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Tyrosine Kinase Activity of Purified Recombinant Cytoplasmic Domain of Platelet-Derived Growth Factor β-Receptor (β-PDGFR) and Discovery of a Novel Inhibitor of Receptor Tyrosine Kinases

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ABSTRACT. Aberrant expression of platelet-derived growth factor and its receptor (PDGFR) has been implicated in various human disorders, including cardiovascular disease and certain types of cancer. Inhibitors of the tyrosine kinase activity of PDGFR are leads in the development of novel agents to combat these diseases. We describe here a novel, potent inhibitor of PDGFR tyrosine kinase, 3-(4-dimethylamino-benzylidenyl)-2-indolinone (DMBI). The compound also inhibits signal transduction through fibroblast growth factor receptor 1 (FGFR1), but is not active towards epidermal growth factor receptor (EGFR) or c-Src tyrosine kinase. The activity of DMBI and other tyrosine kinase inhibitors was compared in a cell-based assay as well as in an assay based on purified recombinant platelet-derived growth factor β-receptor (β-PDGFR) lacking the transmembrane and ligand-binding domain. We showed that this truncated β-PDGFR could dimerize, and that dimerization was required for tyrosine kinase activity. Tyrosine kinase activity was modulated by inhibitors of β-PDGFR autophosphorylation in cells, but not by specific inhibitors of EGFR or c-Src tyrosine kinase. We conclude that β-PDGFR lacking the transmembrane and ligand-binding domain retains the essential properties of the full-length receptor tyrosine kinase. BIOCHEM PHARMACOL **57**;1:57–64, 1999. © 1998 Elsevier Science Inc.

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

PDGF† is a potent mitogen and chemoattractant for cells of mesenchymal origin [1]. There are three PDGF isoforms, denoted PDGF-AA, AB and BB, which are homo- or heterodimers of two related A and B polypeptide chains. The PDGFs bind with different affinities to two distinct types of cell surface receptors, α- and β-PDGFR [2]. β-PDGFR binds only B-chain-containing isoforms, whereas β-PDGFR binds all three PDGF isoforms with similar affinities. According to the current model, activation of PDGFR requires dimerization, followed by *trans*-phosphorylation of distinct tyrosines in the intracellular domain of the receptor [3]. These PTyr sites function as high-affinity binding sites for the Src homology 2 (SH2) domains of downstream signal transduction molecules [4].

PDGF plays a role in wound healing in the adult, and in the formation of blood vessels, kidney glomeruli, and lung alveoli in embryonic development [5–7]. Aberrant expression of PDGF and its receptors has been linked to various human disorders, including pulmonary fibrosis, glomerulonephritis, rheumatoid arthritis, cancer, and cardiovascular disease [1, 8, 9]. Our interest in PDGF stems from its role in restenosis, a complication occurring in 35–45% of patients treated with percutaneous transluminal coronary angioplasty (PTCA) [9]. A characteristic feature of restenosis is the proliferation and migration of smooth muscle cells from the intimal layer to the medial layer of the artery. This process is initiated and regulated by growth factors released from platelets, endothelial cells, and smooth muscle cells at the site of injury. Among these growth factors are PDGF and bFGF [9–11].

There are several indications that activation of β-PDGFR by PDGF-BB is critical for the development of restenosis: (1) The expression of PDGF and β-PDGFR was increased in mechanically injured arteries [12–14]; (2) Infusion of PDGF-BB increased restenosis in a rat model system [15]; and (3) Antibodies against PDGF-BB [10, 16] or (4) an antisense oligonucleotide complementary to β-PDGFR mRNA sequences [17] decreased this process. These studies suggest that inhibition of β-PDGFR tyrosine kinase may attenuate the proliferative effects of PDGF-BB

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† *Abbreviations:* DMBI, 3-(4-dimethylamino-benzylidenyl)-2-indolinone; EGFR, epidermal growth factor receptor; bFGF, basic fibroblast growth factor; FGFR1, fibroblast growth factor receptor 1; PDGF, platelet-derived growth factor; β-PDGFR, platelet-derived growth factor β-receptor; PTyr, phosphorylated tyrosine; AG1296, 3-phenyl-6,7-dimethoxy-quinoline; CN 9, 5,7-dimethoxy-3-(4-pyridinyl) quinoline; PY20, monoclonal PTyr antibody; DMEM, Dulbecco's modified Eagle's medium; and NTA, nitrilotriacetic acid.

