Determination of Drug Residence Time in a HTS Format using Kinetic Analysis with the Transcreener® ADP² Assay

Meera Kumar, Tom Zielinski, Roland Carrillo, and Robert G. Lowery
BellBrook Labs, Madison, WI, USA

Overview

Drug efficacy is influenced by the affinity of the drug to the target and longevity of the drug's interaction with the target, or residence time. In general, longer residence time results in improved efficacy, as the extended contact between drug and enzyme results in extended inhibition of target activity. This in turn allows pharmacological effect to be achieved over protracted time at lower doses, reducing off-target effects. Residence time can be determined by equilibrium binding measurements using methods such as surface plasmon resonance (SPR), which, while highly quantitative data, is low-throughput and requires costly instrumentation. Furthermore, SPR requires that protein targets or drugs to be covalently conjugated to surfaces, which can modify target function and/or drug binding. Here we describe, a "jump dilution" kinetic approach to determine the residence time for protein kinase inhibitor drugs using the Transcreener ADP² Assay, which relies on homogenous immunodetection of ADP, and can be used for continuous monitoring of kinase enzyme activity. In this method the kinase is incubated with the drug at saturating concentrations to allow the formation of the kinase-drug complex, and the complex is then diluted into a solution containing the kinase enzyme reaction components and the Transcreener detection reagents. The recovery of kinase activity is monitored continuously by measuring the change in the fluorescence polarization signal, reflecting formation of ADP. The rate of drug dissociation from the kinase directly correlates to the restoration in the activity of the kinase. In this poster we show a systematic approach to determine the residence time of compounds using Abl1 as the target and inhibitors like imatinib, dasatinib and ponatinib as the interacting drugs. The residence time of dasatinib and ponatinib for Abl1 were both significantly longer than imatinib as has been previously shown in literature. The Transcreener ADP² Assays offers a highly sensitive and versatile method for estimating drug residence times for kinases using a multi-mode fluorescent plate reader.

Transcreener ADP² Assays: Direct, Homogenous ADP Detection with FP, TR-FRET, or FI Readouts

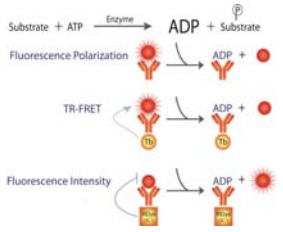
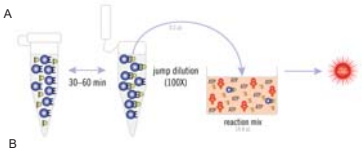


Figure 1. Transcreener® Assays: the Only Direct Detection Method Available for ADP. ADP displaces tracer from a highly specific monoclonal antibody resulting in a change in fluorescence. Three homogenous mix-and-read formats that use a red-shifted tracer to minimize compound interference are available: fluorescence polarization (FP), time resolved FRET (TR-FRET), and fluorescence intensity (FI).

Jump Dilution Method using Transcreener ADP² Assay



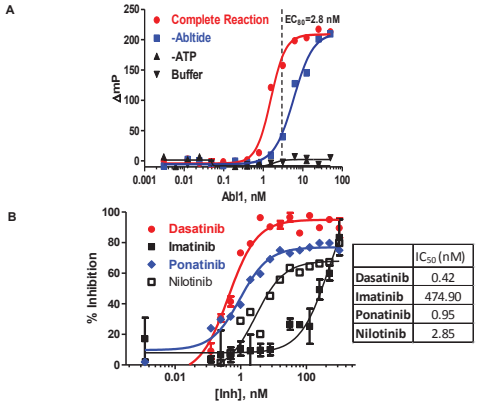
B

- **Step 1** • Pre-incubate Enzyme with saturating concentration of Inhibitor to allow formation of EI complex.
- **Step 2** • Dilute EI complex into a reaction with saturating amounts of ATP and Substrate. • Immediately add Transcreener® Detection reagents.
- **Step 3** • Continuously read diluted samples to monitor changes in fluorescence over time. • Kinase activity increases as the compound dissociates.
- **Step 4** • Determine k_{off} by fitting enzyme progress curves to an integrated rate equation: $[y] = v_s t + (v_0 - v_s/k_{off})(1 - e^{-k_{off} t})/k_{off}$

Figure 2. Using the Transcreener® ADP² Assay for "Jump Dilution". The reversibility of inhibition is determined by measuring the recovery of enzymatic activity after a rapid dilution of EI complex into a reaction mix that contains ATP, acceptor substrate and Transcreener® reagents. This method, known as the "Jump Dilution" method, monitors the recovery of kinase activity over time. **A.** Shows the principle of Jump Dilution experiment and **B** shows a flowchart for running a Jump Dilution experiment using Transcreener assays.

