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Publication Date
2013

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Inhibition of the T-cell Kinase ZAP-70

by

Patrick Ramos Visperas

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular and Cell Biology in the Graduate Division of the University of California, Berkeley

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Spring 2013
Abstract

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor John Kuriyan, Chair

The adaptive immune system responds to foreign antigens to produce a highly specific immune response. T-cells, a type of white blood cell of the adaptive immune system, can either directly kill infected cells or aid in sending signals that regulate the immune response. The T-cell receptor is expressed on the surface of T-cells and recognizes foreign antigens presented by antigen presenting cells. Unlike transmembrane receptor tyrosine kinases, the T-cell receptor does not contain its own kinase activity. Rather, the T-cell receptor recruits a number of non-receptor tyrosine kinases upon extracellular antigen binding to trigger intracellular signaling cascades. Zeta-chain associated protein of 70-kDa (ZAP-70) is one such non-receptor tyrosine kinase that is recruited to regions of the T-cell receptor known as Immuno-receptor Tyrosine-based Activation Motifs (ITAMs). Clinical observations as well as chemical genetic studies suggest that ZAP-70 is a possible target for T-cell inhibition.

Small molecule inhibitors of ZAP-70 may prove to be therapeutically useful for the treatment of autoimmune disease or for organ transplant rejection. Because no such molecules exist today, we hope to exploit the structural insights gleaned from the recently solved autoinhibited structure of ZAP-70 to discover an allosteric inhibitor. In this dissertation, I describe a method of inhibiting the interaction between ZAP-70 and the T-cell receptor. I performed a high throughput screen for inhibitors of ZAP-70:ITAM binding and identified a collection of hit compounds. Inhibition by these compounds was verified in an orthogonal assay and was dose-dependent. I found that the compounds targeted the tandem-SH2 domains of ZAP-70 and were thiol-reactive. I go on to show that covalent cysteine modification underlies the inhibition of ZAP-70 and that individual compounds are specific for different cysteine residues.
For everyone who believed in me, for everyone who took a chance on me,
for everyone who supported me, this is for you.
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I would like to thank all those in the Kuriyan lab who provided the mentorship that enabled me to succeed. Luke Chao was my rotation mentor and introduction to the lab. Sebastian Deindl provided a tremendous amount of instruction while I stumbled through the first years of graduate school and performed the lab’s pioneering work on ZAP-70. Markus Seeliger instructed me in crystallography, running the lab’s undergraduate research program, and offered invaluable encouragement. Qingrong Yan was my first cohort on the ZAP-70 project and his insatiable curiosity led to many fruitful discoveries. Finally, Jonathan Winger, without his tutelage and support none of this would have been possible.

Xiaoxian Cao facilitated this work through countless hours spent on sf9 expression. David King in the UC Berkeley HHMI mass spectrometry and peptide synthesis facility synthesized reagents essential for this work. Steven Jacques maintained an ideal work environment through efficient management of the lab. I would also like to acknowledge my undergraduate students, Kevin Lin and Diane Aum for their tireless endeavors, effort, and enthusiasm. I hope they learned as much from me as I did from them.

Our collaborator, Art Weiss was a perpetual source of optimism and his encyclopedic knowledge of the immune system was a major driving force for this work. Chris Wilson and Michelle Arkin at the Small Molecule Discovery Center were instrumental in developing and implementing the high throughput screen and provided technical guidance and expertise. Mark Gallup at Nurix provided insights on the screening results that changed how we studied the compounds.

Finally, I would like to thank my advisor, John Kuriyan, for encouraging and nurturing my intellectual development. By acting as a model of rigorous and thoughtful scientific investigation, John led me to think more profoundly about my experiments and in doing so set a very high academic standard. I wish to thank him for providing the opportunity and the patience for me to grow as a scientist and also for second chances.
Chapter 1:
Introduction
1.1 Introduction

During the first steps of activation of a T-cell, the T-cell receptor (TCR) recognizes foreign peptide fragments bound to the Major Histocompatibility Complex (MHC) on an Antigen Presenting Cell (APC). This binding event triggers calcium release and results in the activation of intracellular signaling cascades, such as the MAP kinase pathway, ultimately leading to an immune response (Au-Yeung, Levin et al. 2010, Wang, Kadlec et al. 2010). Early experiments demonstrated that portions of the T-cell receptor exhibited two separate activities: the α/β chains of the TCR functioned as the ligand binding portion and the γ, δ, ε, and ζ chains of a TCR-associated membrane protein complex known as CD3 functioned as the signaling portion (Brenner, Trowbridge et al. 1985, Ohashi, Mak et al. 1985). While it was known that following T-cell receptor stimulation there is robust and rapid phosphorylation of tyrosine residues on both the T-cell receptor itself as well as downstream effector proteins, neither the T-cell receptor nor the CD3 coreceptor were found to contain an intracellular catalytic domain like those found in membrane-spanning receptor tyrosine kinases.

Studies of T-cells defective in T-cell receptor signaling showed that these cells failed to exhibit tyrosine phosphorylation following T-cell receptor stimulation. Further experiments found that the cells lacked a protein called Lck, a Src-family tyrosine kinase (Straus and Weiss 1992). Lck associates with the T-cell receptor upon T-cell activation and was found to be responsible for phosphorylation of the CD3 ζ-chain (Iwashima, Irving et al. 1994). A tyrosine-phosphorylated protein of 70-kDa was found to be associated with the phosphorylated ζ-chain, and was later discovered through sequence analysis to contain a kinase domain (Chan, Irving et al. 1991, Chan, Iwashima et al. 1992). Biochemical studies confirmed that this zeta-associated protein of 70-kDa (ZAP-70) has tyrosine kinase activity, is specifically recruited to the T-cell receptor upon T-cell receptor phosphorylation, is activated by Lck-mediated tyrosine phosphorylation (Chan, Iwashima et al. 1992, Wange, Kong et al. 1992, Iwashima, Irving et al. 1994), and is indispensable for downstream T-cell signaling (Arpaia, Shahar et al. 1994, Chan, Kadlec et al. 1994, Elder, Lin et al. 1994).

A protein that functions similarly in B-cells was found to be a close relative of ZAP-70, known as Syk (Geahlen 2009, Mocsai, Ruland et al. 2010). Subsequent studies revealed that ZAP-70 specifically phosphorylates the proteins Linker for Activation of T-cells (LAT) (Zhang, Sloan-Lancaster et al. 1998) and SH2-containing Leukocyte Protein of 76-kDa (SLP-76) (Bubeck Wardenburg, Fu et al. 1996). LAT and SLP-76 act as adaptors that recruit various signaling molecules to propagate T-cell signaling downstream [Figure 1.1].

As a key intermediary in the tyrosine phosphorylation cascade triggered by the T-cell receptor, ZAP-70 recognizes and is activated by phosphorylation signals upstream, and also carries out phosphorylation reactions to further propagate the T-cell receptor signal downstream. ZAP-70 is well suited for this role, containing a pair of SH2 domains for phosphopeptide recognition and a kinase catalytic domain for phosphotransfer to downstream targets. Here, we review the current structural and mechanistic understanding of ZAP-70 activation and autoinhibition.
Figure 1.1 A model for T-cell activation

(1.). MHC-peptide complex binds to the TCR (2.), leading to recruitment of CD4-associated Lck. Lck phosphorylates intracellular ITAMs (3.) that then interact with the tandem-SH2 domains of ZAP-70 to recruit ZAP-70 to the TCR. Lck phosphorylates and activates TCR-bound ZAP-70, which subsequently phosphorylates the adaptor proteins LAT and SLP-76, leading to T-cell activation, proliferation, and differentiation (4.).
1.2 ZAP-70 is a non-receptor tyrosine kinase

The 500 or so protein kinases in the human genome share at their core a highly conserved catalytic kinase domain (Manning, Whyte et al. 2002). The kinase domain is an independently folding domain of approximately 300 residues that catalyzes the transfer of the γ-phosphate from ATP to serine, threonine, and tyrosine residues on substrate proteins. This phosphorylation event often acts as a switch to toggle a substrate protein between “ON” and “OFF” states.

Kinases that phosphorylate tyrosine residues can be grouped into two broad categories. The first category includes the receptor tyrosine kinases, which are often involved in the transfer of signals across the cell membrane in response to binding of an extracellular ligand such as a growth factor. The second category is comprised of the non-receptor tyrosine kinases, which are generally found in the cytoplasm and are involved in propagation of signals throughout the cell. ZAP-70 is a member of this second group.

Kinase domains exhibit a bi-lobed architecture composed of a primarily β-sheet N-terminal lobe (N-lobe) and a primarily α-helical C-terminal lobe (C-lobe) (Taylor, Knighton et al. 1992). A linker connects the two lobes and acts as hinge about which the two lobes of the kinase swivel according to the domain’s activation state. The active site (ATP-binding pocket) is located at the interface of the N-lobe and C-lobe, and both lobes contribute catalytic residues to the active site. Kinase domains assume a common active conformation that is optimal for phosphotransfer (Adams 2001) [Figure 1.2A]. A glutamate residue from a structurally conserved helix (helix αC) in the N-lobe forms an absolutely conserved salt bridge with a lysine that interacts with the α- and β-phosphates of ATP. An aspartate residue from the C-lobe coordinates magnesium, which is required for ATP binding and the phosphotransfer reaction. The N- and C-lobes assume a closed conformation that aligns all the catalytic residues in the active site. A glycine-rich loop formed between strands β1 and β2 folds down to complete the ATP-binding pocket.

Tyrosine kinases are often regulated by phosphorylation on a region of the catalytic domain called the activation loop. In inactive kinase domains, the activation loop is often folded inwards to occlude the active site. Phosphorylation of tyrosine residues in the activation loop causes it to unfurl, exposing the active site and acting as a docking site for substrate. The activation loop of ZAP-70 contains two tyrosine residues, Tyr 492 and Tyr 493, and biochemical and mutation studies have shown that phosphorylation of Tyr 493 is required for activation, while the effect of phosphorylation of Tyr 492 on kinase activity is more complex (Chan, Dalton et al. 1995, Wange, Guitian et al. 1995).

The X-ray crystal structure of the kinase domain of ZAP-70 bound to staurosporine, an ATP-competitive inhibitor, was determined in 2004 (Jin, Pluskey et al. 2004). The ZAP-70 kinase domain was found to be in an active conformation, likely due to the absence of the regulatory N-terminal domains. Indeed, biochemical studies indicate that the isolated kinase domain has a much higher activity than does full-length ZAP-70 (Visco, Magistrelli et al. 2000). The overall fold of the active kinase domain of ZAP-70 strongly resembles that of other active kinases [Figure 1.2B]. The N-lobe and C-lobe are in a conformation that allows the completion of the active site by placing all key residues in position for catalysis. The activation loop adopts an unfurled, active
conformation, and the structurally conserved helix αC is rotated inward, enabling formation of the catalytically critical salt bridge between Glu 386 and Lys 369. The side chain of Asp 479, part of an Asp-Phe-Gly motif conserved in all kinases, is positioned to coordinate a magnesium ion important for ATP binding and catalysis.
Figure 1.2 The isolated ZAP-70 kinase domain adopts an active conformation

(A) Cartoon comparison of active and inactive kinase domains. In an active kinase domain, helix αC (orange) is rotated and swung inward relative to the inactive conformation. This allows a salt bridge required for catalysis to form between a conserved N-lobe lysine and glutamate. The activation loop (magenta) unfurls from the active site clef, and the N- and C-lobes adopt a closed conformation that aligns catalytic residues in the active site.

(B) Ribbon diagram of the ZAP-70 kinase domain bound to staurosporine (Sta) (teal). Active ZAP-70: PDB ID 1U59.
1.3 ZAP-70 contains a pair of accessory SH2 domains

Because of the central involvement of tyrosine phosphorylation in cell signaling, tyrosine kinases are subject to multiple levels of regulation. In addition to an activation loop that requires phosphorylation for kinase activation, non-receptor tyrosine kinases often contain accessory domains that maintain the kinase domain in an inactive state. For example, members of the Src-family kinases, the canonical non-receptor tyrosine kinase family, contain two N-terminal accessory domains, a phosphotyrosine-binding SH2 domain and a poly-proline type II (PPII) helix-binding SH3 domain. Src-family kinases are maintained in an autoinhibited state by two intramolecular interactions: an intramolecular interaction between the SH2 domain and a phosphorylated tyrosine in the C-terminal tail of the kinase domain, and an intramolecular interaction between the SH3 domain and a PPII-type helical conformation assumed by the SH2-kinase domain linker. Together, these interactions stabilize a conformation in which helix αC of the N-lobe is rotated outward, disrupting the arrangement of active site residues and holding the kinase domain in an inactive conformation.