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in the development of vascular disease. Inhibition of PDGFR tyrosine kinase activity was previously shown for substituted quinolines [18–20], phenylamino-pyrimidine analogs [21], and biarylhydrazones [22]. We describe here the identification of an indolinone-based tyrosine kinase inhibitor, DMBI. The compound inhibits both PDGF- and bFGF-stimulated cellular responses, but does not react with EGFR and c-Src tyrosine kinase. We also show that purified recombinant β-PDGFR lacking the transmembrane and ligand-binding domain is a tyrosine kinase. The inhibitor profile of the truncated receptor is compared with that of full-length β-PDGFR expressed in cells.

MATERIALS AND METHODS

Materials

The rat pulmonary artery smooth muscle cell line PAC-1 was kindly provided by Dr. A. Rothman (University of California, San Diego) [23]. The human epidermoid carcinoma cell line A-431 and the murine fibroblast line NIH/3T3 were obtained from the American Type Culture Collection. Human β-PDGFR cDNA [24] was provided by Dr. C.-H. Heldin (Ludwig Institute for Cancer Research). AG 1296 [18] and CN 9 [19] were synthesized at N.V. Organon. Growth factors were purchased from ReproTech. Immunoreagents and suppliers were: PY20 and rabbit β-PDGFR antiserum (Santa Cruz Biotechnology); horseradish peroxidase-conjugated PY20 (Pierce); europium-labeled PY20 and Delphia enhancement solution (EG&G Wallac); biotinylated anti-mouse antibody and streptavidin–peroxidase complex (Vector Laboratories); and peroxidase-conjugated anti-rabbit immunoglobulins (Promega). Purified EGFR and c-Src were from Upstate Biotechnology Inc. Poly(Glu, Tyr)(4:1) was from Sigma. The TKP-2 peptide was from Pierce.

Cellular PDGFR Autophosphorylation Assay

The effect of compounds on PDGF-BB-stimulated autophosphorylation of β-PDGFR in PAC-1 cells was determined in an ELISA format assay. PAC-1 cells were seeded at a density of 3.10^4 cells per well in poly-L-lysine-coated 96-well tissue culture plates and allowed to adhere overnight in DMEM culture medium containing 10% fetal bovine serum. The medium was replaced by serum-free medium containing the compounds to be tested, and the incubation was continued for 1 hr. Tyrosine phosphorylation was stimulated by adding PDGF-BB to a final concentration of 150 ng/mL. After 5 min, the cells were fixed with 4% paraformaldehyde. The paraformaldehyde was removed and the cells were dehydrated by rinsing successively with 50%, 75%, and 100% ethanol. The fixed cells were air-dried at room temperature and incubated for 1 hr at 37° with PBS containing 5% (w/v) BSA and 0.2% Tween-20. Tyrosine phosphorylation was detected by incubation with PY20 (diluted 1:500 in PBS, 5% BSA), biotinylated anti-mouse antibody (1:800 in PBS, 5% BSA), and streptavidin–peroxidase complex (prepared as recommended by the supplier in PBS) for 1 hr at 37°. After several washes with PBST, the wells were stained with 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) (Boehringer Mannheim). The reaction was stopped by adding an equal volume of 2% (w/v) SDS, and the amount of color development was read in an ELISA plate reader at 405 nm. A similar procedure was followed to measure the effect of compounds on EGF-stimulated autophosphorylation of EGFR in A-431 cells. IC_{50} values were determined from concentration-response curves of three independent experiments, with each determination performed in triplicate.

