Site Specific Biotinylated Protein Kinase is Easy-to-Use Solution in the SPR Technology for Kinase Inhibitor Evaluation

Masaki Gouda [1)], Takeshi Kumagai [2)], Yasuyuki Kirii [1)], Masanori Nakamura [1)], Mariko Hatakeyama [1)], and Koichi Yokota [1)].

1) Carna Biosciences, Inc., 2) Bio-Rad Laboratories K.K. Research Solution Center

Carna Biosciences, Inc., BMA 3F 1-5-5 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047 Japan

Abstract

In characterizing kinase inhibitors for drug discovery research, biophysical assays are increasingly of value for the measurement of parameters such as the rates of association (k_a) and dissociation (k_d) and the dissociation constant (K_D). The Surface Plasmon Resonance (SPR) is the gold standard method to determine K_D values for protein and small molecule interaction. So far, the amine coupling, avidin-biotin interaction and antibody capturing has been utilized to immobilize the protein on the sensor chip. However, the immobilization using amine coupling and the preparation of the biotinylated protein have been one of the bottlenecks. The amine coupling requires acidic condition which leads to lose or lowers kinase activity or disrupts steric structure. The chemical biotinylation probing is also problematic, because it is difficult to control the sites and numbers of the biotinylation and additional purification is also required before use. In addition, antibody capturing lowers the sensitivity because of the larger molecular weight.

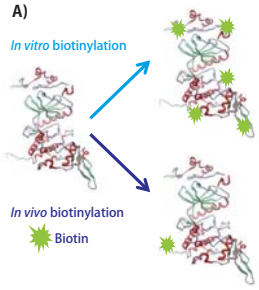
Carna Biosciences recently launched novel tool for the kinase binding assay; BTN-Kinase, which has a single biotinylation at N-terminus region to avoid interference of compound binding and to maintain the kinase activity and an overall functional structure. They are easy-to-use solution for (neutr)avidin coated sensor chip for the SPR measurement.

The kinetic data using some clinical kinase inhibitors, Dasatinib, Lapatinib, and Sunitinib, besides non-specific kinase inhibitor Staurosporine, was measured on a protein interaction array system (ProteOn™ XPR36, Bio-Rad Laboratories). In this system, only we have to do is just diluting the kinase before use with the running buffer. The immobilized BTN-kinases (ALK, BTK, EGFR, FMS, IGF1R, and PLK1) and compounds gave ideal sensorgram in this One-Shot Kinetics™ approach. The combination of the BTN-kinases ligand and One-Shot Kinetics™ provides an effective and easy system for generating robust K_D values and simplifies the assessment of kinase inhibitors with a single analyte injection.

In summary, it was proved that the site specific biotinylated protein kinase is the great tool in the SPR measurement to characterize kinase inhibitor.

Preparation of BTN-Kinase Proteins

A)



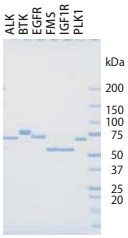
Chemical Reaction

Biotin-NHS reacts with several Lys including ATP binding site.
The modification may result in reduced kinase activity and alteration of structure.
Non-reacted Biotin-NHS may interfere with the binding to avidin or one more purification step necessary.

Enzymatic Reaction

During expression N-terminal specific site is modified by biotin ligase.
Kinase domain is not modified: maintains native kinase activity and structure.
Suitable for both activity- and affinity-based assays.

B)



Preparation of BTN-Kinase Proteins

All human recombinant BTN-kinases were made at Carna Biosciences, Inc. (Kobe, Japan) as indicated in its web site (http://www.carnabio.com).

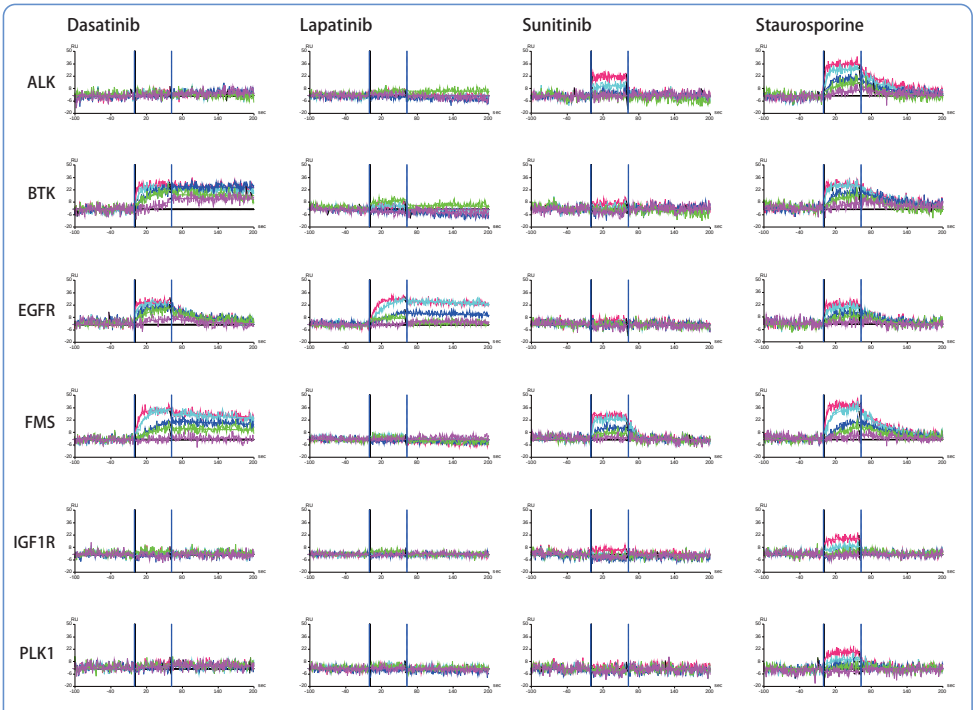
A) Biotinylation of Protein

Biotinylation scheme is shown in figure A). Generally, biotinylation is performed by an *in vitro* chemical reaction method (Light Blue).
In this study, we developed *in vivo* enzymatic reaction (Dark Blue).

