Potent Pseudosubstrate-based Peptide Inhibitors for p60<sup>c-src</sup> Protein Tyrosine Kinase

Qiang Lou, Margaret E. Leftwich, R. Trent McKay, Sydney E. Salmon, Lenka Rychetsky, and Kit S. Lam

Department of Medicine, Arizona Cancer Center (Q. L, M. E. L, R. T. M., S. E. S., L. R., K. S. L.) and Department of Microbiology and Immunology (K. S. L.), University of Arizona College of Medicine, Tucson, Arizona 85724

ABSTRACT

We recently reported the identification of GIYWHHY as an efficient and specific substrate for p60<sup>c-src</sup> protein tyrosine kinase (PTK) by screening a secondary random peptide library (Q. Lou et al., Bioorg. Med. Chem., 4: 677-682, 1996). Based on the primary structure of GIYWHHY, we designed and synthesized several pseudosubstrate-based peptide inhibitors. Some of these peptide inhibitors are highly potent and specific with IC<sub>50</sub> in the low micromolar range. Because both YIYGSFK and GIYWHHY are efficient and specific substrates for p60<sup>c-src</sup> PTK, chimeric branched peptides based on these two sequences were synthesized. These branched peptides inhibit p60<sup>c-src</sup> PTK with high potency, indicating that the enzyme-active site of p60<sup>c-src</sup> PTK can accommodate more than a linear motif. This may explain why seemingly several peptides with very different linear structures can all be phosphorylated by this enzyme.

INTRODUCTION

PTKs mediate important signaling events associated with cellular growth, differentiation, and mitogenesis (1, 2). Overactivation or mutation of PTKs may result in malignant transformation. Examples include amplification of c-erb-B-2 in human breast cancer (3), chromosomal translocation of c-abl in chronic myelogenous leukemia (4) and Ph<sup>+</sup>-positive acute lymphocytic leukemia (5), overexpression of the epidermal growth factor receptor in squamous cell carcinoma (6), and activation of p60<sup>c-src</sup> PTK in colon (7), breast (8), and bladder carcinoma (9). Therefore, PTKs have been considered as important targets for the design of antiproliferative drugs (10, 11). Specific and potent tyrosine kinase inhibitors not only represent a new class of antitumor agents but may also be used as a tool for studying the role of PTK-dependent cellular pathways in normal or tumor cell growth. Several PTK inhibitors have been developed by screening natural products (12) and through synthetic chemistry (13), but it was not until very recently that some potent and more specific PTK inhibitors have been reported (11, 14-23). The biology and biochemistry of p60<sup>c-src</sup> PTK have been recently reviewed (24).

In spite of the great interest in PTK as a drug target (12, 23), there has been a lack of information on the substrate specificities of these enzymes. However, recently we and others were able to identify efficient substrates for PTKs using combinatorial peptide library methods (25, 26). Songyang et al. (26) synthesized a biased 15-mer peptide library, MAXXXXYXXXXAKKK (wherein X = 15 eukaryotic amino acids, excluding Cys, Trp, Tyr, Ser, and Thr), and phosphorylated this solution phase library with p60<sup>c-src</sup> PTK. They then used a ferric chelation column to isolate a collection of phosphopeptides. Microsequencing of this peptide mixture led to the conclusion that EEIYGEFF was the predominant substrate motif for p60<sup>c-src</sup> PTK. Using a totally different approach, the “one-bead-one-compound” combinatorial peptide library method (27, 28), we identified YIYGSFK as an efficient and specific substrate for p60<sup>c-src</sup> PTK (25). In this method, we incubated a random heptapeptide-bead library with [<sup>32</sup>P]ATP and p60<sup>c-src</sup> PTK. The 32P-labeled beads were identified by autoradiography and isolated for microsequencing.

