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STRUCTURE-BASED DESIGN OF NOVEL, DIPEPTIDE LIGANDS TARGETING THE pp60^{Src} SH2 DOMAIN[1]

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Abstract: Based on the X-ray structures of the pp60^{Src} (Src) SH2 domain complexed with two high affinity phosphopeptide ligands (Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu, and Ac-pTyr-Glu-D-Hcy-NH_2; Hcy = homocyclohexylalanine), we report herein the design and structure-activity relationships of a series of novel dipeptide ligands targeting the Src SH2 domain. © 1997 Elsevier Science Ltd.

Introduction:

Protein tyrosine kinases (PTKs) play an important role in the signal transduction pathways that regulate cellular growth, development, and differentiation.[2] The protein pp60^{Src} (Src) may play a role in disregulated cell growth, as elevated activity of this PTK has been associated with certain cancers.[3] Three domains in the Src protein-SH3, SH2, and catalytic tyrosine kinase domain-are involved in cellular signal transduction pathways. These domains consist of approximately 60, 100, and 250 amino acids, respectively. The Src SH2 domain displays high affinity for phosphotyrosine (pTyr) containing proteins and prefers a ~pTyr-Glu-Glu-Ile~ sequence.[4,5]

We and others have previously described the structure-activity relationships for a series of phosphorylated tripeptides,[4,6,7] as exemplified by compounds **1-3** (Figure 1), and these results have impacted our studies reported herein. Also, X-ray structures of the Src SH2 domain have previously shown two well-defined binding pockets for the pTyr-Glu-Glu-Ile type phosphopeptide ligands.[4,8] Specifically, one pocket (P site) binds pTyr and the second (P+3 site) binds Ile. Guided by the comparative structure-activity relationships of the tripeptides **1-3** and the 3-dimensional (3-D) structural information of the Src SH2-phosphopeptide complexes (i.e., Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu[8] and Ac-pTyr-Glu-D-Hcy-NH_2[9] ; Hcy = homocyclohexylalanine), we focused an effort on the structure-based design of novel dipeptides ligands for the Src SH2 domain.

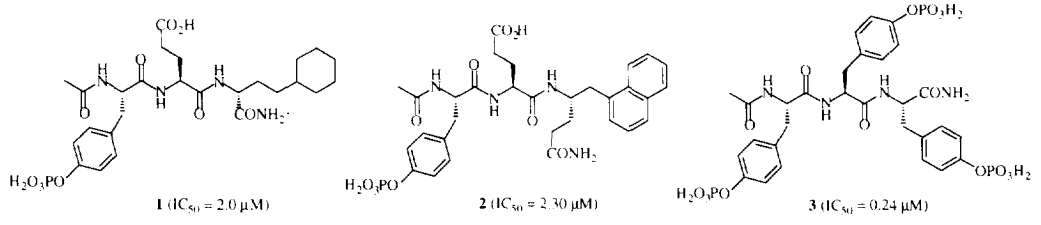


Figure 1. Chemical structures of phosphopeptides: **1** (Plummer et al.[6]), **2** (Rodriguez et al.[7]), and **3** (Gilmer et al.[4])

The co-crystal structure of **1** with the Src SH2 domain shows the C-terminal carboxamide extends into solvent, which allows this carboxamide (Figure 2) to be removed to yield dipeptide ligands without compromising binding affinity.[9,10] We have further examined this prototype peptidomimetic series by varying the P+1 amino acid, the C-terminal hydrophobic ring, and by sidechain transposition (P+1 Cα→P+2 Nα) to identify potent lead compounds.

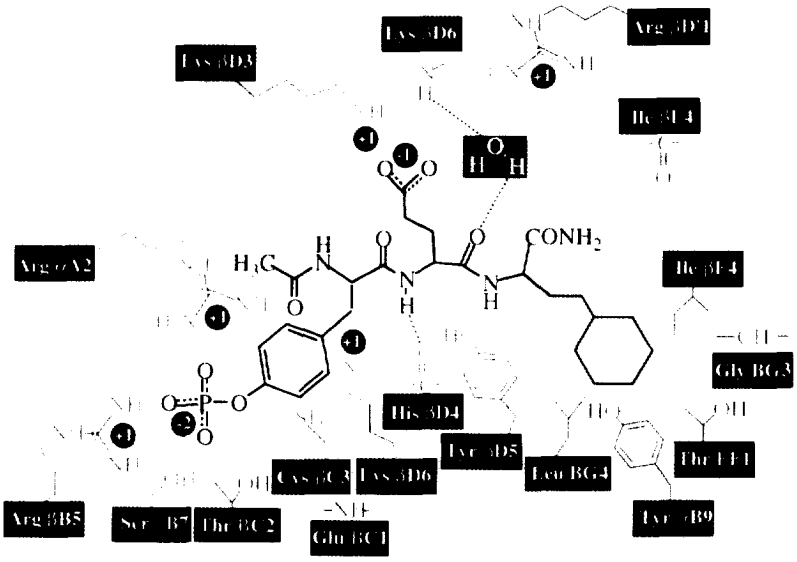


Figure 2. Schematic diagram showing interaction of Ac–pTyr–Glu–D–Hcy–NH_2 complexed with Src SH2 and illustrating the two key binding sites for pTyr and Hcy, respectively.

