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Indolocarbazoles

Potent and Selective Inhibitors of Platelet-Derived Growth Factor Receptor Autophosphorylation

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ABSTRACT. A quantitative assay for measuring the autophosphorylation of platelet-derived growth factor (PDGF) receptors in intact vascular smooth muscle cells has been developed and used to screen for novel tyrosine kinase (TK) inhibitors. Several novel inhibitors of PDGF receptor autophosphorylation have been identified from the indolocarbazole series, including the 3,9 dimethoxy derivative, 3744W (IC_{50} = 14.5 ± 2 nM). Tested against a panel of tyrosine and serine/threonine kinases, 3744W is at least 1,000 fold selective for the PDGF receptor tyrosine kinase and was found to inhibit autophosphorylation of both the α and β isoforms of the PDGF receptor in human smooth muscle cells. PDGF-BB-stimulated DNA synthesis in quiescent cultures of human smooth muscle cells was blocked in a concentration-dependent manner by 3744W, IC_{50} = 10 nM. Binding studies showed that 3744W did not block the binding of PDGF-BB to cell surface receptors on human airway smooth muscle cells. Furthermore, inhibition of bone marrow stem cell proliferation by 3744W was only observed at concentrations 100–1,000 times greater than those needed to block PDGF-driven DNA synthesis in human smooth muscle cells. 3744W represents a novel, potent and selective inhibitor of PDGF receptor autophosphorylation and a powerful biochemical probe for investigating PDGF-dependent responses *in vitro*. BIOCHEM PHARMACOL **55**;3:261–271, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. platelet-derived growth factor; receptor autophosphorylation; tyrosine kinase; A10 cells; human smooth muscle cells

Platelet-derived growth factor (PDGF)¶ is an important competence factor that regulates the proliferation of many cell types including fibroblasts, vascular smooth muscle cells, mesangial cells and glial cells [1–3]. PDGF is also a powerful chemotactic agent for cells of mesenchymal origin e.g. fibroblasts and vascular smooth muscle cells [4–6]. PDGF has been implicated in many cancers, either as an autocrine growth factor or in regulating tumour stroma formation [7, 8]. PDGF also appears to be involved in some fibroproliferative disorders such as glomerulosclerosis and lung fibrosis as well as vascular diseases such as atherosclerosis and restenosis following angioplasty [9–13]. PDGF functions as either homo- or heterodimers, which are formed from PDGF-A and PDGF-B polypeptide chains. PDGF-AA homodimers have high affinity for the α isoform of the PDGF receptor, but bind only weakly to the PDGF-β receptor. In contrast, PDGF-BB and PDGF-AB bind to both α and β PDGF receptors [2, 3]. Cell surface PDGF receptors have intrinsic tyrosine kinase activity and the binding of PDGF induces receptor dimerization and activation of the tyrosine kinase. Receptor dimerization results in the phosphorylation of specific tyrosine residues within the cytoplasmic domain of the receptor, an event referred to as receptor autophosphorylation. Site-directed mutagenesis experiments have shown that the tyrosine kinase activity of PDGF receptors is absolutely required for both mitogenic and chemotactic signalling [14, 15]. Furthermore, inhibitors of PDGF receptor tyrosine kinase have been shown to block PDGF-driven proliferation *in vitro* and *in vivo* [16–18]. Thus, the tyrosine kinase activity of the PDGF receptor is an important therapeutic target. Selective receptor tyrosine kinase inhibitors may provide a valuable treatment for disorders where the unregulated activation of PDGF receptors has been implicated in disease pathology.

Several PDGF receptor kinase inhibitors have been described to date including staurosporine and its analogues, tyrphostins (RG 50872 and RG 13291), 3-substituted quinolines and 2-phenylaminopyrimidines [16, 18–21]. In

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¶ *Abbreviations:* PDGF, platelet-derived growth factor; EGFr, epidermal growth factor receptor; TK, tyrosine kinase; PKC, protein kinase C; HVSMC, human vascular smooth muscle cells; HASMC, human airway smooth muscle cells; GM-CSF, granulocyte-macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; IL-1, interleukin-1; SH-2, src homology-2; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; FCS, foetal calf serum; BSA, bovine serumm albumin; DMSO, dimethyl sulphoxide; TCA, trichloroacetic acid.

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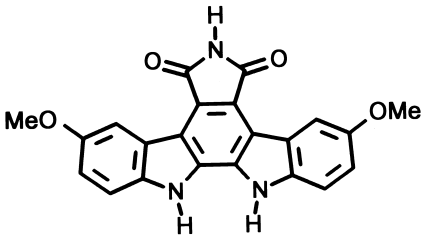

3744W

FIG. 1. Molecular structure of 3744W.

general, the staurosporine class exhibits poor selectivity for protein kinases [22, 23]. RG 50872 displays modest selectivity for PDGF receptor tyrosine kinase over the epidermal growth factor (EGF) receptor tyrosine kinase [16]. In contrast, a 2,3 dimethoxy substituted quinoline was found to be selective for the PDGF receptor tyrosine kinase [21]. The 2-phenylaminopyrimidine (CGP 53716) is highly selective for PDGF receptor tyrosine kinase when compared against a panel of receptor and non-receptor tyrosine kinases and serine/threonine kinases [18]. Herein, we describe the activity of the indolocarbazole, 3744W (see Fig. 1) a novel, potent and selective inhibitor of PDGF receptor autophosphorylation in intact cells.

MATERIALS AND METHODS

Compound Synthesis

Synthesis of indolo[2,3-a]pyrrolo[3,4-c]carbazoles was performed according to the methods of Bergman and Pelcman, 1989 [24]. All compound stock solutions were in DMSO and the final concentration in each assay did not exceed 0.1% v/v.

