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13. Abstract (Maximum 200 Words) The primary focus of this work is the identification of molecules that block the interaction of growth factors with their receptor tyrosine kinases (RTKs). We plan to design, synthesize and evaluate a novel series of synthetic agents that bind to the surface of growth factors and block their interaction with their RTKs. We have now prepared two classes of protein binding agents. The first involves the attachment of four peptide loops to a central scaffold based on the calix[4]arene unit. The second is based around a tetraphenylporphyrin unit in which different recognition groups are attached. We have identified one molecule that binds to the surface of platelet derived growth factor and shows potent antitumor activity in a mouse xenograft model of a human cancer that is activated by PDGF.				
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Introduction

The primary focus of this project is the first step in the aberrant cell signaling pathways that lead to uncontrolled proliferation and cancer, namely the interaction of growth factors with their receptor tyrosine kinases (RTKs). Overexpression of RTKs as well as high serum levels of the activating growth factor are seen in certain breast and ovarian carcinomas. Furthermore, elevated levels of RTKs in breast cancer patients correlate with poor response to chemotherapy and shorter survival times. The design of growth factor antagonists that can inhibit ligand-induced receptor activation is a potentially novel route to new anti-cancer drugs. In recent years this strategy has been supported by the development of antibodies (e.g. Herceptin) against RTKs that have been shown to be active against breast cancer in the clinic. The main goals of this project are to design, synthesize and evaluate a novel series of synthetic agents that bind to the surface of growth factors and block their interaction with their RTKs. We have now prepared two classes of protein binding agents. The first involves the attachment of four peptide loops to a central scaffold (based on the calix[4]arene unit). The second is based around a tetraphenylporphyrin unit in which different recognition groups are attached. A key component of the second year of this project was to further confirm the proof of concept that molecular entities can be designed to bind to growth factor targets and block their function in living cells.

Body of Text

Task 1. To further refine the first generation of protein surface recognition agents based on four β -turn cyclic peptide mimetics linked to a calix[4]arene core scaffold (months 1-12).

In the past year we have made important steps in the wider application of this approach with the preparation of a series of unsymmetrical receptors in which two different loops are attached to the core calixarene (Figure 1).

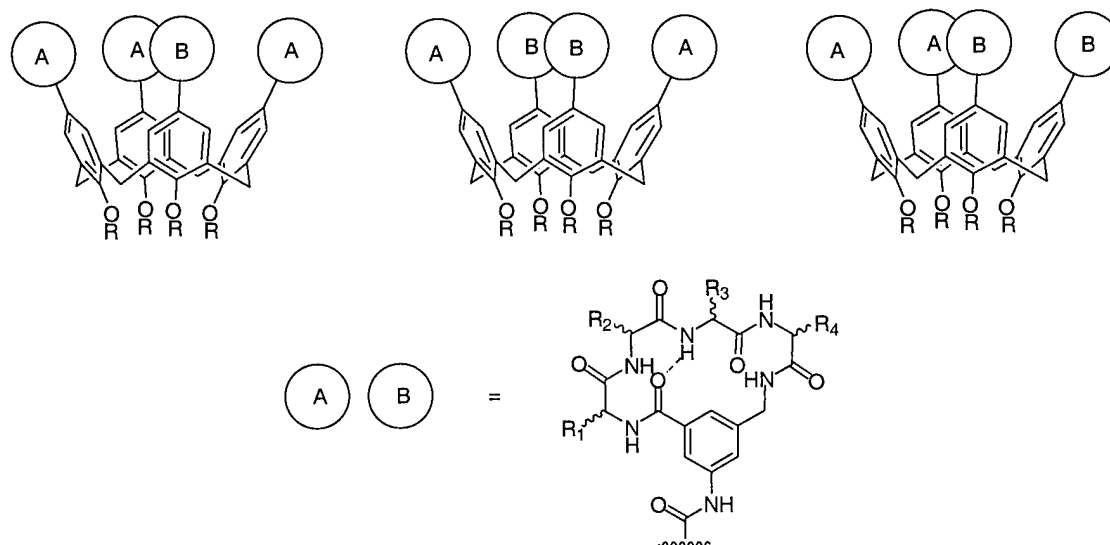
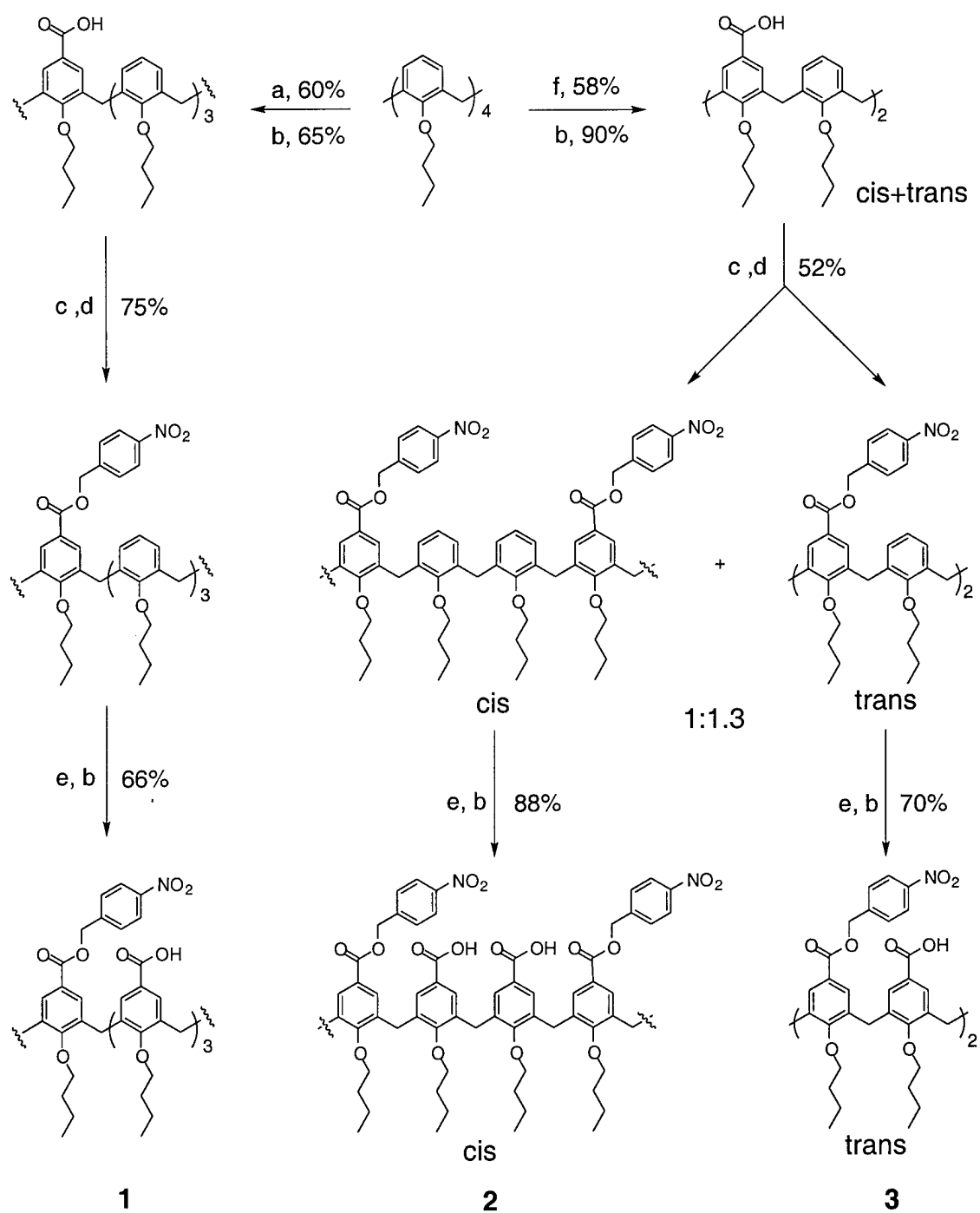


Figure 1. Design of unsymmetrical ligands based on a calix[4]arene scaffold.

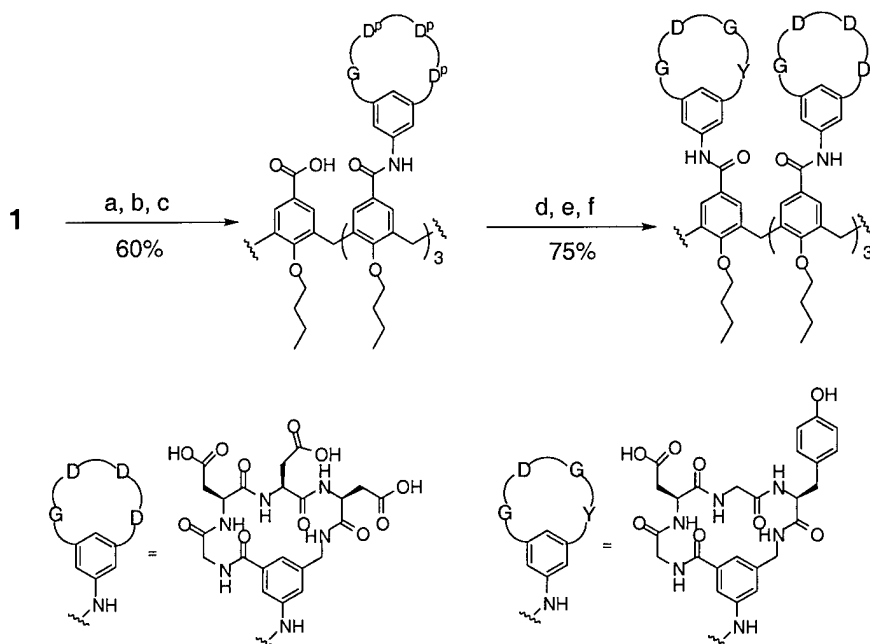
Calix[4]arene has been widely used in the field of supramolecular chemistry due to its well-defined shape.¹ However, few examples exist in which the upper rim of calixarene has been differentially functionalized.² In our effort to attach two different peptide loops onto the calixarene scaffold, we envisioned that three partially protected calix[4]arene tetracarboxylic acid derivatives, such as (1), (2), (3) (Scheme 1), should lead to unsymmetrical receptors through sequential coupling steps. Initial attempts to selectively protect butoxycalix[4]arene tetracarboxylic acid were unsuccessful, resulting in inseparable mixtures. However, stepwise functionalization of the upper rim of calix[4]arene could be carried out efficiently and gave all three partially protected derivatives in good yields (Scheme 1).³ Treating butoxycalix[4]arene with 1 eq. $\text{Cl}_2\text{CHOCH}_3$ in the presence of 1 eq. TiCl_4 gave primarily mono-formylated product in 60% yield. Subsequent oxidation and protection afforded a mono-calix[4]arene carboxylester. The p-nitrobenzyl ester was chosen due to its easy removal by hydrogenation after coupling to the peptide loops (Scheme 2). Further formylation with excess TiCl_4 and oxidation with NaClO_2 furnished the final product as a mono-protected butoxycalix[4]arene tetracarboxylic acid (1). The other scaffolds were prepared in an analogous manner. Formylation of butylcalix[4]arene with CH_2Cl_2 and SnCl_4 gave a mixture of bis-formylated derivatives which could not be separated by flash chromatography. The mixture was carried on to the bis-ester stage after which the two isomers can be easily separated by column in a 1:1.3 cis:trans ratio. The bis-acids were protected as p. nitrobenzyl esters. Further formylation and oxidation afforded the final products, cis-calix[4]arene bis-acid bis-ester (2) and trans-calix[4]arene bis-acid bis-ester (3), which set up all three required scaffolds for our receptor constructions.

The use of the scaffold 1 for the preparation of the unsymmetrical receptors is illustrated in Scheme 2. The first coupling proceeded using a normal acid chloride method.⁴ After removal of the p. nitrobenzyl protecting group under hydrogenation condition, the acid was converted to Yamaguchi-type anhydride⁵, and subsequently reacted with the second peptide loop to give the fully protected calix[4]arene tetracyclic peptide receptor. TFA treatment gave the final product. A series of X-3-XYZW-1-X'Y'Z'W' receptors were prepared in good yields by this route (Table 1).

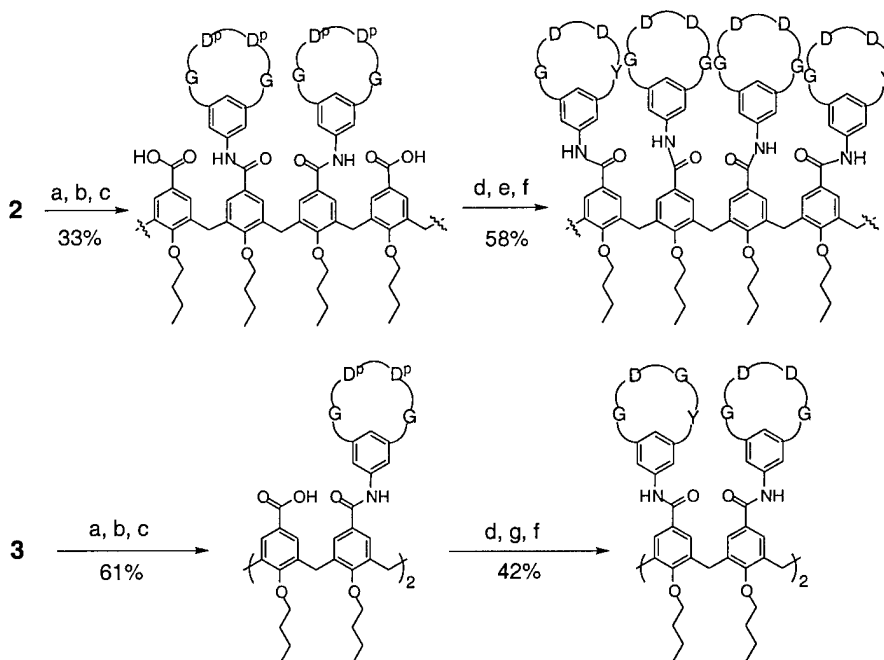
Similar routes were successfully performed on the preparations of cis-A2B2 and trans-A2B2 types of receptors (Scheme 3). In summary, we have developed an efficient route for stepwise functionalizations of calix[4]arene upper rim. It should be noted that functional groups other than carboxylate can also be incorporated into the synthetic scheme. A series of unsymmetrical receptors based on partially protected calix[4]arene tetra carboxylic acid was thus constructed. The biological testing of unsymmetrical receptors for various growth factor targets are underway.



