Broad Kinome Selectivity and Residence Time Analysis in Live Cells with NanoBRET

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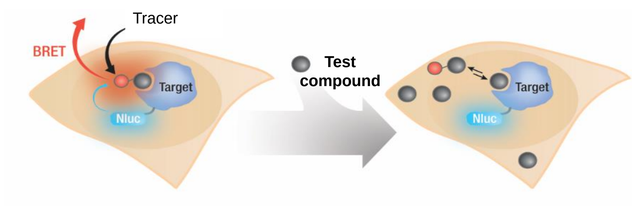
1. Introduction

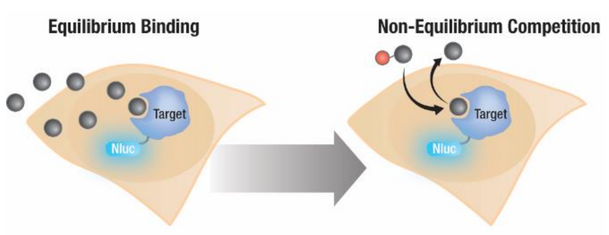
We present the first technique to broadly and quantitatively determine kinase inhibitor potency as well as profile kinase target engagement under physiological conditions, without disruption of cellular membrane integrity. NanoBRET™ enables a biophysical assessment of compound engagement and residence time for chosen intracellular targets. A quantitative capability is achieved in living cells, via energy transfer from cell-permeable tracers reversibly engaged to selected NanoLuc®-tagged target proteins. As the specificity of the BRET signal is dictated by the placement of NanoLuc on the chosen target, a diverse set of broad-coverage tracers support an HTS-compatible method to profile the isozyme-specific affinity and binding kinetics over entire enzyme classes. This technique has enabled a quantitative analysis of compound binding against >200 individual full-length protein kinases, including a key panel of integral membrane receptors. In-cell potency determinations for various types of kinase inhibitors were achieved, including type I, II, and allosteric compounds.. Time-dependent target-compound occupancy (or residence time) can also be obtained with this method. An assessment of kinetic and equilibrium selectivity of various clinically-relevant kinase inhibitors revealed different residence times for compounds with similar equilibrium affinity. The assay was extended to live cell broad kinome selectivity profiling using over 170 kinases. These cellular profiling results revealed an improved selectivity of the compound compared to results obtained by biochemical profiling.

2. Target Engagement (TE) using BRET

Affinity / Potency Determinations

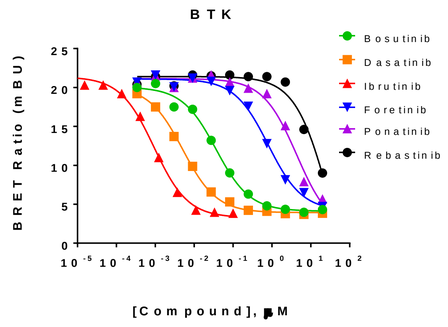


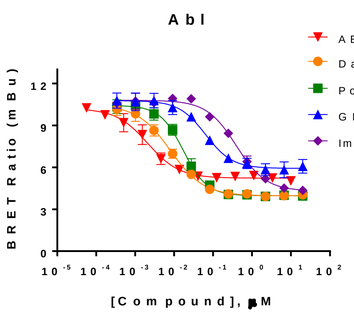
Residence Time Determinations

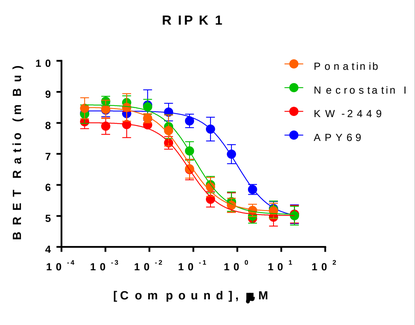


- Target fractional occupancy can be quantified in live cells with NanoBRET. Using the tracer at or below its apparent affinity in a competitive displacement mode, results in a compound IC50 that's a constant value and quantitative.
- Intracellular residence time can be evaluated in a simple format, where test compound is added prior to the tracer.

3. Interrogating Type I, Type II, and Allosteric Kinase Inhibitors





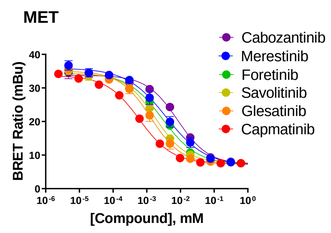


NanoBRET TE experiments were performed with HEK293 cells transiently transfected with Kinase/NanoLuc® fusion proteins. A fixed concentration of tracer approximating the apparent Kd was used.

