In Silico and NMR Identification of Inhibitors of the IGF-I and IGF-Binding Protein-5 Interaction

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Received March 20, 2002

Recently we have determined the crystal structure of the insulin-like growth factor-I (IGF-I) in complex with the N-terminal domain of the IGF-binding protein-5 (IGFBP-5). Here we report results of computer screening for potential inhibitors of this interaction using the crystal coordinates. From the compounds suggested by in silico screens, successful binders were identified by NMR spectroscopic methods. NMR was also used to map their binding sites and calculate their binding affinities. Small molecular weight compounds (FMOC derivatives) bind to the IGF-I binding site on the IGFBP-5 with micromolar affinities and thus serve as potential starting compounds for the design of more potent inhibitors and therapeutic agents for diseases that are associated with abnormal IGF-I regulation.

Introduction

The insulin-like growth factors (IGF-I and IGF-II, ca. 50% identity with insulin) are potent mitogens that promote cell proliferation and differentiation.[1,2] Most of the effects of IGF-I (70 amino acids) are mediated by binding to the type I IGF receptor (IGF-IR), a heterotetramer that has tyrosine kinase activity. The level of free systemic IGF is modulated by the extent of binding to IGF binding proteins (IGFBPs).[3,4] Signaling at the target organ is induced by proteolytic cleavage of IGFBP in the IGF/IGFBP complex by kallikreins, cathepsins, and/or matrix metalloproteinases, which releases IGF from the fragmented IGFBP and enables binding of IGF to the receptor.[1,3−5]

The IGFBP family comprises six proteins (IGFBP-1 to -6) that bind to IGFs with high affinity and a group of IGFBP-related proteins (IGFBP-rP 1−9), which bind IGFs with lower affinity. The proteins are produced in all tissues, typically however with tissue specific relative amounts of the various IGFBPs.[2] A key conserved structural feature among the six IGFBPs is a high number of cysteines (16−20 cysteines), clustered at the N-terminus (12 cysteines) and also, but to a lesser extent, at the C-terminus. The proteins share a high degree of similarity in their primary protein structure (identities around 30−40%), with highest conservation at the N- and C-terminal regions. It has been shown that these regions participate in the high-affinity binding to IGFs.[6,7] Full length IGFBP-5 is a 29 kDa protein. It is expressed mainly in the kidney and is found in substantial amounts in connective tissues. Unlike other IGFBPs, IGFBP-5 strongly binds to bone cells because of its high affinity for hydroxyapatite.

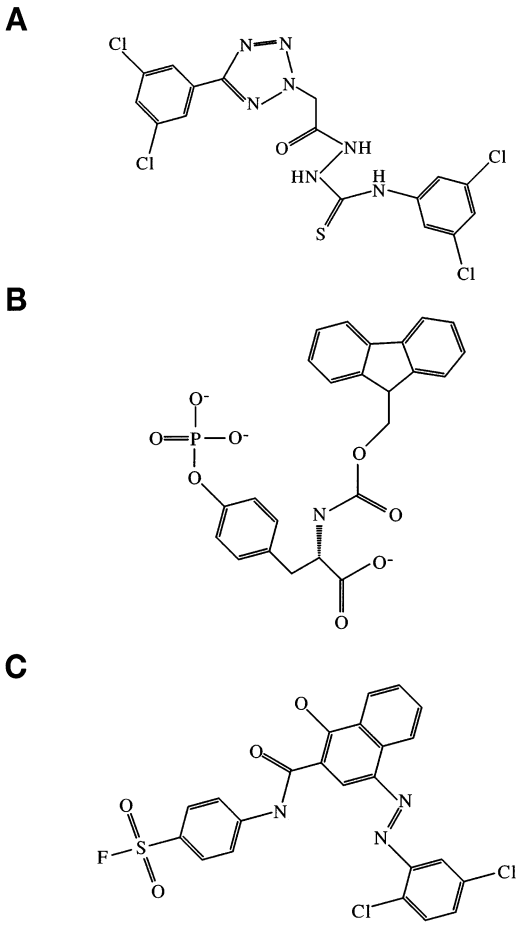

Figure 1. Formula of the compounds proposed by FlexX screening. (A) N^1-(3,4-Dichlorophenyl)-2-{2-[5-(3,5-dichlorophenyl)-2*H*-1,2,3,4-tetraazol-2-yl]acetyl}hydrazine-1-carbothioamide. (B) *N*-$^{\alpha}$-FMOC-*O*-phospho-L-tyrosine. (C) 4-(2,5-Dichlorophenylazo)-4′-fluorosulfonyl-1-hydroxy-2-naphthanilide.