Detailed structure-activity relationship study of YIYGSFK led us to conclude that -Ile-Tyr- were the two critical residues as a peptide substrate for p60<sup>c-src</sup> PTK (29). Based on this dipeptide motif, a secondary peptide library (approximately 1 million beads), one of the identified peptides, GIYWHHY, was found to be a more efficient substrate for p60<sup>c-src</sup> PTK than the parental compound YIYGSFK (30). In the cyclic AMP-dependent protein kinase system, potent and specific pseudosubstrate-based inhibitors have been developed by replacing the phosphorylated seryl residue with Ala (31). In this article, we report on the development and characterization of several potent and highly selective pseudosubstrate-based peptide inhibitors for p60<sup>c-src</sup> PTK using GIYWHHY as the template.

MATERIALS AND METHODS

Materials. Human p60<sup>c-src</sup> PTK, Lyn PTK, and Lck PTK were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). [γ<sup>32</sup>P]ATP was purchased from ICN Biomedicals, Inc. (Irvine, CA). Fmoc-2'-Nal and Fmoc-Na-Tyr were purchased from Advanced ChemTech (Louisville, KY). Fmoc-α-methoxybenzyl and Fmoc-2'-naphthylalanine were obtained from BioMol Research Laboratories (Strasbourg, France). Standard Fmoc-protected amino acids, Boc-protected amino acids, TFA, piperidine, BOP, HOBr, DIEA, and Rink resin were purchased from Advanced ChemTech. DMF, methyl t-butyl ether and acetonitrile were purchased from Baker (McGaw Park, IL). Fmoc-L-lys(aloc)-OH was obtained from Millipore Co. (Bedford, MA). DMF, NMM, and Tetrais(triphenylphosphine)-palladium was purchased from Aldrich Chemical Co. (Milwaukee, WI). Anion exchange TLC plate (Cellulose MN 300 polyethyleneimine-impregnated, catalogue no. 801053) was purchased from Fisher Scientific. [γ-<sup>32</sup>P]ATP was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Phosphoric acid was purchased from Fisher Scientific (Fair Lawn, NJ).

Linear Peptide Synthesis. All carboxyl amide peptides were synthesized with Fmoc chemistry (32, 33) and Rink resin (0.3 mmol/g). Fmoc-protected amino acids and the coupling reagents BOP, HOBt, and DIEA were added to the resin by Fmoc chemistry (32, 33) and Rink resin (0.3 mmol/g). Fmoc-protected amino acids and the coupling reagents BOP, HOBr, and DIEA were added to the resin in 1:3 molar excess. Average coupling time at room temperature was 1 h. Completion of coupling was confirmed with the ninhydrin test. The Fmoc group was deprotected with 20% piperidine (v/v) in DMF (one treatment for 5 mm followed by another one for 15 min). The completed peptides were cleaved from the resin by TFA/ethanediol (95:5 v/v) for 2 h at room temperature. The cleaved peptides were precipitated by methyl t-butyl ether, washed, and lyophilized. The crude peptides were then purified by reversed-phase high-performance liquid chromatography (protein and peptide C<sub>18</sub> column, Vydac). The purity of the peptides was assessed by analytical reversed-phase high-performance liquid chromatography and was determined.
to be >98% pure. Mass spectrometry analysis was used to confirm the molecular weights of the desired peptides.

**Branched Peptide Synthesis.** Synthesis of the branched peptides was performed by Fmoc chemistry as described above except that Fmoc-L-Lys(alk-loc)-OH was used at the branched point and NH$_2$-terminal amino acids (Tyr and lle) were Boc protected. After completion of the synthesis of the main structure, the alloc group of the lysine was removed from Fmoc-L-Lys(alloc)-OH by adding 6.5 ml of DMF/acetonitrile/NMM (10:2:1, v:v:v) with N$_2$ bubbling for 15 min, followed by adding 150 mg of tetraethylphosphonium-palladium/0.1 g of resin with N$_2$ bubbling for 5 min, then shaking for about 4 h. The resin was then washed with DMF (10×), DCM (10×), and DMF (10×). After the synthesis of the branched structure was completed, the resin was treated with TFA/ethanedithiol (95:5, v/v) for 2 h to remove the side chain and NH$_2$-terminal protecting groups and to cleave the completed peptides from the resin. The cleaved peptides were precipitated by methanol-isobutyl ether, washed, lyophilized, and purified as described above.