Immunoprecipitation and Immunoblotting

Confluent cultures of PAC-1 or NIH/3T3 cells were exposed to DMBI and stimulated for 5 min with 100 ng/mL of PDGF-BB or 10 ng/mL of bFGF. After washing with PBS containing 1 mM sodium orthovanadate, the cells were collected by scraping and centrifugation in a table centrifuge. The pellet was resuspended in lysis buffer [150 mM NaCl, 2 mM $MgCl_2$, 50 mM Tris (pH 8.0), 0.5 mM dithiotreitol, 0.5 mM EDTA, 1 mM sodium orthovanadate, 1 mg/mL of Pefabloc™ protease inhibitor (Boehringer Mannheim), 10% (v/v) glycerol, 1% (v/v) Triton X-100] and set on ice for 15 min. The suspension was cleared by centrifugation for 10 min at 14,000 rpm and 4° in an Eppendorf centrifuge, and then used for SDS-PAGE (10 μg per lane) or immunoprecipitation experiments. Protein concentration was determined using a BCA protein assay kit (Pierce). Immunoprecipitation reactions were carried out as follows: 50 μg of protein and 2 μg of β-PDGFR polyclonal antiserum in a final volume of 500 μL of lysis buffer were mixed overnight at 4°. The next day, 50 μL of protein G-Sepharose (Pharmacia LKB) was added, and the incubation was continued for another hour. The immunoprecipitate was collected by centrifugation for 3 min at 3000 rpm and 4° in an Eppendorf centrifuge. The precipitate was washed five times with lysis buffer, resuspended in 50 μL of 1 × SDS-PAGE sample buffer and incubated for 5 min at 95°. Twenty microliters of the samples was loaded on a 7.5% polyacrylamide gel, size-fractionated, and transferred onto a polyvinylidene difluoride membrane by electroblotting. The sheet was incubated for 2 hr in PBST containing 1% (w/v) BSA and 1% (w/v) skim milk powder. Incubations with HRP-conjugated PTyr antibody (1:1000) or β-PDGFR antiserum (1:500) and peroxidase-conjugated anti-rabbit immunoglobulins (1:1000) was for 1 hr in PBST. After a wash with PBST, the sheets were developed for 1 min in enhanced chemiluminescent detection reagent (Amersham International) and exposed for varying times (10 sec to 1 min) to Kodak X-OMAT films (Eastman Kodak).

Expression and Purification of β-PDGFR Tyrosine Kinase

A cDNA fragment encoding the cytoplasmic domain of human β-PDGFR (starting at amino acid codon 525 according to Ref. 24) was expressed as a *His*-tagged fusion protein in Sf9 insect cells using the Bac-to-Bac™ expression system (Life Technologies, Inc.). Sf9 cells were infected with β-PDGFR containing baculovirus and lysed with Tris-based buffer, as described in the system manual. After centrifugation, the supernatant was purified by Ni-NTA resin chromatography. Two mL of Ni-NTA resin was poured into a K10, 10 (∅, 10 mm; ↕, 10 cm) column (Pharmacia) and washed with 20 volumes of 20 mM Tris–Cl, pH 8.0, 500 mM KCl, 20 mM of imidazole, 10 mM of β-mercaptoethanol, 10% (v/v) glycerol (wash buffer A) (flow speed: 0.25 mL per min). Cell lysate from a 300 mL Sf9 culture was added to the column. Subsequently, the column was washed with 15 mL of wash buffer A, followed by 5 mL of wash buffer B (composition identical to A, except that B contained 1 M of KCl), and again with 10 mL of wash buffer A. The protein was eluted with 20 mM Tris–Cl, pH 8.0, 100 mM KCl, 10 mM of β-mercaptoethanol, 10% (v/v) glycerol, containing 100 mM of imidazole. Absorbance at 280 nm was monitored with a Pharmacia UV-1 detection unit. The *His*-tagged β-PDGFR cytoplasmic domain appeared as a single band on SDS/PAGE as detected by Coomassie blue staining.

Size Exclusion Chromatography

The *His*-tagged β-PDGFR cytoplasmic domain was subjected to size exclusion chromatography on a Pharmacia Superdex-75 HPLC column using the Beckman System Gold. The column was equilibrated with 50 mM Tris–Cl, pH 8.0, containing 200 mM NaCl to avoid aggregation of proteins. Elution fractions were analyzed for tyrosine kinase activity in a microtiter plate assay, as described below.