IGFBPs regulate not only IGF action but appear also to mediate IGF-independent actions, including inhibition or enhancement of cell growth and induction of apoptosis. Recently, the presence of specific cell-surface

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10.1021/jm0208828 CCC: $22.00
Published on Web 11/23/2002

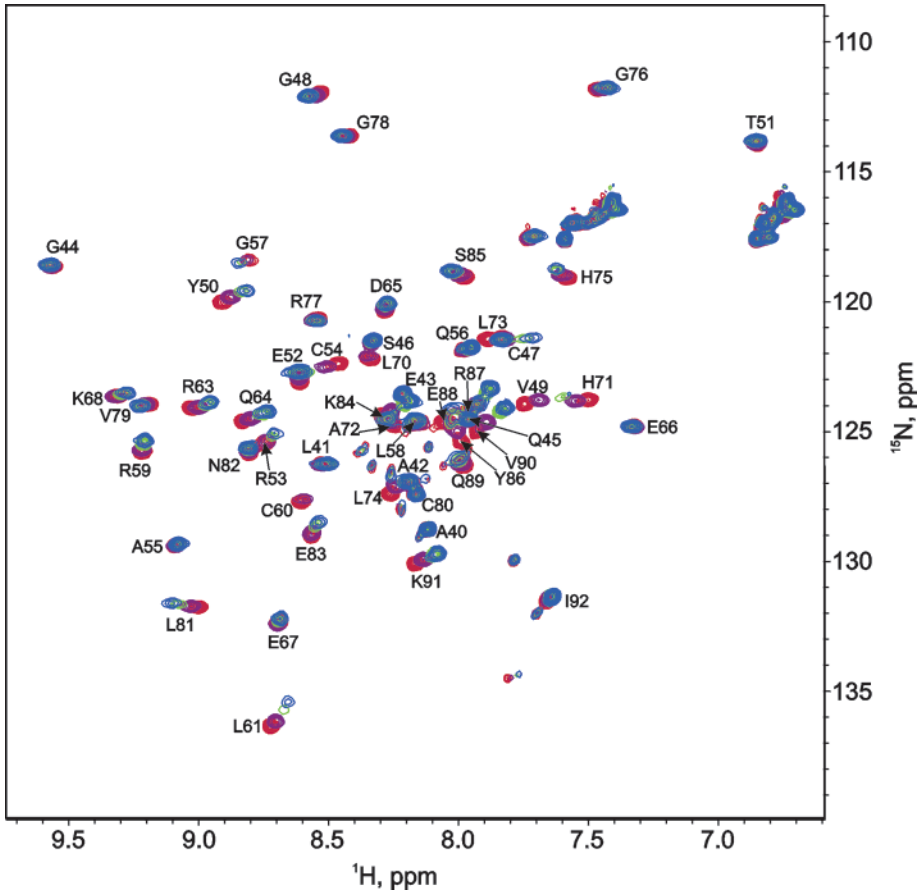

Figure 2. ^{15}N HSQC spectrum illustrating the titration of the mini-IGFBP-5 with the increasing amounts of compound B. The reference is shown in red. 1:1, 1:5, and 1:10 titration steps (protein:ligand) in purple, green, and blue, respectively.

IGFBP receptors were discovered. IGFBP-3 and IGFBP-5 have recently been shown also to be translocated into the nucleus, compatible with the presence of a nuclear localization sequence (NLS) in their mid-region. This raises the possibility that nuclear IGFBP may directly control gene expression.[8] IGFBPs were also shown to bind to important viral oncoproteins such as HPV oncoprotein E7.[1]

The IGFs, with their potent mitogenic and antiapoptotic effects, have been widely studied for their role in cancer.[5,9−11] Serum IGF-I and IGFBP-3 have been proposed as candidate markers for early detection of some cancers. In addition, IGF-I and IGF-II exhibit neuroprotective effects in several forms of brain injury and neurodegenerative disease.[12] This implies that targeted release of IGF from their binding proteins in brain tissue, for example, might have therapeutic value for stroke and other neurodegenerative diseases.[12] Compounds which disrupt the IGFBP−IGF interaction thus represent potential drugs. This idea has been explored by Liu and co-workers,[13] who screened successfully a large library of compounds to identify molecules that could displace IGF from its binding proteins. Here we describe a structure-based attempt to identify IGF releasing substances. The computer docking program FlexX identified IGFBP-5 ligands, FMOC derivatives, that bind to the IGF-I binding site on IGFBP-5 with a micromolar affinity. These results should aid the search for more potent inhibitors of the IGF-I and IGFBP-5 interactions and thus potential IGF-I releasing therapeutics.