Experimental Example: Determining the Dissociation Rate of Abl1 Inhibitors

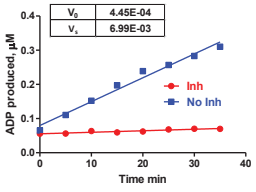
Determining Optimal ABL1 and Drug Concentrations for the "Jump Dilution" Experiments



	IC_{50} (nM)
Dasatinib	0.42
Imatinib	474.90
Ponatinib	0.95
Nilotinib	2.85

Figure 3. Determining the Optimal concentration of Abl1 and Drugs for the "Jump Dilution" Experiment. A. Titration of Abl1 enzyme, performed in the presence of 5 µM ATP and 10 µM Abltide, determined an optimal EC_{80} concentration as 2.8 nM. **B.** A dose response curve for Abl1 enzyme (2.8 nM) in the presence of 5 µM ATP and 10 µM Abltide. The reaction was run in a typical kinase buffer (50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.01% Brij).

Determining Enzyme Velocities-Initial and Steady state velocities to create the Integrated Rate Equation.



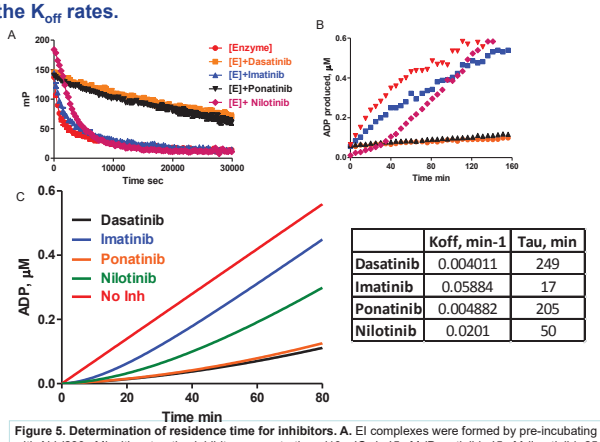
V_0	4.45E-04
V_s	6.99E-03

$$Y = Vst - (V_0 - V_s)/K_{off}\,(1 - \exp(-K_{off} * t))$$

$$Y = 0.006985X - 0.00654/K(1 - \exp(-K*X))$$

Figure 4. Determining Enzyme velocity V_0 and V_s. A. V_0 was determined for fully inhibited reactions using Abl1 enzyme in the presence of 400 nM Dasatinib **B.** Integrated rate equation used to determine the residence time values. **C.** Substituting the values of V_0 and V_s to create an integrated equation

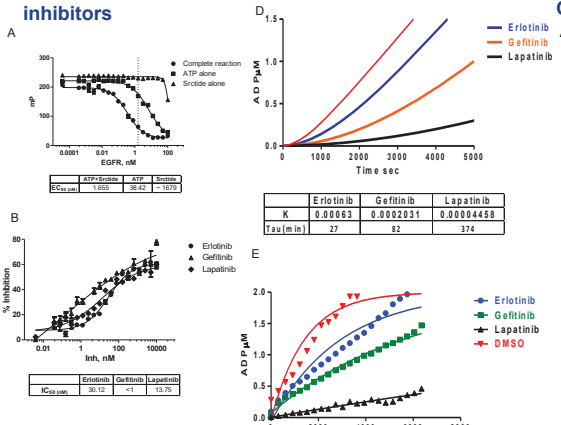
Jump Dilution Step: Recovery of Kinase Activity and Determining the K_{off} rates.



	Koff, min-1	Tau, min
Dasatinib	0.004011	249
Imatinib	0.05884	17
Ponatinib	0.004882	205
Nilotinib	0.0201	50

Figure 5. Determination of residence time for inhibitors. A. EI complexes were formed by pre-incubating with Abl (280 nM) with saturating inhibitor concentrations (10 x IC_{50}): 45 nM (Dasatinib), 45 µM (Imatinib), 25 µM (Nilotinib) or 100 nM (Ponatinib). EI mixtures were diluted 100-fold by adding 0.2 µL into 19.8 µL containing 10 µM Abltide and 5 µM ATP and Transcreener ADP² detection reagents; plates were read at 5 min intervals **B.** Raw polarization was converted into product formed (ADP) using a standard curve. **C.** Progress curves were fit to the integrated rate equation shown in Figure 4C and K_{off} values were determined using Graph Pad Prism. Residence times (Tau values) are reciprocal of K_{off} values.

Dissociation Rate and residence times for EGFR inhibitors



	ATP+Srctide	ATP	Srctide
EC_{80} (nM)	1.655	38.42	~ 1679

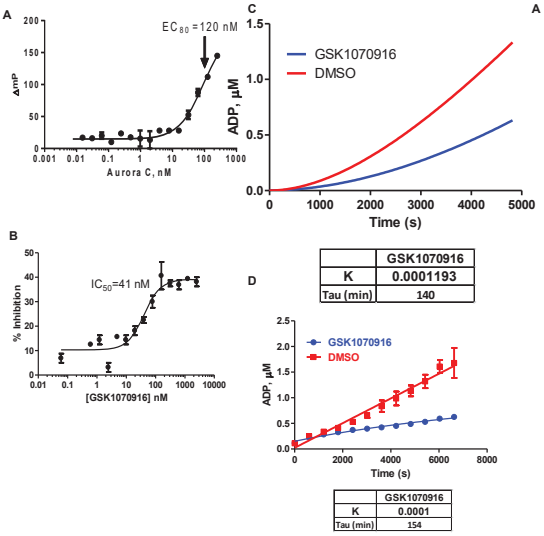
	Erlotinib	Gefitinib	Lapatinib
IC_{50} (nM)	30.12	<1	13.75

	Erlotinib	Gefitinib	Lapatinib
K	0.00063	0.0002031	0.00004458
Tau (min)	27	82	374

	Erlotinib	Gefitinib	Lapatinib
K	0.0003412	0.0001693	0.00003341
Tau (min)	49	98	498

Figure 6. Residence Times for EGFR Inhibitors. A. An optimal (EC_{80}) concentration of 1.5 nM EGFR was determined in the presence of 5 µM ATP and 10 µM Srctide. **B.** Dose response curve for EGFR inhibitors **C.** An integrated rate equation for determining residence time for inhibitors. **D.** Progress curves after jump-dilution of EI complexes into reaction containing ATP, Srctide and Transcreener ADP² detection reagents. Inhibitors were pre-incubated with EGFR at 10 x IC_{50} to allow EI formation. **E.** An approximate determination of time occupied at the target can also be determined by fitting the converted data to one phase decay. This can be used to rank-order compounds residence times.

Dissociation Rate and residence time for GSK1070916 with Aurora C

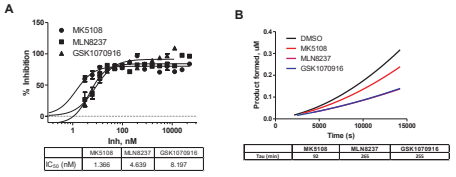


	GSK1070916
K	0.0001193
Tau (min)	140

	GSK1070916
K	0.0001
Tau (min)	154

Figure 7. Residence Times for GSK1070916 with Aurora C. A. Aurora C was titrated in the presence of 20 µM ATP and 10 µM Kemptide to determine an optimal EC_{80} concentration of 120 nM. **B.** A dose response curve for Aurora C enzyme yielded an IC_{50} value of 42 nM. **C.** Residence time for GSK1070916 was determined for Aurora C in a "jump dilution" experiment using the Transcreener® ADP² Assay. Incubations were performed with Aurora C concentration of 1200 nM and inhibitor concentrations of 400 nM. A 40-fold jump dilution was made into 10 µM Kemptide, 20 µM ATP and Transcreener detection reagents. **D.** An approximate determination of time occupied at the target can also be determined by fitting the converted data to one phase decay.

Dissociation Rate and residence time with Aurora B



	MK5108	MLN8237	GSK1070916
IC_{50} (nM)	1.366	4.639	8.197

	MK5108	MLN8237	GSK1070916
Tau (min)	92	265	265

Figure 8. Residence Times for Inhibitors with Aurora B. A. A dose response curve for Aurora B enzyme yielded an IC_{50} value of 1.4 nM, 4.6 nM and 8 nM for MK5108, MLN8237 and GSK1070916 respectively. **B.** Residence time for inhibitors were determined for Aurora B in a "jump dilution" experiment using the Transcreener® ADP² Assay. Incubations were performed with Aurora B concentration of 2500 nM and inhibitor concentrations of 10* IC_{50} concentrations. A 100-fold jump dilution was made into 10 µM MBP, 5 µM ATP and Transcreener detection reagents.

Conclusions

- ***The Transcreener® ADP² Assay relies on highly selective immunodetection of ADP with FP, TR-FRET, and FI readouts. Direct detection has advantages over enzyme-coupled assays in terms of simplicity of use and resistance to compound interference.***
- ***The assays can be tuned for detection of kinases or ATPases at any ATP concentration from 0.1 to 1000 µM, making it well suited for profiling inhibitors with enzymes that have diverse ATP requirements.***
- ***The ability to run reactions in kinetic mode enables determination of inhibitor residence times using a jump dilution method.***
- ***The residence times determined for Abl1, EGFR and Aurora C inhibitors using the Transcreener ADP² Assay in jump dilution experiments were consistent with literature values.***

BellBrook Labs, 5500 Nobel Drive, Suite 230, Madison, WI 53711
866.313.7881 or 608.443.2400