Like Src-family kinases, ZAP-70 contains a pair of accessory domains N-terminal to the kinase domain. The accessory segment of ZAP-70, however, contains two SH2 domains in tandem, instead of the SH2-SH3 module found in Src-family kinases [Figure 1.3]. The two SH2 domains are connected by an inter-SH2 linker (interdomain A) to form the tandem-SH2 unit, which is joined to the C-terminal kinase domain by an SH2-kinase linker (interdomain B). The SH2-kinase linker contains a pair of tyrosine residues, Tyr 315 and Tyr 319, whose phosphorylation by Lck is a critical step in ZAP-70 activation (Di Bartolo, Mege et al. 1999, Brdicka, Kadlec et al. 2005).
Figure 1.3 Comparison of domain organization of Src family kinases and ZAP-70

Comparison of the domain organization of Src and ZAP-70, both non-receptor tyrosine kinases. While the two proteins contain similar kinase domains, each has a different set of accessory domains. Regulatory tyrosine residues discussed in the text are shown.
1.4 Phosphorylation of the SH2-kinase linker is required for ZAP-70 activation

While phosphorylation of the activation loop of ZAP-70 is required for catalytic domain activation, additional phosphorylation is required for activation of the full-length enzyme. Two tyrosine residues in the SH2-kinase linker, Tyr 315 and Tyr 319, are phosphorylated upon T-cell activation (Di Bartolo, Mege et al. 1999). Using stably-transfected Jurkat T cells, in which stimulation of T-cell receptor signaling leads to ZAP-70-mediated activation of the transcription factor NFAT, studies with a series of ZAP-70 constructs demonstrated that the Y319F mutation results in a dramatic decrease in NFAT activity, while the Y315F mutation causes a modest decrease in NFAT activity (Wu, Zhao et al. 1997). Furthermore, murine T-cell hybridomas stably transfected with the Y319F mutant showed greatly reduced phosphorylation of the ZAP-70 substrates SLP-76 and LAT, compared to hybridomas stably transfected with wild-type ZAP-70 (Di Bartolo, Mege et al. 1999). These data indicate that phosphorylation of Tyr 315 and Tyr 319 is a key step in the activation of ZAP-70.

The requirement for phosphorylation of Tyr 315 and Tyr 319 in ZAP-70 activation suggests two possible roles for the SH2-kinase linker in regulation of the kinase domain: phosphorylation of these residues might be required for recruitment of downstream effector molecules, or the SH2-kinase linker might be involved in stabilizing the inactive state of ZAP-70. While the two tyrosine residues can simply serve a scaffolding function when phosphorylated (Wu, Zhao et al. 1997, Gelkop and Isakov 1999, Williams, Irvin et al. 1999), a more direct role for the SH2-kinase linker in ZAP-70 autoinhibition was supported by subsequent studies. In a ZAP-70-deficient Jurkat cell line, a ZAP-70 construct featuring a deleted SH2-kinase linker restored NFAT expression in response to stimulation of the T-cell receptor. The activity of this ZAP-70 deletion construct was even greater than the activity of wild-type ZAP-70. Crucially, recruitment of ZAP-70 to phosphorylated CD3 ζ-chains was unaffected by deleting the SH2-kinase linker (Zhao, Williams et al. 1999). Together, the data demonstrate that, while the SH2-kinase linker plays a role in regulating ZAP-70 activity, it is not required for recruitment to the T-cell receptor, nor is it required for downstream T-cell signaling.

One possible mechanism by which the residues Tyr 315 and Tyr 319 in the SH2-kinase linker might regulate ZAP-70 activation is by formation of an intramolecular autoinhibitory interaction, in a manner analogous to that observed for the receptor tyrosine kinase EPH receptor B2 (EPHB2). Structural and biochemical studies of EPHB2 kinase revealed that a pair of regulatory tyrosine residues in the juxtamembrane segment forms an intramolecular autoinhibitory interaction that is relieved upon phosphorylation. Using ZAP-70-deficient Jurkat cells, Brdicka and coworkers demonstrated that when Tyr 315 and Tyr 319 were mutated to alanine, or when the SH2-kinase linker was removed completely, ZAP-70 remained competent to rescue NFAT-driven luciferase activity. Furthermore, phosphorylation of transiently transfected ZAP-70 on Tyr 319 required co-transfection of Lck, as did phosphorylation of the ZAP-70 substrate LAT. However, the ZAP-70 Y315/319A double mutant was able to phosphorylate LAT in the absence of Lck (Brdicka, Kadlecak et al. 2005). Together, these data indicated that phosphorylation of Tyr 315 and Tyr 319 by Lck is required for activation of ZAP-70, and suggested that these residues participate in an autoinhibitory interaction that is relieved by mutation of the tyrosine residues to alanine.
The tandem-SH2 unit undergoes a conformational change upon ITAM binding

A great deal of work has demonstrated that the tandem-SH2 unit is instrumental in the high-affinity recruitment of ZAP-70 to specific motifs in the activated T-cell receptor (Wange, Malek et al. 1993, Weissenhorn, Eck et al. 1996, Ottinger, Botfield et al. 1998). These Immuno-receptor Tyrosine-based Activation Motifs (ITAMs) have the sequence YXX(L/I)X(6–8)YXX(L/I), in which the pair of tyrosine residues is phosphorylated by the Src-family kinase Lck upon T-cell receptor activation, and X can be any amino acid. While we focus on the ZAP-70 tandem-SH2 domains here, much has also been learnt about the Syk tandem-SH2 domains, and that information complements the information on ZAP-70 (Futterer, Wong et al. 1998, Grucza, Futterer et al. 1999, Grucza, Bradshaw et al. 2000, Kumaran, Grucza et al. 2003, Groesch, Zhou et al. 2006, Zhang, Oh et al. 2008).

The mechanism of high-affinity ITAM binding and recognition by the tandem-SH2 domain unit of ZAP-70 was revealed in 1995, when Hatada and coworkers solved the structure of the tandem-SH2 unit of ZAP-70 bound to a doubly phosphorylated ITAM peptide (Hatada, Lu et al. 1995). In the ITAM-bound structure (Figure 1.4A), the tandem-SH2 unit resembles the letter Y, with the two SH2 domains adjacent to each other, connected by a coiled-coil-like stalk formed by the 65-amino acid inter-SH2 linker. The doubly phosphorylated ITAM peptide binds to both SH2 domains, which also interact with each other. The coiled-coil formed by the inter-SH2 linker stabilizes the SH2 domains in the proper orientation for ITAM binding, and may constrain the distance between the phosphotyrosine binding sites, limiting the tolerated number of residues between phosphotyrosines in an ITAM motif. The C-terminal SH2 domain interacts with the N-terminal phospho-YXXL sequence of the ITAM in a canonical phosphopeptide-SH2 binding mode. However, the phosphotyrosine residue of the C-terminal phospho-YXXL ITAM sequence binds at the junction of the two SH2 domains and interacts with residues from both, leading to speculation that the N-terminal SH2 domain has an incomplete phosphotyrosine binding site (Hatada, Lu et al. 1995).

A key conclusion drawn from the structure of the ITAM-bound tandem-SH2 unit was that the interaction between the SH2 domains is likely quite weak, being mediated by a relatively small number of contacts, and both the inter-SH2 coiled-coil and the ITAM itself likely provide much of the stabilization seen in the crystal structure. These observations suggested that the tandem-SH2 domain unit in the absence of ITAM might exist in an entirely different conformation, and that a conformational switch in the tandem-SH2 unit upon ITAM binding might drive allosteric regulation of the ZAP-70 kinase domain.

Evidence that such a conformational switch was possible was obtained in 2002 when the crystal structure of the unliganded (apo) tandem-SH2 domains of ZAP-70 was published, along with an NMR study describing the conformational dynamics of the apo tandem-SH2 unit (40). Compared to the ITAM-bound structure, the C-terminal SH2 domain of the apo tandem-SH2 unit is shifted away from the N-terminal SH2 domain so that the Y-shape of the ITAM-bound complex has now become more like an “L”, and the tandem-SH2 domains are not in a conformation competent to bind an ITAM (Figure 1.4B). The inter-SH2 coiled-coil is in a similar conformation in the apo structure as it is in the ITAM-bound structure, except for one notable change. Part of the C-terminal helix of the coiled-coil linker has melted to become an unstructured region, and the
original position vacated by the melted helix is now occupied by one of the helixes of the C-terminal SH2 domain. The net result of this change is an increase in the buried surface between the inter-SH2 linker and the C-terminal SH2 domain, and it was postulated that this increase helps to compensate for the lack of stabilization provided by the binding of an ITAM peptide (Folmer, Geschwindner et al. 2002).

The instability of the inter-SH2 linker was further demonstrated by the accompanying NMR studies, which showed that in the apo state the resonances corresponding to the amino acid residues in the coiled-coil region disappeared due to broadening, indicative of conformational exchange (Folmer, Geschwindner et al. 2002). The conformational flexibility of the inter-SH2 linker demonstrated by the studies described above is consistent with previous experiments that showed that an antibody against the inter-SH2 linker no longer bound to the tandem-SH2 domains or to full length ZAP-70 in the presence of doubly phosphorylated ITAM peptide or ITAM-containing ζ-chains (Grazioli, Germain et al. 1998). Together, these data suggested a possible activation mechanism in which ITAM binding would result in the disruption of any contacts between the tandem-SH2 unit and the kinase domain of ZAP-70, possibly leading to release of the tandem-SH2 unit and relief of autoinhibition.
Figure 1.4 Structures of the apo and ITAM-bound tandem-SH2 domains

(A) Ribbon diagram of the ITAM-bound ZAP-70 tandem-SH2 domain, PDB ID 2OQ1. 
(B) Ribbon diagram of the apo ZAP-70 tandem-SH2 domain, PDB ID 1M61. The inter-SH2 linker region is depicted in green, the N-terminal SH2 domain in yellow, and the C-terminal SH2 domain in red. The ITAM peptide is shown in teal. 
(C) Comparison of the structures of the apo (gray) and ITAM-bound tandem-SH2 domains of ZAP-70. The molecules were superimposed by aligning the N-terminal SH2 domains. The arrow represents the direction of the large structural rearrangement that occurs upon ITAM binding.
1.6 Overview of the structure of autoinhibited ZAP-70

While it was clear from structural and biochemical studies that the ZAP-70 kinase domain can be fully active in the absence of the tandem-SH2 unit, and that the tandem-SH2 unit can undergo large conformational rearrangements upon ITAM binding, it was unclear how the tandem-SH2 unit inhibited the catalytic activity of the kinase domain, and how conformational changes in the tandem-SH2 unit upon ITAM binding might result in relief of autoinhibition. Progress towards understanding regulation of the ZAP-70 kinase domain by the tandem-SH2 unit was made in 2007, when, in collaboration with Art Weiss and coworkers, our lab solved the structure of a nearly full-length ZAP-70 construct containing the tandem-SH2 unit, interdomains A and B, the kinase domain, and most of the C-terminal tail (Deindl, Kadlec et al. 2007). Expression and crystallization of the construct was facilitated by the mutation of two tyrosine residues in the SH2-kinase linker to phenylalanine (Y315F and Y319F), and mutation of a catalytically important aspartate residue (Asp 479) to asparagine. Together, these mutations render this ZAP-70 construct, termed ZAP-70-FF, inactive. Our lab would later solve the structure of a ZAP-70 construct with the wild type SH2-kinase linker sequence containing tyrosine residues in position 315 and 319 (Yan, Barros et al. 2013). The new structure corrected a five-residue sequence register error in the SH2-kinase linker. However, the overall architecture is preserved in both structures, and the new structure is very similar to that recently solved of Syk (Gradler, Schwarz et al. 2013). In the ZAP-70 structure, the tandem-SH2 unit is observed to pack against the distal side of the kinase domain, with the SH2 domains facing away from the kinase domain and the coiled-coil of the inter-SH2 linker in contact with the C-lobe of the kinase domain [Figure 1.5]. Part of the SH2-kinase linker is disordered, while the remainder is sandwiched between the inter-SH2 coiled-coil and the N-lobe of the kinase. The tandem-SH2 unit itself exhibits a conformation very similar to that seen in the structure of the isolated tandem-SH2 unit solved previously (Folmer, Geschwindner et al. 2002).

