues were visualized by western blotting as described in Materials and Methods. The data are from a typical experiment which was repeated three times. (C) Effect of WIN 72039 on PDGF receptor autophosphorylation. $hVSMC_i$ were incubated with PDGF-BB (100 ng/mL) in the absence or presence of WIN 72039, as indicated. Phosphotyrosine residues were visualized by western blotting as described in Materials and Methods.

several tyrosine and serine/threonine kinases (Table 2). WIN 41662 was inactive against the serine/threonine kinases, PK-C and PK-A, and was also inactive against the EGF receptor and *erb*B_2 tyrosine kinases. Although WIN 41662 did produce modest inhibition of $p56^{lck}$ tyrosine kinase activity, it was a much more potent inhibitor of the PDGF receptor tyrosine kinase. The IC_{50} for inhibition of $p56^{lck}$, under conditions where the $[S]/K_m$ ratios for the PDGF and $p56^{lck}$ tyrosine kinases were similar, was 30 μM (Fig. 6). This was approximately 500-fold less potent than the IC_{50} of WIN 41662 for the PDGF receptor tyrosine kinase. These data suggest that WIN 41662 is selective for the PDGF receptor.

DISCUSSION

There have been significant advances in the discovery and development of selective tyrosine kinase inhibitors (for a

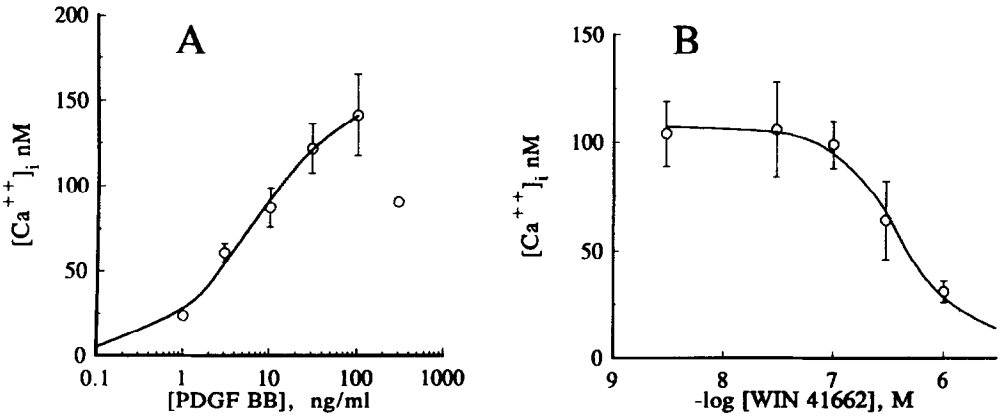


FIG. 4. (A) PDGF-stimulated Ca^{2+} mobilization in hVSMC. (A) $hVSMC_i$, preloaded with INDO-1 as described in Materials and Methods, were incubated with increasing concentrations of PDGF-BB. The Y-axis represents the concentration of Ca^{2+} mobilized over basal levels in response to PDGF stimulation. The data points are means (±SEM) from 5 independent experiments. (B) WIN 41662 inhibition of PDGF-stimulated Ca^{2+} mobilization in hVSMC. $hVSMC_i$ were preincubated for 5 min with WIN 41662 at the indicated concentrations prior to stimulation with 30 ng/mL PDGF-BB. The data are means ± SEM of 5 independent experiments from which an IC_{50} = 430 nM was determined.

recent review, see Ref. 22). Some of the earliest compounds were natural products based on a flavonoid nucleus [23, 24] as exemplified by quercetin-like analogues. While these compounds demonstrate tyrosine kinase inhibitory activity, they are generally nonselective in that they also inhibit serine/threonine kinases.

Recently two groups have reported unique chemical series based on substituted quinoline derivatives. Maguire *et al.* [25] reported that 3-substituted pyridinyl quinolines, derived from a cyclized 2,3-diarylacrylonitrile nucleus, demonstrate potent inhibition of human PDGF receptor autophosphorylation. In a series of these analogues, IC_{50} values in the 8–800 nM range were found. These compounds were selective for the PDGF receptor tyrosine kinase with respect to the EGF receptor tyrosine kinase; activities against other tyrosine kinases were not reported.

Dolle *et al.* [14] reported a similar series of compounds based on a 4-substituted pyridinyl quinoline nucleus. The most active analog in this series, 5,7-dimethoxy-3-(4-pyridinyl)quinoline, inhibits PDGF receptor autophosphorylation with an IC_{50} of 80 nM. This compound appears to be competitive for the ATP site and demonstrates a K_i of 14 ± 2 nM for inhibition of enzyme activity. Compounds in this series are selective for the PDGF receptor with respect to the EGF receptor, $p56^{lck}$ or *erb*B_2 tyrosine kinases and other protein kinases such as PK-C and PK-A. Inhibition of PDGF-mediated hVSMC proliferation was also observed with analogs in this series (IC_{50} range = 0.9 to >10 μM).

