[124] A New Method to Determine Drug-Target Residence Time of Kinase Inhibitors in Living Cells.

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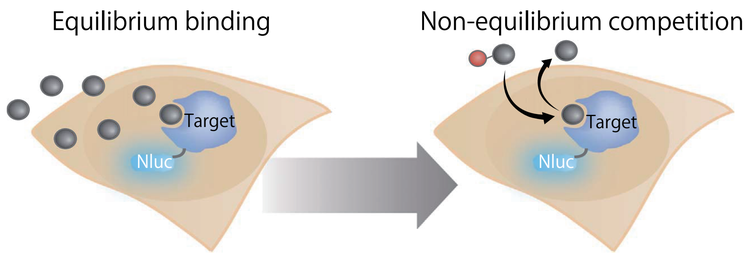
Abstract: Recently, it has become clear that drug-target residence time, in addition to affinity, often drives pharmacodynamic activity and disease efficacy *in vivo*. In the aim of determining the dissociation properties of test compounds, the kinase field has traditionally utilized purified proteins in various competitive probe displacement formats. We propose the method by which the data in the compound washout experiments are fitted to the kinetic equation of Malany as a simple and quantitative approach to determine intracellular residence time of kinase inhibitors.

Intoroduction:

- Kinetic parameters of drug-target binding have been traditionally measured using purified proteins or protein fragments in various competitive probe displacement formats and the data have been analyzed using the approaches described by Motulsky-Mahan.
- A simple and quantitative method to determine the residence time of compounds inside living cells is highly desirable to obtain relevant results which translate with cellular efficacy.



The NanoBRET™ (TE) Intracellular Kinase Assay utilizes BRET in living cells by molecular proximity of the NanoBRET™ Tracer to the NanoLuc® luciferase-fused kinase. After equilibrating with a near-saturating concentration of compound, the cells are washed to remove unbound compound and treated with a near-saturating concentration of a tracer [1].

Data Analysis:

1) One-phase association equation

$$Y = Y_0 + (Y_m - Y_0) \times (1 - \exp(-k_d \times t))$$

Y_0 : BRET ratio at time zero
Y_m : BRET ratio after it reaches a plateau
k_d : Dissociation rate constant of a test compound
t : time

2) Kinetic equation developed by Malany et al. [2, 3]

$$[RL] = k_1 \cdot [R_{tot}] \cdot [L] \cdot (1-\exp(-k_{obs} \times t))/k_{obs} + k_1 \cdot [RI]_{t=0} \cdot [L] \cdot (\exp(-k_{obs} \times t) - \exp(-k_d \times t))/(k_{obs} - k_d)$$

[L] : Tracer concentration
[RL] : Amount of kinase-tracer complexes
$[R_{tot}]$: Total kinase concentration
$[RI]_{t=0}$: Amount of compound-bound kinases at the onset of the incubation with a tracer
k_{obs} : Apparent rate constant of a tracer

From this, the following equation is derived.

$$\frac{[RL]_{Imax}}{[RL]_{I=0}} = \frac{\{(1-\exp(-k_{obs} \times t))/k_{obs} + (\exp(-k_{obs} \times t) - \exp(-k_d \times t))/(k_{obs} - k_d)\}}{(1-\exp(-k_{obs} \times t))/k_{obs}}$$

$[RL]_{Imax}$: Amount of kinase-tracer complexes when cells are pre-incubated with saturating concentration of inhibitor
$[RL]_{I=0}$: Amount of kinase-tracer complexes when cells are pre-incubated with vehicle

At a time point immediately before the BRET ratio of a sample reached its plateau, the k_d was calculated by numerical calculations along with the k_{obs} determined in the same experiment.

