APPLICATION NOTE

HTRF kinase binding assays: Sunitinib-Red validation.

This note presents a new HTRF® kinase binding assay that combines the usage of a Sunitinib-Red ligand with a tagged kinase enzyme and its corresponding anti-tag- Europium labeled detection reagents.

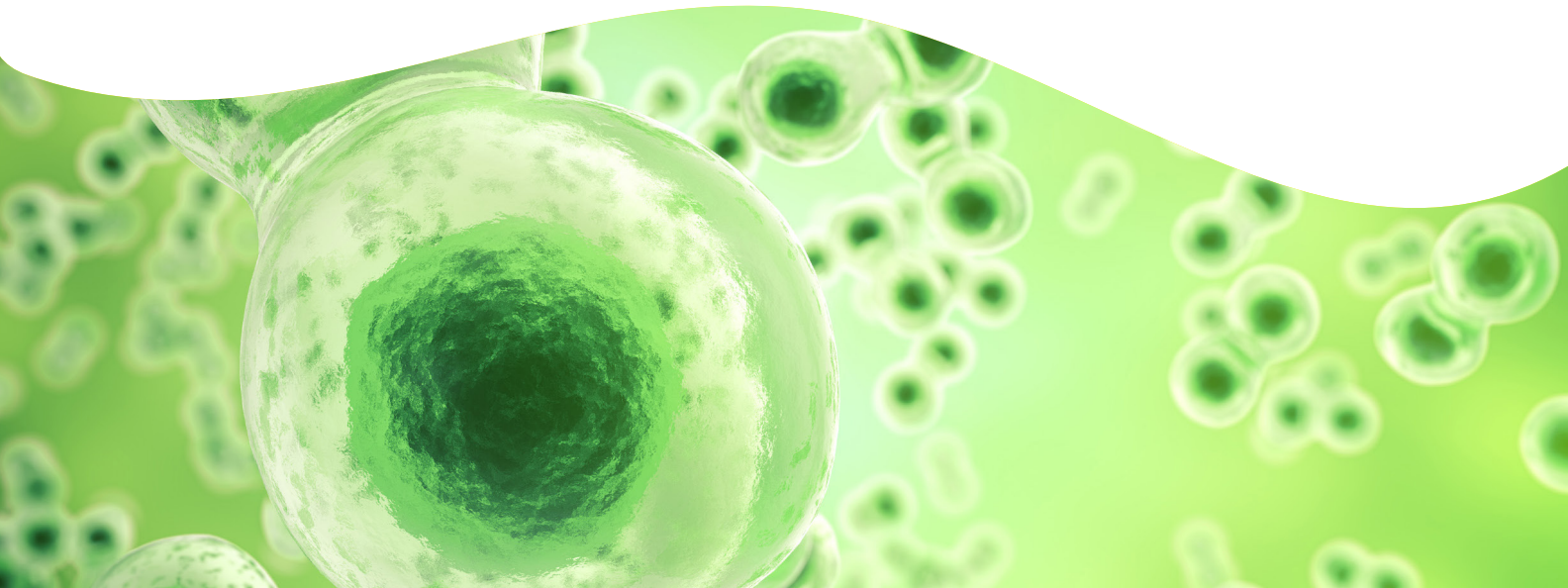
Abstract

Selection of kinase inhibitors has resulted in numerous success stories for novel drug identification especially related to cancer treatment and metabolic diseases. Biochemical enzymatic assays such as KinEASE® are sensitive and efficient methods for drug screening, but the need for robust orthogonal assays to study or further validate the inhibitors' binding mode still remains high.

This application note presents a new HTRF kinase binding assay that combines the usage of a Sunitinib-Red ligand with a tagged kinase enzyme (GST, 6His, or Biotin tagged) and its corresponding anti-tag-Europium labeled detection reagents. Here, 4 examples representative of the different tag formats are investigated in depth.

The data obtained for PDGFRb and KIT demonstrate that the Sunitinib-Red analog enables the accurate characterization of known type-I/II inhibitors independently of the enzyme detection format, confirming the pharmacological relevance of the kinase binding platform.

Revvity's comprehensive offer includes 3 Discovery Kits comprising three fluorescent tracers, Kinase Binding Buffer and either Eu cryptate labeled anti-GST, anti-6HIS Mab, or streptavidin. The individual components are also offered as spare reagents for extended pharmacological studies. With the three fluorescent tracers we estimate that 80% of the Kinome is covered. For further details, see the HTRF Kinase Binding User Guide.