Cells and Cell Lines and Materials

The A10 rat thoracic aortic smooth muscle cell line was purchased from ECACC, Porton Down Wiltshire. A431 Human epidermoid carcinoma cell line, Spodoptera frugiperda clone 21 (Sf21) insect cell line, murine WEHI-3 myelomonocyte and human 5637 bladder carcinoma cell lines were obtained from the American Type Culture Collection. Human primary vascular smooth muscle cells (HVSMC) were established from hepatic arteries using the explant technique described by Fager *et al.*, [25] and subcultured in a growth factor-supplemented medium (Clonetics TCS). Human primary coronary artery smooth muscle cells (HCASMC) were also purchased from Clonetics TCS. Human primary airway smooth muscle cells (HASMC) were established and maintained according to the procedure of Hirst *et al.* [26]. Media was supplied by Gibco, and TCS Biologicals. Foetal calf serum was from Hyclone Labs and growth factor supplements from R&D Systems. Antiphosphotyrosine and receptor antibodies were supplied by UBS/TCS Biologicals or Santa Cruz Biotechnology, radioiodinated antibodies were supplied by Amersham International. PDGF-AA and PDGF-BB were supplied by Boehringer Mannheim.

Ethical Approval

Tissue from human airways was obtained following surgery for the resection of bronchial carcinoma. Permission to use material not required by the pathologist was granted by the West Lambeth Area Health Authority, London, UK. Human vascular tissue was obtained from sections of hepatic artery that were removed by the surgeon from donor livers during transplantation. Ethical approval was obtained from the Ethics Committee, Kings College Hospital, London, UK.

Clones and Expression Systems

PKCβ11 baculovirus system was kindly donated by Dr. Christy King, Burroughs Wellcome Co., Research Triangle Park, North Carolina. c-erbB-2 and v-src were cloned and expressed in insect cells (Sf21), using the baculovirus system. The clones and expression systems were developed in house by Dr. Martin Page (not published). Cloning of c-abl, bcr-abl and their baculovirus expression in insect cells was according to Peakman *et al.* [27]. The cell lines used for transfection experiments were donated by Dr. Peakman.

Sf21 Insect Cell Transfection

Cells at 10^6/mL were incubated for 2 hr at 28° to adhere to a flask. The growth media was replaced, virus stock added to the flask and the cells incubated for a further hour at 28°. Fresh media was added and the cells incubated for 48 hr at 28°.

Cell Culture

A10 cells and A431 cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% v/v foetal calf serum (FCS) and 2 mM Glutamax 1. HCASMC and HVSMC were maintained in Clonetics Smooth Muscle Basal Medium (MCDB131) supplemented with 5% v/v FCS, 5 μg/mL insulin, 0.5 ng/mL human recombinant Epidermal Growth Factor (Hu-EGF), 2 ng/mL human recombinant basic Fibroblast Growth Factor (HubFGF) and 100 μg/mL gentamycin. Human airway smooth muscle cells were maintained in DMEM, 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 1.5 mg/mL amphotericin B and 50 mg/mL gentamycin. Sf21 insect cells were maintained at 21° in TC100 media supplemented with 10% v/v FCS, 2 mM glutamine, 20 μg/mL gentamycin at a concentration of between 5×10^5–10^6 cells/mL.

PDGF Receptor Autophosphorylation Assay in Intact Smooth Muscle Cells

A10 smooth muscle cells were grown for 48 hr in DMEM + 10% v/v FCS and the serum concentration reduced to 0.2% v/v 24 hr prior to the assay. Cell monolayers were incubated with 1 nM PDGF for 5 min in HEPES-buffered Hanks balanced salt solution, pH 7.2. The assay was terminated by washing the monolayer with ice-cold PBS (phosphate-buffered saline) and cells scraped from the dish in PBS containing leupeptin, 100 μg/mL; aprotinin, 25 μg/mL; 4-(2-Aminoethyl)benzenesulphonylfluoride (AEBSF), 1 mM and sodium orthovanadate, 200 μM. Samples were centrifuged at 17,000 × *g* for 1 min, the supernatant removed and pellets resuspended in lysis buffer (TRIS, 50 mM pH 8.0, NaCl 150 mM, NP40 1% v/v, leupeptin 100 μg/mL, aprotinin 25 μg/mL, AEBSF 1 mM and sodium orthovanadate 200 μM. SDS-PAGE sample buffer was added and the samples boiled. Proteins were separated using discontinuous, single-dimension polyacrylamide gel electrophoresis (7.5% gels at pH 8.8) After separation, proteins were transferred to nitrocellulose membranes by electrophoretic transblotting. The membranes were equilibrated in PBS + 5% v/v FCS containing 0.2% v/v Tween 20 and blocked in 3% w/v BSA for 1 hr. After washing, nitrocellulose membranes were incubated with a mouse antiphosphotyrosine monoclonal antibody (4G10), 1:2000 dilution, which was subsequently identified using a radioiodinated sheep anti-mouse monoclonal antibody, 1:1000 dilution. Phosphotyrosine-containing proteins were visualised and quantified using a PhosphorImager, Molecular Dynamics.

Assay for DNA Synthesis in SMC

A10 smooth muscle cells were seeded at 10^5 cells per well in 12 well dishes (Falcon) and cultured in complete growth medium for 48 hr. HVSMC were seeded at 4×10^5 cells per well. The FCS concentration was reduced to 0.2% v/v 18 hr prior to assay. Cell monolayers were washed with HEPES/DMEM + 0.2% v/v FCS and then stimulated with PDGF (10 ng/mL) in the presence of 1 μCi/mL [^{3}H]thymidine for 24 hr (A10 cells) or 48 h (HVSMC). Incorporation of thymidine into DNA was measured by washing the wells with 10% w/v TCA, followed by a further 3 washes in 5% w/v TCA. Precipitated material was solubilised in 0.2 M NaOH and radioactivity determined by liquid scintillation counting.

^{125}PDGF-BB Cross-Competition Binding Analysis

PDGF binding was measured using a procedure based on the methods of Seifert *et al.*, [28]. Near-confluent quiescent HASMC were cultured in 24 well plates, washed twice with ice-cold binding buffer (Hanks buffered salt solution, HEPES 10 mM, BSA 0.25% w/v, pH 7.4) and left for 30 min to cool. ^{125}I-PDGF-BB (1000 Ci/mmol, 33.33 μCi/μg) was diluted to 0.45 ng/mL in binding buffer and added to cells alone or in the presence of increasing concentrations of unlabelled PDGF isoforms. After 4 hr at 4° cells were washed four times with ice-cold binding buffer and then solubilised in 1% v/v Triton X-100, 0.1% w/v BSA at room temperature for 30 min. Cell-bound radioactivity was counted in a gamma counter. Non-specific binding was determined as the ^{125}I-PDGF-BB binding seen in the presence of 300 ng/mL unlabelled PDGF-BB and varied between 10–15% of the total counts bound.

c-abl and bcr-abl Protein Kinase Substrate Phosphorylation Assay

Tyrosine kinase activity was isolated and measured according to the method described by Reynolds *et al.*, [29].

