Introduction

The development of ATP-site ligands as protein kinase inhibitors raises questions about the scope of their off-target effects as well as possibilities for the therapeutic targeting of other ATP-utilizing enzymes. Adenine nucleotides are interconverted by diverse proteins including, in addition to kinases, other types of transferases, phosphodiesterases, membrane transporters, DNA modifying enzymes and molecular chaperonins. Despite high diversity in the sequence motifs and folds that bind adenine nucleotides, there are commonalities in the ATP interaction networks across functionally diverse enzymes. To explore the ligand selectivity of ATP-binding sites, we screened a kinase-focused library across diverse ATP-utilizing enzymes using adenine nucleotide detection as a generic assay method. The assays rely on highly selective antibodies that distinguish between nucleotides on the basis of a single phosphate group. Homogenous fluorescent polarization assays have been developed for both ADP and AMP, making it possible to interrogate a diverse panel of otherwise intractable ATP-utilizing enzymes in an HTS format. Of the eight ATP-utilizing enzymes used in the study, we found micromolar interactions of protein kinase inhibitors with one mammalian target and one bacterial target, indicating the potential for off-target effects. The approach used provides a framework for more systematic efforts to map the ligand selectivity of ATP-utilizing enzymes. It also raises the possibility of leveraging the large body of kinase inhibitor chemoselectivity data to address other target families in the purinome.

Figure 1  Experimental Strategy

Substrate + ATP  →  Product + ADP/AMP

Far red fluorescence polarization detection

ADP-Producing Enzymes

Protein Kinase A (Human):  ATP + Kemptide  →  ADP + P-Kemptide
PI3α Kinase (Human):  ATP + PI(4,5)P2  →  ADP + PI(3,4,5)P3
Hexokinase (Yeast):  ATP + Glucose  →  ADP + Glucose 6-P
HSP73 (Bovine):  ATP + H2O  →  ADP + Pi
Acetyl CoA Carboxylase (Rat):  ATP + HCO3- + Ac-CoA  →  ADP + Pi + Mal-CoA
Glutamine Synthetase (M. tuberculosis):  ATP + Glu + NH4+  →  ADP + Pi + Gln

AMP-Producing Enzymes

Ubiuitin Ligase E1 (Human):  ATP + ubiquitin  →  AMP + PPI + E1-ubiquitin
Acyl CoA Synthetase (Psuedo. sp):  ATP + CoA + olate  →  AMP + PPI + oleoyl-CoA
Transcreener Assay: Homogenous Nucleotide Detection

Transcreener™ ADP monoclonal Ab binds ADP selectively

Transcreener™ AMP/GMP Ab binds AMP selectively

Competition curves with various adenine nucleotides demonstrate the selectivity of the Transcreener antibodies used for ADP and AMP detection. The ADP antibody discriminates ADP with greater than 100-fold selectivity versus ATP. The AMP/GMP Ab discriminates AMP with >1000-fold selectivity versus ATP or cAMP and also discriminates GMP with >1000-fold selectivity versus cGMP. The Transcreener assays are competitive fluorescence polarization immunoassays. The product of ATP utilizing enzymes, ADP or AMP, compete with the tracer for antibody binding. Increased nucleotide concentrations result in lower polarization values.

Table 1  ATP-Utilizing Enzyme Inhibitor Profile

<table>
<thead>
<tr>
<th>ATP-utilizing Enzyme</th>
<th>Transcreener Assay</th>
<th>Number of Hits (50% Inhibition at 10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>ADP</td>
<td>19</td>
</tr>
<tr>
<td>PI3Kα</td>
<td>ADP</td>
<td>3</td>
</tr>
<tr>
<td>Acetyl CoA Carboxylase</td>
<td>ADP</td>
<td>2</td>
</tr>
<tr>
<td>Glutamine Synthetase</td>
<td>ADP</td>
<td>1</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>ADP</td>
<td>0</td>
</tr>
<tr>
<td>Hsp73 ATPase</td>
<td>ADP</td>
<td>0</td>
</tr>
<tr>
<td>Acyl CoA Synthase</td>
<td>AMP/GMP</td>
<td>0</td>
</tr>
<tr>
<td>Ubiquitin E1</td>
<td>AMP/GMP</td>
<td>0</td>
</tr>
</tbody>
</table>

Eight ATP-Utilizing Enzymes were screened using the BIOMOL Kinase Inhibitor Library (at both 1 μM and 10 μM). In-house control compounds supplemented to increase library to 97 compounds. Micromolar inhibition seen for four of eight enzymes in the study. The number of compounds in the library that achieved 50% inhibition at 10 μM compound are listed.
PKA reactions containing 1 μM compounds were performed in 50 mM HEPES (pH 7.5), 4 mM MgCl₂, 2 mM EGTA, 1% DMSO, 50 μM Kemptide, 10 μM ATP, and 15 ng/mL PKA (n=3). PKA control reactions (n=16) were run to 8% ATP conversion, Z'=0.8. Positive hits in the compound library screen were titrated to determine IC₅₀ values.

