Mechanism of Action: Characterization of Enzyme-Inhibitor Interactions using Microfluidic Mobility-Shift Assays

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Introduction

Current drug discovery efforts typically focus on developing small molecule inhibitors of target enzymes involved in regulating specific cellular processes. Mechanism of Action (MOA) studies are necessary to characterize and compare potential drug candidates. The ability to follow reaction rates in real time greatly facilitates the determination of inhibition mechanisms and the calculation of binding coefficients. With real time kinetics capability, Caliper's DeskTop Profiler and LabChip 3000 provide ideal platforms for these studies. MOA experiments were conducted to characterize enzyme-inhibitor interactions for known inhibitors of two protein kinases: PKA and ERBB4. Multiple wells within a microtiter plate were sampled repeatedly during the course of each experiment, allowing for accurate determination of initial velocities from the resulting reaction progress curves. Data obtained using the DeskTop Profiler were used for determination of IC_{50} values, titration of active enzyme concentration, discrimination between ATP- and peptide-competitive inhibition, confirmation of reversible/irreversible inhibition, and calculation of K_i values. In addition to the advantages of running MOA experiments in real-time, the mobility-shift format provides direct detection of both product and substrate. This produces consistent, high quality data, and minimizes the potential for indirect effects of test compounds.

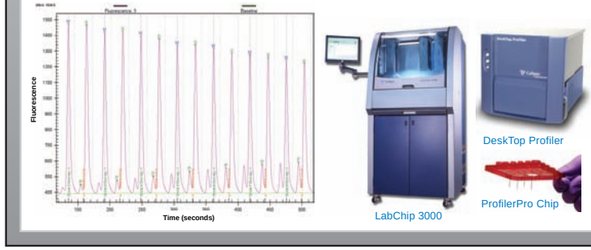
Materials and Methods

All reactions were assembled in 384-well microtiter plates (Corning #3656) and read on the DeskTop Profiler. Product and substrate peak heights were analyzed with DeskTop Profiler software to obtain the ratio of product/ (product+substrate) (P/P+S) and percent conversion.

Stopped reactions were assembled with 1 µL inhibitor in DMSO, 15 µL 2X Enzyme, 7.5 µL 4X Peptide, and 7.5 µL 4X ATP. After incubation, the reactions were stopped by the addition of 45 µL Stop Buffer. Kinetic reactions were assembled with a final volume of 75-80 µL, as indicated for the individual experiments.

Enzymes were obtained from Millipore (PKA, #14-440) and Invitrogen (ERBB4, #PV3626). Inhibitor compounds were purchased from Calbiochem (H89 #371963, PKI 6-22 amide #539684, Staurosporine #539396, Erb Inhibitor [N-(4-((3-Chloro-4-fluorophenyl)amino)pyrido [3,4-d]pyrimidin-6-yl)2-butynamide] #324840).

	PKA	ERBB4
Substrate	5'FAM-**LRRASLG**-$CONH_2$	5'FITC-**KKKKEEIYFFF**-$CONH_2$
Reaction Buffer	50 mM Hepes pH 7.0 0.002% Brij-35 10 mM $MgCl_2$ 2 mM DTT	50 mM Hepes pH 8.0 0.002% Brij-35 2.5 mM $MnCl_2$ 2 mM DTT
Stop Buffer	100 mM Hepes pH 7.5 0.03% Brij-35 40 mM disodium EDTA 0.2% CR-3	100 mM Hepes pH 7.5 0.03% Brij-35 30 mM disodium EDTA 0.2% CR-3
Separation Buffer	100 mM Hepes pH 7.5 0.015% Brij-35 10 mM disodium EDTA 0.1% CR-3	Caliper ProfilerPro Separation Buffer with CR-8
Separation Conditions	Downstream Voltage -500 V Upstream Voltage -2100 V Pressure -1.5 psi Minimum 20 sec between sips	Downstream Voltage -500 V Upstream Voltage -2500 V Pressure -1.7 psi Minimum 25 sec between sips



LabChip 3000

DeskTop Profiler

ProfilerPro Chip

Results

Determination of ATP-Competitive Inhibition

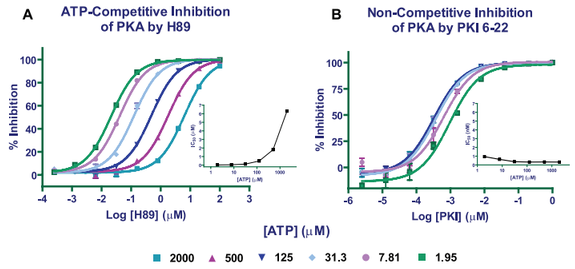


Figure 1. Duplicate inhibitor titrations were run at 6 different ATP concentrations. Reactions were assembled with final concentrations of 0.2 nM PKA and 750 nM peptide substrate, incubated at 28 ºC, and stopped after 45 or 90 min. IC_{50} values were calculated with GraphPad Prism software using the sigmoidal dose-response function. The increase in IC_{50} value with increasing ATP concentration demonstrates competition between ATP and H89 (A, inset). No such effect is observed with PKI 6-22 (B, inset).

