CBI Student Sabbatical Proposal

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10-Week Sabbatical (4 weeks at Stony Brook University, NY; then 6 weeks at CCG, UofM)


My current research focuses on ways to develop selective kinase inhibitors, as both chemical probes and potential cancer therapeutics. Consequently, my proposed sabbatical looks to use my previously developed selective irreversible c-Src inhibitors to explore kinase P-loop conformations through protein crystallization. Also, I am looking to obtain a crystal structure of the c-Yes enzyme to confirm a kinked P-loop structure, seeing as there is no reported crystal structure of this protein to date.
PROPOSAL LETTER

Engineering selective protein tyrosine kinase inhibitors to characterize signal transduction pathways involved in cancer progression.

BACKGROUND

Protein tyrosine kinases (PTKs) control a variety of important human biological processes including cell proliferation, differentiation, adhesion, invasion and motility (1). These ATP-dependent processes are highly regulated, but can become problematic when a particular kinase becomes dysregulated leading to the creation of an oncogenic cell. Approximately half of these oncogene products are PTKs (2). Consequently, PTKs have become attractive small molecule drug targets for the treatment of several cancers such as lung, colon, breast and bladder (3). Current research efforts have focused on designing inhibitors to compete with ATP in the catalytically active site of the kinase. There are, however, more than 500 protein kinases in the human kinome that share nearly identical ATP-binding domains, making selectivity and subsequent cellular characterization difficult to achieve. This non-specificity, coupled with the tumor’s ability to develop mutations within the kinase’s catalytic domain, has lead to the creation of multi-pathway inhibitors that are generally limited in scope to late-stage cancer treatments (4). Although this pan-kinase activity has been effective for several approved inhibitors in treating cancer, the ability to limit off-target toxicity with selective compounds remains a significant challenge in the development of cancer therapeutics.

To overcome this issue, my lab looks to develop strategies for the design of selective chemical probes to initially characterize their effects upon a single kinase, prior to using them to study a larger cellular signaling system. The use of small molecules to study kinase function provides several advantages over genetic techniques such as RNA interference, including the ability to act in fast, dose-dependent manner to achieve varying levels of inactivation (5). Furthermore, chemical means can inhibit catalytic activity without affecting the other domains of the kinase necessary for essential protein-protein interactions. One specific way to accomplish this is through utilizing covalent modification of a non-conserved cysteine residue of the kinase.

Irreversible inhibitors have been used to study a number of different signaling systems, seeing as approximately 40% of the human kinome contains a cysteine residue that could potentially be modified by an ATP-binding site inhibitor (6). Because of their ATP-noncompetitive nature once bound, covalent inhibitors can be provided at lower doses with less circulating time in the blood needed to produce an efficacious result (7). Some of the kinases that have been targeted previously include RSK (8), EGFR (9,10,11), VEGFR2 (12), bruton’s tyrosine kinase (13), FGFR (14) and JNK (15). These past irreversible inhibitors generally start with an inherent level of non-covalent selectivity that improves upon targeting the non-catalytic cysteine residue of interest. In contrast, our work demonstrates the ability to start with a known promiscuous kinase inhibitor (pyrazole-pyrimidine) core scaffold (16,17) where selectivity for a single kinase can be engineered in through covalent modification. This concept was applied to the C280 residue of the PTK

Figure 1. Kinase domain of c-Src with targeted C280 circled (blue).
c-Src, which is located in the ceiling or P-loop of the ATP pocket and is found in only 8 other kinases (Figure 1). Shokat and coworkers have previously used chemical genetic strategies as a selectivity filter to study c-Src activity (18,19), but they did not operate on the wild type enzyme system or target the P-loop, instead choosing to mutate specific residues of interest to cysteines.