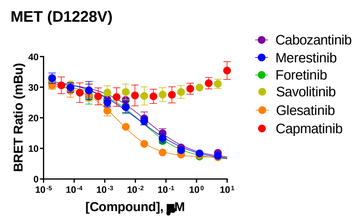
- **Top:** Characterization of type I & II kinase inhibitors at BTK
- **Bottom:** Characterization of type I, type II, & allosteric inhibitors at Abl and RIPK1 kinases

4. Diverse Applications to Explore Inhibitor Pharmacology

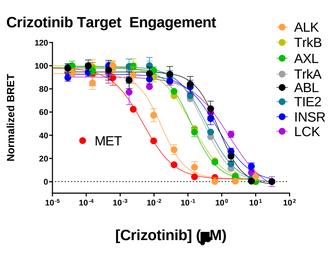
Evaluating diverse chemical matter



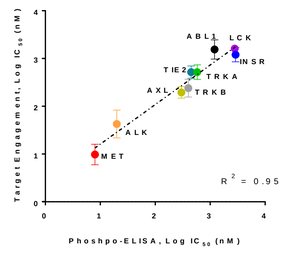
Impacts of clinical mutations



Selectivity among similar targets

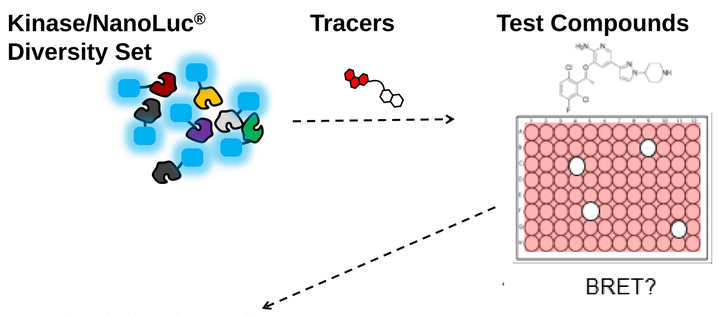


Correlation to phenotype

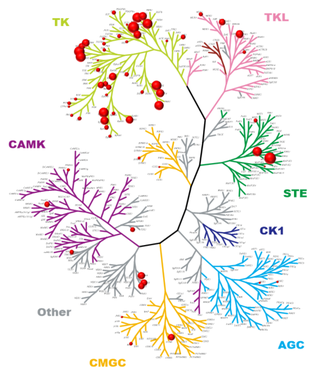


- NanoBRET can be used to evaluate both wildtype and mutant kinases, as shows for MET kinase
- In live cells, NanoBRET data can correlate well with cellular functional assays such as phospho-ELISA

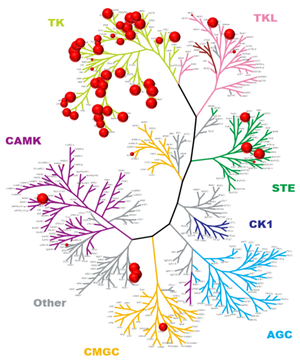
5. Broad Kinome Profiling In Live Cells Reveals Improved Selectivity



1 μM Crizotinib, Live Cell NanoBRET TE



1 μM Crizotinib, QuickScout™ Enzyme Assay

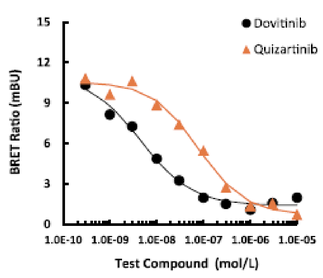


Broad kinome profiling workflow with NanoBRET TE:

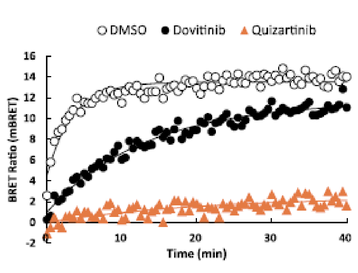
- A kinase library of NanoLuc fusion constructs (178) is reverse-transfected into cells of interest, with each well expressing a unique kinase/NanoLuc fusion. On next day,, target occupancy is determined via NanoBRET. Once occupancy is determined, compound affinity can be determined subsequently.
- A comparison of occupancy vs inhibition of 1μM crizotinib in NanoBRET versus cell-free (QuickScout™, Carna Biosciences). In live cells, crizotinib is more selective for MET and ALK.