**Phosphorylation Assays.** All of the phosphorylation assays were performed at 25°C in a final volume of 20 μl of MES buffer (30 mM MES, 10 mM magnesium chloride, and 0.4 mg/ml BSA, pH 6.8) containing a specific PTK, 10 μM [γ-$^{32}$P]ATP (specific activity, 25 Ci/mmol), a specific peptide substrate with or without an inhibitor. The concentrations of PTKs used were as follows: 30 units/ml for p60$^{5c}$ PTK (900,000 units/mg), 90 units/ml for Lyn (6157 units/mg), and 90 units/ml for Lck (2500 units/mg). The reactions were initiated with the addition of a PTK. Reactions were allowed to proceed for 10 min and were then stopped with 20 μl of 150 mM phosphoric acid. The terminated reaction mixtures (5 μl) were then spotted onto the anion exchange TLC plates which had previously been soaked with 0.5 M NaCl for about 5 mm and rinsed with double-distilled water. The loaded TLC plates were air dried for about 10 min. Chromatography was performed using 15 mM phosphoric acid and 0.25 M NaCl as the mobile phase for about 15 min at room temperature. The plates were exposed to the storage phosphor screen for 1 h at room temperature. The exposed screen was read by the 425S Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and the spots corresponding to the phosphopeptides were quantitated. For the background correction, [γ-$^{32}$P]ATP and a PTK were mixed together in the absence of any peptide substrate and incubated under the same conditions as described above.

**Data Analysis.** The Michaelis constants ($K_m$) for peptides were estimated using the GraFit computer program (Erithacus Software Ltd., Staines, United Kingdom). The IC$_{50}$ and $K_m$ values were also estimated by a nonlinear regression data analysis program of GraFit.

**RESULTS**

Using a solution phase phosphorylation assay, the $K_m$ values of four known peptide substrates were determined (Table 1). Of the four peptides tested, GIYWHHY has the lowest $K_m$ (21 μM). This peptide was therefore chosen as the template to develop pseudosubstrate-based peptide inhibitor for p60$^{5c}$ PTK.

From our previous structure-activity relationship studies on GIYWHHY, we determined that Tyr$^3$ was the phosphorylation site and Tyr$^7$ was not critical for its activity as a substrate for p60$^{5c}$ PTK (30). A series of peptide analogues of GIYWHHY were synthesized and their half inhibition concentrations (IC$_{50}$) were determined using GIYWHHY as a substrate (Table 2). In these pseudosubstrate-based peptide analogues, Tyr$^3$ and in some cases Tyr$^7$ were substituted with another aromatic amino acids such as (L and D) 2'-naphthylalanine, aminobenzoic acid, or D-Tyr. Fig. 1 showed the representative inhibition curves of three selected inhibitors for p60$^{5c}$ PTK. The best three inhibitors from this series were GI(2'-Nal)WHH(2'-Nal) (C), GI(2'-Nal)WHHY (•), and GI(2'-Nal)WHH (○). The reactions were carried out in a final volume of 20 μl containing the assay buffer, 0.6 units of p60$^{5c}$ PTK, 55 μg (equal to the $K_m$) of GIYWHHY, and 20 μM GI(2'-Nal)WHH in the presence and absence of an inhibitor. The reactions were allowed to proceed for 10 min and were stopped with 20 μl of 150 mM phosphoric acid. The phosphorylation of GIYGSFK by p60$^{5c}$ PTK in the absence of any inhibitors was used as a control (100%).

