# Identification of a cyclic peptide inhibitor of platelet-derived growth factor–BB receptor-binding and mitogen-induced DNA synthesis in human fibroblasts

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**Abstract Peptides corresponding to residues from Loops I and III of platelet-derived growth factor–BB (PDGF-BB) were examined for their potential to act as PDGF antagonists. We have identified two peptides which directly stimulated DNA synthesis in human dermal fibroblasts and a cyclic peptide which inhibited PDGF-induced DNA synthesis. The inhibitory action of cyclic PDGF-BB$^{73-81}$, on DNA synthesis was shown to be restricted to cells which express PDGF receptors. Also cyclic PDGF-BB$^{73-81}$ specifically competed for $^{125}$I-labelled PDGF-BB but not for $^{125}$I-labelled EGF binding to their respective cellular receptors. The cyclic peptide therefore provides a minimum structure to investigate PDGF/receptor interactions and our findings confirm the importance of the loop configuration of PDGF-BB$^{73-81}$ in the native molecule. The cyclic peptide may constitute a basis for developing more potent inhibitors of PDGF action.**



*Key words:* Cyclic peptide; Platelet-derived growth factor; Human dermal fibroblast

## 1. Introduction

Platelet-derived growth factor (PDGF) is a major mitogen and chemotactic factor in connective tissues [1,2]. These properties provide PDGF with important roles in: wound healing, development of atherosclerotic lesions, restenosis of arteries after angioplasty [1,3], tumorigenesis [4,5], glomerulonephritis [6] and rheumatoid arthritis [7].

The three PDGF isoforms, homo- or heterodimers of related A and B polypeptides exert their biological effects through binding to α and β cell surface receptor subunits causing their dimerisation to homo- and heterodimers. Subsequent receptor trans-autophosphorylation leads to activation of the intrinsic tyrosine kinase activity (see [8] for review). PDGF-A and -B chains have 60% amino acid identity with full conservation of 8 cysteine residues [9], but whilst PDGF-BB binds to both α- and β-receptors with different affinities, the PDGF-A chain binds only to the α-receptor with a high affinity [8].

The X-ray crystal structure of the homodimer of human PDGF-BB shows that loops I and III are extremely flexible, exposed to solvent and held in close proximity to each other possibly through hydrophobic interactions between Arg$^{27}$ and Leu$^{38}$ at the ends of loop I, and Arg$^{73}$, Ile$^{75}$, Ile$^{77}$, Pro$^{82}$ and Phe$^{84}$ at the periphery of loop III [10]. Site-directed mutagenesis studies have revealed the importance of PDGF-BB residues Arg$^{28}$, Leu$^{29}$, Asn$^{34}$, Asn$^{36}$, Phe$^{37}$, Leu$^{38}$ (Loop I) and Arg$^{73}$, Ile$^{77}$, Lys$^{80}$ (Loop III) in receptor binding [11–15].

Antagonists of PDGF receptor interactions would be expected to block or retard lesion formation and might be clinically beneficial in chronic inflammatory conditions as well as in atherosclerosis. Anti-PDGF antibodies have been shown to inhibit the binding and mitogenic effects of PDGF on murine embryo fibroblasts [16], to inhibit the chemotactic effect of PDGF on pulmonary artery fibroblasts [17] and to inhibit intimal lesion development after de-endothelialisation of the carotid arteries of rats [18]. Further inhibitors of PDGF-induced mitogenesis include Trapidil [19], 2-bromomethyl-5-chlorobenzene sulfonylphthalimide [20] and Suramin [21], although all suffer a lack of specificity. A 13-amino-acid peptide ANFLVW**EIVRKKP** which combines PDGF-BB$^{35-40}$ (loop I) and PDGF-BB$^{76-82}$ (loop III) containing a tryptophan residue modified with thianisole has also been identified as a possible antagonist [22].

We have examined the agonist/antagonist properties of a series of peptides corresponding to residues in loops I and III of PDGF-BB using human dermal fibroblasts. We have identified two peptides which are relatively potent in stimulating DNA synthesis and a cyclic peptide derived from these which inhibits PDGF-induced DNA synthesis. We demonstrate specificity of the cyclic peptide in terms of inhibition of growth factor binding and show a correlation of its ability to inhibit cellular proliferation with the presence of PDGF receptors on cells.