Determination of Peptide-Competitive Inhibition

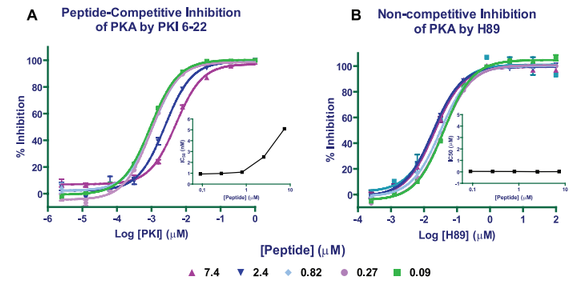


Figure 2. Duplicate inhibitor titrations were run at 5 different peptide substrate concentrations. Reactions were assembled with final concentrations of 0.2 nM PKA and 5 µM ATP, incubated at 28 ºC, and stopped after 1 hour. IC_{50} values were calculated with GraphPad Prism software using the sigmoidal dose-response function. For PKI 6-22, the IC_{50} value increased with increasing peptide concentration (A, inset). No change in IC_{50} value was observed for H89 (B, inset).

Reversibility of PKA Inhibition

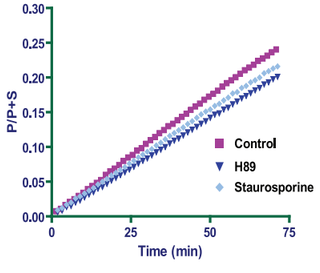


Figure 3. Rapid dilution experiments were used to demonstrate reversibility of PKA-inhibitor binding. 100X Enzyme (20 nM) was incubated with inhibitors at 10X the IC_{50} value (300 nM for H89, 50 µM for Staurosporine) for reactions containing 5 µM ATP. After a 30 min incubation at room temperature, the enzyme and inhibitor were diluted 1:100 into 1X peptide (750 nM) and ATP (5 µM). The reactions were immediately placed on the DeskTop Profiler to obtain progress curves. As expected for reversible inhibition, reactions containing inhibitor (diluted to 0.1X the IC_{50}) progressed at about 90% the velocity of no inhibitor control reactions.

Results (cont)

Tight-Binding Inhibition of ERBB4

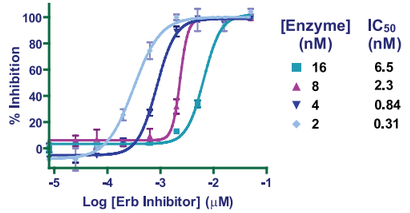


Figure 4. Duplicate titrations of Erb Inhibitor (N-(4-((3-Chloro-4-fluorophenyl)amino)pyrido [3,4-d]pyrimidin-6-yl)2-butynamide) were run at 4 different concentrations of ERBB4. Stopped reactions containing the indicated concentration of enzyme, 1 µM peptide, and 3 µM ATP were assembled and incubated at room temperature for 60 minutes. The shift in IC_{50} value with changing enzyme concentration is indicative of a tight-binding mode of inhibition with dependency on enzyme:inhibitor stoichiometry rather than absolute inhibitor concentration. In all cases, the IC_{50} values are less than the enzyme concentration, indicating that only a portion of the enzyme is active.

Determination of ERBB4 Active Enzyme Concentration

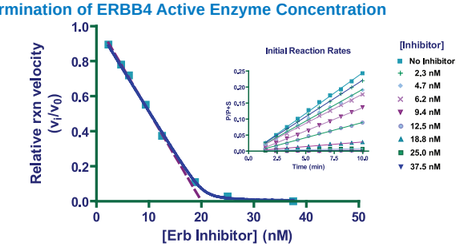


Figure 5. Initial velocities were measured in a titration of Erb Inhibitor with 50 nM total enzyme ([E]/Ki>200) in reactions containing 1 µM peptide and 3 µM ATP (inset). The v_i/v_0 (velocity inhibited/velocity uninhibited) values from reactions containing ≤12.5 nM inhibitor were fit to a line intersecting the X axis at 20 nM. This represents the point at which [Inhibitor] = [Active Enzyme], showing that the enzyme preparation contains 40% active enzyme. The blue line shows the curve generated by fitting the data to the Morrison equation. Curve fitting calculated a K_i^{app} value of 0.044 nM and an active enzyme concentration ($[E]_T$) of 20.4 nM.