Tyrosine Kinase Assays

The tyrosine kinase activities of the purified β-PDGFR intracellular domain, EGFR, and c-Src were determined in microtiter plate assays using exogenous peptide substrates. Ninety-six-well reaction plates were coated overnight at room temperature with 25 μg/mL of poly(Glu, Tyr) in 150 mM NaCl, 22.5 mM Na_2CO_3, and 27.5 mM $NaHCO_3$, pH 9.6. The next day, the plates were washed three times with wash buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 0.2% (v/v) Tween-20). Subsequently, tyrosine kinase diluted in 50 mM Tris–Cl, pH 7.4, 10 mM $MgCl_2$, and 1 mM of dithiotreitol was added to the wells. The phosphorylation reaction was started by adding ATP to a final concentration of 1 mM. After incubation for 1 hr at room temperature, the wells were washed with wash buffer containing 1% (w/v) BSA, and 200 ng/mL of europium-labeled PY20 was added. After several washes, Delphia enhancement solution was added and time-resolved fluorescence was measured in a fluorimeter. Tyrosine kinase assays were also performed with the TKP-2 peptide, corresponding to amino acids 1 to 17 of human gastrin. This peptide was bound to the wells of a streptavidin-coated microtiter plate via a biotin group. Phosphorylation reactions and detection of tyrosine phosphorylation was performed as described above for the assays with poly(Glu, Tyr) substrate. IC_{50} values found for the tyrosine kinase inhibitors in the assay with TKP-2 were similar to those found in the assay with poly(Glu, Tyr) peptide substrate.

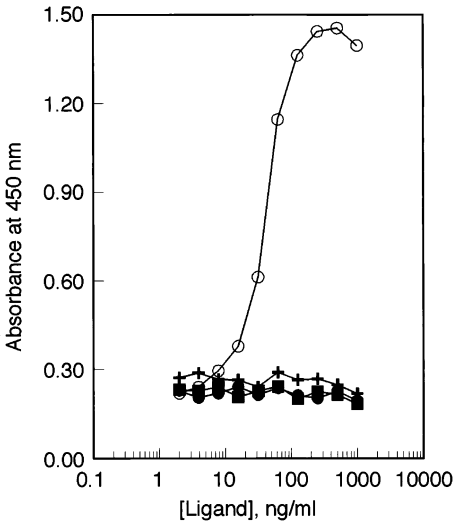

FIG. 1. Tyrosine phosphorylation of PAC-1 cells in response to growth factors. PAC-1 cells were incubated for 5 min with PDGF-AA (●), PDGF-BB (○), EGF (X), or bFGF (■) and fixed. The amount of tyrosine phosphorylation was measured with an ELISA, as described under Materials and Methods.

Proliferation and Cytotoxicity Assay

PAC-1 or NIH/3T3 cells were growth-arrested by incubation for two days in serum-free DMEM (PAC-1) or DMEM containing 1% fetal bovine serum (NIH/3T3). The cells were preincubated for 1 hr with tyrosine kinase inhibitors prior to stimulation with 30 ng/mL of PDGF-BB, 5 ng/mL of bFGF or 10% fetal bovine serum. After four days, cell number was determined indirectly by measuring intracellular acid phosphatase activity (Abacus™ cell proliferation kit, Sigma). The effect of compounds on cell viability was determined with a lactate dehydrogenase release assay (Sigma).

RESULTS

Identification of an Inhibitor of β-PDGFR Autophosphorylation

A cell line derived from rat pulmonary artery smooth muscle, PAC-1 [23], was used to develop an ELISA for the identification of inhibitors of PDGFR autophosphorylation. Figure 1 shows the effect of various growth factors on

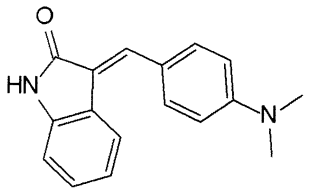

FIG. 2. Structure of 3-(4-dimethylamino-benzylidenyl)-2-indolinone (DMBI).

protein tyrosine phosphorylation in PAC-1 cells. Tyrosine phosphorylation was measured with PY20. PDGF-BB increased tyrosine phosphorylation 8-fold (Fig. 1). In contrast, PDGF-AA did not stimulate tyrosine phosphorylation. This suggests that the increase in tyrosine phosphorylation by PDGF-BB was mediated by the β-type receptor. EGF and bFGF did not stimulate tyrosine phosphorylation in PAC-1 cells (Fig. 1).