Examination of the structure of ZAP-70 reveals that it is in an autoinhibited state: the kinase domain adopts an inactive conformation [Figure 1.6], similar to that observed for the structures of Src-family kinase catalytic domains in the presence of their regulatory domains (Sicheri, Moarefi et al. 1997, Xu, Harrison et al. 1997). Compared to its position in the active conformation, helix αC in the N-lobe is rotated outward, resulting in the removal of the conserved Glu 386 from the active site. As a consequence, the catalytically crucial residue Lys 369 is not in a position to interact with the phosphates of ATP. Instead, Lys 369 forms a salt bridge with Asp 479 of the conserved Asp-Phe-Gly motif, keeping the aspartate side chain from adopting a position competent to bind the catalytically required magnesium ion. The N-lobe is swung away from the C-lobe, further disrupting the active site. Part of the activation loop forms a short helix characteristic of the inactive conformation, which packs against and stabilizes the displaced conformation of helix αC. The remainder of the activation loop is unstructured.
**Figure 1.5 Crystal structure of autoinhibited ZAP-70**

The N-terminal SH2 domain (yellow), inter-SH2 linker (green), C-terminal SH2 domain (red), SH2-kinase linker (orange), and kinase domain (blue) are shown in a ribbon diagram. The ATP analogue AMPPNP is depicted in magenta, and magnesium is depicted as a grey sphere. Disordered regions are depicted as dotted lines. Full-length ZAP-70: PDB ID 2OZO.
Figure 1.6 Comparison of active and inactive ZAP-70 kinase domains with the inactive kinase domain of Hck.

The conformation of the kinase domain of autoinhibited full-length ZAP-70 resembles that of inactive Hck, a Src-family kinase. Helix αC is shown in orange, the activation loop is shown in magenta, and the short 310 helix in the activation loop following the conserved Asp-Phe-Gly motif is shown in cyan. A disordered part of the activation loop is shown as a dotted line. Active ZAP-70: PDB ID 1U59. Inactive ZAP-70: PDB ID 2OZO. Inactive Hck: PDB ID 1QCF.
1.7 The role of interdomain linkers in ZAP-70 autoinhibition

The inactive conformation of the kinase domain is maintained by interactions between the inter-SH2 linker, the SH2-kinase linker, and the N-lobe of the kinase domain. The SH2-kinase linker emerges from the N-terminus of the kinase domain, traces along the distal side of the N-lobe, and then interdigitates with the inter-SH2 linker. This packing of the linkers between the tandem-SH2 unit and the kinase domain is referred to as the linker-kinase sandwich [Figure 1.7]. The inter-SH2 linker and N-terminal SH2 domain also interact directly with the C-lobe of the kinase domain.

The hydrophobic residues, Trp 131 in the inter-SH2 linker, Met 310 and Tyr 315, contributed by the SH2-kinase linker, and Pro 369 from the kinase domain, form a hydrophobic core that stabilizes the linker-kinase sandwich. The importance of the hydrophobic core to the stability of the linker-kinase sandwich was confirmed by mutational analysis in vitro and in vivo (Deindl, Kadlec et al. 2009). Additionally, a number of hydrogen bonds also stabilize the linker-kinase sandwich. Because the linker-kinase sandwich interaction is largely hydrophobic, it is reasonable to assume that phosphorylation of the regulatory tyrosine residues Tyr 315 and Tyr 319 in the SH2-kinase linker would result in disruption of the hydrophobic core that stabilizes the linker-kinase sandwich.

Comparisons between the kinase domain of the autoinhibited ZAP-70 and the active-like state of the ZAP-70 kinase domain solved in isolation show how the SH2-kinase linker stabilizes the autoinhibited kinase domain to be in an inactive state. Residues Pro 322 to Lys 326 form a short segment of α-helix that positions Tyr 319 to insert into the kinase N-lobe, acting as a wedge to stabilize helix αC in an “out” or “Src-like” inactive conformation. A network of hydrogen bonds as well as a hydrophobic cluster fortifies the kinase inactive conformation enforced by insertion of Tyr 319. Tyr 319 of ZAP-70 plays an analogous role to Trp 260 in Src kinase (chicken numbering used). Trp 260 acts as a wedge to limit rotation of helix αC and stabilizes the Src inactive conformation (Sicheri and Kuriyan 1997, Xu, Harrison et al. 1997). Leu 226 is in a structurally similar location to Trp 260 in ZAP-70, but kinase activity assays demonstrate that Tyr 319, not Leu 226, actually plays an analogous functional role (Yan, Barros et al. 2013).
Figure 1.7 Linker-kinase sandwich interaction

The linker-kinase sandwich of autoinhibited ZAP-70 (circled) is composed of the inter-SH2 linker (green), the SH2-kinase linker (orange), and the kinase domain (blue). Autoinhibited ZAP-70: PDB ID 2OZO.
1.8 The mechanism of activation of ZAP-70

The arrangement of key regulatory features in the structure of ZAP-70 suggests a mechanism for activation of ZAP-70 via sequential disruption of the interactions that maintain the autoinhibited conformation [Figure 1.8]. Upon activation of the T-cell receptor and phosphorylation of TCR-associated ζ-chains by Lck, ZAP-70 is recruited to the membrane by binding of the tandem-SH2 unit to phosphorylated ITAMs. Engagement of the ITAM by the tandem-SH2 unit reorganizes the SH2 domains, changing the conformation of the inter-SH2 coiled-coil and resulting in the disengagement of the tandem-SH2 unit from the kinase domain and disassembly of the linker-kinase sandwich.

Upon disassembly of the linker-kinase sandwich, the SH2-kinase linker peels away from the N-lobe of the kinase domain, allowing the structural elements of the kinase domain to assume a catalytically competent conformation and exposing the regulatory tyrosine residues Tyr 315 and Tyr 319. However, removal of the tandem-SH2 unit alone is not sufficient to fully catalytically activate the kinase (Yan, Barros et al. 2013). Rather, Lck phosphorylation of the regulatory tyrosine residues Y315/319 is required to prevent the re-formation of the inhibitory interaction between the SH2-kinase linker and the N-lobe of the kinase domain. The activation loop is also phosphorylated by Lck, resulting in the exposure of the active site of ZAP-70 for substrate binding and catalysis.
Figure 1.8 Structural insights suggest a model for the activation of ZAP-70.

Top: The structure of the ITAM-bound tandem-SH2 domains overlaid on the structure of full-length autoinhibited ZAP-70 by aligning the N-terminal SH2 domains of both structures. ITAM binding to the tandem-SH2 domains of ZAP-70 (N-terminal SH2 domain in yellow, inter-SH2 linker in green, C-terminal SH2 domain in red, and ITAM peptide in teal) results in a large conformational shift relative to the tandem-SH2 domains of inactive ZAP-70 (grey). The inactive ZAP-70 kinase domain (blue), and the SH2-kinase linker (orange) are also shown. Arrows depict structural rearrangements proposed to occur during ZAP-70 activation. Bottom: Schematic diagram of ZAP-70 conformational changes upon ITAM binding and Lck activation, as described in the text. Yellow circles indicate phosphorylation events on critical tyrosine residues. Inactive ZAP-70: PDB ID 2OZO. ITAM-bound tandem-SH2 domains: PDB ID 2OQ1. Apo tandem-SH2 domains: PDB ID 1M61.
1.9 ZAP-70 in disease, target validation, and inhibitor studies

Correct regulation of ZAP-70 activity and expression is crucial to normal immune function as both hyper- and hypomorphic ZAP-70 results in a disease phenotype. Characterization of a strain of mice, denoted SKG mice, that exhibited autoimmune rheumatoid arthritis revealed that it was a result of a missense mutation (W163C) in ZAP-70 (Sakaguchi, Takahashi et al. 2003). ZAP-70 is used as a marker for the severity of B-cell Lymphocytic Leukemia (CLL), among the most prevalent forms of cancer. In patients with CLL, high ZAP-70 expression is often correlated with a highly aggressive form of CLL and often fatal prognosis (Orchard, Ibbotson et al. 2004). While ZAP-70 is known to be able to transduce signals in B-cells (Kong, Bu et al. 1995), the relationship between ZAP-70 expression and CLL is not well understood (Chiorazzi, Rai et al. 2005). Conversely, the absence of ZAP-70 results in very different disease state. Patient samples with Severe Combine Immune Deficiency (SCID), a disease in which there is a total lack of immune function, were shown to be lacking functional ZAP-70 (Elder, Hope et al. 1995) or contain a non-functional ZAP-70 mutant (Chan, Kadlecck et al. 1994, Elder, Lin et al. 1994).

In addition to having been shown to play an important role in a number of diseases, much work has been done to validate ZAP-70 as a bona fide drug target. Early studies with the just the tandem-SH2 domains of ZAP-70 or a catalytically dead kinase domain showed that their overexpression results in a dominant negative effect where impaired T-cell signaling is observed (Northrop, Pustelnik et al. 1996, Qian, Mollenauer et al. 1996). Mutations that impair SH2-phosphotyrosine binding prevent ZAP-70 phosphorylation which is required for ZAP-70 to be fully catalytically active (Iwashima, Irving et al. 1994). Studies that utilized a synthetic phosphatase-resistant ITAM peptide analogue to compete with ITAMs for tandem-SH2 binding also saw a decrease in T-cell signaling (Wange, Isakov et al. 1995). This demonstrates quite concretely that preventing ZAP-70 from localizing to the T-cell receptor is a viable means of inhibiting the protein.

Experiments in which ZAP-70 is rendered catalytically inactive have also been informative as validation of ZAP-70 inhibition as a means of down regulating T-cell activity. Transfection of kinase dead ZAP-70 shows marked decrease in total tyrosine phosphorylation (Iwashima, Irving et al. 1994, Qian, Mollenauer et al. 1996). In a chemical genetic approach, the bump-hole strategy (Bishop, Shah et al. 1998) has been used to develop a kinase inhibitor variant that selectively and reversibly inhibits a mutant ZAP-70 that is sensitive to the kinase inhibitor variant (Levin, Zhang et al. 2008, Au-Yeung, Levin et al. 2010). These studies demonstrated that the bulk of T-cell signaling is inhibited by catalytically inhibiting ZAP-70, but that the kinase plays an additional role in signaling by acting as an adaptor for SH2 domain containing proteins.

The traditional method of inhibiting enzymes is by developing substrate competitors. Kinase inhibitors would compete with either a protein substrate or ATP. A peptide library screen identified a consensus sequence for ZAP-70 active site binding. This optimized binding peptide was then converted into a peptide inhibitor by substituting the substrate tyrosine for phenylalanine (Nishikawa, Sawasdikosol et al. 2000). ATP-competitive inhibitors have been developed which inhibit both ZAP-70 and Syk (Hirabayashi, Mukaiyama et al. 2008, Hirabayashi, Mukaiyama et al. 2008), as well as ZAP-70 specific inhibitors (Hirabayashi, Mukaiyama et al. 2009) through synthesis.
and Structure Activity Relationships. These compounds have been primarily tested in vitro and have shown limited efficaciousness in cells or mouse models. Attempts at designing ZAP-70 ATP-competitive inhibitors through virtual screening and docking studies may one day prove to be the way forward (Sanam, Vadivelan et al. 2009, Kaur, Kumari et al. 2013).

Upon solving the structure of the isolated tandem-SH2 domains of ZAP-70, the authors commented that each SH2 domain was different enough from known SH2 domain structures that ZAP-70 could provide an ideal candidate for phosphotyrosine-mimetic inhibitors that could potentially prevent ZAP-70 localization to the T-cell receptor (Hatada, Lu et al. 1995). In the same year, the first ITAM-competitive synthetic peptide was used in in vitro experiments to prevent ZAP-70 from binding to the T-cell receptor and from phosphorylating its substrates (Wange, Isakov et al. 1995). This peptide had difluorophosphonomethyl phenylalanyl residues substituted for phosphotyrosine to confer protection from phosphatases. Following that work, efforts were made to develop not-peptidic inhibitors of the ZAP-70 tandem-SH2-domains (Vu, Corpuz et al. 1999, Vu, Corpuz et al. 1999, Vu 2000). These inhibitors were designed with non-peptidic scaffolds to evade peptidases in the cell. Unfortunately, these inhibitors contained cell-impermeable phosphate groups to target the phosphotyrosine binding pocket, although they achieved good affinity (IC50 values below 10 µM) and specificity (up to 500 times more specific to the tandem-SH2-domains of ZAP-70 compared to the SH2 domains of Src, Grb2, or Syk).
1.10 Conclusion

Structural and biochemical studies have revealed that the ZAP-70 kinase domain is stabilized in an inactive conformation by autoinhibitory interactions with its accessory domains. The inactive conformation of the ZAP-70 kinase domain is similar to the inactive conformation of Src-family kinases and many others. However, the arrangement of the SH2 domains and associated linkers, and the conformational changes that occur upon ITAM binding, provide ZAP-70 with a mechanism for maintaining the specificity of interaction and control of activation required for its central role in T-cell signaling.