Truncated Cytoplasmic Domain c-erbB-2 Kinase Phosphorylation Assay

Truncated c-erbB-2 was expressed in insect cells using the baculovirus expression system. Isolated erbB-2 receptor tyrosine kinase was obtained by pelleting the infected insect cells and resuspending the pellet in a lysis buffer (40 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl, 10% v/v glycerol, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 20 μg/mL pepstatin, 100 μM sodium vanadate, 0.005% w/v phenylmethylsulphonyl Fluoride (PMSF). The suspension was freeze-thawed in dry ice/methanol and homogenised using a Dounce homogeniser. Finally, the homogenate was centrifuged at 100,000 × *g* for 1 hr and the supernatant stored at −70°. Using a 96 well format, isolated enzyme and compound were incubated for 15 min at ambient temperature before the addition of 100 μM [^{33}P]-γ-ATP, 10 mM $MnCl_2$ and 1 mg/mL poly$Glu_6Ala_3Tyr_1$ substrate. After 1 hr at room temperature the reaction was terminated by the addition of EDTA. Aliquots were spotted onto P81-type Beta-sheet, washed with orthophosphoric acid and dried for 1 hr at 50°. The Beta-sheets were then sealed in plastic bags with Beta-plate scintillant and monitored for [^{33}P].

Epidermal Growth Factor Receptor Autophosphorylation Assay

A high throughput whole cell assay described by King *et al.* [30] was used to measure EGFr kinase activity in A431 human epidermoid carcinoma cells.

v-src TK Substrate Phosphorylation Assay

Baculovirus-expressed, non-myristoylated v-src was purified from Sf21 insect cells based on a method described by Chang et al., [31]. Briefly, infected cells were lysed in 20 mM Tris-HCl pH 7.4, 10% v/v glycerol, 5 mM EDTA, 1% v/v Triton X-100 and the supernatant passed down a poly $Glu_6Ala_3Tyr_1$ affinity column. v-src tyrosine kinase activity was eluted using a stepwise salt gradient. v-src protein kinase activity was mainly found in the 0.3–0.4 M NaCl

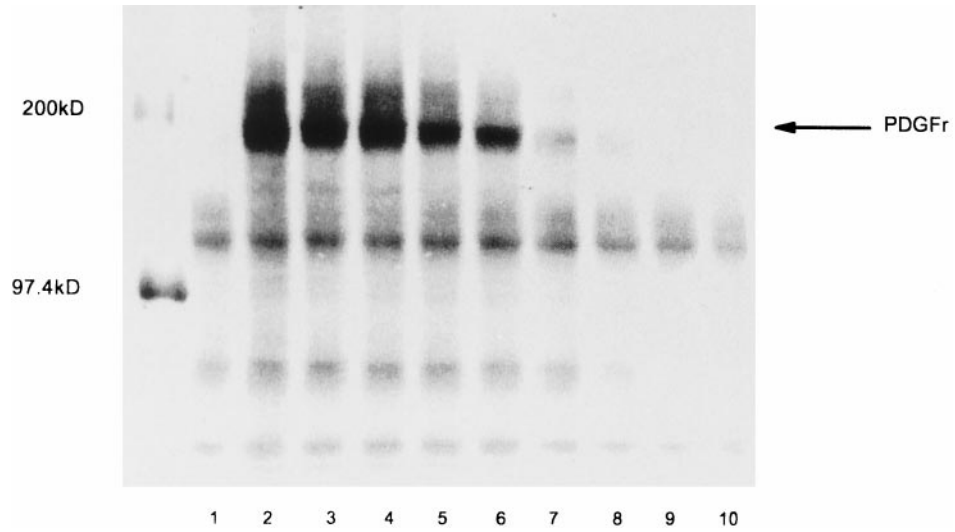

FIG. 2. Effect of staurosporine on the phosphotyrosine content of proteins in PDGF-stimulated A10 cells. Serum-starved A10 cells were treated with (lanes 2–10) or without (lane 1) PDGF-BB (1 nM) for 5 min in the presence (lanes 2–10) and absence (lane 1) of staurosporine. Proteins were separated by SDS electrophoresis, blotted onto nitrocellulose and probed with an anti-phosphotyrosine antibody. A second radioiodinated antibody was used to label phosphotyrosine-containing proteins. PhosphorImager analysis was used to identify and quantitate any changes in phosphotyrosine content. Lane 1 = control, lane 2 = 1 nM, lane 3 = 3 nM, lane 4 = 10 nM, lane 5 = 30 nM, lane 6 = 100 nM, lane 7 = 300 nM, lane 8 = 1 μM, lane 9 = 3 μM, lane 10 = 10 μM.

fractions. Tyrosine kinase activity was assayed by measuring the phosphorylation of a $polyGlu_4Tyr_1$ substrate in 25 mM Tris-HCl buffer pH 7.5 in the presence of 10 mM $MgCl_2$ and 100 μM [^{33}P]-γ-ATP [32]. Aliquots were spotted onto P81 paper washed with orthophosphoric acid and dried for 1 hr at 50°. The paper strips were sealed in a plastic bag with Beta plate scintillant and counted for [^{33}P].

Protein Kinase CβII Substrate Phosphorylation Assay

The kinase activity of baculovirus expressed human PKCβII was monitored by measuring the phosphorylation of a glycogen synthase peptide substrate according to the method described by Bonser *et al.* [33]. Phosphopeptides were separated on phosphocellulose paper and measured by liquid scintillation counting using the method described by Roskoski [34].

