INVESTIGATING THE SRC KINASE HCK FUNCTIONS IN CHRONIC MYELOGENOUS LEUKEMIA USING CHEMICAL GENETICS METHODS

by

Teodora Pene Dumitrescu

PharmD, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania, 1996

Master of Science in Pharmacology, University of Ottawa, 2001

Submitted to the Graduate Faculty of

The University of Pittsburgh School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2009

UNIVERSITY OF PITTSBURGH

School of Medicine

This dissertation was presented

by

Teodora Pene Dumitrescu

It was defended on

February 16th, 2009

and approved by

Thomas E. Smithgall, Ph.D., Major Thesis Advisor, Microbiology and Molecular Genetics

Billy W. Day, Ph.D., Pharmaceutical Sciences and Chemistry

Jennifer Grandis, M.D., Pharmacology and Chemical Biology

Daniel Johnson, Ph.D., Pharmacology and Chemical Biology

Jack Yalowich, Ph.D. Pharmacology and Chemical Biology

INVESTIGATING THE SRC KINASE HCK FUNCTIONS IN CHRONIC MYELOGENOUS LEUKEMIA USING CHEMICAL GENETICS METHODS

Teodora Pene Dumitrescu, PhD

University of Pittsburgh, 2009

The hallmark of chronic myelogenous leukemia (CML) is a chromosomal translocation between the c-*abl* gene (chromosome 9) and the *bcr* gene (chromosome 22). This event gives rise to Bcr-Abl, a chimeric protein with constitutive tyrosine kinase activity that drives the pathogenesis of the disease. Imatinib, a Bcr-Abl kinase inhibitor is the frontline therapy in CML. Although imatinib is very effective in the chronic phase of CML, patients in advanced stages develop resistance. An increased understanding of the signaling pathways implicated in CML pathogenesis and imatinib resistance is critical to the development of improved therapies.

Previous work in our laboratory found that A-419259, a broad-spectrum Src family kinase (SFK) inhibitor induces growth arrest and apoptosis in CML cells, suggesting that SFKs are required for Bcr-Abl transformation of myeloid progenitors. Additionally, Hck couples Bcr-Abl to Stat5 activation in myeloid cells, which may contribute to survival. Furthermore, studies on samples from some imatinib-resistant patients found increased expression and activity of Hck and Lyn. In this dissertation, using two chemical genetic methods, I addressed the contribution of Hck to Bcr-Abl signaling and imatinib resistance.

To explore the individual contribution of Hck to Bcr-Abl signaling, I developed an A-419259-resistant mutant of Hck (Hck-T338M). Expression of Hck-T338M fully protected K562 CML cells from A-419259-induced apoptosis, an effect that correlated with sustained Stat5 activation. In addition, the Hck-T338M partially protected CML cells against the growth

inhibition induced by A-419259. These studies suggest that Hck plays a non-redundant role as a key downstream survival partner for Bcr-Abl.

I also tested whether Hck overexpression was sufficient to induce imatinib resistance in CML cells. For this study, I developed a mutant of Hck (Hck-T338A) that is uniquely sensitive to NaPP1, an analog of the generic SFK inhibitor pyrrazolo-pyrimidine 1. Overexpression of Hck or Hck-T338A in K562 cells induced resistance to imatinib-dependent apoptosis and growth arrest. Furthermore, NaPP1 reversed imatinib resistance in K562-Hck-T338A cells, suggesting that Hck-induced imatinib resistance requires Hck kinase activity. Taken together, my work validates Hck as a target for the development of apoptosis-inducing drugs and that are likely to be effective in imatinib-resistant patients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abl	Abelson tyrosine kinase
Akt	v-akt murine thymoma viral oncogene homolog
AML	Acute myeloblastic leukemia
AP	Accelerated phase
AP-1	Activator protein 1
APC	Adenomatosis polyposis coli
Arg	Abl-related gene
B-ALL	B lineage acute lymphoblastic leukemia
BC	Blast crisis
BCIP	5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
Bcl-X_L	Basal cell lymphoma-extra large
Bcr	Breakpoint cluster region
Blk	B-cell lymphocyte kinase
Brk	Breast tumor kinase
Cbl	Cas-Br-M (murine) ecotropic retroviral transforming sequence
CCyR	Complete cytogenetic remissiion
CD34	Cluster of differentiation 34
CD38	Cluster of differentiation 38

CD4	Cluster of differentiation 4
CD45	Cluster of differentiation 45
CD8	Cluster of differentiation 8
CDK	Cycline-dependent kinase
Chk	Csk-homologous kinase
CHR	Complete hematological response
CLL	B cell chronic lymphocyte leukemia
CML	Chronic myelogenous leukemia
CMR	Complete molecular response
CREB	cAMP response element binding
Crk	v-crk sarcoma virus CT10 oncogene homolog (avian)
CrkL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like
Csk	C-terminal kinase
CXCR4	Chemokine (C-X-C motif) receptor 4
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
Fgr	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FITC	Fluorescein isothiocyanate
FLT3	Fms-related tyrosine kinase 3
Frk	Fyn-related kinase
Fyn	FYN oncogene related to SRC, FGR, YES
G-CSF	Granulocyte colony stimulating factor

GM-CSF	Granulocyte monocyte colony stimulating factor
GSK3	glycogen synthase kinase 3β
Hck	Hematopoietic cell kinase
HIV-1	Human immunodeficiency virus type 1
HLA-DR	Human Leukocyte Antigen DR-1
HMA	2-hydroxymyristic acid
HSC	Hematopoietic stem cell
HXMS	Hydrogen exchange mass spectrometry
IL-3	Interleukin-3
IL-6	Interleukin-6
INF-α	Interferon-α
Jak	Janus family of non-receptor tyrosine kinases
Kit	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
Lck	Lymphocyte-specific protein tyrosine kinase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide
Myc	v-myc avian myelocytomatosis viral oncogene homolog
NBT	Nitro blue tetrazolium chloride
Nef	Negative factor
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PDGFR	Platelet-derived growth factor receptor

PDK1	Phosphoinositide-dependent protein kinase-1
PEP	Proline-enriched tyrosine phosphatase
Ph	Philadelphia chromosome
PI3K	Phosphatidylinositol-3 kinases
PTPase	Protein tyrosine phosphatase
SCF	Stem cell factor
SDF-1	Stromal-derived factor 1
SFK	Src family kinase
SH	Src homology
SHP1	SH2 domain-containing protein tyrosine phosphatase
Src	v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
Srm	src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites
STAT	Signal transducer and activator of transcription
STI571	Signal transduction inhibitor 571
Yes	Yamaguchi sarcoma viral (v-yes) oncogene homolog

ACKNOWLEDGEMENTS

The moment of the thesis defense is almost here. It is a great feeling of accomplishment to receive the final reward after so much hard work. This is the most pleasant but also in a way the hardest section I have to write since I feel words are not sufficient to express my feelings.

As I take a moment to ponder over the people that contributed to my development and my research, I am humbled by the realization that part of this is as much their accomplishment as it is mine. I am just the lucky recipient of their support, patience, encouragement and love that carried me through this endeavor.

My utmost gratitude goes to my thesis advisor, Dr. Thomas E. Smithgall for his guidance and support. When I think of Dr. Smithgall and his mentorship, there are two things that first come in my mind. First, no matter how frustrated I would be over my research, I would always come out of his office with a new clue or direction, renewed motivation and much higher spirits. It is this talent of inspiring and motivating me that made the passing through the graduate years so much easier. Second, I have to say that any conversation I have with him, I never cease to be impressed by his infectious enthusiasm for research, sharp insights and especially by the breadth of his knowledge. For Dr. Smithgall, science and rigorous thinking are a state of mind and it is a privilege to know him and especially to have him as a mentor.

My thanks and appreciation go to my thesis committee for taking the time to work with me during this endeavor, and for their constructive advice and support. Also, I would like to

thank Dr. Billy Day for suggesting the Interdisciplinary Biomedical Sciences graduate program at a time when I was only considering the graduate program within the School of Pharmacy. Without his timely advice, I wouldn't be here now.

Furthermore, I would like to thank the current members of Smithgall laboratory: Dr. Lori Emert-Sedlak, Dr. Sabine Hellwig, Dr. Malcolm Meyn, Jamie Morocco, Purushottam Narute, Dr. Linda O'Reilly, Shoghag Panjarian, Jerrod Poe, Terri Robison, Dr. Jonathan Shaffer, Xiong Zhang who have provided me with great support and suggestions, for their help and last but not least for their friendship. Many warm thanks to Lori and Shoghag for taking time away from their families to endlessly proofread my thesis. Many thanks also to my former lab mates: Dr. Frank Delfino, Dr. Edwina Lerner, and Anthony Schiavone, for helping getting me started in the lab.

I cannot help thinking back to the people who jumpstarted me in biomedical research. I am deeply indebted to my former supervisor at the University of Ottawa, Dr. José-Maria Trifaró for giving me the chance to work in his laboratory especially at a time when my English speaking skills were not so good. It was a particular pleasure to work under the direct guidance of Dr. Tatiana Lejen and Dr. Sergio Rosé who have been great friends and mentors in more than research problems, and whom I truly miss. My tenure at the University of Ottawa would not have been as enjoyable without them.

My thoughts turn to my friends from Romania: Afrodita Alexe, Irina Lipsa, and Doina Mormenschi. Keeping in contact with them is in many ways therapeutic as it is the only thing that alleviates my homesickness. Also, I am grateful to my friends from Pittsburgh: Adina Soaita, Roxana Teisanu, and Elena Sirbu for their friendship and the many Romanian cookouts (with or without a special occasion) that I enjoyed with them. Special thanks to Adina for her

help with statistical analysis. Thanks to my other friends from Pittsburgh: Monique Van Damme and Gregg Rosen, Jana Conn and John Sanderson for their constant presence and regular parties (especially Monique).

Obviously, I would not be sitting in front of my monitor typing these acknowledgements without my Mom and Dad, Viorica and Tudor. My parents have always put my education as a first priority in their life, and in mine. They taught me to set high goals for myself and instilled the diligence to achieve them. Much of what I become I owe to their selflessness, hard work, trust and unconditional love. Although I only learnt the motto "work hard and play hard" when I came to North America, in retrospect, my life as a child and teenager gravitated around this idea (without the negative implications!). I credit my parents for opening the doors to the outdoors for me at a very early age. The many month-long backpaking "expeditions" in the Carpathian Mountains first with my class and biology teacher, and later with just a small group of friends have had a tremendous impact on me. Besides the fantastic memories that will always accompany me, they taught me love and respect for nature, independence, loyal friendship and self-confidence.

Lastly, I could not finish without thanking my husband, Mick. He has helped me stay sane and preserve the contact with the real world. My life would have been empty without his passionate guitar playing and wonderful cooking: his seafood and winter soups are the best I ever had (sorry Mom!), and he can make the most boring vegetables taste so delicious. One of my favorite pastimes is to walk the creeks with him and fish. Over the years, we fished with so many friends or family members and I can honestly say that he has an uncanny ability to guess where the fish is. This is why I am still so annoyingly proud on those rare days when I manage to catch more (or bigger) fish than him. It is this shared excitement, love for the outdoors, and his great

sense of humor that give me freedom from my daily routine. Above all, he showed me unconditional love, support and enormous patience and has been a constant reminder that there are other things in life more important than my research.

1.0 INTRODUCTION

1.1 SRC FAMILY OF KINASES (SFKs)

1.1.1 Overview

Almost one century ago, Peyton Rous discovered that injection of cell extracts from chicken tumors could cause tumors in healthy chickens (1). This led to the revolutionary idea that cancer could be caused by transmissible agents such as viruses, for which Rous received the Nobel Prize in Physiology and Medicine in 1966. This pioneering work led to the identification of viral Src (v-src), the transforming element of the Rous sarcoma virus in chickens and the first retroviral oncogene to be described (2, 3). The realization that v-src was derived from a cellular gene, the proto-oncogene c-src, earned J. Michael Bishop and Harold E. Varmus the Nobel Prize in Physiology and Medicine in 1989 (4, 5). Since its discovery, c-src and the related family members have been the subject of intensive investigation for nearly three decades (6, 7).

Like its viral counterpart, c-src encodes a non-receptor tyrosine kinase, c-Src - the archetype of the Src family kinases (SFKs) (8, 9). In humans, SFK consists of eight 52-62 kDa members (Blk, Fgr, Fyn, Hck, Lck, Lyn, c-Src, and c-Yes) (10). In addition, the human genome encodes three closely related kinases, frk, srm, and brk, with similar domain organization that

were recently classified as SFKs. However, these kinases, together with Src42A have recently been re-classified into the Brk family of tyrosine kinases (11).

Several SFK members (c-Src, Fyn, c-Yes) are ubiquitously expressed (See Table 1 for expression pattern of SFKs) (12). The remaining SFKs have a more restricted expression pattern and are found mainly in hematopoietic cells (13). In the case of Src, Fyn, Lyn and Hck more than one isoform has been identified arising from alternative splicing, separate genetic loci (Lyn), or alternative use of translational initiation sites (Hck) (14-17). Taken together, these observations indicate that SFKs have a complex and overlapping pattern of expression and that all cells, with the exception of erythrocytes, express multiple SFKs and potentially multiple isoforms of an individual member. The significance of the SFK isomers or their redundancy in various tissues remains elusive.

Table 1: SFK expression patterns

SFK	Cell type	Reference
Hck	Myeloid	(18, 19)
Lyn	B cells, myeloid cells, natural killer cells	(20)
Fgr	Monocytes, macrophages	(21)
Src	Platelets, neural, fibroblasts, mammary	(22)
Fyn	T cells (FynT), brain (FynB)	(23)
Lck	T cells	(24)
Blk	B cells	(25)
Yes	Neural, Gastrointestinal	(26)

1.1.2 Structure and Intramolecular Regulation

1.1.2.1 Overview of the structure

All SFK members share a similar domain organization, which includes six distinct regions: a Src-homology (SH) 4 domain, a unique region, an SH3 domain, an SH2 domain, an SH1 (kinase catalytic domain) and a C-terminal tail containing a regulatory phospho-tyrosine residue (Figure 1) (27).

1.1.2.2 The SH4 domain

The SH4 domain encodes a 9-15 amino acid sequence that contains signals for lipid modifications (28). All SFKs contain a consensus myristoylation signal sequence (Met-Gly-X-X-X-Ser/Thr). Myristoylation directs their localization to the cytoplasmic face of the plasma membrane or to other intracellular membranes such as endosomes, perinuclear membranes or secretory vesicles (28, 29). In addition, all members of the family except for Src and Blk contain cysteine residues within their SH4 domain that are sites of palmitoylation (29, 30). Palmitoylation of SFKs is believed to be important for targeting to lipid rafts, which are specialized plasma membrane microdomains critical for the clustering of receptor signaling complexes (31, 32).

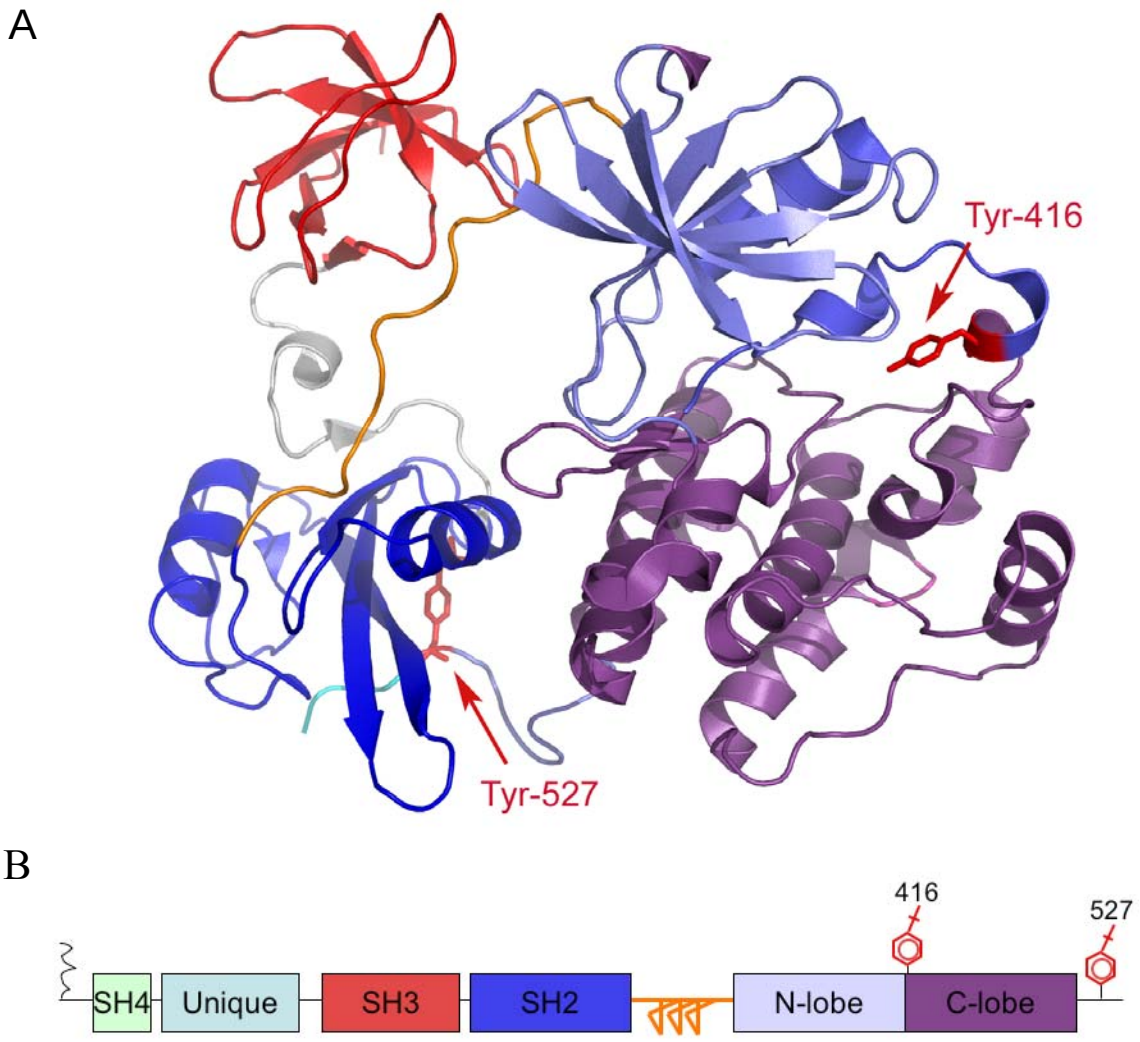


Figure 1: The domain organization of the SFKs

(A) Crystal structure of Hck is shown as a representative model of SFK in downregulated state (PDB 1QCF). The SH4 and unique domains were deleted for crystallization purposes. Domains in the structure are color-coded and correspond to the schematic in B. The activating Tyr-416 and inhibitory Tyr-527 residues are indicated in red. Phosphorylation at Tyr-416 in the kinase domain is required for activation. The engagement between the SH3 and SH2:kinase linker as well as phospho-Tyr-527 in the C-terminal tail interaction with the SH2 domain, contribute to the maintenance of the downregulated conformation. By convention, amino-acid residues are numbered as in chicken c-Src. (B) Schematic of domain organization of SFKs.

While lipid modification is unimportant for the maintenance of kinase activity in vitro, it is often essential for the SFK biological activity (28). The importance of SFK targeting to its appropriate intracellular location for their proper function and signal transduction, is highlighted by the use of myristoylation or palmitoylation inhibitors as effective inhibitors of SFKs (33). For example, the most common N-myristoylation inhibitor is 2-hydroxymyristic acid (HMA) (34, 35). HMA treatment induces relocation of Lck or Fyn to the cytosolic fraction, or the displacement of Lyn from lipid rafts. This in turn leads to their inactivation due to their inability to associate with their signaling partners (33).

In addition to lipid attachment, a modification unique to Fyn was recently described. The SH4 domain of Fyn was shown to be tri-methylated at Lys residues 7 and/or 9 (36). Mutants of Fyn at these residues failed to induce cell adhesion and spreading, suggesting that methylation is required for its proper localization and/or function (36).

1.1.2.3 The Unique Domain

Following the SH4 domain, the unique domain encompasses 50-70 amino acids. Unlike the rest of the molecule, which displays high sequence homology between all the members of the family, the unique domain is highly divergent among the family members. Various functions have been attributed to the unique domain of various SFKs. For instance, in Lck, the unique domain mediates its interaction with the cytoplasmic tails of CD4 and CD8 surface molecules (37), indicating that this region has a role in the recruitment of member-specific signaling partners. In the case of Hck, it has been shown that autophosphorylation at a residue in the unique domain modulates the catalytic activity of this SFK through an unknown mechanism (38). Furthermore, Fyn and Lyn are efficiently cleaved in a caspase-dependent manner after an Asp residue at position 19 or 18, respectively (39). This process occurs in hematopoietic cells undergoing

apoptosis. Cleavage of the N-terminal domain of Fyn and Lyn was linked to increased enzymatic activity, suggesting a novel mechanism for the regulation of SFKs with possible functional consequences (39). Last but not least, member-specific serine and threonine phosphorylation sites were identified in the unique domains of Src or Lck. However, their role in the modulation of protein-protein interaction or regulation of catalytic activity remains unclear (40-43).

1.1.2.4 The SH3 Domain

In general, SH3 domains contain an average of 40 to 70 amino acids, and bind conserved proline-rich sequences initially defined as PXXP motifs, where P is proline and X is a variable, usually hydrophobic amino acid (44, 45).

SH3 domain structure has been extensively studied using both X-ray crystallography and NMR spectroscopy (46, 47). A typical SH3 domain has a β-barrel arrangement of five anti-parallel β-strands that form two β-sheets packed at almost right angles (47-49). In addition, SH3 domains contain two loops termed RT and n-Src loops (47). The ligand-binding surface of the SH3 domains is highly hydrophobic and displays three shallow pockets flanked at either end by the RT or n-Src loops (46-48). These loops often contain charged residues that regulate specificity and orientation of ligand binding.