ZAP-70 has been validated in cells as an ideal target for therapeutics and inhibitor development for the treatment of autoimmune diseases. Attempts at developing ATP-competitive or phosphotyrosine mimetics as inhibitors of ZAP-70 have not yet come to fruition. Here, I describe my attempts to discover such an inhibitor. Chapter 2 is concerned with the method development and implementation of a biophysical screen to detect small organic compounds that disrupt ZAP-70 interaction with the T-cell receptor. A detailed study of the mechanism of action of these inhibitors is presented in Chapter 3. I discover that ZAP-70 is redox sensitive, as the hit compounds from the screen covalently modify cysteine residues. There is a degree of selectivity between the various compounds and the concomitant cysteine residues required for their inhibition. Of particular note is that the functional conservation of these cysteines in Syk, an analogous kinase in B-cells, suggests a means by which ZAP-70 could be regulated in response to oxidative stress in cells.
Chapter 2:
A High Throughput Screen for the Disruption of ZAP-70 Binding to the T-cell Receptor
2.1 Introduction

ZAP-70 plays an essential role in T-cell signaling. Its misregulation also plays a crucial role in immune-related diseases. Overactivation of ZAP-70 can result in autoimmune disease (Sakaguchi, Takahashi et al. 2003) while its absence can cause Severe Combined Immune Deficiency (SCID) (Chan, Kadlec et al. 1994, Elder, Lin et al. 1994). ZAP-70 overexpression in Chronic Lymphocytic Leukemia (CLL) is correlated with poor prognosis (Chiorazzi, Rai et al. 2005). Inhibitors of ZAP-70 could be used as treatments for autoimmune disease and organ transplant rejection. Traditionally, kinase inhibitors have been ATP-competitive molecules that target the active site. Efforts to develop such a drug toward ZAP-70 have not yet yielded results (Wang, Kadlec et al. 2010).

The interaction of ZAP-70 with the ζ-chain of the T-cell receptor plays a critical role in T-cell signal transduction by connecting the extracellular ligand binding event to intracellular signaling cascades. Binding of the tandem-SH2 domains of ZAP-70 to a dually-phosphorylated ITAM motif on the T-cell receptor facilitates T-cell activation in two ways. First, recruitment of ZAP-70 to the ζ-chain localizes ZAP-70 to the plasma membrane and in proximity to its substrates LAT and SLP-76. Second, ITAM binding to ZAP-70 relieves autoinhibition of the kinase by effecting an intramolecular domain reorientation that is not compatible with the autoinhibited structure. Blocking ZAP-70 recruitment has been verified as a means of inhibiting T-cell signaling as overexpression of just the tandem-SH2 domains of ZAP-70 has a dominant-negative effect that blocks downstream signaling (Iwashima, Irving et al. 1994, Northrop, Pustelnik et al. 1996, Qian, Mollenauer et al. 1996).

Previous work from our lab has revealed that ZAP-70 assumes an inactive autoinhibited structure in the absence of ITAM (Deindl, Kadlec et al. 2007). In this structure, the tandem-SH2 domains of ZAP-70 pack against the distal side of the kinase domain, and the conformation of the tandem-SH2 domains is nearly identical to that seen in the crystal structure of the isolated domains (Folmer, Geschwindner et al. 2002). The inter-SH2 linker forms the bulk of the interaction between the tandem-SH2 domains and the kinase domain. The SH2-kinase linker contributes a number of hydrophobic residues that interact with the kinase domain and the inter-SH2 linker to form a hydrophobic core. Our lab described this unique arrangement as the linker-kinase sandwich.

I sought to exploit the insight gleaned from our lab’s structural and functional studies of ZAP-70 to discover allosteric inhibitors of ZAP-70. Toward this end, I developed a fluorescence polarization-based assay [Figure 2.1] that detects an ITAM-derived phosphopeptide binding to ZAP-70. In this assay, ZAP-70 would be bound to a fluorescently-labeled phosphorylated peptide derived from the first ITAM motif in the ζ-chain [CGNQLpYNELNLGRREEpYDVLD; where pY indicates phosphorylated tyrosine and the fluorophore is attached to the cysteine]; this peptide is denoted 2pY. In the absence of an inhibitor, the bound peptide would produce a high FP value. The addition of compound would inhibit peptide binding and the FP value would decrease. This assay was applied to a high throughput screen against a library of 132,842 compounds at the Small Molecule Discovery Center (SMDC) at UCSF. Inhibitors of this interaction could function by directly competing with ITAM binding or by stabilizing the autoinhibited conformation and allosterically preventing ITAM binding. In this chapter I describe the
development, implementation, and verification of hit compounds obtained from this screen.
Figure 2.1 Schematic of FP-based assay

ZAP-70 is bound to a fluorescently-labeled phosphorylated ITAM-derived peptide (left). The addition of an inhibitory compound would disrupt peptide binding and result in a decrease in the FP value (right).
2.2 Results and discussion

2.2.1 Assay development to screen for inhibitors of ZAP-70:ITAM interaction

Fluorescence polarization (FP) is a common biophysical technique used to assay interactions between biological macromolecules. It provides a cost-effective means of measuring protein-protein interactions in homogenous solutions which makes it an ideal method for high throughput screening (Owicki 2000). Our lab has previously reported an FP assay for measuring the ZAP-70:ITAM interaction (Deindl, Kadlecek et al. 2009). Here, I describe the further development and optimization of this assay for use in a high throughput screen for allosteric ZAP-70 inhibitors.

As a first step toward developing an assay to measure ZAP-70:ITAM disruption, I measured the affinity between ZAP-70 and a fluorophore-labeled peptide derived from the first (most N-terminal) ITAM motif in the CD3-ζ chain (Irving, Chan et al. 1993). This measurement had been performed previously with fluorescein, a green fluorescent dye (Deindl, Kadlecek et al. 2009). I replaced fluorescein with Tetramethylrhodamine (TAMRA), a red-shifted dye, as fluorescent compounds in screening libraries tend to interfere in the green wavelengths (Turek-Etienne, Small et al. 2003). Additionally, I miniaturized this assay from 200 µl volumes to 20 µL volumes for use in 384-well plates, as necessary for a high throughput screen.

Using the TAMRA labeled 2pY peptide, I measured the value of the dissociation constant, K_D, for ZAP-70 to be 41.7 nM. This is comparable to the previously measured K_D value of 76.6 nM [Figure 2.2A]. To further examine the sensitivity of this assay, I measured the K_D value between this ITAM peptide and ZAP-70 Y315F/Y319F, in which two tyrosine residues crucial to the autoinhibited conformation are mutated to phenylalanine. These mutations should stabilize ZAP-70 in its autoinhibited conformation and reduce the affinity of the protein for ITAM. The experimental results supported this idea and yielded a K_D value of 79.8 nM [Figure 2.2B]. Though not a large change, this was a repeatable result. In a final verification step, I measured the affinity between the TAMRA-2pY peptide and a chimeric protein that replaced the kinase domain of ZAP-70 with that of Src kinase [Figure 2.2C]. Because this chimeric protein should not be able to adopt a compact inhibited tertiary structure, it should exhibit increased affinity for TAMRA-2pY and therefore a lower K_D value. The experimental results were as expected and the binding constant for ITAM peptide and the ZAP-Src chimera was 15.8 nM. To maximize the signal window, I chose a protein concentration above the K_D and set the assay ZAP-70 concentration at 100 nM which corresponds to 80% binding (EC80).

In addition to apparently unsuccessful attempts at obtaining ATP-competitive ZAP-70 kinase inhibitors, much work in the field had been put into developing phosphotyrosine-competitive SH2 inhibitors of ZAP-70 (Vu, Corpuz et al. 1999, Vu, Corpuz et al. 1999). Like other attempts at inhibiting SH2 domains, these compounds are primarily phosphotyrosine-mimetics and demonstrated moderate specificity and low affinity. In order to select against phosphotyrosine competitors in this screen, I included an additional peptide which was a truncation of the ITAM peptide [CGNQLpYNELNLGRREE] denoted 1pY. This peptide was truncated at the residue preceding the second phosphotyrosine in the ITAM motif.
The rationale for the use of the 1pY peptide was that if I were to discover an allosteric inhibitor of ZAP-70 which stabilized the protein in the autoinhibited conformation, then the affinity between ZAP-70 and the doubly phosphorylated peptide (2pY) would be reduced to a value similar to that for a singly phosphorylated peptide. The 1pY peptide would most likely bind the more complete and accessible C-terminal SH2 domain of ZAP-70, which binds the N-terminal YxxL/I-motif in a doubly-phosphorylated ITAM (2pY). By having an excess of 1pY, I could outcompete any residual 2pY binding and further increase the assay signal window and assay sensitivity. This is possible because of the three order of magnitude difference in 1pY and 2pY affinity for ZAP-70. A secondary purpose adding of 1pY to the assay would be to have it in such an excess that it would outcompete any phosphotyrosine-pocket binding compounds.

To determine the appropriate concentration of the singly-phosphorylated peptide that would only outcompete fluorescent peptide in the presence of an inhibitor, I titrated in 1pY to inhibit the 2pY-ZAP-70 binding reaction [Figure 2.3]. I found the IC$_{50}$ value for inhibition by 1pY to be 158 µM. I chose a 1pY concentration of 30 µM, which corresponds to the IC$_{20}$ value. Because this concentration is at the cusp of the inhibition curve, it increases the sensitivity of the assay by facilitating a larger change in FP in response to an inhibitor.

To further adapt this assay for a high throughput screen, I needed to optimize buffer conditions. FP values are dependent on the tumbling rate of the fluorophore in solution. By decreasing the amount of glycerol in the buffer, I was able to lower the baseline FP value in this assay. This, however, is complicated by the fact that glycerol is a stabilizing agent for proteins and so I did not wish to remove glycerol entirely. By testing different glycerol concentrations over the course of several hours I could identify a minimum concentration of glycerol for which ZAP-70, and therefore the assay, was stable, but which maximized the signal window. The assay was originally developed with 10% glycerol. I tested 5%, 2%, and 0% glycerol. Each decrease in glycerol concentration increased the signal window by further decreasing the bottom FP value. After a 16 hour incubation, I found that 2% glycerol was sufficient to maintain assay stability.

In addition to maximizing protein stability, buffer optimization was necessary for compound delivery. Compounds in screening libraries are composed primarily of large drug-like hydrophobic conjugated ring systems. These compounds have low solubility in water and so the addition of detergent has been shown previously to generally alleviate problems with compound solubility (Thorne, Auld et al. 2010). I tested the detergents Tween-20, TritonX-100, and CHAPS over a small span of concentrations. These concentrations are below the critical micelle concentration (CMC) for each of the detergents tested. Mixing detergents allows one to maximize total detergent concentration, while maintaining each detergent below its respective CMC. I found that a combination of 0.01% Triton and 0.01% Tween had no effect on the assay whereas the addition of CHAPS, the only ionic detergent I tested, rendered the data uninterpretable.
Figure 2.2 Binding curves of TAMRA-2pY:ZAP-70 measured by fluorescence polarization.

(A) ZAP-70 WT
(B) ZAP-70 FF: The more inhibited ZAP-70 FF has a lower affinity for ITAM than WT ZAP-70.
(C) ZAP-Src chimera: The uninhibited ZAP-Src chimera has a higher affinity for ITAM compared to wild type ZAP-70.
Figure 2.3 Competition of doubly phosphorylated peptide (2pY) with singly phosphorylated peptide (1pY)

(A) Schematic of 1pY competing off TAMRA-2pY peptide from ZAP-70
(B) Inhibition curve of 2pY-ZAP-70 binding by 1pY peptide. The IC₅₀ value for the 1pY peptide is determined to be 158 µM and is marked by a dashed vertical line. The numerical fit was derived by including the expected FP value of a free peptide.
2.2.2 Assay verification

In order to test the reliability of the screen, I used the three constructs previously mentioned: ZAP-WT, ZAP-Src chimera, and ZAP-FF. As ZAP-FF has a higher $K_D$ for TAMRA-2pY, I used it as a proxy for an inhibited ZAP-70. The lower $K_D$ of ZAP-Src for ITAM (tighter binding) allowed me to use it to simulate an activated ZAP-70 in the assay. The chimeric protein is also easier to produce than actual activated ZAP-70. In a 1pY dose-response inhibition assay, an activated ZAP-70 would demonstrate a right shifted inhibition curve. Because of the increased affinity of activated ZAP-70 for 2pY peptide, more 1pY is required to displace it. An inhibited ZAP-70 would show a left shifted binding curve due to the lower affinity for 2pY, thus requiring less 1pY to displace 2pY. In the single measurement screening assay using a fixed concentration of 1pY, this would correspond to an increase in FP for Src-ZAP relative to wild type and a decrease in FP for ZAP-FF compared to wild type, which is indeed what I observe when I replace wild type ZAP-70 for either mutant construct in the FP assay.

