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

Introduction

Current drug discovery efforts typically focus on developing small molecule inhibitors of target enzymes involved in regulating specific cellular processes. Mechanisms of Action (MOA) studies are necessary to characterize and compare potential drug candidates. This ability to follow reaction rates in real time greatly facilitates the determination of inhibition mechanisms and the calculation of binding constants. With real-time readout capability, Caliper’s DeskTop Profile and LabChip 3000 provide ideal platforms for these studies. MOA experiments were conducted to characterize enzyme-inhibitor interactions for known inhibitors of two protein kinase, 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 Profile were used for determination of IC₅₀ values, titration of active enzyme concentration, discrimination between ATP- and peptide-competitive inhibition, calculation of reversible/inhibitor concentration, and calculation of Kᵢ values. In addition to the advantages of running MOA experiments in reactions, the mobility-shift format provides direct detection of both product and substrate. This produces consistent, high quality data, and minimizes the assay for indirect effects of test compounds.

Materials and Methods

All reactions were assembled in 384-well microtiter plates (Corning #3595) and read on the DeskTop Profile. Product and substrate peak heights were analyzed with the GraphPad Prism software to obtain the ratio of product/product-inhibitor (P/P₀) 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 45 μM ATP. At the end of the run, the reactions were stopped for visualization of 4S 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, #14-441 and InTrovize (ERBB4, #RVP326), Inhibitor compounds were purchased from Calbiochem (PKA #ABT195, PKI-22 amide #335844, Staurosporine #539396, Erb Inhibitor [N-(4-((3-Chloro-4-fluorophenyl)amino)pyrido [3,4-d]pyrimidin-6-yl)2-butynamide] #372840).

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 50 nM PKA and 750 nM peptide substrate. Incubated at 28 ºC, and stopped after 45 or 90 min. IC₅₀ values were calculated with GraphPad Prism software using the sigmoidal dose-response function. The IC₅₀ in μM ATP with increasing ATP concentration demonstrates competition between ATP and H89 (A, inset). Such effect is observed with PKI-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. Assays were incubated at 28 ºC, and stopped after 1 hour. IC₅₀ values were calculated with GraphPad Prism software using the sigmoidal dose-response function. For PKI-22 amide, the IC₅₀ value increased with increasing peptide concentration (A, inset). No change in IC₅₀ value was observed for H89 (B, inset).

Reversibility of PKA Inhibition

Figure 3. Rapid inhibition experiments were used to determine reversibility of PKA inhibition binding. 10 nM PKA (20 μM) was incubated with inhibitors at 10 μM, 50 μM, 100 μM, or 200 μM ATP. Reactions were measured at 0.5, 10, 30, and 60 minutes. Reactions were stopped by the addition of 4S μL Stop Buffer. Kinetic reactions were assembled with a final volume of 75-80 μL, as indicated for the individual experiments. H89 and PKI-22 amide were incubated at 28 ºC, and stopped after 45 min or 90 min. IC₅₀ values increased with increasing ATP concentration (inset), as expected for ATP-competitive inhibition.

ATP-Competitive Inhibition by Erb Inhibitor

Figure 4. Duplicate reactions were run to test the effect of PKA and ATP on Erb Inhibitor. PKA was incubated with 1 μM ATP, and 200 μM ATP. The reactions were measured at 0.5, 10, 30, and 60 minutes. Reactions were stopped by the addition of 4S μL Stop Buffer. Kinetic reactions were assembled with a final volume of 75-80 μL, as indicated for the individual experiments. PKA (20 μM) was incubated with inhibitors at 10 μM, 50 μM, 100 μM, or 200 μM ATP. Reactions were measured at 0.5, 10, 30, and 60 minutes. Reactions were stopped by the addition of 4S μL Stop Buffer. Kinetic reactions were assembled with a final volume of 75-80 μL, as indicated for the individual experiments. PKA (20 μM) was incubated with inhibitors at 10 μM, 50 μM, 100 μM, or 200 μM ATP. Reactions were measured at 0.5, 10, 30, and 60 minutes. Reactions were stopped by the addition of 4S μL Stop Buffer.

Results (cont.)

Reversibility of ERBB4 Inhibition

Figure 5. Initial velocities were measured in a titration of Erb Inhibitor with 50 nM total enzyme (30 μM ATP) in medium containing 1 μM peptide and 3 μM ATP (inset). The IC₅₀ (velocity inhibited/velocity uninhibited) indicates competitive inhibition. IC₅₀ values from reactions containing 2 μM Erb Inhibitor were 10 to 20 times lower ATP. This represents the point at which the PKA-inhibitor binding. 100X Enzyme (20 nM) was incubated with inhibitors at 10 μM, 50 μM, 100 μM, or 200 μM ATP. Reactions were measured at 0.5, 10, 30, and 60 minutes. Reactions were stopped by the addition of 4S μL Stop Buffer. Kinetic reactions were assembled with a final volume of 75-80 μL, as indicated for the individual experiments. PKA (20 μM) was incubated with inhibitors at 10 μM, 50 μM, 100 μM, or 200 μM ATP. Reactions were measured at 0.5, 10, 30, and 60 minutes. Reactions were stopped by the addition of 4S μL Stop Buffer. Kinetic reactions were assembled with a final volume of 75-80 μL, as indicated for the individual experiments.

Conclusions

- **Mobility-Shift Assays** provide an ideal platform for kinase mechanism of action studies.
- High-quality, normalized data provide 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.