Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry
ELSEVIER
Bioorganic & Medicinal Chemistry 12 (2004) 779–787

Design of tetrapeptide ligands as inhibitors of the Src SH2 domain

Nguyen-Hai Nam,[a] Rebecca L. Pitts,[a] Gongqin Sun,[b] Soroush Sardari,[a] Amie Tiemo,[a] Mingxing Xie,[a] Bingfang Yan[a] and Keykavous Parang[a,*]

[a]*Department of Biomedical Sciences, College of Pharmacy, The University of Rhode Island, Kingston, RI 02881, USA*
[b]*Department of Cell and Molecular Biology, The University of Rhode Island, Kingston, RI 02881, USA*

Received 15 May 2003; accepted 28 October 2003

Abstract—Src homology-2 (SH2) domains are noncatalytic motifs containing approximately 100 amino acid residues that are involved in intracellular signal transduction. The phosphotyrosine-containing tetrapeptide pTyr-Glu-Glu-Ile (pYEEI) binds to Src SH2 domain with high affinity ($K_d = 100$ nM). The development of five classes of tetrapeptides as inhibitors for the Src SH2 domain is described. Peptides were prepared via solid-phase peptide synthesis and tested for affinity to Src SH2 domain using a fluorescence polarization based assay. All of the N-terminal substituted pYEEI derivatives (class II) presented binding affinity (IC_{50} = of 2.7–8.6 μM) comparable to pYEEI ($IC_{50} = 6.5$ μM) in this assay. C-Terminal substituted pYEEI derivatives (class III) showed a lower binding affinity with IC_{50} values of 34–41 μM. Amino-substituted phenylalanine derivatives (class IV) showed weak binding affinities ($IC_{50} = 16$–153 μM). Other substitutions on phenyl ring (class I) or the replacement of the phenyl ring with other cyclic groups (class V) dramatically decreased the binding of tetrapeptides to Src SH2 ($IC_{50} > 100$ μM). The ability of pYEEI and several of the tetrapeptides to inhibit the growth of cancer cells were assessed in a cell-based proliferation assay in human embryonic kidney (HEK) 293 tumor cells. The binding affinity of several of tested compounds against Src SH2 domain correlates with antiproliferative activity in 293T cells. None of the compounds showed any significant antifungal activity against *Candida albicans* ATCC 14053 at the maximum tested concentration of 10 μM. Overall, these results provided the structure–activity relationships for some FEEI and YEEI derivatives designed as Src SH2 domain inhibitors.

1. Introduction

The Src tyrosine kinases are non-receptor tyrosine kinases; a number of these have become key targets for both basic research and anticancer drug discovery over recent years. The $pp60^{src}$ (Src) is a member of Src family kinases featuring several functional domains, including N-terminal (NT), Src homology 3 (SH3), Src homology 2 (SH2), kinase (catalytic), and the C-terminal (CT) noncatalytic domain.[1] Src is involved in intracellular signal transduction.[2] Src has been implicated in the development of osteoporosis and inflammation-mediated bone loss.[3,4] In addition, Src kinases have been associated with several different cancers including colon[5] and breast[6] cancers for which transformed phenotypes have been correlated with Src mutations and/or overexpression of Src tyrosine kinase activities.[7–9] Therefore, Src exists as an important target for drug discovery.

SH2 domains are modules of approximately 100 amino acids that have evolved to recognize and bind specifically to tyrosyl-phosphorylated sequences located on proteins in response to extracellular signals.[10–12] The SH2 domain controls the range of proteins interacting with the Src family kinases. The Src SH2 domain has been shown to interact with FAK, $p130^{cas}$, p85, PI3K, and $p68^{sam}$.[13–16] The phosphotyrosyl group binds in a deep pocket where it is stabilized by a large number of hydrogen bonds and electrostatic interactions. The binding of SH2 domains to their targets propagates downstream signaling by recruiting the SH2 domain-containing protein to its proper signaling complex and/or by altering its enzymatic activity. Inappropriate signaling through tyrosine kinases has been linked to many pathological conditions, providing an impetus to understand the mechanism of SH2 domain recognition and to design SH2 domain-binding inhibitors.[17] Ligands having the ability to disrupt cellular signal transduction pathways by antagonizing SH2 domain-dependent protein–protein interactions provide possible therapeutic agents. The identification of antagonists against SH2 domain–target interaction has been an active area of research.[17–21]

Keywords: Src SH2 domain; pYEEI; Tetrapeptides; Fluorescence polarization.
* Corresponding author. Tel.: +1-401-874-4471; fax: +1-401-874-5048; e-mail: kparang@uri.edu

0968-0896/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2003.10.060

The Src SH2 domain preferentially binds peptides with the sequence phosphotyrosine–glutamic acid–glutamic acid–isoleucine (pTyr-Glu-Glu-Ile, pYEEI) with high affinity ($K_d = 100$ nM).[12] Crystallographic studies[22–24] between pYEEI tetrapeptide and Src SH2 domain have revealed two crucial binding pockets that interact with the ligand. The first binds the phosphotyrosine moiety (P site) and the second accommodates the hydrophobic side chain of an isoleucine residue (P + 3 site). The glutamic acid residues in the P + 1 and P + 2 sites contribute less significantly to the binding by forming ion pairs with lysine and arginine side chains, respectively. The positively charged pTyr binding pocket interacts with phenyl phosphate pTyr residue and is formed by part by Arg 12, Arg 32, and Lys 60.[22–24] Thus strong ionic interactions between doubly negatively charged phosphate and the two Arg residues are observed. The hydrophobic alkyl side chain of Lys 60 interacts with the phenyl ring.