A more in depth characterization of SH3 binding partners led to the description of two classes of ligands that bind to SH3 domains in opposite orientations. Class I ligands have a RxxPxxP motif while class II ligands have a PxxPxR canonical sequence (50-52). When bound to the SH3 domain, both motifs acquire a polyproline type II helical structure (PPII helix). This helix has a triangular cross-section with the two prolines at the base of the triangle. The two prolines occupy two of the three shallow pockets in the SH3 domain ligand-binding surface. In

addition, the third shallow pocket is occupied by a basic Arg residue situated distal from the PxxP core (46).

In SFKs, SH3 domains fulfill two important roles. First, SH3 domains were shown to contribute to substrate recruitment (53, 54). In addition, SH3 domains are critical for the regulation of the kinase activity. Crystallography studies of several SFKs in a downregulated state showed that the SH3 domain binds intramolecularly to a proline-rich sequence in the SH2:kinase linker (55, 56). This interaction results in the tethering of the SH3 domain to the back of the kinase domain and helps in generating an inactive conformation of the kinase. Figure 1 shows the molecular model of the SFK Hck in its downregulated conformation, with the SH3: linker interaction intact.

1.1.2.5 The SH2 domain

SH2 domains contain on average 100 amino acid residues and bind specific phosphotyrosine-containing peptides (57, 58). Structurally, SH2 domains contain a central β-sheet, with one alpha helix packed against each side. These features give rise to two binding pockets, one that coordinates the phosphotyrosine by means of a conserved arginine residue, and the second that accommodates one or more hydrophobic residues C-terminal to the phosphotyrosine (59). While the first pocket is highly conserved, the second recognition pocket is much more divergent, serving as a specificity determinant amongst different SH2 domains (57). The SFK SH2 domains bind preferentially to a phosphorylated YEEI motif, with phosphotyrosine occupying the first pocket and the isoleucine occupying the second (60).

Similar to the SH3 domain, the SH2 domain in SFKs serves a dual role. First, binding interactions mediated by the SH2 domain, contribute to substrate recruitment and facilitate the transmission of signals to downstream effectors. Second, within the inactive SFKs, the SH2

domain binds a phosphotyrosine residue located in the C-terminal tail of the protein (Tyr-527 in chicken c-Src). This interaction was recently shown to be stabilized by an additional interaction between the SH2 domain and the SH2:kinase linker (61). This intra-molecular interaction helps to lock the kinase in an inactive conformation and represents an additional autoregulatory mechanism (56, 62-64).

1.1.2.6 The SH3/SH2 clamp

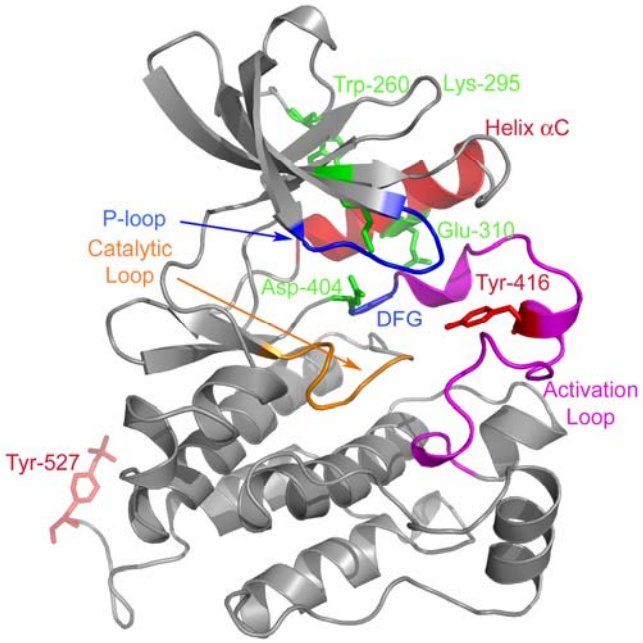
Multiple studies suggest that through their intramolecular interactions, the SH3 and SH2 domains cooperate to induce a downregulated conformation of the kinase domain. Thus, the SH3 and SH2 domains are turned inward and bind to the distal face of the kinase domain locking it into a rigid conformation (55, 56, 62-64). The effectiveness of the SH3/SH2 clamp was shown to depend on a rigid conformational coupling between these domains as well as on the SH2 - C-terminal tail interaction. Mutation of the SH3-SH2 connector residues to glycines, is sufficient to induce a release of the phospho-tyrosine tail of the kinase and to induce activation (65). Conversely, the release of the tail from the SH2 domain upon dephosphorylation, induces a collapse of the connector that allows individual movement of the SH3 and SH2 domains (65). The binding of the SH3/SH2 clamp to the back of the kinase domain may decrease the overall flexibility of the kinase domain, a feature important for the enzyme function (66, 67).

1.1.2.7 Tyrosine kinase domain

The catalytic domains of Ser/Thr and Tyr kinases contain about 300 residues and, as revealed by X-ray crystallography, share a common, bilobed architecture (Figure 2, A)(68, 69). The smaller, amino-terminal lobe (N-lobe) is composed of a five-stranded antiparallel beta-sheet and an α helix (αC). The larger, carboxy-terminal lobe (C-lobe) consists mainly of α-helices with only a

few beta strands. Catalytic activity occurs in a cleft between the two lobes, which are connected by a flexible hinge region. This hinge allows the two lobes to move relative to each other during activation or inactivation events.

A



B

```
  260             P-Loop                  295             310
KDAWEIPRESLKLEKKLGAGQFGEVWMATYNKHTKVAVKTMKPGSMSVEAFLAEANVMKTLQHDKLVKLHAVVTKEPIYIITEFM
             ß1              ß2        ß3            helix C                ß4       ß5

                                                Catalytic loop        DFG         416  A-Loop
AKGSLLDFLKSDEGSKQPLPKLIDFSAQIAEGMAFIEQRNYIHRDLRAANILVSASLVCKIADFGLARVIEDNEYTAREGAKFPI
    helix D         helix E                          ß7      ß8

KWTAPEAINFGSFTIKSDVWSFGILLMEIVTYGRIPYPGMSNPEVIRALERGYRMPRPENCPEELYNIMMRCWKNRPEERPTFEY
 helix EF          helix F                 helix G             helix H

                527
IQSVLDDFYTATESQYQQQP
helix I   helix J
```

Figure 2: The structure of the inactive kinase domain of an SFK

(A) Cartoon representation of the inactive conformation of Hck kinase domain and C-terminal tail (PDB: 1QCF). This cartoon was prepared using Pymol. The elements important for the regulation of activity are color-coded as follows: alpha C helix - red; P-loop – blue; catalytic loop – orange; DFG motif – blue; activation loop (which starts with the DFG motif) - magenta; Tyr-416 and Tyr-527- red; Lys-295, Glu-310, and Asp-404 - green. (B) Sequence of the Hck kinase domain and C-terminal tail, indicating the position of the structural and regulatory elements represented in A. All these elements are color-coded consistent with the structure in A.

Each of the two lobes contain highly conserved elements that are important for catalysis or for the correct conformation of the catalytic site (figure 2, B) (70). In the N-lobe of SFKs, these key elements are: Trp-260 at the N-terminus, the phosphate-binding loop (P-loop, amino-acids 273-281), the αC helix, Lys295 and Glu310 (chicken c-Src numbering). In addition, the C-lobe elements important for the kinase structure and function are: the catalytic loop (amino-acids 386-392) with strictly conserved Asp and Asn residues, the activation loop (amino-acids 404-432) containing the DFG motif (amino-acids 404-406) and Tyr-416, the site of autophosphorylation. All of these elements undergo significant modifications in their orientation, phosphorylation status or interaction with various partners, and this in turn modulate the transition between the inactive to active conformation of the kinase domain, or the catalysis of phosphate group transfer. The role of each of these key structural elements is described in more detail in the next two sections.

The inactive conformation

The structures of downregulated human and chicken Src kinases and human Hck have been solved by X-ray crystallography (56, 62, 64). Several elements contribute to the maintenance of the inactive conformation of SFKs. For example, Asp-404, which lies at the N-terminus of the activation loop within the DFG motif, forms an ionic interaction with Lys-295. This interaction induces the side chain of Phe-405 to come into steric clash with the αC helix in N-lobe, and pushes it away from the catalytic center (63). In addition, residues 413-418 of the activation loop form a short α helix that is located under the αC helix of the N-lobe, and which positions the unphosphorylated Tyr-416 into the catalytic site of the kinase domain where it forms hydrogen bonds with Arg-385 and Asp-386 (62, 63). Consequently, Tyr-416 is well protected from

autophosphorylation and the activation loop occludes the entry to the catalytic domain and effectively prevents the binding of peptide substrates.

Another stabilizing factor for the outward, inactive position of the αC helix is Trp-260, a highly conserved residue within SFKs and other non-receptor tyrosine kinases. This residue lies at the interface between the SH2:kinase linker and the N-lobe of the kinase and points into a hydrophobic region at the C-terminus of the αC helix (64). The role of Trp-260 in the stabilization of the αC helix in a non-catalytic conformation is also supported by the fact that this residue does not contact the αC helix in the structure of the active Lck kinase domain (71). In addition, Leu-255 within the SH2:kinase linker also points into a hydrophobic pocket in the back of the N-lobe and may have a role in the stabilization of the inactive αC helix (72).

The active conformation

Activation of SFKs occurs upon phosphorylation at Tyr-416 in the activation loop (73, 74). Phosphorylation at this residue induces the activation loop to adopt an extended conformation away from the catalytic cleft, allowing contacts between Tyr-416 and Arg-385/363 or Arg-409/387 residues that lie outside of the cleft (71, 75). The change in the orientation of the activation loop, in particular of the Arg-404 within the DFG motif induces an inward rotation of the αC helix in the N-lobe. This conformational change is stabilized by an ionic interaction between Lys-295 and Glu-310, an interaction that is critical for kinase activity (62, 63).

Another putative mechanism of activation involves the release of the SH3/SH2 clamp. The release of the SH3/SH2 clamp may induce a displacement of Trp-260 from the hydrophobic pocket in the N-lobe, inducing a destabilization of the non-catalytic αC helix conformation. Mutagenesis studies support the notion that Trp-260 acts as a switch to communicate the position of the SH3/SH2 clamp to the kinase domain (76). In addition, SH3/SH2 clamp release is likely to

disrupt the conformation of the activation loop and to remove the steric clash with the αC helix, allowing it to adopt an active conformation (27).

The transfer of γ-phosphate from the ATP molecule to the tyrosine substrate proceeds by direct attack of the hydroxyl oxygen of the tyrosine residue on the γ-phosphate and requires the presence of a divalent metal ion (77). This reaction is facilitated by several motifs or individual residues located within both the N and C-lobes. The P-loop, which lies between β1 and β2 strands, coordinates the phosphates in the ATP molecule and contains a highly conserved glycine-rich motif (GXGXΦG) where Φ is a Tyr or Phe (78, 79). The Gly residues coordinate the phosphates in the ATP molecule via backbone interactions while the Φ residue caps the phosphate transfer site. In addition, Asp-386 within the catalytic loop induces a catalytically-competent orientation of the tyrosyl group. Lastly, Asp-404 within the activation loop binds Mg^{2+}, which in turn coordinates the β- and γ-phosphate groups of ATP (80).

1.1.2.8 C-terminal tail

The C-terminal tails of SFKs (residues 521 to 535 in c-Src) contain a Tyr residue (Tyr-527) that is phosphorylated by either C-terminal Src kinase (Csk) or by the Csk-homologous kinase (Chk) (65, 81). Phosphorylation at this residue creates a low-affinity SH2-binding site and induces intramolecular binding of the C-terminal tail to the SH2 domain (64, 81, 82). Although this SH2 binding site is suboptimal, purified SFKs show highly stable association of the SH2 domain and the C-terminal tail, with only 2% displaying the SH2 domain released from the tail (83). This stable association may also be promoted by an additional interaction between the SH2 domain and the SH2:kinase linker (61). Disruption of the SH2:tail interaction is sufficient to activate the

kinase. Mutation of Tyr-527 to phenylalanine or deletion of the C-terminal tail (as in v-Src), results in a constitutively active tyrosine kinase (84-86).

1.1.3 Extrinsic regulators of SFK activity

1.1.3.1 Regulation by phosphorylation

Phosphorylation at Tyr-416 in the activation loop is required for maximal activation of SFKs (87). Phosphorylation at this residue is believed to occur mostly by trans-auto-phosphorylation upon clustering of at least two identical molecules in the same area. In addition, it has been suggested that different SFKs can transphosphorylate each other on this residue and even that other tyrosine kinases can phosphorylate this site (88). While phosphorylation at Tyr-416 induces tyrosine kinase activation, phosphorylation of the C-terminal Tyr-527 residue induces intramolecular interaction with the SH2 domain and promotes an inactive conformation (see section 1.1.2.8).

1.1.3.2 Regulation by dephosphorylation

Dephosphorylation can either activate or inactivate SFKs, depending on the phospho-Tyr residue involved. Dephosphorylation of the C-terminal phospho-Tyr was shown to be mediated by several protein tyrosine phosphatases (PTPases) including proline-enriched tyrosine phosphatase (PEP), tandem SH2 domain-containing protein tyrosine phosphatase or SHP1, or by the transmembrane receptor-like protein phosphatase CD45 (89-91). However, these PTPases were also shown to have the ability to dephosphorylate the phospho-Tyr-416 within the activation loop (90-93). Interestingly, the same phosphatases have been associated with either activation or inhibition of SFKs. Which Tyr residue is dephosphorylated at any given time may depend on the

cell type, subcellular compartment, or on the SFK conformation or interaction partners (83). In addition, a downregulated conformation of SFKs could be induced by dual action of a kinase and a phosphatase. For example, PEP was found to dephosphorylate the Tyr in the activation loop while bound to the SH3 domain of Csk that phosphorylates the tail tyrosine (91, 94).

1.1.3.3 Regulation by interaction with binding partners

Another mode of SFK activation takes place upon ligand binding to their SH3 domain, which induces a disruption of the intramolecular downregulatory interaction with the SH2:kinase linker (81). Work in our laboratory and others showed that displacement of the SH3 domain due to mutations in the linker PXXP motif or due to binding of HIV-1 Nef to the SH3 domain of Hck is sufficient to activate Hck and to induce a transformed phenotype in Rat-2 cells (95-97). Other SFK physiological substrates such as p130Cas, Stat3 or Ras act in a similar manner (98).

Another mode of activation due to interaction with binding partners is through the disruption of the SH2:tail interaction. For example, SFKs have been implicated in signal transduction by both cytokines and growth factors. The mechanism of SFK activation by growth factor receptor tyrosine kinases may involve SH2-dependent recruitment to the activated, autophosphorylated form of the receptor. Binding of the SH2 domain to the receptor may induce Src activation by displacing the negative regulatory tail leading to phosphorylation of the receptor in some cases (81, 99).

Work in our laboratory suggests that the two modes of activation discussed above may occur independently of each other. On one hand, a High Affinity Linker mutant of Hck (HAL) in which the SH3 domain is tightly bound to the SH2:kinase linker can be activated through the release of the SH2 domain via mutation of the tail Tyr-527 (86). On the other hand, replacement of the wild-type tail of Hck with a high-affinity SH2-binding sequence did not affect Hck

activation by Nef, suggesting that activation through SH3 recruitment occurs without SH2 release from the tail (100).

In addition, several lines of evidence suggest that tandem engagement of both SH3 and SH2 domains acts cooperatively to induce the activation of SFKs. For example, addition of Nef to the tail-mutant of Hck further activated the kinase above the level obtained with only the tail release (97, 100). Cas, a Src substrate that possesses both SH3 and SH2 –binding motifs was shown to require both to induce Src autophosphorylation (101). Furthermore, Focal adhesion kinase (Fak) contains closely placed proline and phosphotyrosine motifs shown to bind simultaneously to the SH3 and SH2 domains of Fyn (102).

Collectively, these lines of evidence suggest four possible conformations of SFKs: i) a completely downregulated conformation in which both the SH3 and SH2 domains are engaged with their intramolecular ligands (linker and tail, respectively); ii) an active conformation in which only the SH3 domain is released; iii) an active conformation in which only the SH2 domain is released and iv) a "fully open" conformation in which both the SH3 and SH2 domains are disengaged. Elucidation of these distinct conformations may provide important clues for the development of more selective Src inhibitors (103).

1.1.4 SFK Biological Functions

1.1.4.1 Overview

SFKs play critical roles in regulating cellular responses to a variety of stimuli including: growth factors, cytokines, G-protein coupled receptors, oxidative stress, signals that trigger mitosis and adhesion signals from extracellular matrix (10, 99, 104, 105). As explained in detail in the previous section, all members of the family are highly regulated, exhibiting little or no activity in

the absence of an activating signal. Constitutively active SFKs are known to be capable of inducing malignant transformation of a variety of cell types [reviewed in (106)]. This section will focus on the involvement of SFKs in normal hematopoiesis and hematological malignancies (a multi-article, comprehensive review of SFK signaling in normal and malignant cells was published in 2004, in issue 23 of Oncogene).

1.1.4.2 Normal hematopoiesis and the role of SFKs in hematopoietic cells

Hematopoiesis

Hematopoiesis is a tightly controlled, hierarchical process in which pluripotent stem cells develop into various types of mature blood cells [reviewed in (106)]. Mature blood cells develop upon stem cell commitment to hematopoietic common lymphoid or myeloid progenitors. These progenitor cells maintain some stem cell-like properties but they can only differentiate into a particular lineage.

Hematopoietic cell growth and differentiation are controlled by growth factors and cytokines, which include interleukins, colony-stimulating factors, and hematopoietins (107). With a few exceptions, the receptors for the majority of these hematopoietic growth factors are transmembrane proteins that do not encode a tyrosine kinase catalytic domain (108, 109). Upon cytokine stimulation, the cytoplasmic or signal-transducing subunit of the receptor recruits cytoplasmic kinases, which results in propagation of the signal to downstream target proteins. Some of the kinases recruited and activated by the cytoplasmic subunit of the cytokine receptors include the Janus family of kinases (Jak), the SFKs, and the Tec family of kinases (110-115).

The role of SFKs in hematopoietic cells

Consistent with the high number of interacting partners, SFKs play multiple roles in the process of hematopoiesis. Several lines of evidence suggest a critical role for SFKs in blood cell

function. First, many of the eight family members are found predominantly or exclusively in various hematopoietic compartments (Table 1). Second, some SFK knock-out mice display prominent hematological abnormalities. For example, deletion of the Hck gene induces a defect in phagocytosis, although hematopoiesis proceeds normally (116). Fyn-defective mice present reduced TcR signaling (117), while Lck knock-out mice have defective T-cell maturation (118). Double Hck/Fgr knock-out mice have defective host defense against intracellular macrophage pathogens such as Listeria (116). Mice deficient in Lyn have a reduced number of mature B-cells. Third, a patient with T-cell acute lymphoblastic leukemia has been identified with a fusion gene of *lck* locus and the β-subunit of T-cell receptor gene, suggesting that SFK defects could induce hematological diseases (119). Fourth, several SFKs, including Fyn, Hck and Lyn, have been described to co-precipitate with hematopoietin/cytokine receptors (112, 120-122).

SFKs act downstream of cytokine receptors by activating various pathways involved in cell growth and differentiation, migration and adhesion. Within these signaling pathways, SFKs either phosphorylate the signal transduction molecule itself (such as FAK) or phosphorylate an adaptor protein such as Cbl and Shc that link the SFK to the specific signaling molecule (Ras or PI3K) (123-127).

1.1.4.3 Role of SFKs in hematological malignancies

Constitutively active SFK variants were shown to induce malignant transformation in a variety of cell types [reviewed in (106)]. Activation of one or more members of the family has been linked to breast, colorectal, ovarian, gastric, head and neck, pancreatic, lung, brain, or blood cancers. In this context, they appear to be involved in multiple aspects of tumor progression, including proliferation, migration, invasiveness, angiogenesis, survival and resistance to

apoptosis (106). This section will focus on the role of SFKs in hematological malignancies other than CML. The roles of SFK in CML will be discussed in detail in section 1.2.4.1.

Increasing evidence points to a role for SFKs in hematological diseases. Lyn kinase is predominantly expressed in B-lymphocytes and monocytes/macrophages and several reports suggest a role of Lyn in cancers that arise from these cells. For instance, Lyn kinase activity was specifically upregulated in response to IL-3 or IL-6 in myeloid leukemia cell lines or multiple myeloma cells, respectively (115, 128). This suggests that Lyn may be involved in IL-3 and IL-6-dependent signal transduction and proliferation in these malignancies. In addition, Choi et al. showed the involvement of Lyn kinase in human malignant lymphomas of B cell origin (129).

The Lck tyrosine kinase is expressed mainly in T cells. However, Lck may be involved in the growth and survival of cancers that arise from multiple blood lineages. For example, Lck mRNA was detected in B cell chronic lymphocytic leukemia (130). In addition, it was suggested that Lck has a role in B lineage acute lymphoblastic leukemia (ALL) and in B cell chronic lymphocyte leukemia (CLL) (131, 132).

The Fyn tyrosine kinase is expressed as two main isoforms: FynT found in hematopoietic T cells and FynB found in brain (16). However, a recent report shows abnormal FynB mRNA expression in fresh cells isolated from ALL and CLL (133). Although the FynB protein was undetectable by isoform-specific immunoprecipitation, a possible role of minute levels of FynB in these blood malignancies could not be ruled out (133).

1.2 CHRONIC MYELOGENOUS LEUKEMIA (CML)

1.2.1 Overview

Chronic myelogenous leukemia (CML) is a myeloproliferative disease that arises from neoplastic transformation of a hematopoietic stem cell (See section 1.2.1.2 for details on the stem cell hypothesis of CML). CML accounts for 15-20% of human leukemias and has an incidence of approximately 1 to 5 in 100,000 individuals per year, affecting 4,500 Americans per year, or about 0.3% of the 1.200.000 new US cancer patients (134, 135). The median age of diagnosis is 65 to 75 years, with males having a higher incidence (136).