In this report, we present a structurally distinct class of PDGF receptor tyrosine kinase inhibitors based on a biarylhydrazone nucleus. The most active compound in this series, WIN 41662, inhibited PDGF receptor tyrosine kinase activity with an IC_{50} of 60 ± 2 nM. WIN 41662 appeared to be competitive for the ATP binding site of the PDGF receptor tyrosine kinase; a calculated K_i value of 15 ± 5 nM was observed for inhibition of kinase activity by this proposed mechanism. The parent hydrazine, WIN 45838, had only modest affinity in the isolated PDGF receptor tyrosine kinase assay (IC_{50} = 7 μM) and had no effect on PDGF-stimulated hVSMC proliferation. The hydrazide analog WIN 72039 was inactive in all PDGF receptor assays in which it was tested. In molecular modeling studies, when the pyridyl-pyrimidine nucleus of WIN 41662 and WIN 72039 were maintained in the same plane, a different orientation of the phenyl group was observed. We believe this change in orientation may result in the decreased tyrosine kinase activity observed for WIN 72039.

Consistent with the activity of the molecule against the isolated receptor, WIN 41662 inhibited PDGF-stimulated receptor autophosphorylation and Ca^{2+} mobilization in intact hVSMC with IC_{50} values of less than 1 μM. These cellular inhibitory effects were not observed with the inactive analog, WIN 72039. In addition, WIN 41662 was selective for the PDGF receptor with respect to serine/

TABLE 2. Selectivity of WIN 41662 for the PDGF receptor tyrosine kinase

Protein kinase	% Inhibition	$[S]/K_m$
PDGF receptor	98	2.5
EGF receptor	3	13.0
$p56^{lck}$	23	1.5
erbB_2	-7	1.0
PK-A	8*	5.0
PK-C	14*	5.0

The activity of WIN 41662 at the PDGF receptor tyrosine kinase was determined as described in Materials and Methods. The activities for WIN 41662 against the other kinases were determined as described previously [21]. The substrate K_m (μM) for ATP in each assay was: PDGF (4); EGF (11); $p56^{lck}$ (15); *erb*B_2 (0.5); PK-A (14); and PK-C (112). WIN 41662 was tested at a concentration of 10 μM except where indicated (*: 200 μM).

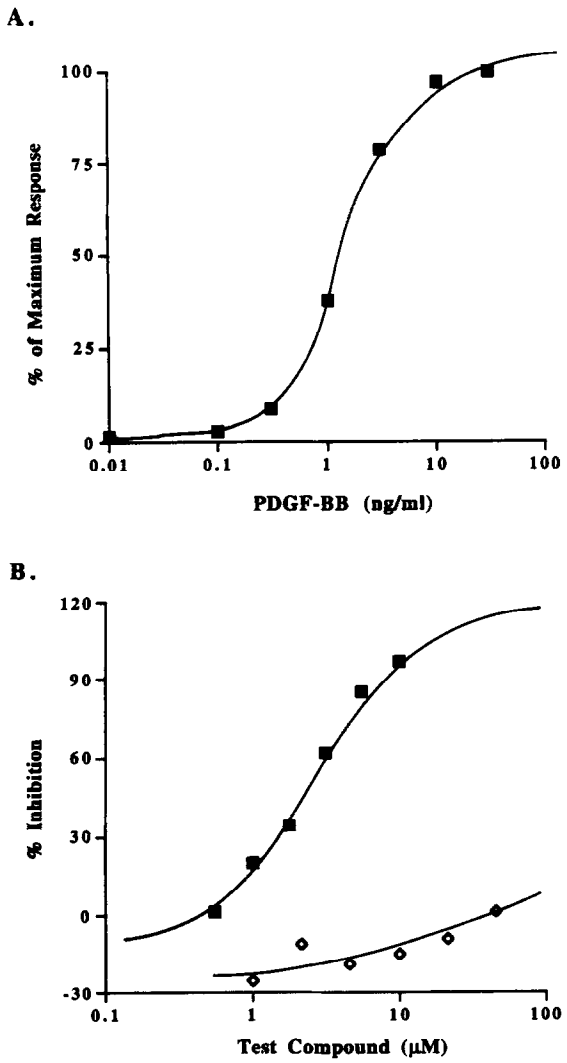


FIG. 5. (A) PDGF stimulation of [^{3}H]TdR incorporation into hVSMC$_p$. The EC_{50} for the PDGF effect in this experiment was 2 ng/mL. (B) Inhibition of PDGF-stimulated [^{3}H]TdR incorporation into hVSMC$_p$ by WIN 41662. hVSMC$_p$ were incubated with PDGF-BB (10 ng/mL) and increasing concentrations of WIN 41662 (■) or WIN 72039 (◇), as indicated. Data are from a representative experiment, which was repeated three times.

threonine kinases and three other tyrosine kinases. This implies that inhibition of PDGF-mediated cellular events by WIN 41662 is related to inhibition of the PDGF receptor tyrosine kinase. However, the effect of WIN 41662 on representatives of other families of tyrosine kinases should be examined to further define the selectivity profile for this inhibitor.