To select inhibitors of β-PDGFR autophosphorylation, the PAC-1 cells were incubated for 1 hr with compounds from synthetic chemical libraries prior to stimulation with PDGF-BB. Screening of 20,000 compounds yielded three novel inhibitors of β-PDGFR autophosphorylation with potencies in the low micromolar range. One of these inhibitors, DMBI (Fig. 2), we describe in this report. DMBI inhibited tyrosine phosphorylation in PAC-1 cells with an IC_{50} value of 4 μM. To verify that the decreased signal in the ELISA was caused by decreased phosphorylation and not by decreased expression of β-PDGFR, immunoblotting was performed. Cells were treated as described for the ELISA, lysed, and β-PDGFR was immunoprecipitated with a polyclonal antiserum. Tyrosine phosphorylation of β-PDGFR was analyzed by immunoblotting with PY20 (Fig. 3A). The amount of receptor in the immunoprecipitates was analyzed by incubation of the blots with the β-PDGFR antiserum (Fig. 3B). The blots show that DMBI inhibited the autophosphorylation, but not the expression of the β-PDGFR.

Tyrosine Kinase Activity of Truncated β-PDGFR

There are at least three mechanisms by which a compound might decrease the amount of PDGFR autophosphorylation in cells: (1) inhibition of binding of growth factor to the receptor; (2) inhibition of receptor dimerization; and (3) inhibition of receptor tyrosine kinase activity. To examine the latter possibility, we performed experiments with purified recombinant β-PDGFR lacking the transmembrane and ligand-binding domain, which was expressed as a *His*-tagged fusion protein using the baculovirus expression system. The protein was purified by Ni-NTA resin chromatography under reducing conditions and analyzed by size exclusion chromatography (Fig. 4). The column effluent was examined for tyrosine kinase activity in a microtiter plate assay in which the tyrosine phosphorylation of a random polymer of glutamate and tyrosine residues [poly-(Glu, Tyr)] was measured with europium-labeled PY20. In the chromatogram in Fig. 4B, two peaks were detected. The first (retention time 25 min) corresponds to the monomeric *His*-tagged β-PDGFR cytoplasmic domain, which has a calculated molecular weight of 62 kDa. The second (retention time 21–22 min) corresponds to the dimeric *His*-tagged β-PDGFR cytoplasmic domain, which has a molecular weight of 125 kDa. Approximately 90% of the fusion protein was present as a dimer, and tyrosine kinase activity was only detected in this fraction (Fig. 4C). We conclude that β-PDGFR lacking the transmembrane and ligand-binding domain is active as a tyrosine kinase. Truncated β-PDGFR can form a dimer, and dimer formation is required for enzymatic activity.

DMBI inhibited the tyrosine kinase activity of truncated β-PDGFR in the microtiter plate assay with an IC_{50} value of 0.3 μM (Table 1). DMBI also inhibited the autophosphorylation of truncated β-PDGFR, as determined by PY20 immunoblotting (data not shown). These data confirm that DMBI is an inhibitor of β-PDGFR tyrosine kinase and show that the compound acts by interfering with β-PDGFR enzymatic activity.

Cross-reactivity of DMBI with FGFR1

The selectivity of DMBI for β-PDGFR tyrosine kinase was examined by testing the compound against other tyrosine kinases. DMBI did not inhibit the tyrosine kinase activity of EGFR or c-Src in the concentration range tested (up to 100 μM). We also examined the ability of DMBI to decrease signal transduction through FGFR1. Since PAC-1