Before performing the screen, I had to first confirm that it would perform as well at the screening facility as it did using our laboratory instruments. I began by comparing the assay FP values in the Greiner 384-well microplates that I had developed the assay in to the Corning 384-well plates that are used at the UCSF Small Molecule Discover Center. The two plates gave nearly identical FP values, indicating it was safe to proceed with testing the assay. Using the liquid dispensing robots at the SMDC, I tested the assay’s behavior with automation by measuring a “Z’ plate”. A Z’ value or Z-factor, is the most common statistical measure of assay robustness in high throughput screening [Table 2.1] where $Z' = 1$ is a perfect assay and a $Z'$ value of 0.5 is accepted as the minimum threshold for a high throughput screening assay (Zhang, Chung et al. 1999). I divided a 384-well plate in half and measured the FP values of all the assay components (ZAP-70, 1pY, and TAMRA-2pY) as the top value or negative control of the assay. The second half of the plate contained only TAMRA-2pY. I used this as a positive control and minimum FP value for the assay. The $Z'$ plate reported a $Z'$ value of 0.78, which is an excellent value to proceed with the assay. I also tested the assay’s sensitivity to DMSO, as it is used as the delivery vehicle for the compounds. Testing a range of 10% to 0.01% DMSO, I found that this assay is tolerant of up to 5% DMSO.
Figure 2.4 Predicted inhibition curves of ZAP-70 variants

Illustrative 1pY inhibition curves of ZAP-70 variants mimic different activation states. In the presence of an inhibitor (here, represented by ZAP-70 FF and shown in green), ZAP-70 would exhibit a left-shifted inhibition curve. In the presence of an activator (here, represented by ZAP-Src and depicted in red), ZAP-70 would show a right shifted 1pY inhibition curve relative to wild type ZAP-70 (drawn in blue). At a fixed concentration of 1pY peptide (chosen as the IC20 value of 1pY peptide), as is the case for the screening assay, one would predict an increased FP value for the ZAP-Src chimera and a decreased FP value for ZAP-70 FF relative to wild type. Inset box (right) shows actual measured FP values for each ZAP-70 variant at the assay conditions. This verifies that the FP screening assay is reporting on activated or inhibited states of ZAP-70.
Table 2.1 Calculation of assay Z’

\[
Z' = 1 - \frac{3 \cdot (\sigma \text{ of sample} + \sigma \text{ of control})}{|\bar{x} \text{ of sample} - \bar{x} \text{ of control}|}
\]

\(\sigma\) = standard deviation
\(\bar{x}\) = mean
2.2.3 Pilot Screen

To test the utility of the assay in an actual screen, I performed a pilot screen against a library of 2000 molecules known to be bioactive. The pilot screen yielded 12 hit compounds that showed a decrease in FP after filtering for fluorescence artifacts. Of those 12 compounds, a single compound, SMDC compound ID #130796, showed dose-responsive inhibition. Further investigation into the identity of this compound revealed it to be Pyrithione zinc, a common treatment for fungus and yeast found in topical creams and dandruff shampoos (Brauer, Opdyke et al. 1966, Box, Sangha et al. 1980, Faergemann and Fredriksson 1980, Veien, Pilgaard et al. 1980). The compound showed a dose-responsive inhibition curve with an IC$_{50}$ value of 126 ± 1.1 nM [Figure 2.4A], which is very close the protein concentration of 100 nM. The compound was able to inhibit ITAM binding to full-length ZAP-70 as well as ITAM binding to ZAP-Src with a similar IC$_{50}$ value, suggesting that the compound acted on the tandem-SH2 domains alone [Figure 2.4D].

Dose-responsive FP inhibition assays testing a variety of metals showed that ZAP-70 inhibition is specific to zinc [Figure 2.4C]. Further experiments with the metal chelator, ethylenediaminetriacetic acid (EDTA), showed that the inhibition was reversible [Figure 2.4B]. The zinc concentration at which one would recover half of the maximum binding, EC$_{50}$, corresponded roughly to the concentration of Pyrithione zinc. Competition assays with the zinc-binding dye, 4-(2’-Pyridylazo)resorcinol (PAR), demonstrated that zinc was binding to ZAP-70 and not to the 2pY peptide [Figure 2.4E]. I attempted to understand the mechanism of inhibition through structural studies. I set crystal trays with the tandem-SH2 domains of ZAP-70 with saturating amounts of zinc. I immediately saw precipitation which suggested a mechanism of inhibition. However, I could not reconcile this precipitation with the reversible inhibition I had previously characterized.

To gain insight into the relationship between zinc and ZAP-70 precipitation, I performed a zinc titration to examine precipitation. By spinning down the precipitated protein at different zinc concentrations and measuring the concentration of soluble protein, I saw that precipitation was dependent on zinc concentration [Figure 2.4F]. I then titrated EDTA into a precipitated ZAP-70/zinc solution and observed resolubility of the precipitate [Figure 2.4G]. I spun down precipitated protein to quantify the amount of protein left in solution and found that it was proportional to the EDTA concentration in the reaction. This suggests zinc inhibition is due to reversible precipitation. This phenomenon has been previously observed in a number of proteins (Yang, Cleland et al. 2000, Lee, Schulman et al. 2007). Whether or not this inhibition mechanism is physiologically relevant is debatable as intracellular levels of zinc are actively increased upon T-cell activation (Yu, Lee et al. 2011) while zinc plays both stimulatory as well as inhibitory roles in T-cell signaling (Honscheid, Rink et al. 2009). I chose to not pursue this further.
**Figure 2.4 Characterization of ZAP-70 inhibition by zinc**

(A) Titration of Pyrithione zinc into the FP assay shows dose-dependent inhibition.
(B) Titration of EDTA into the Pyrithione zinc-inhibited reaction shows recovery of binding activity.
(C) Titration of various metals shows that zinc metal is sufficient for dose-dependent ZAP-70-2pY binding inhibition.
(D) Replacement of ZAP-70 with the ZAP-Src chimera shows that both Pyrithione zinc as well as zinc chloride only require the tandem-SH2 unit for inhibition.
(E) PAR bound to zinc shows high absorbance at 500 nm. Titration of either 2pY or ZAP-70 shows that ZAP-70 and not 2pY competes with PAR for zinc binding and results in a decrease in 500 nm absorbance.
(F) Measuring soluble protein after the addition zinc and centrifugation demonstrates that precipitation is zinc concentration-dependent.
(G) Titration of EDTA is able to reverse zinc precipitation in a dose-dependent manner.
2.2.4 Hit compounds identified by screening results and secondary assays

I performed the high throughput screen at UCSF’s Small Molecule Discovery Center, and screened against the SMDC’s diversity library, a collection of 132,842 compounds. The assay performed well, with an overall Z’ score of 0.7. I first removed fluorescence artifacts by removing from further consideration all compounds that exhibited fluorescence values that were within 1σ above and below the mean fluorescence of the library [Figure 2.5A]. I further filtered the results based on a cutoff of greater than or equal to 3σ inhibition above the mean (~20% inhibition) [Figure 2.5B]. This left 428 compounds, corresponding to a ~0.3% hit rate. I then took the most potent 320 compounds from the primary screen and tested them in a dose-response FP assay. 102 of these compounds exhibited dose dependent inhibition, minimal cooperativity (Hill slope, nH ≤ 2), and an IC50 value less than 100 µM [Figure 2.6A-B]. The chemical structures of these compounds are presented in the following chapter [Table 3.1].

In order to verify the results of the primary screen and FP dose-response assay, I developed an orthogonal screen using time-resolved FRET (TR-FRET) as an assay of ZAP-70:ITAM binding which I performed in a dose-response format. This assay utilizes terbium as the FRET donor. This has the advantage of using a far red-shifted excitation wavelength that should avoid fluorescence artifacts from the library compounds. Furthermore, the lifetime of the terbium excitation exceeds that typically seen for organic molecules. These two aspects of the TR-FRET assay make it an ideal method for screening small molecule libraries (Bazin, Preaudat et al. 2001). For this assay, I engineered a ZAP-70 construct that could be specifically biotinylated on a lysine residue on a genetically encoded tag sequence known as an AviTag (Avidity). The biotin ligase, BirA, then adds biotin to the AviTag lysine residue. Biotinylated ZAP-70 was then labeled by the addition of streptavidin labeled with a terbium cryptate (CisBio) to produce a fluorescent ZAP-70 FRET donor. The addition of AlexaFlour488-labeled ITAM, denoted AF488-2pY, provides a FRET acceptor upon binding to ZAP-70-biotin [Figure 2.7A]. In order to account for acceptor emission through solution FRET or donor excitation bleed-through, I performed a titration of AF488-2pY into Streptavidin-Tb in the absence of ZAP-70. This titration generated a linear background FRET response which I was able to subtract from the ZAP-70:ITAM binding isotherm.

Using this method, I determined the binding constant between ZAP-70 and phosphorylated ITAM to be 55 nM; this is very similar to the value I derived from the FP assay [Figure 2.7B]. I further characterized the assay by competing off the doubly phosphorylated 2pY peptide with singly phosphorylated 1pY and obtained an IC50 value of 134 µM [Figure 2.7C] which was comparable to that seen in the FP assay.

The TR-FRET dose-response assay was performed similarly to the FP dose-response assay. This assay also proved to be extremely robust and I calculated a Z’ value of 0.8 using this TR-FRET assay. The most potent 320 compounds from FP-based primary screen were delivered in DMSO in a range of 0.102-33.33 µM. 125 compounds showed total fluorescence within one standard deviation of the mean fluorescence at 33 µM of compound, as well as reasonable IC50 values. 86 hit compounds scored positively in both the FP and TR-FRET dose-response assays. As a personally gratifying result, the majority of the IC50 values generated in the TR-FRET dose response assay were remarkably similar to those obtained in the FP dose response assay [Figure 2.6A-D].
Figure 2.6 Screening results

(A) Graph of screen total fluorescence by well shown. Each blue dot represents an assay well. One standard deviation above and below the mean total fluorescence value of the library is represented by the red horizontal lines.

(B) Graph of screen inhibition by well shown. Each blue dot represents an assay well. Three standard deviations above the mean inhibition value of the library (~20% inhibition) is represented by the red horizontal line.
Figure 2.7 Dose-response assays of hit compounds from screen

(A-B) FP dose response curves of SMDC compounds 180893 (left) and 41457 (right). 
(C-D) TR-FRET dose response curves of SMDC compounds 180893 (left) and 41457 (right). IC$_{50}$ values are similar in both FP and TR-FRET assays for each compound.
Figure 2.8 Diagram and development of TR-FRET assay

(A) Schematic of TR-FRET ZAP-70:ITAM binding assay is shown. Addition of an inhibitory compound would cause AlexaFluor488-2pY to dissociate and result in a decrease in TR-FRET signal.

(B) TR-FRET measurement of AviTagged ZAP-70 binding to AlexaFluor488-2pY produces a $K_D$ value of 55.4 nM.

(C) Competition assay with 1pY displacing AlexaFluor488-2pY produces IC$_{50}$ value of 134 ± 7.1 nM which is marked on the graph by a dashed vertical line.
2.3 Conclusions

ZAP-70 is an attractive target for drug discovery due to the well characterized role of ZAP-70 in autoimmune disease and cancer. Efforts toward developing an ATP-competitive kinase inhibitor of ZAP-70 have not yet succeeded (Wang, Kadlecek et al. 2010). I designed an assay of ZAP-70:ITAM binding by taking advantage of our knowledge of the mechanism of ZAP-70 regulation. Our lab had previously determined the autoinhibited structure ZAP-70. This structure showed the kinase domain in a catalytically incompetent conformation. This conformation was stabilized by the docking of the tandem-SH2 unit to the distal side of the kinase. The inter-SH2 linker and SH2-kinase linkers form the crux of the interaction surface and formed a hydrophobic core which stabilized the interaction. In its autoinhibited state, ZAP-70 is unable to bind to a doubly phosphorylated ITAM motif. ITAM binding effects a conformation change that relieves autoinhibition and allows tyrosine phosphorylation on interdomain B and the activation loop of the kinase domain which is required for full ZAP-70 catalytic activation, and acts to localize the kinase to the membrane and the T-cell receptor.