Scheme 1 a) 1 eq. $\text{Cl}_2\text{CHOCH}_3$, 1 eq. TiCl_4 , CH_2Cl_2 , -10°C ; b) NaClO_2 , $\text{H}_2\text{NSO}_3\text{H}$, CH_2Cl_2 , Acetone, H_2O ; c) $(\text{COCl})_2$, cat. DMF, CH_2Cl_2 ; d) *p*-nitrobenzyl alcohol, DIEA, CH_2Cl_2 ; e) $\text{Cl}_2\text{CHOCH}_3$, TiCl_4 (excess), CH_2Cl_2 , -10°C ; f) $\text{Cl}_2\text{CHOCH}_3$, SnCl_4 , CH_2Cl_2 , -10°C .



Scheme 2. a) $(\text{COCl})_2$, cat. DMF, CH_2Cl_2 ; b) *cyclo*-(Gly-Asp(O^tBu)-Asp(O^tBu)-Spc)-NH₂, DIEA, CH_2Cl_2 ; c) 10% Pd/C, H₂, MeOH; d) 2,4,6-trichlorobenzoyl chloride, TEA, THF; e) *cyclo*-(Gly-Asp(O^tBu)-Gly-Tyr(O^tBu)-Spc)-NH₂, DMAP, Benzene/ CH_2Cl_2 ; f) 25% TFA/ CH_2Cl_2 .



Scheme 3. a) $(\text{COCl})_2$, cat. DMF, CH_2Cl_2 ; b) *cyclo*-(Gly-Asp(O^tBu)-Asp(O^tBu)-Gly-Spc)-NH₂, DIEA, CH_2Cl_2 ; c) 10% Pd/C, H₂, MeOH; d) 2,4,6-trichlorobenzoyl chloride, TEA, THF; e) *cyclo*-(Gly-Asp(O^tBu)-Asp(O^tBu)-Tyr(O^tBu)-Spc)-NH₂, DMAP, Benzene/ CH_2Cl_2 ; f) 25% TFA/ CH_2Cl_2 ; g) *cyclo*-(Gly-Asp(O^tBu)-Gly-Tyr(O^tBu)-Spc)-NH₂, DMAP, CH_2Cl_2 .

Table 1 Unsymmetrical Receptors.

sequence	yield	molecular weight	
		expected(M+H ⁺)	determined(ES-MS)
X-3(GDDD)-GDGY	45%	2945.82	2925.50 ± 0.55
X-3(GDGY)-GDDG	45%	2867.88	2867.37 ± 0.46
X-3(GDGY)-GDDY	52%	2974.00	2973.75 ± 0.87
X-3(GDGY)-GDDD	51%	2925.91	2926.01 ± 0.31
X-3(GDGD)-GDGY	43%	2771.71	2771.22 ± 1.30
X-3(GDGD)-GDDD	57%	2778.98	2778.22
X-3(GDGD)-GDDY	54%	2829.75	2828.74 ± 0.45
trans-X-2(GDDG)-2(GDGY)	26%	2819.80	2818.79 ± 0.72
cis-X-2(GDDG)-2(GDDY)	19%	2935.88	2935.62 ± 0.07

In addition to our work on calixarene based protein surface binding agents we have also continued to develop our strategy that utilizes alternatives to the calixarene unit. In particular we have established the tetraphenylporphyrin group as a powerful scaffold for targeting the surface of cytochrome c (as a model for highly charged growth factor surfaces). This design is based around a tetraphenylporphyrin unit in which different recognition groups are attached. Details of this approach have recently been published in *Organic Letters* (**Ref.1**, Reportable Outcomes) and a copy of the paper is attached.

Task 2. To develop combinatorial libraries of protein surface binding agents that can be targeted to a range of growth factor structures (months 12-24).

This task has been a major focus of the current year and we have made good progress. The work described under task 1 summarizes our progress towards parallel libraries based on unsymmetrically substituted calixarenes. In the search for an alternative synthetic route to receptor libraries, an obvious choice is a solution phase two-step mixture coupling method. In this method, n different cyclic peptides are put into the coupling solution. If we assume they have equal reactivities, we should anticipate a mixture library being generated that contains a large number of receptors whose percentage in the library should follow a statistical rule. If we look more closely at this mixture, we should find all six possible unsymmetric receptors (Figure 2) in addition to the four-fold symmetric ones, which are otherwise very difficult to prepare. However, at the same time, the *degeneracy* occurs as a result of the scaffold symmetry (Table 2). For example, we may have six different arrangements (six different compounds) for the ABCD-type receptor. Considering the degeneracy and the combinatorial rule, a theoretical calculation of the size of a receptor library is shown in Table 2, and exemplified in Table 3. For example, a library of 1,044 receptors can be generated by mixing only 8 different cyclic peptides with the activated calixarene scaffold.

To identify the effective protein surface receptors from the mixture libraries, a simple detection method is needed. Mass spectrometry has proved itself highly suitable for such applications for its sensitivity, speed of analysis, and automation capability with solution phase techniques such as HPLC and CE. The success of a quantitative mass monitoring will critically depend on that the individual mass peaks of library compounds being distinguishable, and having similar physical properties such as laser desorption abilities. To test the viability of the MALDI-TOF method in our library monitoring process, a library was generated by coupling four cyclic peptides: *cyclo*-GDGDSp, *cyclo*-GDNDSp, *cyclo*-aDGDSp, *cyclo*-GDDYSp, and one equivalent of H₂O with the scaffold. The above cyclic peptides were selected from a pool of cyclic peptides with the aid of a computer program (MASP) such that the resulting library mixture does not have overlapped peaks in the mass spectrum.

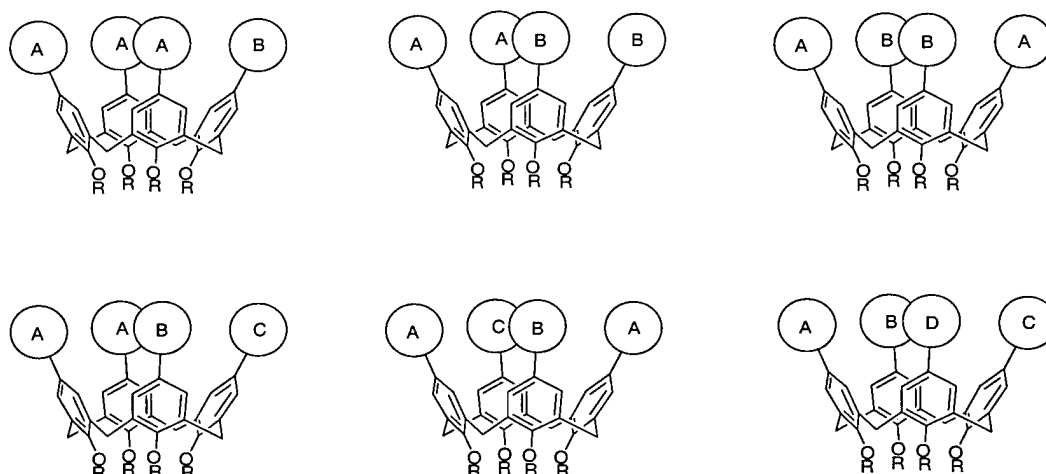


Figure 2 Six types of unsymmetrical receptors in mixture libraries.

Table 2 Calculated number of receptors produced with n cyclic peptides.^a

Combination type	Degeneracy	Combinatorial rule	Combinations
AAAA	1	n	n
AAAB	1	$n(n-1)$	$n(n-1)$
AABB	2	$n(n-1)/2$	$n(n-1)$
AABC	3	$n(n-1)(n-2)/2$	$3n(n-1)(n-2)/2$
ABCD	6	$n(n-1)(n-2)(n-3)/4!$	$n(n-1)(n-2)(n-3)/4$

^aThe total number of compounds equals the sum of the number of compounds of all five combination types.

Table 3 Examples of library sizes for n cyclic peptides.

Combination type	$n = 4$	$n = 6$	$n = 8$	$n = 10$	$n = 20$
AAAA	4	6	8	10	20
AAAB	12	30	56	90	380
AABB	12	30	56	90	380
AABC	36	180	504	1,080	10,260
ABCD	6	90	420	1,260	29,070
Total	70	336	1,044	2,530	40,110

Calculation of the expected mass distribution shows that a 170-membered multi-loop library can be generated with four cyclic peptides and one equivalent of water. Specifically, 26 bis-loop receptors, 74 tris-loop receptors, and 70 tetra-loop receptors are in the library (Figure 3A). Indeed, the MALDI-TOF mass spectrum for this library (Figure 3B) showed an exact distribution of multi-loop receptors as well as peak intensity as predicted by calculation, indicating the similar reactivities of the cyclic peptide amines.

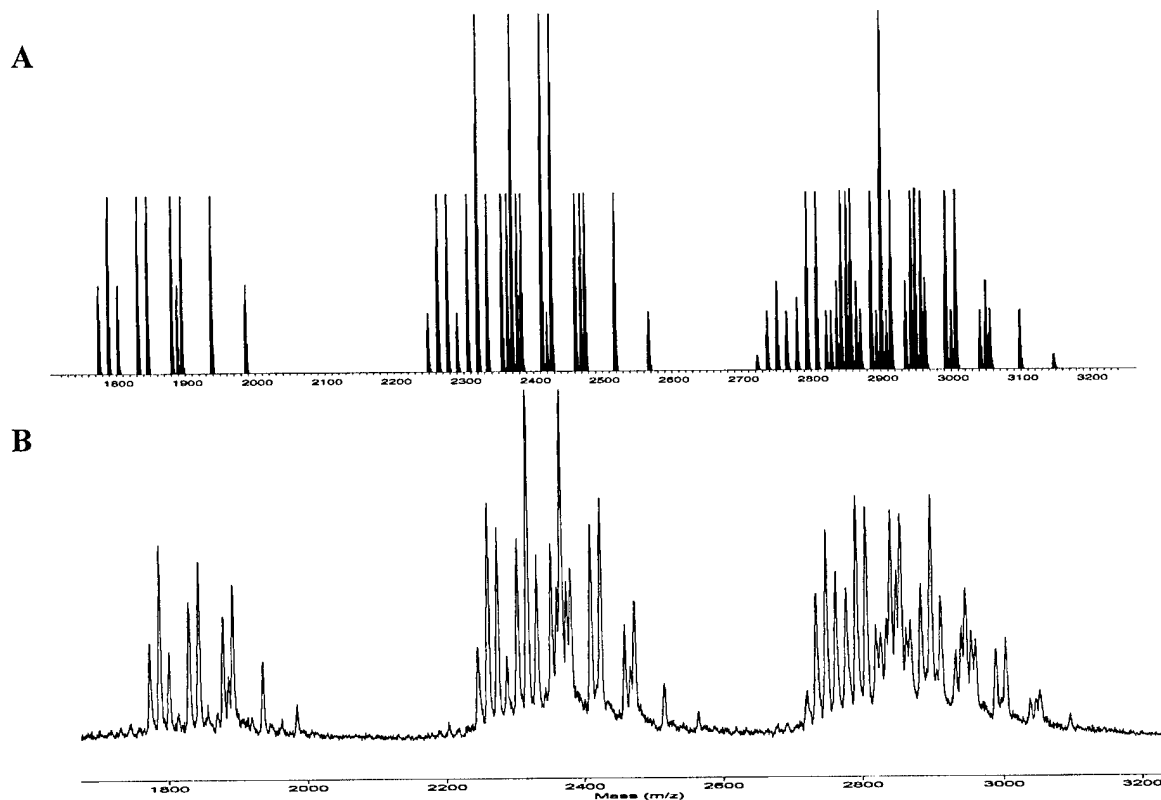


Figure 3. MALDI-TOF mass spectra of a mixture library: (A) the predicted mass spectrum using a computer program MASP; (B) the observed mass spectrum.

These results provide good support for the strategy of solution generation of libraries. We are currently investigating mass spectrometric methods that allow us to selectively detect which members of the library are binding to protein targets.

Task 3. To develop synthetic antagonists for PDGF activation of its receptor tyrosine kinase that are active both in purified protein and in whole cell assays (months 1-18).

We have continued to make excellent progress in the completion of task 3. We have prepared a large number of cyclic peptide derivatives based on the 3-aminomethylbenzoic acid scaffold and attached them to the core calixarene scaffold. These have been tested for their ability to interact with platelet derived growth factor using fluorescence spectroscopy, non-denaturing gel electrophoresis and protein binding assays. We have further shown that one key molecule can disrupt the growth of PDGF activated human tumor cell growth in a nude mouse

model. This work has recently been published in *Nature Biotechnology* (**Ref. 2**, Reportable Outcomes) and in *Oncogene* (**Ref. 3**, Reportable Outcomes) and copies of the papers are appended.

Task 4. To target other growth factors involved in aberrant breast cell proliferation pathways, including VEGF, EGF and heregulin (months 18-36).

This task will be a major focus in the next year of this project.

Key Research Accomplishments

- Developed stepwise synthetic routes to unsymmetrical calixarene derivatives containing different peptide loop recognition domains.
- Designed, synthesized and evaluated a novel series of protein surface receptors based on a tetraphenylporphyrin scaffold and showed that certain compounds can bind with high affinity to cytochrome c.
- Developed parallel and solution phase methods for the preparation of combinatorial libraries of protein surface binding agents. Established a mass spectrometric approach to determine the diversity of the solution based libraries.
- Showed that one calixarene tetraloop derivative is able to bind to platelet derived growth factor and inhibit it from binding and activating its receptor tyrosine kinase. This compound was also shown to have potent anti-tumor activity in a nude mouse xenograft model.

Reportable Outcomes

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3) Sebt, S. M.; Hamilton, A. D. "Design of Growth Factor Antagonists with Antiangiogenic and Antitumor Properties" *Oncogene*, **2000**, 19, 6566-6573.

