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A facile, click chemistry-based approach to assembling fluorescent chemosensors for protein tyrosine kinases

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ABSTRACT

A group of fluorophore-labeled peptide substrates of Src kinases have been synthesized with the aid of click chemistry. Some of the generated peptides exhibit an increase in fluorescence upon phosphorylation and are capable of detecting Src kinases with high sensitivity and specificity. Their availability permits real-time activity measurement of aberrantly activated oncogenic Src kinases in the crude lysate of chronic myelogenous leukemia cells. These new chemosensor peptides are highly useful tools that can be used for high-throughput screening to search for small molecule inhibitors of Src kinases as potential therapeutics for cancer treatment.

Protein tyrosine kinases are enzymes which catalyze the transfer of the γ-phosphate of ATP to a specific tyrosine residue of a protein substrate.[1] A particularly well-studied and therapeutically important subcategory of this group of enzymes is the Src-family protein tyrosine kinases (SFKs). Aberrant activation and expression of SFKs is known to contribute to the formation and disease progression of many types of cancers, such as leukemia, colon and breast carcinoma.[2,3] For example, SFKs cooperate with the oncogenic tyrosine kinase Bcr-Abl to induce oncogenic growth and proliferation in chronic myelogenous leukemia (CML).[4] Consequently, small molecule compounds capable of specific inhibition of SFKs are potential therapeutics for cancer treatment.[5]

To facilitate the search for, and development of therapeutic small molecule SFK inhibitors, suitable screening assays are required. Radioactive assays represent the gold standard for measuring kinase activity, however they are discontinuous and require tedious procedures to separate the phosphorylated peptides from other starting materials.[6] Fluorescent peptide-based kinase substrates that are able to signal their phosphorylation state through a change in fluorescence offer a particularly attractive alternative. These may be used to monitor kinase activity in a simple, sensitive and continuous manner, and are therefore more amenable to high-throughput screening applications. Several different designs for such chemosensor peptides have been reported in recent years, amongst the simplest, most elegant and successful of which are those of the Lawrence group.[7] These incorporate either an environment-sensitive fluorophore,[8] or a fluorophore whose fluorescence is initially quenched by a tyrosine residue, but then restored upon phosphorylation.[9]

Herein, we describe the development of a series of new fluorescent chemosensor peptides that may be used for the real-time measurement of SFK activity. Our approach is novel in that we have made use of the Cu(I)-assisted Huisgen cycloaddition reaction ('click reaction') as an efficient means of conjugating a library of fluorophores to a SFK peptide substrate, thereby facilitating the search for fluorophores capable of sensing the phosphorylation state of the tyrosine residue within this peptide. In addition to outlining the optimal conditions for click assembly of the fluorophore–peptide conjugates, we present the results of our examination of the efficiency and specificity of the phosphorylation of the peptides by a SFK member, Lyn (referred to as Lyn kinase) as well as the extent of fluorescence enhancement of these peptides upon phosphorylation by Lyn kinase. Furthermore, we detail the successful use of one of the chemosensor peptides for rapidly assaying SFK activity in the crude cell lysate of CML cells, both in the presence and absence of the CML therapeutic compound Imatinib.

The Src optimal peptide (SOP), with the sequence AEEEIYGEFEA, was previously found to be a selective and efficient peptide substrate of SFKs.[10] The phenylalanine at the Y+3 position was determined to be an essential determinant dictating efficient phosphorylation of SOP by SFKs. Replacement of phenylalanine by alanine significantly reduced the efficiency of phosphorylation

