

The design and synthesis of a novel fluorogenic probe targeting inactive forms of Bruton's tyrosine kinase for high throughput binding assay

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Introduction

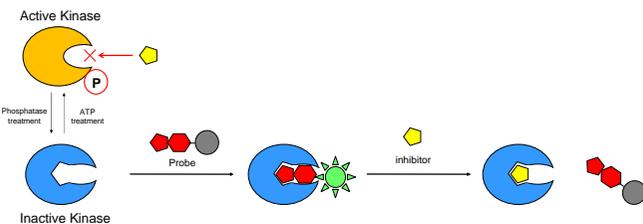
The aberrant activation of B-cell receptor (BCR) signaling is thought to promote cancer cell expansion and survival in B cell lymphoma and chronic lymphocytic leukemia. The Bruton's tyrosine kinase (BTK) is a member of the Tec family of cytoplasmic tyrosine kinase and plays a crucial role in the BCR signaling which is essential for B-cell development and activation. Therefore inhibitors of BTK are considered as potential therapeutic agents for the treatment of B cell lymphoma, chronic lymphocytic leukemia and autoimmune diseases.

The majority of the reported kinase inhibitors are targeting ATP binding site of its active conformation. The ATP-binding sites of protein kinases in the active conformation are very similar, therefore it is difficult to obtain the kinase selectivity by these classical type inhibitors. On the other hand, some papers reported that an inhibitor showing a high selectivity was found to bind to an inactive conformation of kinase, that is believed that structures of inactive states are much diverse than that of the active states.

Therefore targeting the inactive conformations of kinases is an attractive approach for drug discovery to develop a selective kinase inhibitor. However, it is difficult to identify such "inactive form binders" because commonly used activity-based assays cannot measure the activity of the inactive states of kinases.

To identify compounds that preferentially bind to the inactive conformation of BTK, we have developed a novel competitive binding assay using a newly synthesized fluorogenic probe compound.

Concept of fluorogenic competitive binding assay targeting an inactive state of BTK



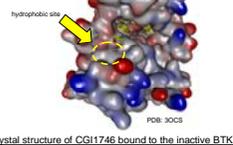
Design of fluorogenic probe based on CGI1746

As CGI1746 is reported to bind to the inactive form of BTK with 32-fold greater affinity than the activation form ($K_d=2.9$ nM, and 94.1 nM, respectively), CGI1746 was used to probe the active site of the inactive conformation of BTK.

To detect the probe bind to the BTK active site, we attached an environmentally-sensitive fluorophore, nitrobenzoxadiazole (NBD) to CGI1746 via various linkers. The fluorescence of NBD is extremely sensitive to the local environment around the chromophore.

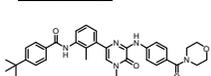
NBD shows unique fluorescence properties:

- > almost non-fluorescent in an aqueous solution.
- > highly fluorescent in non-polar solvents or when binding to proteins.

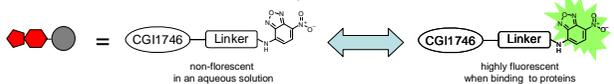


Crystal structure of CGI1746 bound to the inactive BTK

Structure of CGI1746

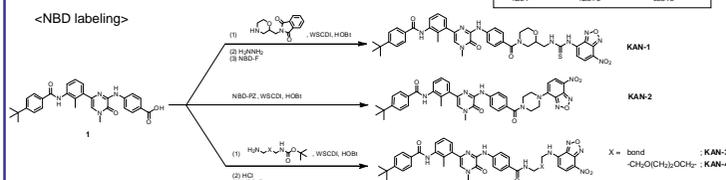


NBD chromophore

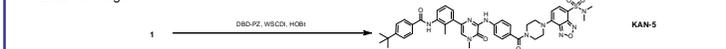


Synthesis of fluorogenic probes

<NBD labeling>



<DBD labeling>



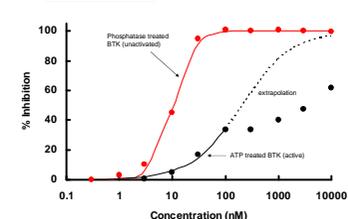
Inhibitory profiles of probe compounds for BTK

BTK inhibition activity of probe compounds

Probe	IC ₅₀ (nM)	
	ATP-treated BTK (activated)	Phosphatase treated BTK (inactivated)
KAN-1	249	14
KAN-2	202	10
KAN-3	1219	156
KAN-4	719	15
KAN-5	287	9

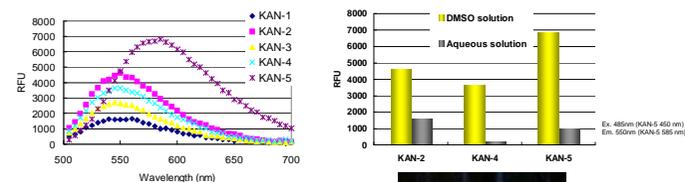
IC₅₀ values determined by measuring the inhibition of the substrate phosphorylation with Caliper mobility shift assay

Inhibition curve of KAN-2

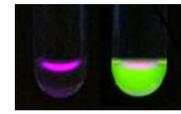


Fluorescence properties of probe compounds

The emission spectra of the probe compounds were measured in DMSO and in water.