In 1845, three independent pathologists described patients with massive splenomegaly and leukocytosis, which we now know as two clinical hallmarks of CML [reviewed in (137)]. Today, the clinical features of the disease are well characterized. CML has three distinct clinical phases: a chronic phase that may last approximately 2 to 5 years, accelerated phase that lasts 6 to 18 months, and blast crisis phase that lasts only about 3 to 6 months (136, 138). In the chronic phase patients are mostly asymptomatic but develop a tremendously increased number of mature granulocytes in peripheral blood, weight loss, and splenomegaly. Both accelerated and blast crisis phases are characterized by a severe reduction in hematopoietic differentiation and accumulation of immature blasts in the bone marrow and in peripheral blood ($\geq$ 30% blasts in peripheral blood or bone marrow) (138, 139). The blast crisis phenotype varies, with approximative 50% of the patients entering a myeloid blast stage similar to acute myeloblastic leukemia (AML); 30% of the patients entering a pre-B blast stage similar to acute lymphoid leukemia (B-ALL); and 10% of the patients developing erythroid blasts (137).

1.2.1.1 Philadelphia Chromosome

The first landmark discovery in the pathogenesis of CML came in 1960 when Nowell and Hungerford associated CML with the consistent appearance of a chromosomal abnormality, later termed the Philadelphia (Ph) chromosome (140). This discovery was the first demonstration that a cancer may be associated with a chromosomal rearrangement. In 1973, the Ph chromosome was further characterized as a reciprocal translocation between chromosome 9 and chromosome 22, now described as t(9;22)(q34;q11) (141). In the 1980s, the Ph chromosome was shown to give rise to a unique chimeric gene, Bcr-Abl, due to the fusion between the "break point cluster region gene" (Bcr) on chromosome 22 with sequences of the c-abl protooncogene on chromosome 9 (142, 143).

A closer look at Bcr-Abl revealed that there are three breakpoint regions [minor (m); major (M); micro (μ)] in the Bcr gene that gives rise to three variants of Bcr-Abl differing in the amount of Bcr included in the fusion protein (Figure 3, B) (143). These three Bcr-Abl proteins are named p190, in which the junction occurs between the e1 region of Bcr and a2 region of abl; p210 in which the junction occurs between b2/a2 or b3/a2 regions of Bcr and Abl, respectively; and p230 in which the junction occurs between e3/a2 regions of Bcr and Abl, respectively (Figure 3, B) (144). P190 encompasses the oligomerization and SH2 domain of Bcr; p210 has the PH and Dbl domains in addition to the oligomerization and SH2 domains; and p230 includes an addition of the calcium/phospholipid binding domain of Bcr (145-147). Consistent with the differences in the content of Bcr, the three forms of Bcr-Abl display distinct biological properties and are associated with different diseases (144). Specifically, p190 Bcr-Abl is associated with ALL; p210 with CML, and p230 with chronic neutrophilic leukemia (CNL) and CML with thrombocytosis (148, 149).

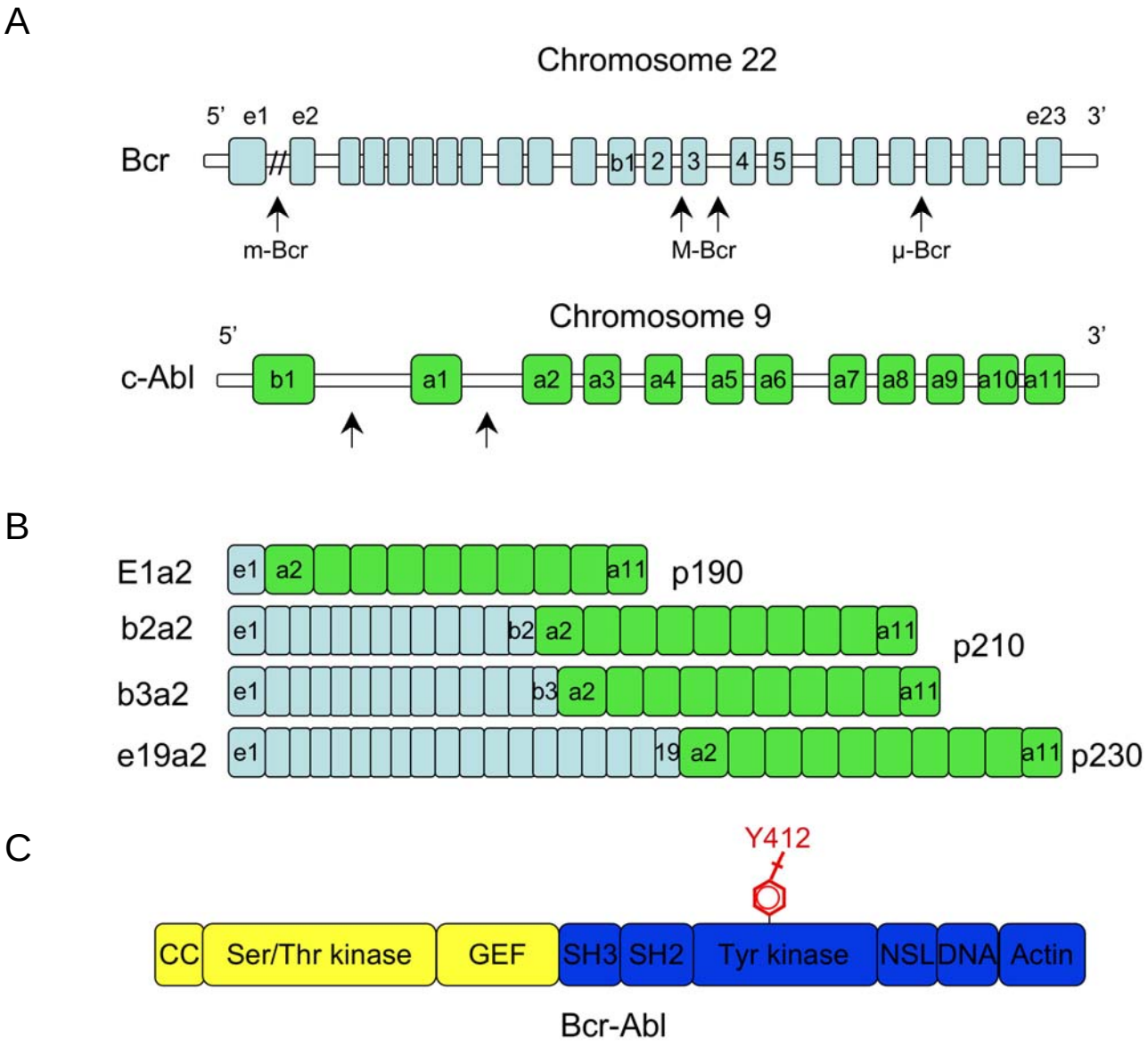


Figure 3: Schematic diagram showing the Bcr and c-Abl genes and the formation of Bcr-Abl variants

(A) Breakpoints location in Bcr and c-Abl genes; (B) Composition of the chimeric Bcr-Abl mRNA transcripts; (C) Domain organization of p210 Bcr-Abl. Regulatory Tyr-412 is illustrated in red. NSL: Nuclear Localization Signal; DNA: DNA-binding domain; Actin: Actin-binding domain [Modified from (150)].

The mechanism of Ph chromosome formation is not known. However, people exposed to high dose irradiation have a higher risk of developing CML (151). In addition, *in vitro* high-dose irradiation of myeloid cell lines induces the expression of Bcr-Abl transcripts similar to CML (152). These observations suggest that high dose irradiation may be one of the risk factors for CML.

Numerous studies in mice confirmed the prediction that Bcr-Abl can induce leukemia *in vivo*. For example, enriched hematopoietic stem cells (HSC) were collected from mice pretreated with 5-fluorouracil. These cells were then induced to cycle *in vitro* using cytokine mixtures, and were infected with retroviral vectors carrying p210 Bcr-Abl (153). Subsequently, these HSC carrying p210 Bcr-Abl were transplanted in lethally irradiated mice, which later developed CML-like disease. More recently, p210 Bcr-Abl was found to induce a CML-like disease upon expression under the control of the tec gene promoter, a gene that is preferentially expressed in hematopoietic cells (154). In a similar approach, Bcr-Abl expression under the control of PCMV promoter of the murine stem cell virus induced a myeloproliferative disease similar to CML (150).

1.2.1.2 Stem Cell Hypothesis

Stem cells are defined by three distinctive properties: self-renewal, the potential to develop into multiple lineages and the ability to proliferate indefinitely. More than 30 years ago, Fialkow et al. made the observation that although only myeloid cells are expanded in the chronic phase of CML, both granulocytes and erythroid lineage cells contain the Ph chromosome. This suggested that the Ph chromosome originally occurs in a HSC and is passed down to both myeloid and erythroid lineages (155). In 1977, Fialkow et al. provided evidence supporting the HSC origin of the Ph chromosome in an elegant study using X-linked polymorphic glucose-6-phosphate

dehydrogenase (G-6-PD) loci in selected female chronic phase patients as markers for the monoclonal origin of Ph^+ cells (155). Since then, the development of sensitive cytogenetic and molecular biology techniques has allowed the detection of Ph chromosome and Bcr-Abl transcripts in all hematopoietic lineages except natural killer cells, further supporting the HSC origin of Ph chromosome (156).

Upon the acquisition of the Ph chromosome, the HSC undergoes proliferation and self-renewal giving rise to a population of cells called leukemic stem cells or leukemia-initiating cells that initiate the chronic phase of CML (157). These leukemic stem cells share many properties with normal HSCs. For example, leukemic stem cells at steady-state are quiescent, have a long life-span and show great proliferative and self-renewal potential that enables them to maintain the cancer cell population (158). In addition, they display resistance to drugs and express typical HSC markers (159). Similar to the normal HSC, the immunophenotype of CML stem cells is $CD34^+$ $CD38^-$ (160). However, there is evidence that contrary to HSC, which are negative for HLA-DR antigens, in CML stem cells the HLA-DR antigen may be expressed aberrantly (161).

Using long-term cultures, Coulombel et al. showed that normal HSCs persisted in patients during early stages of CML (162). The relative frequency of circulating Ph^+ $CD34^+$ cells remains under debate and ranges between 60% to 97% (163, 164). Much effort is being made to optimize protocols for purging the Ph^+ cells and enriching the Ph^- stem cells from chronic phase CML patients for the purpose of autologous stem cell transplantation (163, 165).

More recently, increasing evidence suggests that although the Ph^+ CML stem cells (leukemia initiating cells) are important for the initial expansion of myeloid progenitors during the chronic phase, other cells with stem cell-like properties support the progression to blast crisis. Specifically, it was shown that expression of Bcr-Abl in myeloid progenitor cells can induce

myeloproliferative disease in transgenic mouse models (166). In addition, Jamieson et al. brought evidence that the granulocyte-macrophage progenitor (GMP) pool from patients in blast crisis CML have increased levels of ß-catenin, as compared to the levels in normal progenitor cells (167). Because the GMP cells display enhanced self-renewal activity, and since in normal hematopoietic stem cells the process of self-renewal requires ß-catenin, the authors propose that granulocyte-macrophage progenitor cells are the stem-like cells that drive the progression to blast crisis. Lastly, a recent mathematical model of CML blast crisis also suggests that CML blasts are likely to result from more differentiated leukemic progenitors (168).

1.2.2 c-Abl

The Abl family of non-receptor tyrosine kinases consists of c-Abl (Abelson tyrosine kinase) and a single homolog, Arg (Abl-related gene) (169). Similar to other proto-oncogenes, c-Abl was discovered as the normal cellular form of the v-abl oncogene from the Abelson murine leukemia virus (170). c-Abl has two alternative splicing sites that generate two proteins with different amino-termini: a myristoylated (1b) splice variant, and a 1a splice variant that is 19 amino acids shorter and lacks the myristoylation site (171). c-Abl is localized at multiple intracellular sites, including the nucleus, cytoplasm, mitochondria, or endoplasmic reticulum, where it interacts with a multitude of cellular proteins, including adaptors, protein kinases and phosphatases, cell-cycle regulators, transcription factors, and cytoskeletal proteins. c-Abl functions in a range of cellular processes such as cell proliferation and survival, oxidative stress, DNA damage responses, and actin dynamics [reviewed in (172)].

c-Abl consists of approximately 1150 residues and is comprised of an N-terminal “cap”, followed by an SH3 domain, an SH2 domain, a tyrosine kinase domain, and a long C-terminal

region (Figure 4) (173, 174). The structural arrangement of SH3, SH2 and kinase domains of c-Abl closely resemble the corresponding domains of SFKs (175). The C-terminal region encompasses an actin binding domain, DNA and SH3 binding elements, one nuclear export and three nuclear localization signals (172, 174, 176).

1.2.2.1 Structure and Regulation

Crystallographic analysis of the c-Abl 1b core (N-terminus, SH3, SH2 and kinase domain) revealed the structural basis of its regulation (Figure 4) (177). Similar to SFK structure, the c-Abl core assumes an autoinhibited conformation stabilized by a complex set of intramolecular interactions among its SH3 and SH2 domains and the kinase domain (178, 179). These modular domains were shown to dock onto the back of the kinase domain, acting as a clamp and restricting its conformational flexibility (177, 178). The SH3 domain interacts with the N-terminal lobe of the kinase domain through an atypical PXXP motif within the SH2:kinase linker that encompasses the residues $P_{242}TVY_{245}$ (177). Contrary to SFKs, this interaction is maintained in the absence of the SH2 domain (180). The tethering of the SH2 domain is mediated through the Tyr-245 side chain that points away from the SH3 domain and interacts with the N-lobe of the kinase (177). Mutation of Pro-242 or phosphorylation of Tyr-245 can induce an increase in Abl kinase activity, suggesting that this "sandwich" interaction is essential for the maintenance of the downregulated conformation (181-183).

In SFKs, the SH2 domain and the C-lobe of the kinase are kept spatially close to each other through an interaction between the SH2 and the phospho-Tyr C-terminal tail (184). Although c-Abl lacks a phosphorylated ligand, the SH2 domain is docked tightly against the C-lobe (177). This interaction requires the binding of myristic acid to a hydrophobic pocket in the C-lobe, which in turn induces a specific conformational change in helix αI in the C-terminus of

the C-lobe. SH2 binding is promoted by the myristate-induced conformational change and is stabilized through a series of hydrogen bonds described in detail by Nagar et al. (177). Interestingly, although c-Abl 1a is not myristoylated, deletion of the N terminus in both c-Abl isoforms induces kinase activation, indicating that 1) the myristoyl moiety is required for the stabilization of the downregulated conformation of c-Abl 1b, and 2) in c-Abl 1a, there must be other compensatory interactions that stabilize this conformation (173, 185). Engagement of the SH2 domain by other proteins induces kinase activation due to the disruption of the downregulated conformation (186).

The short eight-residue connector between the SH3 and SH2 domains forms a rigid structure highly similar to that seen in SFKs, a structure stabilized by a network of hydrogen bonds (177). This connector dynamically couples the SH3-SH2 into a regulatory "clamp" (177).

In addition to the SH3-SH2 clamp, the N-terminal "cap" (N-cap) is believed to compensate for the lack of the phospho-Tyr tail and to further stabilize the kinase in an inactive conformation. For example, when expressed in HEK293, an N-cap deletion mutant of c-Abl displayed increased kinase activity as measured by the total cellular phosphotyrosine levels (173). The mechanism by which the N-cap provides an extra layer of stabilization is illustrated by recent crystallographic and hydrogen exchange mass spectrometry (HXMS) studies (187, 188). The crystal structure of the c-Abl kinase core shows that residues within the cap region interact with both the SH3 and SH2 domains through a network of hydrogen bonds and appear to stabilize their docking onto the kinase domain (188). In addition, Ser-69 within the cap region is phosphorylated and interacts with the SH3-SH2 rigid linker. Mutations at this residue induce kinase activation, presumably due to N-cap destabilization (188). Lastly, studies using HXMS,

Chen et al. showed that the N-cap stabilizes the dynamics of the SH3 domain and has implications for the SH3 binding and downregulation of Abl kinase activity (187).

Similar to SFKs, c-Abl kinase activity is also regulated by tyrosine phosphorylation and this process has been extensively studied (Figure 4). In the absence of activating stimuli, endogenous c-Abl was found to be unphosphorylated at Tyr residues (181, 189). Crystallographic studies showed that when unphosphorylated at Tyr-412, the activation loop folds into the active site, preventing substrate and ATP binding (190). Tyr-412 autophosphorylation in trans or by SFKs was shown to induce kinase activation (191, 192). In addition to Tyr-412, Tyr-245 in the SH2:kinase domain linker was shown to be required for full activation of the kinase (183). However, the exact mechanism by which Tyr-245 participates in kinase activation is not well understood, HXMS studies showed that phosphorylation at this residue does not induce disruption of SH3-SH2:linker interaction (183).

Phosphorylation at several other Tyr residues has been implicated in controlling c-Abl activity. These residues include Tyr-134 in the SH3 domain, which is directly involved in binding the PXXP motif in the SH2:kinase linker, and Tyr-283 in the amino-terminal lobe of the kinase domain, which comes into close contact with Ser-94 of the SH3 domain (193). Phosphorylation at any of these residues was predicted to disrupt the SH3-linker-N-lobe of the kinase domain interaction and to activate c-Abl (178). In addition, c-Abl is phosphorylated by SFKs at various residues such as Tyr-89 in the SH3 domain (194). Phosphorylation at this residue results in the disruption of the negative regulatory interaction with the linker and SH2 domain (195).

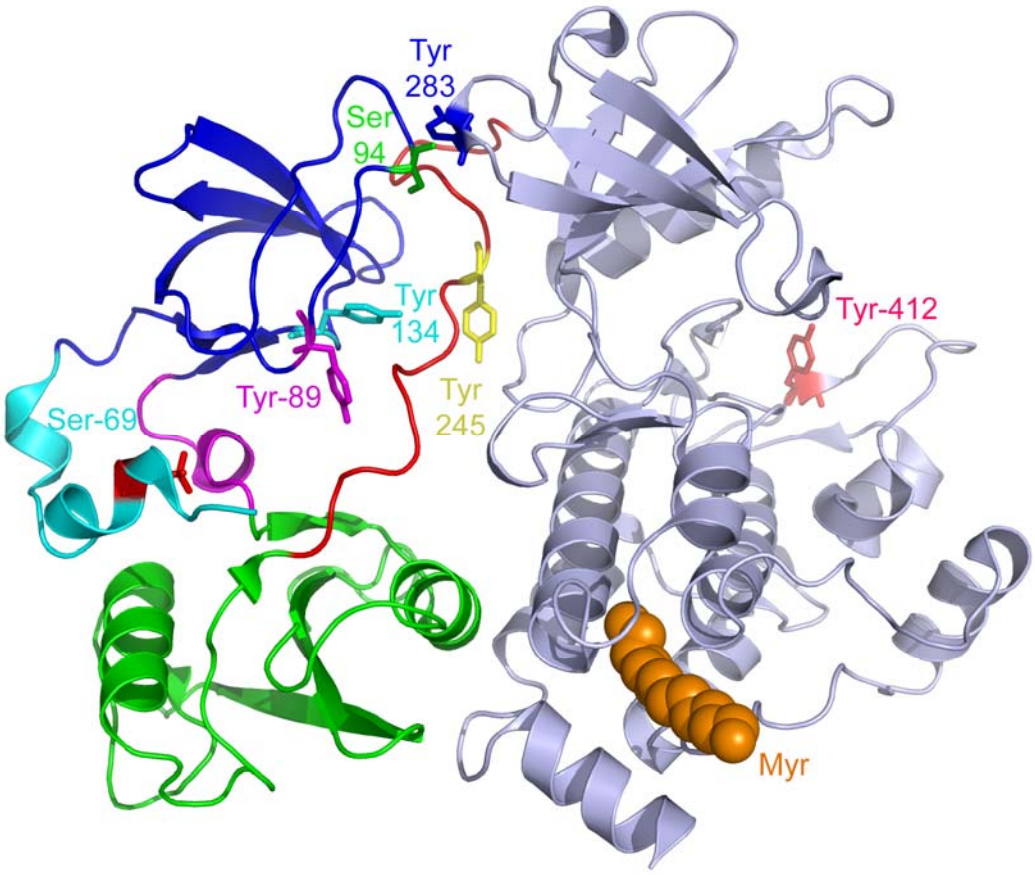


Figure 4: Crystal structure of the c-Abl kinase core

The crystal structure begins at Ala-65. c-Abl regions are color-coded: cyan for the Ncap; blue for the SH3 domain; yellow for the SH3 domain; purple for the SH3:SH2 linker; green for the SH2 domain; light bleu for the kinase domain; orange for myristoyl moiety. Some of the tyrosine residues shown to be important for the regulation of kinase function are displayed: red for Tyr-412 and Ser-69; yellow for Tyr-245; purple for Tyr-89; cyan for Tyr-134; green for Ser-94; bleu for Tyr-283 (PDB: 2FO0).

1.2.3 Bcr-Abl

As described in section 1.2.1.1., Bcr-Abl is a chimeric protein with constitutive tyrosine kinase activity, which arises upon a chromosomal translocation between chromosome 9 and chromosome 22 (Figure 3, A). There are three forms of Bcr-Abl: the 190 kDa Bcr-Abl that induces ALL, the 210 kDa that induces CML, and the 230 kDa Bcr-Abl that induces CNL (Figure 3, B) (196, 197).

Structurally, Bcr-abl contains multiple domains, as shown in Figure 3, C in section 1.2.1.1. The Bcr region is comprised of a coiled-coil oligomerization domain, a serine/threonine kinase domain, a pleckstrin homology domain, a Dbl/cdc24 guanine exchange factor homology domain, serine/threonine and tyrosine phosphorylation sites, and binding sites for the Abl SH2 domain and Grb2 (198, 199). Because of N-terminal Bcr fusion, Bcr-Abl lacks the regulatory N-terminal "cap" of c-Abl, but includes the SH3 and SH2 domains, the tyrosine kinase domain, and the large C-terminal region.

The fusion of Bcr sequences upstream of c-Abl constitutively activates the Abl tyrosine kinase and is essential for Bcr-Abl oncogenicity (200, 201). The N-terminal oligomerization domain of Bcr is required for Bcr-Abl kinase activation (202). Zhao et al. showed that structurally, the first 72 amino acids of Bcr form N-shaped monomers that dimerize through the formation of an antiparallel coil-coil, and that two dimers associate to form tetramers (203).

