Protein Kinases

Goal: Generate a potent and selective inhibitor for every protein kinase in the human kinome.

Challenge: The ATP-binding pockets of protein kinases are highly conserved.

Catalytic Domain

- P-loop
- helix $\alpha C$
- DFG-motif
- activation loop

Completely non-selective

Completely selective for EGFR
Chemical Genetic Methods for Selective Kinase Inhibition

• Answer basic biological questions
• Drug target validation

Pharmacological Inhibition

1. Fast time scale
2. Only perturbs targeted sub-domains
3. graded dose response - tunability
4. Most drugs are small molecules

In many cases, RNAi and pharmacological inhibition do not phenocopy
Selective Inhibition of Kinases

A

Adenosine triphosphate (ATP)

PP1

1-NM-PP1

3-MB-PP1

B

catalytic domain  bulky analog

as-kinase  +  

inhibited

wt-kinase  +  

resistant
Bivalent Kinase Inhibitors

• Create a bivalent inhibitor consisting of an ATP-competitive inhibitor and a ligand for a 2° binding domain
• Capable of enhancing inhibitor selectivity and potency

\[ \Delta G_{AB} = \Delta G_A + \Delta G_B + \Delta G_L \]

Goal: To develop a method that will allow the assembly of bivalent kinase inhibitors in living cells

Initial Targets

Src kinase

SH3 Domain

SH2 Domain

Catalytic Domain

Abl kinase

Highly homologous tyrosine kinases with a similar N-terminal domain architecture

Generating selective inhibitors is challenging

PDB ID: 2H8H
PDB ID: 1OPL
Almost all ATP-competitive inhibitors are equipotent for Src and Abl.
SNAPtag Labeling Method

Human $O^6$-alkylguanine DNA alkyltransferase (SNAPtag)

Small, self-labeling protein

Engineered to selectively and rapidly react with $O^6$-benzylguanine derivatives

Allows proteins to be rapidly labeled with fluorophores in living cells
SNAPtag-Small Molecule Conjugates

Strategy
Use the SNAPtag labeling technique to generate selective kinase inhibitors

Requirements
1. SNAPtag fusion proteins that contain a ligand that targets a secondary binding domain
2. An ATP-competitive inhibitor that is linked to O\textsuperscript{6}-benzylguanine
SNAPtag-Small Molecule Conjugates

SNAPtag fusion proteins provide kinase selectivity
Selective polyproline motifs have been identified for many SH3 domains
N- and C-terminal fusions with variable linker lengths were generated for each polyproline ligand.

All fusion proteins have the same catalytic activity as SNAPtag.
Several 4-anilinoquinazolines are potent inhibitors of Src and Abl.

\[ IC_{50} (\text{Src}) = 640 \text{ nM} \]
\[ IC_{50} (\text{Abl}) = 710 \text{ nM} \]

ATP-Binding Site Ligand

Structure of a 4-anilinoquinazoline derivative bound to Src kinase (PDB ID: 2H8H)
In vitro Activities of BG Derivatives

Attaching an AGT-labeling group does not affect inhibitor potency.
Conjugation to SNAPtag causes a modest drop in potency for the 4-anilinoquinazoline inhibitor
Src-Selective Conjugates

Src-selective SH3 ligand
APPLPPRRNRPRL

PP1 and PP2 are N-terminal fusions
PP3 and PP4 are C-terminal fusions

IC$_{50}$ (nM)

<table>
<thead>
<tr>
<th></th>
<th>Src</th>
<th></th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT(wt)-A</td>
<td>&gt;5000</td>
<td>AGT(wt)-B</td>
<td>2000 ± 300</td>
</tr>
<tr>
<td>AGT(PP1)-A</td>
<td>13 ± 3</td>
<td>AGT(PP1)-B</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>AGT(PP2)-A</td>
<td>25 ± 6</td>
<td>AGT(PP2)-B</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>AGT(PP3)-A</td>
<td>72 ± 10</td>
<td>AGT(PP3)-B</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>AGT(PP4)-A</td>
<td>34 ± 3</td>
<td>AGT(PP4)-B</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>
**Abl-Selective Conjugates**