Stem Cell Cytotoxicity Assay

An *in vitro* soft-agar gel assay for measuring compound toxicity was developed from assays previously described [35, 36]. Briefly, a human bladder carcinoma cell line, 5637 that constitutively produces colony stimulating factors was grown in DMEM + 10% v/v FCS. The conditioned growth medium contains granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and interleukin-1 (IL-1). Murine myelomonocyte, WEHI-3 cells grown in DMEM + 10% v/v FCS produced conditioned medium containing interleukin-3 (IL-3). Stem cells isolated from human and murine bone marrow samples were plated out in 10% w/v agar in conditioned media which was supplemented with 30% v/v FCS, 1% w/v BSA, 2 μg/mL human recombinant erythropoetin, 0.64 mM sodium selenite and 40 mM monothioglycerol. After 8–10 days the colonies were counted; groups of cells in excess of 50 were defined as colonies.

RESULTS

Receptor Autophosphorylation Assay

Autophosphorylation of PDGF receptors on tyrosine residues in the rat A10 smooth muscle cell line was detected using the monoclonal anti-phosphotyrosine antibody, 4G10. In PDGF-stimulated A10 cells the phosphotyrosine content of several proteins increased significantly (Fig. 2). In particular, a protein band with a molecular size of approximately 180 kDa was heavily phosphorylated after addition of PDGF-BB (Fig. 2). The PhosphorImager provided a method for accurately quantitating the phosphotyrosine content of the 180 kDa protein band (Fig. 2). PDGF-stimulated phosphorylation of the 180 kDa band in A10 cells was concentration dependent, although concentrations of PDGF-BB up to 2 nM did not saturate the response (Fig. 3). PDGF-induced tyrosine phosphorylation of the 180 kDa protein was rapid, reached a peak at 5 min and then declined slowly (Fig. 4). The 180 kDa band was therefore positively identified as the PDGF-β receptor on the basis of the following parameters (1) molecular size, (2) increases in phosphotyrosine content were PDGF-dependent (3) kinetics of tyrosine phosphorylation matched those reported for PDGF receptors in other cell types [16] and (4) antibody to the β isoform of the PDGF receptor (Santa Cruz) co-localised with the phosphorylated 180 kDa band but antibody to the α isoform of the PDGF receptor

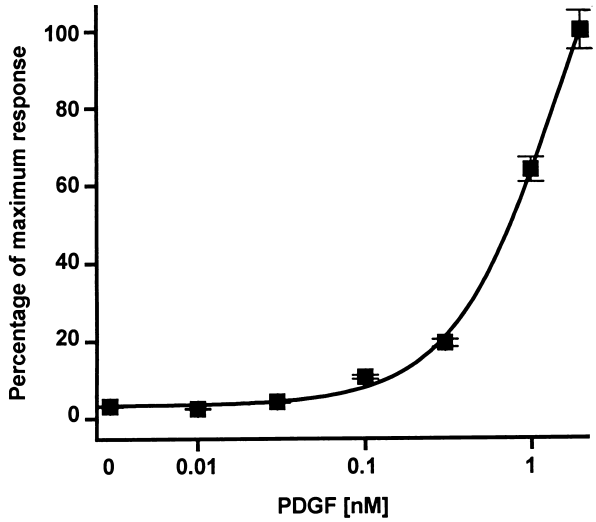

FIG. 3. Concentration-dependent activation of PDGF receptor autophosphorylation in A10 cells by PDGF-BB. Serum-starved A10 cells were stimulated for 5 min in the presence of increasing concentrations of PDGF-BB and PDGF receptor autophosphorylation analysed as described in "Materials and Methods." Results are expressed as mean ± SEM for 3 separate experiments.

(Santa Cruz) did not cross react with the 180 kDa protein (results not shown).

PDGF Receptor Tyrosine Kinase Inhibitors

Staurosporine blocked PDGF receptor autophosphorylation in a concentration-dependent manner with an IC_{50} value of 30 nM (Figs. 2 and 5). The staurosporine analogue, K252a [37], was also a potent inhibitor of PDGF receptor tyrosine kinase, blocking the autophosphorylation response in a dose-dependent fashion with an IC_{50} value of 20 nM (Fig. 5). The indolocarbazole series contains a number of PDGF receptor kinase inhibitors, including 3744W, one of the most potent compounds tested. 3744W inhibited PDGF receptor autophosphorylation in the intact A10 cell assay with an IC_{50} value of 14.5 ± 2nM (Fig. 6). Pretreatment experiments with 3744W (results not shown) indicated that exposure periods as short as 30 sec resulted in complete inhibition of PDGF receptor autophosphorylation. Details of structure/activity relationships within the indolocarbazole series will be published elsewhere.

Selectivity of 3744W for the PDGF Receptor Tyrosine Kinase

The inhibitory profile of 3744W tested against a panel of receptor and non-receptor tyrosine kinases was also determined. 3744W had no effect on EGF receptor tyrosine kinase activity even at concentrations up to 50 μM (Table 1). A related receptor tyrosine kinase, erbB-2, was also relatively insensitive to 3744W, inhibition at 50 μM 3744W reached 55% (Table 1). The inhibition of two non-receptor tyrosine kinases, c-abl and bcr-abl, by 3744W also required high drug concentrations (Table 1). 3744W blocked bcr-abl tyrosine kinase activity with an IC_{50} value of 30 μM and similar concentrations were required to block c-abl tyrosine kinase activity, inhibition at 50 μM 3744W reached 79%. Likewise, significant inhibition of the serine/threonine kinase PKCβII was only seen with high concentrations of 3744W. The IC_{50} value for inhibition of PKCβII by 3744W was calculated to be 60 μM (Table 1). 3744W had no effect on the tyrosine kinase activity of the v-src protein at concentrations up to 30 μM.