Although Bcr-Abl exhibits constitutive tyrosine kinase activity, the kinase domain may retain some of the intra-molecular constrain and inhibitory interactions of c-Abl (178). An important clue in Bcr-Abl regulation that supports this idea comes from a non-biased mutagenesis screen performed by Azam et al. to identify mutants of Bcr-Abl that induce imatinib resistance (204). In this screen, in addition to identifying residues that directly contact imatinib,

the authors also identified residues that map at the interface between the kinase domain and the SH3-SH2 clamp that had been previously shown to regulate c-Abl kinase activity (173, 181, 186, 193, 205). The fact that mutations at residues that disrupt c-Abl inhibition also induce imatinib resistance in Bcr-Abl strongly suggests that mechanisms that govern c-Abl autoinhibition are preserved in Bcr-Abl (178).

1.2.4 Bcr-Abl Oncogenic Signaling

Bcr-Abl promotes leukemic transformation through several mechanisms that include: induction of constitutive mitogenic signaling; induction of growth-factor independence; impairment of cell adhesion properties; promotion of resistance to apoptosis; and disruption of the DNA-repair response mechanisms. Bcr-Abl oncogenic potential relies on its constitutive tyrosine-kinase activity and its ability to activate many different signal transduction pathways, which are discussed in the following sections.

1.2.4.1 SFK-dependent signaling

The pathological involvement of SFKs in Bcr-Abl signaling has been studied extensively and various groups report seemingly controversial findings (206). This controversy arises most likely from the different specific phases of the disease in which their role has been probed or due to assay differences (disease onset vs. chronic phase vs. blast crisis). In this section, I will give an overview of the arguments that either support or contest a role of SFKs in CML pathogenesis.

Based on the fact that Bcr-Abl induces cytokine independence of myeloid cells and that SFKs are activated by these cytokines, Danhauser-Riedl et al. investigated for the first time whether Bcr-abl induces activation of SFKs (207). The authors showed that Bcr-Abl associates

with and activates Hck and Lyn kinases in murine 32D cells transformed with Bcr-Abl and in CML cell lines such as K562 (207). The interaction between Bcr-Abl and Hck is mediated by the SH3, SH2, kinase domain, and the C-terminus of Bcr-Abl, and the SH3 and SH2 domains of Hck (208, 209). Interestingly, although the interaction between the isolated SH2 domain of Hck and Bcr-Abl requires autophosphorylation of Bcr-Abl (209), the interaction of full-length Hck and Bcr-Abl did not require Abl kinase activity (210). These studies suggest that Bcr-Abl may activate SFKs through the displacement of one or more intra-molecular inhibitory interactions in Hck, and that multiple interaction modes may exist, depending on the activation status of Bcr-Abl.

Some SFKs activated by Bcr-Abl act as intermediates between Bcr-Abl and downstream pathways involved in leukemic transformation. For example, co-immunoprecipitation experiments in Bcr-Abl-transformed murine 32D myeloid cells, show that Bcr-Abl, Hck and Stat5 form a stable complex, in which Stat5 is phosphorylated at Tyr-699 and thereby activated. Expression of a kinase-dead Hck mutant suppressed Stat5 activation, suggesting that Stat5 is a substrate of Hck and not of Bcr-Abl (211). Importantly, work in our laboratory had shown that expression of this kinase-dead mutant of Hck blocks Bcr-Abl-induced transformation of myeloid cells (209). Together, these data suggest that Hck couples Bcr-Abl to Stat5 signaling and this Bcr-Abl→Hck→Stat5 pathway is required for Bcr-Abl-induced transformation.

Lyn activation by Bcr-Abl is another example of an SFK acting as an intermediate to activate downstream signaling pathways (212). Bcr-Abl-induced activation of Lyn triggers G-protein coupled receptor CXCR4-dependent signaling and results in loss of responsiveness to stromal-derived factor 1 (SDF-1) and activation of PI3K. This pathological pathway blocks the response to the SDF-1 chemokine and disrupts chemotaxis, thus increasing the ability of

hematopoietic progenitor cells to escape the bone marrow and potentially contributing to oncogenicity of Bcr-Abl (212). Interestingly, the CXCR4 expression level was shown to be highly downregulated in $CD34^+$ cells from patients in blast crisis phase compared to either control individuals or chronic phase patients (213). Also, in a recent study, Diaz-Blanco et al. compared the molecular signature of $CD34^+$ cells from chronic phase CML patients with normal $CD34^+$ using microarrays that covered more than 8700 genes (214). In this study, the authors found Lyn and Yes to have a significantly higher mRNA expression than in normal $CD34^+$ cells, providing a rationale for using dual Abl/Src inhibitors for the eradication of primitive progenitor cells in CML.

Work in our laboratory performed in the CML cell lines K562 and Meg01 showed that A-419259, a SFK selective inhibitor, blocks CML cell proliferation and induces apoptosis without affecting the growth and survival of Ph-negative myeloid cells (215). The effects of this compound correlate with downregulation of both Stat5 and ERK activation, suggesting once again that SFKs couple Bcr-Abl to downstream signaling pathways. Importantly, as a continuation of these studies, work presented in this thesis shows that A-419259 induces growth arrest and apoptosis in $CD34^+$ cells isolated from CML patients in chronic phase at a level comparable to imatinib (Chapter 2) (216). This strongly argues for a role of SFKs in proliferation and survival of CML progenitor cells, suggesting that SFKs may be an important therapeutic target for the eradication of CML stem cells. Indeed, dasatinib, a dual Bcr-Abl/SFK inhibitor was shown to be more effective against an earlier progenitor population than imatinib in primary CML samples (217).

Multiple studies have implicated SFKs in phosphorylation of various sites in c-Abl or Bcr-Abl, which may affect their regulation, signaling or oncogenicity. For example, Hck and

Lyn can phosphorylate Bcr-Abl on Tyr-177 in the Bcr part of the molecule (194, 210, 218). Since Tyr-177 is a known binding site for Grb2, these SFKs link Bcr-Abl to the Ras and MEK/ERK oncogenic signaling cascades (194, 210, 218). Furthermore, Tyr-245 and Tyr-412 were shown to represent sites of SFK-mediated phosphorylation in c-Abl (191, 192). Phosphorylation at Tyr-412 is necessary for the catalytic activity of c-Abl (191), while phosphorylation at Tyr-245 strongly up-regulates c-Abl activity, possibly due to the disruption of the SH3-linker interaction (183). In addition, recent work in our laboratory demonstrated that Hck, Lyn, and Fyn phosphorylate multiple Tyr residues in the SH3-SH2 region of Bcr-Abl, possibly by inducing dissociation of the autoregulatory interactions that may be retained in Bcr-Abl (194, 195). Importantly, phosphorylation at these sites is required for full Bcr-Abl oncogenicity (194). Finally, Hck was shown to induce desensitization of c-Abl to inhibition by imatinib due to induction of an active conformation upon phosphorylation at Tyr-412 (182, 190).

Several lines of evidence suggest a role of SFKs in CML progression to blast crisis. For example, Donato et al. showed that overexpression and/or activation of Hck and Lyn occur during CML progression (219). Furthermore, using a mouse model of CML, Hu et al. demonstrate that the transition of CML to lymphoid blast crisis requires the presence of Lyn, Hck, and Fgr (220). In addition, downregulation of Lyn using RNAi induced apoptosis in cells from both myeloid and lymphoid blast crisis patients (221). Lastly, Bcr-Abl was shown to induce elevated IGF-1 expression in blast crisis cells through the activation of Hck and Stat5b, suggesting a role of these proteins in the transition to blast crisis (222).

SFKs may play an additional role in CML through a direct effect on ß-catenin, which in turn was implicated in CML stem and progenitor cell self-renewal, and in progression to blast crisis (167). Briefly, Src was shown to phosphorylate the 72 amino-acid cytoplasmic domain of

Muc1 (Muc1-CD), a transmembrane glycoprotein, shown to be overexpressed in various cancers, including CML (223, 224). Src-induced phosphorylation promotes binding of Muc1-CD to ß-catenin and targeting of ß-catenin to the nucleus (224).

Some of the evidence that disputes a role of SFKs in CML pathogenesis comes from genetic studies. For example, Hu et al. addressed the requirement of SFKs in Brc-Abl-dependent induction of CML using SFK-knockout mice (225). The authors used a Bcr-Abl retrovirus to transduce bone marrow from mice lacking the myeloid-expressed SFKs (Hck, Lyn, and Fgr), and determined that these marrow cells were still able to induce a CML-like syndrome in recipient animals. Conversely, these cells did not induce a B-cell acute lymphoblastic leukemia (B-ALL)-like syndrome, which is also dependent on Bcr-Abl. In addition, a SFK selective inhibitor was effective in blocking the growth of ALL cells *in vitro* and in B-ALL mice, but had no effect in blocking CML progression (225). Together, these data suggest that Hck, Lyn, and Fgr are required for the onset of B-ALL but not of CML. However, it should be pointed out that knockout of one or more SFKs may induce functional compensation due to overexpression of other SFKs. Therefore, these studies do not persuasively rule out a requirement of Hck, Lyn, or Fgr or of other SFKs in CML induction.

In summary, the data discussed above represent an overwhelming body of evidence for the various roles of SFKs in Bcr-Abl signaling and CML pathogenesis. These include a role in linking Bcr-Abl to mitogenic and survival pathways; modulation of Bcr-Abl oncogenicity; disruption of chemotaxis that in turn may favor the escape of CML progenitor cells from the bone marrow; or a role in disease progression. In addition, an increasing number of studies point to a role for SFKs in induction of Bcr-Abl – independent resistance to imatinib. This topic will be discussed in detail in section 1.2.5.3.

1.2.4.2 SFK-independent signaling

Ras/Raf/MEK/ERK signaling

The MAPK pathway (Ras/Raf/MEK/ERK) is activated by many growth factors and cytokines and has important mitogenic and antiapoptotic roles in hematopoietic cells via downstream transcription factors including NF-kB, CREB, Ets-1, AP-1 and c-Myc (226-228).

Some of the first reports linking Bcr-Abl to Ras showed that phosphorylation of Tyr-177 in the Bcr-derived part of Bcr-Abl generates a binding site for Grb2 adapter protein. This in turn recruits the Sos guanine nucleotide exchange factor and facilitates Ras activation (229-232). The importance of Tyr-177 for Bcr-Abl oncogenicity has been widely debated. For example, mutation of Tyr-177 to Phe reduces Ras activation and fibroblast transformation (229) and prevents the induction of myeloproliferative disorder in a CML mouse model (233, 234). However, this mutant retains the ability to render hematopoietic cell lines growth-factor independent (230), suggesting the existence of alternate Bcr-Abl-dependent pathways that allow continuous activation of Ras (198, 235). Indeed, it was shown that Bcr-Abl activates Ras through another adapter molecule Shc (236, 237). Goga et al. demonstrated that recruitment of Shc by Bcr-Abl requires the SH2 domain of Bcr-Abl, while Cortez et al. showed that this interaction also required Bcr-Abl kinase activity (230, 238). Lastly, Bcr-Abl can activate Ras by binding and phosphorylating CrkL-C3G adapter complex (239, 240). Interestingly, to illustrate the importance of this complex in Bcr-Abl-induced activation of the Ras pathway, Oda et al. used a cell-permeable CrkL-SH3-domain blocking peptide and showed that this peptide inhibits proliferation of blast cells from CML patients (240).

STAT signaling

The signal transducer and activator of transcription proteins (Stats) regulate many aspects of cell growth, survival and differentiation [reviewed in (241)]. Stats are monomeric cytoplasmic transcription factors that require phosphorylation at a specific, conserved Tyr residue for activation (241). Phosphorylation at this Tyr residue induces oligomerization, nuclear transport, and DNA binding. Stats were shown to be activated downstream of receptor tyrosine kinases (such as EGFR or PDGFR), or receptors without tyrosine kinase activity that recruit various cytoplasmic tyrosine kinases (such as Jaks or SFKs) (242, 243).

The major Stats activated by Bcr-Abl are Stat1, Stat3 and Stat5 (244-246). The role of Stat1 in Bcr-abl-induced transformation is less clear since disruption of the stat1 gene in mice leads to compromised immune function and unresponsiveness to interferon (247). In addition, Bcr-Abl-induced transformation of growth-factor-dependent 32Dcl3 murine myeloid cells induced a very robust activation of Stat5, while Stat1 was only weakly activated and Stat3 activation was not detected (248).

Stat3 was shown to be constitutively active in Bcr-Abl–expressing embryonic stem (ES) cells and to promote self-renewal even in the absence of LIF (244). This constitutive activation of Stat3 in ES cells is also dependent on MEK kinase 1 (249). More importantly, Stat3 was found to be active in primary $CD34^+$ cells from CML patients, suggesting that it might be involved in the maintenance of an undifferentiated phenotype in CML stem cells (244).

The importance of Stat5 activation in Bcr-Abl leukemogenesis is supported by multiple observations. For example, ectopic expression of a dominant-negative Stat5 mutant decreases Bcr-Abl-dependent cell proliferation of Ba/F3 cells, and blocks Bcr-Abl-dependent transformation of primary mouse bone marrow cells (248, 250). In addition, Stat5 activation is

consistently observed in CML (246) and it was proposed to play a role in disease progression to blast crisis (251). Activation of Stat5 by Bcr-Abl was shown to require both SH3 and SH2 domains of Bcr-Abl and to be Jak2-independent (248, 252). The mechanism of Bcr-Abl induced activation of Stat5 is mediated by SFKs and was discussed in section 1.2.4.1.

Jak2 signaling

Jak2 is a member of the Janus kinase (Jak) family of non-receptor tyrosine kinases, which are important regulators of cytokine growth factor receptors (253). Although Jak2 was not linked to the activation of Stats in response to Bcr-Abl, increasing evidence points to a role of Jak2 in Bcr-Abl leukemogenesis. In collaboration with our laboratory, Xie et al. showed that Bcr-Abl binds and phosphorylates Jak2 at Tyr-1007, a residue required for its activation (254). The same group showed that Bcr-Abl-induced activation of Jak2 induces phosphorylation of Gab2 (found in the same multimolecular complex), hence linking Jak2 to other signaling pathways such as phosphatidylinositol-3 kinases (PI-3K) and Ras (255). In addition, Jak2 activation in Bcr-Abl positive cells was linked to c-Myc protein induction, a transcription factor required for Bcr-Abl transformation (256). Lastly, treatment of CML cell lines and 32D/Bcr-Abl cells with Jak2 inhibitors induced apoptosis, suggesting that Jak2 may be a critical target in CML (255).

PI-3K signaling

The PI-3Ks are a family of proteins that catalyze the transfer of γ phosphate from ATP to phosphoinositides that act as anchors for pleckstrin homology (PH) domain-containing proteins such as Akt or phosphoinositide-dependent protein kinase-1 (PDK1). The class Ia of PI-3Ks are homodimers composed of a p85 regulatory and a p110 catalytic subunit.

Bcr-Abl activates PI-3K through the recruitment of the scaffolding Grb2/Gab2 protein complex to phospho-Tyr-177 in the Bcr part of the molecule. This in turn interacts with the p85

regulatory subunit and induces PI-3K activation (257, 258). Replacement of Tyr-177 with Phe results in a Bcr-Abl mutant that exhibits decreased tyrosine phosphorylation of Gab2 and decreased PI-3K activation (259). In addition, bone-marrow myeloid progenitors from Gab2 (-/-) mice are resistant to transformation by Bcr-Abl, suggesting that Gab2 is a critical mediator of Bcr-Abl-induced activation of PI-3K (260).

Additional scaffolding proteins implicated in the activation of PI-3K are c-Cbl, CrkL and c-Crk. Sattler et al. showed that Bcr-Abl induces the formation of a multimeric complex of signaling molecules that leads to the recruitment of p85. CrkL or c-Crk bind to Bcr-Abl through their SH3 domains and to c-Cbl through their SH2 domains, while p85 binds directly to c-Cbl through its SH3 and SH2 domains (261, 262).

PI-3K kinase triggers Akt activation, an upstream regulator of various transcription factors and pro-apoptotic molecules that have a critical role in Bcr-Abl transformation (263, 264). For example, Akt phosphorylates BAD and inhibits its pro-apoptotic activity by inducing its cytoplasmic sequestration due to interaction with 14-3-3ß (265, 266). In addition, Akt inhibits p53 tumor suppressor function by phosphorylating MDM2, inducing its cytoplasmic export from the nucleus and promoting p53 ubiquitination and degradation (267, 268). Furthermore, Akt phosphorylates inhibitor of NF-kB kinase (I_kB-kinase-α), which in turn induces phosphorylation and proteasomal degradation of I_kB (269, 270). Degradation of I_kB releases NF-kB, allowing its translocation into the nucleus where it functions as a transcription factor for an entire spectrum of target genes that facilitate tumor progression, inflammation, cell survival, angiogenesis, proliferation, and metastasis [reviewed in (271)]. Lastly, the PI-3K/Akt pathway was shown to regulate production of high levels of reactive oxygen species, which is associated with Bcr-Abl transformation (272, 273).

WNT/ß-catenin signaling

The WNT/ß-catenin pathway promotes hematopoietic stem cell renewal since expression of a degradation-resistant ß-catenin mutant induces sustained proliferation in culture and in vivo bone-marrow reconstitution (274). In the absence of WNT signals, ß-catenin is found in a cytosolic complex with APC, Axin, and the Ser/Thr-kinase GSK3. GSK3 constitutively phosphorylates ß-catenin and initiates its degradation by the proteasome (275, 276). WNT factors are lipid-modified proteins that bind to Frizzled (FZD) receptors and induce inhibition of GSK3. This in turn induces accumulation and nuclear translocation of ß-catenin, where it functions as a transctiptional coactivator for genes like c-myc, c-jun and cyclin D1 (277). Several hematological malignancies display increased ß-catenin expression and stabilization in committed myeloid and lymphoid progenitors (278).

Several reports link abnormal ß-catenin function with CML stem cells. For example, Jamieson et al. showed for the first time that granulocyte-macrophage progenitors from blast-crisis and imatinib-resistant CML patients have elevated levels of nuclear ß-catenin (167). More recently, Bcr-Abl was shown to physically interact with ß-catenin and phosphorylate it on Tyr-86 and Tyr-654. Phosphorylation at these Tyr residues induces disruption of ß-catenin association with Axin/GSK3, increasing stability, and ß-catenin transcriptional activity (279). In addition, ß-catenin was shown to be essential for the survival and self-renewal of leukemic stem cells in CML cells and mouse models (167, 280-282).

1.2.5 CML therapy

The effectiveness of CML therapies is measured by the degree of hematological, cytogenetic or molecular responses. CHR, or complete hematological response, denotes the return to a normal

white blood count. CCyR, or complete cytogenetic remission denotes the absence of Ph chromosome within 20 metaphases on karyotype analysis. Lastly, CMR, or complete molecular response denotes the elimination of Bcr-Abl mRNA as measured by real-time quantitative RT-PCR (283).

1.2.5.1 Treatment before the emergence of Bcr-Abl targeted therapy

The only curative treatment for CML remains allogeneic transplantation of normal bone marrow cells following a conditioning phase for the destruction of normal and leukemic cells by chemotherapy or irradiation (284). However, only 20% of patients are candidates for allogeneic bone marrow transplantation due to the lack of available compatible donors or age limitations. Lastly, the 5-year disease-free survival rate varies largely between 30 % to 80 % (284, 285).

Introduced about 25 years ago, interferon-α (INF-α) was for a long time the standard CML therapy in patients with no matched bone marrow donor. INF-α leads to both hematological and cytogenetic responses in chronic phase patients (286). It has been suggested that INF-α induces its antileukemic effects by blocking the Jak1-Stat1 pathway and activation of INF-α responsive genes (287). Despite the prolonged survival, the majority of patients develop resistance and ultimately die of the disease.

The second-line treatment in patients either resistant or intolerant to INF-α was Busulfan or hydroxyurea, which nonspecifically blocks the proliferation of both normal and Ph^+ leukemic cells. These treatments obtained hematological responses only in the chronic phase of CML [reviewed in (135)].

1.2.5.2 Targeted Bcr-Abl kinase inhibitor: Imatinib

Validation of Bcr-Abl as an ideal therapeutic target in CML comes from the massive number of studies showing that Bcr-Abl is required for the onset and maintenance of the disease. Imatinib (Signal Transduction Inhibitor – STI571) is a derivative of 2-phenylaminopyrimidine, and was originally discovered in a screen for inhibitors of the platelet-derived growth factor receptor tyrosine kinase (PDGF-R). Further testing showed that imatinib specifically inhibits the proliferation of Bcr-Abl positive cells in vitro and the growth of Bcr-Abl positive tumors in vivo (288). In contrast, imatinib does not inhibit immortalized or transformed cell lines that do not express Bcr-Abl. Similar selective depletion of Bcr-Abl-positive cells has been observed in long-term bone marrow cultures (289). More detailed testing revealed that imatinib inhibits c-Abl, v-Abl, Bcr-Abl, PDGF-R α and ß, c-Kit, Arg, and c-Fms (290-294). This promiscuity was shown to be key to the therapeutic effects of Bcr-Abl. Wong et al. engineered an inhibitor analog-sensitive mutant of Bcr-Abl that allowed selective inhibition of this kinase without the concomitant inhibition of the other target kinases, and showed that simultaneous inhibition of Bcr-Abl and c-Kit was required for the potent cytotoxic effects of imatinib on CML cells (295).

In an initial, phase I clinical study, imatinib proved remarkably successful with 53 out of 54 patients displaying CHR after only 4 weeks of treatment (296). 96% percent of these patients maintained CHR for over a year (297). In addition, 55% of the patients in accelerated phase or blast crisis achieved hematological responses (21/38) (297). In a phase II clinical study, 55% of the patients showed CCyR and 91% of the patients in chronic phase obtained CHR. From these, 89% showed no disease progression (297). However, the rate of CHR in patients in accelerated phase of blast crisis was 69% and 29%, respectively (282, 297). In a phase III clinical study

conducted in CML chronic phase patients, only 3.3% showed disease progression after 18 months (298).

To elucidate the mechanism of imatinib binding and specificity towards Abl, numerous studies described the crystal structure of c-Abl core or c-Abl kinase domain in complex with imatinib (179, 190). Schindler et al. showed that imatinib binds to an inactive conformation of the kinase domain in which the activation loop is not phosphorylated and points inward (190). A close evaluation of these crystal structures shows that the drug is sandwiched between the N- and C-terminal lobes of the kinase domain. More specifically, the compound tightly fits between the activation loop and helix αC, locking the kinase in an inactive conformation. The binding of compound is stabilized through 6 hydrogen bonds with Met-318, Thr-315, Glu-286, His-361, Ile-360, and Asp-381, and through numerous van der Waals interactions (179).