Conclusions

During the past project period we have continued to make progress in establishing the concept that synthetic agents can be used to bind to growth factor surfaces and to block their oncogenic function in activating tumor cell growth. We have significantly advanced our synthetic work on the design of agents for the complexation of protein exterior surfaces. We have extended our design strategy with the synthesis of unsymmetrical calixarene derivatives with different peptide loops arrayed around the upper rim. We have also developed parallel and solution phase approaches to libraries of protein surface binding agents. These methods should allow us to rapidly screen for strong binding agents against a range of protein targets. We have identified one molecule that not only binds to the surface of platelet derived growth factor with high affinity ($IC_{50} < 250nM$) under physiological conditions but also blocks its activation of PDGF receptor tyrosine kinase. This molecule also shows potent antitumor activity in a mouse xenograft model of a human cancer that is activated by PDGF. These compounds have the potential to be the first in a new class of rationally designed antitumor agents that function similarly to new antibody based therapies in blocking growth factor function but that are based around relatively low molecular weight synthetic derivatives.

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Protein Surface Recognition by Synthetic Receptors Based on a Tetraphenylporphyrin Scaffold

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ABSTRACT



synthetic
receptor

protein
surface

Receptors based on a tetraphenylporphyrin scaffold bearing different charged and hydrophobic groups have been synthesized. The interactions of these with horse heart cytochrome *c* were studied by fluorescence spectroscopy. Receptor 4 was identified to be the strongest synthetic receptor ($K_d = 20$ nM) for cytochrome *c*. The differences in affinity among the receptors reflected a dependence on the number of anionic and hydrophobic groups.

The past two decades have seen enormous progress in the design of synthetic molecules targeted to disrupt protein–ligand interactions.¹ The majority of these medicinally important molecules disrupt interactions occurring inside well-defined cavities on the proteins. In contrast, synthetic molecules that mediate protein function through binding to the solvent-exposed *exterior surface* are largely unexplored.² This is surprising since such molecules may provide a fundamentally different mechanism for modulating protein function, by sterically preventing protein–ligand and protein–protein interactions. This strategy may not only result in the discovery of novel drug candidates but may also provide opportunities to systematically understand the role of protein exterior surfaces in molecular recognition.

Recently, we have reported a novel family of synthetic receptors that target protein exteriors.³ The initial design,

involving four peptide loops arrayed around a central calix-[4]arene core, was shown to inhibit the approach of small molecule substrates to cytochrome *c* and chymotrypsin at submicromolar concentrations. However, the calixarene unit suffers several complications, including difficulty of synthesis and tendency to aggregation. We sought to improve this synthetic approach by studying different organic scaffolds onto which recognition sites can be attached. Porphyrins, owing to their large size, rigidity, and photophysical properties, have been used in numerous artificial receptors and model systems in bioorganic and bioinorganic chemistry.⁴ In this Letter, we report on the design, synthesis, and recognition properties of a series of tetraphenylporphyrins that bind, in certain cases with high affinity, to the surface of cytochrome *c*.

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Cytochrome *c* is a particularly attractive target since it plays key roles in electron transfer and apoptosis, which are mediated by complex formation to other proteins (cytochrome *c* oxidase, Apaf1, etc.). One critical recognition region involves an array of lysine and arginine residues surrounding the exposed heme edge surface.⁵ The tetraphenylporphyrin scaffold closely matches the arrangement of hydrophobic and charged domains present in this region (Figure 1). In pioneering work, Fisher recognized this



Figure 1. A space filling representation of horse heart cytochrome *c* based on its X-ray crystal structure.⁷ The heme group which directly faces the reader is drawn in gray spheres. Black spheres represent positively charged lysine and arginine residues. White spheres correspond to all other residues. A tetraphenylporphyrin scaffold (black cylindrical bonds) is drawn in the center.

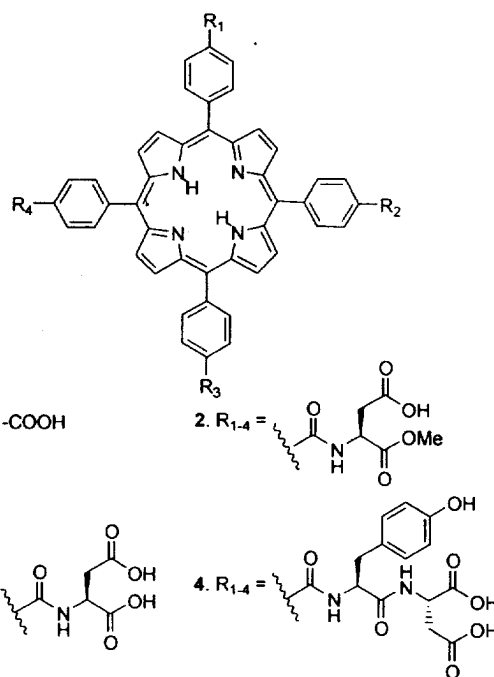
geometrical relationship and showed that tetracarboxyphenyl porphyrin **1** binds to cytochrome *c* with a K_d of $\sim 5 \mu\text{M}$.⁶

To determine the optimum combination of recognition features in synthetic receptors of this type, we designed a series of tetraphenylporphyrins with various amino acid derivatives attached. The resulting receptors contain a large, flat, and semirigid molecular surface of approximately $300\text{--}400 \text{ \AA}^2$ in area. The receptors were prepared by the generation of the tetra-acid chloride $((\text{COCl})_2, \text{DMF})$ starting from *m*-tetrakis(4-carboxyphenyl) porphyrin **1** and subsequent coupling with the corresponding *tert*-butyl-protected amino acid or peptide amines. Deprotection of the *tert*-butyl groups with trifluoroacetic acid provided receptors **2–4**. Acidic and hydrophobic attachments were chosen to complement the cationic and hydrophobic surface surrounding the heme edge of cytochrome *c*.

Compounds **1–4**, and coproporphyrin I, a naturally occurring tetraanionic porphyrin, were initially screened for binding to horse heart ferricytochrome *c* using fluorescence spectroscopy. Addition of cytochrome *c* to solutions containing **1–4** resulted in quenching of porphyrin fluorescence emission ($\text{ex} = 420 \text{ nm}$, $\text{em} = 650 \text{ nm}$) due to the enforced proximity of the Fe(III) heme that results from complex formation between the protein and the receptors.

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In contrast, titrations with tetracationic *m*-tetrakis(4-trimethylaminophenyl) porphyrin (TTMAPP), showed no quenching even at high concentrations, indicating the absence of nonspecific binding. Typical titrations of **3**, **4**, and TTMAPP with cytochrome *c* are shown in Figure 2 (see Supporting Information for others).

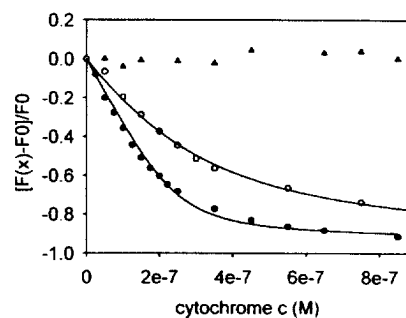


Figure 2. Fluorescence quenching of **4** (●), **3** (○), and TTMAPP (▲) upon addition of cytochrome *c*. The curve fit indicates a K_d of $20 \pm 5 \text{ nM}$ for **4** and $160 \pm 20 \text{ nM}$ for **3**. Titrations were carried out under 250 nM initial receptor concentration in 5 mM sodium phosphate buffer, $\text{pH} = 7.4$, at 298 K .

Dissociation constants were derived by curve fitting to a 1:1 binding equation⁸ with stoichiometries being fixed at $n = 1$ for all compounds. Preference for 1:1 binding was confirmed for receptors **3** and **4** by Job's method⁹ (see Supporting Information). K_d values for all compounds are summarized in Table 1.

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Table 1. Dissociation Constants^a and Structural Properties of Synthetic Receptors and Other Water-Soluble Porphyrins

compound	K_d (nM)	charge	aryl groups
1	950 ± 250	-4	4
2	860 ± 90	-4	4
3	160 ± 20	-8	4
4	20 ± 5	-8	8
coproporphyrin I	7700 ± 270	-4	0
uroporphyrin I	1000 ^b	-8	0

^a Determined at 5 mM sodium phosphate, pH 7.4, 298 K. ^b From ref 10, at $m = 4$ mM sodium phosphate, pH 7.26, 298 K.

Significant changes in affinity for cytochrome *c* were observed by altering the relative proportions of acidic and aromatic functionalities in the receptors (Table 1). Receptors **2** and **3** differ only by the substitution of four methyl esters by carboxylic acids, respectively, providing controls for probing the charge requirements for cytochrome *c* recognition. A ~5-fold increase in affinity was seen, on going from receptor **2** to receptor **3**, indicating a preference for octaanionic receptors over their tetraanionic counterparts. Receptors **1** and **2**, with the same number and type of charged groups, show little difference in their binding affinities. Similar trends were observed when aromatic groups were incorporated into the receptor while keeping the number of charged groups constant. Rodgers had earlier shown that uroporphyrin I, containing eight carboxylate groups, binds cytochrome *c* with μ M affinity.¹⁰ However, receptor **3** which has eight anionic groups and 4 phenyl groups binds to cytochrome *c* approximately 6-fold stronger than uroporphyrin. In a similar analysis, receptor **2** binds cytochrome *c* 9-fold tighter than tetraanionic coproporphyrin I. These results suggested that an appropriate combination of charged and hydrophobic groups on the porphyrin periphery would give a molecule with exceptionally high affinity for cytochrome *c*. To confirm this, we designed receptor **4** to contain eight negatively charged groups and eight phenyl groups. The titration curve (Figure 1) shows a sharper achievement of saturation with **4** compared to **3**. This corresponds to a K_d of 20 ± 5 nM for **4** binding to cytochrome *c* and represents an 8-fold increase in affinity compared to **3** (Table 1). To our knowledge, **4** is one of the strongest synthetic receptors for cytochrome *c*, under these experimental conditions. The binding experi-

ments between **4** and cytochrome *c* were carried out in 5 mM phosphate buffer at pH 7.4. Remarkably, receptor **4** with a molecular weight of ~1900 Da can bind to cytochrome *c* 100 fold stronger than its natural protein partners such as cytochrome *c* peroxidase, which has a K_d of 2.4 μ M measured at 5 mM phosphate buffer, pH = 7.¹¹ The combination of many electrostatic and hydrophobic interactions over a large contact surface is primarily responsible for the formation of high-affinity protein-protein complexes in nature.¹² The synthetic receptors reported here behave similarly, attaining large enhancements in affinity through the incorporation of both anionic and aromatic groups. The tetraphenylporphyrin scaffold appears to provide a template in which the peripheral anionic and aromatic groups take up a good geometrical relationship to the cationic and hydrophobic side chains of cytochrome *c*. On the basis of these findings, it should be possible to extend this approach to identify new receptors for protein targets that are known to interact with simple porphyrin derivatives.¹³

In summary, we have designed and synthesized synthetic receptors that recognize a protein surface with high affinity in aqueous medium. Analogues of receptor **4** with less symmetrical substitution patterns may provide a clearer understanding of the detailed recognition properties of the cytochrome *c* protein surface. In addition to providing a new strategy for protein surface recognition, these findings may have important medicinal consequences. Cytochrome *c* has been shown to interact with Apaf1, leading to the activation of programmed cell death or apoptosis.¹⁴ Receptor **4** may serve as a valuable lead in the search for efficient disruptors of Apaf1-cytochrome *c* interaction.

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Supporting Information Available: Experimental procedure and characterization for compounds **2**–**4**. Fluorescence titration plots for **1**–**2** and coproporphyrin I and Job's plots for **3** and **4**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Design of GFB-111, a platelet-derived growth factor binding molecule with antiangiogenic and anticancer activity against human tumors in mice

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We have designed a molecule, GFB-111, that binds to platelet-derived growth factor (PDGF), prevents it from binding to its receptor tyrosine kinase, and blocks PDGF-induced receptor autophosphorylation, activation of Erk1 and Erk2 kinases, and DNA synthesis. GFB-111 is highly potent (IC₅₀ = 250 nM) and selective for PDGF over EGF, IGF-1, aFGF, bFGF, and HRGβ (IC₅₀ values > 100 μM), but inhibits VEGF-induced Flk-1 tyrosine phosphorylation and Erk1/Erk2 activation with an IC₅₀ of 10 μM. GFB-111 treatment of nude mice bearing human tumors resulted in significant inhibition of tumor growth and angiogenesis. The results demonstrate the feasibility of designing novel growth factor-binding molecules with potent anticancer and antiangiogenic activity.

Keywords: platelet-derived growth factor, angiogenesis, oncogenesis, cancer drug discovery

Platelet-derived growth factor (PDGF) is a potent inducer of growth and motility in several cell types such as fibroblasts, endothelial cells, and smooth muscle cells. It induces cell proliferation, angiogenesis, wound healing, and chemotaxis, and inhibits apoptosis¹⁻³. Additionally, PDGF has been directly implicated in malignant diseases involving uncontrolled cell proliferation such as cancer, where overexpression of PDGF and/or PDGF receptors is common in human tumors including glioblastomas and sarcomas². Also, PDGF stimulates the proliferation and migration of endothelial cells leading to formation of new blood vessels, a process that is required for the growth of tumors⁴⁻⁷. Furthermore, PDGF has also been shown to stimulate endothelial cells to express high levels of vascular endothelial growth factor (VEGF), a potent angiogenesis inducer^{7,8}. Finally, the importance of PDGF in angiogenesis has also been documented by the fact that mice deficient in PDGF-BB or its receptor β are defective in blood vessel development^{9,10}.

To elicit the above biological response, PDGF binds to its cell surface receptor, PDGFR, a tyrosine kinase¹¹⁻¹³. Binding results in dimerization, receptor auto (cross) phosphorylation, and recruitment by means of the resulting phosphotyrosine of SH2 domain-containing signaling proteins such as Grb2/Sos1, PLC-γ, PI-3 kinase, and Src^{11,12}. These proteins trigger several arms of the PDGF signal transduction pathways that are involved in different cellular responses. For example, Grb2/Sos1 activates the GTPase Ras, resulting in the activation of a cascade of mitogen-activated protein kinases (MAPK), such as Erk1 and Erk2, and this contributes to the proliferation arm of the PDGF signaling pathway¹³. Others, such as PI3 kinase, activate yet another kinase, AKT, which is responsible for the survival or the anti-apoptotic arm of PDGF signaling pathways¹.