Results and Discussion

We used the FlexX program[14,15] and the crystal structure of the IGF-I complex with the N-terminal mini-IGFBP-5 fragment[16] to identify potential inhibitors of the N-terminus-IGFBP-5/IGF-I interaction. Screening through the ACD database identified three dissimilar compounds (Figure 1) with a theoretically predicted binding capacity to the IGFBP-5 region responsible for IGF-I interaction. We then applied NMR to test for the predicted ligand−protein interactions.[17,18] Titrations of the ^{15}N-labeled mini-IGFBP-5 with the potential inhibitors revealed no binding affinity for compounds A and C to mini-IGFBP-5. This is not unexpected and is a common drawback of in silico screenings as the produced possible binding modes do not necessarily reflect real ligand binding. For this reason hits from virtual screening must be verified by other methods. Compound B, however, clearly altered the ^{15}N HSQC spectrum of the protein, indicating binding of this compound to mini-IGFBP-5 (Figure 2).

Compound B, because of its low solubility in water, was initially dissolved in DMSO. Titration of the protein with DMSO (e.g., lacking compound B) as a control was also performed. To investigate the influence of DMSO on the compound B binding to the protein, compound B dissolved in PBS buffer (at a lower concentration) was also titrated. Dissociation constants were estimated by monitoring several amino acid residues that display ligand induced changes in ^{15}N−^{1}H chemical shifts (Figures 2−4). The values of the dissociation constants for ligand B dissolved in DMSO and in PBS were similar

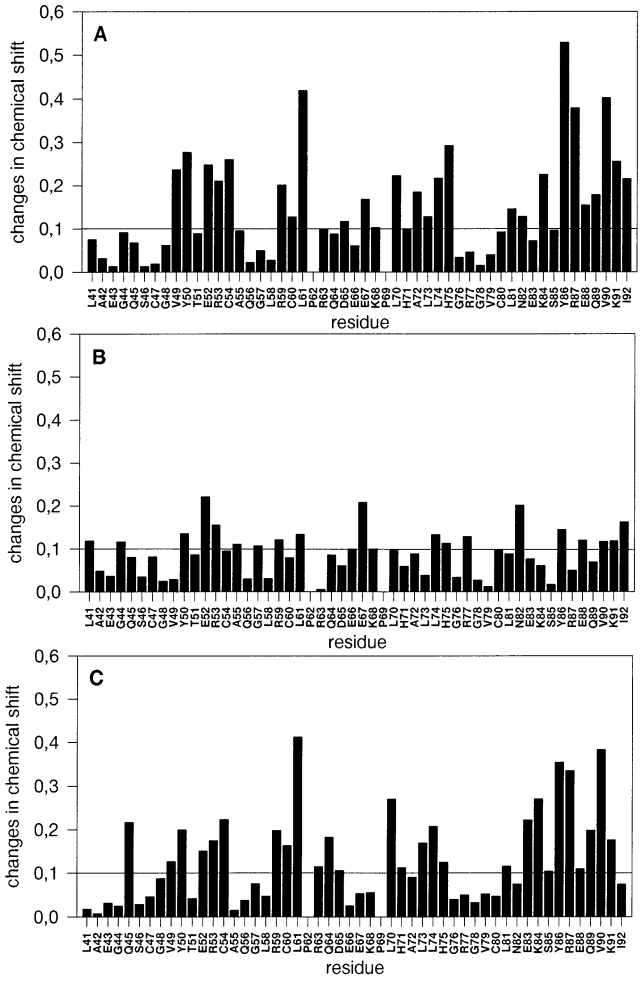

Figure 3. Differences in chemical shifts of free and inhibitor B-complexed mini-IGFBP-5 for all residues. Large shifts indicate residues involved in the compound B binding. Data for (A) ligand dissolved in DMSO; (B) DMSO; (C) ligand dissolved in PBS.