I developed a screen that detects ZAP-70:ITAM binding and provides a means of screening for compounds that block ITAM binding. I optimized the assay for application in a high throughput format and carried out the screen against a library of 132,842 compounds at UCSF’s SMDC. The assay demonstrated good robustness with a Z’ value of 0.7 and a hit rate of ~0.3% or 428 compounds. I subsequently performed a dose-response FP assay against the most potent hit compounds and found that 102 compounds inhibited in a dose-dependent manner. To confirm inhibition by the hit compounds, I developed an orthogonal TR-FRET assay of ZAP-70:ITAM binding. I performed a TR-FRET dose-response assay and found that 125 compounds of the 438 hit compounds inhibited in a dose-dependent manner, with 86 compounds scoring positive in both FP and TR-FRET dose-response assays. These hit compounds could provide promising leads toward therapeutics for autoimmune disease or organ transplant rejection as ZAP-70 inhibitors. These compounds can also be useful chemical probes of ZAP-70’s conformational and activation state. Before these things can be realized, a careful study of their mechanism of inhibitions is warranted, which is described in the following chapter.
2.4 Methods

2.4.1 Protein Preparation

A construct spanning ZAP-70 residues 1-606, a Gly-Ser-Gly linker, a PreScission Protease recognition sequence, and a 6xHis tag were cloned into pFastBac1 (Invitrogen) with the BamHI and EcoRI restriction sites. A catalytically necessary aspartate residue (Asp 416) was mutated by QuickChange site directed mutagenesis (Stratagene) to asparagine to maintain the kinase in a catalytically inactive state to aid in expression. ZAP-70 FF was prepared as previously described (Deindl, Kadlec et al. 2009). Avitag ZAP-70 was prepared by engineering a C-terminal AviTag (GLNDIFEAQKIEWHE) (Avidity) onto the ZAP-70 construct previously mentioned. This was then inserted into pFastBacHTb with the BamHI and EcoRI restriction sites. Expression is as previously described (Deindl 2007). Sf9 cell pellets were resuspended in a buffer containing 50 mM NaCl, 50 mM Tris pH 8.0, and protease inhibitor cocktail including PMSF. Cells were lysed with a french press and centrifuged at 45,000 x g for 1hr to pellet insoluble cell debris. Cell lysate was filtered using 5.0 µm and 0.45 µm syringe filters before being loaded on 5 mL Ni-NTA columns (GE). ZAP-70 was eluted with a buffer containing 500 mM NaCl, 500 mM imidazole, 20 mM Tris pH 8.0, and 5% glycerol. 1mM tris(2-carboxyethyl)phosphine (TCEP) and PreScission Protease (GE) were added and the cleavage reaction allowed to proceed for 16 hrs at 4°C to remove the 6xHis tag. The protein was then buffer exchanged into 20 mM Tris pH 8.5, 150 mM NaCl, and 5% glycerol. This solution was then passed over a Ni-column and Q ion-exchange column (GE) and the flow through collected. ZAP-70 does not bind to the Ni-column or Q-column at this point and under these conditions which allows us to utilize these columns as subtractive step. The protein was then run over an S200 gel filtration column equilibrated with 20 mM Tris pH 8.0, 150 mM NaCl, 5%glycerol, and 1 mM TCEP and the protein concentrated to 10 mg/mL.

2.4.2 Peptide labeling

Peptide corresponding to ITAM ζ (XGNQL(pY)NELNLGRREE(pY)DVLD) including an N-terminal propargylglycine and glycine linker (prepared by David King, UC Berkeley HHMI) was resuspended in 20 mM Tris buffer to a peptide concentration of 1.5 mM and the pH adjusted to 7.5. This peptide was then conjugated via the alkyne group of propargylglycine to an azide moiety on Tetramethylrhodamine (TAMRA) by a [3+2] “Click” cycloaddition (Invitrogen). Similarly, Peptide corresponding to ITAM ζ (CGNQL(pY)NELNLGRREE(pY)DVLD; where pY is phosphotyrosine) including an N-terminal cysteine and glycine linker (prepared by David King, UC Berkeley) was resuspended in 20mM Tris and pH adjusted to 7.5. The peptide was reduced by incubation with 3-fold amount of TCEP and incubated at 50°C for 30 min. This peptide was then conjugated via the thiol group of the cysteine residue to a maleimide moiety on AlexaFluor488 (Invitrogen). Labeled peptides were purified by High Performance Liquid Chromatography (HPLC) by reverse phase chromatography on a C18 column using a water and acetonitrile gradient with 0.01% trifluoroacetic acid. Correct labeling was confirmed by mass spectrometry.
2.4.2 Fluorescence polarization assays

Fluorescence polarization binding assays were performed in 80 µL reaction volumes with 2 nM TAMRA-2pY, 150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 1 mM TCEP, and serial dilutions of ZAP-70 ranging from 1500 nM to 0.003 nM. This volume was aliquoted in 20 µL volumes to a 384-well plate (HE) and read as pseudo-triplicates in a Biotek Synergy H4 fluorescence plate reader. For 1pY (CGNQL(pY)NELNLGRREE) competition assays, reactions were carried out in 80 µL volumes with 2 mM TAMRA-2pY, 150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 1 mM TCEP, 100 nM ZAP-70, and serial dilutions of 1pY.

2.4.3 High throughput screen

The high throughput screen was performed at the UCSF Small Molecule Discovery Center. A reaction master-mix was prepared with 20 mM Tris pH 8.0, 150 mM NaCl, 2% Glycerol, 1 mM TCEP, 100 nM ZAP-70, 30 µM 1pY, and 2 nM TAMRA-2pY. Reaction mixture was dispensed into 384-well plates with Matrix Wellmate Bulk Dispenser. Compounds were delivered in DMSO (final DMSO concentration = 0.25%) at 25 µM while shaking on a Beckman FXp Liquid Handling Robot. Plates were incubated with compounds at room temperature for 20 min before reading in a Molecular Devices Analyst HT Plate Reader. Hit compound dose-response inhibition assays were performed in a similar manner, with multiple compound additions steps to increase the concentration of compound in the assay.

2.4.4 TR-FRET assay

Time-resolved Fluorescence Resonance Energy Transfer assays were performed in 80 µL reaction volumes with 2 nM biotinylated ZAP-70-AviTag, 30 µM 1pY, 150 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 1 mM TCEP, and 100µM 2pY-AlexaFluor488. This volume was aliquoted in 20 µL volumes to a 384-well plate (HE) and read as pseudo-triplicates in a Biotek Synergy H4 fluorescence plate reader.
Chapter 3:
Analysis of Mechanism of ZAP-70 Inhibition by Screening Hits
3.1 Introduction

The ability of ZAP-70 to bind phosphorylated ITAMs is closely coupled to its activation state. Previous work from our lab (Deindl, Kadlec et al. 2007, Yan, Barros et al. 2013) and others (Folmer, Geschwindner et al. 2002) has shown that ZAP-70 assembles into an autoinhibited “off” conformation where the kinase domain assumes a catalytically inactive conformation while the tandem-SH2 unit adopts a conformation that is not conducive to ITAM binding. At the heart of the autoinhibited arrangement is a hydrophobic core of residues formed by close packing of interdomain A and the kinase domain as well as crucial residues from interdomain B interdigitating the interface. Our lab has denoted this intramolecular packing interaction the “linker-kinase sandwich”.

Mutational analysis has demonstrated that disrupting the linker-kinase sandwich weakens the autoinhibited conformation and results in increased affinity for ITAM. Conversely, mutations that strengthen the linker-kinase sandwich reduce the affinity between ZAP-70 and ITAM (Deindl, Kadlec et al. 2009). Allosteric inhibitors of ZAP-70 might act in a similar manner by stabilizing ZAP-70 in an inhibited conformation and therefore reducing the affinity of ZAP-70 for ITAM. Additionally, they could directly prevent ZAP-70 recruitment to the T-cell receptor. This would prevent ZAP-70 from being fully catalytically activated, and prevent colocalization with ZAP-70 substrates LAT and SLP-76, thereby blocking downstream signaling.

The FP-based assay was used to screen against a small molecule library and selected for compounds that produced the desired output of decreased fluorescence polarization. However, the screen does not provide any insight into the mechanism by which these compounds achieve this effect. In this case, the FP screen I described in the previous chapter was designed to identify compounds that prevent a fluorescently labeled ITAM-derived phosphopeptide from binding to ZAP-70. More detailed mechanistic studies are necessary to elucidate how the compounds are acting on ZAP-70 to cause the peptide to dissociate. One can envision three mechanisms by which this could be accomplished: 1. The compound directly competes with peptide for binding the tandem-SH2 unit, 2. the compound acts allosterically at a site distinct from the tandem-SH2 domains, but in a way that reinforces the autoinhibited conformation, or 3. the compound acts directly on the tandem-SH2 unit and acts as a wedge to prevent domain rearrangement necessary for ITAM binding.

In this chapter, I present a series of experiments that build on the results of the high throughput screen by presenting observations about requirements for hit compound activity. I show that removal of the singly phosphorylated peptide, which was used as a competitor in the screen, does not have an effect on the ability of the compounds to inhibit the peptide binding. Surprisingly, every hit compound acts on the tandem-SH2 unit alone and does not require the kinase domain or the SH2-kinase linker for inhibitory activity. The compounds also inhibit the tandem-SH2 domains of Spleen Tyrosine Kinase (Syk), a non-receptor tyrosine that plays an analogous role to ZAP-70 in B-cells. This suggests that the compounds could be acting by a similar mechanism on both proteins. In another, perhaps more surprising, result, I found that all the compounds are covalent modifiers of the tandem-SH2 domains and that covalent modification of cysteine residues is an essential aspect of the mechanism. Furthermore, there is a degree of compound selectivity towards different cysteines.
3.2 Results and discussion

3.2.1 Confirmation of potency of repurchased compounds

Following the dose-response assay performed at the SMDC, I reordered those hit compounds that scored positive in the FP and FRET dose-response that also performed well in a cell-based assay performed by our collaborators. I ordered these molecules in quantities (10-20 mg) sufficient for further investigation. I began by confirming the identity and purity of all the compounds that I had ordered by liquid chromatography followed by mass spectrometry (LC/MS). This step confirmed that all of the compounds that I had received were of a mass that corresponded to the identity of the compounds in the SMDC library and that the compounds were of sufficient purity that I could proceed with these experiments.

As a preliminary experiment, I performed a dose-response assay identical to the screening assay using the newly purchased compounds to confirm their identity through a functional assay [Figure 3.1A]. These hit compounds were renamed “IOZ” for Inhibitor Of ZAP-70, and the compounds are listed according to this nomenclature in Table 3.1. Using concentrations ranging from 500 µM to 0.24 µM, I found that 20 of the 24 IOZ compounds that I received showed dose-dependent inhibition [Figure 3.1B-C] (representative date shown). Nearly all compounds exhibited a Hill slope greater than 1; this apparent cooperativity is in disagreement with the dose-response curves that I had generated at the SMDC. Hill slopes greater than 1 are often an indicator that a compound is not acting through a reversible mechanism such as precipitation or covalent inhibition.

Those compounds that did not show a dose-response failed to show any inhibition at all. Differences in the identity of the compound that actually exists in the SMDC screening library compared to what I had reordered and received could account for this discrepancy. These differences in chemical matter could arise due to compound degradation, the presence of impurities in the screening solution, or the incorrect identification of the chemical matter of the compounds in the screening library. The IC₅₀ values I obtained from these experiments did not necessarily correlate with the IC₅₀ values from the SMDC dose-response data. This could be due to incorrect measurements when preparing compound solutions. This could also be due to compound precipitation or degradation in the SMDC compound library. Here I present data for compounds IOZ1.4 and IOZ5.2. I chose these compounds as they are very chemically distinct, but showed similar inhibitory characteristics. Nearly all of the following experiments will be directed toward characterizing IOZ1.4 and IOZ5.2.
Table 3.1 Chemical structure of hit compounds

Hit compound IOZ nomenclature, SMDC ID #, and molecular mass (Da) shown. Compounds are grouped by common scaffold.

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47
Figure 3.1 Dose-response assay of IOZ compounds confirms inhibition

(A) Schematic of Fluorescence Polarization assay. Addition of compound causes displacement of doubly phosphorylated TAMRA-2pY peptide and results in a decrease in FP.

(B-C) Titration of IOZ1.4 and IOZ5.2 shows dose-responsive inhibition with large Hill slopes and IC$_{50}$ values of 2.9 µM and 10.0 µM, respectively.
3.2.2 Compounds target tandem-SH2 domains

The screening assay was originally designed with a singly phosphorylated ITAM peptide, denoted 1pY, present in excess to outcompete any compounds that might bind one of the phosphotyrosine binding sites and also to increase the sensitivity of the assay by competing off residual binding from doubly-phosphorylated peptide in the presence of an inhibitory compound. As a first step toward understanding the requirements of the hit compounds for inhibition, I omitted the singly phosphorylated peptide from the assay and performed a dose-response assay to examine the effect on the compounds’ ability to disrupt ZAP-70:ITAM binding. Removing the 1pY peptide from the reaction did not significantly affect the IC$_{50}$ values compared to the results of assays that did include the 1pY peptide. The compounds therefore do not require singly phosphorylated 1pY peptide for their inhibitory mechanisms [Figure 3.2B-C].