The involvement of PDGF overactivity in proliferative diseases prompted many researchers to target PDGF as a therapeutic strategy¹⁴⁻²². The approaches used to interfere with aberrant PDGF function in disease include PDGFR antibodies that block PDGF binding¹⁴, linear or cyclic peptides corresponding to areas of PDGF that bind its receptor^{15,16}, receptor dimerization antagonists¹⁷, and inhibitors of the tyrosine kinase activity of the receptor^{18,19}. An under-exploited area in the search for antagonists of PDGF overactivity is that of rationally designed agents that bind to PDGF and inhibit it from activating its receptor. In this area only a few strategies such as antibodies against PDGF²⁰, soluble forms of PDGFR²¹, and DNA aptamers²² have been tried with marginal success. These are all based on biosynthetically derived compounds that are limited in structural building blocks to the four nucleosides or 20 amino acids. Synthetic derivatives that antagonize PDGF binding have the potential advantages of being stable to proteolytic or nuclease cleavage, having smaller molecular weights and utilizing a wider range of structural scaffolds that might complement the protein surface. To date there have been no examples of nonnatural systems that antagonize PDGFR activation by discrete binding to the growth factor surface. This is primarily due to the difficulty of designing agents to disrupt protein-protein interactions that are mediated over large surface binding areas. We have previously reported the synthesis of a protein-binding molecule that interacts with strongly cationic regions on chymotrypsin²³ and cytochrome c²⁴. In the present paper we significantly expand this strategy with the synthesis of a library of binding agents from which we identify a molecule (GFB-111) that targets the regions of PDGF (loops I and III) that are involved in binding to its receptor. GFB-111 treatment of nude mice bearing human tumors resulted in significant inhibition of tumor growth and angiogenesis.

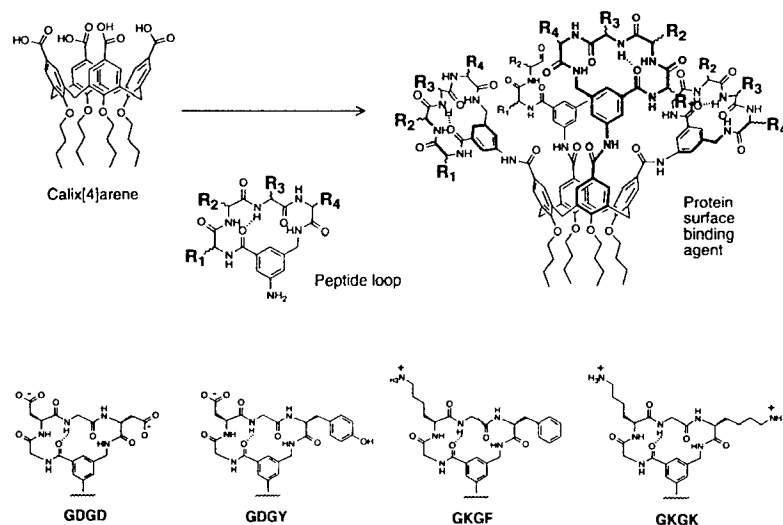


Figure 1. Structure of growth factor binders (GFBs). GFBs were synthesized as described^{23,24}.

Results

Design of GFB-111, a synthetic molecule that binds PDGF. To design molecules that bind PDGF and disrupt its signaling function, we have prepared a novel series of protein surface binding agents. The molecules are composed of a central calix[4]arene scaffold to which are attached four peptide loop domains (Fig. 1). The peptide loop component is based on a cyclic hexapeptide in which two residues were replaced by a 3-aminomethylbenzoate dipeptide mimetic containing a 5-amino substituent for attachment to the scaffold. The resulting molecules (Fig. 1 and Table 1) contain a functionalized and variable surface

~500 Å² in area. Several peptide loop sequences were synthesized (Table 1) to provide molecular surfaces with negatively and positively charged as well as hydrophobic regions designed to bind to complementary areas on the target growth factor. To identify PDGF-binding molecules from this library, we first used a cell-based screening assay with NIH 3T3 cells to detect molecules capable of blocking PDGF-BB-induced tyrosine autophosphorylation of the PDGFR. The ability of the identified molecules to bind PDGF-BB and inhibit interaction with its receptor was then confirmed biochemically. Starved NIH 3T3 cells were pretreated for 5 min with the synthetic molecules before stimulation with PDGF for 10 min. The effects of the molecules on PDGF-stimulated receptor tyrosine phosphorylation were determined by antiphosphotyrosine western blotting. Screening the library for potential PDGF binders identified GFB-111 as a potent ($IC_{50} = 250$ nM) inhibitor of PDGF-BB stimulation of PDGFR tyrosine autophosphorylation (Table 1). GFB-111 has four-fold symmetry, containing four identical peptide loops with negative and hydrophobic residues, GDGY, that are designed to be complementary to the PDGF surface involved in binding to

PDGFR. The area of PDGF-BB that binds PDGFR is composed primarily of positive and hydrophobic residues^{25,26}. Substitution of aspartic acid in the loop by positively charged lysine is not tolerated (compare GFB-111 (GDGY) ($IC_{50} = 250$ nM) to GFB-115 (GKGF) ($IC_{50} = 50$ μM) and GFB-116 (GKVK) ($IC_{50} = 40$ μM)), indicating that negatively charged residues are important for the inhibitory activity of GFB-111. However, negatively charged residues are not sufficient, and the presence of an aromatic hydrophobic residue in the loop is also critical (compare GFB-111 (GDGY) to GFB-107 (GDGD) ($IC_{50} = 2.5$ μM), as well as GFB-122 (GDDY) ($IC_{50} = 1.7$ μM) to GFB-106 (GDDD) ($IC_{50} = 8$ μM)) (Table 1).

The structure/activity relationship results in Table 1 are consistent with GFB-111 being a PDGF-BB-binding molecule. To confirm this, we determined whether GFB-111 binds PDGF-BB by gel electrophoresis and also inhibits binding of ¹²⁵I-labeled PDGF (¹²⁵I-

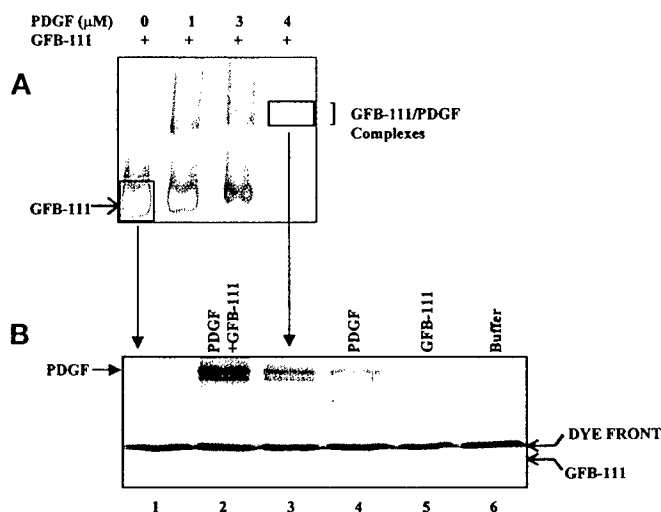


Figure 2. GFB-111 binds PDGF and inhibits ¹²⁵I-PDGF binding to its receptor on NIH 3T3 cells. (A) GFB-111 was incubated with increasing concentrations of PDGF-BB and the mixtures were then loaded onto native PAGE. Bands corresponding to the boxed area were cut out of the lanes of the gel, denatured, and loaded onto SDS-PAGE gel. (B) Lanes 1–6 correspond to GFB-111 from the native gel; PDGF + GFB-111 standards; PDGF + GFB-111 complex from the native gel; PDGF standard; GFB-111 standard; and buffer, respectively. Data are representative of three independent experiments. (C, D) NIH 3T3 (PDGF) or NIH 3T3 cells overexpressing EGFR (EGF), IGF1R (IGF), or Flk-1 (VEGF) were incubated with ¹²⁵I-PDGF, ¹²⁵I-EGF, ¹²⁵I-IGF, or ¹²⁵I-VEGF, respectively. (C) Specific ¹²⁵I-PDGF binding to NIH 3T3 cells with increasing concentrations of GFB-111 (◆) or GFB-116 (■) were used. (D) Specific binding with GFB-111 at 10 μM. Empty and filled bars represent control and GFB-111 treated samples, respectively. Cells were incubated at 4°C and washed. Lysates plus wash were counted. Excess of cold growth factors was used to obtain nonspecific binding levels. Data are representative of three independent experiments.

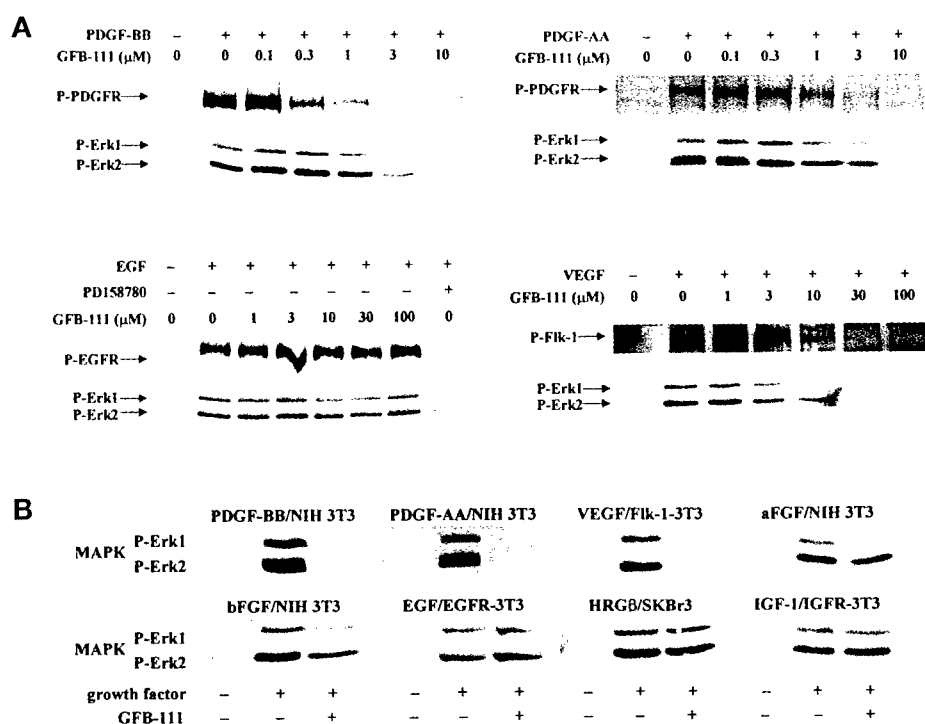


Figure 3. GFB-111 inhibits selectively PDGF-stimulated receptor tyrosine autophosphorylation and activation of MAPK. (A) Starved NIH 3T3 cells (PDGF-BB and PDGF-AA), and NIH 3T3 cells overexpressing either EGFR (EGF) or Flk-1 (VEGF) were pretreated with GFB-111 before stimulation with PDGF-BB, PDGF-AA, EGF, and VEGF. Cell lysates were run on SDS-PAGE gels, then transferred to nitrocellulose and western blotted with anti-phosphotyrosine or anti-phosphorylated Erk1/Erk2. (B) Starved NIH 3T3 cells (PDGF-AA, PDGF-BB, aFGF, bFGF), NIH 3T3 cells overexpressing EGFR, IGF-1R, or Flk-1, and the human breast carcinoma SKBr3 that overexpresses ErbB2 (HRG β) were pretreated with GFB-111 (100 μ M) for 5 min before 10 min stimulation with the indicated growth factors. Cell lysates were then immunoblotted with anti-phosphorylated Erk1/Erk2 as described for (A). Data are representative of three independent experiments.

PDGF) to its receptor on NIH 3T3 cells. Figure 2A shows that mixing GFB-111 with PDGF-BB resulted in a concentration-dependent disappearance of GFB-111 that was paralleled by the appearance of a slower migrating band on native polyacrylamide gel electrophoresis (PAGE). In this native PAGE system, PDGF-BB itself migrates toward the cathode and hence does not penetrate the gel. To demonstrate that the slower migrating band is formed by the complex between GFB-111 and PDGF-BB, the band was cut out of the native gel, processed and loaded onto denaturing SDS-PAGE gel. Figure 2B shows that the GFB-111 standard (lane 5), as well as the GFB-111 band cut out of the Figure 2A gel (lane 1), migrated on SDS-PAGE as a single band that eluted faster than the dye front. Mixing PDGF-BB standard (lane 4) and GFB-111 standard (lane 5) confirmed their separation on SDS-PAGE (lane 2). Most important is that the band cut out of the native gel presumed to contain the complex did indeed

contain both PDGF-BB and GFB-111 (Fig. 2B, lane 3). The interaction was structure-dependent in that the positively charged derivative GFB-116 (Table 1) did not bind to PDGF-BB as demonstrated by gel electrophoresis (data not shown). Furthermore, GFB-111 did not bind to the related peptide growth factors epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1), demonstrating that GFB-111 is selective for PDGF (data not shown).

GFB-111 blocks ^{125} I-PDGF binding. The complementarity of the recognition domain of GFB-111 (negatively charged and hydrophobic residues) with the receptor binding surface of PDGF (positively charged and hydrophobic sites) suggested that GFB-111 would block binding of PDGF to PDGFR. To support this, we determined whether GFB-111 can block ^{125} I-PDGF-BB specific binding in NIH 3T3 cells. Figure 2C shows that in the absence of GFB-111, NIH 3T3 cells bound ^{125} I-PDGF-BB. However, pretreatment with increasing concentrations

Table 1. Inhibition of PDGF-dependent receptor tyrosine phosphorylation by growth factor binders (GFBs)^a

Compounds	Loop sequence	IC ₅₀ (μ M)
GFB-105	GDFD	2.4
GFB-106	GDDD	8
GFB-107	GDGD	2.5
GFB-108	d-ADGD	9
GFB-109	GDLD	7.5
GFB-110	GDAD	1.7
GFB-111	GDGY	0.25
GFB-112	ADGD	29
GFB-113	GDSD	2.8
GFB-115	GKGF	50
GFB-116	GK GK	40
GFB-117	GDND	5.8
GFB-119	PDGD	20
GFB-120	GDDG	1.3
GFB-122	GDDY	1.7

^aStarved NIH 3T3 cells were pretreated with the various GFBs for 5 min before stimulation with PDGF. The cells were then harvested and lysed, and the proteins from the lysates separated by SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody. The phosphotyrosine PDGF receptor bands were scanned and the concentrations of GFBs that inhibited the ability of PDGF to stimulate PDGFR tyrosine phosphorylation by 50% were determined. Data for each compound are representative of at least two independent experiments.