Assay principle

The assay is based on an HTRF sandwich format using either Anti-Tag-Eu cryptate Mab or Streptavidin-Eu cryptate and a fluorescent derivative of Sunitinib (Sunitinib-Red, 62KB03REDC/E).

When a GST, 6HIS, or biotinylated kinase is present an HTRF signal is generated. Upon adding competitive type I/II inhibitors of the ATP binding site or allosteric type III inhibitors, the fluorescent Sunitinib is displaced and the HTRF signal disappears (see below).

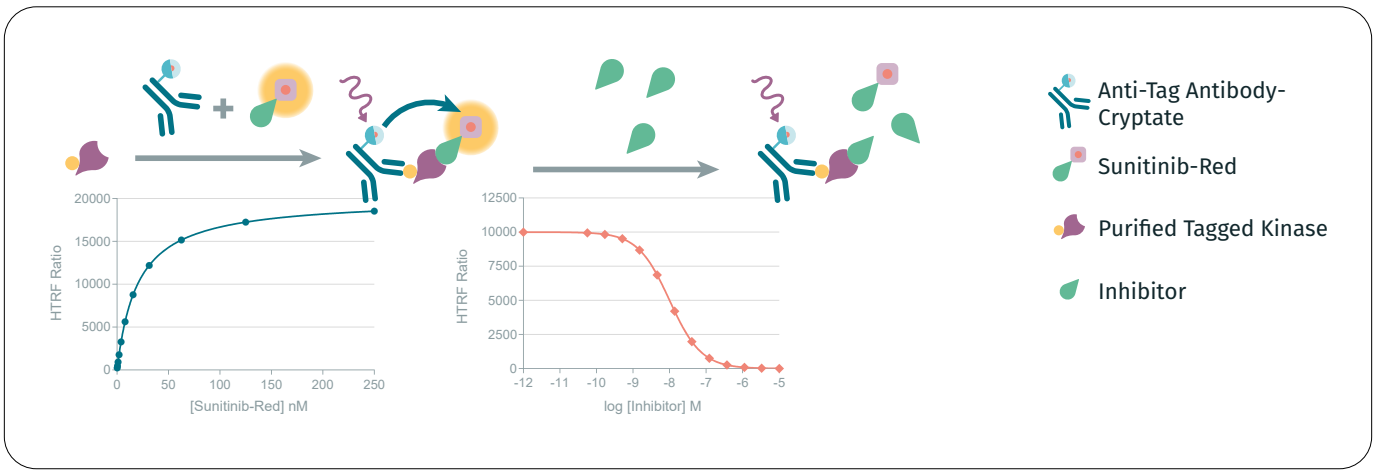


Figure 1: HTRF assay principle In a typical kinase binding assay, a purified GST-tagged Kinase at 5 nM is incubated with MAb Anti-GST Eu cryptate and a 2-fold dilution series from 0-250 nM of Sunitinib-Red, all diluted in the Kinase binding buffer. In order to determine non-specific binding, the kinase is removed. An HTRF signal is generated which is dependent on the concentration of Sunitinib-Red bound to the GST-Kinase. After determining the Kd of the tracer to your GST-Kinase of interest, the affinity of type I and II inhibitors to the ATP binding site can be assessed. A competitive binding assay is carried out by adding a fixed concentration of Sunitinib-Red at or near the Kd and a dilution series of your compound of interest

Saturation binding assay: Kd determination on GST-kinases

Determination of the Sunitinib-Red's Kd on the tagged kinase of interest

The first step in the development of the kinase inhibitor assay is to identify the optimal tracer concentration for the kinase of interest.

The dynamic range of the assay will depend on the kinase concentration. We have used kinase concentrations of 5 nM and a concentration range from 0-250 nM of fluorescent tracer to determine the tracer Kd on several tagged kinases.