A comparison of active and inactive conformations of different classes of kinases suggested that whereas the conformations of protein kinases in active state are very similar amongst different kinase families, there are important differences in their downregulated conformations (179). Therefore, it has been proposed that the high selectivity towards Abl displayed by imatinib stems from its ability to exploit a fairly unique conformation of the inactive kinase.

1.2.5.3 Mechanisms of resistance to Imatinib

Despite the high rates of hematological and cytogenetic responses to imatinib, emergence of resistance upon exposure to the drug is a major problem for the treatment of CML (299). There are two main types of resistance to imatinib: Bcr-Abl-dependent and – independent mechanisms.

Bcr-Abl-dependent mechanisms of resistance

One Bcr-Abl-dependent mechanism of resistance is due to gene amplification. For example, using dual-color fluorescence in situ hybridization (FISH), Gorre et al. reported the presence of multiple copies of Bcr-Abl gene in metaphase spreads from 11 patients. This amplification occurred within a unique inverted duplicate Ph chromosome. Interestingly, one patient displayed this unique Ph chromosome before imatinib exposure (300).

A second and very well characterized Bcr-Abl-dependent mechanism of resistance is due to the presence of Bcr-Abl point mutations that renders it refractory to imatinib. Gorre et al. reported for the first time a substitution of Thr-315 with Ile in 6 patients that relapsed on imatinib treatment (300). This substitution not only precludes formation of a critical hydrogen bond with imatinib, but also, since Ile has an extra hydrocarbon group in the side chain, induces a steric clash with imatinib (300). Consequently, the binding of imatinib is prevented.

Since this first report, more than 50 additional mutations inducing imatinib resistance have been described (301-303). These mutations tend to cluster in certain areas of the kinase domain such as the imatinib-binding interface (Thr-315), within the phosphate-binding loop (for example Glu-255, Tyr-253), activation loop (for example His-396), or at the SH2-kinase interface (Met-351) [reviewed in (304)]. However, many of these mutants are relatively rare in clinical specimens. The most common mutations, accounting for 60-70 % of all mutations, occur at residues Gly-250, Tyr-253, Glu-255, Thr-315, Met-351, and Phe 359 (305). Interestingly, some of these mutations were shown to exist prior to the onset of treatment with imatinib (303, 306). Furthermore, two of the most clinically frequent imatinib-resistant mutants (Y253F, E255K) have a greater transforming potential than the wild type Bcr-Abl in cell-based assays and are associated with a poor prognosis (307-309). Lastly, mutation at the c-Abl residue

corresponding to T315 in Bcr-Abl was recently reported to induce kinase activation and transformation activity (310). This mutation was also associated with a much poorer prognosis (311).

In an elegant study, Azam et al. used random mutagenesis on Bcr-Abl and in vitro selection for imatinib resistance to identify additional imatinib-resistant Bcr-Abl mutants (204). It is interesting that many of these mutations occur at sites that do not directly contact the drug. For example, some of these mutations mapped to the SH2:kinase linker, or others at the SH3-SH2 domain linker, many of them in positions required to maintain inactive states of the enzyme (204, 302). Although many of these mutants were not clinically relevant and conferred only low resistance to imatinib, they are important for understanding Abl kinase regulation and mechanisms of resistance.

Bcr-Abl-independent mechanisms

Bcr-Abl-independent resistance has been attributed to many different mechanisms including enhanced drug efflux from target cells through P-glycoprotein-mediated active transport, or reduced intracellular drug delivery due to the presence of α1 acid glycoprotein in the membrane (312, 313). Other mechanisms such as activation of secondary tyrosine kinases or leukemic stem cells refractoriness and residual disease will be discussed below.

In a series of articles published in the last 5 years, Donato et al. suggested a mechanism of resistance to imatinib involving SFKs. First, the authors showed that K562 CML cells cultured in increasing concentrations of imatinib displayed upregulated Lyn activity (219). Inhibition of Lyn kinase reduced proliferation and survival of these cells, while it did not have any effect in imatinib-sensitive K562 cells. In addition, analysis of clinical samples from patients in the advanced stages of CML that relapsed upon imatinib treatment showed an association between

imatinib-resistance and upregulation of either Hck or Lyn in the absence of Bcr-Abl mutations (219, 314). Reducing Lyn by siRNA in mononuclear cells isolated from a patient with increased Lyn levels induced a reduction in cell survival (314).

Although the vast majority of patients in chronic phase achieve CCyR when treated with imatinib, relapses are commonly observed after imatinib cessation (315, 316) due to the persistence of a limited, "residual" population of CML stem and progenitor cells that are intrinsically resistant to imatinib. Determination of the mutation status of Bcr-Abl in these cases showed that usually the relapse is not accompanied by a mutated Bcr-Abl phenotype. This refractoriness to imatinib was causatively associated with the stemness character of the leukemia initiating cells and their relative quiescence. Refractory CML cells were shown to upregulate genes for proteins responsible for drug efflux, elimination and detoxification (317). Numerous studies have addressed the cause of imatinib resistance in this population. In a recent report, Diaz-Blanco assessed differential gene expression in total $CD34^+$ from CML patients in chronic phase compared to normal $CD34^+$ cells, using microarrays covering over 8700 genes (214). Apart from some known transcriptional changes previously shown to take place upon Bcr-Abl expression in this cellular compartment (activation of MAPK and PI-3K pathways), this analysis revealed some novel transcriptional changes. For example, the data showed higher expression levels of proliferation-associated genes such as CDKs and various cyclins, as well as downregulation of proapoptotic factors such as interferon regulatory factor 8 (214). Interestingly, the SFKs Lyn and Yes were found in significantly higher levels (1.56 and 1.38 times, respectively), suggesting a potential role for SFKs in $CD34^+$ CML cells (214).

1.2.5.4 Strategies to circumvent resistance to current therapies

The discovery of imatinib resistance has prompted a quest for the development of alternative therapies to override this resistance. Within these new therapies, Bcr-Abl kinase activity remains a valid target since many cases of resistance occur due to the accumulation of a leukemic clone with Bcr-Abl point mutations. However, in the case of residual disease or other types of resistance, it is important to direct these new therapies towards novel targets in alternative pathways.

Improved Bcr-Abl inhibitors recently developed include: 1) selective Abl inhibitors such as nilotinib (Novartis) that is about 30 times more potent than imatinib and inhibits 32 of 33 Bcr-Abl imatinib-resistant mutants, with the exception of T315I mutants (318, 319); 2) non-ATP competitive inhibitors such as GNF-2 which binds to the myristoyl binding site and inhibits the kinase activity allosterically (320); and 3) Aurora kinase inhibitors such as MK-0457 (Merck) which inhibits Aurora kinase, Flt3, Abl, and Jak2. Importantly, MK-0457 also inhibits T315I mutant of Bcr-Abl (321). Although some of these inhibitors have improved activity towards some Bcr-Abl imatinib-resistant mutants, in many cases this improved activity is accompanied with a wider spectrum of activity that historically has been associated with increased side effects or intolerance.

Given the importance of SFKs in Bcr-Abl signaling and resistance to imatinib, dual Abl and SFK inhibitors such as dasatinib (Bristol-Myers Squibb) are very promising compounds. The dual character of dasatinib lies in its flexibility in binding to different conformations of Bcr-Abl, some of which are similar to the SFK active conformations (322). It has been shown that dasatinib inhibits 21 out of 22 imatinib-resistant Bcr-Abl mutants, with the sole exception once again being the T315I mutant (323, 324).

Another unique mechanism of inducing Bcr-Abl inhibition was recently described by Bartholomeusz et al. and consists of induction of a Bcr-Abl-destruction pathway by WP1130. WP1130 is a second-generation tyrphostin derivative (degrasyn) discovered during screens for AG490-like molecules that suppress IL-6 and IL-3 activation of Stats (325). Although WP1130 was shown to induce rapid downregulation of Jak kinases, the mechanism of Bcr-Abl destruction is currently unknown.

Finally, due to the general resistance and refractoriness of CML stem cells, targeting the "residual disease" has proved very challenging. However, recently two approaches show significant promise. First, BMS-214662, a farnesyl transferase inhibitor developed by Bristol-Myers Squibb shows apoptotic activity not only against imatinib-resistant or blast crisis CML cells, but also against CML stem and progenitor cells (326). Second, based on proven cell-surface presentation of Bcr-Abl epitopes encompassing the fusion region, immunotherapy of residual disease is also promising (327). Multiple immunotherapy clinical trials are currently ongoing.

1.3 HYPOTHESES AND SPECIFIC AIMS

1.3.1 Hypotheses

The Src family of non-receptor tyrosine kinases (SFK) is comprised of eight members in humans: c-Src, Hck, Fgr, Blk, Lck, Lyn, c-Yes, and Fyn. Although some Src members are ubiquitously expressed, Hck, Lyn, Fgr, Lck, and Blk, are restricted to a few hematopoietic cell types. SFKs critically regulate a variety of cellular processes such as proliferation, motility,

adhesion, angiogenesis, and survival (7). However, overexpression and/or aberrant activation of SFKs have been linked to various cancers (7, 328). In the context of chronic myelogenous leukemia (CML), Bcr-Abl, the oncogenic protein-tyrosine kinase responsible for the onset of CML, binds to both Hck and Lyn, and this interaction leads to an increase in Lyn and Hck tyrosine kinase activity (207). Work in our laboratory has shown that general Src-kinase inhibitors of the pyrrolo- and pyrazolo-pyrimidine (PP) classes block the transforming activity of Bcr-Abl in CML-derived cell lines (215). In addition, Hck has been shown to couple Bcr-Abl to Stat5 activation in leukemia cells, which may increase survival (211). Given the involvement of Hck in the pathogenesis of CML, I propose the **hypothesis that Hck cooperates with Bcr-Abl in CML pathogenesis.** Furthermore, although imatinib, the frontline therapy in CML, proved remarkably effective in patients in the initial phase of CML, patients in advanced phases develop resistance. In 50 % to 70 % of the cases, resistance to imatinib occurs due to point mutations in the abl kinase domain of Bcr-Abl that interfere with imatinib binding (329). Interestingly, in other patients with wild type Bcr-abl, resistance has been associated with either overexpression or overactivation of the SFKs Hck and Lyn (330, 331). Based on this association, I also propose the **hypothesis that Hck overexpression in CML cells induces imatinib resistance**. To address this hypotheses, I used the power of chemical genetics and pursue the following aims (1) to investigate the independent contribution of Hck to Bcr-Abl signaling and cellular transformation in K562 cells using a mutant that is genetically engineered to be resistant to the general SFK inhibitor, A-419259; and (2) to investigate the effect of Hck overexpression on imatinib resistance in the context of a wild-type Bcr-Abl.

1.3.2 Specific Aims

Aim 1. To investigate the independent contribution of Hck to Bcr-Abl signaling and cellular transformation in K562 cells using a mutant that is genetically engineered to be resistant to the general SFK inhibitor, A-419259.

In this aim, I adapted a chemical genetics approach to develop an A-419259-resistant mutant of Hck by replacing the gatekeeper residue (Thr-338) in the inhibitor-binding site with a bulkier methionine residue (Hck-T338M). This substitution reduced Hck sensitivity to A-419259 by more than 30-fold without affecting kinase activity in vitro. Expressing this mutant in CML cellular models and pairing it with the global SFK inhibitor, A-419259, I was able to show that Hck plays a non-redundant role as a key downstream antiapoptotic signaling partner for Bcr-Abl.

Aim 2. To investigate the effect of Hck overexpression on imatinib resistance in the context of a wild-type Bcr-Abl.

In this aim, I showed that Hck overexpression in CML cells is sufficient to induce resistance to imatinib. In addition, to establish the mechanism of Hck-induced resistance to imatinib, I used a second chemical genetic strategy, originally developed by Shokat and co-workers (332). This strategy introduces a silent mutation into the ATP-binding pocket of Hck (T338A) to produce unique sensitivity to NaPP1, a bulky analog of global SFK inhibitors of the pyrazolo-pyrimidine class. Using this inhibitor-sensitive mutant of Hck, I determined that Hck-kinase activity is required to induce imatinib-resistance in K562 cells.

2.0 AN INHIBITOR-RESISTANT MUTANT OF HCK PROTECTS CML CELLS AGAINST THE ANTI-PROLIFERATIVE AND APOPTOTIC EFFECTS OF THE BROAD-SPECTRUM SRC-FAMILY KINASE INHIBITOR A-419259

2.1 ABSTRACT

Chronic myelogenous leukemia (CML) is driven by Bcr-Abl, a constitutively active protein-tyrosine kinase that stimulates proliferation and survival of myeloid progenitors. Global inhibition of myeloid Src-family kinase (SFK) activity with the broad-spectrum pyrrolo-pyrimidine inhibitor A-419259 blocks proliferation and induces apoptosis in CML cells, suggesting that transformation by Bcr-Abl requires SFK activity. However, the contribution of Hck and other individual SFKs to Bcr-Abl signaling is less clear. Here we developed an A-419259-resistant mutant of Hck by replacing the gatekeeper residue (Thr-338) in the inhibitor binding site with a bulkier methionine residue (Hck-T338M). This substitution reduced Hck sensitivity to A-419259 by more than 30-fold without affecting kinase activity in vitro. Expression of Hck-T338M protected K-562 CML cells and Bcr-Abl-transformed TF-1 myeloid cells from the apoptotic and anti-proliferative effects of A-419259. These effects correlated with persistence of Hck-T338M kinase activity in the presence of the compound, and were accompanied by sustained Erk and Stat5 activation. In contrast, control cells expressing equivalent levels of wild-type Hck retained sensitivity to the inhibitor. We also show for the first

time that A-419259 induces cell-cycle arrest and apoptosis in primary CD34+ CML cells with equal potency to imatinib. These data suggest that Hck plays a non-redundant role as a key downstream signaling partner for Bcr-Abl and may represent a potential drug target in CML.

2.2 INTRODUCTION

Chronic myelogenous leukemia is an acquired genetic hematological malignancy that affects 1 in 100,000 people each year and is characterized by clonal expansion of transformed multipotent hematopoietic stem cells (135). The hallmark genetic anomaly of CML is the Philadelphia chromosome (Ph^+) that results from a reciprocal chromosomal translocation between the c-*abl* locus on chromosome 9 and the *bcr* locus on chromosome 22 (141, 333). Bcr-Abl, the protein product of this translocation, is a 210 kDa chimeric tyrosine kinase with abnormal cytoplasmic localization (334). Bcr-Abl transforms fibroblasts, growth factor-dependent hematopoietic cell lines and primary bone marrow cells in culture (335-337) and induces a myeloproliferative disorder that closely resembles CML in mice (153, 338).

Constitutive tyrosine kinase activity and cytoplasmic relocalization underlie the ability of Bcr-Abl to activate numerous signal transduction pathways and promote cell proliferation and survival (261). For example, Bcr-Abl was shown to activate the Stat5 transcription factor, inducing its nuclear translocation and transcription of cell growth and survival genes such as Cyclin-D1 and Bcl-X_L (245, 246, 339, 340). Bcr-Abl also activates the PI3K/Akt pathway upon phosphorylation of Tyr-177 in the Bcr region and recruitment of Grb2/Gab2 adapter proteins (261). Additionally, PI3K is also activated by Bcr-Abl via other adapter proteins such as Shc (341, 342), Crkl and c-Cbl (261) or by direct binding of the p85 subunit of PI3K to Bcr-Abl

(263, 343). Activation of PI3K leads to suppression of programmed cell death via Akt and other pathways. Similarly, the Ras/Erk pathway is activated by Bcr-Abl either upon phosphorylation of Bcr-derived Tyr-177 and direct Grb2/Sos recruitment or via the Shc adaptor protein (230, 259). By stimulating the Ras/Erk pathway, Bcr-Abl increases growth-factor independent cell proliferation.

Members of the Src-kinase family have been strongly linked to Bcr-Abl signaling and leukemogenesis (209, 211, 225). Several reports have demonstrated that Bcr-Abl binds to multiple Src-family members including Hck, Lyn and Fyn leading to their activation (194, 207, 209, 210). Activation of SFKs may have a positive feedback effect on Bcr-Abl signaling, as SFKs directly phosphorylate Bcr-Abl at key Tyr residues critical for regulation and function. These sites include Tyr-177 in the Bcr part of the protein, Tyr-89 and Tyr-134 in Abl-derived SH3 domain, Tyr-245 in SH2-kinase linker, and Tyr-412 in the activation loop of the Abl kinase domain (194, 210, 229, 233, 234, 344). On the other hand, SFKs may serve as key intermediates linking Bcr-Abl with downstream effectors. For example, Hck has been shown to couple Bcr-Abl to Stat5 activation in myeloid leukemia cells, which may contribute to survival (211). Furthermore, global inhibition of SFK activity with the ATP-competitive pyrrolo-pyrimidine compound A-419259 blocks Stat5 and Erk signaling, leading to growth arrest and apoptosis in CML cell lines (215). Here we show for the first time that A-419259 also blocks proliferation and induces apoptosis in primary $CD34^+$ CML cells. Other studies have shown that Hck and Lyn are overexpressed and activated in CML blast-crisis patients, and up-regulation correlates with disease progression and drug resistance (330, 331). Taken together, these studies emphasize the significant role of SFKs in Bcr-Abl signaling.

Consistent with the critical role for Bcr-Abl in CML, the Abl kinase inhibitor imatinib mesylate produces dramatic hematological and cytogenetic remission in most chronic phase CML cases (135, 345). However, patients with advanced disease often acquire drug resistance and continue to progress despite imatinib therapy. In addition, residual Bcr-Abl$^+$ primitive progenitor cells (CML stem cells) can persist in patients achieving complete cytogenetic remission (346), highlighting the need for additional therapeutic targets. Given their important role in Bcr-Abl signaling and in imatinib resistance, SFKs have recently emerged as novel targets for CML treatment. However, the relative contribution of individual SFKs to Bcr-Abl signaling is not fully understood.

In this study we investigated the independent contribution of Hck to Bcr-Abl signaling using a mutant (Hck-T338M) with engineered resistance to the broad-spectrum SFK inhibitor, A-419259. Expression of this mutant in the CML cell line K562 and in TF-1 myeloid cells acutely transformed with Bcr-Abl allowed persistence of Hck kinase activity in the presence of A-419259 at concentrations that inhibited all endogenous SFK activity. Remarkably, Hck-T338M rescued both K562 and TF-1/Bcr-Abl cells from the apoptotic effects of A-419259 treatment. This result correlated with sustained activation of Stat5 in the presence of the inhibitor and provides direct evidence that Hck alone is able to transmit anti-apoptotic signals from Bcr-Abl. In addition, Hck-T338M had a partial protective effect on A-412959-induced growth arrest that correlated with rescue of Erk signaling in K562 cells. Taken together, our data demonstrate the utility of engineered inhibitor-resistant mutants to dissect the roles of individual members of a closely related family of protein kinases in oncogenic signaling, and point to Hck as a clinically relevant target for CML therapy.

2.3 RESULTS

2.3.1 Design of an inhibitor-resistant mutant of Hck

Many ATP-competitive inhibitors of protein kinases access a small hydrophobic pocket adjacent to the ATP-binding site in kinase domain. Accessibility of inhibitors to this hydrophobic pocket is controlled by a non-conserved amino acid often referred to as the "gatekeeper" residue (347). Numerous studies have shown that natural variation in the gatekeeper residue is one of the main structural determinants of kinase sensitivity to small molecule inhibitors (300, 332, 348-350). Tyrosine kinases that possess a threonine at this position are sensitive to various classes of inhibitors that access the hydrophobic pocket (Table 1). However, replacement of the gatekeeper residue with more bulky amino acids such as methionine or isoleucine has been reported to induce resistance to these inhibitors (Table 1 and Discussion).

Kinase	Gatekeeper Residue	Gatekeeper Mutation	Inhibitor	Class	Refs
c-Abl	FYII**T**$_{315}$EFMTYGN	T→I	Imatinib	2-phenylaminopyrimidine	(300)
Kit	TLVI**T**$_{670}$EYCCYGD	T→I	Imatinib	2-phenylaminopyrimidine	(351)
PDGFR alpha	IYII**T**$_{674}$EYCFYGD	T→I	Imatinib	2-phenylaminopyrimidine	(352)
EGFR	VQLI**T**$_{766}$QMPFGD	T→M	Gefitinib	anilino-quinazoline	(353)
Src	IYIV**T**$_{338}$EYMSKGS	T→ I	PP58/PP1	pyrido/pyrazolo-pyrimidine	(349, 354)
Hck	IYII**T**$_{338}$EFMAKGS	T→M	A-419259	pyrrolo-pyrimidine	(this study)

Table 2: Peptide sequences surrounding the gatekeeper threonine at the ATP-binding site of several tyrosine kinases

Mutations of this residue to methionine or isoleucine account for a common mechanism of resistance to inhibitors of various classes as described in the text.

To determine whether we could artificially engineer resistance to A-419259 in Hck by increasing the size of the gatekeeper residue, we examined the crystal structure of Hck in complex with a related pyrazolo-pyrimidine inhibitor, A-420983 (Figure 5). The crystal structure revealed close contact between the Hck gatekeeper residue [Thr-338; human c-Src crystal structure numbering (62)] and the inhibitor, suggesting that replacement of Thr-338 with a bulkier methionine residue would result in A-419259 resistance due to steric clash without affecting kinase activity.