6. Intracellular Residence Time and Affinity May Not Always Correlate

FLT3: Equilibrium Binding

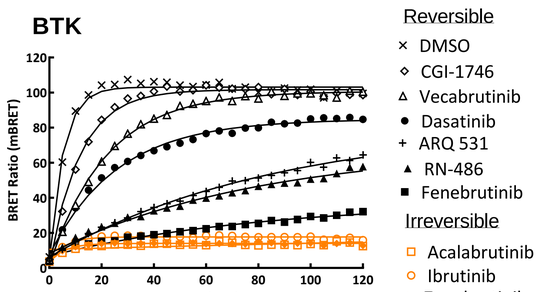


FLT3: Residence Time



- Equilibrium (steady-state) binding may not always correlate with binding kinetics.
- Despite weaker affinity, quizartinib exhibits more durable inhibition than dovitinib following a washout of live cells.

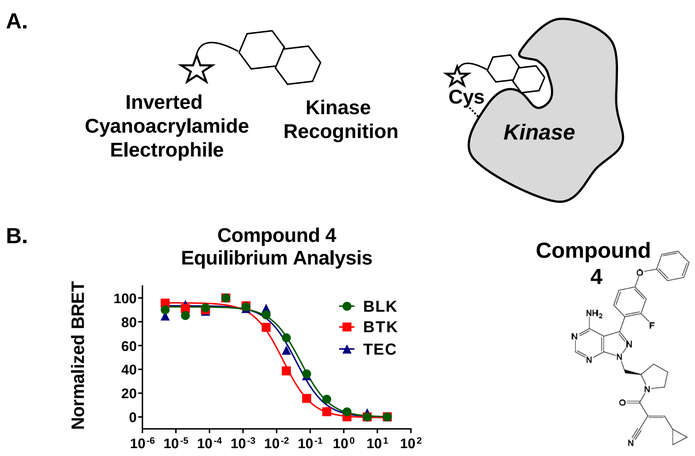
7. Residence Time Can Be Measured with Reversible and Irreversible Inhibitors

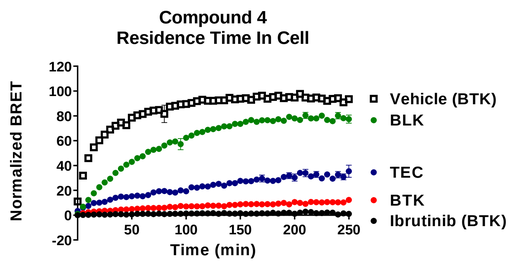


Compound	Binding mode	IC_{50} (nM)	Test concentration (nM)
Dasatinib	reversible	20.0	100
Ibrutinib	irreversible	0.84	3
Fenebrutinib	reversible	6.42	30
Acalabrutinib	irreversible	33.5	100
Zanubrutinib	irreversible	1.93	10
CGI-1746	reversible	66.6	1000
RN-486	reversible	6.46	30
Vecabrutinib	reversible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

HEK293 cells expressing BTK were preincubated with test compounds or vehicle (DMSO) for 2hrs at 37°C followed by a brief washout. NanoBRET Tracer K-4 was then added and BRET was repeatedly measured with the GloMax® Discover Multimode Reader (Promega) equipped with injector.

8. Kinetic Selectivity with a Reversible Covalent Inhibitor





Kinase	τ (hrs)
BTK	>10
TEC	9.1
BLK	1.1

A. The reversible covalent inhibitor compound 4 (Nature Chem. Bio. 11:525 (2015)), provided by Michael Bradshaw of Principia Biopharma, targets a non-catalytic cysteine in BTK.

B. It shows similar cellular potencies for BLK, BTK, & TEC using NanoBRET TE kinase assays.

C. Compound 4 shows kinetic selectivity for BTK using NanoBRET TE kinase residence time analysis. Ibrutinib was used as a control, as it's a covalent BTK inhibitor targeting the same cysteine in BTK.

9. Conclusions

Cell permeable Tracers have been developed that allow NanoBRET TE assays for >200 full length kinases. Carna Biosciences offers services to support kinase target engagement with NanoBRET.

NanoBRET TE Kinase assays broadly enable the quantitative determination of compound affinity inside cells, including various Type I, II, and allosteric kinase inhibitors.

Monitoring both potency and residence time in cells using NanoBRET TE assays can reveal equilibrium and kinetic selectivity of kinase inhibitors, offering unique opportunities.

For kinases, intracellular selectivity and affinity profiles can differ dramatically from those determined biochemically, underscoring the need for quantitative methods to measure compound engagement and occupancy in live cells.

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