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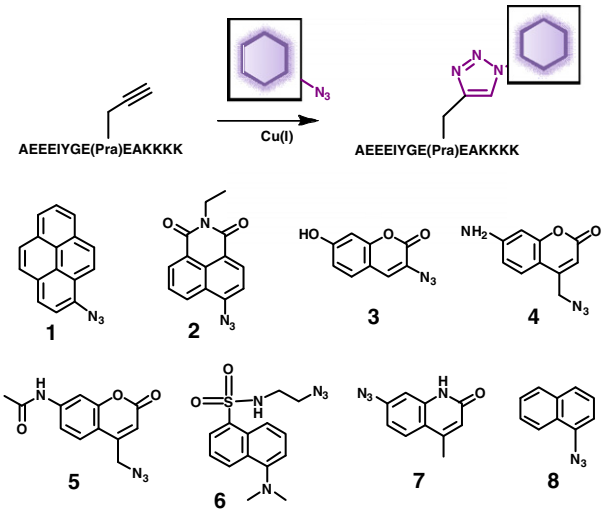
of SOP by SFKs, indicating that SFKs possess a specific binding pocket for a hydrophobic residue at the Y+3 position of the protein and peptide substrates.[11] Based upon these findings, we proposed to introduce a number of aromatic fluorophores at the Y+3 position of a peptide scaffold derived from SOP. Our strategy was to first synthesize a peptide with the sequence AEEEIYGE(Pra)EAKKKK, in which the Pra represents a propargylglycine residue (containing an alkyne side chain), and then to conjugate a series of azide-derivatized fluorophores to this peptide scaffold using the click reaction (Scheme 1).

In the first instance, we investigated solution phase conjugation of the fluorophores to propargylglycine-modified SOP. After exploring a range of conditions, a 3:1 (v/v) mixture of DMF and H_2O was found to be the best solvent mixture in which to achieve dissolution and subsequent reaction of the azide-derivatized fluorophores (see Table S1 of Supplementary data for a complete list of trialed reaction conditions). The click reaction was initiated by the addition of 0.1 equiv of copper(II) sulfate as the copper source, sodium ascorbate as the reducing agent (to reduce Cu(II) to Cu(I)) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA) ligand as a copper(I) stabilizing ligand.[12] Complete conversion of the peptide substrate (as judged by ESI-MS; see Fig. S17) was achieved after stirring overnight at room temperature, and the pure fluorophore-labeled product isolated from the reaction mixture using reverse-phase HPLC. Faster rates of conversion could be achieved by increasing the amounts of the click reagents to 1 equiv each, however reverse-phase HPLC failed to effectively separate the excess Cu(I)-TBTA complex from the labeled peptide.

Next, in order to further streamline synthetic access to libraries of fluorophore–peptide conjugates, we sought to establish whether coupling of the fluorophores to propargylglycine-modified SOP could be achieved prior to cleavage of the peptide from the resin following solid phase peptide synthesis. Initially, we trialed the optimal conditions established for the solution phase click reaction, however we failed to detect any product formation under these conditions. Further experimentation eventually revealed that a mixture of 10 equiv each of $[Cu(CH_3CN)_4]PF_6$, sodium ascorbate, TBTA and DIPEA in DMF could drive the solid phase click reaction to completion, when used in conjunction with microwave heating at a temperature of 80 °C for 30 min (Table 1; see Table S2 of Supplementary data for a complete list of trialed reaction conditions). The relatively large amounts of click reagents could be readily removed by washing the resin prior to cleavage of the final peptide



Scheme 1. Assembly of fluorophore-labeled SFK peptide substrates via click reaction between propargylglycine-modified Src optimal peptide (SOP) and a small library of azide-derivatized fluorophores (labeled **1**–**8**).

Table 1
Optimization of conditions for click reaction between azide-derivatized fluorophore **1** and resin-bound SOP in DMF

Cu source (equiv)	Reaction time	Microwave energy	Yield (%)
$CuSO_4$ (10)	24 h	No	0
$CuSO_4$ (10)	30 min	Yes	60
$[Cu(CH_3CN)_4]PF_6$ (0.1)	24 h	No	5
$[Cu(CH_3CN)_4]PF_6$ (0.1)	5 min	Yes	5
$[Cu(CH_3CN)_4]PF_6$ (1)	30 min	Yes	60
$[Cu(CH_3CN)_4]PF_6$ (10)	30 min	Yes	100

product. The identity and purity of all fluorophore-bearing SOP analogues produced were confirmed by ESI-MS and RP-HPLC analyses (Table S3 and Fig. S17).