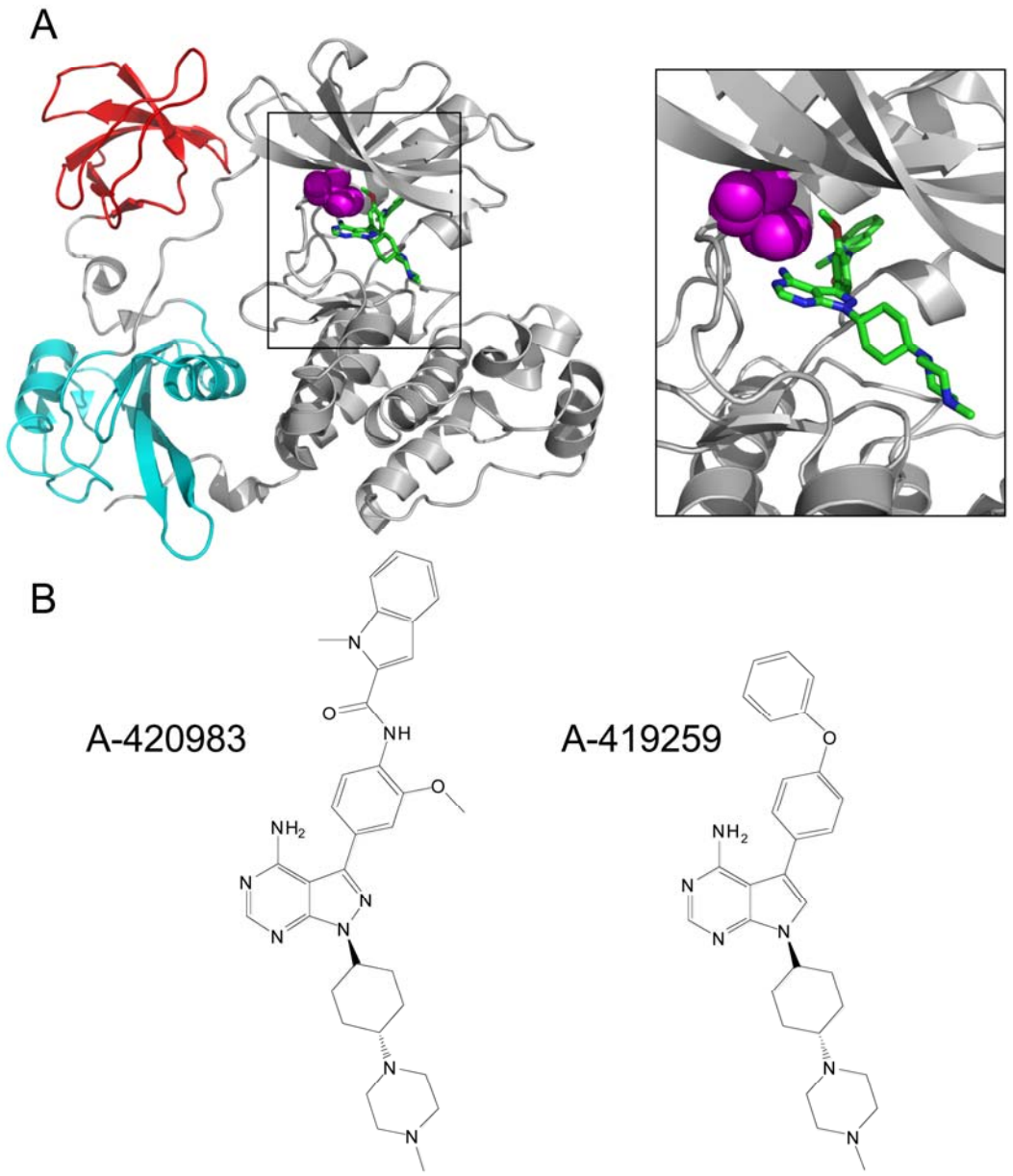


Figure 5: Structure-based design of Hck inhibitor-resistant mutant

(A) Orientation of A-420983, an analog of A-419259, in the Hck nucleotide-binding pocket. The overall structure of Hck is shown on the left, with the SH3 domain in red, the SH2 domain in blue, and the kinase domain in grey. The side chain of the gatekeeper residue (T338; c-Src numbering) is highlighted in magenta. The relationship of the gatekeeper residue to the pyrazolo-pyrimidine moiety of A-420983 is enlarged on the right. This model was produced using PyMol and the PDB file 2COI. (B) Chemical structures of A-419259 and A-420983.

To address whether the T338M substitution induced Hck resistance to A-419259, wild-type and Hck-T338M were expressed in Sf9 insect cells and purified to homogeneity in their downregulated conformations. Normally, Hck as well as other SFKs adopt an inactive, downregulated conformation in vivo due to phosphorylation of a conserved tyrosine residue in the C-terminal (Tyr-527) by the regulatory kinases Csk and Chk (27). To attain this conformation while avoiding the co-expression of the regulatory kinases, both forms of Hck were altered at the C-terminal tail from Tyr-527-Gln-Gln-Gln-Pro to Tyr-527-Glu-Glu-Ile-Pro (referred to hereafter as Hck-YEEI). This modification promotes autophosphorylation of the tail independently of Csk, and was previously shown to have a higher affinity for the SH2 domain, stabilizing the downregulated conformation (27, 63, 82). Importantly, Hck-YEEI undergoes autophosphorylation and exhibits substrate phosphorylation kinetics similar to wild-type Hck (27, 355). The sensitivity of recombinant Hck-YEEI and Hck-T338M-YEEI to A-419259 were compared in an in-vitro kinase assay using a peptide substrate. As shown in Figure 6, the T338M mutation induced dramatic resistance to A-419259, increasing the IC_{50} value by almost 30-fold from 11.26 ± 1.23 nM for wild-type Hck to 315.6 ± 80.3 nM for the T338M mutant. Kinetic analysis showed a modest difference in the K_m for ATP in the T338M mutant compared to wild-type Hck (10.7 ± 2.5 μM for wild-type vs. 3.9 ± 0.8 μM for T338M).

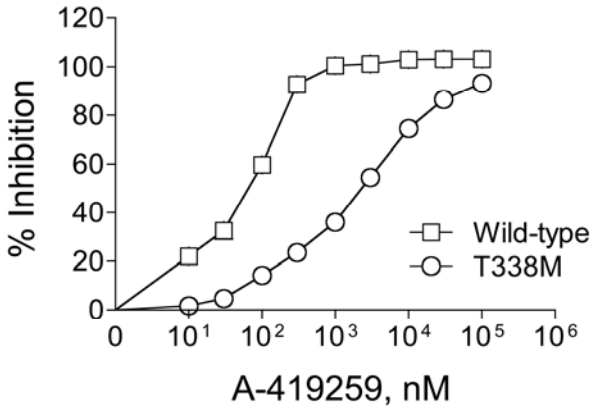


Figure 6: In vitro kinase assay of recombinant wild-type and T338M forms of Hck

Recombinant wild-type Hck and Hck-T338M were purified from Sf9 insect cells in their downregulated conformations and assayed for kinase activity with a peptide substrate in vitro in the presence or absence of the indicated concentrations of A-419259. A representative experiment is shown and the extent of inhibition is expressed as mean ± S.D. of four assays. The entire experiment was repeated twice with comparable results. Data from two independent experiments were best-fit by non-linear regression analysis and yielded IC_{50} values of 11.26 ± 1.23 nM for wild-type Hck and 315.6 ± 80.3 nM for Hck-T338M.

2.3.2 Hck-T338M retains its activity and A-419259-resistance in fibroblasts

We next investigated whether the T338M resistance mutation influenced Hck biological signaling and if resistance to A-419259 was maintained in intact cells. To address these questions, both wild-type Hck and Hck-T338M were activated by replacing the C-terminal Tyr-527 with phenylalanine (YF mutation). This mutation was previously shown to up-regulate Hck kinase activity and to induce oncogenic transformation of fibroblasts, which do not express endogenous Hck (86, 95, 96, 356). Rat-2 fibroblasts expressing Hck-YF, Hck-T338M-YF, as well as wild-type Hck or the *neo* resistance marker as negative controls were plated in soft-agar

in the presence or absence of increasing concentrations of A-419259. As shown in Figure 7A, the T338M mutation did not impair biological activity, as cells expressing Hck-YF or Hck-T338M-YF produced similar numbers of transformed colonies in the absence of the inhibitor. To determine whether transformation correlated with constitutive activation of the kinase, cell lysates were tested for reactivity with the phosphospecific antibody pY418. This antibody recognizes the conserved phosphotyrosine residue in the activation loop of active Hck and other SFKs (215, 356, 357). Consistent with the transformation results, both Hck-YF and Hck-T338M-YF reacted strongly with this phosphospecific antibody (Figure 7B). Furthermore, activation loop phosphorylation correlated with phosphorylation of the endogenous Hck substrate protein, pp40 (95, 356) as shown by anti-phosphotyrosine immunoblots of the cell lysates (Figure 7B). In contrast, wild-type Hck did not exhibit kinase or transforming activity. Taken together, these results show that the T338M mutation is functionally silent and does not influence Hck biological activity.

Next, we investigated the sensitivity of Rat-2 cells transformed by each form of Hck to A-419259. As shown in Figure 7A, 0.3 μM A-419259 almost completely blocked colony formation by cells expressing the Hck-YF mutant. This was accompanied by complete inhibition of Hck-YF autophosphorylation and pp40 substrate phosphorylation (Figure 7B). In contrast, the same concentration of A-419259 induced only a 25% decrease in colony formation in cells expressing Hck-T338M-YF mutant with no detectable change in kinase activity (Figure 7A). Interestingly, the activity of Hck-T338M-YF was also unaffected by 1 μM A-419259, despite a further decrease in colony-forming activity. This finding suggests that endogenous A-419259-sensitive SFKs may cooperate with Hck to induce the transformed phenotype.

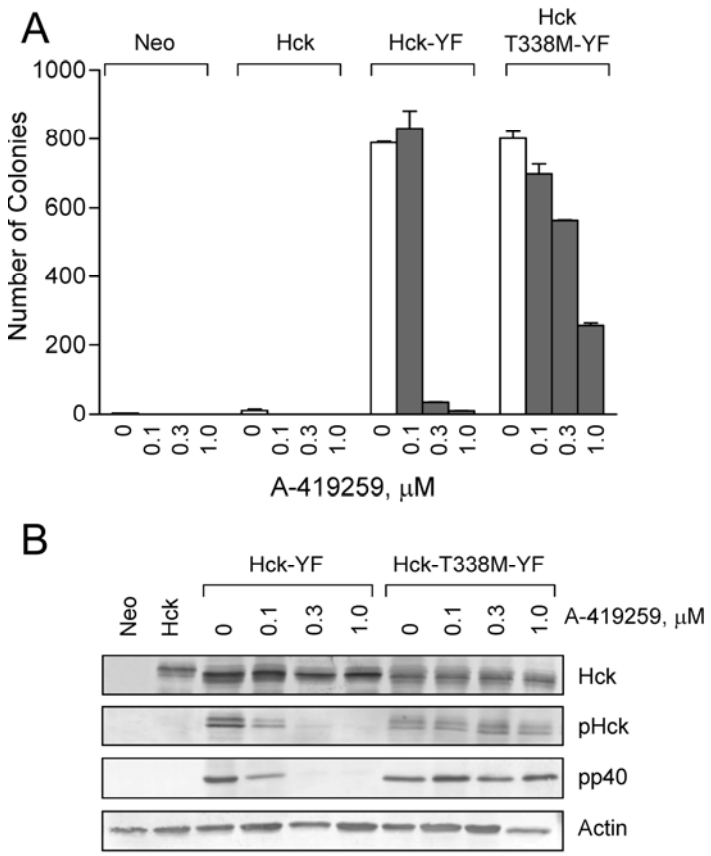


Figure 7: Hck-T338M maintains its biological activity and A-419259 resistance in fibroblasts

Rat-2 fibroblasts were infected with recombinant Hck-YF and Hck-T338M-YF retroviruses. Cells infected with a virus carrying only the selection marker (Neo) or wild-type Hck served as negative controls. Upon G418 selection, cells were plated in soft agar in the presence or absence of the indicated concentrations of A-419259 for 10 to 14 days. Transformed colonies were visualized using MTT staining. (A) Colony numbers for each cell line were determined using scanned images of the plates and BioRad QuantityOne colony-counting software. Results from a representative experiment are shown as the mean number of colonies ± S.D. The entire experiment was performed twice and yielded comparable results. (B) Lysates from each of the cell lines shown in (A) were probed with phosphospecific antibodies against the Hck activation loop phosphotyrosine residue (pHck). Replicate membranes were probed with a general anti-phosphotyrosine antibody to determine phosphorylation of the endogenous Hck substrate pp40, with an anti-Hck antibody to determine Hck expression levels, and with anti-actin as a loading control.

2.3.3 Hck-T338M expression in K562 cells confers resistance to A-419259-induced growth arrest and apoptosis

The in vitro and cell-based assays described above support the idea that the T338M mutation induces Hck resistance to A-419259 without affecting kinase activity, providing a unique probe to test the singular contribution of Hck to Bcr-Abl signaling in CML cells. To test whether Hck-T338M is resistant to A-419259-induced inhibition in the context of a CML-derived cell line, we used K562 cells, a Ph^+ human CML cell line in which endogenous Hck expression is not detectable by immunoblot (Figure 8). K562 cells were infected with recombinant retroviruses carrying wild-type Hck, the Hck-T338M mutant, or a control virus carrying only the *neo* resistance marker. Following G418 selection, these three cell populations (K562-Hck, K562-Hck-T338M, and K562-Neo) were treated in parallel with various concentrations of A-419259, and the effect of inhibitor treatment on Hck activity was assessed with the phosphospecific antibody, pY418, as described above. As shown in Figure 8, A-419259 caused partial inhibition of wild-type Hck at 0.1 μM and complete inhibition at 0.3 μM in K562-Hck cells. This observation is consistent with previous findings that A-419259 inhibits overall SFK activity in K562 and other CML cell lines with an IC_{50} value of 0.1-0.3 μM (215). In contrast, little change in Hck-T338M pY418 phosphorylation was observed at these A-419259 concentrations, although partial inhibition was observed at 1 μM A-419259.

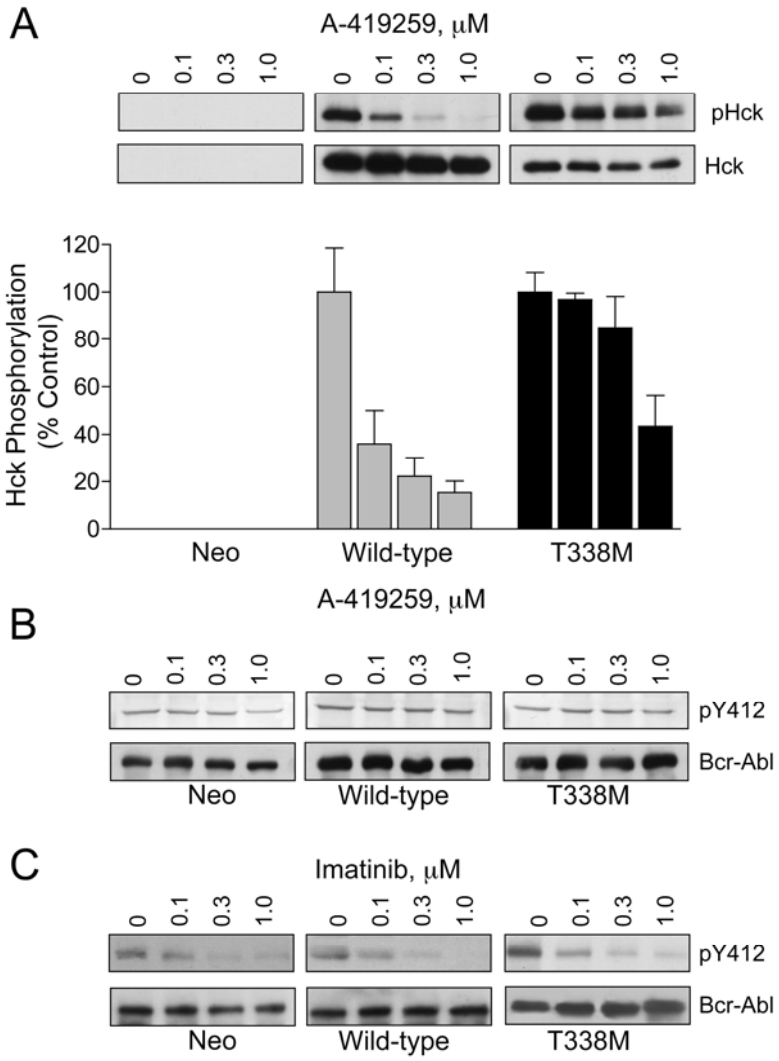


Figure 8: Hck-T338M is resistant to inhibition by A-419259 in K562 cells

Wild-type and Hck-T338M proteins were expressed in K562 cells using recombinant retroviruses. K562-Hck wild-type and K562-Hck-T338M cells were treated with A-419259 at the indicated concentrations for 5 h. Hck activity was assessed by immunoprecipitation of Hck from clarified cell lysates and immunoblotting with phosphospecific antibodies against the Hck activation loop phosphotyrosine residue (pHck). Duplicate blots of the immunoprecipitates were blotted with the anti-Hck antibody to insure equal loading. Representative blots are shown at the top. Phosphotyrosine signal intensities from the blots of two independent experiments were normalized to the levels of Hck. Results are presented as percent of control levels ± S.D. in the bar chart.

Previous work has shown that A-419259-dependent inhibition of SFK activity in K562 cells induces growth arrest and apoptosis (215). In addition, this prior study found that A-419259 is 300 to 1000 times more potent against SFKs compared to c-Abl in vitro. To determine whether A-419259 has a direct inhibitory effect on Bcr-Abl in our experimental system, we probed the lysates from the inhibitor-treated K562 cell populations with a phosphospecific antibody against the phosphotyrosine residue in the activation loop of active Bcr-Abl (pY412). As shown in Figure 8B, A-419259 did not significantly inhibit Bcr-Abl phosphorylation at the autoactivation site. In contrast, imatinib caused a dose-dependent inhibition of the pY412 signal (Figure 8C). These data support the selectivity of A-419259 for SFKs in K562 cells, in agreement with our previous data in this cell line (215).

To investigate the contribution of Hck alone to Bcr-Abl-induced transformation and signaling in CML, K562-Hck, K562-Hck-T338M, and K562-Neo cells were treated with 0.1 to 1.0 μM A-419259 and cell proliferation was measured over 72 hours. Figure 9 shows that 0.1 μM A-419259 induced 75-80% growth arrest of K562-Hck and K562-Neo cells. Conversely, K562-Hck-T338M cells exhibited only a 50% reduction in cell proliferation at this inhibitor concentration. This result correlates with sustained Hck-T338M kinase activity compared to the wild-type Hck in the presence of the inhibitor (Figure 8). Given that 0.1-0.3 μM A-419259 completely inhibits endogenous SFK activity in CML cells (215), this result suggests that Hck alone is able to partially sustain Bcr-Abl-induced cell proliferation (see Discussion).

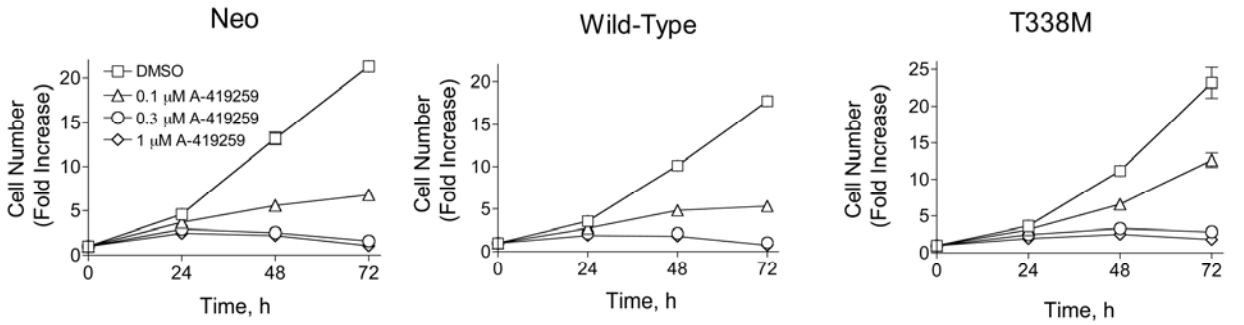


Figure 9: Hck-T338M expression in K562 cells confers resistance to A-419259-induced growth arrest

K562 cells expressing wild-type Hck, the T338M mutant, as well as the vector control cells (Neo) were treated with A-419259 at the indicated concentrations. Cell proliferation was monitored 24, 48 and 72 h later using the CellTiter-Blue cell viability assay as described under "Materials and Methods". Three replicate wells were monitored for each dose in three independent experiments and gave comparable results; a representative example is shown.

We next investigated whether expression of Hck-T338M also confers resistance to A-419259-induced apoptosis in CML cells. To address this issue, K562-Neo, K562-Hck and K562-Hck-T338M cells were treated with A-419259 for 72 h and apoptosis was measured using an Alexa-Fluor-conjugated anti-phosphatidylserine (PS) antibody and flow cytometry. As shown in Figure 10, A-419259 potently induced dose-dependent apoptosis in K562-neo and K562-Hck cells starting at 0.3 μM. In contrast, Hck-T338M expression completely rescued K562 cells from apoptosis induced by 0.3 μM A-419259. Furthermore, K562-Hck-T338M cells also displayed significant resistance to apoptosis induced by 1 μM A-419259. These data correlate with Hck-T338M kinase activity, which is not affected by 0.3 μM A-419259 and only partially inhibited by this compound at 1 μM (Figure 8).

