Proposal of ATP Competitive Study
- HotSpot Kinase Assay -

This proposal is to test whether the customer's compound is competitive with respect to ATP or not. The results include kinetic constants, K_m for ATP and K_i for the inhibitor, as well as mode of inhibition at a constant substrate concentration. The assay designed here is based on the previous data RBC performed for the customer. **If the compound has not been tested with the target kinase by RBC, the IC_{50} determination is required prior to proposal.**

Materials and reagents:

Compound information from the customer

Kinase reaction buffer: 20 mM HEPES-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.1 mM Na_3VO_4, 0.02 mg/ml BSA, 2 mM DTT, and 1% DMSO.

Kinases: (Example)
ROCK2; Recombinant Human protein (amino acids 5-554), N-terminal GST-tagged, expressed in Sf9 cells. Mw=88 kDa.

Substrate for kinase: (Example)
For ROCK2; Long S6 kinase substrate peptide,
[KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK], Mw=3,630 Da

Standard reaction conditions (unless otherwise specified):
1 nM ROCK2, 20 μM peptide substrate, and varied ATP

Experimental Procedures:

The kinase assay will be performed at room temperature. Compounds will be added 10-dose IC_{50} mode into Enzyme/substrate mixture using acoustic technology, and pre-incubated for 20 min* to ensure compounds are equilibrated and bound to the enzyme. Then 5 concentrations of ATP will be added to initiate the reaction. The activity will be monitored every 5-15 min for a time course study. ATP and compound concentrations will be tested as follows:

1. K_i determination for kinase

ATP concentrations will be tested: 5 concentrations of ATP; generally at 0.25xKm, 0.5xKm, 1xKm, 2xKm, and 4~5xKm if possible.

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Compound concentrations will be tested: 10-dose IC_{50} with 3-fold serial dilution started at ? μM (the 10-dose range will be determined under standard conditions (10 μM ATP) prior to MOA studies).

Time points will be measured: 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min

* Standard pre-incubation time is 20 min.

Data Analysis:

The reactions will be monitored every 5-15 min to obtain progress curves with time course. At each time point, radioisotope signal (^{33}P) will be converted into "μM phosphate transferred to substrate", and will be plotted against time. The slopes of initial linear portion of progress curves will be obtained by linear regression in Excel. The slopes (or velocity; μM/min) will be then plotted against ATP concentrations for Michaelis-Menten plot, and subsequent Lineweaver-Burk plot (double-reciprocal plot), using GraphPad Prism software. The results will be further analyzed by global fit using GraFit software with the "Mixed Inhibition" equation (1).

$$v = \frac{V_{max}.[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+\left(1+\frac{[I]}{K_i'}\right)[S]} \quad (1)$$

Where v is velocity, [S] is substrate (ATP) concentration, [I] is inhibitor concentration, K_i is inhibitor affinity for enzyme, and K_i' is inhibitor affinity for enzyme/ATP complex. High number (infinity) of K_i' values mean almost no inhibitor affinity for enzyme/ATP complex, which turns pure competitive inhibition. If the compound is noncompetitive with respect to ATP, K_i and K_i' values will be equal.

The report in PDF file will contain detailed assay performed, data and figures, analyses results, and summary table of kinetic constants and mode of inhibition.

Limitations of this proposal:

1. This proposal is designed for "simple and reversible" inhibitors. If the inhibitor is as follows, further studies may be needed:
   a. Time dependent inhibitor: The progress curves will be non-linear, which makes the analyses difficult. Since the compound is pre-incubated for 20 min already, the curve fitting for on-rate will not be accurate. It needs different assay design for determination of on-rate (i.e., k observe).

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b. Tight-binding inhibitor: The tight-binding inhibitor binds to the enzyme at 1:1 molar ratio, and apparent IC_{50} or K_i will be lower than the enzyme concentration. It needs different assay design for K_i determination.

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