## 2. Materials and methods

### 2.1. Materials

All chemicals were of the highest grade and were from Sigma Chemical Company Ltd., Poole, UK, unless otherwise stated. All reagents for peptide synthesis were from PerSeptive Biosystems, Hertford, UK. Myoclone fetal bovine serum (FBS), Dulbecco's modified Eagles medium (DMEM), medium M199, L-glutamine, sodium pyruvate and gentamicin were from Life Technologies Ltd., Paisley, Scotland. Tissue culture plastics (Falcon plastics) were from Marathon Laboratory Supplies, London, UK. Methyl-[$^3$H]-thymidine (1.85 TBq/mmol) was from ICN Biomedicals Ltd., Thame, UK. Recombinant human PDGF-BB, EGF and FGF (146-amino-acid isoform) were from R&D Systems Europe Ltd., Abingdon, UK. $^{125}$I-Labelled recombinant human growth factors were from Amersham Interna-

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*Abbreviations:* PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell(s)


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tional plc, Amersham, UK. The cell lines HFFF2 (human dermal foreskin fibroblast), A431 (squamous carcinoma) and COS-1 (transformed African green monkey kidney) were obtained from the European Tissue Culture Collection, Porton Down, UK.

*2.2. General methods for peptide synthesis*

Peptides were synthesised by continuous-flow solid-phase synthesis using the Fmoc strategy [23]. N-terminal blocking was performed using 45% acetic anhydride in *N,N*-dimethyl-formamide for 30 min prior to cleavage and deprotection. Cyclisation of peptides was carried out on line using allyl based protection of Glu. Following removal of the Nα-Fmoc group, head to tail cyclisation was performed using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and 1-hydroxy-7-azabenzotrizole as per manufacturer's instructions. Peptides were purified by reverse-phase HPLC on a Vydac C18 column (22×250 mm) using a 30 min gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid. Identification and characterisation of the purified peptides were performed using analytical reverse-phase HPLC and ESI mass-spectrometry. Peptide sequences and defined peptide numbers are given in Table 1.

*2.3. Cell culture*

Complete DMEM and complete M199 are defined as the respective medium supplemented with 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. HFFF2 (passage numbers 11–15) and COS-1 cells were maintained in complete DMEM containing 10% FBS, at 37°C in a humidified chamber containing 10% $CO_2$, and 5% $CO_2$, respectively. A431 were maintained in complete M199 containing 10% FBS under 5% $CO_2$. Cells were kept in a subconfluent condition and were passaged following mild trypsinisation every 3–4 days into 75 $cm^2$ flasks.

*2.4. Effects of peptides on DNA synthesis*

HFFF2 cells were seeded into 48-well flat-bottom tissue culture plates at a cell density of 8000 cells/well, and grown until approximately 50% confluent (15000 cells/well, 2 days). Cells were rendered quiescent by replacing the medium with complete DMEM containing 0.2% or 0.5% FBS, for 64–72 h. The agonist behaviour of the peptides was studied by replacing the medium, at time zero, with complete DMEM containing 0.5% FBS and varying concentrations of the different peptides listed in Table 1. The antagonist effects were examined by coaddition of peptides with PDGF (15 ng/ml, 0.5 nM) in complete medium containing 0.2% FBS. In one set of experiments the medium was replaced after 4 h with complete DMEM containing 0.2% FBS. Each concentration was performed in triplicate, and experiments repeated at least twice. [$^3$H]Thymidine (11.1 Kbq/well) was added at time 22 h and incubated until time 28 h. Medium was aspirated, cells were washed twice with cold PBS (magnesium and calcium free), fixed by the addition of ice-cold 10% TCA (30 min), and washed with cold 70% ethanol. Following solubilisation of cells in 0.1 N NaOH (30 min), incorporated [$^3$H]thymidine was quantified by scintillation counting. Inhibition of cellular proliferation was calculated as % reduction in [$^3$H]thymidine incorporation with respect to the stimulation achieved in the absence of peptide.

*2.5. Receptor-binding inhibition assay*

The general methodology of Bowen-Pope and Ross [24] was followed. Briefly, 24-well flat-bottomed tissue culture plates were seeded with human fibroblasts, 25000 cells per well in DMEM containing 10% FBS, and cultured until confluent (2 days). The medium was replaced with DMEM containing 0.2% FBS for a period of 24 h. Wells were washed once with PBS and incubated for 10 min at 4°C with 500 μl of binding buffer (PBS/20 mM HEPES, pH 7.4 containing 5% BSA). Varying concentrations of peptide plus $^{125}$I-labelled PDGF (0.202 nM, 22.94 Tbq/mmol) or $^{125}$I-labelled EGF (0.09 nM, 51.54 TBq/mmol) in binding buffer were added to the cells in a total volume of 300 μl/well, and incubated for 2 h at 4°C on an orbital shaker. Wells were washed 3 times with cold binding buffer, and then cells were solubilised by incubation with 500 μl of PBS containing 1% (w/v) Triton X-100 and 1% (w/v) BSA for 1 h prior to determination of radioactivity using a gamma counter. For controls, wells were incubated with an additional amount of cold PDGF-BB or EGF (200 molar excess over labeled growth factor).

## 3. Results and discussion

Functional activity of protein molecules is often linked to dynamic conformational variations [25,26]. This may explain why loops I and III of PDGF are found to be very highly flexible with associated high temperature coefficients found in the X-ray crystal structure [10]. Peptides corresponding to these regions of PDGF which had previously been shown to be crucial for receptor binding were chosen for this study [11–15]. Recognition by receptors of flexible linear oligopeptides is sensitive to the entropic penalty incurred when the peptide becomes constrained to a well-defined region of its conformational space upon receptor binding [27,28]. We therefore reasoned that cyclisation of the peptides would enable them to mimic their natural conformations within PDGF-BB, and would be predicted to provide improved binding affinity to the PDGF receptors, as compared to their linear counterparts.

The series of peptides corresponding to PDGF-BB$^{73-81}$ include combinations where end-terminal charge groups have been modified by either acetylation of the N-terminus or amidation of the C-terminus, or Cys included at the C-terminus to allow peptide dimerisation. In the design of **P8**, 3 Gly were included in the sequence. These form a pliant linker which is estimated to be able to bridge the distance of 91.3 nm between Ile$^{25}$ and Leu$^{38}$, as defined by the crystal structure [10], and also maintain the flexibility of this loop I peptide.

Peptides were initially tested for their effects on growth-arrested human fibroblasts. **P1**, **P2**, **P3**, **P5**, and **P8** induced a direct stimulatory effect on these cells, but only at a concentration of 100 μM (Fig. 1). **P4** and **P6** induced cellular proliferation even lower than 6.25 μM this correlated with the presence of Cys in both peptides and the lack of N-terminal acetylation. The presence of Cys in both these peptides and subsequent thiol cross-linking thus allows them to cause PDGF receptor dimerisation, a factor which is crucial for the normal PDGF-induced mitogenic response [8]. The cyclic loop III construct, **P7**, showed no stimulatory effect, but reduced DNA synthesis below that observed with the growth-arrest

Table 1
Peptides used in this work

| Peptide number | Sequence and corresponding residue numbers |
|---|---|
| 1 | $^{73}$RKIEIVRKK$^{81}$ |
| 2 | Ac-$^{73}$RKIEIVRKK$^{81}$ |
| 3 | Ac-$^{73}$RKIEIVRKK$^{81}$-$NH_2$ |
| 4 | $^{73}$RKIEIVRKK$^{81}$-C |
| 5 | Ac-$^{73}$RKIEIVRKK$^{81}$-C |
| 6 | $^{77}$IVRKK$^{81}$-C-$^{73}$RKIE$^{76}$ |
| 7 | $^{77}$IVRKK$^{81}$-C-$^{73}$RKIE$^{76}$ (cyclic) |
| 8 | GG-$^{25}$I(S)RRLIDRTNANFL$^{38}$-CG (cyclic) |

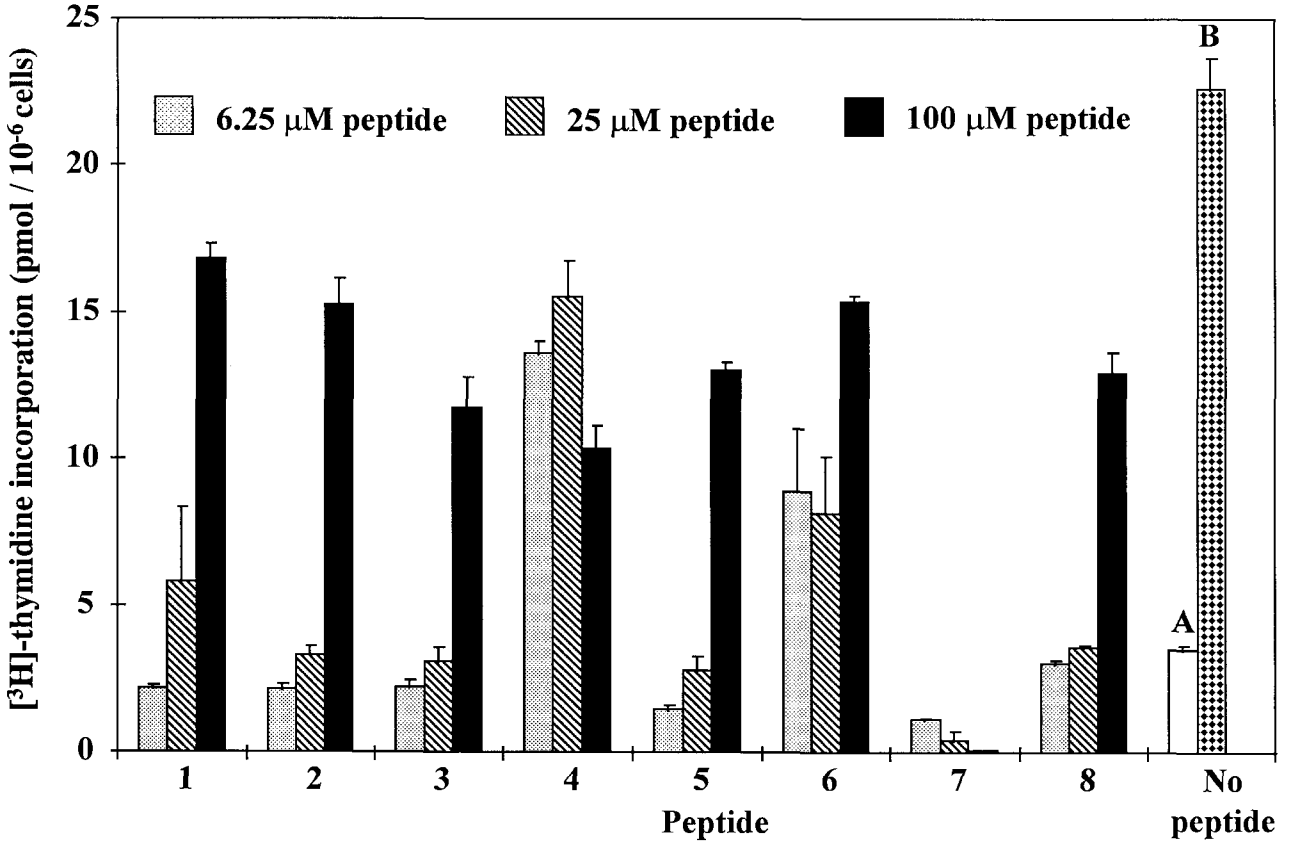


Fig. 1. The ability of peptides to directly induce DNA synthesis in growth-arrested human fibroblasts. Peptides in growth-arrest medium were incubated with cells at varying concentrations and the [$^3$H]thymidine uptake measured as described in Section 2. Comparison is made to controls grown in the absence (A), and presence (B) of PDGF-BB (0.5 nM). Results represent means ± SEM ($n = 3$).

control, throughout the range of peptide concentrations tested.

The $K_d$ for the interaction of PDGF-BB with purified PDGF extracellular receptor domains and with cells having very low copy numbers of receptor is approximately 1 nM [29,30]. By contrast on cells expressing the receptor in high