Phosphorylation of the tyrosine residue within the fluorophore-labeled peptides by the SFK member, Lyn kinase resulted in enhanced fluorescence in five out of eight cases (Table 2). The peptide constructed using the pyrene derivative **1** gave the greatest fluorescence enhancement (~fourfold after ~75% phosphorylation, as judged by HPLC analysis) (Fig. 1), a result in line with the findings reported by Wang et al.[13] for a similar amide-linked pyrene-peptide construct. The fluorescence of the pyrene moiety is initially quenched by the phenol group of the tyrosine via a dynamic H abstraction mechanism.[14] Upon phosphorylation, this quenching ability is eliminated, leading to restoration of fluorescence. The second best chemosensor peptide, which displayed ~2.5-fold fluorescence enhancement within the same time period, was that generated from the naphthalimide derivative **2** (Fig. S20). The peptides incorporating coumarin fluorophores **3**, **4**, and **5** yielded much more modest increases in fluorescence (10–30%).

One of the major advantages of employing fluorescent chemosensor peptides for assaying SFKs over other methods is the capability to monitor kinase activity in real-time. As shown in Figure 2, incubation of fluorophore **1**-labeled SOP with Lyn kinase induced a time-dependent increase in fluorescence intensity from ~20 to

Table 2
Fluorescence enhancements observed after 60 min incubation of the fluorophore-labeled peptides with Lyn kinase

Clicked azide fluorophore	λ_{ex} (nm)	λ_{em} (nm)	Fold change in fluorescence after 60 min reaction
1	345	380	4
2	346	426	2.5
3	392	475	1.1
4	360	467	1.3
5	335	417	1.3
6	330	564	0
7	329	475	0
8	280	330	0

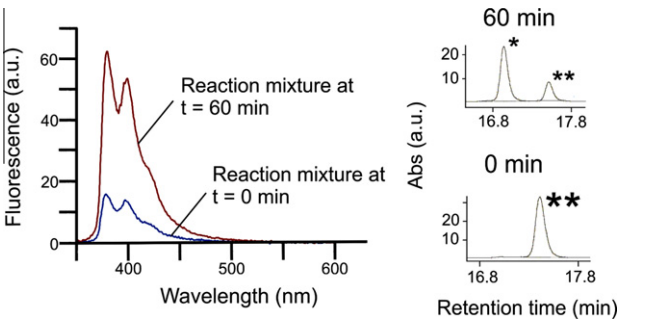


Figure 1. Left: fluorescence emission spectra of fluorophore **1**-labeled SOP before, and 60 min after addition of Lyn kinase (λ_{ex} = 345 nm). Right: HPLC elution profiles showing ~75% conversion of the peptide substrate (**) to its phosphorylated form (*) after 60 min.

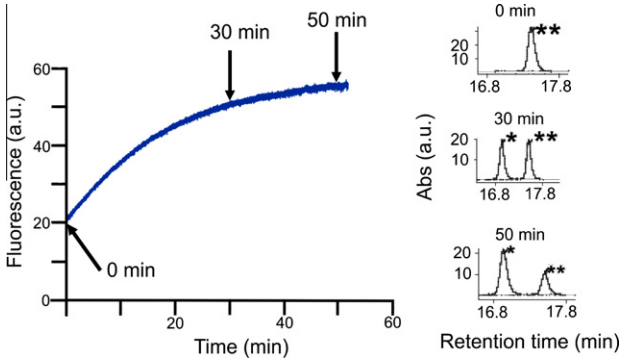


Figure 2. Left: real-time fluorescence measurements (λ_{ex} = 345 nm, λ_{em} = 380 nm) accompanying phosphorylation of fluorophore **1**-labeled SOP by Lyn kinase. Right: HPLC elution profiles showing conversion of the peptide substrate (**) to its phosphorylated form (*).

