One-Pot Measurement of the Kinetic Parameters K_I, k_{inact}, and Time-Dependent IC_{50} for Analysis of Covalent Small Molecule Kinase Inhibitors

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Background

Drug discovery has historically concentrated on inhibitors which reversibly bind their targets. However, the success of compounds such as ibrutinib have renewed interest in covalent inhibitors, which form a covalent bond and thus irreversibly inhibit their target.[1] These drugs dissociate pharmacokinetics from pharmacodynamics and have a prolonged duration of action compared to a reversible inhibitor due to their kinetic profile. The two step process of loading onto the target followed by covalent adduction lends interesting kinetics to the reaction (fig. 1).[2]

$$K_I = (k_{off} + k_{inact})/k_{on}$$

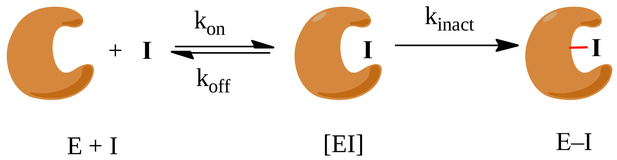

Figure 1 Kinetic scheme of kinase (orange shape) and irreverisible inhibitor (**I**); [EI] denotes the inhibitor-enzyme complex whereas E-I denotes the inhibitor-enzyme adduct.

Describing the potency of inhibitors is necessary for comparison between two small molecule constructs. Reversible inhibitors are readily described by IC_{50} assays, however the kinetics of irreversible inhibitors make IC_{50} measurements time-dependent and thus inappropriate for description of covalent inhibitors. Instead, k_{inact}/K_I is considered the standard of measurement for covalent inhibitors because it accounts for both reactivity and potency in a time-independent manner.[2,3] Unfotunately, current assay methodology for determning k_{inact} and K_I require cumbersome endpoint analysis over many hours and represent an inefficient means of analysis.[4] We therefore strove to design and implement a higher-throughput method for measureing covalent kinase inhibitors utilizing an off-chip mobility shift assay.

Methods

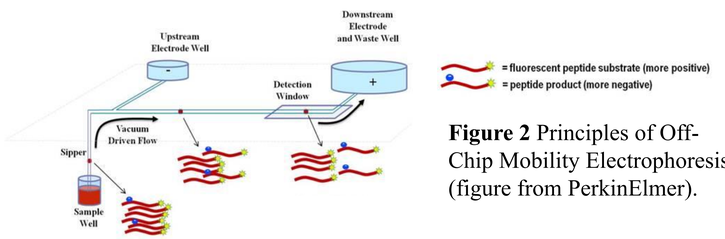

Figure 2 Principles of Off-Chip Mobility Electrophoresis (figure from PerkinElmer).

The LabChip3000 EZ Reader (PerkinElmer) was utilized for these experiments. All dilutions and reactions were carried out in a kinase reaction buffer (100 mM Hepes pH 7.3, 0.015% Brij-35, 0.004% Tween-20, 10 mM $MgCl_2$ and 2 mM DTT) and electrophoretic separations were achieved using separation buffer (100 mM Hepes pH 7.3, 0.015% Brij-35, 1 mM disodium EDTA, 0.1% coating reagent 3, 5% DMSO and 1X coating reagent 8). A 384-well plate was loaded with inhibitor, peptide substrate (final concentration = 2 uM), [ATP] = K_M, and enzyme at a concentration which gave 30% conversion over an hour. As the peptide is phosphorylated by functional enzyme, product and substrate can be separated by electrophoresis and detected by a fluorescent tag (6-carboxyfluoroscein).

Results

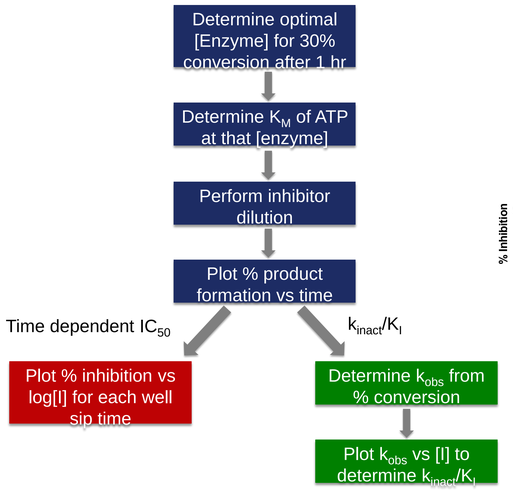

Figure 3 Workflow for determining k_{inact}, K_I, and time-dependent IC_{50}

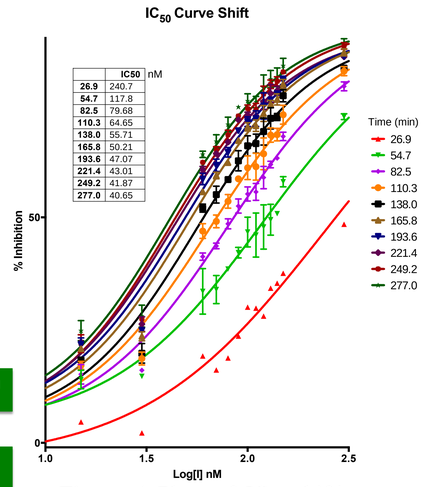

Figure 4 Plots of IC_{50} shifting over time for BMX-IN-1 and BMX. IC_{50} values are reported in the insert.

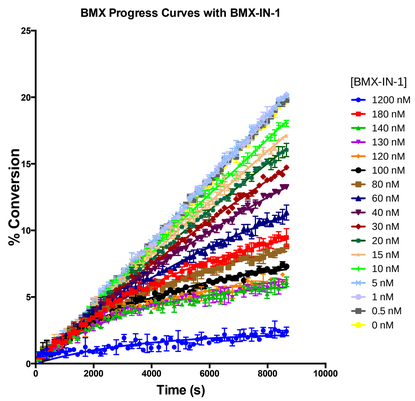

Figure 5 Progress curve of BMX doped with indicated concentrations of covalent inhibitor BMX-IN-1. These data were fit to equation and best fits by least squares were found:

$$\%P = (V_0/k_{obs})^* (1\text{-}\exp(-k_{obs}t))$$

	Best-fit values		Std. Error		
[BMX-IN-1]	V_0	k_{obs}	V_0	k_{obs}	R^2
1200 nM	0.00065	0.000278	5.31E-05	3.78E-05	0.6029
180 nM	0.002039	0.000168	3.12E-05	5.94E-06	0.9885
140 nM	0.001815	0.000293	4.38E-05	1.14E-05	0.9654
130 nM	0.001822	0.000292	3.93E-05	1.02E-05	0.9732
120 nM	0.001856	0.000271	3.68E-05	9.08E-06	0.9778
100 nM	0.001892	0.000231	3.42E-05	7.78E-06	0.9819
80 nM	0.001983	0.00019	3.13E-05	6.35E-06	0.9869
60 nM	0.00199	0.00011	2.85E-05	5.06E-06	0.991
40 nM	0.001985	6.3E-05	1.71E-05	2.82E-06	0.9971
30 nM	0.002024	4.22E-05	1.67E-05	2.62E-06	0.9975
20 nM	0.002046	2.16E-05	1.82E-05	2.72E-06	0.9973
15 nM	0.002104	1.36E-05	2.08E-05	2.99E-06	0.9967
10 nM	0.002166	7.14E-06	1.77E-05	2.44E-06	0.9978
5 nM	0.00224	-1.02E-05	1.59E-05	2.07E-06	0.9984
1 nM	0.002162	-1.94E-05	1.63E-05	2.17E-06	0.9982
0.5 nM	0.002184	-1.32E-05	1.39E-05	1.85E-06	0.9987
0 nM	0.002101	-2.13E-05	1.35E-05	1.84E-06	0.9987