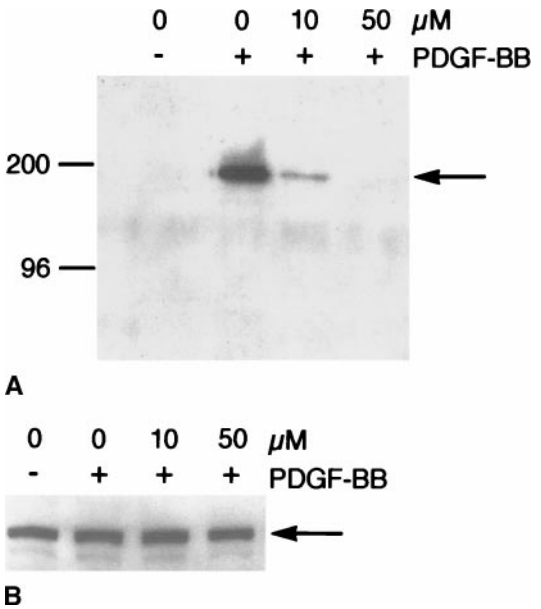

FIG. 3. Inhibition of β-PDGFR autophosphorylation in PAC-1 cells after exposure to DMBI. PAC-1 cells were incubated for 1 hr with DMBI and stimulated for 5 min with PDGF-BB. β-PDGFR was immunoprecipitated from cell lysates and analyzed by immunoblotting with PY20 (A) or anti-β-PDGFR antiserum (B). The position of migration of β-PDGFR is indicated by an arrow on the *right*, and molecular mass markers (kDa) are indicated on the *left*.

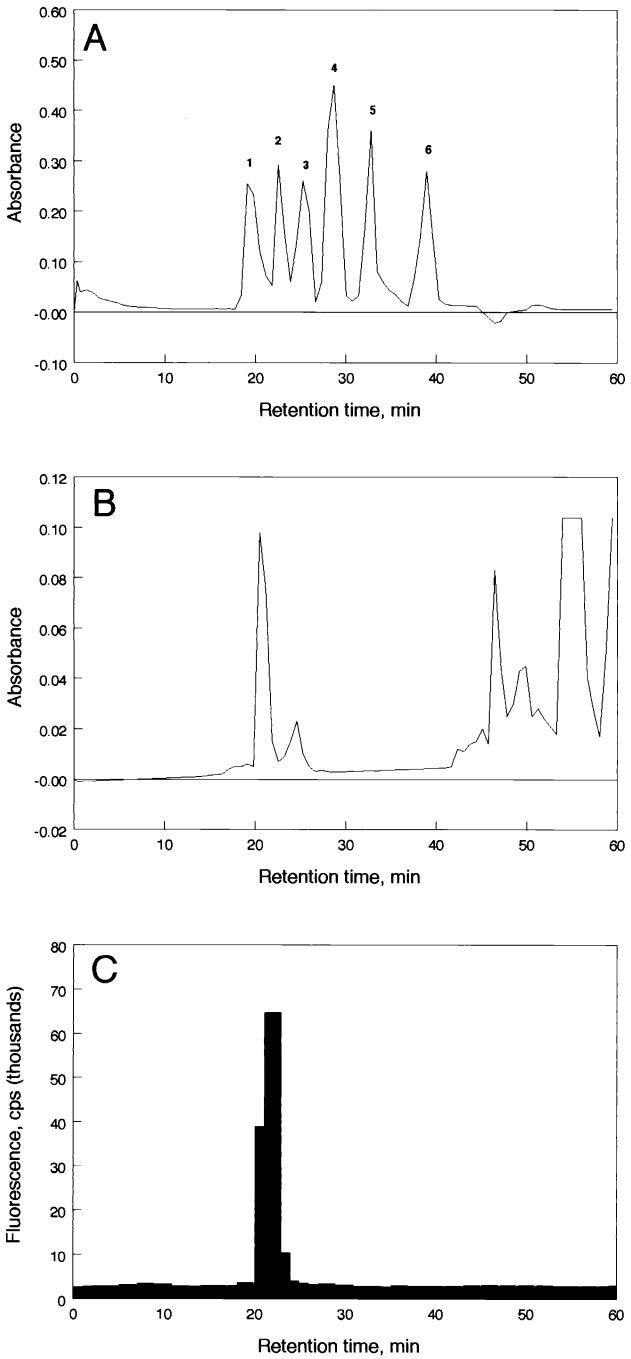

FIG. 4. Chromatographic analysis of the *His*-tagged β-PDGFR cytoplasmic domain. (A) Chromatogram of six standards: (1) γ-globulin (160 kDa); (2) serum albumin (68 kDa); (3) ovalbumin (43 kDa); (4) carbonic anhydrase (31 kDa); (5) cytochrome C (12 kDa); and (6) aprotinine (6.5 kDa). (B) Chromatogram of the *His*-tagged β-PDGFR cytoplasmic domain. (C) Tyrosine kinase activity of column effluent fractions.

cells showed no obvious changes in tyrosine phosphorylation upon exposure to bFGF on immunoblots (not shown) or in an ELISA (Fig. 1), we used NIH/3T3 fibroblasts. In this cell line, bFGF increased the tyrosine phosphorylation of a number of proteins (Fig. 5). The tyrosine phosphorylation of a 90 kDa protein (p90) was the most prominent alteration (Fig. 5) [25], and was used to determine the effect of DMBI on FGFR1 tyrosine kinase activity. DMBI inhibited tyrosine phosphorylation of p90 with an IC_{50} value of approximately 5 μM (Fig. 5).