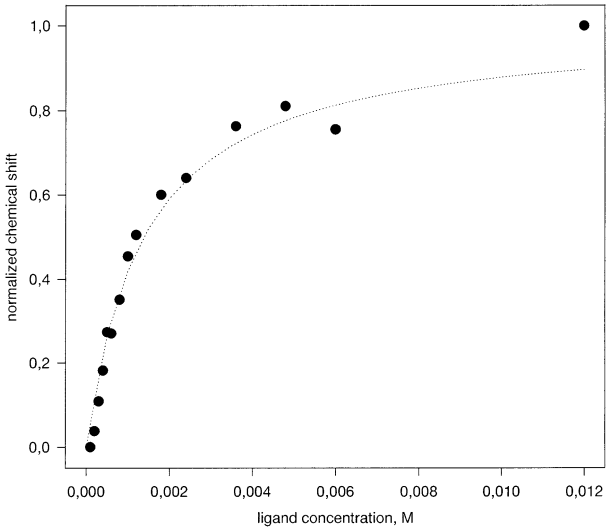

Figure 4. Titration with compound B in DMSO. Data for residue S85. Protein concentration 1 mM.

(1.86 and 2.31 mM, respectively; Table 1 and Figure 4). The affected residues are concentrated mostly in a contiguous region of the three-dimensional structure of the mini-IGFBP-5 (Figure 5A) which comprise the binding site of IGF-I. The binding surface between IGF-I and mini-IGFBP-5 appears mostly hydrophobic.[16] The principal IGF-I mini-IGFBP-5 interaction is a hydrophobic sandwich that consists of interlaced protruding side chains of IGF-I and solvent exposed hydrophobic side chains of the mini-IGFBP-5 (Figure 5A, and Figure 2b in Zeslawski et al.[16]). The side-chains of IGF-I Phe 16, Leu 54 and also Glu 3, are inserted deep into a cleft on the mini-IGFBP-5. This cleft is formed by side chains of Arg 53, Arg 59 on the solvent-exposed side of mini-IGFBP-5 and by Val 49, Leu 70, Leu 74 on the opposite inner side, with a base formed by residues Cys 60 and Leu 61. Phe 16 makes direct contacts with the backbone and side chain of Val 49, and with Cys 60 of mini-IGFBP-5. The other type of an important IGF/IGFBP-5 interaction consists of a network of hydrogen bonds that include side chains of Glu 3 and Glu 9 of IGF-I and His 71 and Tyr 50 of mini-IGFBP-5; in addition Arg 59 of mini-IGFBP-5 makes hydrogen bonds with Glu 58. The binding of ligand B to IGFBP-5 is partly hydrophobic (FMOC region of the ligand and Leu70, Leu74, Tyr50, His71, and Leu 61 of IGFBP-5) and partly hydrophilic (phosphate and carboxylic groups of the ligand and Arg59, Glu83, Gln45, Cys54, Arg53, and Cys60 of IGFBP-5).

Table 1. Dissociation Constant Calculations for Compound B or DMSO Binding to IGFBP-5 Using Data from Distinct Amino Acid Residues. Given Errors Are Due to the Fitting Procedure

residue	ligand in DMSO K_D [mM]	ligand in PBS K_D [mM]	DMSO K_D [mM]
Y50	1.58 ± 0.09	1.82 ± 0.95	648 ± 370
L73	1.31 ± 0.17	2.93 ± 1.41	541 ± 306
L81	2.78 ± 0.30	2.88 ± 1.18	610 ± 343
S85	1.38 ± 0.10	2.33 ± 0.94	650 ± 373
Y86	1.90 ± 0.17	1.72 ± 0.99	783 ± 498
R87	1.64 ± 0.12	2.36 ± 1.00	921 ± 662
K91	2.42 ± 0.18	2.12 ± 1.03	719 ± 434
average	**1.9 ± 0.5**	**2.3 ± 0.4**	**700 ± 100**

Dissociation constants for compound B and mini-IGFBP-5 interactions are significantly higher than the constants for interactions of the mini-IGFBP-5 with IGF-I, which are in the nanomolar range.[19] In the gel filtration studies compound B was not able to abolish the IGF-I/IGFBP-5 interactions (data not shown). Compound B was, however, used as a starting lead compound in search for higher affinity inhibitors for the IGF-I and IGFBP-5 interaction. Analyses of the IGFBP-5 residues involved in the compound B binding, as resolved by the present NMR study (Figures 3 and 5) and confirmed by molecular modeling predictions (Figure 5B), show that the binding region is in a similar location to that responsible for interactions with IGF-I.[16] We decided to find derivatives of compound B with enhanced binding to IGFBP-5. Analogues of compound B are commercially available as they are commonly used in peptide synthesis.

