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Development of a novel fluorescent probe for fluorescence correlation spectroscopic detection of kinase inhibitors

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ABSTRACT

We have developed a fluorescently labeled probe for high-throughput screening of kinase inhibitors using fluorescence correlation spectroscopy. With this probe, we have successfully evaluated the inhibitory activities of known inhibitors of a model kinase, ASK1. Because the probe contains a general kinase inhibitor, staurosporine, we believe that this homogeneous, high-throughput, and simple method can be applied to the inhibitor screening of other kinases as well.

Protein kinases play pivotal roles in virtually all aspects of cellular physiology, including intercellular communication associated with physiological responses, homeostasis, and the functioning of the nervous and immune systems.[1] Since kinases are involved in cancer and many other diseases, they are attractive targets for therapeutic agents. Indeed, imatinib mesilate, which is a Bcr-Abl tyrosine kinase inhibitor, has already been applied to treat chronic myeloid leukemia (CML).[2]

Several methods to screen kinase inhibitors have been reported, such as scintillation proximity assay (SPA) and time resolved-fluorescence resonance energy transfer (TR-FRET).[3] These methods, however, require radioisotope (RI)-labeled ATP and luminescent lanthanide complex-labeled antibody, respectively. Hence, there is still a need for a novel methodology that is non-RI, low-cost, convenient, and homogeneous. Preferably the method should be generally applicable to any kinase.

Here, we propose a novel methodology for high-throughput screening (HTS) of kinase inhibitors based on fluorescence correlation spectroscopy (FCS), which should fulfill all of the above requirements. FCS is a single molecule detection technique using a fluorescent probe. It sensitively measures fluctuations of the fluorescence intensity emitted from only a few fluorescent molecules (3–5 particles) that diffuse in and out of a small volume element at the subfemtoliter level in solution. The obtained parameter, diffusion time, is dependent on molecular weight and molecular structure.[4]

A schematic representation of this assay is shown in Figure 1. The assay is based on competition between a standard fluorophore-labeled inhibitor, or a probe, and an inhibitor candidate.[5]

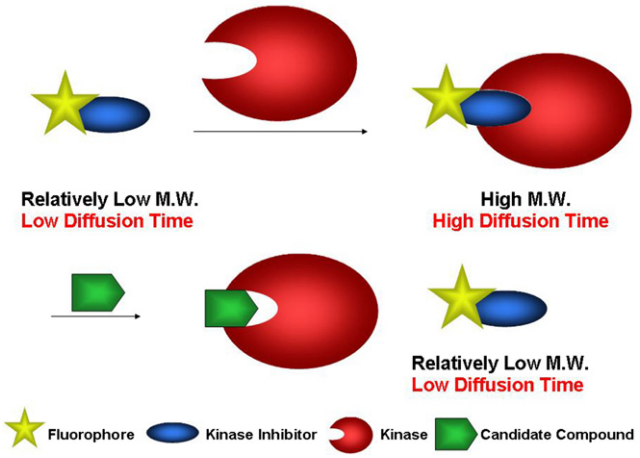

Figure 1. Schematic illustration of the assay.

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In the absence of a candidate, or if a candidate has low affinity for the kinase, the probe interacts with the target enzyme, resulting in a high diffusion time. If the candidate compound has a high binding affinity to the enzyme, the probe is released from the enzyme, resulting in a low diffusion time. This method is expected to have several advantages over previously available methods because the measurement can be done in a very small volume (~1 μL), without the use of expensive antibody or RI. Moreover, it is based on binding, not kinase, assay. That is, it is also applicable to inactive kinases, whose structures are often unique in terms of their interactions with the ligand in comparison to the active kinases.[6]