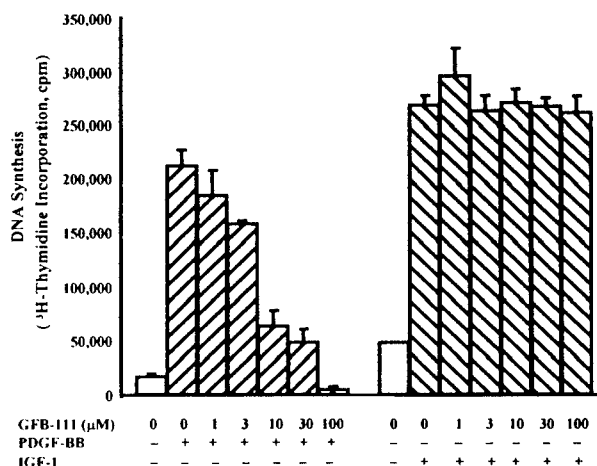


Figure 4. GFB-111 selectively inhibits PDGF-stimulated DNA synthesis. Starved NIH 3T3 or NIH 3T3 cells overexpressing IGFR were treated with GFB-111 and either PDGF or IGF-1, respectively, then labeled with [^{3} H]thymidine. [^{3} H]thymidine incorporation was quantified by scintillation counting. Data are representative of three independent experiments.

Table 2. GFB-111 inhibits tumor growth in nude mice*

Cell line	Tumor origin	Tumor growth inhibition (%)			
		1	2	3	4
U87MG	Human brain	61% (35)	56% (32)	81% (32)	88% (32)
		50mpk	50mpk	100mpk	200mpk
A-549	Human lung	62% (30)	53% (31)	64% (27)	N/D
		50mpk	50mpk	50mpk	
C6	Rat brain	54% (15)	64% (15)	46% (18)	N/D
		50mpk	50mpk	50mpk	
DaOY	Human brain	21% (48)	0% (37)	N/D	N/D
		50mpk	50mpk		

*The antitumor studies were performed exactly as described under legend to Figure 5A except that the human lung adenocarcinoma (A-549), human brain medulloblastoma (DaOY) or rat glioma (C6) were implanted s.c. in nude mice. The number in each parenthesis represents the day the % inhibition was calculated which is also the day the experiment was terminated (N/D, not determined; mpk, mg/kg.)

of GFB-111 resulted in a concentration-dependent decrease in PDGF-BB binding. The IC_{50} value of binding inhibition was 250 nM (Fig. 2C), which is similar to the IC_{50} value measured for GFB-111 inhibition of PDGF-BB-stimulated PDGFR tyrosine phosphorylation (Table 1). Figure 2C also shows that PDGF-BB binding to its receptor was blocked by GFB-111 but not GFB-116. Similar experiments with EGF, IGF-1, and VEGF demonstrated that GFB-111 (10 μ M) had no significant effects on ^{125} I-EGF or ^{125} I-IGF-1 binding but inhibited ^{125} I-VEGF binding by 50% in NIH 3T3 cells overexpressing EGFR, IGF-1R, and VEGFR (Flk-1), respectively (Fig. 2D).

GFB-111 selectively blocks PDGF-induced receptor tyrosine phosphorylation and MAP kinase activation. We next determined the selectivity of GFB-111 by evaluating its ability to block growth factor-stimulated tyrosine phosphorylation of several receptor tyrosine kinases and subsequent activation of two MAPKs, Erk1 and Erk2. Figure 3A shows that PDGF-BB stimulation of starved NIH 3T3 cells in the absence of GFB-111 resulted in PDGFR autophosphorylation and Erk1/Erk2 activation. However, pretreatment of these cells with GFB-111 (0.1–10 μ M) for 5 min resulted in a concentration-dependent inhibition of PDGF-BB stimulation of receptor tyrosine phosphorylation with an IC_{50} of 250 nM and a complete block at 3 μ M GFB-111. Figure 3A also shows that GFB-

111 is a potent inhibitor of PDGF-dependent activation of Erk1 and Erk2 (IC_{50} of 0.8 and 1.2 μ M, respectively). PDGF-AA stimulation of PDGFR phosphorylation and Erk1/Erk2 activation was also blocked with GFB-111 with a similar potency. In contrast, GFB-111 at concentrations as high as 100 μ M did not affect the ability of EGF to stimulate EGFR tyrosine phosphorylation and Erk1/Erk2 activation. For comparison, PD158780, a known EGFR tyrosine kinase inhibitor, blocks EGFR tyrosine phosphorylation and Erk1/Erk2 activation (Figure 3A). To further evaluate GFB-111 selectivity, we have demonstrated that this compound was able to inhibit VEGF-stimulated Flk-1 tyrosine phosphorylation and MAPK activation, but at concentrations higher (IC_{50} = 10 μ M) than those required for PDGF (IC_{50} = 250 nM). Furthermore, activation by IGF-1, acidic or basic fibroblast growth factors (aFGF, bFGF), and HRG β of Erk1/Erk2 was minimally affected by GFB-111 at concentrations as high as 100 μ M (Fig. 3B). Thus, GFB-111 blocks PDGF signaling at very low concentrations, inhibits VEGF signaling at higher concentrations (10 μ M), but has little effect on EGF, IGF-1, aFGF, bFGF, and HRG β signaling at very high concentrations.

GFB-111 selectively inhibits PDGF-stimulated DNA synthesis. To confirm whether this selectivity applies to PDGF-induced cellular responses, we next evaluated the selectivity of GFB-111 to inhibit growth factor-stimulated DNA synthesis using a [^{3} H]thymidine incorporation assay. In NIH 3T3 cells PDGF-BB stimulated DNA synthesis by 10-fold, and GFB-111 suppressed this stimulation in a dose-dependent manner with an IC_{50} of 6.5 μ M (Fig. 4). In contrast GFB-111 was ineffective at inhibiting IGF-1-stimulated DNA synthesis at concentrations as high as 100 μ M (Fig. 4). These data further confirm the highly selective character of GFB-111 toward PDGF.

GFB-111 inhibits the growth of human tumors in nude mice. The ability of GFB-111 to potentially and selectively block PDGF-dependent signaling and DNA synthesis, coupled with the ability of PDGF to stimulate angiogenesis and contribute to cancer cell growth, prompted us to determine the in vivo antitumor efficacy of GFB-111 in the nude mouse human xenograft model. Daily intraperitoneal administration with GFB-111 (50, 100, and 200 mg/kg) to nude mice implanted subcutaneously with the human

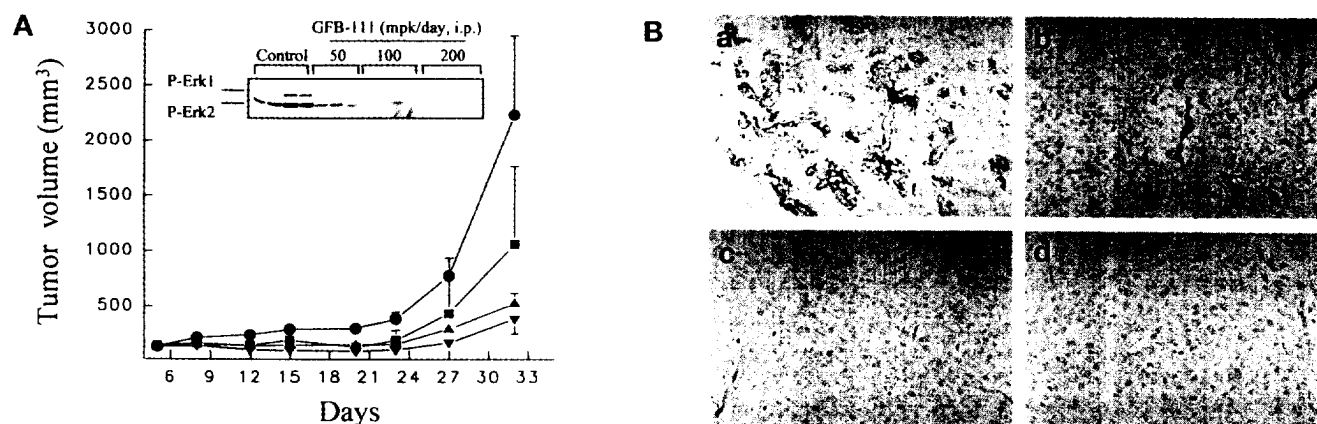


Figure 5. GFB-111 inhibits tumor growth and angiogenesis in nude mice. (A) GFB-111 inhibits the growth of the human glioblastoma U87MG in nude mice. U87MG cells were injected subcutaneously into nude mice, and when tumors reached about 100 mm³, animals were dosed intraperitoneally with 0.2 ml once daily. Control animals (●) received a saline vehicle, whereas treated animals were injected with GFB-111 at 50 (■), 100 (▲), or 200 (▼) mg/kg/day. For detection of phosphorylated/activated Erk1/Erk2 in tumors in vivo, the tumors were processed as described in the Experimental Protocol. The lysates were electrophoresed on SDS-PAGE, and immunoblotted, with anti-phospho-p44/p42 MAPK (Thy202/Tyr204). Data are representative of three independent experiments. (B) GFB-111 inhibits angiogenesis. On the indicated termination day of each experiment (see Table 2), the U87MG tumors were extracted. Tissue sections were stained with H&E and also subjected to immunostaining for Factor VIII. Control (panel a), 50 mg/kg (panel b), 100 mg/kg (panel c), and 200 mg/kg (panel d). Data are representative of two independent experiments.

Table 3. GFB-111 inhibits tumor angiogenesis^a

Tumor	Group	Microvessel Count (Mean $\pm$ sd)	
		250X	400X
A-549	Control	15.66 $\pm$ 7.00	7.74 $\pm$ 2.88
	GFB-111 (50 mg/kg)	3.63 $\pm$ 1.36	2.34 $\pm$ 1.01
U87MG	Control	66.40 $\pm$ 1.36	31.53 $\pm$ 7.58
	GFB-111 (50 mg/kg)	23.13 $\pm$ 15.69	12.33 $\pm$ 8.37
U87MG	Control	23.48 $\pm$ 6.21	15.68 $\pm$ 3.25
	GFB-111 (50 mg/kg)	10.50 $\pm$ 2.12	6.00 $\pm$ 1.13
	GFB-111 (100 mg/kg)	7.73 $\pm$ 2.61	5.20 $\pm$ 1.71
	GFB-111 (200 mg/kg)	5.13 $\pm$ 3.10	3.07 $\pm$ 1.94
C6	Control	20.23 $\pm$ 7.84	9.77 $\pm$ 3.13
	GFB-111 (50 mg/kg)	14.27 $\pm$ 4.79	7.97 $\pm$ 2.83
DaOY	Control	8.07 $\pm$ 1.47	4.73 $\pm$ 0.90
	GFB-111 (50 mg/kg)	7.20 $\pm$ 2.52	4.80 $\pm$ 1.37

^aFor micro blood vessel counting, the five areas of highest tumor neovascularization were microscopically selected using a low-power view (4 $\times$). After the areas of highest neovascularization had been identified, individual microvessels were counted on a 250 $\times$ (25 $\times$ objective lens and 10 $\times$ ocular lens), and a 400 $\times$ (40 $\times$ objective lens and 10 $\times$ ocular lens) field. Brown-staining endothelial cells or endothelial cell clusters were considered as a single, countable microvessel following Weidner et al. criteria³⁴. Vessel lumina and/or red blood cells were not used to define a microvessel³⁴. Data represent average value from at least two independent experimental groups for each tumor type.

glioblastoma U87MG resulted on day 32 in a dose-dependent inhibition of tumor growth of 56, 81, and 88%, respectively (Fig. 5A and Table 2). Extraction and processing of tumors on day 32 show that Erk1/Erk2 activation was blocked in the tumors from the animals treated with 100 and 200 mg/kg GFB-111 (Fig. 5A, inset). The ability of GFB-111 to inhibit tumor growth was not limited to U87MG. GFB-111 was also effective at inhibiting the tumor growth in nude mice of the human lung adenocarcinoma A-549 and the rat glioma C6 (Table 2). However, the growth of the human medulloblastoma DaOY was minimally affected (Table 2).

GFB-111 inhibits angiogenesis in vivo. Because GFB-111 was effective at inhibiting the tumor growth of human cancer cells that express a PDGF/PDGFR autocrine loop (U87MG) as well as those that express neither PDGF nor PDGFR (A-549), and because PDGF promotes angiogenesis, we next explored whether GFB-111 inhibits angiogenesis in vivo. Tissue sections from U87MG tumor biopsy specimens collected on day 32 were immunostained with the vascular marker Factor VIII. Tumors from control animals showed very marked staining (Fig. 5B). In contrast, the Factor VIII immunostaining was decreased in a dose-dependent manner in tumors from animals treated with 50, 100, and 200 mg/kg GFB-111 (Fig. 5B). Quantification of microvessels at two field magnifications (250 $\times$ and 400 $\times$) clearly demonstrated that GFB-111 inhibited angiogenesis strongly in U87MG and A-549 tumors, to a lesser degree in C6 tumors, and had no effect on angiogenesis in DaOY tumors (Table 3).

Discussion

In this study we have used a new approach to inhibiting receptor tyrosine kinase signaling by targeting the binding of growth factors to their receptor. The discovery of GFB-111 demonstrates that a designed synthetic molecule can bind to a growth factor, and disrupt its biological activity by preventing it from binding to its receptor. This is a critical finding that supports the disruption of protein-protein interactions as a sound approach for modern anticancer therapy. An advantage of this strategy is the direct access to the target, avoiding problems associated with cellular uptake and intracellular metabolism and degradation. This approach also has the

potential for high selectivity because it targets interactions that do not involve the common adenosine triphosphate (ATP) binding site of receptor tyrosine kinases.

GFB-111 is selective for the disruption of PDGFR binding, tyrosine phosphorylation, and Erk1/Erk2 activation compared to EGF and, to a lesser extent, VEGF binding to their respective receptors. This is consistent with differences in their overall structure, particularly in areas involved in receptor binding. The binding of PDGF to its receptor is mediated by mainly hydrophobic and basic amino acid residues found in the loops connecting the β -sheets of the PDGF peptide^{25,26}. Although VEGF shares some overall structural homology with PDGF, there are critical differences in their receptor binding regions. In particular, the receptor binding loop regions of VEGF are mainly composed of hydrophobic and acidic residues²⁷. Epidermal growth factor has no structural homology with PDGF and VEGF and has a receptor binding domain composed of hydrophobic groups and a single basic residue (Arg 41) located in a hairpin turn²⁸. Similarly, FGF has no structural relationship with PDGF and has a large receptor binding surface composed primarily of hydrophobic and positively charged or hydrogen-bonding (arginine and asparagine) residues²⁹. IGF-1 has strong homology with insulin but bears no similarity to PDGF. The recognition surface of IGF-1 has been shown to include several critical tyrosine residues as well as two important arginine groups³⁰. Finally, heregulin is similar in structure to EGF but bears little sequence homology. Alanine scanning experiments have shown the importance of hydrophobic as well as both positively (Arg 44 and 31) and negatively (Glu 19) charged residues in the binding of heregulin to different ErbB receptors³¹. Overall, these comparisons point to unique shape and surface residue distributions in the receptor binding domain of each of the growth factors that can be exploited in the design of synthetic agents that are selective for binding to PDGF.

The lack of correlation between GFB-111 inhibition of tumor growth and PDGF/PDGFR expression in the cancer cells suggested that tumor growth inhibition may be due to either inhibition of cell proliferation that depends on PDGF/PDGFR and/or angiogenesis. Indeed, PDGF has been directly implicated in promoting angiogenesis by mediating endothelial cell migration and proliferation. Furthermore, PDGF also induces endothelial cells to express VEGF, a very potent angiogenic growth factor. Therefore, by blocking PDGF binding to its receptor on endothelial cells, GFB-111 could inhibit endothelial cell migration and proliferation as well as VEGF expression, hence blocking angiogenesis. In addition, GFB-111 at higher concentrations could block VEGF binding to Flk-1 on endothelial cells and inhibit angiogenesis. Thus, our results demonstrate that selective PDGF binding molecules such as GFB-111 that are capable of blocking signaling, tumor growth, and angiogenesis may lead to the development of a new class of anticancer drugs capable of treating a wide spectrum of human cancers.

Experimental protocol

Inhibition of PDGF-dependent receptor tyrosine phosphorylation by growth factor binders (GFBs). Starved NIH 3T3 cells were pretreated with the various GFBs (0–100 μ M) for 5 min before stimulation with PDGF (10 ng/ml) for 10 min. The cells were then harvested and lysed, and the proteins from the lysates separated by SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Lake Placid, NY) as described by us³². The phosphotyrosine PDGF receptor bands were scanned using a BioRad Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA) and the concentrations of GFBs that inhibited the ability of PDGF to stimulate PDGFR tyrosine phosphorylation by 50% were determined.

PDGF binding. GFB-111 (50 μ M) was incubated with buffer A (phosphate-buffered saline, PBS–0.5% bovine serum albumin, BSA) or PDGF-BB (1, 3, and 4 μ M in buffer A) at room temperature for 10 min. The mixtures were then loaded onto a native 4% PAGE. The gel was then stained with silver nitrate. Duplicate lanes were run in the same gel but were not

stained. Bands corresponding to the boxed area were cut out of the lanes of the nonstained gel, denatured with SDS-PAGE loading buffer, loaded onto a 4% SDS-PAGE gel, and silver stained.

Binding of ^{125} I-PDGF to NIH 3T3 cells and inhibition by GFBs. NIH 3T3 (PDGF) or NIH 3T3 cells overexpressing either EGFR (EGF), IGFR (IGF-1), or Flk-1 (VEGF), were incubated with ^{125} I-PDGF, ^{125} I-EGF, ^{125} I-IGF, or ^{125} I-VEGF, respectively (50,000 c.p.m. per well) and increasing concentrations of GFB-111 or GFB-116. Cells were incubated at 4°C for 1–3 h, then washed three times with PBS and three times with 25 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 1% SDS. Lysates plus wash were counted. Excess of cold growth factors was used to obtain nonspecific binding levels.

PDGF-stimulated receptor tyrosine autophosphorylation and activation of MAPK. Starved NIH 3T3 cells (PDGF-BB and PDGF-AA), NIH 3T3 cells overexpressing either EGFR (EGF) or Flk-1 (VEGF) were pretreated with the indicated concentrations of GFB-111 for 5 min before 10 min stimulation with PDGF-BB (10 ng/ml), PDGF-AA (10 ng/ml), EGF (100 ng/ml), and VEGF (50 ng/ml). Cell lysates were run on SDS-PAGE gels, then transferred to nitrocellulose and western blotted with anti-phosphotyrosine (4G10) or anti-phosphorylated Erk1/Erk2 (New England Biolabs, Beverly, MA)³².

In other experiments, starved NIH 3T3 cells (PDGF-AA, PDGF-BB, aFGF, bFGF), NIH 3T3 cells overexpressing EGFR, IGF-1R, or Flk-1 and the human breast carcinoma SkBr3 that overexpresses ErbB2 (HRG β) were pretreated with GFB-111 (100 μ M) for 5 min before 10 min stimulation with the indicated growth factors at the concentrations shown above and aFGF (10 ng/ml), bFGF (50 ng/ml), IGF-1 (50 ng/ml), and HRG β (10 ng/ml). Cell lysates were then immunoblotted with anti-phosphorylated Erk1/Erk2 (New England Biolabs).

PDGF-stimulated DNA synthesis. Starved NIH 3T3 or NIH 3T3 overexpressing IGFR were treated for 16 h with indicated concentrations of GFB-111 and either 10 ng/ml PDGF or 50 ng/ml IGF-1, respectively, then labeled for 2 h (NIH 3T3) or 4 h (IGFR) with [^{3} H]thymidine. Cells were then washed in PBS, lysed in 0.1 N NaOH, and spotted onto glass fiber filters. DNA was precipitated with ice-cold ethanol and then [^{3} H]thymidine incorporation quantified by scintillation counting.

Antitumor efficacy studies in nude mice. U87MG cells (human glioblastoma) were harvested, resuspended in PBS, and injected subcutaneously into the right and left flank (10 $\times$ 10⁶ cells per flank) of eight-week-old female nude mice. When tumors reached about 100 mm³, animals were dosed intraperitoneally with 0.2 ml once daily. Control animals received saline vehicle whereas treated animals were injected with GFB-111 (50, 100, or 200 mg/kg/day). The tumor volumes were determined by measuring length (l) and width (w) and calculating volume ($V = lw^2/2$) as described³³. For detection of phosphorylated/activated Erk1/Erk2 in tumors in vivo, the tumors were extracted 20 min after the last GFB-111 injection on day 32, rinsed, and the tissue homogenized in N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) lysis buffer (30 mM HEPES, pH 7.5, 1% Triton X-100, 10% glycerol, 10 mM NaCl, 5 mM MgCl₂, 25 mM NaF, 1 mM EGTA, 2 mM Na₂VO₄, 10 μ g/ml trypsin inhibitor, 25 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, PMSF). The lysates (50–100 μ g) were electrophoresed on a 15% SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted, with anti-phosphorylated Erk1/Erk2 (New England Biolabs).

Angiogenesis studies. On the indicated termination day of each nude mouse experiment described above, the tumors were extracted and fixed in 10% neutral buffered formalin for 6 h. After fixation the tissue samples were processed into paraffin blocks. Tissue sections (4 μ m thick) were obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E) using standard histological techniques. Tissue sections were also subjected to immunostaining for Factor VIII (Dako, Carpinteria, CA) using the avidin biotin peroxidase complex technique. Rabbit polyclonal FVIII antibody was used at 1:400 dilution, following microwave antigen retrieval (four cycles of 5 min each on high in 0.1 M citrate buffer).

Acknowledgments

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Design of growth factor antagonists with antiangiogenic and antitumor properties

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This review describes our recent efforts in the development of novel therapies for cancer. Our primary approach is to design synthetic agents that antagonize the function of growth factors that are critically involved in oncogenesis and angiogenesis. We achieve this by designing synthetic molecules that can recognize the exterior surface of the growth factor and so block the interaction with its receptor tyrosine kinase. A key step is the construction of synthetic agents that contain a large (>400 Å²) and functionalized surface area to recognize a complementary surface on the target growth factor. In the course of this work we have discovered a molecule, GFB-111, that binds to PDGF, prevents it from binding to its receptor tyrosine kinase, blocks PDGF-induced receptor autophosphorylation, activation of Erk1 and Erk2 kinases and DNA synthesis. The binding affinity for PDGF is high (IC₅₀ = 250 nM) and selective over EGF, IGF-1, aFGF, bFGF and HRGβ. In nude mouse models GFB-111 also shows significant inhibition of tumor growth and angiogenesis. *Oncogene* (2000) 19, 6566–6573.

Keywords: platelet-derived growth factor; angiogenesis; oncogenesis; cancer drug discovery

Introduction

The search for new therapies in the war against cancer remains a top priority in biomedical research. In many cases current clinical therapies have changed little in several decades. The key to successfully developing novel clinical strategies against these cancers is twofold. First, critical protein targets that are known to be essential in the growth of tumors must be identified and validated as cancer therapy targets. Second, a novel approach to disrupting the function of these essential targets must be developed and shown to selectively block the growth of transformed cells. Our strategy is to disrupt the interaction of key growth factors with their receptor tyrosine kinase (RTK) targets in the cell membrane. To this end, we use an entirely novel strategy based on the design of synthetic agents that selectively and strongly bind to the surface of the growth factor and so block its oncogenic signaling function (Figure 1).

Our primary goal is to design synthetic agents that can recognize the exterior surface and antagonize the function of growth factors that are critically involved in oncogenesis. In our early work we have placed

particular focus on platelet derived growth factor (PDGF) and its complementary receptor tyrosine kinase, platelet derived growth factor receptor (PDGFR). Overexpression of PDGFR is seen in many carcinomas and some cancer patients have high serum levels of PDGF. These elevated levels of PDGF and PDGFR in cancer patients also correlate with poor response to chemotherapy and shorter survival times. Synthetic agents that can block the uncontrolled signaling function of overexpressed receptor tyrosine kinases have the potential to slow the growth of tumors and greatly improve the quality of life for cancer patients (Figure 1).

Receptor tyrosine kinases as targets for cancer chemotherapy

Interaction of a quiescent cell with an external growth factor such as PDGF or epidermal growth factor (EGF) results in the activation of a complex network of signaling pathways that lead ultimately to cell division. The first step in this pathway involves the binding of the growth factor to the extracellular region of a membrane bound protein receptor tyrosine kinase (PDGFR or EGFR). Binding leads to dimerization of the receptor and activation of autophosphorylation of tyrosines on the receptor surface (Rockwell and Goldstein, 1995). This initiates the recruitment of several signaling proteins (including phosphatidylinositol-3-kinase, Src, Grb2, m-SOS-1 (a nucleotide exchange factor for Ras) and signal transducers and activators of transcription (STATs)) each of which contains src-homology-2 (SH-2) domains. Binding of the SH-2 regions of these proteins to the phosphotyrosines on the RTKs activates several pathways that are critical for triggering the cell cycle machinery.

The most well studied pathway passes through the GTP-binding protein Ras and activates the mitogen activated kinase (MAPK) cascade and subsequently transcription factors in the nucleus (Figure 2). Mutations in the Ras protein are present in 30% of human cancers and have been the subject of intense recent interest. We have reported extensively over the past seven years on the design of inhibitors for farnesyltransferase, a key enzyme in the posttranslational modification of Ras (Sebti and Hamilton, 2000). These compounds not only disrupt Ras farnesylation in whole cells but also block the growth of human tumors in nude mice models, and are on the verge of phase I clinical trials (Sun *et al.*, 1999). None of this work will be discussed here, however it provides a proof of

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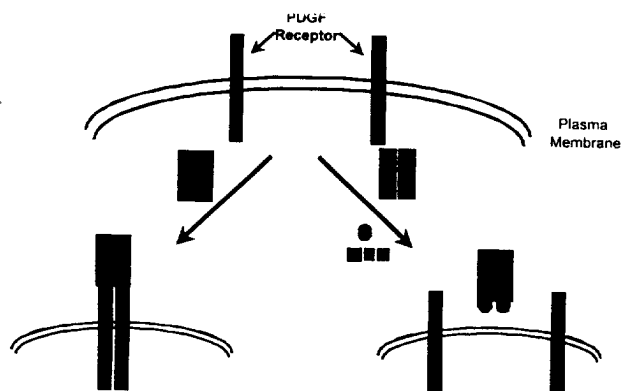


Figure 1 Schematic approach to growth factor binding agents (GFBs)

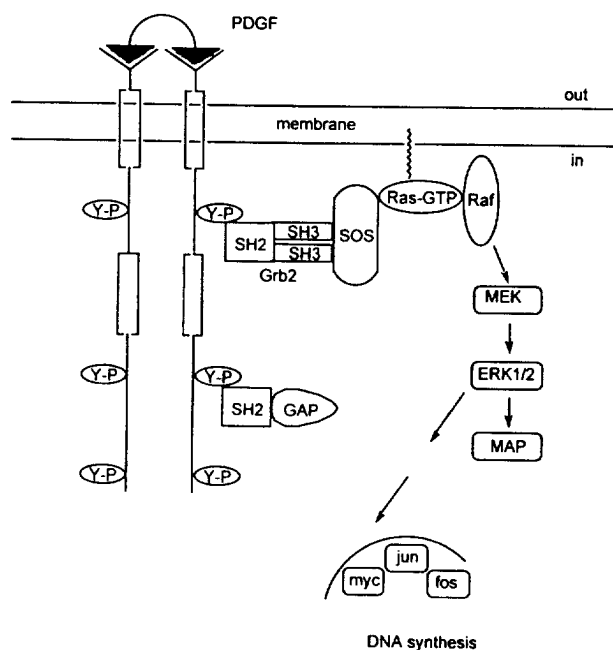


Figure 2 Simplified PDGF-activated signaling pathway

principle for targeting RTK signaling pathways in cancer therapy.

The primary focus of this review is the first step in the aberrant cell signaling pathways that lead to uncontrolled proliferation and cancer, namely the interaction of growth factors with RTKs. For example, PDGF, which plays an important role in many human malignancies (Heldin, 1992) exists in three isoforms (homodimers PDGF-AA and PDGF-BB and heterodimer PDGF-AB) and exerts its biological effects via specific binding to PDGF receptors (PDGFRs) (Hart *et al.*, 1988). Overexpression of PDGFR is seen in certain breast (Seymour *et al.*, 1993) and ovarian (Henriksen *et al.*, 1993) carcinomas as well as high serum levels of PDGF. Furthermore, elevated levels of PDGF and PDGFR in breast cancer patients correlate with poor response to chemotherapy and shorter survival times (Seymour *et al.*, 1993). The design of PDGF antagonists that can inhibit ligand-induced receptor activation has been suggested as a route to

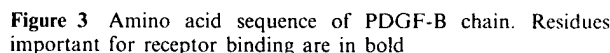
new anti-cancer drugs (Fiona and William, 1994). This strategy is supported by antibodies against PDGF which show anti-mitogenic activity (Johnson *et al.*, 1985) and a 110 kDa soluble form of the receptor has been shown to inhibit both the binding of PDGF-BB to PDGFR and the receptor tyrosine kinase activity (Duan *et al.*, 1991). These results strongly suggest that PDGF and PDGFR represent compelling targets for anti cancer drug design. Furthermore, PDGF has been shown to be critically involved in the angiogenesis process. PDGF can not only stimulate the proliferation of endothelial cells but also can stimulate the secretion of vascular endothelial growth factor. Moreover, PDGF knockout mice are deficient in processes of angiogenesis (Blaskovich, 2000).

The crystal structure of PDGF shows it to be a head-to-tail dimer, with each peptide folded into two long antiparallel pairs of β -strands with three intramolecular disulfide bonds (Figure 3) (Oefner *et al.*, 1992). The three surface loops connecting the strands (designated as I, II and III) are clustered at one end of the elongated dimer upon dimerization. Mutational analyses of individual amino acid residues or short peptide sequences in PDGF-B (Fenstermaker *et al.*, 1993; Ostman *et al.*, 1991; LaRocelle *et al.*, 1992) have indicated that the key binding regions on PDGF occur in loops I and III. There is also some indication that residues from loop II from the other subunit of the dimer may also be involved in receptor binding (Anderson *et al.*, 1995). Inspection of the X-ray structure of PDGF-BB shows that the hairpin turns in loops I and III are separated by 10-12Å. Loop I projects a hydrophobic sequence (ANFLVW), loop III contains a highly cationic region (EIVRKKP) and loop II has a combination of both (NNRNV) (Engstrom *et al.*, 1992).

Design of novel synthetic agents for protein surface binding

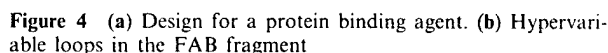
Protein recognition can be divided into two categories in terms of the interaction sites: (1) interactions that occur inside proteins, e.g. enzyme active sites; (2) interactions that occur on the surface of proteins. During the past two decades large numbers of synthetic molecules targeted to disrupt interior protein interactions have been shown to have medically important biological activities (Babine and Bender, 1997). However, artificially designed molecules that target the protein surface and disrupt its biological activity are rare (Hartwell *et al.*, 1997). Considering the unique composition of charged, hydrophobic and hydrophilic domains on every protein's surface, synthetic molecules that match the electrostatic features and topology of the protein targets might be expected to bind to the exterior and sterically prevent protein-protein interactions.

A model for protein surface recognition is provided by the immune system which generates a large number of antibodies that show high sequence and structural selectivity in binding to a range of protein surfaces (Brandon and Tooze, 1991). Our approach to growth factor binding agents involves the attachment of several peptide loops onto a core scaffold (Figure 4a) in direct analogy to the six hypervariable loops that are



We have recently prepared the first of a new class of protein surface receptors involving the attachment of four peptide loops to a central scaffold (based on the calix[4]arene unit **1**) (Hamuro *et al.*, 1997). The peptide loop component was based on a cyclic hexapeptide **2** in which two residues were replaced by a 3-aminomethylbenzoyl (3 amb) dipeptide mimetic (Bach *et al.*, 1994) containing a 5-amino substituent for linkage to the scaffold. The resulting molecule **3** contains a functionalized surface approximately 400Å² in area. The GDGD sequence in **2** was chosen to provide a surface containing both negatively charged and hydrophobic regions that would bind to a complementary surface on a target protein. We have prepared analogs of **3** in which the sequence of the cyclic peptide is varied to include anionic, hydrophobic and cationic residues, such as GDGD (**3**), GDGY (**5**), GKGF (**6**) and GK GK (**7**). This provided us with a first generation library of protein binding agents that were screened for their ability to bind to the surface of PDGF (Scheme 1).

Our first assessment of whether these molecules were able to interact with growth factors involved testing



Similarly, there is a loss in binding ability when the key Asp and Tyr residues in the cyclic peptides in GFB-111 are changed. For example, substitution of negatively charged Asp by positively charged Lys, as with GFB-115 (GKGF) ($IC_{50} = 50 \mu M$) and GFB-116 (GKGK) ($IC_{50} = 40 \mu M$), causes an almost 200-fold reduction in antagonist potency. Changing the hydrophobic Tyr for Asp, as in GFB-107 (GDGD) ($IC_{50} = 2.5 \mu M$), leads to an order of magnitude loss in activity. The specific sequence of the cyclic peptide also appears to be important. Rearranging the anionic and hydrophobic residues in the four cyclic peptides as in GFB-122 (GDDY) ($IC_{50} = 1.7 \mu M$) leads to a sevenfold reduction in binding activity compared to GFB-111 (GDGY) (Table 1).

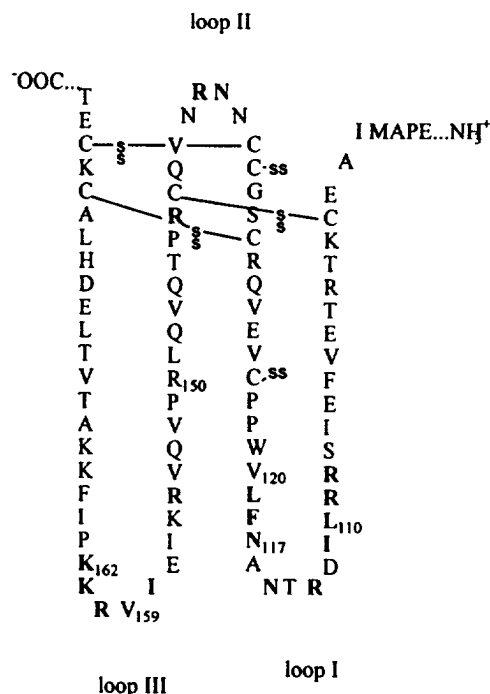


Figure 3 Amino acid sequence of PDGF-B chain. Residues important for receptor binding are in bold

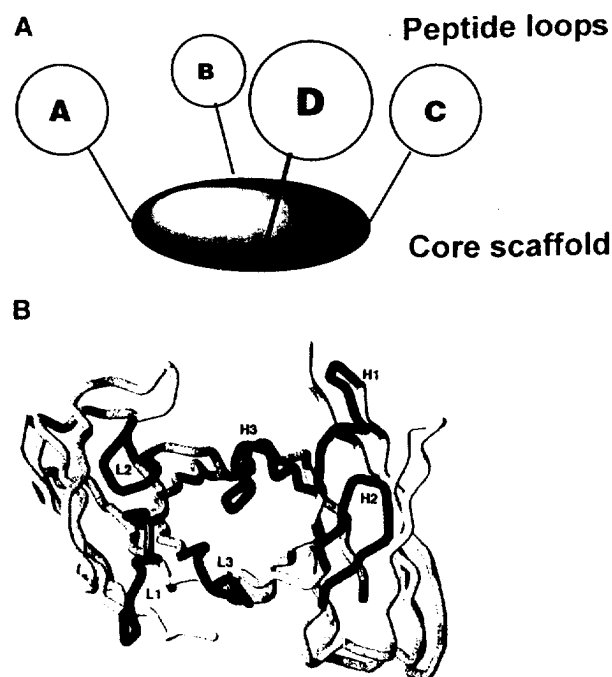


Figure 4 (a) Design for a protein binding agent. (b) Hypervariable loops in the FAB fragment

found in the antigen recognition region of the antibody FAB fragment (Figure 4b). The advantages of using this design for the recognition of protein surfaces are fourfold: (1) large surface areas can be created ($>400 \text{ \AA}^2$); (2) modular design enables us to generate a large diversity from a small number of cyclic peptide or scaffold components; (3) various non-natural groups can be incorporated into the loop structures and (4) scaffolds are relatively flexible which allows an 'induced fit' mechanism of binding.

We have recently prepared the first of a new class of protein surface receptors involving the attachment of four peptide loops to a central scaffold (based on the calix[4]arene unit 1) (Hamuro *et al.*, 1997). The peptide loop component was based on a cyclic hexapeptide 2 in which two residues were replaced by a 3-aminomethyl-benzoyl (3 amb) dipeptide mimetic (Bach *et al.*, 1994) containing a 5-amino substituent for linkage to the scaffold. The resulting molecule 3 contains a functionalized surface approximately $400 \text{ \AA}^2$ in area. The GDGD sequence in 2 was chosen to provide a surface containing both negatively charged and hydrophobic regions that would bind to a complementary surface on a target protein. We have prepared analogs of 3 in which the sequence of the cyclic peptide is varied to include anionic, hydrophobic and cationic residues, such as GDGD (3), GDGY (5), GKGF (6) and GKGG (7). This provided us with a first generation library of protein binding agents that were screened for their ability to bind to the surface of PDGF (Scheme 1).

Disruption of growth factor signaling by synthetic protein binding agents

Our first assessment of whether these molecules were able to interact with growth factors involved testing

their ability to block PDGF-induced autophosphorylation of PDGFR. NIH3T3 cells were treated with the synthetic growth factor binding agents and PDGF (30 ng/ml) for 10 min, followed by lysis of the cells. SDS-PAGE and Western blot analysis using an antiphosphotyrosine antibody. Figure 5 shows the ability of the first generation GFB series at $5 \mu\text{M}$ concentrations to inhibit PDGF stimulation of the receptor tyrosine phosphorylation. We found that the molecule containing GlyAspGlyTyr (GDGY) sequences in each of the four peptide loops of (GFB-111) blocked PDGF-BB-stimulation of PDGF receptor tyrosine autophosphorylation with an IC_{50} value of 250 nM (Table 1). This fourfold symmetrical arrangement of negative and hydrophobic residues shows a strong complementary to the region of PDGF that interacts with the recognition surface of PDGFR. The critical receptor binding area on PDGF-BB contains lysine and arginine residues as well as a significant number of amino acids with hydrophobic side chains. Mutational analysis has shown that growth factor binding to its receptor diminishes when these residues are changed (Oefner *et al.*, 1996; Anderson *et al.*, 1995).

Similarly, there is a loss in binding ability when the key Asp and Tyr residues in the cyclic peptides in GFB-111 are changed. For example, substitution of negatively charged Asp by positively charged Lys, as with GFB-115 (GKGF) ($\text{IC}_{50} = 50 \mu\text{M}$) and GFB-116 (GKGG) ($\text{IC}_{50} = 40 \mu\text{M}$), causes an almost 200-fold reduction in antagonist potency. Changing the hydrophobic Tyr for Asp, as in GFB-107 (GDGD) ($\text{IC}_{50} = 2.5 \mu\text{M}$), leads to an order of magnitude loss in activity. The specific sequence of the cyclic peptide also appears to be important. Rearranging the anionic and hydrophobic residues in the four cyclic peptides as in GFB-122 (GDDY) ($\text{IC}_{50} = 1.7 \mu\text{M}$) leads to a sevenfold reduction in binding activity compared to GFB-111 (GDGY) (Table 1).

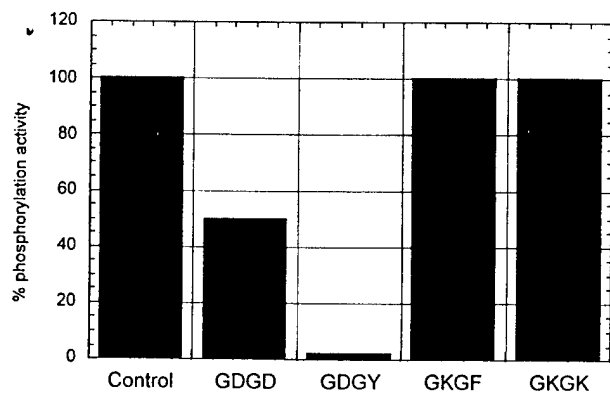


Figure 5 Inhibition of PDGF-stimulated tyrosine phosphorylation of PDGFR by the first generation of GFBs

Table 1 Inhibition of PDGF-dependent receptor tyrosine phosphorylation by growth factor binders (GFBs)

Compounds	Loop Sequence	IC ₅₀ (μM)
GFB-105	GDGD	2.4
GFB-106	GDDD	8
GFB-107	GDGD	2.5
GFB-108	D-ADGD	9
GFB-109	GDLD	7.5
GFB-110	GDAD	1.7
GFB-111	GDGY	0.25
GFB-112	ADGD	29
GFB-113	GDSD	2.8
GFB-115	GKGF	50
GFB-116	GKKG	40
GFB-117	GDND	5.8
GFB-119	PDGD	20
GFB-120	GDDG	1.3
GFB-122	GDDY	1.7

series of hydrophobic and positively charged amino acids in loops I and III of one monomer, and loop II of the head-to-tail linked second monomer in dimeric PDGF (Oefner *et al.*, 1996; Anderson *et al.*, 1995). VEGF shares some structural homology with PDGF, but its receptor binding regions are made up principally of hydrophobic and negatively charged residues (Muller *et al.*, 1997). EGF has a completely different structure to PDGF and VEGF. The critical receptor binding region has been shown to contain several hydrophobic groups and a positively charged arginine residue (Arg41) (Campion and Niyogi, 1994). The receptor binding domains of these three growth factors are thus quite different suggesting that the negatively charged and hydrophobic surface of GFB-111 achieves its selectivity by matching the positive and hydrophobic surface of PDGF.