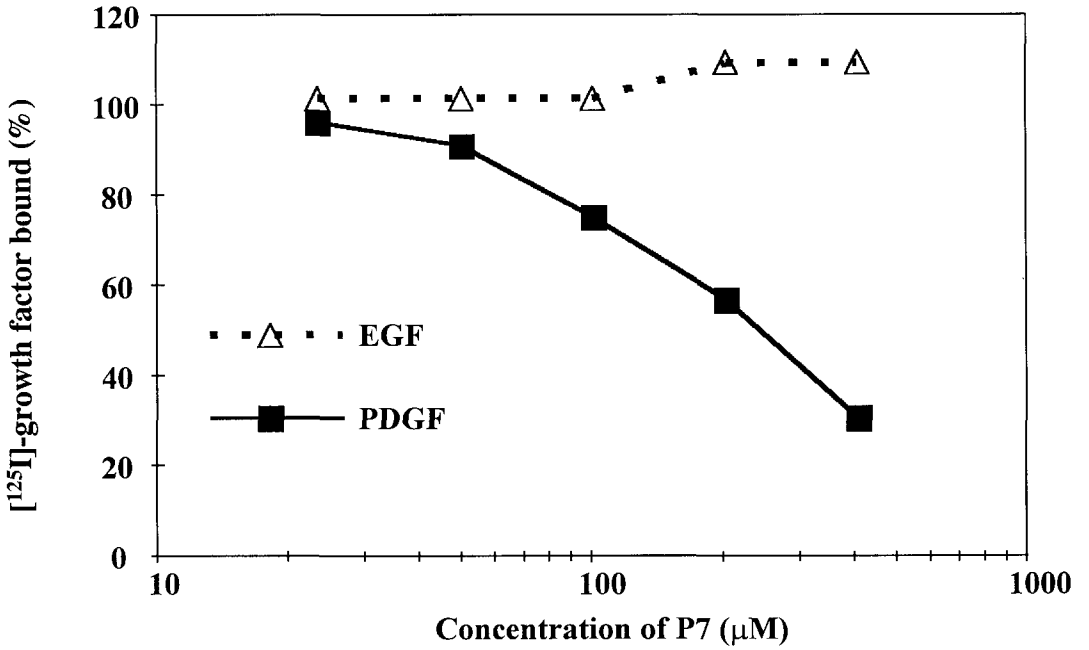


Fig. 2. The ability of peptide **P7** to influence the binding of $^{125}$I-labelled PDGF-BB or EGF to respective receptors on human dermal fibroblasts. Experimental procedures are described in Section 2. SEM was < 5% for all values shown.

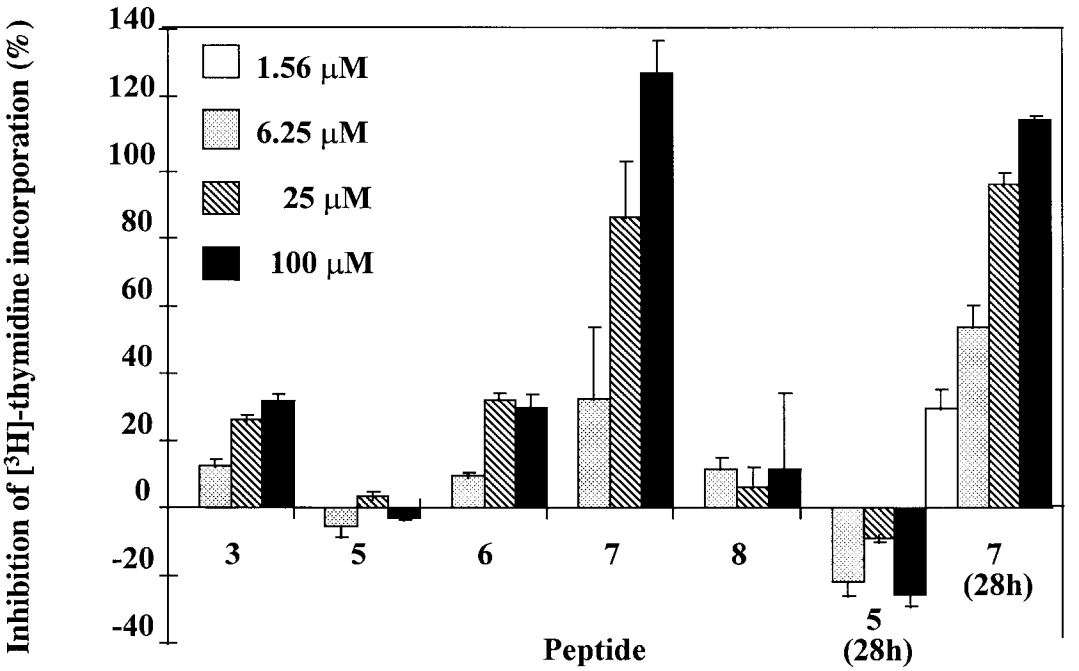


Fig. 3. The ability of peptides to inhibit PDGF-induced DNA synthesis in growth-arrested human fibroblasts. Co-incubation of PDGF with varying concentrations of peptides was either for 4 or 28 h (indicated on the $x$ axis), as described in Section 2. % Inhibition of [$^3$H]thymidine incorporation is calculated with respect to the uptake measured in the absence of peptide. Results represent means ± SEM ($n = 3$).