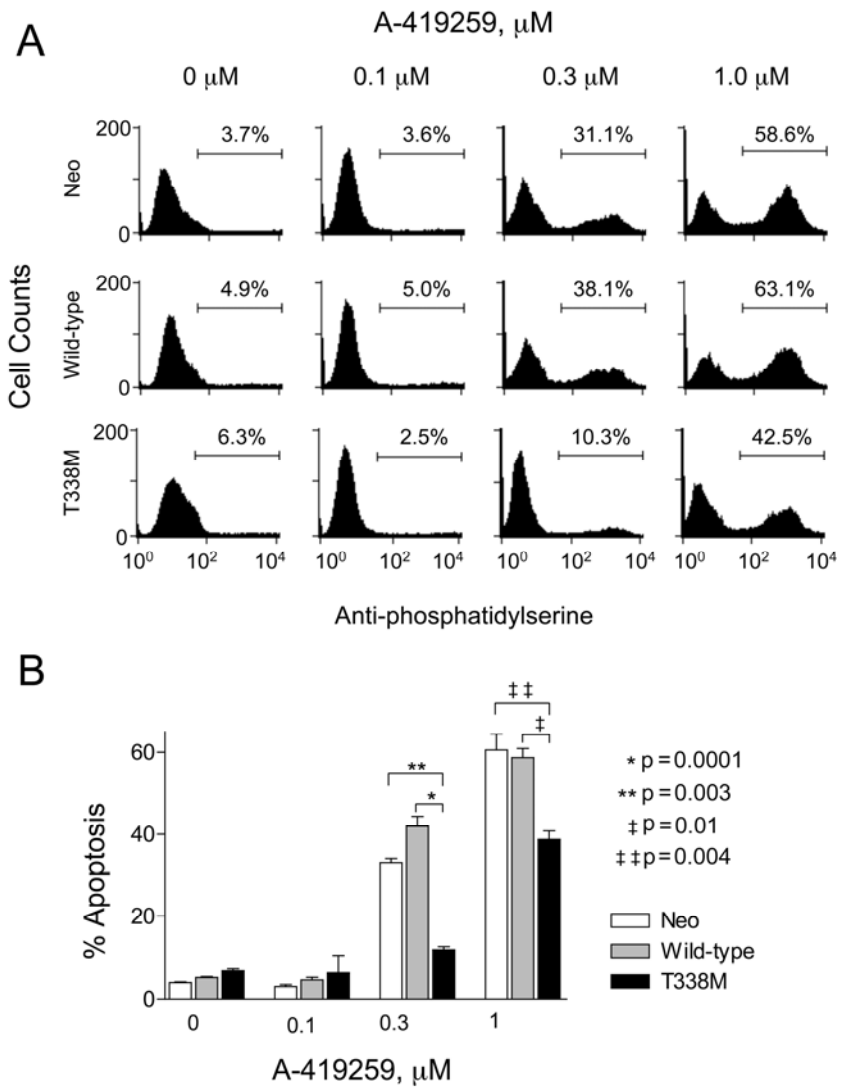


Figure 10: Hck-T338M protects K562 cells against the apoptotic effect of the SFK inhibitor A-419259

K562 cells expressing wild-type Hck, the T338M mutant, as well as the vector control (Neo) cells were plated in the absence or presence of the indicated concentrations of A-419259 for 72 h. Apoptotic cells were detected by anti-phosphatidylserine-Alexa Fluor 488 conjugated antibody staining and flow cytometry. (A) Representative experiment with the percentage of apoptotic cells in each population shown above the bar. (B) Bar graph showing the average of three independent experiments and plotted ± S.D. Statistical analyses were performed at each drug concentration between Neo and Wild-type, Neo and T338M, and Wild-type and T338M, using two tailed Student's T test. Only statistically significant differences are displayed.

2.3.4 Expression of Hck-T338M in K562 cells rescues the inhibitory effects of A-419259 on Stat5 and Erk activation

Previous reports suggest that Hck may couple Bcr-Abl to Stat5 and Erk activation in myeloid leukemia cells (210, 211). In addition, work in our laboratory has shown that inhibition of SFK activity in K562 cells using A-419259 induces apoptosis and growth arrest that correlates with decreased Ras/Erk and Stat5 activation (215). To assess the individual contribution of Hck to the activation of growth and survival pathways downstream of Bcr-Abl, we focused on the ability of Hck-T338M to rescue Ras/Erk and Stat5 activation from the inhibitory effects of A-419259. K562-Neo, K562-Hck, and K562-Hck-T338M cells were treated with A-419259 and cell lysates were probed with phosphospecific antibodies for activated Erk by immunoblotting. As shown in Figure 11, A-419259 induced a dose-dependent inhibition of Erk phosphorylation in K562-Hck and K562-Neo cells. In both cases, the phospho-Erk signal was partially reduced at 0.3 μM and completely absent at 1.0 μM. However, expression of Hck-T338M completely rescued Erk activation at 0.3 μM A-419259, and showed a partial effect at 1.0 μM. While these data are consistent with the partial reversal of A-419259 growth-arrest observed in K562-Hck-T338M cells (Figure 8), they suggest that Hck-independent and Erk-independent pathways also contribute to Bcr-Abl-driven proliferation.

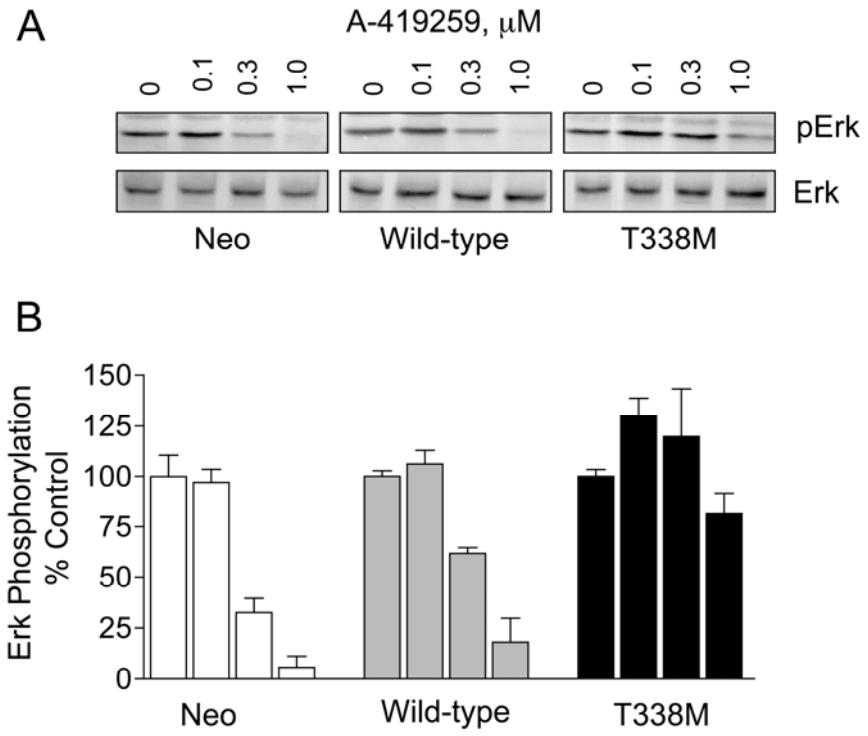


Figure 11: Expression of Hck-T338M in K562 cells reverses the inhibitory effects of A-419259 on Erk activation

K562 cells expressing wild-type Hck, the T338M mutant, as well as the vector control cells (Neo) were treated with the indicated concentrations of A-419259 for 5 h. (A) Cell lysates were prepared and analyzed for the presence of active Erk by immunoblotting with phosphospecific antibodies (pErk). Duplicate blots were probed with antibodies to Erk2 as a loading control. Representative blots are shown. (B) Phospho-Erk signal intensities from the blots of two independent experiments were normalized to the levels of Erk, and are presented as percent of control levels ± S.D.

Next, we examined the effect of Hck-T338M on Stat5 activation. To address this question, Stat5 was immunoprecipitated from lysates of A-419259-treated cells and probed with phosphospecific antibodies for active Stat5 by immunoblotting. As shown in Figure 12, A-419259 induced a dose-dependent inhibition of Stat5 activation in K562-Neo and K562-Hck

cells. In contrast, only a modest change in Stat5 phosphorylation was observed in K562-Hck-T338M cells, indicating that Hck plays a major role coupling Bcr-Abl to Stat5 activation. This result correlates with the change in Hck-T338M activity (Figure 8A) and with the ability of Hck-T338M to rescue K562 cells from A-419259-induced apoptosis.

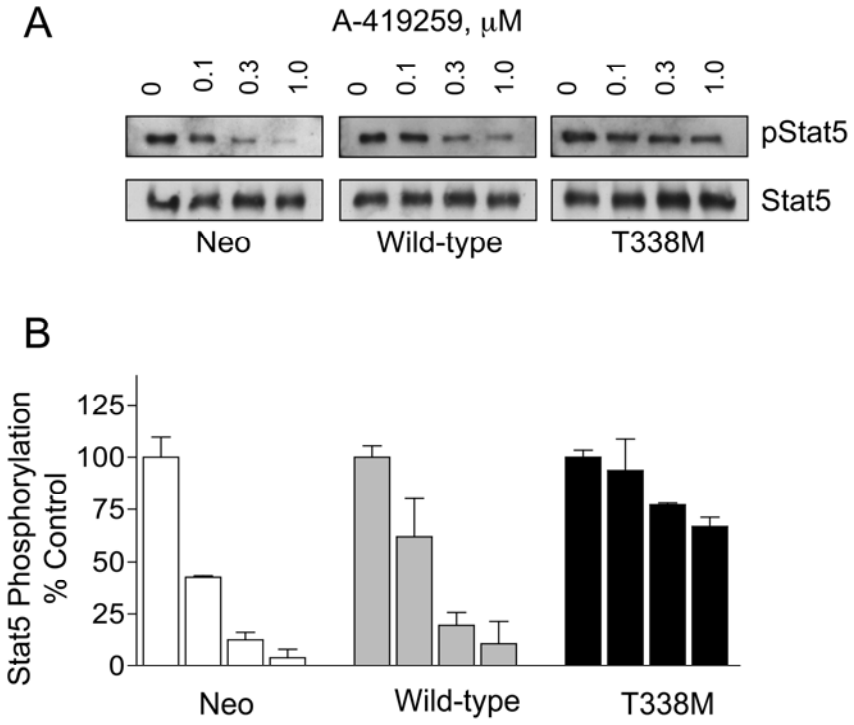


Figure 12: Expression of Hck-T338M in K562 cells opposes the inhibitory effects of A-419259 on Stat5 activation

K562 cells expressing wild-type Hck, the T338M mutant, as well as the vector control cells (Neo) were treated with the indicated concentrations of A-419259 for 5 h. Stat5 tyrosine phosphorylation was assessed by immunoprecipitation of Stat5 from clarified cell lysates and immunoblotting with anti-phosphotyrosine antibodies (pStat5). Duplicate membranes were blotted with anti-Stat5 antibody to insure equal loading (Stat5). Representative blots are shown. (B) Stat5 phosphotyrosine signal intensities from the blots of two independent experiments were normalized to the levels of Stat5 protein, and the results are presented as percent of control levels ± S.D.

2.3.5 Effect of Hck-T338M on Bcr-Abl Tyrosine Phosphorylation

Recent data from our laboratory and others show that Src kinases directly phosphorylate Bcr-Abl at sites including Tyr-89 located in the SH3 domain, Tyr-245 in the SH2-kinase linker, and Tyr-412 in the kinase domain activation loop (215) [(Abl residue numbering as per the human c-Abl crystal structure (188).] Tyr-89 and Tyr-245 lie along the interface between the SH3-SH2 clamp and the back of the kinase domain, and phosphorylation of these sites may destabilize these intra-molecular interactions and promote an active conformation of the kinase (194). Mutation of Tyr-89 to phenylalanine diminished transformation of TF-1 cells to cytokine independence by Bcr-Abl, while mutation of Tyr-245 to phenylalanine abolished fibroblast transformation by Bcr-Abl (194, 344). Similarly, phosphorylation at Tyr-412 is required for full activation of Bcr-Abl and mutation of this site was sufficient to abolish Bcr-Abl-induced fibroblast transformation (344). Moreover, previous work showed that Hck phosphorylates the Bcr component of Bcr-Abl at Tyr-177 (Bcr numbering) in transfected COS cells (210) creating an SH2-binding site for the Grb2-Sos guanine nucleotide exchange factor for Ras (229, 231). Phosphorylation at this site was shown to be required for transformation of fibroblasts and for efficient induction of CML-like myeloproliferative disease by Bcr-Abl in mice (229, 233, 234). Given the importance of these phosphorylation sites for Bcr-Abl regulation and leukemogenic potential, we decided to investigate the contribution of Hck to their phosphorylation in K562 cells using our unique A-419259-resistant form of Hck.

To address this question, lysates from A-419259-treated K562-neo, K562-Hck, and K562-Hck-T338M cells were first probed with phosphospecific antibodies for Tyr-89 in the SH3 domain. As shown in Figure 13, A-419259 completely blocked phosphorylation of Tyr-89 in K562-neo and K562-Hck cells at concentrations of 0.3 μM or higher. In contrast, K562-Hck-

T338M showed only a partial inhibition of the pY89 signal at these concentrations. This result suggests that while Hck is partially responsible for phosphorylation of Tyr-89, additional Src-family members may also trans-phosphorylate Bcr-Abl at this site consistent with previous findings (194).

Similar to phosphorylation at Tyr-89, 0.3-1.0 μM A-419259 treatment induced complete inhibition of Tyr-245 phosphorylation in K562-neo and K562-Hck cells but only a partial inhibition of the pY245 signal in K562-Hck-T338M cells. This result suggests that while Hck contributes to transphosphorylation at this regulatory site, other SFKs present in K562 cells are also involved, consistent with earlier data (194). Importantly, incubation with A-419259 did not have any effect on total Bcr-Abl protein levels or on Tyr-412 autophosphorylation in the kinase domain activation loop. As a positive control for the anti- pY412 antibody, we performed immunoblots on cells incubated with increasing concentrations of the Abl-selective inhibitor, imatinib. As expected, imatinib induced a dose-dependent inhibition of Bcr-Abl phosphorylation at Tyr-412 in all three cell lines (Figure 8C). Taken together, these data are consistent with the report that A-419259 is a Src-selective inhibitor and at these concentrations, it does not inhibit Bcr-Abl activity in vivo (215).

We also surveyed the phosphorylation of Bcr-Abl at Bcr-derived Tyr-177. As shown in Figure 13, A-419259 induced a partial reduction in pY177 signal intensity in K562-neo and K526-Hck cells at the highest concentration tested (1 μM). In contrast, the pY177 signal remains unchanged in K562-Hck-T338M cells, suggesting that while Hck may contribute to Tyr-177 phosphorylation in vivo, this site may be primarily a Bcr-Abl autophosphorylation site or may be targeted by other tyrosine kinase families in K562 CML cells.

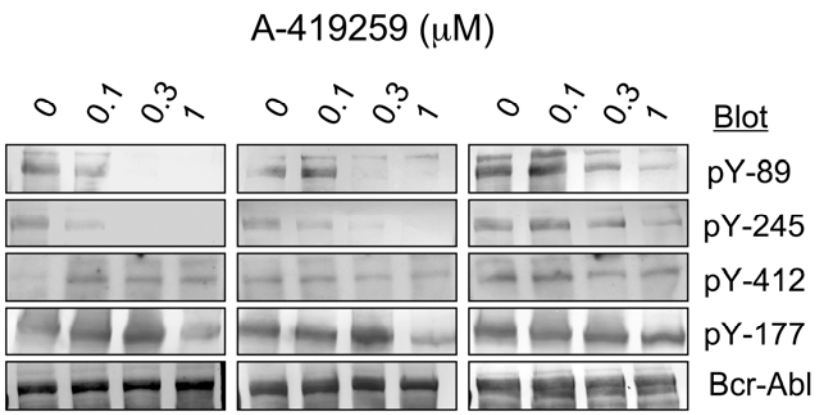


Figure 13: Hck-T338M transphosphorylates Bcr-Abl on multiple tyrosine residues in K562 cells

(A) K562-neo, K562-Hck and K562-Hck-T338M cells were treated with the indicated concentrations of A-419259 for 5 h. Cell lysates were prepared and probed with phosphospecific antibodies against Abl pY89, Bcr pY177, Abl pY245, and Abl pY412 as well as Bcr-Abl protein levels by immunoblotting. (B) K562-neo, K562-Hck and K562-Hck-T338M cells were treated with the indicated concentrations of imatinib for 5 h. Cell lysates were immunoblotted with antibodies for Abl pY412 and Abl protein as indicated.

2.3.6 Expression of Hck-T338M in Bcr-Abl-transformed TF-1 cells confers resistance to the apoptotic effects of A-419259 and correlates with sustained Stat5 activity

All of the data presented thus far are derived from the K562 CML cell line. To rule out the possibility that our observations are unique to this particular system, we turned to the human GM-CSF-dependent myeloid cell line, TF-1 (358, 359). Expression of Bcr-Abl in TF-1 cells results in cytokine-independent survival and proliferation (359), providing a CML model system that lacks the secondary genetic aberrations present in K562 cells. TF-1 cells were first infected with a Bcr-Abl retrovirus, which resulted in transformation to a cytokine-independent phenotype

as expected (Figure 14A). These TF-1/Bcr-Abl cells were then infected with the wild-type and T338M Hck retroviruses as well as the control virus carrying only the drug selection marker. Expression of these Hck proteins did not significantly affect the cytokine-independent growth of these cells (Figure 14A). We also expressed the Hck proteins in TF-1 cells alone, and observed that they were unable to stimulate GM-CSF-independent proliferation on their own (Figure 14A). All of the cell lines grew in the presence of GM-CSF (data not shown).

Next, we examined the effect of A-419259 treatment on survival of TF-1/Bcr-Abl cells. As shown in Figure 14B, incubation of the TF-1/Bcr-Abl vector control cells with A-419259 induced apoptosis in a dose-dependent manner. Note that parental TF-1 cells are completely unresponsive to A-419259 in this assay (215). Expression of wild-type Hck partially reversed apoptosis in response to 0.3 and 1 μM A-419259 in the TF-1/Bcr-Abl population. However, TF-1/Bcr-Abl/Hck-T338M cells displayed much more resistance to A-419259-induced apoptosis, with an almost complete reversal of apoptosis at 0.3 μM. This effect closely parallels that observed in K-562 cells expressing Hck-T338M (Figure 10).

We next assessed Hck and Stat5 activity in each TF-1/Bcr-Abl cell population following A-419259 treatment. As shown in Figure 14B, wild-type Hck activity was partially inhibited with 0.1 and 0.3 μM A-419259 and completely blocked at 1 μM. Conversely, Hck-T338M showed significant resistance to A-419259, with partial inhibition observed only with 1 μM A-419259. Similarly, expression of wild-type Hck partially rescued Stat5 activation at 0.1 μM A-419259 compared to control TF-1/Bcr-Abl cells. More importantly, expression of Hck-T338M rescued Stat5 activation at 0.3 and 1 μM A-419259. Taken together, these data are consistent with the partial reversal of A-419259-induced apoptosis by wild-type Hck and the more pronounced effect observed with Hck-T338M (Figure 14B).

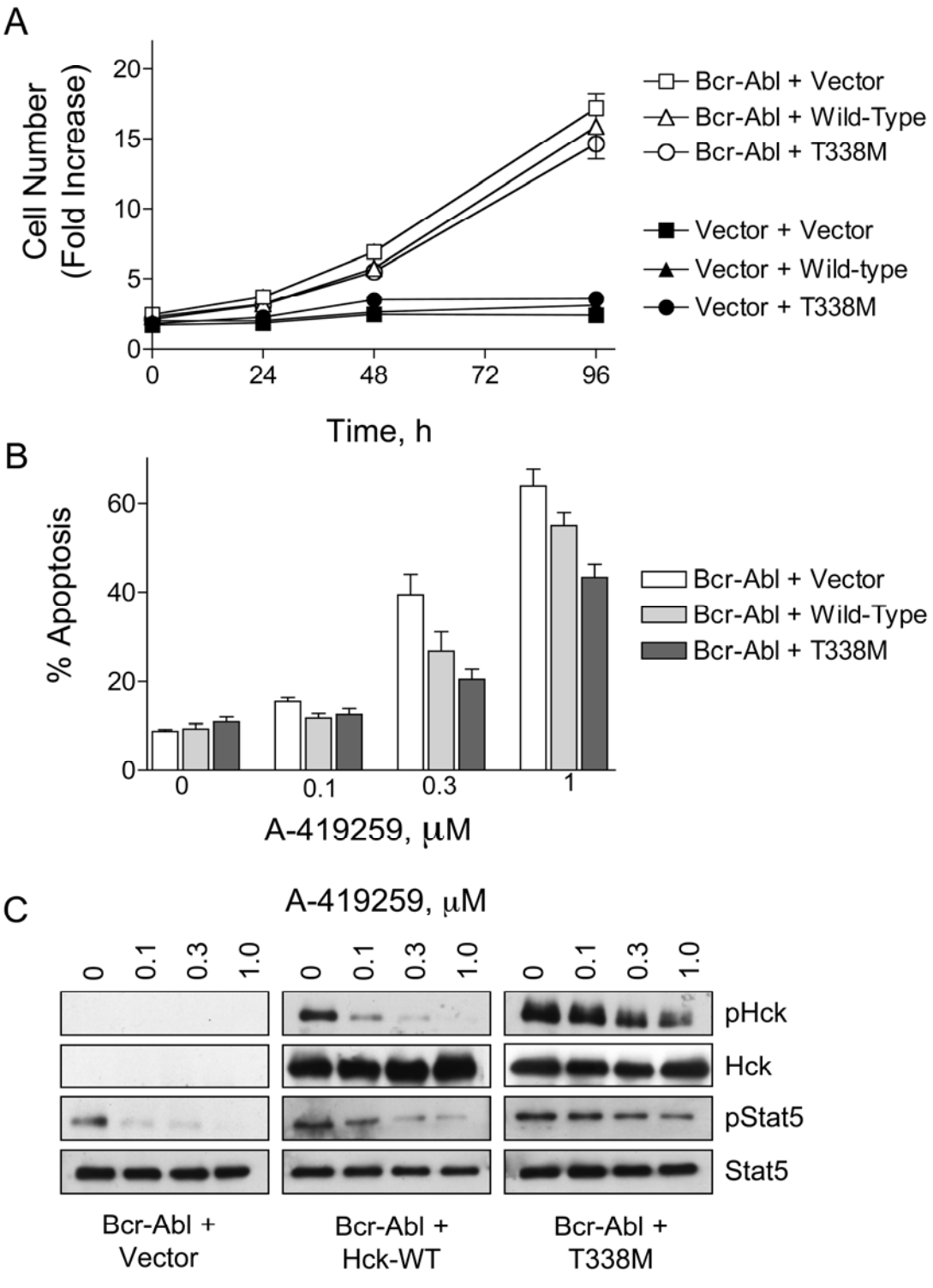

A
Cell Number (Fold Increase)
20
15
10
5
0
0
24
48
72
96
Time, h
Bcr-Abl + Vector
Bcr-Abl + Wild-Type
Bcr-Abl + T338M
Vector + Vector
Vector + Wild-type
Vector + T338M
B
% Apoptosis
60
40
20
0
0
0.1
0.3
1
A-419259, μM
Bcr-Abl + Vector
Bcr-Abl + Wild-Type
Bcr-Abl + T338M
C
A-419259, μM
0
0.1
0.3
1.0
pHck
Hck
pStat5
Stat5
Bcr-Abl + Vector
Bcr-Abl + Hck-WT
Bcr-Abl + T338M

Figure 14: Expression of Hck-T338M protects TF-1/Bcr-Abl cells against the apoptotic effects of A-419259

(A) Transformation of TF-1 cells to cytokine independence with Bcr-Abl. TF-1 cells were first transduced with Bcr-Abl retroviruses or the corresponding vector control and selected with G-418. The resulting populations were then transduced with wild-type Hck, Hck-T338M, or vector control retroviruses and selected with puromycin. The six resulting cell populations were then tested for growth in the absence of GM-CSF using the CellTiter Blue assay (see Materials and Methods). The average fold increase in the relative number of cells from three replicate wells is shown ± S.D. Two separate experiments from independently derived cell populations gave comparable results; a representative example is shown. (B) TF-1/Bcr-Abl cells expressing the wild-type and T338M Hck proteins along with vector control cells were plated in the absence or presence of the indicated concentrations of A-419259 for 72 h. Apoptotic cells were detected by anti-phosphatidylserine-Alexa Fluor 488 conjugated antibody staining and flow cytometry as described in "Materials and Methods". The bargraph shows the average of two independent experiments plotted ± S.D. (C) TF-1/Bcr-Abl cells expressing wild-type and T338M Hck along with the vector control were treated with the indicated concentrations of A-419259 for 5 h. Hck and Stat5 were immunoprecipitated from clarified cell lysates and immunoblotted with an anti-Hck phosphospecific pY418 antibody (pHck) or an anti-phosphotyrosine antibody (pStat5). Duplicate membranes were blotted with anti-Hck or anti-Stat5 antibody to insure equal loading. Representative blots are shown.