![Fig. 1. Inhibition of p60$^{5c}$ PTK activity by three selected inhibitors derived from the structure of GIYWHHY. GI(2'-Nal)WHH(2'-Nal) (C), GI(2'-Nal)WHHY (•), and GI(2'-Nal)WHH (○). The reactions were carried out in a final volume of 20 μl containing the assay buffer, 0.6 units of p60$^{5c}$ PTK, 55 μg (equal to the $K_m$) of GIYWHHY, and 20 μM GI(2'-Nal)WHH at concentrations of 1, 2.5, 5, 10, and 20 μM. The reactions were allowed to proceed for 10 min and were stopped with 20 μl of 150 mM phosphoric acid. The phosphorylation of GIYGSFK by p60$^{5c}$ PTK in the absence of any inhibitors was used as a control (100%).](image-url)

**Table 1** Kinetic studies of peptide phosphorylation by p60$^{5c}$ PTK

<table>
<thead>
<tr>
<th>Peptide (carboxylamide)</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIYWHHY$^{ab}$</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>EIYEEYE$^{ab}$</td>
<td>448 ± 20</td>
</tr>
<tr>
<td>YIYGSFK</td>
<td>55 ± 18</td>
</tr>
<tr>
<td>EEYGEFF$^{ab}$</td>
<td>45 ± 8</td>
</tr>
</tbody>
</table>

$^{ab}$ GIYWHHY and EIYEEYE were identified by Lou et al. (30) as substrates for p60$^{5c}$ PTK using a one-bead, one-combinatorial peptide library (XIXX...XX). $^{ab}$ EEYGEFF was identified by Songyang et al. (26) as a predominant substrate motif for p60$^{5c}$ PTK using a different combinatorial peptide library (MAXXXXYXXX-AKKK) approach. See text for detail.

**Table 2** Pseudosubstrate-based peptide inhibitors for p60$^{5c}$ PTK derived from the structures of GIYWHHY

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$ (μM)$^{ab}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI(2'-Nal)WHH(2'-Nal)</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>GI(2'-Nal)WHH(2'-Nal)</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>GI(2'-Nal)WHH(2'-Nal)</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>GI(2'-Nal)WHHY</td>
<td>7.0 ± 0.7</td>
</tr>
<tr>
<td>GI(2'-Nal)WHH</td>
<td>16 ± 0.9</td>
</tr>
<tr>
<td>GI(2'-Nal)WHH</td>
<td>23 ± 1.3</td>
</tr>
<tr>
<td>GI(2'-Nal)WHH</td>
<td>26 ± 1.4</td>
</tr>
<tr>
<td>GI(2'-Nal)WHH</td>
<td>26 ± 1.9</td>
</tr>
<tr>
<td>GlyWHH</td>
<td>50 ± 2.3</td>
</tr>
<tr>
<td>AcGI(2'-Nal)WHH</td>
<td>63 ± 8.3</td>
</tr>
<tr>
<td>L(2'-Nal)WHH</td>
<td>76 ± 9.4</td>
</tr>
<tr>
<td>GI(2'-Nal)W</td>
<td>108 ± 18.7</td>
</tr>
<tr>
<td>I(2'-Nal)WH</td>
<td>127 ± 21.7</td>
</tr>
<tr>
<td>GI(2'-Nal)WH</td>
<td>130 ± 17.7</td>
</tr>
</tbody>
</table>

$^{ab}$ The values represent the mean of two to four independent experiments. The substrate used in these experiments was YIYGSFK at a concentration of 55 μM.

$^{ab}$ Aab, aminobenzoic acid.
YIYGAFK was used as the substrate. The double-reciprocal plot for inhibition by these two inhibitors shows straight lines intersecting off the Y axis but on the X axis (intersection of the lines at the 1/v = 0, and I/[S] < 0), consistent with noncompetitive inhibition. The Kₐ values for GI(2'-Nal)WHHY and GI(2'-Nal)WHH were 9 and 12 μM, respectively.