Vascular smooth muscle cell proliferation is a critical part of the development of an atherosclerotic plaque [10]. Inhibition of this proliferative response may also be beneficial in preventing the development of lesions that contribute to restenosis following angioplasty or arterial bypass grafts. As a measure of hVSMC proliferation, we assessed the stimulation of incorporation of [^{3}H]TdR into hVSMC by PDGF and the effect of WIN 41662 on this response. PDGF stimulated [^{3}H]TdR incorporation in these cells with an EC_{50} of 2 ng/mL. Inhibition of this response was observed with WIN 41662; an IC_{50} value of 2 μM was determined from concentration–response curves. These data suggest that attenuation of PDGF-mediated proliferative responses in hVSMC may be achieved *in vivo* with WIN 41662. The data presented on [^{3}H]TdR incorporation were obtained with hVSMC$_p$ (fifth passage), whereas all of the other assays were performed with hVSMC$_i$. We observed high levels of basal [^{3}H]TdR incorporation by the immortalized cells, making quantitation of PDGF stimulation and inhibition of the cellular response by test compounds difficult.

Cell cytotoxicity was assessed by measuring the level of LDH activity in cell culture supernatants under test conditions employed in the hVSMC proliferation assay. WIN 41662 and WIN 72039 (3–30 μM) did not produce an increase in LDH activity compared with control during 48 hr of incubation with hVSMC. These results suggest that the *in vitro* cytotoxicity of this series of compounds may be negligible.

At present, we do not understand the observed decrease in potency of WIN 41662 in the cell proliferation assay compared with PDGF-stimulated autophosphorylation and Ca^{2+} mobilization. In part, this discrepancy may be explained by the different cell lines that were used. It is possible that the concentration of PDGF receptors, or the local concentration of ATP in the vicinity of the receptor, may be higher in hVSMC$_p$ than in hVSMC$_i$, either of which could decrease the apparent potency of this ATP-competitive inhibitor [26]. Conversely, it may be necessary to occupy a greater percentage of PDGF receptors to pro-

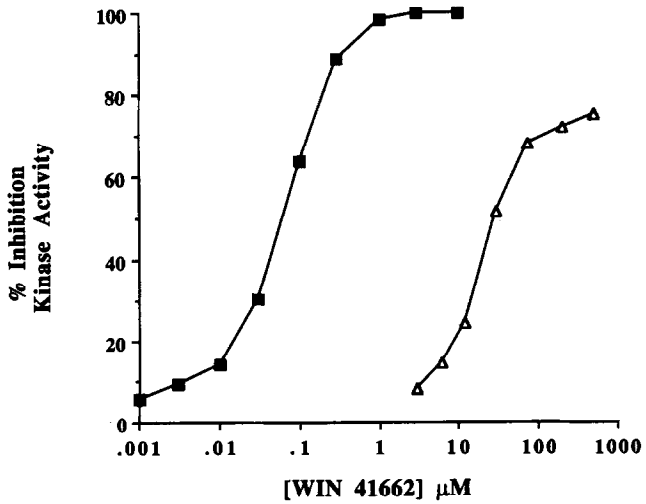


FIG. 6. Comparison of inhibition of PDGF receptor and p56^{lck} tyrosine kinase activities by WIN 41662. The PDGF receptor (■) and p56^{lck} (△) were incubated with increasing concentrations of WIN 41662 and assayed as described in Materials and Methods. The concentration of ATP was 10 μM for the PDGF receptor tyrosine kinase and 20 μM for p56^{lck}, resulting in similar [S]/K_m ratios for each enzyme. The control OD values (±SD) in the PDGF and p56^{lck} ELISA assays were 1.08 ± 0.08 and 1.52 ± 0.07, respectively, from duplicate experiments.

duce the cell proliferative response compared with either receptor autophosphorylation or PDGF-stimulated Ca^{2+} mobilization. Regardless of the biochemical explanation, similar differences in potency between biochemical models and intact-cell models are seen with other selective PDGF receptor tyrosine kinase inhibitors [14].

In conclusion, we present a novel series of PDGF receptor tyrosine kinase inhibitors and the respective *in vitro* activities of the lead compound in the series, WIN 41662. Further studies to determine the efficacy of this series *in vivo* in models of restenotic plaque formation are warranted to determine the value of these molecules as potential therapeutic entities.

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