B) SDS-PAGE Analysis

Purity was determined by SDS-PAGE analysis (Figure B) with densitometer software. Six purified proteins used in this study were loaded (1μg/lane).
Kinase activities were measured by off-chip mobility shift assay using EZ reader II (Data is not shown, Caliper Life Science).

Result



The concentrations of inhibitor are 1( ), 0.33( ), 0.11( ), 0.037( ), and 0.012( ) μM.

◀ Figure 1 | Kinase inhibitors interaction kinetics to the six different BTN-kinases.

BTN-Kinase Protein Immobilization

Pre-immobilization of Neutravidin (Thermo Scientific) was carried out using ProteOn™ Amine coupling kit (Bio-Rad). Neutravidin was immobilized to a high density on all channels of a ProteOn™ GLH sensor chip (Bio-Rad) by the standard method. PBS-T solution (PBS with 0.005% Tween20) was used as an immobilizing step and temperature was set to 25 ℃. 50 μg/ml of neutravidin in 10mM Sodium Acetate pH 5.0 was injected for 5 min at a flow rate of 30 μl/min.
Immobilization levels for neutravidin were approximately 15,000 to 20,000 RU.
Six BTN-kinase proteins were immobilized on the neutravidin coated sensor chip.
All BTN-kinase proteins were diluted to 50 μg/ml in buffer A (10 mM Tris-HCl, pH7.5, 0.15 M NaCl, 10 mM $MgCl_2$, and 0.05 % Tween 20).
BTN-kinase proteins were injected in all channels for 5 min. at a flow rate 30 μl/min. Injected BTN-kinase proteins showed immobilization levels sufficiently to detect binding of small molecules. After the immobilization of BTN-kinase proteins, running buffer was exchanged to buffer B (10 mM Tris-HCl, pH7.5, 0.15 M NaCl, 10 mM $MgCl_2$, 0.05% Tween 20, and 2% DMSO).

Interaction Analysis

Kinase inhibitor interactions with six immobilized BTN-kinase proteins were examined using One-Shot Kinetics approach at 25 ℃. As kinase inhibitors 3 clinical kinase inhibitors (i.e. Dasatinib, Lapatinib, Sunitinib) and the non-specific kinase inhibitor Staurosporine were chosen.
All inhibitors were serially diluted to five different concentrations from 1 to 0.012 μM, and a blank sample (0 μM) was used as reference. Injection was done simultaneously with six different concentrations of the same inhibitor for 1 min. (Association time) at a flow rate of 50 μl/min., inhibitor dissociation was measured for 3 min. (dissociation time) with a buffer B. This approach did not require additional regeneration step of sensor chip.
The obtained sensorgrams were processed and fitted to determine the dissociation constants using ProteOn Manager™ software (Bio-Rad). For the interactions of the inhibitors, the obtained sensorgrams were globally fitted to the 1:1 biomolecular binding model to calculated kinetic rate constants (k_a, k_d).
All the interaction analysis between the BTN-kinases and inhibitors are illustrated in figure 1 and k_a, k_d, and K_D are shown in table 1.

Table 1 | ▶ Interaction kinetic parameters of kinase inhibitors and BTN-kinases.

	Dasatinib			Lapatinib			Sunitinib			Staurosporine		
BTN-Kinase	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]
ALK	-	-	-	-	-	-	-	-	1.49×10^{-6}	4.86×10^{5}	0.02	3.93×10^{-8}
BTK	4.90×10^{5}	4.17×10^{-4}	8.50×10^{-10}	-	-	-	-	-	-	6.32×10^{5}	0.01	2.11×10^{-8}
EGFR	7.81×10^{5}	0.01	1.74×10^{-8}	9.79×10^{4}	1.20×10^{-3}	1.22×10^{-8}	-	-	-	4.93×10^{5}	0.03	5.12×10^{-8}
FMS	1.62×10^{5}	1.40×10^{-3}	8.64×10^{-9}	-	-	-	8.33×10^{5}	0.08	9.78×10^{-8}	1.95×10^{5}	0.02	8.85×10^{-8}
IGF1R	-	-	-	-	-	-	-	-	-	7.39×10^{4}	0.03	4.54×10^{-7}
PLK1	-	-	-	-	-	-	-	-	-	9.21×10^{4}	0.04	4.52×10^{-7}

K_D value of Sunitinib vs. ALK was calculated by affinity analysis. (-)s were judged to " no-interaction."

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Conclusion

- Carna Biosciences Inc., has successfully developed one-site specific biotinylated protein kinases (BTN-kinases) using *in vivo* biotinylation.
- These BTN-kinases can easily be used for SPR measurement system.
- Six BTN-kinases were analyzed and showed interaction with target kinase inhibitors.
- Staurosporine showed binding to all six BTN-kinases, whereas the kinase inhibitors Dasatinib, Lapatinib and Sunitinib specifically bound with the target kinases.
- Combination of the ProteOn™ XPR36 system and BTN-kinases is suitable for inhibitor selectivity profiling. Robust K_D values could be generated using this application.

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