Five classes of tetrapeptides (Fig. 1) were synthesized and tested against Src SH2 domain. The purpose of this study was to understand the structure–activity relationships of these compounds in order to develop novel Src SH2 inhibitors. Tetrapeptides were derived from (i) substitution on the phenyl ring of YEEI or FEEI (class I), (ii) N- or C-terminal substitution of pYEEI (classes II and III), (iii) replacing the *O*-phosphate of tyrosine in pYEEI with nonhydrolyzable groups such as *N*-sulfondiamides, phosphoramidates, and *N*-malonates (class IV), or (iv) replacing the pTyr moiety with other aromatic or non-aromatic carboxylic acids (class V). A fluorescence polarization (FP)-based competitive binding assay was used to determine the binding affinity of the compounds for the Src SH2 domain.[25]

In class I, the substitution of groups affecting various steric, electronic and hydrophobic features were examined by the replacement of pTyr in pYEEI with a number of substituted phenylalanine and tyrosine derivatives. Classes II and III represent pYEEI derivatives having the N- or C-terminal substitution of short and long chain groups such as aminoacyl or diaminoalkyl groups. Keeping cell permeability considerations in mind, it was of interest to know whether it would be possible to use a purely hydrophobic side chain to create new favorable contacts with the protein or the binding affinity of pYEEI can be dramatically improved by attaching the N- or C-terminus of pYEEI to short or long chain alkyl groups. The possible hydrolysis of the phosphate ester bond by phosphatases render pTyr-containing compounds unsuitable for biological activity and in vivo cell studies. Replacing pTyr in by phosphatase-resistant mimics without losing high binding affinity for the Src-SH2 domain is therefore desirable. In class IV, the effect of the replacement of *O*-phosphate group of tyrosine with phosphoramidates, a sulfondiamide, and an *N*-malonate was tested. The two negative charges of pTyr at physiological pH can, in principle, impair cell membrane penetration. In class V of compounds, the tyrosine phosphate group is replaced with other cyclic groups having carboxylic acid moiety.

2. Results and discussion

2.1. Chemistry

The syntheses of the peptides are shown in Figures 2–5. Tetrapeptides **1–33** were synthesized by automated solid phase peptide synthesis and purified by preparative

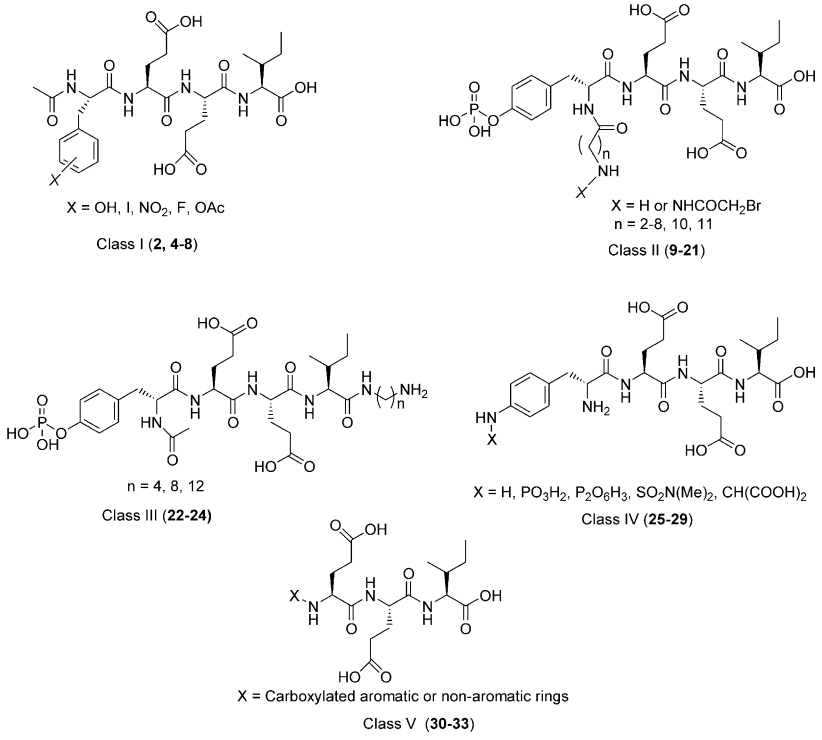


Figure 1. General structures for five classes of synthesized compounds assayed for binding affinity to Src SH2 domain.

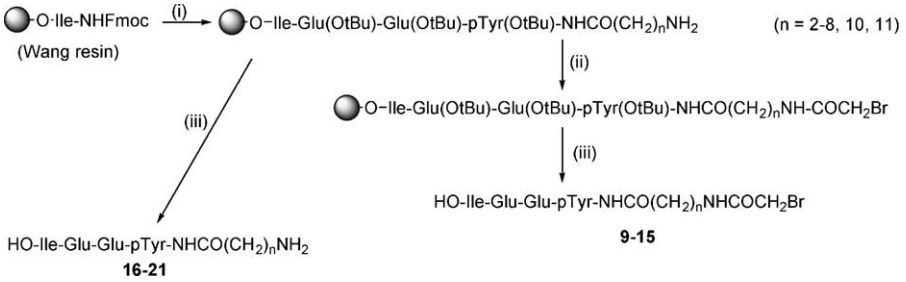


Figure 2. The synthesis of N-terminal-substituted pYEEI derivatives: (i) Fmoc peptide chemistry: RCOOH [e.g., Fmoc-AA-OH, FmocNH(CH_2)*n*-COOH)], HBTU, NMM, piperidine, DMF; (ii) Fmoc peptide chemistry: HBTU, NMM, piperidine, DMF, $BrCH_2COOH$; (iii) TFA, anisole, water.