Abl-selective SH3 ligand

APTYSPPPP

PP5 and PP6 are N-terminal fusions

PP7 and PP8 are C-terminal fusions

<table>
<thead>
<tr>
<th><strong>IC\textsubscript{50} (nM)</strong></th>
<th><strong>Abl</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AGT(wt)-A</strong></td>
<td>3200 ± 100</td>
</tr>
<tr>
<td><strong>AGT(PP5)-A</strong></td>
<td>18 ± 6</td>
</tr>
<tr>
<td><strong>AGT(PP6)-A</strong></td>
<td>15 ± 4</td>
</tr>
<tr>
<td><strong>AGT(PP7)-A</strong></td>
<td>18 ± 7</td>
</tr>
<tr>
<td><strong>AGT(PP8)-A</strong></td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>IC\textsubscript{50} (nM)</strong></th>
<th><strong>Abl</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AGT(wt)-B</strong></td>
<td>1300 ± 200</td>
</tr>
<tr>
<td><strong>AGT(PP5)-B</strong></td>
<td>&lt; 6</td>
</tr>
<tr>
<td><strong>AGT(PP6)-B</strong></td>
<td>&lt; 6</td>
</tr>
<tr>
<td><strong>AGT(PP7)-B</strong></td>
<td>&lt; 6</td>
</tr>
<tr>
<td><strong>AGT(PP8)-B</strong></td>
<td>&lt; 6</td>
</tr>
</tbody>
</table>
All conjugates are equipotent inhibitors of Src KD and Abl KD

Interaction with the SH3 domain is the main contributor to increased potency
Kinase Selectivity of Conjugates

AGT conjugates are selective for kinases that contain SH3 domains
No inhibition of 20 additional kinases
The catalytic domains of protein kinases contain diverse ligand binding sites. Signaling specificity is achieved through these sites. These binding sites can be effectively targeted with bivalent inhibitors.
Summary

1. SNAPtag is an efficient scaffold for displaying bivalent kinase inhibitors

2. Any interaction site for which a suitable ligand has been identified ($K_D < 10 \, \mu M$) can be used to construct bivalent inhibitors

Challenge

There are not well defined interaction sites for most kinases
Solution: Intracellular Antibodies

- Antibodies can be developed to recognize a single antigen
  - Not functional in the reducing cellular environment
- Potent and selective protein binders have been designed
  - Termed “intracellular antibodies”
  - Stable structure not dependent on disulfide

A potential general solution to finding secondary binding ligands
DARPins

- Antibody-like protein binding molecules
- Stable tertiary structure allows extensive mutation of the variable loop regions
- Libraries are panned using ribosome display

Monobodies

Very similar to DARPins

- Stable β-sandwich structure
- Loops can be extensively mutated to select for binding interactions (phage display)

Bivalent Inhibition with Monobodies and DARPins

- Selective monobody has been developed to bind SH2 domain of Abl
- Monobody does not affect non-activated Abl

Bivalent SNAPtag fusions containing Monobodies or DARPins are highly selective
Bivalent Inhibitors Can Be Assembled in Cells

Cell Permeability Assay

1. Transfect cells with AGT
2. Incubate cells with linkable inhibitor (or DMSO)
3. Incubate cells with linkable Rhodamine
4. Lyse cells, SDS-PAGE, and determine labeling efficiency

Low cell permeability (Cos-7, HeLa, HEK293, K562, NIH-3T3)

High cell permeability (Cos-7, HeLa, HEK293, K562, NIH-3T3)
Sub-Cellular Pharmacology

Phenotype

Phosphoproteomic Analysis

Wash cells

Stimulate

inhibitor =

Labeling Moeity Linker Kinase Inhibitor
Methods for Studying Signaling Enzyme Function

Loss-of-function studies:

Protein of Interest → RNAi or gene knock-out → phenotype

Small-molecule inhibitors provide rapid, reversible, and dose-dependent control over protein function
Methods for Studying Signaling Enzyme Function

Gain-of-function studies:

over-expression or constitutively active mutant

GOAL: Develop a general method for activating signaling enzymes with a small molecule probe
Release of intra-molecular auto-inhibition is a common mechanism of enzyme activation. Allows tight control over enzymatic activity coupled with rapid activation kinetics.

Kuriyan and Eisenberg *Nature* 2007, 450, 983-90
Regulation of Signaling Enzyme Activity

Example: Src-family kinases

An inter-molecular binding event releases intra-molecular auto-inhibition
Proposed Method

replace regulatory domains with a protein-protein interaction that can be disrupted with a small molecule inhibitor

The same small molecule inhibitor can be used to control multiple enzymes
Bcl-xL is an anti-apoptotic protein that can be minimized to a functional domain of 20-25 kDa.

20-30 amino acid peptides from BH3-only proteins (BH3 peptides) bind to Bcl-xL.