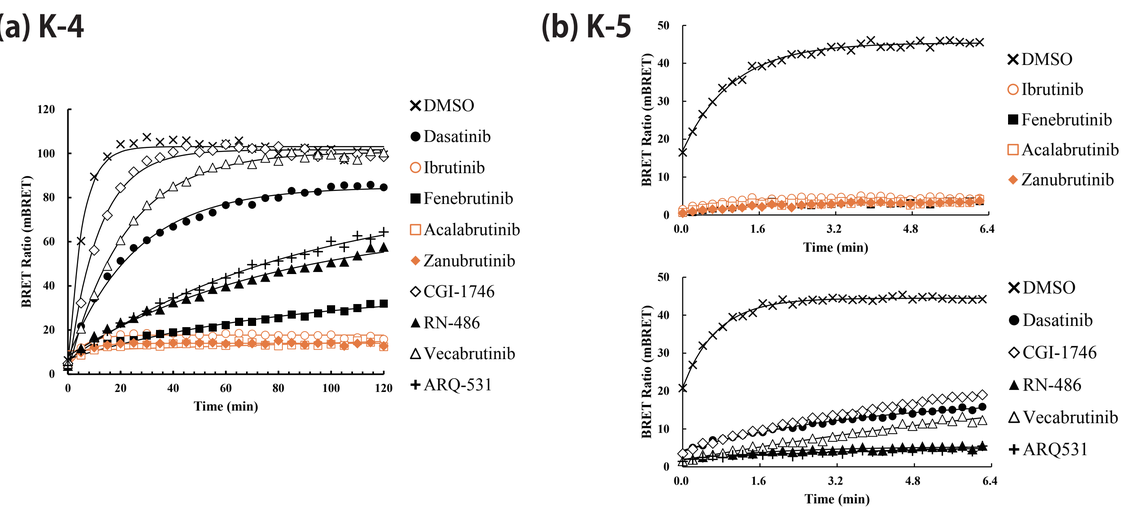
In both methods, the residence time of a test compound is,

$$\tau = 1/k_d$$

Figure 1. The NanoBRET™ assay revealed the longer residence time for the covalent/irreversible BTK inhibitors and slow dissociating reversible inhibitors than for the other reversible inhibitors.

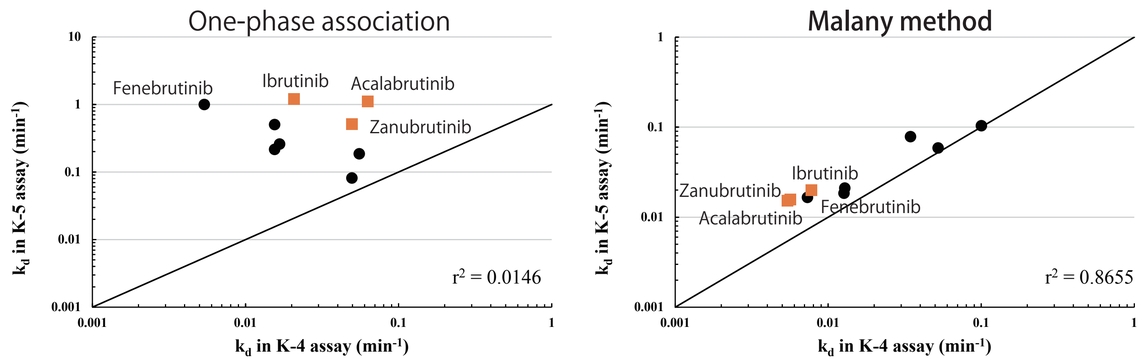
Compound	Binding mode	IC_{50} (nM)	Test concentration (nM)
Dasatinib	reversible	20.0	100
Ibrutinib	irreversible	0.84	3
Fenebrutinib	reversible	6.42	30
Acalabrutinib	irreversible	33.5	100
Zanubrutinib	irreversible	1.93	10
CGI-1746	reversible	66.6	1000
RN-486	reversible	6.46	30
Vecabrutinib	reversible	28.1	300
ARQ-531	reversible	33.4	300

IC_{50} concentrations and test concentrations in the residence time measurement of nine BTK inhibitors tested in this study.



HEK293 cells expressing BTK were pre-incubated with test compounds or vehicle (DMSO) for 2 hours at 37°C followed by a brief washout. The NanoBRET™ Tracer (a) K-4 or (b) K-5 was then added and BRET was repeatedly measured with the Glomax® Discover Multimode Microplate Reader (Promega) equipped with injectors for 120 and 6.2 minutes, respectively.

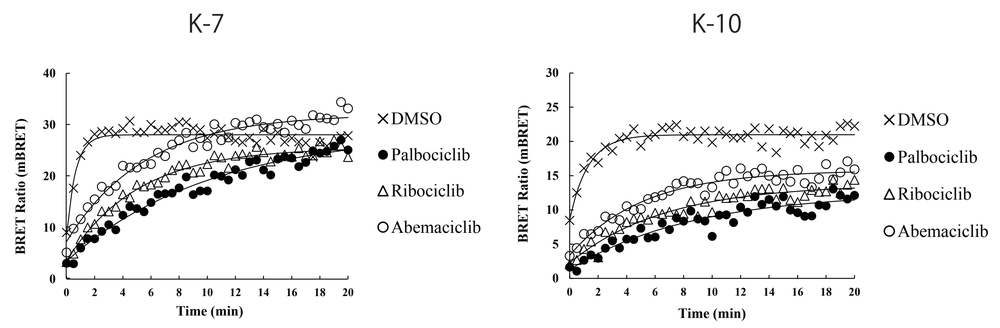
Figure 2. The dissociation rate constants of BTK inhibitors as determined using the Malany approach shows gratifying agreement irrespective of the tracer used.