For this method, we designed a novel chemical probe (STS–PEG–TAMRA). This probe consists of three moieties: fluorophore, linker, and inhibitor. For the inhibitor moiety, we selected staurosporine (STS) because it is a well-known promiscuous kinase inhibitor.[7] TAMRA was chosen as a fluorophore, due to its water solubility, resistance to photobleaching, and low conversion to the excited triplet state.[8] For the linker, we used polyethyleneglycol (PEG) to give sufficient structural flexibility and water solubility. The synthetic route to the probe is shown in Scheme 1. Staurosporine was modified at a secondary amine, which may participate in hydrogen bonding to kinases, but orients to the outside upon binding (Fig. 2).[9] It was reported that this amine can even be converted to amide without loss of the activity against various kinases.[10]

As a target kinase, we focused on apoptosis signal-regulating kinase 1 (ASK1). ASK1/MAPKKK5 is a ubiquitously expressed MAPKKK that activates the JNK and p38 pathways by directly phosphorylating and thereby activating their respective MAPKKs.[11] ASK1 is activated by oxidative stress, endoplasmic reticulum (ER) stress, and calcium overload. Activated ASK1 induces proliferation, differentiation, and apoptosis. The function of ASK1 has not been fully uncovered, but ASK1 has already been reported to be involved in various diseases, such as cancer, Alzheimer's disease, ischemic cardiomyopathy, and septic shock.[12] Therefore, the development of ASK1 inhibitors is of great interest in connection with treatment of these diseases.

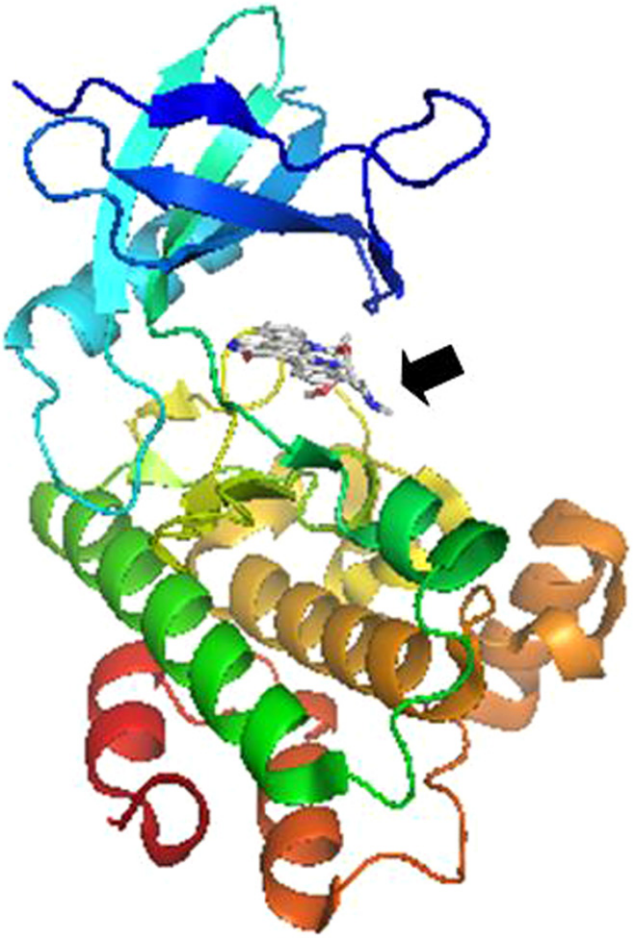
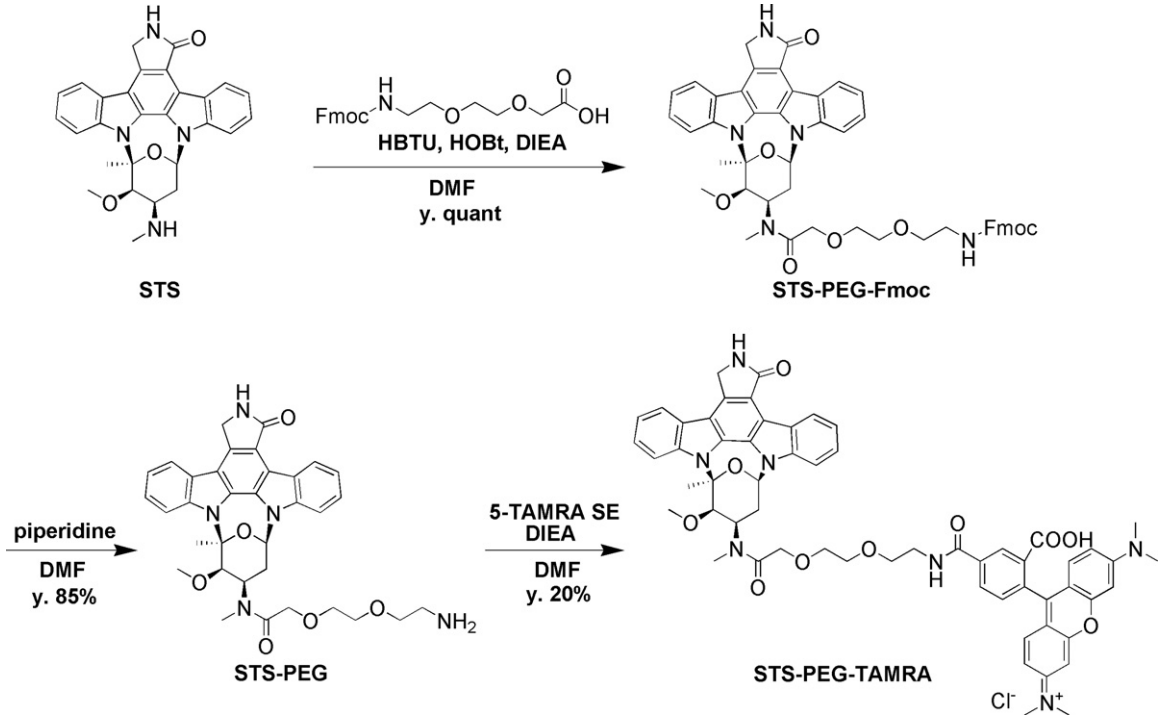