Bcl-xL has a high affinity for BH3 peptides ($K_D = 1-20$ nM).
Bcl-xL/BH3 Peptide Interaction Inhibitors

ABT-737 and A-385358 disrupt the interaction between Bcl-xL and BH3 peptides

Both inhibitors bind tightly to Bcl-xL ($K_D = 0.1 - 5 \text{ nM}$)
Bcl-xL/BH3 Peptide Interaction Inhibitors

ABT-737 and A-385358 bind to the same hydrophobic groove as BH3 peptides

(PDB: 2BZW)

(PDB: 2YXJ)
Target = Small GTPases

Small GTPases act as molecular switches in cell signaling

22 Rho-family GTPases in humans
Small GTPases act as molecular switches in cell signaling

22 Rho-family GTPases in humans
Dbl-Family Guanine Nucleotide Exchange Factors (GEFs)

69 Dbl-family members in humans

Multi-domain proteins that activate Rho-family GTPases

All members contain a Dbl homology (DH) domain that catalyzes nucleotide exchange in Rho-family GTPases

Truncated mutants are constitutively active – regulated by intra-molecular auto-inhibition

Intersectin – activates Cdc42

Tim – activates RhoA

Synthetic Intersectins can activate Cdc42 (GTPase) in a small molecule-dependent manner
The DH domain of Intersectin catalyzes nucleotide exchange for the GTPase Cdc42.

2 DH domain constructs:
- DH1 = grey and red (residues 1229-1445)
- DH2 = grey (residues 1229-1429)
Synthetic Intersectin (sITSN) Constructs

Four synthetic Intersectin (sITSN) constructs containing artificial regulatory domains were generated.

- sITSN1 = Bcl-xL GT2 ITSN DH1 GT WWWW
- sITSN2 = Bcl-xL GT2 ITSN DH2 GT WWWW
- sITSN3 = Bcl-xL GT2 ITSN DH2 WWWW
- sITSN4 = WWW GT ITSN DH1 GT Bcl-xL

All constructs are monomeric (confirmed by size exclusion chromatography).
A fluorescent nucleotide exchange assay was used to determine the catalytic activity of the sITSN constructs.

Nucleotide exchange activity is determined by fluorescence loss (release of mant-GDP from Cdc42).
sITSN Constructs Show Reduced GEF Activity

All four sITSN constructs are highly auto-inhibited

Constructs showed little activation in the presence of competitor (5 µM Bad peptide ($K_D$ (Bcl-xL) = 5 nM))
BH3 Peptides with Reduced Affinity for Bcl-xL

**BH3 Peptide:** NLWAAQRYGRELRRMSDEFLVDSFKKG

Two hydrophobic residues (Leu151 and Phe158) make a major contribution to the binding affinity of the BH3 peptide.

3 mutations:
- Leu151Glu – no affinity
- Phe158Ser – medium affinity
- Phe158Gly – low affinity
Characterization of sITSN Constructs

Fold Activation


sITSN1  sITSN2  sITSN3  sITSN4

↑  ↑  ↑  ↑

Bcl-xL  GT2  ITSN DH2  GT  Bcl-xL  GT2  GT  Bcl-xL  GT2  GT  Bcl-xL  GT2  GT
Higher concentrations of the BH3 competitor increase the catalytic activity of the sITSN constructs (12-fold activation).
Generality of Strategy

12-fold activation of Cdc42
Generality of Strategy

12-fold activation of Cdc42

20-fold activation of RhoA
Generality of Strategy

12-fold activation of Cdc42

20-fold activation of RhoA

16-fold activation of Rac1
Generality of Strategy

Engineering of GEFs allows small molecule activation of the Rho GTPases Cdc42, RhoA, and Rac1.
Activation of GTPases (Cdc42) in Cells

Cells were transfected with sITSN Phe158Gly (or control) and treated with **A-385358** (or DMSO)

NIH 3T3
Dose-Dependent Activation of Cdc42

Cells were treated with A-385358 for 30 minutes
Rapid Activation of Cdc42

Similar activation kinetics are observed for RhoA and Rac1.

Live cell imaging confirms activation kinetics.
Live Cell Imaging of Cdc42 Activation

TIRF imaging of MEFs expressing GFP-LifeAct (Actin marker)
Confirms rapid activation kinetics
Location-Specific Activation

Example: Src-family kinases

Regulatory domains often provide proper localization for cellular function
Location-Specific Activation of Cdc42

Activating different populations of Cdc42 leads to varying phenotypes
Activation of Other GTPases

Additional GEFs allow selective and rapid activation of other GTPases (Rac and Rho) in cells.

The ssTIAM construct activates Rac1 (lamellipodia)
Summary

The interaction between Bcl-xL and a BH3 peptide can be used as artificial regulatory domains for GEFs.

Small molecule disruptors of the Bcl-xL/BH3 peptide interaction can be used to activate synthetic GEFs.

Tuning the affinity between Bcl-xL and the BH3 peptide is essential for generating synthetic GEFs with a dynamic range of activities.

Synthetic GEFs can be used to activate Cdc42, RhoA, and Rac1 (GTPases) in cells.

Cellular activation is rapid and dose dependent.