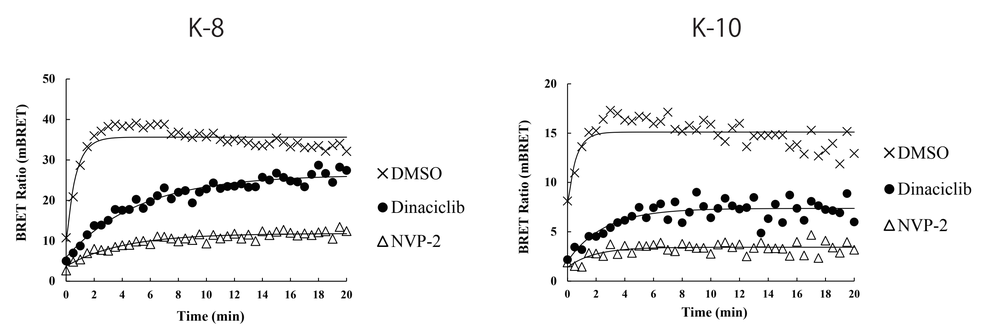
- The dissociation rate constants for irreversible inhibitors (■) i.e. ibrutinib, acalabrutinib, and zanubrutinib were more reasonably quantitated using the Malany method as evidenced by their small rate constants.
- Fenebrutinib showed protracted residence time, which was comparable to that of irreversible inhibitors in the evaluation with the Malany method.

Figure 3. The kinetic properties of CDK inhibitors are successfully evaluated in the NanoBRET™ assays using different tracers.

(1) CDK6/Cyc D1

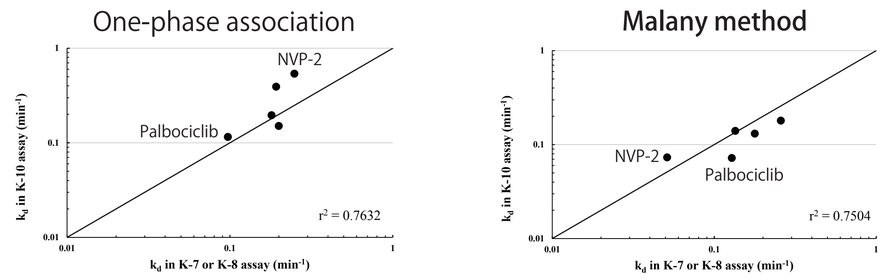


(2) CDK9/Cyc K



HEK293 cells expressing (1) CDK6/Cyc D1 or (2) CDK9/Cyc K were pre-incubated with test compounds or vehicle (DMSO) for 2 hours at 37°C followed by a brief washout. The NanoBRET™ Tracer K-7 or K-10 was utilized for the CDK6/Cyc D1 assay and Tracer K-8 or K-10 for the CDK9/Cyc K assay, respectively. The concentrations of test compounds were as follows; 30 nM (palbociclib), 100 nM (Abemaciclib), 30 nM (Ribociclib), 100 nM (Dinaciclib), and 10 nM (NVP-2).

Figure 4. Correlation of dissociation rate constants determined with the Malany method is also confirmed in the NanoBRET™ CDK6/Cyc D1 and CDK9/Cyc K assays using different tracers.



- Similar dissociation rate constants were obtained for each tracer by using either the one-phase association equation or kinetic equation developed by Malany due to similar kinetic properties of different tracers.
- However, rank order of rate constants was different between these two methods.

References:

[1] Robers MB, et al. Nat Commun. 2015;6:10091.
[2] Malany S, et al. J Recept Signal Transduct Res.2009;29(2):84-93.
[3] Packeu A, et al. Br J Pharmacol. 2010;161(6):1311-28.

Conclusions:

Carna Biosciences is now offering target engagement cellular kinase assay services not only for affinity but also for residence time measurement with high expertise in collaboration with Promega using NanoBRET™ technology.

1. NanoBRET™ (TE) technology enables the compound residence time measurement in living cells.
2. The kinetic equation by Malany well describes similar dissociation rate constants and bridges the difference of tracers.