Effect on Cell Proliferation

DMBI was also examined for its ability to inhibit cell proliferation. Consistent with the data from the cellular phosphorylation experiments, DMBI inhibited PDGF-BB- and bFGF-stimulated proliferation of cells (Table 2). Inhibition of serum-stimulated cell proliferation was significantly less efficient, supporting the conclusion that DMBI is a specific inhibitor of growth factor receptor tyrosine kinases.

Inhibitor Profile of Truncated β-PDGFR

Table 1 also show the activities of several other tyrosine kinase inhibitors in the cellular β-PDGFR autophosphorylation assay and in the tyrosine kinase assays. AG 1296 and CN 9 are quinoline-based inhibitors of PDGFR tyrosine kinase [18, 19]. Neither AG 1296 nor CN 9 inhibited the bFGF-stimulated phosphorylation of p90 in NIH/3T3 cells (data not shown). PD 153035 is an inhibitor of EGFR tyrosine kinase [26]. Genistein is a nonspecific tyrosine kinase inhibitor [27]. The data show that the inhibitor profile of truncated β-PDGFR is similar to that of the full-length receptor expressed in PAC-1 cells; i.e. the tyrosine kinase activities of both truncated and full-length receptors were inhibited by AG 1296 and CN 9, but not by PD 153035 nor by an inhibitor of c-Src tyrosine kinase (Table 1). We conclude that β-PDGFR lacking the transmembrane and ligand-binding domain retains the essential properties of the full-length receptor.

DISCUSSION

There is ample evidence for a role of PDGF-BB and β-PDGFR in the development of restenosis [10, 12–17]. bFGF has also been implicated in this disease [11]. A recent study showed that a mixture of antibodies against PDGF-BB and bFGF reduced neointimal thickening in injured rat carotid arteries by 85%, whereas the individual antibodies decreased thickening by approximately 50% [16]. We have described here a novel inhibitor of β-PDGFR tyrosine kinase, DMBI, that also reacts with FGFR1 tyrosine kinase. Two distinct tyrosine kinase inhibitors based on an indolinone core were recently described by Mohammadi *et al.* [28]. Both compounds inhibited tyrosine kinase activity of FGFR1 and showed different specificities toward other growth factor receptor tyrosine kinases [28]. Crystal structures of the tyrosine kinase domain of FGFR1 in complex with these compounds indicate that the indolinone moiety can occupy the site where the adenine of ATP binds [28]. This suggests that the indolinones are compet-

TABLE 1. Inhibitor activity in cell-based β-PDGFR autophosphorylation assay and in tyrosine kinase assays with poly(Glu, Tyr) peptide substrate for truncated β-PDGFT, EGFR, and c-Src

Compound	PDGFR (in cells)	PDGFR (*in vitro*)	EGFR (*in vitro*)	c-Src (*in vitro*)
DMBI	4 ± 1	0.3 ± 0.2	>100	>100
AG 1296	5 ± 2	1.7 ± 0.7	>100	>100
CN 9	1 ± 1	0.4 ± 0.3	>100	>100
PD 153035	>100	>100	3 ± 1	>100
Compound A[a]	>100	>100	60 ± 8	5 ± 1
Genistein	>100	7.3 ± 0.7	40 ± 11	50 ± 14

IC_{50} values (in μM) are means ± SD of three separate experiments.
[a]Proprierary inhibitor of Src-family kinases.

itive with ATP, a property they share with several other tyrosine kinase inhibitors [19, 26, 27]. The indolinone-based tyrosine kinase inhibitors are leads in the development of novel agents to combat restenosis, as well as other disorders associated with abnormal cell proliferation.