We first tested a compound B derivative N^{α}-FMOC-*O*-*tert*-butyl-L-tyrosine (FMOC, fluorenylmethyloxycarbonyl), where the hydrophilic phosphate group of B is replaced by a similarly sized hydrophobic *tert*-butoxy group (compound B1). This substitution resulted in an increase of the binding affinity by about 3-fold (Table 2). The next compound tested resembled B1 but the *tert*-butyl group was completely omitted, resulting in N^{α}-FMOC-L-phenylalanine (compound B2). Binding of compound B2 was weaker than of compound B1 but still

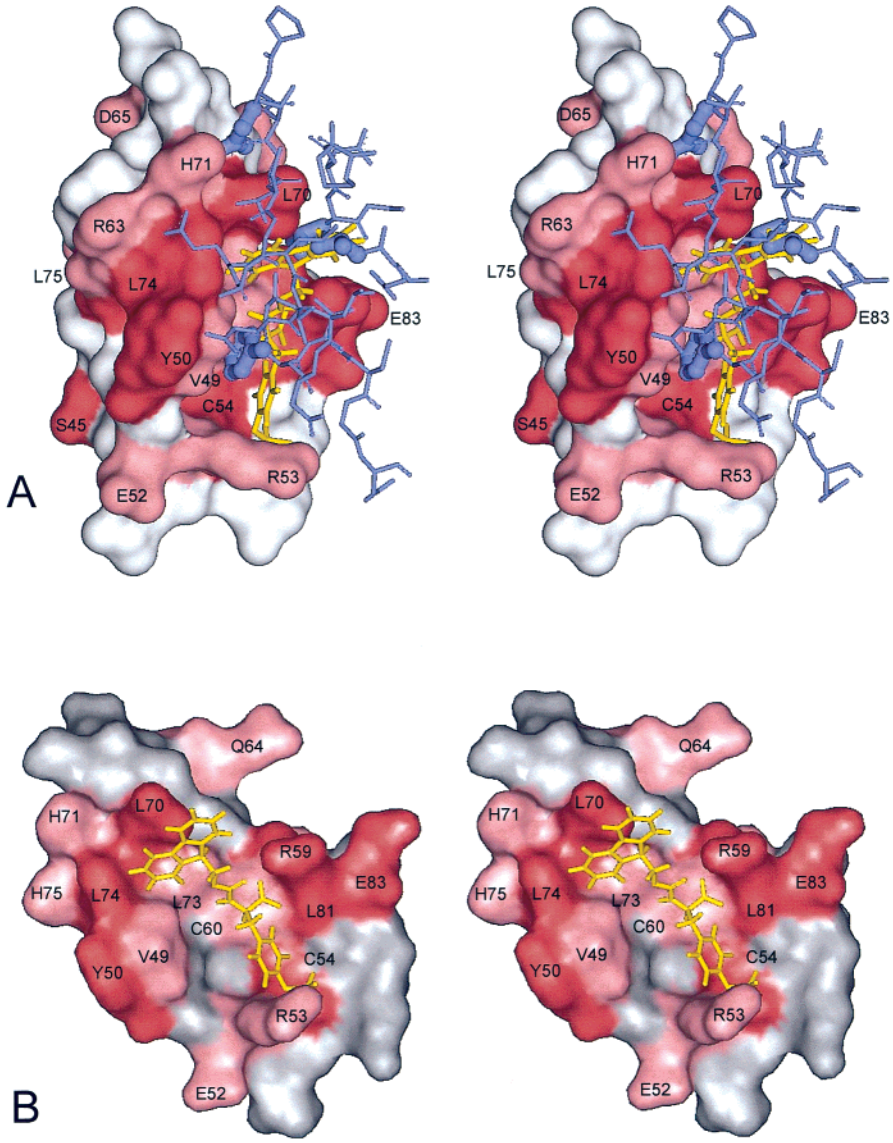

Figure 5. (A) Surface plot of mini-IGFBP-5 as resolved by X-ray crystallography[16] superimposed with the docking result of compound B (yellow) and with the interface residues of the IGF-I/mini-IGFBP-5 complex. IGF is shown in blue. Four IGF-I residues most essential for interactions with IGFBP-5 (from the top: Glu3, Leu57, Leu54, and Phe16, respectively) are shown as blue balls. Residues with chemical shift changes due to binding of compound B binding as revealed by the present study are shown in red (the more intense the color the bigger changes). (B) A close-up of the mini-IGFBP-5 and compound B only.