Figure 4. PI3Kα Screen
ATP + Phosphatidylinositol (4,5) diphosphate → ADP + Phosphatidylinositol (3,4,5) triphosphate

PI3Kα reactions containing 10 μM compound were performed in 50 mM HEPES (pH 7.1), containing NaCl (100 mM), MgCl₂ (4 mM), EGTA (2 mM), DTT (2 mM), DMSO (1%), ATP (30 μM), PI(4,5) P2 C16 (30 μM), and PI3Kα (100 ng/μL). PI3Kα control reactions (n=16) were run to 7%, Z'=0.9. Positive hits in the compound library screen were titrated to determine IC₅₀ values.
Figure 5. Acetyl-CoA Carboxylase Screen

\[
ATP + HCO_3^- + \text{acetyl-CoA} \rightarrow \text{ADP} + \text{Pi} + \text{malonyl-CoA}
\]

10 μM Compounds

Acetyl CoA Carboxylase 1 reactions containing 10 μM compound were performed in 50 mM HEPES (pH 7.5), 4 mM MgCl2, 1 mM EGTA, 2 mM potassium citrate, 50 μM bicarbonate, 50 μM acetyl CoA, 1% DMSO, and 2 ng/μL ACC1 (n=3). Acetyl CoA carboxylase control reactions (n=16) were run to 7% ATP conversion, \( Z' = 0.8 \). Positive hits in the compound library screen were titrated to determine IC\(_{50}\) values.

Figure 6. Glutamine Synthetase Screen

\[
ATP + \text{Glutamic acid} + \text{ammonium} \rightarrow \text{ADP} + \text{glutamine} + \text{Pi}
\]

10 μM Compounds

Glutamine Synthetase (M. tuberculosis) reactions containing 10 μM compound were performed in 50 mM Imidazole (pH 7.1), 4 mM MgCl2, 0.4 mM MnCl2, 2 mM EGTA, 2.5 mM KCl, 1% DMSO, 0.01% Brij-35, 25 μM glutamate, 25 μM ammonium, 500 μM ATP, 800 ng/mL glutamine synthetase (n=3). Glutamine synthetase control reactions (n=16) were run to 3.5% ATP conversion, \( Z' = 0.8 \). Positive hits in the compound library screen were titrated to determine IC\(_{50}\) values.
Transcreener ADP and AMP nucleotide immunodetection assays and the BIOMOL Kinase Library were used to screen eight diverse enzymes that use ATP as a substrate. The enzymes include a protein kinase, a lipid kinase, a carbohydrate kinase, a heat shock protein (ATPase), a carboxylase, a synthase, a ligase, and a microbial target involved in amino acid synthesis.

Protein kinase inhibitors exhibit ‘off target’ effects for PI3Kα, Acetyl-CoA Carboxylase1, and Glutamine Synthetase. Acetyl-CoA Carboxylase was inhibited by several tyrosine kinase inhibitors while the microbial target, Glutamine Synthetase, was inhibited by serine-threonine kinase inhibitors. PI3Kα showed a mixed inhibition profile.

The four ATP-Utilizing enzymes including Hexokinase, Hsp 73, Acyl CoA Synthase, and Ubiquitin E1 were not inhibited.

Materials and Methods

Materials. Glutamine Synthetase was a generous donation from Dr. Guenter Harth (UCLA) and Dr. Marcus Horwitz (UCLA). Protein kinase A (PKA), PI3α, Kinase and ACC1 were purchased from Invitrogen (Carlsbad, CA), Millipore (Dundee, Scotland), and BlueSky (Worcester, MA), respectively. Substrates, Lipids and lipid substrates were purchased from Sigma, Avanti Polar Lipids (Alabaster, AL) or Cellsignals (Columbus, OH). Inhibitors were from BIOMOL Kinase Inhibitor Library or from Sigma, Upstate, or EMD Biosciences (La Jolla, CA).

Standard plate and instrumentation settings. All assays were performed in black Corning® 384 Well Microplates (Corning, NY). Fluorescence intensity and polarization measurements utilizing the AMP/GMP-Alexa Fluor®633 or ADP-Alexa Fluor®633 tracers were performed on a Tecan Ultra plate reader using the following filters and settings: 612 nm excitation filter (10 nm bandwidth), 670 nm emission filter (25 nm bandwidth), 10 flashes per well, 30°C or on the Tecan Safire2™ plate reader using the following settings: 635 nm excitation (LED), 670 nm emission (20 nm bandwidth), 10 flashes per well, 30°C.

Enzyme reactions. Enzyme concentration and reaction time were variable, but always designed to produce <10% ATP conversion. The basic assay protocol is mix and read: enzyme reactions were run in 10 μL volumes in 384-well plates at room temperature followed by the addition of 10 μL ADP Detection Mixture (containing EDTA and antibody/tracer complex). Reactions were equilibrated for one hour at room temperature before reading the plate.

Conclusions

- Transcreener ADP and AMP nucleotide immunodetection assays and the BIOMOL Kinase Library were used to screen eight diverse enzymes that use ATP as a substrate. The enzymes include a protein kinase, a lipid kinase, a carbohydrate kinase, a heat shock protein (ATPase), a carboxylase, a synthase, a ligase, and a microbial target involved in amino acid synthesis.

- Protein kinase inhibitors exhibit ‘off target’ effects for PI3Kα, Acetyl-CoA Carboxylase1, and Glutamine Synthetase.

- Acetyl-CoA Carboxylase was inhibited by several tyrosine kinase inhibitors while the microbial target, Glutamine Synthetase, was inhibited by serine-threonine kinase inhibitors. PI3Kα showed a mixed inhibition profile.

- The four ATP-Utilizing enzymes including Hexokinase, Hsp 73, Acyl CoA Synthase, and Ubiquitin E1 were not inhibited.