A novel binding assay to identify inhibitors that bind to inactive forms of Bruton's tyrosine kinase based on fluorescence resonance energy transfer

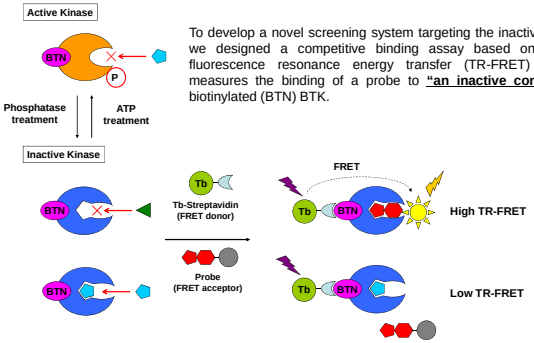
Tokiko Asami, Wataru Kawahata and Masaaki Sawa

Drug Discovery Research, Carna Biosciences, Inc., BMA 3F 1-5-5 Minatojima-Minami, Chuo-ku, Kobe 650-0047 Japan

INTRODUCTION

Bruton's Tyrosine Kinase (BTK) is a member of the Tec family of non-receptor tyrosine kinases. BTK is one of the crucial kinases for the B cell maturation and also involved in mast cell activation through the high-affinity IgE receptor. Therefore, BTK is an attractive target for the potential treatment of multiple therapeutic areas that involve B-cell and/or mast cell activation, such as B cell malignances, asthma, and rheumatoid arthritis. In order to develop a selective BTK inhibitor, it is important to identify a highly selective compound as the drug discovery starting point. Generally, activity-based high-throughput screening (HTS) using active kinase has been used to identify hit compounds by measuring the inhibition of substrate phosphorylation. However, the activity-based HTS campaign frequently results in the enrichment of classical ATP competitive inhibitors, which would often require considerable medicinal chemistry efforts to increase kinase selectivity, because the human protein kinase family consists of over 500 enzymes with very similar active sites. On the other hand, it is believed that inactive kinase structures are more diverse than the active forms. Therefore targeting the inactive conformations may offer advantages in selectivity. Here we describe a novel approach to identify inhibitors of an inactive form of BTK.

Concept of competitive binding assay targeting an inactive state of BTK based on TR-FRET system

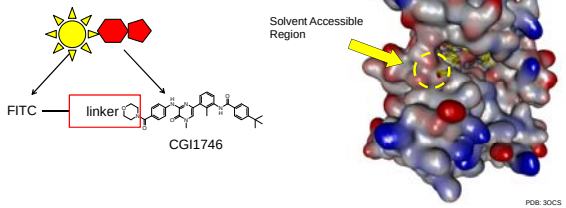


To develop a novel screening system targeting the inactive form of BTK, we designed a competitive binding assay based on time-resolved fluorescence resonance energy transfer (TR-FRET) platform that measures the binding of a probe to **"an inactive conformation"** of biotinylated (BTN) BTK.

MATERIALS & METHODS

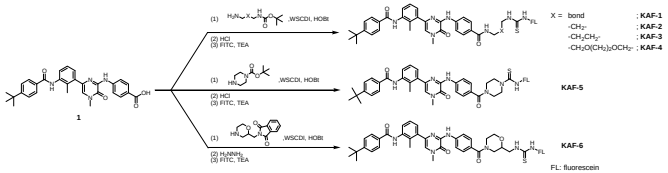
Probe design based on CGI1746

CGI1746 was chosen as a probe of the active site of the inactive conformation of BTK, as CGI1746 is reported to bind to the inactive form of BTK with 32-fold greater affinity than the activated form (*Kd*=2.9 nM, and 94.1 nM, respectively. *Nat. Chem. Biol. 7, 41-50. 2011*).



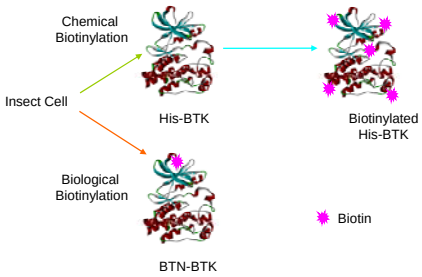
Synthesis of FITC labeled CGI1746

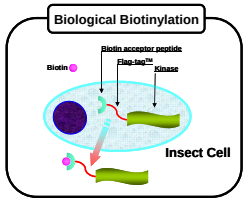
CGI1746 was labeled with FITC via various linker groups to find the optimum length of the linker for FRET.

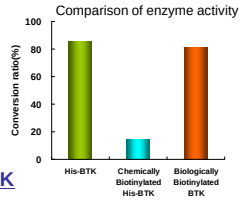


Preparation of Biotinylated BTK (BTN-BTK)

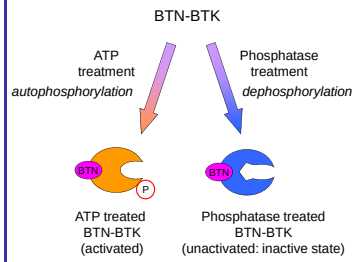
To examine the effect of the biotinylation on enzymatic activity, biotin was conjugated to BTK either by chemically or biologically.