Tracer Kds for GST-tagged kinases

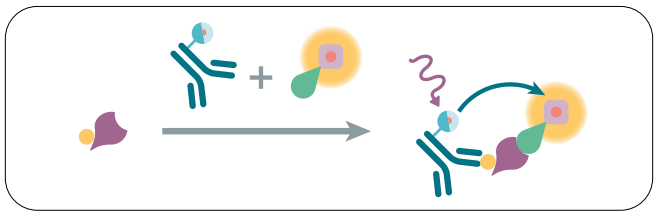


Figure 2: HTRF assay principle for GST-tagged kinases

The dissociation constants (Kd) of Sunitinib-Red (#62KB03REDC/E) on two GST-tagged kinases (5 nM) obtained from Carna Biosciences were measured using anti-GST Eu cryptate MAb (#62KBGSTKAF/B) and a dilution series of Sunitinib-Red. The results are shown below.

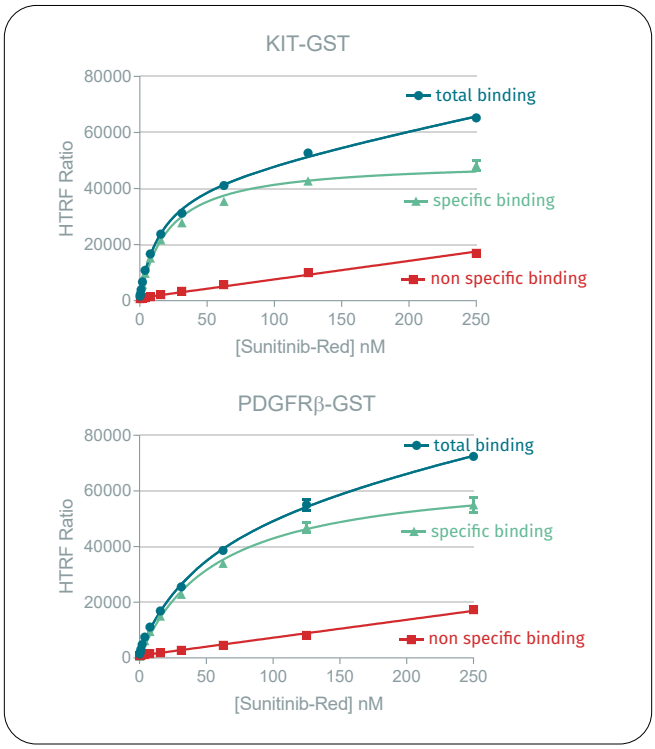


Figure 3: Analysis of KIT-GST and PDGFRβ-GST Saturation binding curves of Sunitinib-Red on 5 nM KIT-GST or PDGFRb-GST measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-GST, 5 µL of MAb anti-GST Eu cryptate, and 5 mL of Sunitinib-Red from 0-250 nM all diluted in the 'Kinase Binding Buffer'. For the non-specific binding signal 5 µL of kinase-GST was replaced by the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation

The Kd values of Sunitinib-Red obtained on the GST tagged kinases are shown in the table below. These results demonstrate that Sunitinib-Red readily binds on these GST-tagged kinases, with good affinities giving high assay windows. Moreover, the signal is specific, as shown by the difference from the signal generated without Kinase.

Kinase	Concentration	Type	Ref carna	Kd (nM)
KIT (CD117)	5 nM	RTK	08-156	21
PDGFRb	5 nM	RTK	08-158	56

*The Kd may vary depending on the source of the GST-kinase used.

Saturation binding assay: Kd determination on 6HIS-kinases and biotin-kinases

Sunitinib-Red's Kds for 6-HIS-tagged kinases

Kd measurements using Sunitinib-Red are not only limited to GST-tagged kinases. Using Cisbio's Anti 6HIS Europium cryptate MAb, elevated assay windows and representative Kd values can be obtained on 6-HIS tagged kinases.

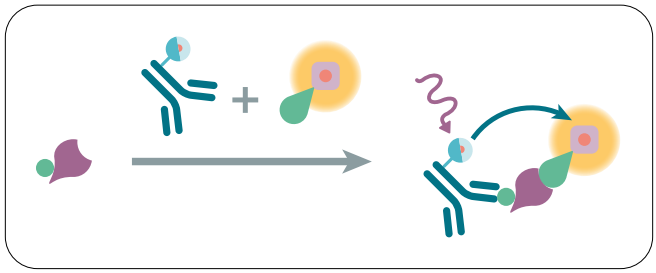


Figure 4: HTRF assay principle for 6-HIS-tagged kinases

The dissociation constant (Kd) of Sunitinib-Red on 6-HIS tagged PDGFRb was measured using anti-6HIS Eu cryptate MAb (#62KBHISKAF/B) and a dilution series of Sunitinib-Red. The results are shown below.