A major aspect of understanding the mode of inhibition of these compounds is determining which domains of ZAP-70 are required for inhibition by the compound. This will establish if the compounds are targeting a binding site present only in the full length protein. I prepared a ZAP-70 construct lacking interdomain B as well as the kinase domain, containing only the two SH2 domains as well as the inter-SH2 linker [Figure 3.2D]. All of the hit compounds inhibited this tandem-SH2 domain construct with IC$_{50}$ values that are similar to those for full length ZAP-70 [Figure 3.2E-F]. Because neither the SH2-kinase linker nor the kinase domain were necessary, it appears that the compounds do not require the intact autoinhibited form of ZAP-70. Rather, it appears that the compounds are directly targeting the tandem-SH2 domains.
Figure 3.2 Dose-response assays localize IOZ compound inhibition to tandem-SH2 unit

(A) Schematic of assay. Addition of compound causes displacement of TAMRA-2pY from ZAP-70 and a decrease in FP signal.

(B-C) Dose-response curves for IOZ1.4 and IOZ5.2 yielded IC$_{50}$ values of 3.2 µM and 14.8 µM, respectively, and display high Hill slopes.

(D) Schematic of assay. Addition of compound causes displacement of TAMRA-2pY from the ZAP-70 tandem-SH2 domains and causes a decrease in FP.

(E-F) Dose-response curves of IOZ1.4 and IOZ5.2 demonstrate similar IC$_{50}$ values to those measured with full length protein.
3.2.3 The IOZ compounds are thiol-reactive

I obtained an early indication that the IOZ compounds were modifying ZAP-70 covalently when I observed that the addition of the reductant dithiothreitol (DTT) to the assay buffers completely abolished inhibition. In actual fact, working this out was far from straight-forward. Rather than recount all the confusing intermediate results that I obtained, I present here a brief description of the situation as I understand it now.

All experiments involving the IOZ compounds included a 20 minute incubation period. The IOZ compounds were selected by filtering for compounds that produced dose-response curves with Hill slopes between 0.5 and 3. Compounds that bind covalently to their targets typically have Hill slopes ≥ 1 and IC$_{50}$ values close to the protein concentration. However, a slowly reactive compound could have an apparently low Hill slope if the binding/inhibition measurement is made before the completion of the covalent reaction.

If a compound were to form a covalent adduct with the protein, I should be able to detect it by mass spectrometry. LC/MS analysis revealed that all of the IOZ compounds are in fact covalent modifiers of ZAP-70 [Figure 3.3D-E]. The screening assay used tris(2-carboxyethyl)phosphine (TCEP), a phosphine-based reductant to maintain reducing conditions. If the compounds were thiol-reactive then I should be able to eliminate inhibition in the assay by replacing TCEP with DTT, a thiol-containing reductant. Indeed, replacement with DTT abolished any inhibition by the IOZ compounds [Figure 3.3B].

Having determined that the compounds are thiol-reactive, I was able to deduce the chemical structures of the adducts for a subset of the compounds because of the mass additions to the protein in the mass spectrometry data [Figure 3.4C-E]. Mass spectrometry also revealed that the C-terminus of the protein was subject to protease degradation. Identification of this cleaved fragment (residues 254-259) revealed that Cys 254 was modified by the IOZ compound because of a mass addition in the uncleaved protein that corresponded to adduct formation. The mass spectrometry data also showed that under the reaction conditions, the IOZ compounds could modify at least two cysteine residues through adduct formation. However, ZAP-70 contains an astonishing 15 cysteine residues with 9 of them being in the tandem-SH2 domains alone [Figure 3.4A]. Determining the mechanism by which covalent cysteine modification inhibits the protein would require further structural and biochemical characterization.
Figure 3.3 IOZ compounds are thiol-reactive

(A) Domain arrangement of tandem-SH2 domains showing the location of all cysteines. 
(B) Replacing TCEP, a phosphine-based reductant, with the thiol-based reductant DTT abolishes compound inhibition. 
(C) Chemical structures of adducts formed on cysteine residue by compounds IOZ5.2 and IOZ7. 
(D) Deconvoluted mass spectrometry data shows an addition in mass of 222 Da once in the truncated tandem-SH2 unit (residues 1-253) and two additions of 222 Da each in the full length tandem-SH2 unit (1-259). 
(E) Deconvoluted mass spectrometry data shows an addition in mass of 186 Da once in the truncated tandem-SH2 unit (residues 1-254) and two additions of 186 Da in full length tandem-SH2 unit (1-259). Tandem-SH2 abbreviated as “tSH2”.
3.2.4 Crystal structures of ZAP-70 tandem-SH2 unit covalently bound to IOZ compounds

In my initial attempt to understand the mode of compound inhibition, I performed crystallization trials with various compounds and the tandem-SH2 domains in the presence and absence of the 2pY peptide. I determined the structure of the tandem-SH2 domain module bound to two IOZ compounds. Although I will describe the structures in this section, in neither case could I explain the mechanism of inhibition on the basis of the structures. In one case (IOZ7), it appears that reaction of the compound induces a conformational change in the tandem-SH2 unit, but I will show later that mutation of the reacted cysteine has no effect on inhibition. In the other case, the compound (IOZ5.2) is bound to the active and ITAM-bound conformation of the tandem-SH2 unit in a way that does not perturb ITAM binding. These structures emphasize the fact that the most reactive cysteines in the tandem-SH2 unit are not the ones responsible for inhibition.

I obtained crystals of IOZ7 bound to the apo tandem-SH2 unit and I subsequently solved this crystal structure to 2.0 Å resolution by molecular replacement. The tandem-SH2 domains adopt a novel active-like conformation in the absence of ITAM [Figure 3.4]. I was at first unable to fit the compound into the density surrounding the position at Cys 117, nor was I able to rationalize the very superficial position of the density relative to the tandem-SH2 structure that I had built. This was one of the first indicators that the compounds were covalent modifiers. It was not until I had performed mass spectrometry of a sample of protein pre-incubated with IOZ7 that I was able to elucidate the nature of the Cys 117-IOZ7 adduct. With the predicted structure of the adduct from the mass spectrometry data, I was able to quickly build into the electron density, and found this structure clearly shows that IOZ7 forms an adduct on Cys 117 [Figure 3.4 (inset)].

It is surprising that the tandem-SH2 unit adopts a nearly active conformation in the absence of an ITAM peptide. This may be partially due to sulfate ions from the crystallization buffer that are bound in the phosphotyrosine binding pockets of the SH2 domains. The high sulfate concentration (1.1 M (NH₄)₂SO₄) likely stabilizes this conformation. But another structure of the apo tandem-SH2 unit crystallized in the presence of sodium phosphate (100 mM sodium phosphate) does not assume the active conformation (Folmer, Geschwindner et al. 2002). The presence of the adduct on Cys 117 results in the acetophenone group from the IOZ7 adduct forming an aromatic-aromatic π-stacking interaction with Tyr 178 contributed by the C-terminal SH2 domain from a crystallographic symmetry mate [Figure 3.4B]. This and other interactions distort the geometry of the helix containing Cys 117 and results in clockwise helical rotation, underwinding the helix at Asp 120. Disruption of the α-helical backbone hydrogen-bond network is compensated for by formation of a new hydrogen bond between the carbonyl of Asp 120 and the secondary amine of the Arg124 sidechain. This interaction also propagates upward and enforces an active-like arrangement in the tandem-SH2 domains.

Overlaying the tandem-SH2:IOZ7 structure on the ITAM-bound structure (Hatada, Lu et al. 1995) (PDBID: 2OQ1) shows that the SH2 domains in the tandem-SH2:IOZ7 structure assume a conformation that is almost competent to bind ITAM [Figure. 3.4C]. While the inter-SH2 linker does not align with that in the tandem-SH2:IOZ7 structure, examination of the structures reveals that the sulfate ions in SH2
domains of the tandem-SH2:IOZ7 structure overlay perfectly with the phosphotyrosine residues in the ITAM peptide-bound tandem-SH2 domain structure.

Crystallization trials of the ZAP-70 tandem-SH2unit, ITAM, and IOZ5.2 resulted in a crystal structure solved to 2.9 Å resolution by molecular replacement nearly identical to that of the ZAP-70 tandem-SH2 unit bound to ITAM solved by Hatada and coworkers (Hatada, Lu et al. 1995) [Figure 3.5A]. IOZ5.2 forms an adduct on Cys 78 which appears to have no effect on the crystal structure or packing [Figure 3.5B (inset)]. The adduct does not interact with neighboring residues in any significant manner. Because of the lack of interaction with the protein and the presence of ITAM peptide bound in the crystal, this structure of IOZ5.2 modification on the protein is indicative that Cys 78 plays no role in inhibition.

Taken together, these data suggest that the compounds attack multiple cysteines on ZAP-70. However, not all of these sites are relevant to inhibition as revealed by the ITAM-bound crystal structure with a covalent modification on Cys 78. Covalent modification of Cysteine 117 also plays no role in IOZ7 inhibition as mutation of this residue to a serine did not affect the ability of the compound to inhibit peptide binding [Figure 3.6]. This further supports the possibility that the conformation seen in the IOZ7:ZAP-70 crystal structure is an artifact of both high sulfate and crystal packing.
Figure 3.4 Crystal structure of IOZ7 bound to tandem-SH2 unit

(A) Structure of IOZ7 bound to ZAP-70 tandem-SH2 domains. Sulfates are bound in the phosphotyrosine binding pockets. IOZ7 has formed a covalent adduct on Cys 177. Inset: Carbonyl of Asp 120 forms hydrogen bond with Arg 124 sidechain as a result of underwinding of the helix.

(B) Cys 117-IOZ7 forms a π-stacking interaction with Tyr 178 in a neighboring molecule (shown in grey).

(C) Tandem-SH2 unit:IOZ7 structure overlayed on ITAM-bound tandem-SH2 structure (PDBID: 2OQ1 shown in grey). SH2 domains in IOZ7 structure overlays on those of ITAM-bound structure. Sulfates occupy sites where phosphotyrosines are located in the ITAM-bound structure.
Figure 3.5 Crystal structure of ITAM and IOZ5.2 bound to tandem-SH2 unit

(A) Overlay of ITAM-bound tandem-SH2 structure ((Hatada, Lu et al. 1995) PDBID: 2OQ1) and IOZ5.2 modified ITAM-bound tandem-SH2 structure; structures are nearly identical.

(B) Modification of Cys 78 plays no role in inhibition as adduct does not perturb ITAM binding in the crystal structure.
Figure 3.6 C117S and C254S mutations do not affect IOZ inhibition

ZAP-70 (1-254) with C117S and C254S mutations. The compounds inhibit this protein with an IC$_{50}$ value similar to the wild type tandem-SH2 domains.
3.2.5 Compounds inhibit through covalent modification and exhibit specificity towards different cysteine residues

Having shown that the compounds are covalent modifiers, I sought to show that the inhibition was time-dependent, which is characteristic of covalent inhibitors. In the case of the FP assay, one would expect a decrease in the polarization value as the peptide disengages from the protein due to covalent inhibitor binding. In a first experiment, I incubated compound with the ZAP-70 tandem-SH2 domains and TAMRA-2pY for an hour before reading in an endpoint assay. As one would expect for a covalent inhibitor, the compounds exhibited an IC\textsubscript{50} value near the assay protein concentration of 100 nM [Figure 3.7A-B]. Following this experiment, I performed a kinetic FP assay with IOZ1.4 and IOZ5.2 and found that both compounds displayed time dependent inhibition [Figure 3.8A]. Interestingly, IOZ5.2 shows incomplete inhibition whereas the FP value in the presence of IOZ1.4 extends down to the baseline.

In order to determine compound specificity, I prepared a truncated construct of Syk, a very closely related tyrosine kinase which plays a similar role to ZAP-70 in B-cells. This truncation is composed of the tandem-SH2 unit of Syk. Syk binds to the T-cell receptor ζ-chain with an affinity similar to that of ZAP-70 (K\textsubscript{D} = 30 nM). I tested IOZ1.4 and IOZ5.2 and found that both compounds also inhibited Syk-ITAM binding in a time-dependent FP assay [Figure 3.8b]. Thus, these compounds are not specific to the tandem-SH2 unit of ZAP-70. If the compounds inhibit ZAP-70 and Syk by a similar mechanism, the compounds could be exploiting a similar conserved binding pocket in both proteins. This result does not preclude the possibility that the compounds are acting as non-specific phosphotyrosine competitive SH2 domain blockers.

Because the compounds are able to disrupt both ZAP-70 and Syk tandem-SH2:ITAM binding, I postulated that the compounds could be inhibiting the two proteins in a similar way. As a corollary of this, one would expect that the locations of cysteine residues in the two tandem-SH2 units would be structurally conserved [Figure 3.9]. Comparison of these two proteins reveals three structurally conserved cysteines: Cys 84, Cys 95, Cys 254 in ZAP-70 and Cys 89, Cys 101, and Cys 259 in Syk. A more careful examination reveals that ZAP-70 contains a cysteine (Cys 39) in the N-terminal SH2 domain phosphotyrosine binding pocket. Syk lacks the equivalent cysteine, containing instead a serine in that position. Rather, Syk contains a cysteine (Cys 206) in its C-terminal SH2 domain phosphotyrosine binding pocket. While, not structurally conserved, the location of this cysteine suggests that it could be functionally conserved and could play an equivalent role in compound inhibition.