ATP-Competitive Inhibition by Erb Inhibitor

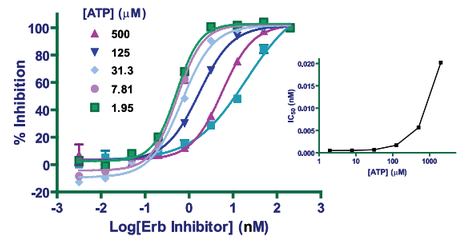


Figure 6. Duplicate reactions were run to titrate Erb inhibitor at 5 different concentrations of ATP. Reactions were assembled with 1.4 nM active enzyme and 1 µM peptide, incubated at room temperature, and stopped after 45 min or 90 min. IC_{50} values increased with increasing ATP concentration (inset), as expected for ATP-competitive inhibition.

Results (cont)

Irreversibility of ERBB4 Inhibition

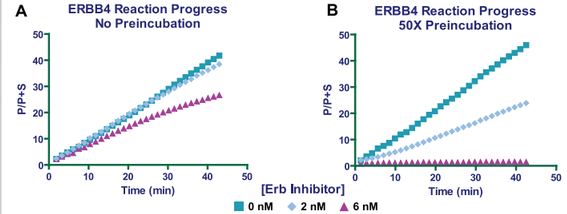


Figure 7. Reaction progress curves were generated with and without preincubation of 50X enzyme and 50X inhibitor. For preincubation, enzyme (100 nM active) was mixed with inhibitors at 100 nM and 300 nM. After 15 min at room temperature, the enzyme and inhibitor mix was diluted 1:50 into peptide (1 µM) and ATP (500 µM). The reaction progress curves showed complete inhibition with 6 nM inhibitor (final concentration) and about 50% inhibition with 2 nM inhibitor (B). In contrast, when enzyme and inhibitor were mixed as the reaction was initiated (A, no preincubation), 2 nM inhibitor showed little inhibition, and 6 nM inhibitor showed only about 40% inhibition. Erb Inhibitor could not be washed out by rapid dilution with excess ATP, indicating irreversible binding to ERBB4.

Calculation of K_i Values for Inhibitors

Inhibitor	Mechanism	K_i (nM)	K_m^* (µM)	Equation	R^2 Value
H89	ATP-Competitive	16 (+/- 0.13)	4.2	$IC_{50} = K_i^*(1 + ([ATP]/K_m))$	0.9997
PKI 6-22	Peptide-Competitive	0.62 (+/- 0.025)	0.98	$IC_{50} = K_i^*(1 + ([Peptide]/K_m))$	0.9926
Erb Inhibitor	Tight-binding ATP-Competitive	0.062 (+/- 0.0014)	6.5	$IC_{50} = K_i^*(1 + ([ATP]/K_m)) + ([E]_T/2)$	0.9998

* K_m^{ATP} for H89 and Erb Inhibitor, $K_m^{Peptide}$ for PKI 6-22.

Table 1. K_i Values for each inhibitor were calculated using XLfit4 software to fit data to the Cheng-Prusoff Equation. For Erb Inhibitor, a term correcting for the effect of active enzyme concentration ($[E]_T$) was factored into the equation.

Summary

- Known inhibitors of PKA and ERBB4 were characterized using Caliper's Mobility Shift Assays.
- H89 was demonstrated to be an ATP-competitive, reversible inhibitor of PKA with a K_i value of 16 nM.
- PKI 6-22 was demonstrated to be a peptide-competitive inhibitor of PKA with a K_i value of 0.62 nM.
- Erb Inhibitor was demonstrated to be an ATP-competitive tight-binding/ irreversible inhibitor of ERBB4. Two different methods were used to obtain K_i values of 0.062 nM and 0.044 nM.
- Erb Inhibitor was used to provide an accurate determination of active ERBB4 enzyme concentration.

Conclusions

- *Mobility-Shift Assays provide an ideal platform for kinase mechanism of action studies.*
- *High quality, ratiometric data produces consistent, reliable results.*
- *Both product and substrate are measured directly, minimizing chances of compound interference and indirect effects.*
- *Kinetic mode allows repeated sampling of wells to easily generate reaction progress curves and measure initial reaction rates.*
- *Stopped mode allows higher throughput for more complex experimental setups.*
- *Assays can be run at any ATP concentration and a wide range of peptide concentrations.*

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