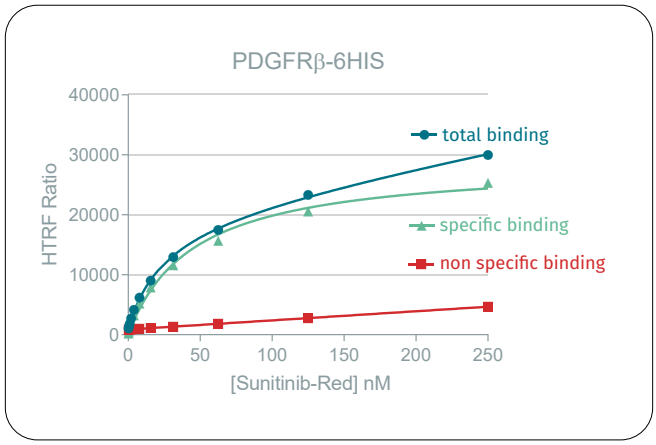


Figure 5: Analysis of PDGFRβ-6HIS Saturation binding curves of Sunitinib-Red on 5 nM PDGFRb-6HIS measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-6HIS, 5 mL of MAb anti-6HIS Eu cryptate, and 5 µL of Sunitinib-Red from 0-250 nM, all diluted in the 'Kinase Binding Buffer'. For the non-specific binding signal, 5 µL of kinase-6HIS was replaced by the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation

Saturation binding assay: Kd determination on 6HIS-kinases and biotin-kinases

Analyses of the data revealed a Kd of 45 nM and an assay window of 10 at Kd. Interestingly, the Kd of the 6HIS tagged kinase is very similar to that of the GST-tagged kinase (56 nM), demonstrating that both tags can be used in the HTRF kinase binding assay.

Sunitinib-Red's Kds for N-terminal biotinylated kinases

Because of the strong biotin-streptavidin interaction the assay format will be particularly advantageous, as an HTRF kinase binding assay can be set up for more advanced applications such as measuring kinetic binding. Although this application is not shown here, we were able to set up a proof-of-concept by combining 5 nM of KIT-BTN (#08-456-21N-Carna Biosciences) and Streptavidin-Eu cryptate (#62KBSAKAF/B).

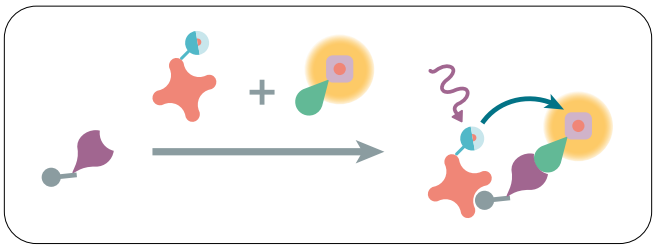


Figure 6: HTRF assay principle for N-terminal biotinylated kinases

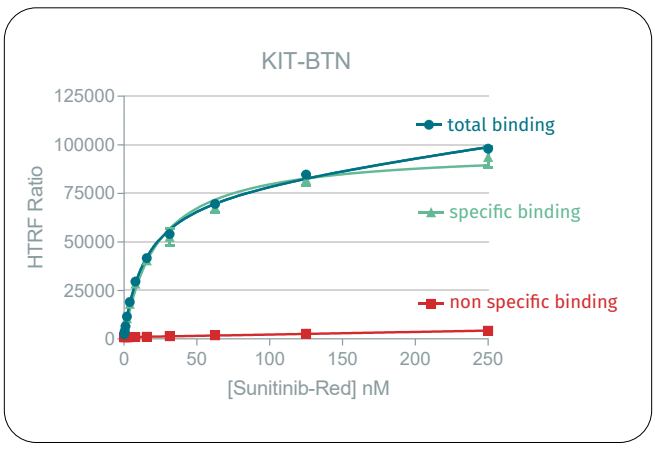


Figure 7: Analysis of KIT-BTN Saturation binding curves of Sunitinib-Red on 5 nM KIT-BTN measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-BTN, 5 µL of Streptavidin Eu cryptate, and 5 µL of Sunitinib-Red from 0-250 nM, all diluted in the 'Kinase Binding Buffer '. For the non-specific binding signal, 5 µL of kinase-BTN was replaced by the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation

Analyses of the data revealed a Kd of 22 nM and an assay window of 45 at Kd. The Kd of the biotinylated kinase is very similar to that of the GST-tagged kinase (21 nM), demonstrating that both tags can be used in the HTRF kinase binding assay.