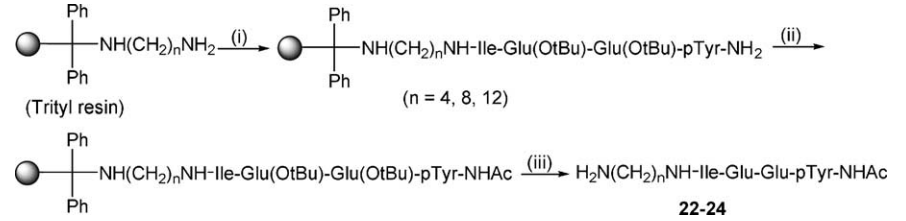


Figure 3. The synthesis of C-terminal-substituted pYEEI with short- and long-chain carboxylic acids: (i) Fmoc peptide chemistry: Fmoc-AA-OH, HBTU, NMM, piperidine, DMF; (ii) Ac_2O, piperidine; (iii) TFA, anisole, water.

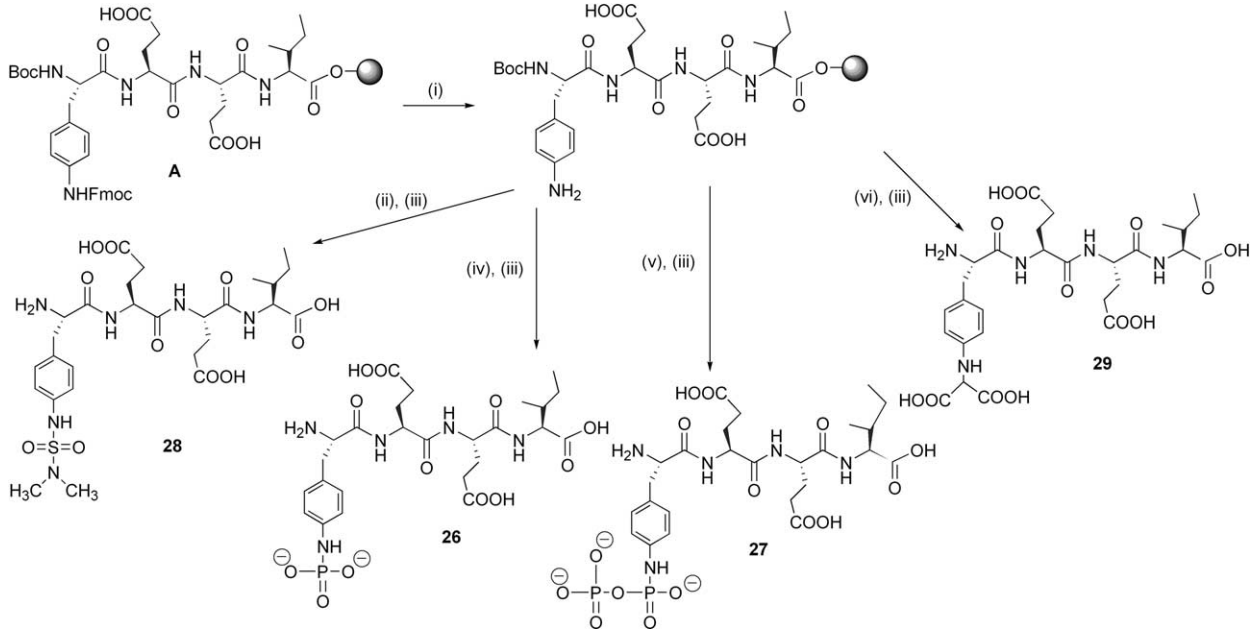


Figure 4. The synthesis of *N*-sulfonamide, phosphoramidates, and *N*-malonate derivatives of pYEEI (class IV): (i) piperidine, DMF, 5 min (2); (ii) dimethylsulfamoyl chloride (*N*-DMSCl), DIPEA, DCM, 3 h, rt; (iii) TFA, anisole, water; (iv) pyridine, $POCl_3$, 3 h, rt; (v) pyridine, $POCl_3$, DCM, 12 h, rt; (vi) $Br(CHCOOEt)_2$, DIPEA, DMF.

reversed-phase high performance liquid chromatography (HPLC) and characterized by electrospray mass spectrometry. N-Terminal-substituted tetrapeptides **9–21** (class II) were synthesized using Fmoc-Ile-Wang-resin utilizing a standard Fmoc solid-phase synthesis protocol as shown in Figure 2. Two series of class II compounds were synthesized. The first series of compounds (*N*-bromoacetylated compounds; **9–15**) were synthesized by the coupling of the *N*-substituted phosphopeptides on solid phase with bromoacetic acid in the presence of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-methylmorpholine (NMM), followed by cleavage with trifluoroacetic acid (TFA)/anisole/water (95:5:5). The second series of class II compounds (**16–21**) were prepared with simple cleavage of *N*-linked phosphopeptides from the resin.

Figure 3 shows the synthetic strategy for the compounds in class III. The protected SH2-directed phosphopeptide pYEEI was synthesized on amino-substituted trityl resin utilizing a standard Fmoc solid-

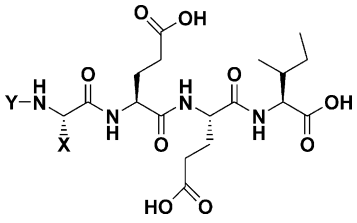
phase synthesis protocol. The N-terminus was protected with acetyl group in the presence of acetic anhydride. The completeness of the segment condensations was monitored by the Kaiser test. The phosphopeptides were cleaved from the resin and deblocked with TFA/water/anisole (95:5:5) and then purified by preparative reversed-phase HPLC to yield **22–24**.

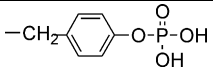
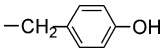
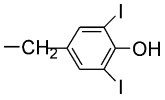
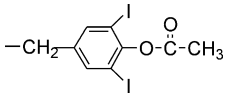
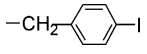
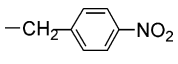
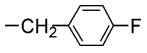
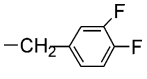
Figure 4 shows the strategy for the synthesis of *N*-substituted analogues (class IV). *N*-Boc-protected *p*-aminophenylalanine derivatized tetrapeptide **A** was synthesized according to standard Fmoc synthetic protocol using Boc-Phe(4-NHFmoc)-OH as the last amino acid. Deprotection and substitutions on amino group on solid phase with phosphorous oxychloride, dimethylsulfamoyl chloride, or diethyl bromomalonate, followed by cleavage with TFA/anisole/water (95:5:5), generated aminophosphates (phosphoramidates **26** and **27**), sulfondiamide **28**, or aminodicarboxylate (*N*-malonate **29**) derivatives, respectively.

2.2. Src SH2 binding affinity of tetrapeptide ligands

The binding affinities of the synthesized compounds, expressed as IC_{50} values (the concentration of the compound that inhibits the binding of fluorescent probe to the Src SH2 domain by 50%), are summarized in Tables 1–4 using a FP competitive binding assay.[25] Also included in Table 1 is the IC_{50} value of the reference pYEEI analogue **1**.

Table 1. Comparative Src SH2 binding affinities (IC_{50} values) for class I compounds



Compd	X	Y	IC_{50} (μM)
1		Ac	6.5
2		Ac	> 100.0
3		H	> 100.0
4		Ac	> 100.0
5		Ac	> 100.0
6		Ac	> 100.0
7		Ac	> 100.0
8		Ac	> 100.0

2.3. Class I: substituted phenyl derivatives of YEEI and FEEI (2–8)

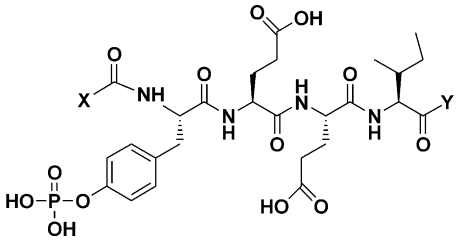
The class I compounds (Table 1) have substituted phenylalanine or tyrosine amino acid residues in place of phosphotyrosine of pYEEI (**1**) to create additional hydrogen bonding, hydrophobic, or electrostatic interactions and to avoid the negatively charged phosphate group of pTyr which is sensitive to phosphatases and blocks cell permeability. Compounds **2–4** contain a substituted tyrosine ring in the pTyr position of the peptide. Compound **1** is the tetrapeptide substrate sequence preferred by Src SH2 ($IC_{50} = 6.5$ μM) and was used as a positive control. Compound **2**, the corresponding unphosphorylated analogue, was used as the negative control. Compounds **3** and **4** are similar in possessing diiodo-substituted tyrosine phenyl rings. The N-terminus of **3** is not acetylated, while both the N-terminus and the tyrosine hydroxyl are acetylated in **4**. In other synthesized pYEEI analogues (**5–8**), the pTyr is replaced by substituted phenylalanine derivatives (Table 1).

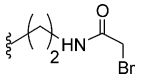
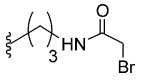
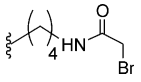
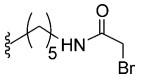
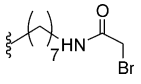
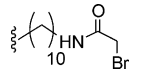
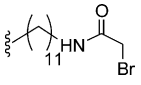
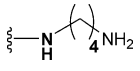
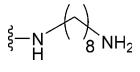
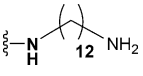
The hydrophobic nature of the iodine atom constitutes the rationale for the synthesis of tetrapeptides **3–5**. However, the bulky iodine molecules in compounds **3–5** ($IC_{50} > 100$ μM) failed to compensate for the electrostatic interaction of the phosphate with positively-charged amino acids, suggesting that the presence of the iodine may have introduced excessive steric strain or only weak hydrophobic interactions.

Tetrapeptide **6** is substituted with 4-nitrophenylalanine in the pTyr position. The strong electron-withdrawing characteristics of the nitro group, along with its partial negative charge, were expected to partially mimic the phosphate group. However, the binding affinity of this compound ($IC_{50} > 100$ μM) suggests that the partial negative charge of the *p*-nitro functionality in tetrapeptide **6** does not mimic the two negative charge of the phosphate group. Furthermore, the nitro group may not be situated properly within the binding pocket in order to interact with two positively charged arginines in the binding pocket of Src SH2 domain.

Fluorine is small and highly electronegative atom, affording substantial hydrogen-bonding possibilities within the SH2 substrate-recognition pocket. Compounds **7** and **8** contain mono- and difluoro-substituted phenylalanine in the pTyr position, respectively. The possibilities of the formation of hydrogen-bonding by the fluorine atoms with the amino acids within the P site of Src SH2 domain may afford peptides potent towards Src SH2. The fluorosubstituted tetrapeptides **7** and **8**, however, showed no significant binding affinity to the SH2 domain of Src ($IC_{50} > 100$ μM), indicating that the possible hydrogen-bonding of fluorine with amino acids within the P site of Src SH2 domain cannot compensate for the loss of electrostatic interactions observed with the phosphate group.

Table 2. Comparative Src SH2 binding affinities (IC_{50} values) for classes II and III compounds



Compd	X	Y	IC_{50} (μM)
Class II			
9		OH	4.0
10		OH	3.5
11		OH	8.6
12		OH	3.2
13		OH	4.8
14		OH	5.3
15		OH	3.7
16		OH	3.1
17		OH	2.7
18		OH	3.9
19		OH	2.5
20		OH	5.1
21		OH	7.3
Class III			
22	NHAc		35.0
23	NHAc		33.7
24	NHAc		41.0

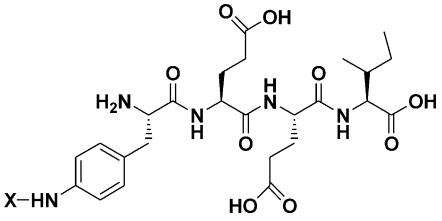
Despite the potential for some of these entities to set up bonding interactions within the binding pocket of the SH2 protein, none of the compounds showed significant binding when tested up to maximum 100 μM concentration. Therefore, the strategy to compensate the loss of negative charge by creating new interactions using substituents on aromatic group was not successful.

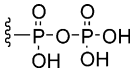
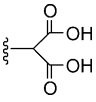
The data confirms the major contribution of the phosphate group for the high binding affinity of pYEEI to Src SH2 domain. The pTyr binding pocket of the Src SH2 domain contains four positively charged residues (Arg 12, Arg 32, His 58, and Lys 60). The most critical of these residues is Arg 32 which forms two H-bonds with the phosphate oxygens of the pTyr side chain. Arg 12 interacts with the phosphate group and the carbonyl group of the *N*-acetylated tyrosine residue.[17,22-24] Tetrapeptides **2–8** lack the phosphate functionality and therefore the SH2 domain failed to recognize these compounds as ligands ($IC_{50} > 100$ μM) since the interactions of Arg 32 and Arg 12 with the phosphate are crucial for SH2 ligand recognition.

2.4. Classes II and III: N- and C-terminal substituted pYEEI derivatives (9–24)

The classes II and III compounds are pYEEI derivatives with the pYEEI scaffold being substituted at the N- or C-terminus, respectively, with short or long chains of varying lengths. Evaluating of the N- and C-terminal substituted pYEEI allows examining the effect of the substituted chain length on the binding affinity, as well as to study of the flexibility and ligand-accomodating properties of the SH2 domain binding pocket. Table 2

Table 3. Comparative Src SH2 binding affinities (IC_{50} values) for class IV compounds



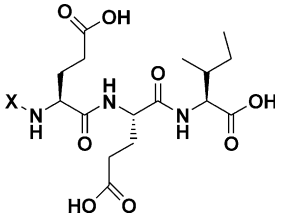
Compd	X	IC_{50} (μM)
25	H	153.0
26		16.0
27		82.1
28		> 100.0
29		37.1

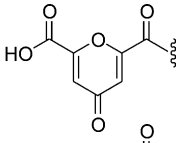
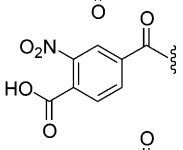
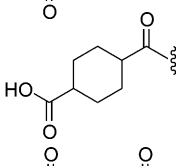
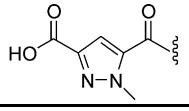
presents results from the competitive binding assay with these compounds.

Incorporation of the preferred tetrapeptide sequence should yield activity towards SH2 domain. *N*-Substitution may restrict or prevent pTyr from interacting with arginine and lysine side chains located within the P site of Src SH2 domain binding pocket. However, results of the assay with the class II show a general trend indicating that increasing the chain length of *N*-substituents does not decrease the affinity towards SH2. All of the *N*-substituted compounds (**9**–**21**) in class II bind to Src SH2 domain with IC_{50} values in the low micromolar range (IC_{50} = 2.7–8.6 μM). Long or short chain substitution on N-terminus of tetrapeptide pYEEI with aliphatic aminoacyls did not cause any significant loss in binding affinity to Src SH2 domain (IC_{50} for pYEEI is 6.5 μM). Therefore, N-terminal substitution does not significantly restrict the binding of phosphopeptides with Src SH2 domain. The results suggest the P site is capable of accommodating short and long hydrocarbon chains. The bromoacetylated *N*-substituted derivatives of pYEEI (**9**–**15**) can be used for reaction with adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) for the development of bisubstrate analogues of Src tyrosine kinases targeting the ATP- and SH2-binding sites.

The class III compounds (**22**–**24**) contain the pYEEI scaffold with substitution of short and long carbon chains on the isoleucine residue. C-Terminal substitution affords compounds that showed lower affinity towards Src SH2 domain. Compounds were found to bind Src SH2 with IC_{50} values of 33.7–41.0 μM. A substituent adjacent to isoleucine presents excessive bulk and therefore cannot be completely accommodated near the hydrophobic P + 3 site. In general, N-terminal substitution is acceptable for ligand binding, but C-terminal substitution will decrease the binding affinity.

Table 4. Comparative Src SH2 binding affinities (IC_{50} values) for class V compounds



Compd	X	IC_{50} (μM)
30		147.6
31		206.6
32		> 200.0
33		170.2

2.5. Class IV: phosphoramidate, *N*-malonate and sulfondiamide tetrapeptides (26–29)

In the search for pTyr mimetic peptides which are stable to phosphatases and may offer the potential for cell membrane penetration, the phosphotyrosine in pYEEI was replaced with *N*-substituted *p*-aminophenylalanines, resulting in class IV tetrapeptides (**26**–**29**). Tetrapeptide **25** with unsubstituted amino group was synthesized as the control. None of the compounds were acetylated on the N-terminus. Table 3 presents results from the competitive binding assay with these compounds.

Phosphoramidate tetrapeptides **26** and **27** which were synthesized to obtain stability towards phosphatases, exhibited weaker Src SH2 binding affinity (IC_{50} values of 16.0 and 82.1 μM, respectively) compared to pYEEI (IC_{50} value of 6.5 μM), possibly due to the improper position of the oxygens of aminophosphate for interaction with positively-charged arginine in the binding pocket of Src SH2 domain or different negative charge distribution by a more electronegative element oxygen in *O*-phosphate to less electronegative nitrogen in aminophosphate. This phenomenon is more evident for diphosphate derivative **27**, in which the three negatively-charged oxygens are probably out of range, not properly aligned to interact with positively-charged amino acids within P site of Src SH2 domain, or have disrupted charge–charge interactions. Sulfondiamide tetrapeptide **28** did not show any binding affinity at 100 μM since the recognition of ligands by the Src SH2 domain critically depends on strong polar interactions by the highly charged groups, which are absent in compound **28**, with positively charged amino acids within the P site of the binding pocket.

N-Malonyl substitution may offer an alternative approach to cell delivery due to stability of carboxylic acid groups to phosphatases. The carboxylate-based tetrapeptide **29** was designed to afford structural alternatives to phosphorous-containing pTyr mimetics. Retention of two carboxyl groups was deemed necessary in order to provide a dianionic 'phosphate pharmacophore' that could interact with two critical arginines within the pTyr binding pocket. The reduced binding potency of *N*-malonate (IC_{50} = 37.1 μM) could potentially indicate that interactions of the two carboxyls with two arginines within the pTyr binding pocket do not optimally approximate that provided by the phosphorous-containing parent structure pYEEI (**1**).

The results from the binding affinity studies of compounds from classes I–III agree with the earlier crystallographic studies,[22–24] indicating that charge–charge and hydrophobic interactions of phosphotyrosine and isoleucine residues, respectively, with SH2 are critical for binding.

Tetrapeptides **25–27** lack an acetyl group on N-terminus that may explain their weaker binding affinity. The interaction of the carbonyl group of *N*-acetyl in acetylated pYEEI with the side chain of Arg 12, which is absent in tetrapeptides **25–27**, contributes significantly to the binding.[22–24]

2.6. Class V: carboxylated derivatives of XEEI (30–33)

The class V compounds are a small number of tetrapeptides XEEI, where X is a carboxylated aromatic, heteroaromatic, or cyclic group. Results of the binding assay with these compounds are presented in Table 4.

Two key features of the chelidonic acid compound (**30**) — the carboxyl and carbonyl groups — both failed to render the molecule as a ligand for Src SH2 (IC_{50} = 147.6 μM). Compound **31**, with the 5-nitroisophthalic acid functionality, contains both carboxyl and nitro groups. The negative charges associated with this peptide do not mimic the phosphate group, resulting in low activity as measured by the FP assay (IC_{50} = 207 μM). Compound **32** also contains a carboxyl group capable of charge–charge interactions, but failed to compete with the fluorescent probe (IC_{50} > 200 μM). This could be, in part, due to incorporation of cyclohexyl group into the molecule, which might restrict the possible interaction of arginines in the binding pocket of SH2 domain with the carboxyl moiety. Furthermore, the hydrophobic interaction between the side chain of Lys 60 and aromatic phenyl is lost in **32**. Compound **33** where the pTyr is replaced by the 3,5-pyrazole dicarboxyl moiety (IC_{50} = 170.2 μM), showed no significant improvement in the binding affinity towards the SH2 domain. These results indicate the importance of phosphate and aromatic phenyl groups in binding with positively charged amino acids in the binding pocket of Src SH2 domain. Carboxylic acid and nitro groups failed to produce sufficient electrostatic interactions with positively-charged amino acids in Src SH2 domain.

2.7. Cell proliferation assay

The ability of pYEEI and several of the compounds to inhibit the growth of cancer cells were assessed in a cell-based proliferation assay in human embryonic kidney (HEK) 293 tumor cells (Fig. 5). The antiproliferative activities detected were correlated with kinase binding affinity studies. Compounds **5**, **6**, and **8** which did not show any significant binding affinity to Src SH2 domain, were not inhibitory on cell proliferation. Compounds **9**, **10**, **12–16** showed antiproliferative activities at the 50–200 μM range. pYEEI (**1**), used as the control, significantly inhibited the tumor cell proliferation at 50 μM. No conclusion can be made that the antiproliferative activity of these compounds are due to the inhibition of Src SH2 domain of PTKs and more experiments are needed to understand the mechanism of the antiproliferative activities of these compounds. The lowered activity of pYEEI derivatives compared to pYEEI could be due to higher lipophilicity of these compounds and their integration into the cell membrane of cancer cells.

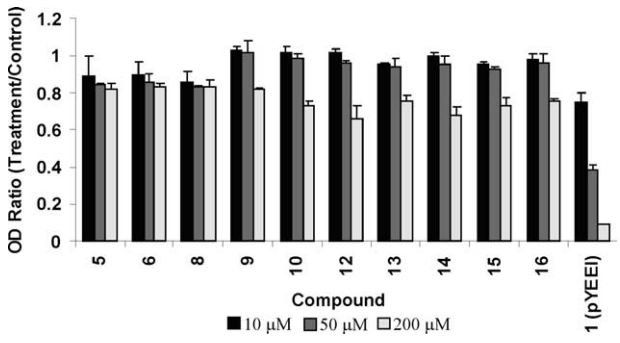


Figure 5. Cell proliferation assay for tetrapeptides and pYEEI in human embryonic kidney (HEK) 293 tumor cells.

The two negative charges of pTyr at physiological pH can in principle impair cell membrane penetration. In spite of having negatively charged phosphate which may have hindered the penetration of these compounds (**1**, **9**, **10**, **12–16**) into cells and susceptibility to cleavage by phosphatases in the cell culture, the antiproliferative activities of these compounds were significant compared to compounds with no phosphate group (**5**, **6**, **8**).

2.8. Antifungal activity

The antifungal activities of several compounds were examined against *Candida albicans*. None of compounds showed any significant antifungal activity against *C. albicans* ATCC 14053 at the maximum concentration of 10 μM. The observed lack of activity is probably due to the lack of penetration into fungal cell as result of existing cell wall and membrane that can make a more stringent than normal barrier for such compounds.

3. Conclusion

The participation of tyrosine kinases in both normal and aberrant signal transduction pathways renders them attractive targets for drug discovery efforts. The specificity of SH2 domains for phosphotyrosine-containing motifs provides a foundation for rational design of PTK inhibitors. This research focused on the synthesis and analysis of phosphotyrosyl mimicking peptides by exploring hydrophobicity, electronegativity, and charge-charge interactions. Long and short chain substitution on N-terminus of pYEEI with aliphatic alkyl derivatives did not cause any significant loss in binding affinity to Src SH2 domain. Therefore, the Src SH2 domain can tolerate substantial steric bulkiness in the N-terminus of pYEEI. These studies allow a richer understanding of the ligand selectivity of the Src SH2 domain and should enhance our knowledge for the design of peptidomimetic tyrosine kinase inhibitors.

4. Experimental

4.1. Chemistry

4.1.1. Peptide Synthesis. In general, all peptides (**1–33**) were synthesized by the solid-phase peptide synthesis

strategy on a PS3 automated peptide synthesizer (Rainin Instrument Co., Inc.) employing *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) based chemistry. HBTU and NMM (0.4 M) in *N,N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively. Fmoc-Ile Wang resin, amino substituted trityl resin, and Fmoc-protected amino acids were obtained from NovaBiochem Company. N-terminal acetylation was accomplished using acetic anhydride. Fmoc deprotection at each step was carried out using 20% piperidine/DMF. A mixture of TFA (90%), anisole (5%), water (5%) was used for side-chain deprotection of amino acids and cleavage of the synthesized peptides from the resin. Crude peptide were precipitated by addition of cold diethyl ether (Et_2O) and purified by preparative HPLC on a Phenomenex® Prodigy 10u ODS reversed-phase column. Separation of peptides was achieved by eluting the crude peptide at 4.0 mL/min using a gradient of 0–100% acetonitrile (ACN; 0.1% TFA) and water (0.1% TFA) over 85 min to yield highly pure compounds (97–99%), which were lyophilized. The purity of final products was confirmed by analytical HPLC on a Shimadzu® 3 μ C-18 column at 0.5 mL/min using the same gradient system. The chemical structures of compounds were confirmed using high resolution PE Biosystems API 2000 electrospray mass spectrometer and amino acid analysis.

The fluorescent probe consists of 5-carboxyfluorescein coupled to a pentapeptide (fluorescein-Gly-pTyr-Glu-Glu-Ile-NH_2). The synthesis, purification, and characterization of the probe were carried out according to previously reported procedures.[25,26]

4.1.2. Expression and purification of Src SH2 domain. The Src SH2 domain was expressed and purified as described previously.[22,27] The protein used was a glutathione-*S*-transferase (GST) fusion protein. The Src SH2 domain was released from the GST affinity column by the addition of 5 mM reduced glutathione (0.1% β-mercaptoethanol) and detected by 5× Bradford reagent. The concentration of protein was determined by reading optical density employing a Pharmacia Biotech Ultrospec 2000 UV/VIS spectrophotometer using bovine serum albumin (BSA, 1.0 mg/mL) as a standard.

4.1.3. Src SH2 domain binding assay. The binding affinities of these ligands for the Src SH2 domain were measured using a competitive FP assay as previously described.[25] The excitation and emission wavelengths were set at 485 and 535 nm, respectively. The reported IC_{50} values are the mean of three separate determinations with a standard deviation of less than ±20%. The concentration of probe and SH2 domain was kept constant (80 and 750 nM, respectively) while the concentration of peptide was varied.

4.1.4. Cell proliferation assay. The cytotoxicity of the test compounds was evaluated based on their effect on cell proliferation. Cells (293T) were seeded in 96-well plates (5000/well) and cultured for 24 h. The cells were then treated with a tetrapeptide at various concentrations (10–200 μM) or the same amount of the vehicle. The treated cells were incubated for additional 12 h and the proliferation rates were determined with a CellTiter kit as described by the manufacturer (Promega). To precisely determine the effect of tetrapeptide on cell proliferation, control plates were seeded and cultured similarly but the absorbance ($l = 570$ nm) was determined at the time right before tetrapeptides were added to the experimental plates. The experiments were performed in quadruplicate and the absorbance from the control plates was subtracted. Data are expressed as a percentage of cells treated with vehicle and presented as means ± SD.

4.2. Antifungal assay

4.2.1. Microorganism. *C. albicans* ATCC 14053 was obtained from American Type Culture Collection, Manassas, VA, USA. Stock cultures were kept on Sabouraud dextrose agar (SDA; Becton-Dickinson and Co., Sparks, MD, USA). Subcultures were prepared on SDA at 35–37 °C. Suspension cultures were prepared by inoculation of single colonies in 7 mL of normal saline solution. Prior to preparation of susceptibility assays, yeast cells were resuspended in normal saline to make a transmittance of 73–75% at 530 nm that provide equivalent concentration of 10^6 cells/mL to produce a similar transmittance at 530 nm compared to the control tube. RPMI (RPMI 1640; ICN Biomedicals, Aurora, OH, USA) adjusted to pH 6.9 was used as the medium.

4.2.2. Susceptibility testing. Stock solutions (15.8 and 17.5 mg/mL, respectively) were in DMSO and stored at 0 °C temperature; the dilutions were prepared in RPMI medium. The final maximum concentration of DMSO in the assays was 2.5% (v/v). Test compounds were dissolved in DMSO (0.563 mg/mL) and working dilutions were made in RPMI or SDB media. DMSO was not inhibitory to the organisms tested. Microdilutions for control experiments with *C. albicans* was the modified method according to the previously described procedures.[28,29] Dilutions were prepared in 0.1 mL of RPMI; the inocula were 10^4 *C. albicans* cells. The tubes were incubated for 24–48 h at 36 ± 1 °C, and turbidity was read visually. MICs were calculated in comparison to growth control as the lowest concentration that shows inhibition (MIC) for amphotericin B and the test compounds.

Acknowledgements

N. H. Nam and R. L. Pitts contributed equally. We acknowledge the financial support from National Center for Research Resources, NIH, Grant number 1 P20 RR16457.

References and notes

1. Schwartzberg, P. L. *Oncogene* **1998**, *17*, 1463.
2. Brown, M. T.; Cooper, J. A. *Biochim. Biophys. Acta* **1996**, *1287*, 121.
3. Lowe, C.; Yoneda, T.; Boyce, B. F.; Chen, H.; Mundy, G. R.; Soriano, P. *Proc. Nat. Acad. Sci. U.S.A.* **1993**, *90*, 4485.

4. Botfield, M. C.; Green, J. *Annu. Rep. Med. Chem.* **1995**, *30*, 227.
5. Weber, T. K.; Steele, G.; Summerhayes, I. C. *J. Clin. Invest.* **1992**, *90*, 372.
6. Ottenholf-Kalff, A. E.; Rijksen, G.; van Beurden, E. A.; Hennipman, A.; Michels, A. A.; Staal, G. E. *Cancer Res.* **1992**, *49*, 4773.
7. Biscardi, J. S.; Tice, D. A.; Parsons, S. J. *Cancer Res.* **1999**, *76*, 61.
8. Avizienyte, E.; Wyke, A. W.; Jones, R. J.; McLean, G. W.; Westhoff, M. A.; Brunton, V. G.; Frame, M. C. *Nat. Cell Biol.* **2002**, *4*, 632.
9. Irby, R. B.; Yeatman, T. J. *Oncogene* **2000**, *19*, 5636.
10. Sadowski, I.; Stone, J. C.; Pawson, T. *Mol. Cell. Biol.* **1986**, *6*, 4396.
11. Cantley, L. C.; Auger, K. R.; Carpenter, C.; Duckworth, B.; Graziana, A.; Kapeller, R.; Soltoff, S. *Cell* **1991**, *64*, 281.
12. Songyang, Z.; Shoelson, S. E.; Chaudhuri, M.; Gish, G.; Pawson, T.; Haser, W. G.; King, F.; Roberts, T.; Ratnofsky, S.; Lechleider, R. J.; Neel, B. G.; Birge, R. B.; Fajardo, J. E.; Chou, M. M.; Hanafusa, H.; Schaffhausen, B.; Cantley, L. C. *Cell* **1993**, *72*, 767.
13. Fukui, Y.; Hanafusa, H. *Mol. Cell. Biol.* **1991**, *11*, 871.
14. Schaller, M. D.; Hilderbrand, J. D.; Shannon, J. D.; Fox, J. W.; Vines, R. R.; Parsons, J. T. *Mol. Cell. Biol.* **1994**, *14*, 1680.
15. Taylor, S. J.; Shalloway, D. *Nature* **1994**, *14*, 1680.
16. Petch, L. A.; Bockholt, S. M.; Bouton, A.; Parsons, J. T.; Burridge, K. *J. Cell. Sci.* **1995**, *108*, 1371.
17. Sawyer, T. K. *Biopolymers* **1998**, *47*, 243.
18. Vu, C. B.; Corpuz, E. G.; Merry, T. J.; Pradeepan, S. G.; Bartlett, C.; Bohacek, R. S.; Botfield, M. C.; Eyermann, C. J.; Lynch, B. A.; MacNeil, I. A.; Ram, M. K.; van Schravendijk, M. R.; Violette, S.; Sawyer, T. K. *J. Med. Chem.* **1999**, *42*, 4088.
19. Vu, C. B.; Corpuz, E. G.; Pradeepan, S. G.; Violette, S.; Bartlett, C.; Sawyer, T. K. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3009.
20. Muller, G. *Topics Curr. Chem.* **2000**, *211*, 17.
21. Cody, W. L.; Lin, Z.; Panek, R. L.; Rose, D. W.; Rubin, J. R. *Curr. Pharm. Des.* **2000**, *6*, 59.
22. Waksman, G.; Kominos, D.; Robertson, S. C.; Pant, N.; Baltimore, D.; Birge, R. B.; Cowburn, D.; Hanafusa, H.; Mayer, B. J.; Overduin, M.; Resh, M. D.; Rios, C. B.; Silverman, L.; Kuriyan, J. *Nature* **1992**, *358*, 646.
23. Waksman, G.; Shoelson, S. E.; Pant, N.; Cowburn, D.; Kuriyan, J. *Cell* **1993**, *72*, 779.
24. Eck, M. J.; Shoelson, S. E.; Harrison, S. C. *Nature* **1993**, *362*, 87.
25. Lynch, B. A.; Loiacono, K. A.; Tiong, C. L.; Adams, S. E.; MacNeil, I. A. *Anal. Biochem.* **1997**, *247*, 77.
26. Adamczyk, M.; Fishpaugh, J. R.; Heuser, K. J. *Bioconjugate Chem.* **1997**, *8*, 253.
27. Bradshaw, J. M.; Grucza, R. A.; Ladbury, J. E.; Waksman, G. *Biochemistry* **1998**, *37*, 9083.
28. Galgiani, J. N. *Antimicrob. Agents Chemother.* **1993**, *37*, 2517.
29. Fernandez-Torres, B. B.; Carrillo, A. J.; Martin, E.; Del Palacio, A.; Moore, M. K.; Valverde, A.; Serrano, M.; Guarro, J. *Antimicrob. Agents Chemother.* **2001**, *45*, 2524.