p60⁴⁻Src PTK can phosphorylate all three peptides, YIYGAFK, YIYGKFK, and EEIYGEFF, and EEIYGEFF rather efficiently. With the exception of Ile² and Tyr³, there are considerable differences in the remaining residues. This suggests that the p60⁴⁻Src PTK binding pocket may be able to accommodate more than a linear peptide. We therefore designed and synthesized four chimeric branched peptides and tested their inhibitory activity toward p60⁴⁻Src PTK using YIYGAFK as a substrate. The result is shown in Table 4. All four branched peptides strongly inhibited p60⁴⁻Src PTK. The best inhibitor was YI(2'-Nal)-GK(N⁵-HHW)FK with an IC₅₀ value of 0.6 μM. Although Tyr³ is present in YIYGK(N⁵-HHW)FK, this peptide was not phosphorylated by p60⁴⁻Src PTK. Instead, it was an inhibitory peptide with an IC₅₀ value of 2.2 μM (Table 4).

We also examined the inhibitory effect of GI(2'-Nal)WHHY toward Src family PTKs. Lyn and Lck PTKs were selected as representatives of Src family PTK because of their structural homology to Src kinase (1, 35). In these experiments, two peptides, YIYGAFK and cdc2(6-20) peptide (KEKIGEGTYGVVYK), were used as the substrate for these PTKs. The IC₅₀ value of GI(2'-Nal)WHHY for p60⁴⁻Src PTK was 27 μM when YIYGAFK was used as a substrate and 32 μM when cdc2(6-20) peptide was used as a substrate (Table 5). In contrast, no inhibition by GI(2'-Nal)WHHY (up to 800 μM) was found for other Src family PTKs such as Lyn and Lck kinases.

![Image](https://example.com/image.png)
DISCUSSION

There has been great interest in using PTK as a target for drug development (10, 11). It was estimated that there are at least 150 PTKs in eukaryotic cells (35), but there is very little information on the substrate specificities of these enzymes. It was not until recently that we (25, 30) and others (26) were able to identify efficient peptide substrates for PTKs using combinatorial peptide library approaches. We used the “one-bead, one-peptide” combinatorial peptide library method (27) and solid-phase phosphorylation approach (28) to screen a completely random heptapeptide library XXXXXX (wherein X = all 19 eukaryotic amino acids except Cys) and identified YIYGSFK as a relatively efficient and specific substrate for p60Src PTK (25). Using YIYGSFK as a template, we developed several pseudosubstrate inhibitors with moderate potency (36). Recently, we reported on the optimization of the initial lead YIYGSFK by screening a secondary one-bead, one-peptide combinatorial peptide library based on the dipeptide motif (XIXXXX, wherein X = all 19 eukaryotic amino acids except Cys). One of the identified peptides, GIYWHHY, was found to be more efficient and specific for p60Src PTK (30). Songyang et al. (26) used a completely different combinatorial peptide library approach. They synthesized a biased solution phase 15-mer peptide library, MAXXXXYXXXXAKKKK (wherein X = all 15 eukaryotic amino acids except Cys, Trp, Tyr, Ser, and Thr). This peptide library was then phosphorylated by p60Src PTK, and the phosphorylated peptides were isolated by a metal chelation column. The eluted peptides were then subjected to microsequencing collectively, and EEIYGEFF was determined to be the predominant motif.

The Michaelis constant ($K_m$) of a substrate reflects its steady-state binding affinity to the enzyme-active site. It is therefore important to determine the $K_m$’s of various peptide substrates prior to the design of pseudosubstrate-based peptide inhibitors. Table 1 shows that GIYWHHY has the lowest $K_m$ value (21 μM). Therefore, it was chosen as the template to develop pseudosubstrate peptide inhibitors for p60Src PTK. The peptide motif determined by Songyang et al. (26), EEIYGEFF, is the system’s $K_m$ value of 45 μM. This is very similar to the value of 33 μM determined by Songyang et al. (26) using AEEEIYGEFEAKKKK as a substrate.

Recently, we designed and synthesized a series of pseudosubstrate inhibitors based on the YIYGSFK template and found that replacement of Tyr4 with 2′-Nal will generate a potent pseudosubstrate inhibitor. However, to our surprise, both EEI(2′-Nal)GEFF and EEIYGEFF were very poor inhibitors for p60Src PTK whether YIYGSFK or EEIYGEFF was used as the substrate (Table 3). This suggests that EEIYGEFF, GIYWHHY, or YIYGSFK interacts very differently with the enzyme, although they are all good substrates for p60Src PTK.