Table 2. Dissociation Constants Calculated for Compound B and Its Derivatives Binding to IGFBP-5 Using Changes in Chemical Shift for the Residue L81

compound	chemical name	K_D [mM]
B	N^{α}-FMOC-*O*-phospho-L-tyrosine	2.78 ± 0.30
B1	N^{α}-FMOC-*O*-*tert*-butyl-L-tyrosine	0.718 ± 0.079
B2	N^{α}-FMOC-L-phenylalanine	1.075 ± 0.507
B3	N^{α}-FMOC-*N*-BOC-L-tryptophan	0.0432 ± 0.0115
B4	N^{α}-FMOC-L-leucine	1.088 ± 0.519

better than for compound B. The decrease in ligand binding affinity correlated with the reduction of compound size suggested that larger hydrophobic substituent may enhance affinity. We therefore decided to test an analogue of compound B with a larger aromatic group (N^{α}-FMOC-*N*-BOC-L-tryptophan; compound B3); the substitution enhances ligand affinity into the micromolar range (43.2 μM; Table 2). Substitution of the aromatic tryptophan by the aliphatic leucine did not improve the affinity of the binding (N^{α}-FMOC-L-leucine, compound B4, Table 2). Compound B3, our best lead, was still to weak in binding to be able to abolish IGF-I/IGFBP-5 interactions at concentrations tested in gel filtration studies (data not shown).

Since it is well-known that DMSO might have a considerable effect on proteins we finally performed two control experiments. Titration of the protein with DMSO (e.g., lacking compound B) as a control revealed very weak binding of DMSO to mini-IGFBP-5 (Figure 3, Table 1). The DMSO interaction is most likely nonspecific, as indicated by the small and similar extent of the chemical shift perturbations of a large number of amino acid residues (Figure 3B). Compound B was soluble in PBS buffer at low concentrations. Comparison of a titration of compound B in PBS and DMSO (Figures 3A and 3B) shows that most significant changes appear at the same amino acid residues. Note that the changes in chemical shift do not necessarily go in the same direction for both experiments. So values in Figures 3C

and 3B might not be simply added to arrive at values in Figure 3A, but will for different residues partially cancel or add up.

IGFs are known for their neuroprotective properties. Brain injury is commonly associated with increase in IGF expression but, paradoxically, also with increased expression of the inactivating binding proteins. Attempts to administer IGF-I exogenously as protective therapy in cases of brain injury[20] may thus be hampered by the increased expression of brain IGFBP. Combined administration of IGFs and IGFBP ligand inhibitors may optimize treatment of neurodegeneration. Alternatively, displacement of the large "pool" of endogenous IGF from the IGF-binding proteins might elevate "free" IGF levels such that administration of IGFBP ligand inhibitors elicit neuroprotective effects comparable to those produced by administration of exogenous IGF. Bayne and co-workers[21] reported an IGFBP ligand inhibitor, [Leu^{24,59,60}, Ala^{31}] IGF-I mutant, with high affinity to IGF-binding proteins (0.3−3.9 nM) but with no biological activity at the IGF receptors (>10 μM). Loddick and co-workers[12] examined effects of this high-affinity IGFBP ligand inhibitor in in vitro studies of release of "free" bioactive IGF-I from rat cerebrospinal fluid and in in vivo studies to evaluate its neuroprotective effects in a rat model of focal ischemia. This successful targeting of IGFBPs suggests that it may be possible to identify nonpeptide small molecules that act as IGFBP ligand inhibitors, with the potential for good blood−brain barrier penetration and oral activity. The data collected by Loddick and co-workers[12] demonstrate that displacement of IGFs from IGFBPs in the brain is a potential treatment for stroke. Moreover, in view of the potent actions of IGFs on survival of neurons and glial cells as well as the widespread protective affects against a variety of brain insults, IGFBP ligand inhibitors may have broader utility for the treatment of various neurodegenerative disorders as well as traumatic brain and spinal cord injury.