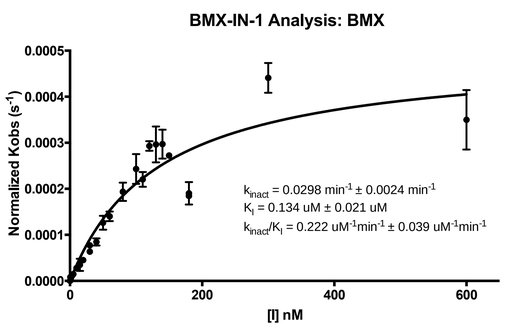

Figure 6 k_{obs} data from fits of several progress curves, such as that found in figure 5, were fitted to the following equation:

$$k_{obs} = k_{inact}[I]/(K_I+[I])$$

Best fits by least squares for k_{inact} and K_I were found, and k_{inact}/K_I was calculated. These values are shown in the insert.

Discussion

The results presented here validate the methodology taken to accomplish a one-pot determination of kinetic parameters of covalent inhibitors. These experiments were able to accurately and reproducibly determine k_{inact} and K_I, in addition to the time-dependency of IC_{50} for a covalent inhibitor of BMX, BMX-IN-1. This process allows for the high-throughput characterization of covalent inhibitors of kinases and can be extrapolated to a plethora of inhibitor-target systems. There are several limitations of this assay that merit discussion. First, the instrument can only take measurements every ~100 seconds due to a thorough wash-out step. Therefore, an extraordinarily fast inhibitor may covalently inhibit the target within a few cycles leaving very few data points to accurately assess k_{inact} and K_I (fig. 7). In this instance, a novel analytical method will be necessary to determine inhibitor potency. Second, an extremely slow enzyme would be very difficult to work with due to the poor accuracy of the EZ Reader when % conversion is very low (fig. 7). A possible solution for this instance is to allow a period of uninhibited enzymatic activity and then add inhibitor in order to raise the % conversion above the threshold of sensitivity. A correction factor would be used in the initial equation to account for the uninhibited activity. Interestingly, a surface generated from the equation $\%P = (V_0/k_{obs})^* (1\text{-}\exp(-k_{obs}t))$ when $k_{obs} = k_{inact}[I]/(K_I+[I])$, where arbitrary values for time, inhibitor concentration, and percent conversion are selected, show the importance of K_I versus k_{inact} (fig. 8). This shows it is easier to make an inhibitor more potent by reducing the K_I relative to an increase in k_{inact} and directs medicinal chemists to prioritize on optimizing K_I before improving k_{inact}.

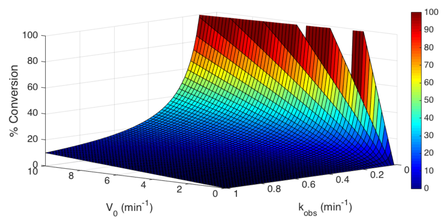

Figure 8 Surface plot of equation $\%P = (V_0/k_{obs})^* (1\text{-}\exp(-k_{obs}t))$ where t = 360 min. Intensity bar is shown on the right (0%-100%).

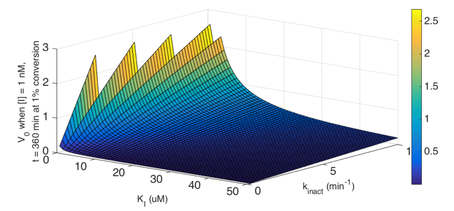

Figure 9 Surface plot of equation $\%P = (V_0/k_{obs})^* (1\text{-}\exp(-k_{obs}t))$ when $k_{obs} = k_{inact}[I]/(K_I+[I])$; arbitrary values are shown on the z-axis. Intensity bar is shown on the right (0-2.5 uM^*min^{-1}).

References

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Special thanks to the Nathanael Grey lab at Dana Farber Cancer Institute for BMX-IN-1 and our funding, CPRIT RP140233.