Competitive inhibition assay

Determination of IC50/Ki of the inhibitor of interest

The simple assay format makes it easy to screen inhibitors without the need of specific substrates nor the presence of ATP. After determination of the tracer Kd, inhibition assays can be set up at tracer concentrations varying between the Kd and 4x Kd to maintain optimal conditions. The optimal tracer concentration depends on the dynamics range observed for the kinase of interest. For further details about the selection of optimum tracer concentration please see the HTRF Kinase Binding User Guide. Either a fixed concentration (for high throughput studies) or a dose response curve can be determined for inhibition and pharmacological studies.

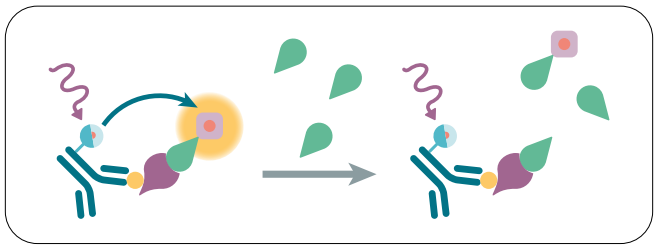


Figure 8: HTRF competitive inhibitor assay principle

Inhibitor testing for GST-PDGFRb

A panel of well known kinase inhibitors was screened on GST-PDGFRb using Sunitinib-Red at 56 nM and the results are shown below. The IC_{50} values measured were used to calculate the Ki potency using the Cheng-Prusoff [1] equation below:

$$K_i = \frac{IC_{50}}{\left(1+\left(\frac{Sunitinib\text{-}Red}{K_d}\right)\right)}$$

The results were compared to data generated by Karaman et al. [2] and a clear correlation with these data is demonstrated proving that these assays give representative Ki values.

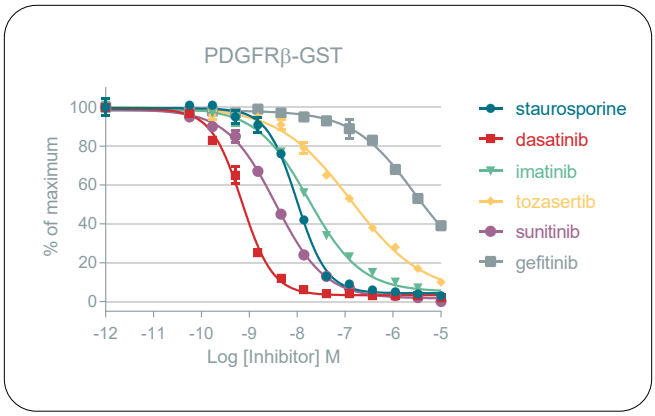


Figure 9: Analysis of Inhibitor screening for PDGFRβ-GST Dose response curves of a panel of kinase inhibitors on 5 nM GST-PDGFRb measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase-GST, 5 µL of anti-GST Eu cryptate MAb, and 5 µL of Sunitinib-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation

Competitive inhibition assay

Inhibitor	GST-PDGFRb		
	IC50 (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	9.8	4.9	1.8 [2]
Dasatinib	0.7	0.35	0.63 [2]
Imatinib	17.3	8.6	14 [2]
Tozasertib	1 325	663	310 [2]
Sunitinib	3.5	1.8	0.08 [2], 5.7 [3]
Gefitinib	2 674*	1 337	>10 000 [2]
Gefitinib	>10 000	>10 000	>10 000 [2]
Sorafinib	145	73	540 [2], 22 [4]

* = incomplete inhibition

Inhibitor testing for 6HIS-PDGFRβ and BTN-KIT

The panel of kinase inhibitors was also screened on 6HIS-PDGFRb and BTN-KIT using Sunitinib-Red at its Kd (45 nM for PDGFRb and 22 nM for KIT), and the results are shown below. An analysis similar to that described for the GST-tagged kinases was performed. Overall, a good correlation between these results and the literature data was observed, confirming the suitability of the Sunitinib-Red tracer for performing pharmacological characterization of inhibitors. Moreover it is worth noting that the Ki results obtained on the panel of inhibitors were in the same range whatever the tag used for PDGFRb (GST-PDGFRb and 6HIS-PDGFRb).