To further interrogate which cysteines are important for inhibition, I created a series of tandem-SH2 domain mutants in which individual cysteines were mutated to serine or alanine depending on their degree of surface exposure in the crystal structure. I had previously mutated cysteines 117 and 254 to serine and had established that they are not relevant for compound inhibition; the following experiments are in the context of that background.

Mutation of Cys 84 and Cys 222 had no effect on compound inhibition and exhibited similar inhibition to the wild-type ZAP-70 tandem-SH2 unit [Figure 3.10a-b]. The C39S mutation was, however, able to protect against IOZ5.2 inhibition. This mutation was not, however, able to protect from IOZ1.4 inhibition [Figure 3.10c]. This is highly suggestive that IOZ1.4 inhibits by modification of another cysteine. I can
rationalize the role of Cys 39 in 2pY binding inhibition by examination of the crystal structure of the ZAP-70 tandem-SH2 unit bound to an ITAM peptide. I saw that Cys 39 lies at the bottom of the phosphotyrosine binding pocket of the N-terminal SH2 domain. Modification of this residue is therefore expected to directly block phosphorylated ITAM binding. IOZ5.2 is negatively charged; this could enable it to better bind the positively charged phosphotyrosine binding pocket and lend compound specificity over IOZ1.4. The incorporation of certain cysteine mutations appears to destabilize the tandem-SH2 unit and identifying the targets of IOZ1.4 requires further study. A more thorough and exhaustive study of the effect of cysteine mutation is ongoing, and will be completed later.
Figure 3.7 Longer compound incubation time results in left-shifted inhibition curves

Increasing compound incubation time to 60 minutes in the dose-response assay showed a dramatic decrease in the IC₅₀ value approaching that of the protein concentration, which is characteristic of a covalent inhibitor.
Figure 3.8 Compound inhibition is time-dependent

(A-B) ZAP-70 and Syk tandem-SH2 domains are inhibited by IOZ1.4 and IOZ5.2 in a time-dependent manner.
Figure 3.9 Location of cysteine residues conserved between ZAP-70 and Syk

Ribbon diagram of ZAP-70 (left) and Syk (right) tandem-SH2 domains in grey with cysteines highlighted in red. Cysteines conserved between both proteins and probable targets of compound inhibition shown in sphere representation. ZAP-70 Cys 254 and Syk Cys 259 not shown because previous experiments showed they play no role in compound inhibition.
Figure 3.10 Mutational analysis identifies Cys 39 as target of IOZ5.2

(A-B) Mutation of C84A and C222S in ZAP-70 tandem-SH2 domains does not protect from compound inhibition.

(C) C39S protects protein from IOZ5.2 inhibition. This suggests a mechanism in which Cys 39 is necessary for IOZ5.2 inhibition.
3.3 Conclusions

The binding of ZAP-70 to phosphorylated ITAMs and the consequent recruitment of ZAP-70 to the T-cell receptor is a key step in T-cell signaling that makes ZAP-70 a potential for small molecule inhibition. As opposed to the traditional mode of inhibiting kinases with ATP-competitive inhibitors, I sought to develop compounds that would prevent ZAP-70 signaling by allosterically preventing its recruitment and activation. The high throughput screen identified a list of compounds that disrupted ZAP-70:ITAM binding. Further characterization of these compounds reveals that they are covalent inhibitors that target key cysteine residues in the tandem-SH2 unit. Mass spectrometry and crystallographic data clearly demonstrate that the compounds react covalently with cysteine residues. Large Hill slopes in dose-response FP assays and time-dependent inhibition in FP assays further suggested covalent binding as the mechanism of compound inhibition. Mutational analysis identified key cysteine residues as important for inhibition and showed a level of specificity of different compounds for various cysteines.

Identification of allosteric protein inhibitors is often complicated by several factors. Compound-induced aggregation can easily lead to false positives by sequestering the protein in the screening assay, therefore killing the enzyme activity and providing a non-relevant mechanism of inhibition. These are often large hydrophobic molecules that form aggregates or micelles that interact promiscuously with proteins (McGovern, Helfand et al. 2003). Furthermore, artificial hits can commonly arise from high throughput screens by compound fluorescence and compound reactivity (Arkin and Wells 2004, Jadhav, Ferreira et al. 2010). The fluorescence polarization screen should be free from compound fluorescence artifacts due to the fact that I had filtered based on total fluorescence intensity. Aggregation may be an issue, but in the case of this FP-based assay, one would expect a large increase in the polarization value if the compound aggregated both protein and peptide. Denaturation of ZAP-70 in the assay and consequent apparent inhibition is also a possible source of false positives.

I have shown in this chapter that for a subset of the hit compounds that compound reactivity is the mechanism of action of ZAP-70:ITAM binding inhibition. While I do not have a detailed understanding of the mode in which the compounds are binding, the selectivity of IOZ5.2 for Cys 39 suggests that there is a degree of specificity conferred by the location of Cys 39 such that IOZ1.4 is not able to inhibit by the same mechanism. It should be noted that IOZ5.2 is actually a known commercially available alkylating agent with the chemical name 4-Chloro-7-nitro-2,1,3-benzoxadiazole or 4-Chloro-7-nitrobenzofurazan (abbreviated NBD-Cl; Sigma catalogue #163260 ALDRICH). Derivatives of this scaffold have been used as selective suicide inhibitors of Glutathione-S-Transferase that show cytotoxic activity in certain cancer cells lines. (Ricci, De Maria et al. 2005, Turella, Cerella et al. 2005, Ascione, Cianfriglia et al. 2009).

The discovery of inhibitors that bind specifically while being facilitated by covalent modification of their target protein has been previously reported (Wiekowski, Prosser et al. 1997, Carter, Scherle et al. 2001). Both of those studies identified compounds that could reversibly bind and were further aided by a non-reversible covalent modification step. They go on to suggest that increasing the reversible binding affinity while decreasing reactivity could produce a drug lead compound.
In an example more closely related to the work I have presented, thiol-reactivity was unknowingly used to identify inhibitors of PAK1 kinase. The group I p21-activated kinase (PAK) family of kinases relies on binding of the 21 kDa small GTPases, Rac and Cdc42, to relieve their autoinhibitory dimers (Lei, Lu et al. 2000, Parrini, Lei et al. 2002). Studies by Peterson and co-workers identified IPA-3, an allosteric disulfide-containing inhibitor of PAK1. The compound inhibited closely related PAK1 isoforms, but not more distantly related PAK-family kinases. Furthermore, IPA-3 was not able to inhibit preactivated PAK1 (Deacon, Beeser et al. 2008). In a follow-up study the authors discovered that IPA-3 binds covalently to the GTPase-binding inhibitory domain, preventing Cdc42 binding and kinase activation (Viaud and Peterson 2009).

While my initial intentions were not to discover a covalent inhibitor of ZAP-70, one can see the utility of such a compound. These compounds could be further optimized to serve as useful laboratory tools. Limiting the reactivity while increasing the specificity of the inhibitors could produce a ZAP-70 specific covalent inhibitor. However, covalently reactive compounds are not without their limitations. For example, the crystal structures of IOZ5.2 and IOZ7 bound to the tandem-SH2 domains of ZAP-70 demonstrate that the compounds will readily react with the most surface exposed cysteines. Perhaps iterations of medicinal chemistry could yield a covalent compound specific to Cys 39 in ZAP-70 that is selectively reactive when noncovalently bound and placed in high local concentration with a specific cysteine.

I have shown that covalent cysteine modification is a viable way of blocking an essential protein-protein interaction in T-cell signaling. By forming an adduct on a residue located in the phosphotyrosine binding pocket, I have created a steric block that prevents ITAM binding, and therefore association of ZAP-70 with the T-cell receptor. Covalent inhibition of protein-protein interactions avoids the difficulty of identifying compounds that bind the flat large surfaces that constitute protein interaction interfaces (Way 2000). Moreover, by showing specificity between compounds and various cysteines in ZAP-70, I have demonstrated a pathway toward developing selective inhibitors of ZAP-70.

The structural and perhaps functional conservation of cysteine residues in ZAP-70 and Syk highly suggest that they play some inhibitory role in the cell and that the two kinases may be subject to redox regulation. Hydrogen peroxide provides a plausible oxidative species produced in immune cells in response to an antigenic stimulus. However, macrophages are thought to be the only immune cell that produce hydrogen peroxide, and only Syk is expressed in macrophages (Forman and Torres 2002). ZAP-70 expression is normally limited to T-cells and Natural Killer Cells (NK cells) which are not known to produce hydrogen peroxide (Au-Yeung, Deindl et al. 2009).

The oxidative inhibition of protein tyrosine phosphatases is a well characterized system in which hydrogen peroxide inhibits a cellular signaling protein through the oxidative sulfenylation of a catalytically essential cysteine residue. (Denu and Tanner 1998). More recent work has shown hydrogen peroxide acts as a signaling molecule that enhances EGFR kinase activity, and thus, EGFR signaling in response to EGF stimulation (Paulsen, Truong et al. 2012, Truong and Carroll 2012).

Hydrogen peroxide may play a downregulatory role in T-cell signaling. How ZAP-70 and Syk are released from ITAMs on the T-cell receptor and B-cell receptor, respectively, is not known. Preliminary studies on Syk suggest that phosphorylation may play an inhibitory role in B-cell signaling (Zhang, Oh et al. 2008). The studies presented
here on small molecule modification of cysteines in ZAP-70 may be a portent of endogenous redox regulation of kinases involved in immune-cell signaling.
3.4 Materials and methods

3.4.1 Cloning and protein preparation

A construct containing ZAP-70 tandem-SH2 domains (residues 1-254 or 259) or Syk tandem-SH2 domains (residues 6-269) was subcloned into pET28 (Invitrogen) with HindIII and BamHI restriction sites. A 6xHis tag, PreScission Protease site, and Gly-Ser-Gly linker were inserted N-terminal to ZAP-70. This construct was grown in BL21(DE3) cells in TB until they reached an OD = 1.0. Cells were then induced with 1 mM IPTG and grown overnight at 18°C. Purification was as described previously for ZAP-70 full length (chapter 2). Site directed mutagenesis was performed with QuickChange (Stratagene).

3.4.2 Protein labeling for mass spectrometry

Protein was incubated with a 3-fold excess of compound and incubated at room temperature overnight. Mass spectrometry was performed in the UC Berkeley QB3 mass spectrometry facility.

3.4.3 Time-dependence FP assays

FP reaction buffer (150 mM NaCl, 20 mM Tris pH 8.0, 2% glycerol, 0.01% TritonX-100, 0.01% Tween-20), 1 mM TCEP, 100 nM ZAP-70, and 2 nM AlexaFluor488-2pY were premixed in 80 µL reaction. Time dependent inhibition was initiated with the addition of compound or DMSO before transfer to a 384-well plate. This reaction was read as triplicates in a Biotek Synergy H4 fluorescence plate reader. Measurements were taken every 3 minutes for 30 minutes.

3.4.4 Tandem-SH2:IOZ7 crystallization

Tandem-SH2 domains at 743 µM were preincubated with 818 µM IOZ7 for 15 minutes before being spun down at 16,000 x g at 4°C for 10 minutes. 1 µL of this tandem-SH2:IOZ7 solution was mixed with 1 µL of well solution (7.75% PEG 3350, 1.10 M ammonium sulfate, 5 mM TCEP) and equilibrated by hanging drop for 16 hours at 20°C. The crystals were soaked in a cryoprotectant of 100% saturated lithium acetate in well solution and frozen in liquid nitrogen. Data collection was performed at the Advanced Light Source (Lawrence Berkeley National Lab, Berkeley, CA) on beamline ALS 8.2.1.

3.4.5 Tandem-SH2:ITAM:IOZ5.2 Crystallization

Tandem-SH2 domains at 177 µM were preincubated with 344 µM IOZ5.2 and 258 µM doubly phosphorylated ITAM peptide for 15 minutes before being spun down at 16,000 x g at 4°C for 10 minutes. 1 µL of this tandem-SH2:ITAM:IOZ5.2 solution was mixed with 1 µL of well solution (24% PEG 6000, 220 mM calcium chloride, 100 mM MES pH 6.0, and 20 mM TCEP) and equilibrated by hanging drop for 16 hours at 20°C. The crystals were soaked in a cryoprotectant of well solution with 30% glycerol and frozen in liquid nitrogen.
liquid nitrogen. Data collection was performed at the Advanced Light Source (Lawrence Berkeley National Lab, Berkeley, CA) on beamline ALS 8.3.1.
References