Inhibition of p60Src PTK activity by YI(2′-Nal)GSFK, as expected, is competitive with respect to YIYGSFK, with a $K_i$ of 24 μM (36). The same kinetic experiments are performed on GI(2′-Nal)-WHHY and GI(2′-Nal)WHH using YIYGSFK as a substrate. The results (Fig. 2) clearly demonstrate that these two inhibitors inhibit p60Src PTK noncompetitively with respect to YIYGSFK, with $K_i$ values of 9 and 12 μM, suggesting that GIYWHHY and YIYGSFK may bind to a different but perhaps overlapping site. On the basis of these results, we hypothesized that a hybrid peptide based on these two motifs may be more potent as an inhibitor for p60Src PTK. To test this hypothesis, we synthesized four branched chimeric peptides by combining portions of these two motifs together (Table 4). These four branched peptides proved to be more potent than their linear counterparts with an $IC_{50}$ value as low as 0.6 μM. The fact that these larger branched structures are more potent inhibitors indicates that the enzyme-active site is relatively big and can accommodate more than one linear sequence. This may explain why seemingly several peptides with very different linear structures can all be phosphorylated by the same enzyme. Although Tyr4 is present in YIYGK(N′-HHW)FK, it is not clear why this peptide could not be phosphorylated by p60Src PTK (Table 4). Instead, it was a rather potent inhibitor with an $IC_{50}$ value of 2.2 μM.

Selectivity may be the major advantage of pseudosubstrate-based peptide inhibitors because they compete at the peptide substrates binding pocket rather than the conserved ATP binding pocket. In this study, we decided to use YIYGAKF as an alternative substrate to evaluate the selectivity of our inhibitors among Src family PTKs. It is because both Lyn and Lck kinase preparations purified from cell membrane do contain some contaminating Ser kinases. Replacement of Ser with Ala will exclude the possibility of phosphorylation by a Ser kinase. We have previously shown that this replacement did not change the level of phosphorylation by p60Src PTK (29, 36). The cdc2(6-20) peptide KVEKIGETTYGVVVK, derived from p34cdc2, has been reported to be a specific and efficient substrate for the Src family PTKs (37), and was also used as an alternative substrate to evaluate the selectivity of our inhibitors. The $IC_{50}$ values of GI(2′-Nal)WHHY for p60Src PTK was between 27 and 32 μM. In contrast, no inhibition by GI(2′-Nal)WHHY (up to 800 μM) was detected in both Lyn and Lck PTKs (Table 5). These data demonstrate our inhibitors could distinguish p60Src PTK from other Src family PTKs, suggesting that pseudosubstrate-based peptide inhibitors do have high selectivity and may provide a useful approach to the design of specific peptidomimetic p60Src PTK inhibitors. We also believe that these selective inhibitors may be useful as tools to study the catalytic mechanism of this important enzyme. For example, structural information obtained in studies involving co-crystallization of p60Src PTK (38) with the peptide substrates shown in Table 1 or with the pseudosubstrate inhibitors shown in Tables 2 and 4 will facilitate the understanding of the structure and function of the catalytic site.

We have also studied the biological effect of several of our pseudosubstrate peptide inhibitors on intact v-src-transfected 3T3 cells (39, 40). However, these peptide inhibitors did not show any significant effect on the Tyr phosphorylation pattern of the cell lysate as analyzed by Western blot using antiphosphotyrosine antibody (data...
not shown). This is probably due to the inability of these peptides to enter intact cells. Work is currently underway in our laboratory to develop peptidomimetics that may be able to cross the cell membrane. Additionally, we are planning to deliver the all L-amino acid peptides into the cell via a retroviral peptide expression system and study their biological as well as biochemical effects on intact cells.

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