Conclusion

Because of their high structure similarity, we assume that all B analogues bind similarly to IGFBP-5. This is supported by the fact that mostly the same residues of IGFBP-5 are affected in the NMR titrations. Figure 5 shows compound B docked in the IGF-I binding site of IGFBP-5. Analysis of the structures shows the prediction that the phenyl group of the compound B mimics Phe16 from IGF-I, and that the FMOC-group binds at the equivalent position of IGF-I-Leu54, Leu 57 (Figure 5A). The IGF "Glu3 hydrogen bonding" region of IGFBP-5 (His 71 and NH of Leu70), however, seems not to be involved in interactions with the B compounds. Thus, this site offers binding interactions for new IGFBP-5 ligands, which when linked with compound B3 could significantly enhance binding affinities. Currently, we are screening for other small molecular weight compounds that would be able to bind to the IGFBP-5 at this or other neighboring sites on the IGFBP-5 surface.

Experimental Section

Molecular Modeling. The protein model for flexible docking was taken from the high-resolution X-ray structure of the IGF-I/mini-IGFBP-5 complex[16] without further modification, i.e., the model neither underwent additional minimization nor were any side chain conformations changed. As the small molecule database, the Available Chemicals Directory (ACD, MDL Information System) of commercially available compounds was used and filtered to include approximately 90 000 compounds with $M_r \leq 550$ Da that contain at least one atom from the set {N, O, F, S}. The stereochemical information was used as provided by ACD. The set of molecule files were converted to the mol2 format with SYBYL (Tripos, St. Louis, MO) with all hydrogens added. This set served as input to FlexX (GMD, St. Augustine, FL) for flexible docking into a binding site on IGFBP-5 to identify small molecules which might bind to IGFBP-5 and thereby block the interaction with IGF-I. The binding site was defined as a sphere around all residues of IGFBP-5 toward the interaction site plus a 5 Å border (taking whole residues). The side chain conformations of mini-IGFBP-5 were not adjusted by the docking protocol. The small molecule conformations for each compound generated by FlexX using the standard FlexX scoring function were clustered by an rmsd of 2.3 Å and each best scoring pose within a cluster was saved as the cluster representative. Analysis of all the saved conformations of all docked ligands was carried out using a distance-based filter defining the following criteria: (1) A substructure of the ligand must interact with the region Val49/Leu70/Leu73/Leu74. (2) A substructure of the ligand must interact in the deep pocket around Cys47/Thr51. As a result, three compounds were selected for an NMR screening (Figure 1).

Materials. Mini-IGFBP-5 (amino acids 40−92 of human IGFBP-5) was expressed and purified using the construct described by Kalus and co-workers.[19] Compounds A, B, and C were purchased from ChemPur (Karlsruhe, FRG), Fluka (Buchs, Switzerland), and Sigma (Deisenhofen, FRG), respectively. Compound B derivatives were generously provided by Prof. Luis Moroder.

Detection of Ligand Binding. Ligand binding was detected by acquiring ^{15}N HSQC spectra. All NMR spectra were acquired at 300 K on Bruker DRX600 spectrometer. The samples for NMR spectroscopy were concentrated and dialyzed against PBS buffer. Typically, the sample concentration was varied from 0.3 to 1.0 mM. Before measuring, the sample was centrifuged in order to sediment aggregates and other macroscopic particles. A 450 μL amount of the protein solution was mixed with 50 μL of D_2O (5−10%) and transferred to an NMR sample tube. The stock solutions of compounds were 100 mM either in water or in perdeuterated DMSO. pH was maintained constant during the whole titration. The binding was monitored by observation of the changes in the ^{15}N HSQC spectrum. Dissociation constants were obtained by monitoring the chemical shift changes of the backbone amide of several amino acid residues (Table 1) as a function of ligand concentration. The precision of the dissociation constants estimated from the induced chemical shifts of several residues is adequately high to justify monitoring a single residue to estimate K_D values. Therefore, for K_D determination of the compounds derived from ligand B we chose the residue L81 to monitor the chemical shift changes. Data were fit using a single binding site model.

Acknowledgment. This research was supported by the German Science Foundation (DFG) project SFB469-A7 to T.A.H.

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JM0208828