~55 intensity units over a period of ~50 min. This change in fluorescence intensity closely mirrored the change in the extent of phosphorylation of the chemosensor peptide as determined by HPLC analysis (Figs. 2 and S18) and, radioactive phosphate incorporation (Fig. S24) indicating that the increase in fluorescence intensity provides a true measure of the extent of phosphorylation (linear correlations were observed, with R^2 values of 0.98 and 0.99, respectively, as shown in Figs. S26 and S28). Similar analyses for SOP labeled with fluorophore **2** (Figs. S19, S25, S27, and S29 and S28) show that it can also be used as a chemosensor for real-time assay of SFK activity. Comparison of the radioactive time-course data for the fluorescent SOP with that for unmodified SOP indicate that the pyrene-modified analogue is phosphorylated at only a slightly reduced rate (~10% lower than SOP), while the naphthalimide version is phosphorylated at about one-half the rate (Figs. S23–25). Thus, attachment of the fluorophores to the SOP scaffold is well tolerated by Lyn kinase.

Having established that the peptides could be used to directly monitor phosphorylation by Lyn kinase, we next investigated the utility of fluorophore **1**-labeled SOP for specifically monitoring SFK activity in the crude lysate of cancer cells. For this purpose we used the K562 CML cell line. As shown in Figure 3, incubation of the chemosensor peptide with the crude lysate furnished a time-dependent increase in fluorescence intensity. The increase was abolished when 10 μM of the selective SFK inhibitor, SU6656,[15] was introduced, suggesting that the chemosensor peptide selectively monitors SFK activity in the lysate. We also performed a radioactive kinase assay of the cell lysate using fluorophore **1**-labeled SOP as the substrate, which confirmed that the increase in fluorescence intensity shown in Figure 3 reflects the phosphorylation of the chemosensor peptide by SFKs in the cell lysate (Fig. S32).

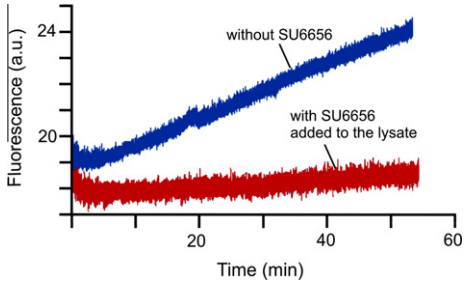


Figure 3. Time-dependent increase in fluorescence (λ_{ex} = 345 nm, λ_{em} = 380 nm) observed upon incubating fluorophore **1**-labeled SOP and K562 CML cell lysate in the absence and presence of SU6656 (10 μM), an SFK inhibitor.

It is well established that SFKs are aberrantly activated by the upstream oncogene kinase Bcr-Abl, however the mechanism by which this occurs has not been fully elucidated.[16] To further demonstrate the applicability of fluorophore **1**-labeled SOP for monitoring SFK activity in cancer cells, we treated the K562 cells with the cell-permeable Bcr-Abl inhibitor, Imatinib, which is the most common drug for CML treatment. Incubation of the peptide with the Imatinib treated K562 cell lysate did not result in an increase in fluorescence from the peptide, confirming that suppression of Bcr-Abl by Imatinib prevents SFK activation in CML cells (Figs. S31–S33). Furthermore, the results lend further credence to our claim that fluorophore **1**-labeled SOP is a highly selective chemosensor peptide substrate of SFKs.

In summary, we have successfully employed click chemistry to help develop fluorescent peptide-based chemosensors for simple, continuous, non-radioactive assay of SFK activity. These peptides will be useful for high-throughput screening applications and could also potentially be further utilized to discern the advancement of Imatinib resistance in CML patients. Given the flexibility of solid phase peptide synthesis and convenience of click chemistry, the approach described herein could be readily adapted to the development of fluorescent chemosensor peptide substrates for other protein kinases.

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Supplementary data

Synthesis of azide-derivatized fluorophores; optimization of solution and solid phase click chemistry conditions; ESI-MS spectra of labeled SOPs; linear correlation analyses (normalized fluorescence vs phosphorylated product concentration determined by radioactive and HPLC analyses); radioactive assay data for Lyn-catalyzed phosphorylation of SOP and fluorophore **1**- and **2**-labeled SOP; and postulated model for activation of SFKs by Bcr-Abl. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.005.

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