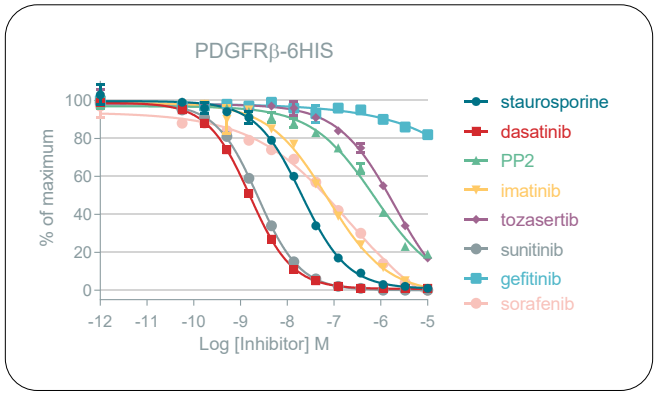


Figure 10: Analysis of Inhibitor screening for PDGFRβ-6HIS Dose response curves of a panel of kinase inhibitors on 5 nM 6HIS-PDGFRb measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase, 5 µL of anti 6HIS-Eu cryptate Mab, and 5 µL of Sunitinib-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1H of incubation

Inhibitor	6HIS-PDGFRb		
	IC50 (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	20.3	10	1.8 [2]
Dasatinib	1.6	0.8	0.63 [2]
PP2	732	366	nr
Imatinib	73	37	14 [2]
Tozasertib	1964	982	310 [2]
Sunitinib	2.4	1.2	0.08 [2], 5.7 [3]
Gefitinib	>10 000		>10 000 [2]
Sorafinib	198	99	37 [2], 57 [4]

nr = not reported

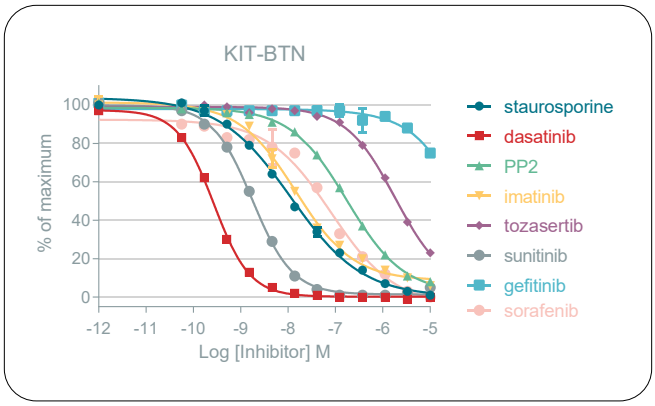


Figure 11: Analysis of Inhibitor screening for KIT-BTN Dose response curves of a panel of kinase inhibitors on 5 nM BTN-KIT measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase, 5 µL of Streptavidin-Eu cryptate, and 5 µL of Sunitinib-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1H of incubation

Inhibitor	BTN-KIT		
	IC50 (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	11.4	5.7	19 [2], 22 [5]
Dasatinib	0.26	0.13	0.62 [2], 0.38 [5]
PP2	174	87	nd
Imatinib	14.6	7.3	14 [2], 26 [5]
Tozasertib	1823	912	240 [2], 1590 [5]
Sunitinib	1.8	0.9	0.37 [2], 0.8 [5]
Gefitinib	>10 000		10 900 [5]
Sorafinib	81.8	41	31 [2], 5.7 [4]

References

1. Y.C Cheng, W.H. Prusoff., Biochem. Pharmacol. 22 (1973) 3099-3108.
2. M.Z. Karaman et al., Nat. Biotechnol. 26 (2008) 127-132.
3. D. Kitegawa et al., Gene Cells. 18 (2013) 110-122.
4. S.M. Wilhelm et al., Cancer Res. 64 (2004) 7099-7109.
5. V. Georgi et al., J. Am. Chem. Soc. 140 (2018) 15774-15782.

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