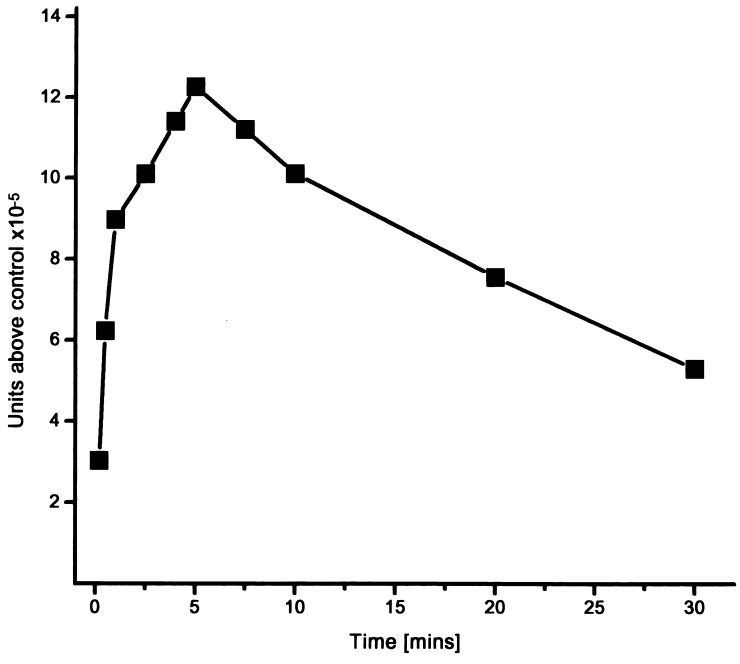

FIG. 4. Time course of ligand-induced PDGF receptor autophosphorylation in A10 cells. Serum-starved A10 cells were stimulated for varying periods in the presence of 1 nM PDGF-BB and PDGF receptor autophosphorylation measured as described in "Materials and Methods." Results are from a single representative experiment.

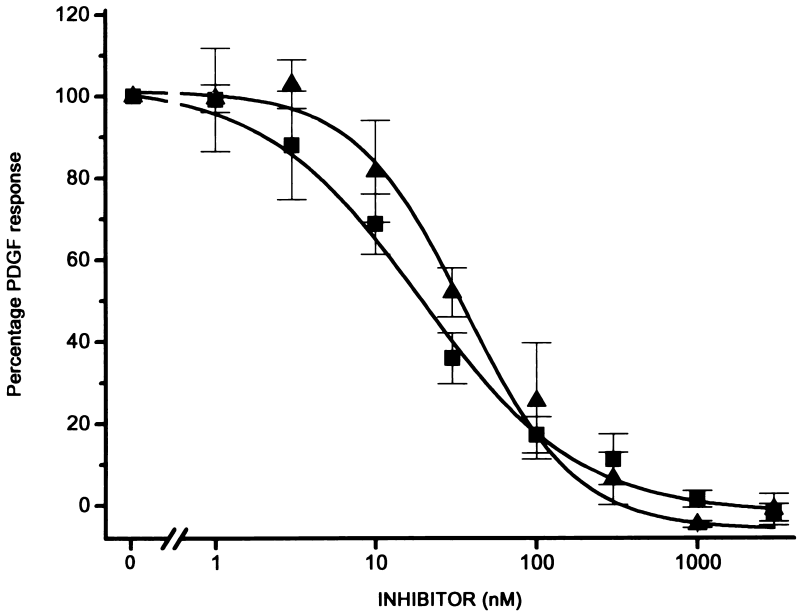

FIG. 5. Concentration-dependent inhibition of PDGF receptor autophosphorylation by staurosporine and K252A in A10 cells. Serum-starved A10 cells were stimulated with PDGF-BB (1 nM) for 5 min in the presence of increasing concentrations of staurosporine and K252A and the phosphotyrosine content of the PDGF receptor (180 kD protein, see Fig. 1) quantitated using the PhosphorImager as described in the "Materials and Methods" section. Results are expressed as the mean ± SEM for 3 separate experiments. ▲, Staurosporine; ■, K252A.

Inhibition of PDGF Receptor Autophosphorylation in Human Vascular Smooth Muscle Cells

Addition of PDGF-BB (1 nM) induced tyrosine phosphorylation of several proteins in HVSMC, including a heavily phosphorylated band with a molecular size of approximately 180 kDa (Fig. 7, lanes 6 and 7). The 180 kDa band was recognised by an antibody specific for the human PDGF-β receptor (results not shown) and thus positively identified as the human PDGF-β receptor. Treatment of HVSMC with PDGF-AA (1 nM) stimulated tyrosine phosphorylation of a protein band that had a slightly faster electrophoretic mobility than the PDGF-β receptor, its molecular size was estimated to be approximately 170 kDa (Fig. 7, lanes 4 and 5). This protein band was identified as the PDGF-α receptor on the basis of the following criteria (1) molecular size, (2) the increase in phosphotyrosine content was stimulated by PDGF-AA, which binds selectively to PDGF-α receptors and (3) the 170 kDa band cross-reacted with an antibody specific for the PDGF-α receptor (results not shown). The increase in the phosphotyrosine content of the PDGF-α receptor was quantitatively much less than that of the PDGF-β receptor. In the presence of 1 μM 3744W, PDGF-stimulated tyrosine phosphorylation of PDGF-α and β receptors was almost completely blocked (Fig. 7, lanes 8–11). PhosphorImager analysis indicated that PDGF-α receptor phosphorylation was reduced by 85% and PDGF-β receptor phosphorylation inhibited by 80%.

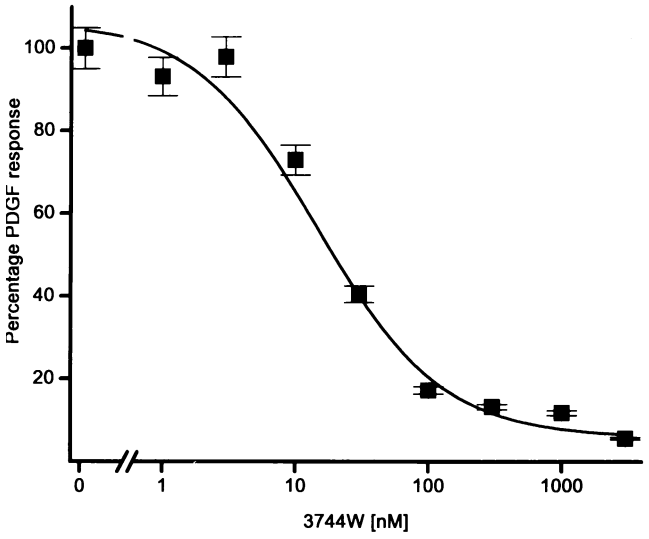

FIG. 6. Inhibition of PDGF receptor autophosphorylation by 3744W. Serum-starved A10 cells were preincubated with increasing concentrations of 3744W for 5 min and then stimulated for a further 5 min with 1 nM PDGF-BB. PDGF receptor autophosphorylation was quantitated as described in "Materials and Methods." Results are expressed as the mean ± SEM for 3 separate experiments.