**PRELIMINARY WORK**

Initial data collection was used to determine an optimal linker length and electrophile combination to covalently react with the C280 residue. As a result, a series of six inhibitors were produced using either a glycine or beta-alanine unit attached to an acrylic-, alpha-chloro ketone- or vinyl sulfone-modifying group. Biochemical kinase activity was assessed via a continuous fluorescence activity assay (20) and confirmed the time dependent inhibition of c-Src for all six compounds, while constant levels of inhibition were observed with a C280S c-Src mutant (Figure 2a). Gel filtration and ESI mass spectrometry further validated irreversibility for each of the electrophiles. The two most potent hits of the series were based off the ethylene spacer scaffold and contained either an alpha-chloro ketone- (FEK-2-109) or vinyl sulfone- (FEK-2-119) reactive moiety. To establish a single lead compound, both inhibitors were subjected to a wide range of pre-incubation times with enzyme to determine their rates of inactivation (k\textsubscript{inact}) and inhibition constants (K\textsubscript{i}) (Figure 2b) according to the procedure by Krippendorff and coworkers (21). FEK-2-119 was proven to have more favorable properties, with both a more potent K\textsubscript{i} value (360 ± 1 nM) and faster rate of inactivation (8.696 ± 1.859 x 10\textsuperscript{-2} min\textsuperscript{-1}) in comparison to FEK-2-109.

<table>
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<th>n</th>
<th>R</th>
<th>Compound</th>
<th>WT c-Src, IC\textsubscript{50} (nM)</th>
<th>C280S c-Src, IC\textsubscript{50} (nM)</th>
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<td>1</td>
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<td>FEK-2-79</td>
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<td>2</td>
<td></td>
<td>FEK-2-119</td>
<td>93 ± 20</td>
<td>1,237 ± 59</td>
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**Figure 2.** Biochemical assay data for irreversible inhibitors of wild type and C280S c-Src. (a) General kinase inhibitor scaffold is pictured along with all electrophile combinations synthesized. Assay data shown is after a two hour pre-incubation with enzyme and inhibitor. (b) Time dependence inhibition of c-Src for both FEK-2-109 and FEK-2-119. K\textsubscript{i} and k\textsubscript{inact} values were determined using XLfit software (21).
With FEK-2-119 in hand, we sought to confirm its selectivity over a reversible-binding analogue, FEK-2-121, by testing both compounds against homologous PTKs, c-Abl and Hck (Figure 3). FEK-2-119 showed a 82-fold increase in potency over c-Abl with a normalized (to FEK-2-121) 28-fold value and a 86-fold increase in potency over Hck with a normalized (to FEK-2-121) 22-fold value.

The cellular activity of the irreversible probe FEK-2-119 was demonstrated through incubation with v-Src stably transfected NIH-3T3 murine fibroblast cells, where growth is dependent on v-Src activity. The v-SRC gene differs from c-SRC by several point mutations (including a T341I gatekeeper mutation) and the deletion of the regulatory C-terminal domain, rendering it constitutively active (1). First, v-Src 3T3 cells were treated with varying concentrations of vinyl sulfone in a 2D cell culture for one hour, prior to cell lysis and western blot analysis (Figure 4a). The irreversible inhibitor at 100 µM (lane 3) reduced global tyrosine phosphorylation levels dramatically in comparison to the vehicle (lane 1) and to a lesser degree at 10 µM (lane 2). Furthermore, continuous 24-hour monitoring of the cells in a 3D v-Src 3T3 cell format using Essen IncuCyte camera imaging allowed for growth velocities and subsequent GI₅₀ curves to be determined for both FEK-2-119 and its reversible analogue FEK-2-121 at various concentrations (Figures 4b & 4c). The irreversible inhibitor displayed a 4-fold increase in potency over this time frame, emphasizing the advantage of covalent modification in regards to a cellular system.

**Figure 4.** Cellular analysis of both irreversible and reversible inhibitors in v-Src stably transfected NIH-3T3 cells. (a) Western blot analysis of phosphotyrosine detection (HRP-chemiluminescence) of FEK-2-119 at 10 µM (lane 2) and 100 µM (lane 3) after 1 hour of incubation with 2D v-Src 3T3 cells, with the vehicle present at 1% DMSO (lane 1). (b) IncuCyte camera images of v-Src 3T3 3D cell culture (using basement membrane extract) after 24 hours of incubation with vehicle at 1% DMSO (left), FEK-2-119 at 10 µM (middle) and FEK-2-121 at 10 µM (right), all contrast enhanced. (c) GI₅₀ plot for the percent cell confluence developed over a 24 hour time period via IncuCyte camera images. FEK-2-119, GI₅₀ = 3.588 µM, is displayed in red (squares) with FEK-2-121, GI₅₀ = 15.857 µM, shown in black (triangles). Each growth velocity point at a given concentration was collected in triplicate.