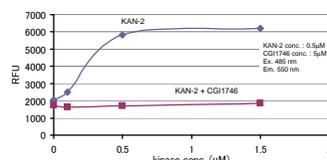
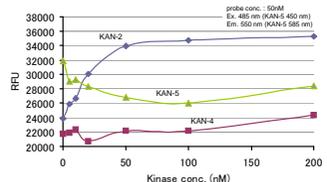


The fluorescence intensity of the probes varied along with the linker structures. KAN-2 showed the highest intensity among the NBD series. $\lambda_{ex}/\lambda_{em}$ of KAN-5 (DBD labeling) is different from those of the NBD series.

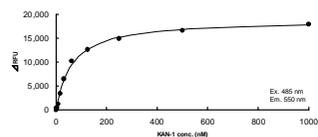


Dramatic decrease in the fluorescence intensity were observed when the solvent was changed into water.

Titration study of probes with BTK



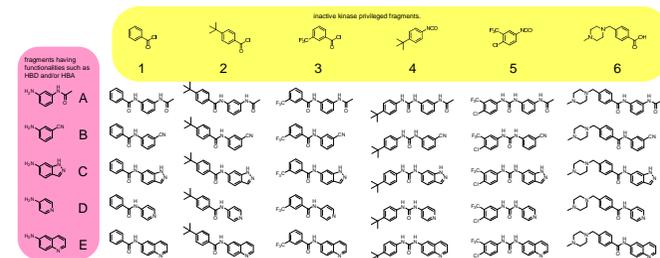
The fluorescence was quenched by adding of free CGI1746 indicating specific binding of KAN-2 to the active site of BTK.



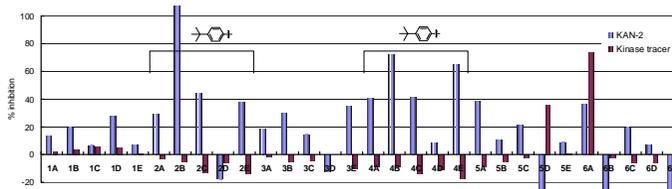
The K_d value of KAN-2 was determined to be 63 nM in this assay system.

Screening of small compound library using fluorogenic probe KAN-2

To validate compound screening using KAN-2, a small compound library was screened. The compound library was designed to bind "the back-pocket of BTK" where the t-butylphenyl group of CGI1746 binds. All compounds were synthesized by combinatorial chemistry.

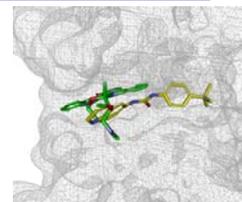


Compounds (30 μ M) were incubated with the dephosphorylated BTK (25 nM) and KAN-2 (50 nM) for 2 hours, and then the fluorescence were measured (Ex. 485 nm / Em. 550 nm). To consider the effects of compounds on the fluorescence, the fluorescence of a mixture of each compound and KAN-2 was measured and used as the background signal. The screening results using Kinase Tracer178 (LanthaScreen® TR-FRET Invitrogen) were also shown (red bar).



KAN-2 could detect various BTK binders compared to Kinase Tracer178. The majority of hit compounds have the t-butylphenyl group suggesting those compounds would bind to the back-pocket of the inactive BTK where CGI1746 uses.

Predicted binding mode of hit compound 4E in the inactive conformation of BTK



Hit compound 4E (yellow) was docked into the inactive conformation of BTK (PDB: 3OCS). 4E could bind to the back-pocket and might not compete with staurosporine (green).

Summary

- We have successfully designed and synthesized a novel "fluorogenic probe" KAN-2 which binds selectively to the inactive form of BTK.
- The fluorescence intensity of KAN-2 was increased by adding BTK in a dose-dependent manner.
- The quenching of the fluorescence by adding of free CGI1746 indicates KAN-2 specifically binds to the active site of BTK.
- A novel competitive assay system using KAN-2 was developed and validated by screening of the inactive kinase focused compound library.
- The methods using the fluorogenic probe KAN-2 have great potential for the discovery of novel lead compounds against inactive states of BTK.

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