TABLE 1. Inhibition of protein kinases by 3744 W

Kinase	Per cent inhibition of kinase activity in the presence of 50 μM 3744W
PDGF receptor	IC_{50} = 15 nM
EGF receptor	0
erbB-2 receptor	55
c-abl	79
bcr-abl	IC_{50} = 30 μM
PKCβII	IC_{50} = 60 μM
v-src	0

Protein kinase inhibition was initially measured in the presence of 50 μM 3744W. Where significant inhibition was observed (i.e. >50%) IC_{50} values were generated from the analysis of concentration-effect curves.

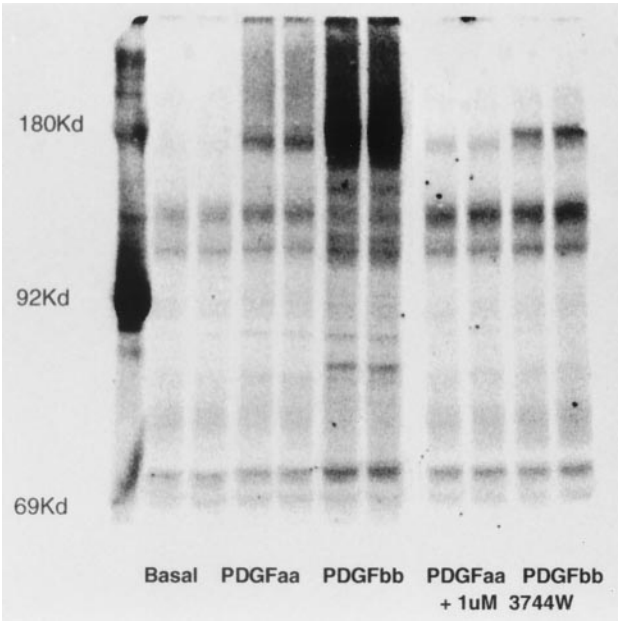

FIG. 7. Inhibition of PDGF receptor-α and -β autophosphorylation by 3744W in human primary smooth muscle cells. Quiescent cultures of human primary vascular smooth muscle cells were stimulated with either PDGF-BB (1 nM) or PDGF-AA (1 nM) in the presence and absence of 3744W (1 μM) for 5 min and phosphotyrosine containing proteins analysed using a PhosphorImager (see "Materials and Methods").

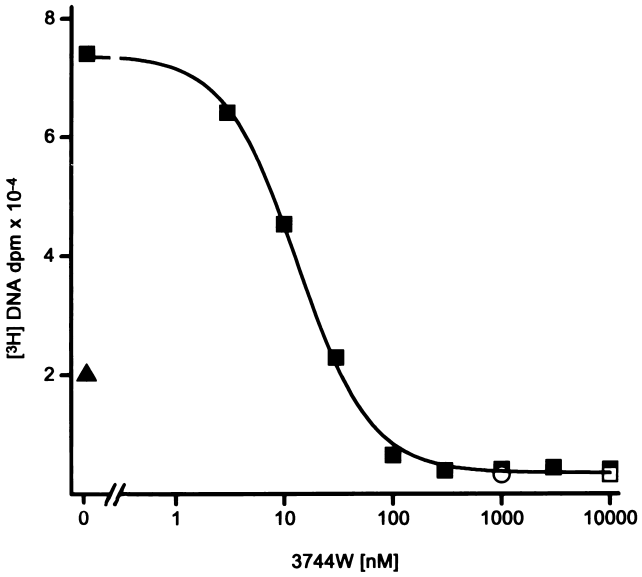

FIG. 8. Concentration-dependent inhibition of PDGF-stimulated DNA synthesis by 3744W in human primary vascular smooth muscle cells. Incorporation of [^{3}H]thymidine into DNA was measured in the presence (■) and absence (▲, ○, □) of PDGF-BB (10 ng/ml) in quiescent cultures of human primary vascular smooth muscle cells treated with (■, ○, □) or without (▲) 3744W. Results are the mean of two separate experiments. Variance from the mean was ≤10% for all values and consequently error bars have been omitted since in most cases they fall within the dimensions of the symbols.

Inhibition of PDGF-Stimulated DNA Synthesis in Human Vascular Smooth Muscle Cells by 3744W

The addition of PDGF-BB (10 ng/mL) to quiescent HVSMC and serum-starved A10 cells stimulated the incorporation of [^{3}H]thymidine into DNA. In A10 cells PDGF-BB-stimulated DNA synthesis increased by as much as 9-fold over basal levels in some experiments (results not shown). In contrast, in HVSMC the response to PDGF-BB was more modest, incorporation of [^{3}H]thymidine into DNA increased only 3–4 fold (Fig. 8). PDGF-AA also stimulated DNA synthesis in HVSMC cultures (results not shown). PDGF-BB-stimulated DNA synthesis in HVSMC was inhibited by 3744W in a concentration-dependent manner (Fig. 8). The IC_{50} value for blockade of PDGF-stimulated DNA synthesis was calculated to be 10 nM. Interestingly, higher concentrations of 3744W also reduced the basal level of [^{3}H]thymidine incorporation, although DNA synthesis was never completely blocked even at 30 μM 3744W.