The activity of DMBI in the cell-based and *in vitro* assays for β-PDGFR tyrosine kinase was similar to that of AG 1296 and CN 9 (Table 1). It may be noted that the IC_{50} values reported previously for AG 1296 [18] and CN 9 [19] are up to 10-fold lower than the values we found (Table 1). As the compounds used in our study were chemically pure in NMR and mass spectroscopy, we attribute these differences to the use of different cell lines and/or experimental procedures. The higher potency of the inhibitors in the assay based on purified β-PDGFR fragment as compared to the assay based on PAC-1 cells indicates that passage through the plasma membrane, or metabolism, precludes full activity of the inhibitors in a cellular context. However, as we used rat cells in the autophosphorylation assay and human β-PDGFR fragment in the tyrosine kinase assay, we cannot exclude the possibility that different sensitivities of rat versus human β-PDGFR were responsible for the different activities of the compounds in the two assays. We showed that purified recombinant β-PDGFR lacking the transmembrane and ligand-binding domain retains tyrosine kinase activity. Recombinant insulin receptor and EGFR cytoplasmic domains are also active as tyrosine kinases [29, 30]. Ligand binding to these receptors increased tyrosine kinase activity, probably by inducing a conformational change that leads to relief of an inhibitory constraint in the kinase domain of the receptor [30, 31]. In contrast, ligand binding to PDGFR is thought to serve primarily to recruit and/or stabilize receptor dimers in order to allow intersubunit *trans*-phosphorylation [32]. Ligand-independent dimerization and autophosphorylation of β-PDGFR tyrosine kinase activity can be induced by cross-linking of purified receptor with antibodies against the extracellular domain [33] or by high-level expression of the full-length receptor in insect cells [34, 35]. Dimerization is regulated by sequences in the ligand-binding domain [36]. Our data suggest that β-PDGFR lacking the transmembrane and ligand-binding domain can also dimerize, and that dimer formation is required for tyrosine kinase activity.

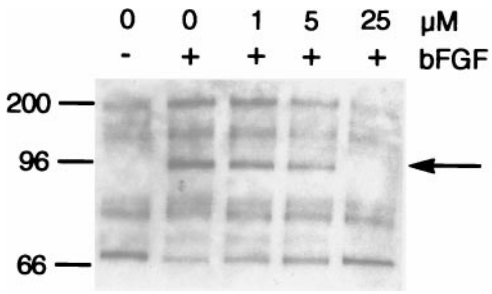

FIG. 5. Inhibition of tyrosine phosphorylation of p90 in NIH/3T3 fibroblasts by DMBI. NIH/3T3 cells were incubated for 1 hr with DMBI and stimulated for 5 min with bFGF, and tyrosine phosphorylation was analyzed by immunoblotting. The position of p90 is indicated by an arrow on the *right*, and molecular mass markers (kDa) on the *left*.

The translation of complex biological processes involved in human disease into simple assays for high-throughput selection of potential inhibitors from compound libraries is a major activity in drug discovery research in the pharmaceutical industry. We have described here two functional assays for β-PDGFR tyrosine kinase, one cell-based and the other based on purified recombinant receptor fragment. The first is relatively laborious (and expensive), while the second is suitable for automated screening. As similar inhibitor profiles were found in both assays, inhibitors selected with the tyrosine kinase assay are expected to decrease PDGF-stimulated autophosphorylation of full-length β-PDGFR as well. For high-throughput screening, the biology of β-PDGFR thus can be caught in a simple enzyme assay (taking into account that inhibitors of ligand-receptor interaction and receptor dimerization will not be selected). Our data are also of interest for the basic

TABLE 2. Inhibition of cell proliferation in response to growth factors or serum

Compound	PDGF-BB	bFGF	Serum
DMBI	2	5	>20
AG 1296	9	>20	>20
CN 9	4	>20	>20

IC_{50} values (in μM) are means of two separate experiments with NIH/3T3 cells.

understanding of β-PDGFR functioning, as they imply that the cytoplasmic domain of β-PDGFR contains the essential properties of the full-length receptor tyrosine kinase. Furthermore, we have described a new tool (DMBI) for examining the role of growth factor receptor tyrosine kinases in cellular physiology and human disease.

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