copy numbers, i.e. NIH 3T3 and human foreskin fibroblasts, the $K_d$ of the interaction is approximately 10 pM. This high affinity is presumed to be due to the simultaneous binding of the PDGF dimer to two receptors, as these cells have about 20 000–30 000 α-receptors and 100 000 β-receptors/cell [31]. We investigated the ability of loop III peptides **P2, P4, P5, P6, and P7** to inhibit the binding of $^{125}$I-labelled PDGF-BB to human dermal fibroblasts. Only peptide **P7** was able to inhibit this binding which was achieved with an $EC_{50}$ of 220 μM (Fig. 2, other data not shown). This ability to inhibit growth factor binding proved to be specific as peptide **P7** was unable to inhibit the binding of $^{125}$I-labelled EGF, which has a reported $K_d$ of 0.33–0.39 nM for binding to its full-length receptors [32]. This increase in the binding affinity of the cyclic peptide (**P7**) as compared to its linear counterparts is in agreement with our original hypothesis. The 67% sequence homology of PDGF-BB$^{73-81}$ with the corresponding 9-amino-acid loop III region PDGF-AA allows for the possibility that the peptides will also mimic PDGF-AA loop III, and this has yet to be examined.

An investigation into the ability of each peptide to affect PDGF-induced DNA synthesis (4 h co-incubation) of human fibroblasts showed that a reduction of 10–30% was induced by peptides **P3, P6,** and **P8** (Fig. 3). In the presence of a relatively high concentration of PDGF-BB (0.5 nM) only **P7** proved to be a very strong inhibitor of PDGF-BB-induced [$^3$H]thymidine incorporation, with an $EC_{50}$ of 9.5 μM. The other peptides (Table 1) were without effect. The reason for **P7** having a 30-fold greater $EC_{50}$ for inhibition of PDGF-BB binding as compared to the $EC_{50}$ for its inhibition of DNA synthesis is as yet unexplained, but may reflect the differences in inhibition of the biological effects of PDGF-BB versus total receptor occupancy. The greater affinity of PDGF-BB, as compared to **P7**, for the PDGF-receptor can be explained by the binding cooperativity provided by loops I and II present in PDGF-BB.

Other studies showed that a 28 h co-incubation of cells with PDGF-BB and anti-PDGF-BB antibodies provides a more rigorous test of the inhibition of mitogen-induced cellular proliferation than a shorter 4 h co-incubation (unpublished data). When PDGF and each of the peptides were incubated with cells during a 28 h period of growth it was observed that inhibition of PDGF-induced thymidine uptake by peptide **P7** still occurred, with an $EC_{50}$ of 6.3 μM (Fig. 3). Peptide **P7**, when incubated at 100 μM with cells not expressing PDGF receptors for 28 h (A431 or COS-1, [33,34]), had no effect upon DNA synthesis.

In summary, from a series of peptides from loops I and III of PDGF-BB we have identified two peptides which are able to stimulate cellular proliferation at a concentration of less than 6.2 μM. These peptides were not, however, able to inhibit the high-affinity interaction of $^{125}$I-labelled PDGF-BB binding to its receptor. A further peptide **P7** was identified which inhibited PDGF-BB-induced mitogenesis, specifically inhibited $^{125}$I-labelled PDGF-BB binding to its cellular receptors, and whose action was restricted to cells expressing PDGF receptors. Peptide antagonists of PDGF may be useful clinically in chronic inflammatory conditions and in preventing restenosis post-angioplasty and peptide **P7** may form a useful initial design step from which further compounds with increased potency can be developed. The mechanistic basis for the inhibition of PDGF activity by peptide **P7** is currently under investigation.

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