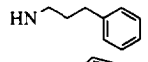
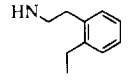
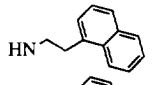
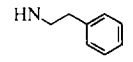
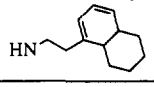
Results and Discussion:

Modifications of The C-Terminal Hydrophobic Group

Based on the interactions observed in the X-ray structure of Src SH2 complexed with the pTyr–Glu–Glu–Ile peptide[8] and Ac–pTyr–Glu–D–Hcy–NH_2[9] (Figure 2), a series of analogs have been synthesized in an attempt to optimize interactions at the hydrophobic P+3 binding pocket (Table 1). Relative to the parent dipeptide **4**, the naphthyl modified analog **5** was designed to provide increased hydrophobicity with the shallow P+3 pocket and to restrict rotational freedom of the aryl–alkyl sidechain. Modeling suggests that these aryl groups cap the P+3 hydrophobic pocket, but do not extend into the pocket. Reduction of **5** to provide the tetrahydronaphthalene derivative **6** showed further increased potency (IC_{50} = 3.2 μM) relative to compound **4**. Similarly, replacement of the fused cyclohexyl ring of compound **6** by an *ortho* ethyl substituent (**7**), in an effort to mimic the branched alkyl chain of Ile which extends into the hydrophobic pocket, results in about two fold increased potency relative to the parent dipeptide **4**. As a reference, compound **8** was essentially equipotent to **4** but illustrates the binding enhancing effects of the fused cyclohexyl ring or *ortho* ethyl substituent of compounds **6** and **7**, respectively.

Table 1. Structure–activity relationships of Ac–pTyr–Glu–NHR analogs.

Ac–pTyr–Glu–NHR

Analog No.	NHR	IC_{50} (μM)	Analog No.	NHR	IC_{50} (μM)
4		10	7		4.6
5		4.8	8		9.1
6		3.2			

P+1 Amino Acid Substitution

In conjunction with modifying the hydrophobic interactions in the P+3 binding pocket to increase the binding affinity of dipeptide **4**, we explored amino acid replacements at the P+1 position (Table 2). Specifically, we were interested in the replacement of the acidic Glu sidechain with an uncharged sidechain. The X–ray structures[8,9] show that the C_β and C_γ of the P+1 Glu side chain conform to the protein surface and reside over a small hydrophobic patch, while the carboxylate extends outward towards Lys–57. In an effort to interact with this small hydrophobic area, we substituted Glu with norvaline (Nvl), giving compound **9**, which did not significantly effect potency. Truncating the P+1 alkyl sidechain of **9** by one methylene unit, essentially replacing the $-CO_2H$ of Glu with a hydrogen atom to give the aminobutyric acid (Abu) analog **10** resulted in essentially identical binding affinity compound **4**. When Ala was substituted for the P+1 Glu (compound **11**) only a slight decrease in affinity occurred. However, replacement of P+1 Glu by Gly, to give compound **12**, effected about a five fold decrease in activity which results from increased rotational freedom and decreased hydrophobic interactions. A branched amino acid at P+1 was less tolerated than the parent as a three fold decrease in potency was observed with the P+1 Val modified analog **13** (IC_{50} = ~30 μM). Finally, both P+1 Tyr and pTyr modified analogs were tested (compounds **14** and **15**, respectively), and the latter dipeptide was found to be about three fold more potent than the parent analog **4**, thus supporting the preference for acidic residues in this position for possible electrostatic interaction with Lys–57 of Src SH2 domain.

Table 2. Structure–activity relationships of Ac–pTyr–Xxx–Ppa[a] analogs.

Ac–pTyr–Xxx–Ppa

Analog No.	Xxx	IC_{50} (μM)	Analog No.	Xxx	IC_{50} (μM)
4	Glu	10	12	Gly	~46
9	Nva	12	13	Val	~30
10	Abu	10	14	Tyr	20
11	Ala	15	15	pTyr	3.9

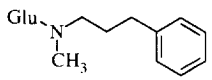
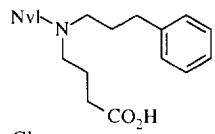
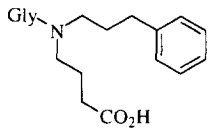
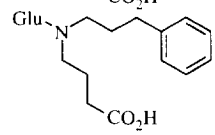
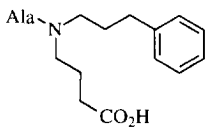
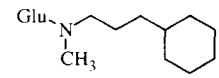
[a]Ppa refers to phenylpropylamide; NH–$(CH_2)_3$–phenyl.