There is a strong possibility that the amphipathic GFB-111 is binding to the cationic and hydrophobic regions of the PDGF dimer and in doing so blocking its association to the receptor surface. A calculated structure for the interaction of this region of PDGF (using the crystal structure coordinates) with GFB-111 is shown in Figure 8. Similar interactions have been proposed for suramin binding to PDGF (Middaugh *et al.*, 1992) and heparin binding to fibroblast growth factor (Thompson *et al.*, 1994).

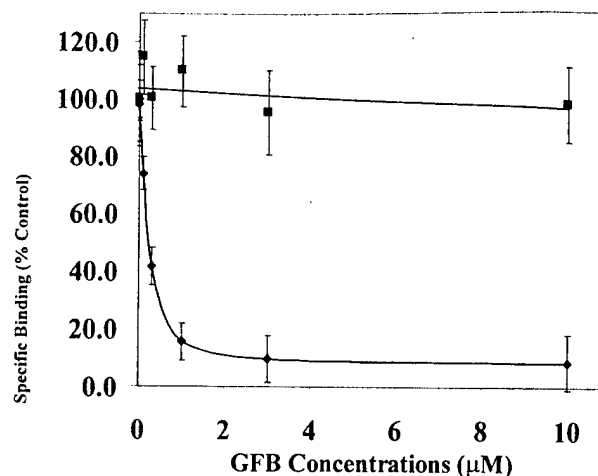


Figure 6 Inhibition of [125-I]PDGF binding to its receptor on NIH3T3 cells with increasing concentrations of GFB-111 (◆) or GFB-116 (■)

These early results clearly pointed to an interaction between GFB-111 and PDGF and a disruption of the initial activation of PDGFR tyrosine phosphorylation. The next question concerned whether this complex formation would lead to a disruption in downstream cell signaling pathways (Figure 2). We assessed the ability of GFB-111 to block growth factor-stimulated tyrosine phosphorylation of several receptor tyrosine kinases and subsequent activation of two mitogen-activated protein kinases (MAPK), Erk1 and Erk2. Treatment of starved NIH3T3 cells with PDGF-BB led to PDGFR autophosphorylation and Erk1/Erk2 activation (Figure 9). However, in the presence of GFB-111 (0.1–10 μM) there was a concentration-dependent inhibition of activation of Erk1 and Erk2 (IC₅₀ of 0.8 and 1.2 μM, respectively). A similar effect was seen on PDGF-AA stimulation of PDGF receptor phosphorylation and Erk1/Erk2 activation. However selectivity among different growth factors was seen since GFB-111 at concentrations as high as 100 μM did not affect the ability of EGF to stimulate EGFR tyrosine phosphorylation and Erk1/Erk2 activation. As a positive control a known EGFR tyrosine kinase inhibitor, PD158780, blocks EGFR tyrosine phosphorylation and Erk1/Erk2 activation (Figure 9). Furthermore, IGF-1-, aFGF-, bFGF- and HRGβ-activation of Erk1/Erk2 was minimally affected by GFB-111 at concentrations as high as 100 μM (Figure 9). The results are consistent with the earlier radiolabeled growth factor data which showed a significant protein binding selectivity in the effects of GFB-111.

Anticancer activity of GFB-111

The above investigations showed that GFB-111 was able to bind to PDGF and block in a potent and selective manner PDGF-dependent signaling. However, a critical test for the strategy concerned the ability of GFB-111 to interfere with the function of PDGF in animal models. PDGF is well known to contribute to cancer cell growth by, in part, stimulating angiogenesis. This prompted us to investigate the *in vivo* anti-tumor

efficacy of GFB-111 in a nude mouse human xenograft model. The experiment involved nude mice implanted s.c. with the human glioblastoma U87MG. The tumor growth of a control group (treated with saline solution) was compared to that of a group treated daily with GFB-111 at 50, 100 and 200 mg per kg. Figure 10 shows that the treated group exhibited a dose-dependent inhibition of tumor growth of 56, 81 and 88%, respectively. Daily treatment with GFB-111 went on for more than one month yet there were no signs of gross toxicity among the animals. This provides good support that the molecules are exerting their effect through interference with a specific growth mechanism rather than through any systemic toxicity. At the end of the experiment the tumors from the mice were extracted and analysed to determine the extent of

PDGF-dependent signaling. Figure 10 shows that Erk1/Erk2 activation was diminished in the tumors treated with 50 mg per kg and almost completely blocked in those treated with 100 and 200 mg per kg, confirming that our designed molecule was causing an inhibition of the PDGF-activated signaling pathway.

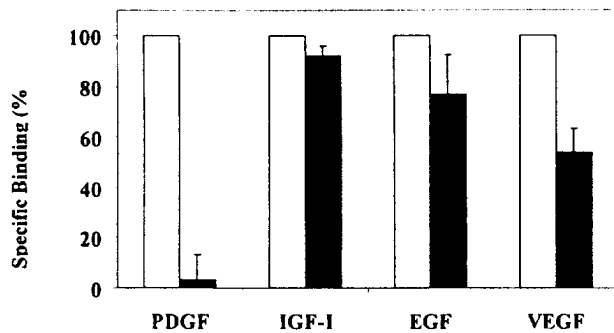


Figure 7 Inhibition of [¹²⁵I]-labeled growth factor binding to NIH3T3 cells overexpressing the complementary receptor tyrosine kinase, for PDGFR (PDGF), EGFR (EGF), IGF-1 (IGF) or Flk-1 (VEGF)



Figure 8 Front view of the postulated interaction between GFB-111 and PDGF

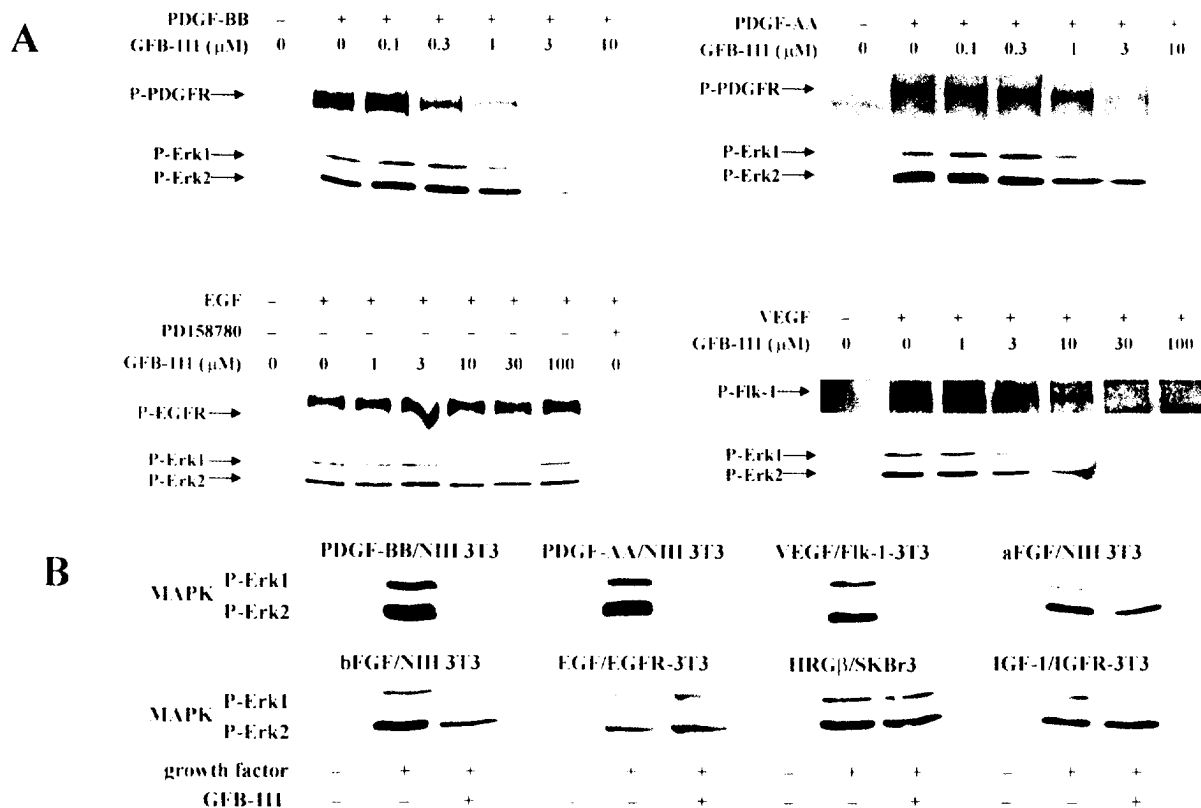


Figure 9 Effects of GFB-111 on growth factor stimulated receptor tyrosine autophosphorylation and activation of MAPK

Similar growth inhibition in nude mice was seen with other tumor cell lines, such as human lung adenocarcinoma A-549 and the rat glioma C6. However, little effect was seen on the growth of the human medulloblastoma DaOY (Table 2).

Although excellent inhibition of tumor growth by GFB-111 was seen, the mechanism by which the synthetic molecule exerts this effect was not clear. An intriguing result is the equipotent effect seen with tumors that depend on PDGF (U87) and those that do not (A549), suggesting an alternative mechanism to direct effects on the tumor cells. One possibility was that GFB-111 interferes with the growth of blood vessels in the tumor since PDGF is known to be important in promoting angiogenesis. In the U87MG nude mouse xenograft experiment, tumor biopsy specimens were taken at the end of the experiment, fixed and processed into paraffin blocks. The tissue sections were immunostained with the vascular marker Factor VIII and are shown in Figure 11. Tumors from animals treated with 50, 100 and 200 mg per kg GFB-111 showed a dose dependent decrease in immunostaining, in marked contrast to tumors from the untreated animals which showed extensive staining (vascularization). A more detailed quantification of microvessels in the tumor preparations showed that U87MG and A-

549 tumors were sensitive to GFB-111 inhibition of angiogenesis whereas DaOY tumors showed no effect.

Conclusion

In this review we have described the recent progress that we have made in establishing a novel approach to disrupting the interaction between a growth factor and its receptor tyrosine kinase. We have outlined a strategy that mimics the use of peptide loop recognition domains as seen in antibody complementary determining regions. The synthetic growth factor binding agents (GFBs) are composed of four synthetic peptide loop domains linked to a core calixarene based scaffold. The potential of the approach has been firmly established by targeting the interaction between PDGF and its receptor tyrosine kinase. We show that one effective member of the GFB family is able not only to block the interaction of PDGF with PDGFR but also to inhibit its signaling function. This potent effect further translates into an ability to slow down the growth of human tumors in nude mouse models through an antiangiogenic mechanism.

This study represents the first example of a potential new approach to controlling cell signaling through the selective disruption of protein-protein interactions. The key to the strategy will be to develop a range of GFBs with different surface recognition characteristics that can be then applied to different growth factors.

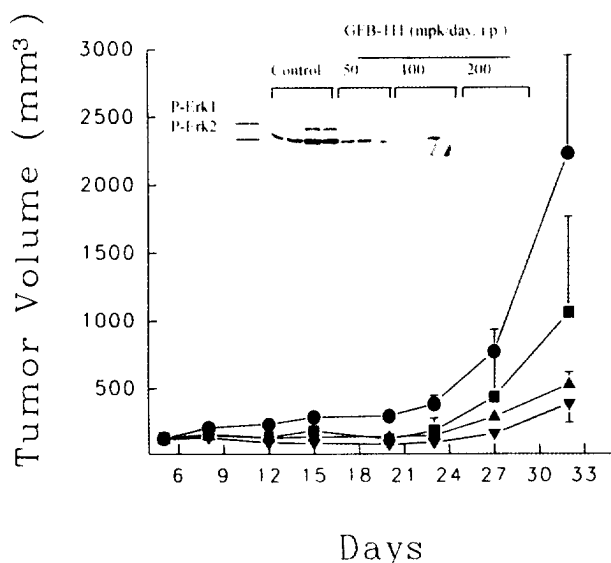


Figure 10 Inhibition of the growth of the human glioblastoma U87MG by GFB-111 in nude mice. Insert: Extent of phosphorylated/activated Erk1/Erk2 in tumors *in vivo*

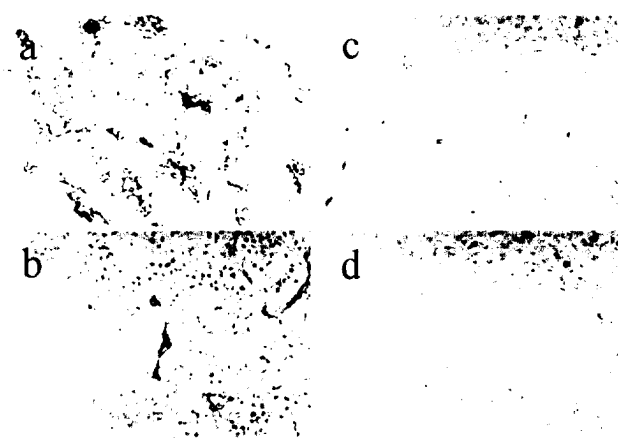


Figure 11 Extent of angiogenesis determined by immunostaining for Factor VIII complex technique: control (a), 50 mg/kg (b), 100 mg/kg (c), 200 mg/kg (d)

Table 2 GFB-111 inhibition of tumor growth in nude mice

Cell line	Tumor Origin	% Tumor Growth Inhibition Experiment			
		1	2	3	4
U87-MG	human brain	61% (35)	56% (32)	81% (32)	88% (32)
		50 mpk	50 mpk	100 mpk	200 mpk
A-549	human lung	62% (30)	53% (31)	64% (27)	N D
		50 mpk	50 mpk	50 mpk	
C6	rat brain	54% (15)	64% (15)	46% (18)	N D
		50 mpk	50 mpk	50 mpk	
DaOY	human brain	21% (48)	0% (37)	N D	N D
		50 mpk	50 mpk		

We are currently investigating different core scaffold structures and alternative recognition elements that will enhance selective binding to growth factor surfaces.

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