Figure 2. Crystal structure of ASK1 (ribbon) with staurosporine (stick, O: red, N: blue). The arrow indicates the modified secondary amine. PDB: 2CLQ. The structure was modified by PyMol.



Scheme 1. Synthesis of STS–PEG–TAMRA. For details, see Supplementary data.

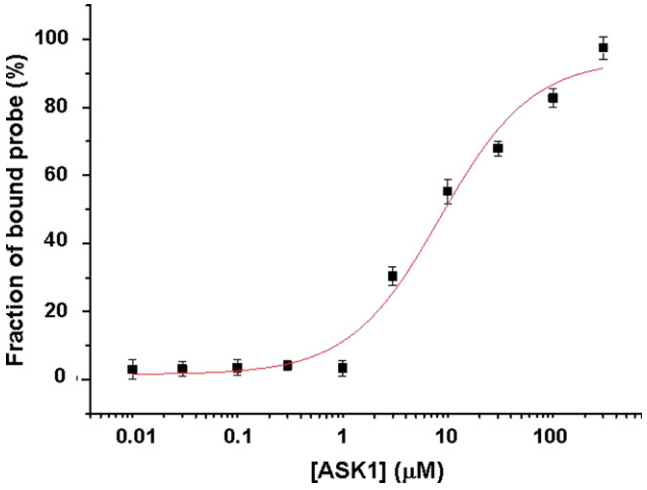


Figure 3. Binding curve of STS–PEG–TAMRA. All measurements were performed in FCS buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20), containing 10 nM probe (STS–PEG–TAMRA), various concentrations of ASK1, and 0.5% DMSO as a cosolvent. The value was calculated by two-component analysis of the autocorrelation function. For details, see supporting information.

Using the synthesized probe, we first performed a binding assay of the probe with ASK1 kinase domain, based on FCS (Fig. 3). The K_d value was calculated to be 8 μM. It has been reported that the K_d value of native staurosporine is 110 nM against ASK1.[7] The difference may partly be explained by the nature of ASK1 we used, which only consisted of the kinase domain (646–940 aa), and by loss of hydrogen bonding of the modified secondary amine. Nevertheless, we believe that the K_d of 8 μM is sufficiently strong for initial screening of inhibitors.