P+1 Sidechain Transposition

With our tripeptide analogs[6] we previously observed that N–methylation of the P+2 Nα provided increased binding affinity. A similar trend was observed in the dipeptide[10] series as exemplified by N–methylation of dipeptide **11** to provide compound **16**, which resulted in increased binding affinity by approximately three fold. Since that N–methylation at the C–terminal amide was advantageous, we explored the concept of sidechain transposition (i.e., P+1 Cα → P+2 Nα) of the Glu sidechain of compound **4** to its C–terminal amide NH (Table 3). This would result in the nitrogen being substituted by propionic acid. Molecular modeling[11] indicated that homologation of this group to butyric acid would allow better mimicry of the P+1 Glu sidechain; as stated above, X–ray structure indicates that this group forms a weak ionic interaction with Lys 57. We chose to evaluate Gly, Ala, and Nvl as P+1 replacements in this series (compounds **17–19**, respectively). The Gly derivative **17** and the Ala derivative **18** were three– and two fold more potent, respectively, than the corresponding NH derivatives **12** and **11**. This increase in affinity was similar to that observed for simple P+2 N–methylation (*vide supra*) and suggests the carboxyalkyl functionality in the transposed sidechain was not contributing to activity in the Ala series. Interestingly, the P+1 Nvl derivative **19** was three fold more potent than the corresponding NH derivative **9**, while analog **20** was five fold more potent than the parent compound **4**. Finally, substitution of the phenyl moiety of analog **4** by a cyclohexyl ring provided dipeptide **21** which was essentially equipotent to the tripeptide lead **1**.

Table 3. Structure–activity relationships of Ac–pTyr–Xxx–NR'R" analogs.

Ac–pTyr–Xxx–NR'R"

Analog No.	Xxx–NR'R"	IC_{50} (μM)	Analog No.	Xxx–NR'R"	IC_{50} (μM)
16		3.5	19		4.3
17		14	20		2.0
18		6.4	21		1.7

Conclusion:

We have examined several prototype peptidomimetics through C–terminal modifications, varying the P+1 sidechain and transposing the P+1 sidechain to the adjacent P+2 Nα position. A series of dipeptides systematically modified to explore P+3 binding site interactions were advanced. Specific modifications of the C–

terminal hydrophobic functionality included aromatic substitution and the alkyl linkage chain length modification to validate molecular modeling studies based on X–ray crystallographic structures of Src SH2–phosphopeptide complexes. This structure–activity study was focused on the P+1→P+3 pockets of Src SH2 domain, and it extends our discovery efforts to identify novel dipeptide ligands. Such information impacts our design of second–generation peptidomimetic and nonpeptide ligands to provide potent, specific, and metabolically–stable lead compounds to explore the effects of Src SH2 blockade in cellular signal transduction pathways.

Experimental, Materials and Methods:

Chemistry. Preparation of dipeptides with generic structure Ac–pTyr–Glu–NHR′, and Ac–pTyr–Xxx–NR′R″ were achieved by convergent synthesis using the appropriate C–terminal fragments NHR′ or NR′R″. Coupling to commercially available Fmoc–Glu(OtBu)–OH in the presence of HOBT/EDCI/NMP/DMF at room temperature and subsequent Fmoc removal (20% piperidine in NMP) provided the intermediates, Glu(OtBu)–NHR′ or Glu(OtBu)–NR′R. Final coupling to commercially available Ac–Tyr and phosphorylation was accomplished according to a published procedure.[12] All phosphopeptides were isolated by preparative reversed–phase HPLC and analytical data (electrospray mass spectrometry, ^{31}P and ^{1}H NMR spectroscopy) were satisfactory to confirm the structural integrity of final products.

Materials. ^{125}I–Radiolabeling of Glu–Pro–Gln–pTyr–Glu–Glu–Ile–Pro–Ile–Tyr–Leu was carried out by Amersham Corporation. Filter plates (0.45 μM hydrophobic PVDF) were purchased from Millipore. Glutathione–sepharose 4B beads were purchased from Pharmacia. Each SH2 protein was expressed as a fusion construct of gluutathione S–transferase (GST) according to a published procedure.[13]

Src SH2 binding assay. Binding of ^{125}I–labeled phosphopeptide to a Src SH2–GST fusion protein was performed in 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.1% NP–40. Assay conditions include Src SH2 fusion protein–glutathione sepharose bead complex, 2.8 nM ^{125}I–labeled phosphopeptide and 2% DMSO test sample concentrations typically ranging from 0.1 to 100 μM. Binding was carried out at room temperature for 20 minutes while continuously inverting the plate. Bound ^{125}I–labeled phosphopeptide was separated from free ^{125}I–peptide by vacuum filtration and twice washing with 100 μL assay buffer/well. The remaining radioactivity was determined by scintillation counting. All measurements were determined in duplicate.

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