Following our past characterization of this first generation of irreversible wild type c-Src inhibitors, we are looking to explore the conformational dynamics of multiple kinases under various conditions to aid in the development of future inhibitors. We propose to use our lead covalent modifier, FEK-2-119, to initially

<table>
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<th>Compound</th>
<th>c-Src, IC₅₀ (nM)</th>
<th>c-Abl, IC₅₀ (nM)</th>
<th>Hck, IC₅₀ (nM)</th>
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<tr>
<td>FEK-2-119</td>
<td>93 ± 20</td>
<td>7,649 ± 1,128</td>
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<td>FEK-2-121</td>
<td>720 ± 162</td>
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**Figure 3.** Selectivity assessment of irreversible inhibitor FEK-2-119 over reversible binding analogue, FEK-2-121.

**Figure 5.** Rates of inactivation (kₙₐₓ) of WT c-Src, Q252C c-Abl & c-Yes.
investigate how the rate of inactivation (1st order loss of enzyme activity) is affected in wild type or mutated kinases (chemical genetic approach) that contain cysteine residues in the P-loop. Early work with a Q252C c-Abl variant (the equivalent C280 residue in c-Src) has shown that the kinked P-loop conformation of the kinase does result in a greater than 2-fold increase in the rate of inactivation (Figure 5) over c-Src (undistorted P-loop), which is consistent with past research performed within the group that has shown P-loop interactions to be critical in determining c-Src selectivity over c-Abl (22). Additionally, work with c-Yes (WT kinase has cysteine residue in the equivalent P-loop position) has displayed a similar rate of inactivation to c-Abl, which suggests that this kinase also adopts a similar kinked P-loop conformation. This is significant since there are no reported crystal structures of the kinase domain of c-Yes.

PROPOSAL & RELATION TO THESIS WORK
Consequently, my proposed sabbatical work at both Stony Brook and the University of Michigan would look to build upon this past collected data and would include three specific aims:

**Aim 1. Obtaining a crystal structure for c-Src covalently bound inhibitor.** The final portion of this first set of c-Src irreversible inhibitors project would involve crystalizing the FEK-2-119 and c-Src complex; not to confirm irreversibility (which has been thoroughly done biochemically) but rather to examine the amount of P-loop movement upon binding as well as to observe any possible distal protein conformational changes. Additionally, future crystal structures of other c-Src mutant forms (T338I & T338M) with FEK-2-119 could be used to further study P-loop dynamics, which could possibly aid in explaining the decrease in $K_m$ for ATP binding in both of these enzymes.

**Aim 2. Confirming c-Yes kinked P-loop.** To validate our earlier conformational dynamics work, it would be extremely beneficial to crystalize the c-Yes PTK. c-Yes is a Src family kinase member, and relatively little is known about how kinase inhibitors are able to differentiate between c-Src and c-Yes binding. I propose to obtain a structure of c-Yes with dasatinib (reversible, type I, ATP-competitive inhibitor of c-Src) in order to visualize its kinked P-loop confirmation.

**Aim 3. Exploring type II kinase inhibitor binding modes.** My final objective is to obtain a crystal structure for a type II kinase inhibitor of c-Src, which binds to the inactive conformation of the enzyme (23). Binding information for this compound will help me design and synthesize a type II irreversible kinase inhibitor that can help explore the cross-talk interactions between the P-loop and activation loop of the kinase. This work will greatly benefit kinase inhibitor development research, seeing as one of the most successful kinase inhibitors to date (imatinib, Novartis) binds to its target via this mechanism.
References: