A rapid and convenient click chemistry method to develop fluorescent based chemosensor peptide substrates for protein tyrosine kinases

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ABSTRACT

A group of fluorescence-based chemosensor peptide substrates of Src kinases were synthesized via the click chemistry approach. Some of the generated chemosensor peptides are capable of detecting Src kinases with high sensitivity and specificity. Their availability permits real-time activity measurement of the aberrantly activated oncogenic Src kinases in crude lysate of chronic myelogenous leukaemia cells. Furthermore, these chemosensor peptides are amenable for high throughput screens to search for small molecule inhibitors of Src kinases as potential therapeutics for cancer treatment

Aberrant activation and expression of Src-family of protein tyrosine kinases (SFKs) are known to contribute to the formation and disease progression of many types of cancer such as leukaemia, colon and breast carcinoma^{1,2}. For example, SFKs cooperate with the oncogenic tyrosine kinase Bcr-Abl to induce oncogenic effects in chronic

myelogenous leukaemia (CML)³. For this reasons, small molecule compounds capable of specific inhibition of SFKs are potential therapeutics for cancer treatment. To

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facilitate the search and development of these therapeutic small molecule SFK inhibitors, we have designed and synthesized fluorescent chemosensor peptides suitable for high throughput assay for activity of SFKs.

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Herein, we describe how we used the click chemistry approach in synthesis and assembly of these fluorescent chemosensor peptides. The approach we used is suitable for rapid syntheses and screening of a small library of peptide substrates derived with different types of fluorophores for use as chemosensor substrates of protein kinases. Furthermore, we used one of the peptide substrates chemosensors and examined its suitability for rapid and convenient assay of SFK activity in crude cell lysate of CML cells with and without treatment with CML therapeutic compound Imatinib.

The Src optimal peptide with the sequence of AEEIYEGEFEA was previously found to be a selective and efficient peptide substrates of SFKs⁴. The phenylalanine at the pY+3 position was determined to be an essential determinants dictating efficient phosphorylation of the Src optimal peptide by SFKs. Replacement of phenylalanine by alanine significantly reduced the efficiency of phosphorylation of Src optimal peptide by SFKs, indicating that SFKs possess a specific binding pocket for a hydrophobic residue at the pY + 3 position of the protein and peptide substrates⁵. Based upon these findings, we proposed to introduce a number of fluorophores at the pY+3 position of a peptide scaffold derived from the Src-optimal peptide. Since the fluorophore moiety of the resultant peptide analogs can potentially bind to the (pY+3)-binding pocket of SFKs, these peptides are expected to be efficient and specific substrates of SFKs. One of the criteria for a Src-optimal peptide analog to serve as a chemosensor peptide of SFKs is its ability to exhibit fluorescence enhancement upon phosphorylation. Lawrence et al. (REF) previously developed a pyrene-based chemosensor peptide substrate of which the phenol group of the tyrosine quenches the fluorescence of the pyrene-moiety. Upon phosphorylation, formation of phosphoester in the resultant phosphotyrosine disrupts the dynamic interaction between the aromatic groups of the phosphotyrosine and the pyrene moiety. In this manuscript, we describe convenient methods to synthesize a library of Src-optimal peptide analogs. We also present results of the examination of the efficiency and specificity of phosphorylation of these peptides by SFKs as well as the extent of fluorescence enhancement of these peptides induced by their phosphorylation by SFKs.

The peptide scaffold was prepared by replacing the hydrophobic phenylalanine at the pY + 3 position of the Src optimal peptide with propargylglycine. The peptide (referred to as propargylglycine – SOP) contains the sequence AEEIYGE(Pra)EAKKKK-NH₂ in which the –NH₂ represents the amidated C terminus and Pra

represents the propargylglycine residue. The reactive alkyne group of the propargylglycine residue allows it to react with a large number of azide derivatized fluorophores to generate multiple Src optimal peptide analogues carrying a wide spectrum of fluorophores at the pY + 3 position. The approach allows the peptide scaffold

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to “click” with a library of azide – derivatized fluorophores as referred as the click chemistry approach. The general scheme for the synthesis of the fluorescent Src optimal peptide analogues is depicted in Scheme 1.

Scheme 1. The scaffold peptide (1) was clicked with various azide derivatized fluorophores (2) to generate the clicked product of chemosensor peptide substrate (3)

The trademark for click chemistry reaction is defined by the specific reaction of azide-alkyne group with addition of Cu(I) species or Cu(II) catalyst that is accompanied by reducing agent and Cu(I) stabilizing ligand^{9,10}. However in our fluorescent based Src optimal peptide chemosensor syntheses, the commonly used click chemistry procedures either did not furnish the desired products or yield a low amount of the desired products due to the highly hydrophobic nature of the azide derivatized fluorophores used as the starting materials. We therefore attempted to

optimize conditions of the solution phase and solid phase click chemistry.

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In the solution phase click chemistry, the solubility of the azide derivatized fluorophores become an issue. The fluorophore quickly precipitated in the presence of water. After much troubleshooting, we identified DMF:H₂O mixture with ratio of 3:1 as the best solvent. The click chemistry is then initiated with the addition of 0.1 equivalents copper (II) sulphate as the copper source, sodium ascorbate as the reducing agent and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA) ligand as the copper (I) stabilizing ligand. The reaction took 24 hours to **complete**. Moreover, we also found that the reaction could reach completion within 2 hours simply by changing the ratio of copper (II) sulphate, sodium ascorbate and TBTA to 1 equivalent each. However, the reverse phase HPLC purification step failed to effectively separate the excess Cu(I)-TBTA complex from the reaction product. Thus, we did not adopt the use of high concentration of click chemistry additives in our syntheses. The various reaction conditions we examined are summarized in Table 1.

Table 1. Conditions of the solution phase click chemistry.

Cu source (eq)	Solvent	Reaction time	Microwave energy	Yield (%)
CuSO ₄ (0.1)	1:2 acetonitrile: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	2:1 acetonitrile: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	1:2 tBuOH: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	2:1 tBuOH: H ₂ O	24 hr	No	5
CuSO ₄ (0.1)	2:1 tBuOH: H ₂ O	5 min	Yes	5
CuSO ₄ (0.1)	2:1 DMSO: H ₂ O	24 hr	No	90*
CuSO ₄ (0.1)	2:1 DMF: H ₂ O	24 hr	No	5
CuSO ₄ (10)	2:1 DMF: H ₂ O	24 hr	No	60
CuSO ₄ (0.1)	3:1 DMF: H ₂ O	24 hr	No	100

*The clicked product obtained 90 % yield from 2:1 DMSO:H₂O reaction could not be purified due to the sulfoxide adduct formed.

We next investigated the solid phase click chemistry approach in our syntheses. Initially, conditions similar to those used in solution phase synthesis was employed but **we failed to detect any product formation with these conditions**. However, when the reaction was enhanced by microwave energy, the resultant product yield is around 5 %. To further improve the efficiency of the reaction, we increase the amount of copper (II) sulphate, sodium

ascorbate and TBTA to 10 equivalents each in addition to using microwave energy. The clicked product yield is significantly improved to around 60 %. We later explored the use of [Cu(CH₃CN)₄]PF₆, a Cu(I) salt that is readily dissolved in organic solvent as the Cu(I) source. This eliminates the presence of aqueous elements in the reaction mixture that may hinder the reaction to proceed. Mixtures of 10 equivalents each of [Cu(CH₃CN)₄]PF₆ salt, sodium ascorbate, TBTA and DIPEA was found to drive the click reaction to completion with microwave energy at temperature of 80 ° C and reaction time of 30 minutes. **The reaction conditions used in our solid phase click chemistry syntheses are shown in Table 2.**

Table 2. Conditions of the solid phase click chemistry.

Cu source	Solvent	Reaction time	Microwave energy	Yield (%)
CuSO ₄ (0.1)	1:2 acetonitrile: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	2:1 acetonitrile: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	1:2 tBuOH: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	2:1 tBuOH: H ₂ O	24 hr	No	0
CuSO ₄ (0.1)	2:1 tBuOH: H ₂ O	5 min	Yes	5
CuSO ₄ (0.1)	3:1 DMF: H ₂ O	24 hr	No	0
Cu[(CH ₃ CN) ₄]PF ₆ (0.1)	DMF	24 hr	No	5
Cu[(CH ₃ CN) ₄]PF ₆ (0.1)	DMF	24 hr	No	5
Cu[(CH ₃ CN) ₄]PF ₆ (0.1)	DMF	5 min	Yes	5
Cu[(CH ₃ CN) ₄]PF ₆ (0.1)	DMF	30 min	Yes	60
Cu[(CH ₃ CN) ₄]PF ₆ (10)	DMF	30 min	Yes	100

Subsequently, fluorescence measurements of the clicked products indicate **that peptide 1 gave the highest fluorescence enhancement (~4 fold)**. This is in line with the similar design reported by Lawrence and colleagues⁸. The second best fluorescent based Src kinase chemosensor is peptide 2 with fluorescence enhancement of around 2.5 fold. Results of fluorescence measurement of the Src optimal peptides derived with the various fluorophores are listed in Table 3

One of the advantages of the assay using fluorescence based Src kinase chemosensor peptide over other types of assay methods is the capability to monitor the Src kinase activity in real time. As shown in Figure 1, the presence of the SFK member Lyn (referred to as Lyn kinase in this

Table 1. Fluorescence measurements of the clicked chemosensor

Clicked Chemosensor	$\lambda_{\text{excitation}}$ (nm)	$\lambda_{\text{emission}}$ (nm)	Fold change in fluorescence
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

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manuscript) induced a **time-dependent** increase in fluorescence intensity from ~ 20 to ~ 54 intensity units. The intensity increase **was** rapid between 0 – 20 minutes and started to reach **the** plateau phase after 40 minutes. The change in fluorescence intensity closely mirrored the change in **the** extent of phosphorylation of the chemosensor peptide as reflected by radioactive phosphate incorporation (SFig. ?) and HPLC analysis (Fig. 1B). The results indicate that the increase in fluorescence intensity is a true measure of the extent of phosphorylation of the chemosensor peptide by the Lyn kinase.

Figure 1. Real time fluorescence measurements of peptide 1 phosphorylation by Lyn kinase and HPLC analysis profiles showing the percentage of substrate consumption at different time interval

In contrast to the fluorescence – based assay method depicted in Figure 1, radioactive assays and other assay methods are discontinuous and they require tedious procedures to separate the phosphorylated peptides from other starting materials¹¹. This further confirm the usefulness of the fluorescence – based Src kinase chemosensor peptide for high throughput screening for potential inhibitors of protein kinases.

The applicability of peptide 1 to specifically monitor the SFK activity in crude cell lysate of cancer cells was investigated. SFKs are aberrantly activated in CML cells. We therefore prepared crude cell lysate of the K562 CML cell lines and examined if peptide 1 can selectively monitor SFK activity in the cell lysates. As shown in Figure 2, incubation of peptide 1 with an aliquot of the cell lysate furnished a time dependent increase in fluorescence intensity. The increase was abolished when

10 μ M of the selective SFK inhibitor SU6656¹² was introduced to the crude cell lysate, suggesting that peptide 1 selectively monitored the SFK activity in K562 cell lysate (data in supplementary section). We also

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performed radioactive kinase assay using peptide 1 as the substrate. The radioactivity shows that the increase in fluorescence intensity as shown in Figure 2 reflects the phosphorylation of the chemosensor peptide by SFK in the cell lysate.

Figure 1. Real time fluorescence spectra of clicked chemosensor (1) upon addition of crude cell lysate of K562R cell

It is well established that SFKs are aberrantly activated by the upstream oncogene kinase Bcr-Abl in CML cells. However, the mechanism of SFK activation by Bcr-Abl has not been fully elucidated¹³. To further demonstrate the applicability of peptide 1 in monitoring SFK activity in cancer cells, we treated the K562 cells in culture with the cell-permeable Bcr-Abl inhibitors Imatinib, which is the frontline therapeutics for the treatment of CML patients. The treatment was able to induce K562 cell death in culture (data not shown). The Imatinib treated K562 cell lysate failed to induce desirable enhancement of fluorescence intensity of peptide 1, confirming that suppression of Bcr-Abl by Imatinib prevents SFK activation in CML cells. Furthermore, the results lend further credence to our claim that peptide 1 is a highly selective chemosensor peptide substrate of SFK. All the data are shown in supplementary section.

In summary, we had developed a rapid method to generate fluorescent based Src kinase chemosensors for use in convenient, non radioactive and real-time assays of SFKs. We demonstrated that these peptides are useful for the search and development of SFK inhibitors which are

potential anti-cancer therapeutics. Since aberrant activation of SFKs contributes to the development of drug resistant CML, the chemosensors can be used to develop the diagnostic test to monitor disease progression of CML patients from the Imatinib-sensitive stage to Imatinib-resistant stage. Given the flexibility in solid phase peptide synthesis and convenience of click chemistry, the procedures we described in this manuscript can facilitate the development of chemosensor substrates to monitor the activity of protein kinases that are involved in other types of diseases.

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