Inhibition of Bone Marrow Stem Cell Colony Formation

Figure 9 illustrates the effects of 3744W on human and murine bone marrow stem cell colony formation. Concentrations of 3744W up to 1 μM had no significant cytotoxic effects on the outgrowth of murine or human primary bone marrow stem cell colonies. Formation of murine stem cell colonies was inhibited 30% by 5 μM 3744W, however, this concentration had no effect on the generation of human stem cell colonies. Higher concentrations of 3744W were cytotoxic and the outgrowth of murine colonies was completely blocked at 50 μM. The cytotoxic effects of the indolocarbazole were slightly less pronounced in human stem cell colonies; colony formation was reduced by 30 and 90% at 10 and 50 μM 3744W, respectively.

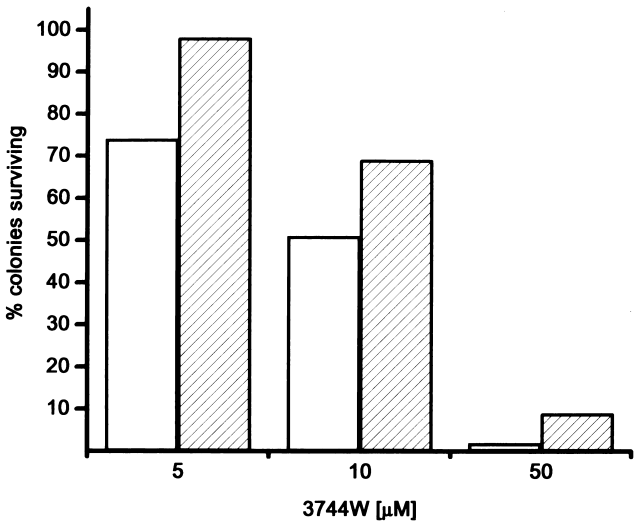

FIG. 9. Effect of 3744W on human and murine stem cell colony formation. Human [hatched bars] and murine [open bars] bone marrow stem cell colony formation in soft agar was measured in the presence of increasing concentrations of 3744W. Results shown are from a single representative experiment. Values plotted are the mean of duplicate determinations.

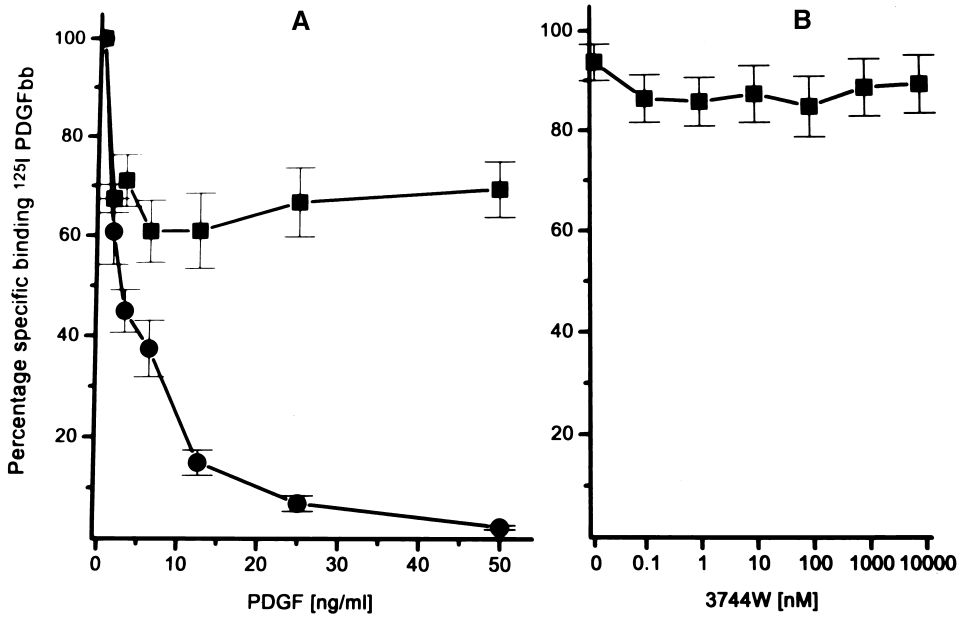

FIG. 10. Effect of 3744W on the binding of PDGF to its receptors in human primary airway smooth muscle cell cultures. (A) Specific binding of radioiodinated PDGF-BB to PDGF receptors on human airway smooth muscle cells was measured in the presence of increasing concentrations of unlabelled PDGF-BB (●) or PDGF-AA (■) [100% binding = specific binding measured in the absence of unlabelled ligands and DMSO and was 3601 ± 829 cpm (n = 4)]. (B) PDGF-BB binding to its receptors on human airway smooth muscle cells was monitored in the presence of increasing concentrations of 3744W. Results are the mean ± SEM for 3 separate experiments using cells from different donors and are expressed as a percentage of the 100% binding value shown in A.

Likewise, the growth of MRC5 human fibroblasts in 10% foetal calf serum was only blocked at the higher concentrations of 3744W i.e. above 1 μM (results not shown). This observation supports the results seen in the colony forming assays and illustrates that the cytotoxic/cytostatic properties of this molecule are only exhibited at concentrations 100–1,000 times greater than those needed to inhibit PDGF receptor autophosphorylation.

PDGF Binding to Human Airway Smooth Muscle Cells

Binding of PDGF to its receptors was measured in primary (early passage) cultures of human airway smooth muscle cells (HASMC). Primary cultures of HASMC bind radiolabelled PDGF-BB with high affinity. PDGF-BB binding was displaced completely with unlabelled PDGF-BB but only partially by PDGF-AA (Fig. 10A). These observations probably reflect the fact that PDGF-α receptors are expressed in lower abundance than PDGF-β receptors by smooth muscle cells in culture. Addition of 3744W to HASMC displaced approximately 15% of the total PDGF-BB bound (Fig. 10B). Part of this displacement was due to the presence of the drug solvent, since specific binding was reduced by 6.9 ± 3.7% in the presence of 0.1% v/v, DMSO (Fig. 10B). Additional displacement of radiolabelled PDGF by 3744W was not concentration-dependent and observed at all 3744W concentrations tested, including those which had no significant effect on receptor autophosphorylation or PDGF-driven DNA synthesis (compare Figs. 6, 8 and 10B). Furthermore, the apparent 3744W-dependent reduction in specific PDGF binding is not statistically significant and falls within the experimental error defined by these experiments. Thus, the evidence clearly shows that the indolocarbazole 3744W does not affect the binding of PDGF to its receptors in intact smooth muscle cells.