Next, to demonstrate the utility of this probe, we used it in an FCS-based competitive assay of known ASK1 inhibitors. Inhibitory activities of these compounds were confirmed by luciferase assay, and the IC_{50} values are shown in Table 1. The results of the FCS-based assay, as well as the structures of the inhibitors, are shown in Figure 4. Staurosporine and compound **2** inhibited the binding of the probe and ASK1 in a dose-dependent manner, while compound **1** showed no inhibition. These results suggest that the probe competes with staurosporine for binding to kinases. In other words, both compounds appear to bind to an identical ATP-binding pocket in kinases, and with this probe as a screening tool, we can detect compounds which bind to the pocket. The results of the FCS-based assay are roughly consistent with those of luciferase assay (Table 1). Note that in this FCS assay, the IC_{50} value is thought to be independent of the probe used, because the concentration of the probe is far lower than the K_d of the probe (see supporting information). Hence, the difference of IC_{50} values between FCS and luciferase assay may derive from the difference of the methods, that is, binding assay for FCS and kinase assay for luciferase assay, and ligand depletion.[13] It might be true that this method has lower sensitivity than luciferase assay due to the high ASK1/probe ratio, but it is advantageous in terms of handling simplicity. It is also well known that kinase assay depends on various factors, such as the concentration of ATP or substrates used.

Table 1
Inhibitory activities of ASK1 inhibitors

Compound	IC_{50}[a] (μM)	IC_{50}[b] (μM)
1	>100	82.7
2	7	0.15
STS	1	0.037

[a] Values were determined by FCS-based competition assay.
[b] Values were determined by luciferase assay. See supporting information.

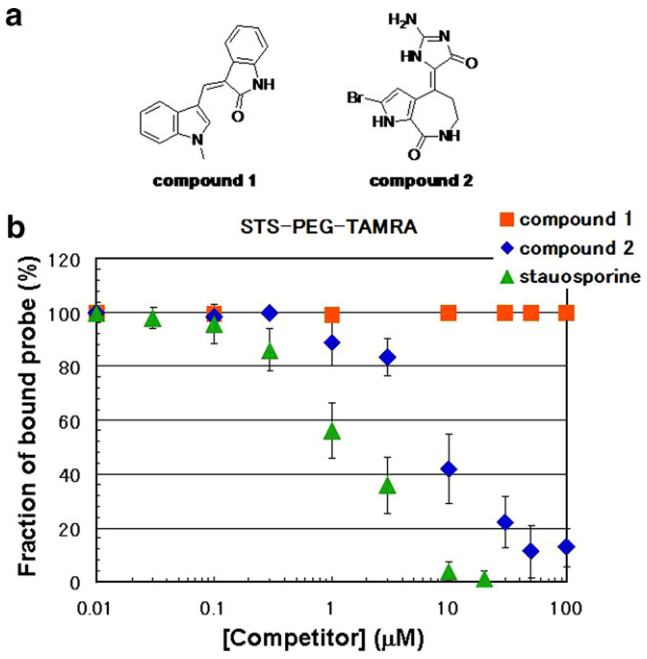


Figure 4. (a) The structures of compound **1** and compound **2**. (b) Competition plot of compound **1**, compound **2**, and staurosporine (STS) with STS–PEG–TAMRA obtained by FCS measurement. Assay was performed in FCS buffer, containing 1% DMSO as a cosolvent. [ASK1] = 5 μM.

To conclude, the results obtained here indicate that our probe can successfully screen kinase inhibitors in a homogeneous and high-throughput manner, without the use of expensive antibody or RI. Since staurosporine is a general kinase inhibitor, this probe should also be applicable to HTS of other kinases. We are currently preparing for large-scale screening of ASK1 inhibitors using this probe, and the results will be reported in the near future.

Supplementary data

Supplementary data concerning (1) the synthetic procedure and characterization of the compounds, (2) expression and purification of ASK1, and (3) method of FCS measurement, are available online. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.040.

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