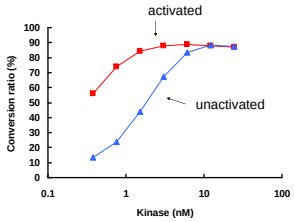






Preparation of active and inactive-states BTN-BTK





RESULTS

Inhibitory profiles of probe compounds for BTK

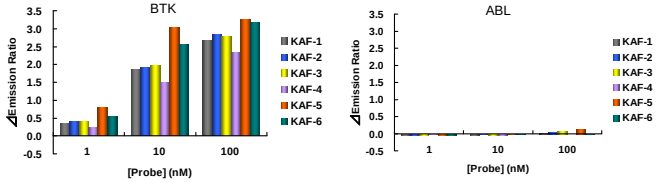
Probe	IC50 (nM) ATP treated BTK (activated)	Phosphatase treated BTK (unactivated)
KAF-1	290	5.6
KAF-2	208	3.2
KAF-3	189	4.3
KAF-4	305	7.8
KAF-5	69	0.61
KAF-6	350	4.8

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

Titration study of probes with inactive form of BTK

Assay condition :
5 nM kinase, 2 nM Tb-Streptavidin, 1-100 nM probe, 1% DMSO or 10 μM CGI1746, 1 hour incubation
probe emission (Ex. 320 nm / Em. 520 nm), donor emission (Ex. 320 nm / Em. 495 nm)

$$\text{Emission Ratio} = \frac{\text{Probe(Ex. 320 nm/Em. 520 nm)}}{\text{Donor(Ex. 320 nm/Em. 495 nm)}}$$

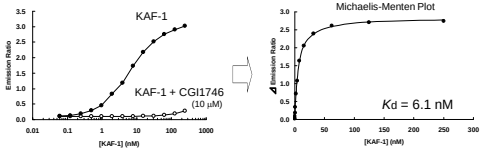


All probe compounds provided excellent TR-FRET signals with the inactive form of BTN-BTK.
No TR-FRET signals were observed with ABL kinase.

Assay development

Determination of *Kd* value

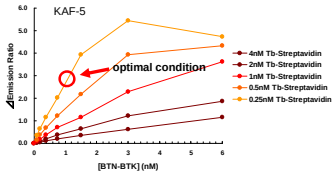
The *Kd* values of probe compounds were determined against the inactive state of BTN-BTK.



Probe#	*Kd* (nM)
KAF-1	6.1
KAF-2	6.2
KAF-3	5.5
KAF-4	8.8
KAF-5	2.5
KAF-6	3.8

Optimization

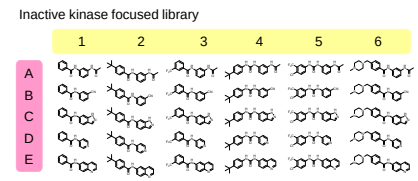
The TR-FRET signals were measured at various concentration of enzyme and Tb-Streptavidin to optimize assay conditions.

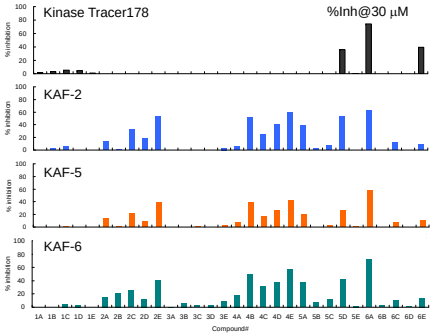


KAF-2, KAF-5 and KAF-6 were selected for further evaluation because the other probes were found to be not stable in a solution.

Validating the TR-FRET assay using KAF-2, 5 and KAF-6

To demonstrate the ability of the novel probes, an inactive kinase focused compound library was screened against inactive form of BTK by optimized the TR-FRET conditions to identify compounds which can bind to the inactive conformation of BTK





Compound	IC50 (μM) KAF-2	KAF-5	KAF-6	Kinase Tracer178
2C	65	73	52	n.d.
2E	23	48	32	n.d.
4B	27	38	28	n.d.
4C	86	157	155	n.d.
4D	31	55	36	n.d.
4E	22	34	25	n.d.
5A	41	50	47	n.d.
5D	30	130	47	47
6A	19	23	13	13
6E	150	163	240	42
CGI1746	0.0032	0.0040	0.0027	0.0028
Staurosporine	0.031	0.066	0.042	0.066

n.d. : signal not detected

KAF-2, 5 and 6 identified 10 hit compounds having potential to bind to the inactive form of BTK.
On the other hand, a commercially available probe Kinase Tracer178 (LanthaScreen® TR-FRET, Invitrogen) detected only 3 hits.

SUMMARY

- We have designed and synthesized novel compounds to probe the inactive conformation of BTK.
- All probe synthesized here retain its affinity to the inactive form of BTK.
- Validation screening study demonstrated that the novel probes could identify several hit compounds which might preferentially bind to the inactive conformation of BTK.
- The methods using novel probes KAF-2, 5 and 6 have great potentials for the discovery of novel lead compounds against inactive states of BTK.

ACKNOWLEDGEMENT

Carna Biosciences Research and Development team and Production and Manufacturing Technology team