DISCUSSION

PDGF has been implicated in many pathological conditions, including fibroproliferative disorders, vascular diseases and several cancers. Pulmonary fibrosis results from fibroblast proliferation and excessive collagen production in the lungs and PDGF has been identified as one of the major mitogens present in the airways of idiopathic pulmonary fibrosis patients [38]. The expression of PDGF and its receptors also correlates with the proliferation of myofibroblasts in chronic liver diseases and the hyperplastic response of vascular smooth muscle cells in atherosclerotic plaques [39–43]. Antibodies to PDGF inhibit fibroproliferative responses in a rat model of glomerulosclerosis and block neointima formation in an animal model of restenosis [44, 45]. Tumours of the brain e.g. glioblastomas and astrocytomas express high levels of PDGF and PDGF receptors, suggesting that PDGF is a major autocrine factor for these highly malignant cancers [46–49]. Other cancers e.g. breast, lung, colon and stomach, do not consistently express receptors for PDGF but do produce large amounts of PDGF-A and/or PDGF-B [50–54]. PDGF released by tumour cells could act as a paracrine growth factor for many cell types including; fibroblasts, smooth muscle cells and

endothelial cells and thus contribute to stroma formation as well as the neovascularization of tumours [8, 53–57].

Ligand-induced dimerization of PDGF receptors results in activation of the intrinsic tyrosine kinase activity of receptor molecules and phosphorylation of specific tyrosine residues within the cytoplasmic domain of the receptor. This phenomenon, referred to as receptor autophosphorylation, is common to a number of tyrosine kinase growth factor receptors [58, 59]. Phosphotyrosine residues, within discrete cytoplasmic domains of the receptor, are specifically recognised by key intracellular signalling molecules that contain src-homology-2 domains (SH2 domains). The assembly of these signal transduction elements is regulated by the phosphorylation state of the receptor and 'kinase-dead' receptor mutants, although able to bind ligand, do not generate intracellular signals [58]. Thus, selectively inhibiting the tyrosine kinase activity of growth factor receptors provides a strategy for blocking biological responses to a wide variety of mitogens and chemotactic factors [60]. The validity of this approach is exemplified by the observation that inhibitors of PDGF receptor tyrosine kinase reduce neointima formation in the rat carotid artery model of restenosis [61] and block the growth of PDGF-driven(c-sis) tumours in nude mice [18]. In addition, selective inhibitors of EGF receptor tyrosine kinase have been shown to inhibit the proliferation of EGF-dependent tumours in nude mice [19, 62].

This report describes the development of a PDGF receptor tyrosine kinase assay which combines the specificity of anti-phosphotyrosine antibody detection with the quantitative analysis provided by a PhosphorImager. This assay has been used to screen for novel and potent inhibitors of PDGF autophosphorylation in intact smooth muscle cells. Staurosporine and its derivatives are potent, non-selective inhibitors of protein kinases [22, 23, 63, 64]. One compound in particular, CGP 41251, was shown to block PDGF receptor autophosphorylation in BALB/c 3T3 cells [19]. This observation prompted us to investigate a chemically-related series of aglycone staurosporine derivatives, the indolocarbazoles. The indolocarbazole series contains a number of inhibitors of PDGF receptor autophosphorylation, the most potent compound identified was 3744W, a 3,9-dimethoxy substituted analogue. Surprisingly, this compound was a very weak inhibitor of PKC and displayed good selectivity when tested against a panel of tyrosine kinases. Unlike staurosporine, 3744W was not toxic to rat A10 or HVSMC in culture. More importantly, 3744W did not inhibit PDGF binding to its receptors on HASMC, but was a potent inhibitor of PDGF-BB-stimulated DNA synthesis in HVSMC. The human vascular smooth muscle cells used in these studies expressed both the α and β isoforms of the PDGF receptor and both PDGF-AA and PDGF-BB were able to stimulate DNA synthesis in these cells. The ability of 3744W to completely block PDGF-BB-driven DNA synthesis reflects therefore, the non-selective inhibition of PDGF-α and -β receptor autophosphorylation by this compound. It is not unreasonable to assume that biological responses to all three homo- and heterodimeric forms of PDGF would be sensitive to 3744W. The cytotoxicity of 3744W was further evaluated by measuring colony formation in human and murine bone marrow stem cell cultures. 3744W concentrations in the range 10–50 μM blocked colony formation, however, it should be noted that these levels are three orders of magnitude greater than are needed to block PDGF-receptor autophosphorylation or PDGF-driven DNA synthesis in vascular smooth muscle cells. In addition, the growth of MRC-5 human fibroblasts, cultured in 10% FCS, was only inhibited at micromolar concentrations of 3744W. Thus, at nanomolar concentrations, 3744W is a selective inhibitor of PDGF-driven responses. Furthermore, 3744W acts rapidly and therefore appears to be freely taken up by vascular smooth muscle cells *in vitro*. Disappointingly, the poor aqueous solubility of this compound has prevented a thorough evaluation of its activity *in vivo*.

The data derived from the PDGF receptor autophosphorylation assay would strongly suggest that 3744W acts by inhibiting the tyrosine kinase activity of the PDGF receptor. Since PDGF binding to its receptor is not blocked by 3744W it seems likely that receptor dimerization also occurs normally. Evidence in support of the later conclusion has not been presented and therefore the precise mechanism of action of 3744W cannot be defined. Inhibition of normal receptor dimerization would provide an alternative mode of action which would result in the apparent inhibition of tyrosine kinase activity. Activation of endogenous phosphotyrosine phosphatases by 3744W provides another plausible explanation for the attenuation of PDGF receptor autophosphorylation in intact vascular smooth muscle cells. Although the precise mode of action of 3744W cannot be unequivocally defined, nonetheless, the biochemical screen described in this report has identified a novel, potent and selective inhibitor of PDGF receptor autophosphorylation in intact vascular smooth muscle cells. 3744W represents an important chemical lead and it is anticipated that analogues with greater solubility could serve as useful therapeutic agents for diseases in which PDGF has been shown to play a significant role.

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