2.3.7 A-419259 inhibits proliferation and induces apoptosis in CML $CD34^+$ progenitor cells

In a final series of experiments, we determined whether selective inhibition of SFK activity affects the growth and survival of primary $CD34^+$ CML cells. Because purified $CD34^+$ progenitor cells undergo rapid differentiation when cultured in the presence of cytokines, we first determined the time course of differentiation as indicated by the loss of $CD34^+$ from the cell surface. As shown in Figure 16, 48 h post isolation, 95 % of the cells still retained CD34 at the

cell surface, while at 72 h and 96 h, CD34 dramatically dropped to 81 % or 41 %, respectively. In order to determine the effects of SFK inhibition on $CD34^+$ cells and to avoid interference from differentiated cells, we conducted the apoptosis experiments at 16 and 48 h time points. Thus, purified $CD34^+$ progenitors from three chronic-phase CML patients were incubated with A-419259 in the presence of cytokines (see Materials and Methods). A-419259 treatment induced a dose-dependent inhibition of cell proliferation in all three patient samples, with caspase activation evident as early as 16 h (Figure 16). The extent of growth inhibition and caspase activation is comparable to that observed with 1 μM imatinib treatment. These results provide the first evidence that inhibition of SFK activity is sufficient to induce cell-cycle arrest and apoptosis in primary $CD34^+$ CML cells.

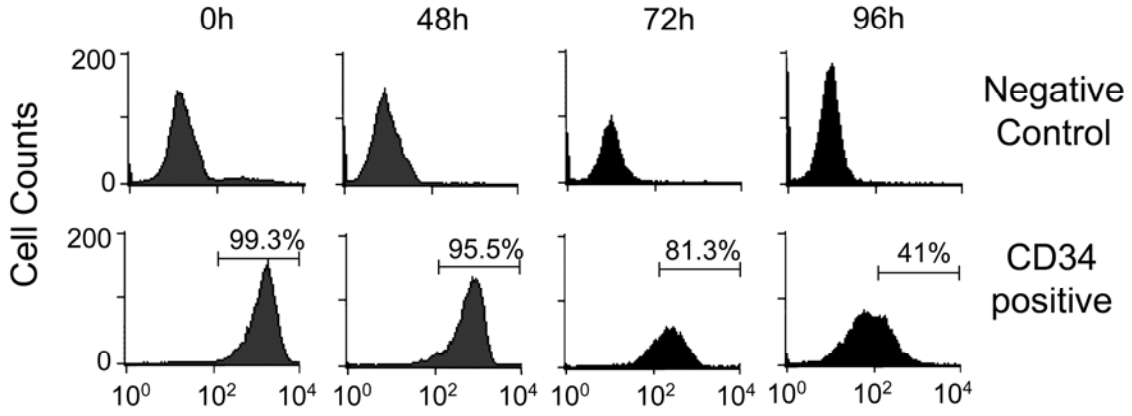


Figure 15: Time course of the loss of $CD34^+$ signal in CML primary cells in culture

Purified $CD34^+$ cells from chronic phase CML patients cultured in the presence of cytokines as described in "Materials and Methods". At each of the indicated time points an aliquot has been removed and processed for $CD34^+$ signal detection by flow cytometry. The experiment was repeated twice with similar results, using samples from two different patients. One representative experiment is shown.

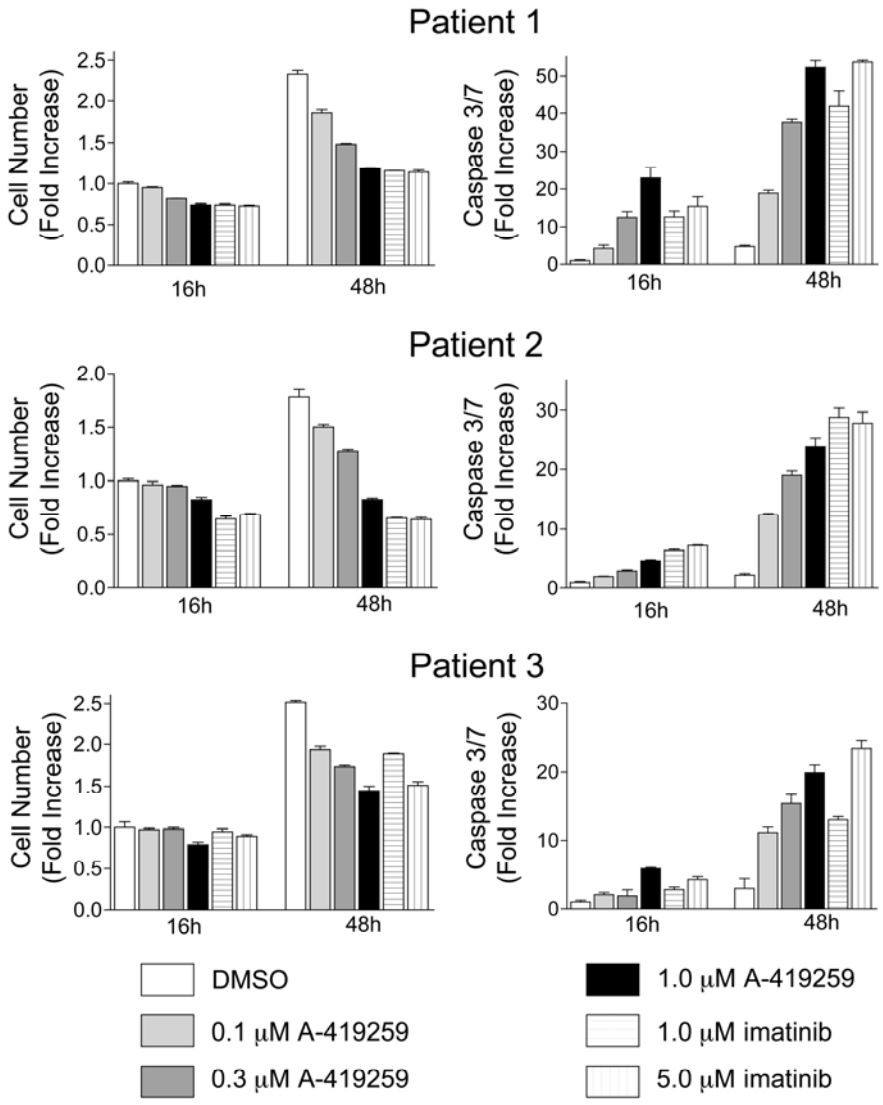


Figure 16: A-419259 induces growth arrest and apoptosis in CD34$^+$ progenitor cells from CML patients

Purified CD34$^+$ cells from three chronic phase CML patients were plated in triplicate in 96-well plates at 10^5 cells/ml and incubated for 16 or 48 h in the presence of A-419259 or imatinib at the concentrations indicated. (A) Cell proliferation was monitored using the CellTiter-Blue viability assay as described in "Materials and Methods". The average fold increase in the relative number of cells from three replicate wells is shown ± S.D. (B) Apoptosis was measured in each well using the Apo-One Caspase-3/-7 assay as described in "Materials and Methods". The average fold increase in caspase-3/-7 signal intensity from three replicate wells is shown ± S.D.

2.4 DISCUSSION

Given the important role of SFKs in Bcr-Abl signaling and imatinib resistance, these kinases have recently emerged as novel therapeutic targets for the treatment of CML (360). This idea has been validated by the success of dual Abl/Src inhibitors such as dasatinib in CML therapy (361, 362). However, these inhibitors have a broad range of molecular targets. For example, dasatinib inhibits not only the SFKs Fgr, Fyn, Hck, Lck, Lyn, Yes (363) and Bcr-Abl, but also c-Kit, PDGFR, and the Ephrin receptor tyrosine kinase (364). Although there is emerging evidence that compound promiscuity is critical to the efficacy of a significant number of approved drugs, this property has traditionally been regarded as undesirable due to possible adverse effects. This concept points to a need for drugs that have "controlled promiscuity" – drugs that specifically target only disease-relevant targets. In the case of CML, one key to effective progress in designing such drugs is a more in-depth understanding of the relative contribution of individual SFK members to Bcr-Abl signaling. Work presented here provides a novel approach to dissect the individual roles of myeloid SFKs to Bcr-Abl signaling and provides new evidence supporting a unique role for Hck in Bcr-Abl-induced proliferation and survival.

In this study, we exploited engineered inhibitor resistance to help elucidate the role of the SFK member Hck in Bcr-Abl signaling. Inhibition of protein kinases by target-selective, ATP-competitive compounds often depends on the presence of a relatively small threonine residue at the gatekeeper position adjacent to the hydrophobic pocket in the catalytic site. Substitution of the gatekeeper residue with amino acids bearing bulkier side-chains can dramatically reduce inhibitor potency without affecting kinase activity (Table 1). Some of the first drugs developed against protein-tyrosine kinases include imatinib, which inhibits Abl, c-Kit, and the PDGFR, as well as and gefitinib and erlotinib, which target the EGFR. In each case, these compounds target

the hydrophobic pocket and in some cases make direct hydrogen bonds to the gatekeeper residue (e.g. imatinib and Thr-315 in the Abl kinase domain). Interestingly, one of the most common mechanisms of clinical resistance to these inhibitors arises from mutations that replace the gatekeeper residue with bulkier amino acids (349). For example, replacement of Thr-315 in Bcr-Abl with isoleucine, Thr-671 in Kit with isoleucine, or Thr-766 in EGFR with methionine leads to clinically relevant insensitivity to imatinib or gefitinib, respectively (353, 365). Furthermore, a change in v-Src versus c-Src at the gatekeeper position induces resistance to the pyrazolo-pyrimidine inhibitor, PP1 (347). Resistance mutants similar to those described above, were first used as research tools for the purpose of identifying off-target effects of various inhibitors (350). Based on these observations, we engineered an A-419259-resistant Hck allele by replacing the gatekeeper residue, Thr-338, with methionine. This mutant allowed us to test the hypothesis that expression of inhibitor-resistant Hck alone is sufficient to rescue CML cells from the anti-proliferative and apoptotic effects of this broad-spectrum SFK inhibitor (215).

Using both in vitro and cellular model systems, we first provide proof-of-principle evidence that the T338M mutation renders Hck resistant to A-419259. Thus, Hck-T338M was 30-fold less sensitive to A-419259 than wild-type Hck in an in-vitro kinase assay (Figure 6). In addition, expression of active forms of wild-type Hck or the Hck-T338M mutant in Rat-2 fibroblasts induced similar levels of transformation, suggesting that T338M mutation does not significantly affect the biological function of Hck in a cell-based system (Figure 7A). The Hck-T338M-YF mutant was at least 10-fold less sensitive to A-419259 than Hck-YF in the fibroblast transformation assay (Figure 7). Interestingly, while Hck-T338M-YF activity was not affected by 1 μM A-419259 in fibroblasts, transformed colony numbers were partially reduced (Figure 7B). This observation suggests that Hck-YF may require endogenous A-419259-sensitive SFKs

to generate a full transforming effect. More importantly, we show that Hck-T338M also maintains resistance to A-419259 upon expression in K562 cells, allowing us to test the individual contribution of Hck to Bcr-Abl transformation in this CML cell line (Figure 8). As described in the results section, the Hck T338M mutation induces a subtle decrease in the K_m for ATP relative to the wild-type kinase. Whether the T338M mutation induces changes in substrate recognition or impacts other biological functions of Hck is not known. This possibility represents a caveat of the inhibitor-resistant mutant approach.

Experiments with A-419259 and other inhibitors as well as a Hck dominant-negative mutant suggest that SFKs contribute to Bcr-Abl-induced cell proliferation (10, 209, 210, 215). A-419259 treatment also led to suppression of Erk activity, a critical component of proliferation signaling downstream of Bcr-Abl (215, 366). In the present study, we show that Hck-T338M expression has a moderate protective effect on the A-419259-induced inhibition of cell proliferation (Figure 9), and this effect correlated with a rescue of Erk activity (Figure 11). The observation that Hck-T338M does not fully reverse A-419259-induced growth arrest and Erk inhibition suggests that other SFKs contribute to Bcr-Abl-mediated cell proliferation.

Previous work in our laboratory has shown that collective SFK inhibition in K562 cells using A-419259 induces apoptosis and that this effect correlates with a decrease in Stat5 activation (215). Here we show that Hck-T338M but not wild-type Hck expression fully protects K562 cells against the apoptotic effects of 0.3 μM A-419259 (Figure 10). This new finding suggests a compelling non-redundant role for Hck in Bcr-Abl survival signaling in CML cells. In addition, the Hck-T338M protective effect correlates with rescue of Stat5 activation, which is completely blocked by A-419259 in control cells as well as cells over-expressing wild-type Hck (Figure 12). These results identify Hck as an important target for the development of apoptosis-

inducing drugs for the treatment of CML. However, our data do not exclude the possibility that additional SFKs may participate in Bcr-Abl anti-apoptotic signaling in other experimental systems. For example, siRNA against Lyn induces apoptosis of CML blast crisis cells, especially of lymphoid origin (221). Furthermore, Lyn overexpression has been identified as an alternative mechanism of imatinib resistance in the absence of Bcr-Abl mutations (314, 330). Taken together, these findings suggest that differences may exist in the role of various SFKs within different hematopoietic lineages transformed by Bcr-Abl and emphasize the need to further investigate the role of other individual SFKs in CML.

Several studies have shown that SFK-dependent trans-phosphorylation modulates the function of both c-Abl and Bcr-Abl, drug sensitivity and signaling. Fursatoss et al. showed that c-Src phosphorylates c-Abl at both Tyr-412 and Tyr-245 (367), while Brasher and Van Etten proposed that phosphorylation at Tyr-245 enhances c-Abl activity by disrupting the negative regulatory interaction between the SH3 domain and the SH2-kinase linker (183). Recently, our laboratory has shown that Hck and other SFKs phosphorylate Bcr-Abl at SH3 domain sites Tyr-89 and Tyr-134 as well as other sites that lie at the interface between the SH3-SH2 domains and the SH2-kinase linker (194). Based on the crystal structure of the downregulated c-Abl core (188) phosphorylation of these sites is predicted to promote displacement of negative regulatory interactions of the SH3-SH2 clamp and sustain an active conformation of the Abl core within Bcr-Abl. Other experiments performed in COS cells have shown that Hck phosphorylates Bcr-Abl in the Bcr-derived portion of the protein at Tyr-177, an event required in leukemogenesis (229, 233, 234). Experiments presented here with Hck-T338M suggest that in CML cells, Hck contributes to Bcr-Abl phosphorylation at Tyr-89, Tyr-177 and Tyr-245. However, Hck-T338M does not completely reverse phosphorylation at these sites in A-419259-treated cells, suggesting

a role for other SFKs in Bcr-Abl trans-phosphorylation. Alternatively, Bcr-Abl may undergo autophosphorylation at these sites, as suggested by previous studies of c-Abl (183).

In contrast to the regulatory sites in the SH3-SH2-linker region, A-419259 treatment did not affect phosphorylation of the Bcr-Abl activation loop (Tyr-412; Figure 13). This observation suggests that SFKs do not contribute to activation loop phosphorylation in K562 cells, and also provides evidence of the selectivity of this compound for SFKs vs. Bcr-Abl directly (368, 369). In other work, high concentrations of A-419259 did suppress Bcr-Abl Tyr-412 phosphorylation, a result that is likely reflective of the different CML cell line used (194). Note that Tyr-412 phosphorylation was completely blocked by imatinib in all three K562 cell lines used here, suggesting that autophosphorylation may be the primary mechanism of activation loop phosphorylation in K562 cells (Figure 13).

There is increasing evidence that some patients that achieve complete cytogenetic remission on imatinib have persistent residual CML stem cells (non-proliferating $CD34^+$ cells) which might be responsible for relapse upon imatinib cessation (370-372). These quiescent $CD34^+$ cells represent less than 1% of total $CD34^+$ cells (217). Here we show for the first time that selective inhibition of SFK activity by A-419259 blocks proliferation and induces apoptosis in primary CML $CD34^+$ progenitor cell populations as effectively as imatinib (Figure 15). This observation suggests that SFKs play a critical role in Bcr-Abl signaling in primary CML progenitors as well as the cell lines examined here. Future work will address the effect of selective SFK inhibition on the non-proliferating $CD34^+$ cell population.

By pairing an inhibitor-resistant SFK mutant with a broad-spectrum SFK inhibitor, we have established new pharmacological evidence for Hck in Bcr-Abl survival signaling in K562 CML cells and in TF-1 cells acutely transformed by Bcr-Abl. This work validates Hck as a

specific target for the development of apoptosis-inducing drugs for the treatment of CML. In addition, these studies demonstrate the utility of mutants with engineered resistance to general inhibitors as an approach to address the contributions of individual members of a highly homologous kinase family to specific signaling pathways.

2.5 MATERIALS AND METHODS

2.5.1 Cell culture

K562 cells, derived from a CML patient in blast crisis (373), were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin (Antibiotic-Antimycotic, Invitrogen). The human GM-CSF-dependent myeloid leukemia cell line TF-1 (358) was obtained from ATCC and grown in RPMI 1640 supplemented with 10% FBS, Antibiotic-Antimycotic (Invitrogen) and 1 ng/ml human recombinant GM-CSF. Rat-2 fibroblasts were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS and Antibiotic-Antimycotic (Invitrogen). Sf9 insect cells were maintained in Grace's medium (Gibco) supplemented with 10% FBS and 50 μg/ml gentamicin (Gibco).

2.5.2 Selection of $CD34^+$ progenitors

Leukapheresis samples from three CML patients were thawed and cultured overnight in RPMI 1640 supplemented with 10% FBS, and Antibiotic-Antimycotic (Invitrogen). $CD34^+$ cells were isolated using an immunomagnetic column separation method (Miltenyi Biotech, Auburn, Ca, USA) following the manufacturer's instructions. Upon isolation, $CD34^+$ cells were cultured in SFEM medium (StemCell Technologies) supplemented with 40 μg/ml LDL (Sigma), and a five cytokine cocktail comprised of SCF, Flt3-L (100 ng/ml each), IL-3 and IL-6 (20 ng/ml each; StemCell Technologies) and 20 ng/ml G-CSF (Peprotech). The enrichment of $CD34^+$ cells was determined by flow cytometry using anti-CD34-FITC (Miltenyi Biotech) and ranged between 95% and 99% (data not shown).

2.5.3 Hck protein purification and kinase assay

The Thr-338 to methionine (T338M) mutation was introduced into human p59 Hck-YEEI (86) via site-directed mutagenesis (QuikChange XL Site-directed Mutagenesis Kit, Stratagene). Human Hck-YEEI and Hck-T338M-YEEI were expressed in Sf9 insect cells as N-terminal hexahistidine fusion proteins and purified as described (63, 374). Kinase assays were performed using the FRET-based Z'-Lyte Src kinase assay kit and Tyr-2 peptide substrate according to the manufacturer's instructions (Invitrogen). The assay was first optimized to determine the amount of each kinase and the incubation time necessary to phosphorylate 50-60% of the Tyr-2 peptide in the absence of inhibitor. Final ATP and Tyr-2 substrate concentrations were held constant at 50 μM and 2 μM, respectively. For inhibition experiments, each kinase was pre-incubated with A-419259 in kinase assay buffer for 30 min, followed by incubation with ATP and Tyr-2 peptide

for 1 h. Fluorescence was assessed on a Gemini XS microplate spectrofluorometer (Molecular Devices).

2.5.4 Retrovirus-mediated expression of Hck constructs in Rat-2 fibroblasts

Active forms of Hck and Hck-T338M were obtained by replacing the negative regulatory tyrosine residue in the C-terminal tail with phenylalanine using a PCR-based strategy (95, 96, 357). The constructs were then subcloned into the retroviral vector pSRαMSVtkneo (375), and used to generate high-titer retroviral stocks as described elsewhere (96, 376). For transformation experiments, Rat-2 fibroblasts (2.5×10^4) were plated in 6-well tissue culture plates and incubated overnight. The following day, the medium was replaced with 5 ml undiluted viral stock containing 4 μg/ml polybrene, and cultures were centrifuged at 1000 x g for 4 h at 18° C. Following infection, the virus was replaced with fresh medium. Forty-eight h later, cells were trypsinized and equally divided into four 60 mm culture dishes and 5 ml of medium containing G418 (800 μg/ml) was added. After 14 days of selection, cells were used in either soft-agar assays or for SDS-PAGE analysis of protein expression and tyrosine kinase activity.

2.5.5 Rat-2 fibroblast transformation assay (soft-agar assay)

Soft-agar assays were performed in 35 mm Petri dishes (Falcon) using Seaplaque Agarose (FMC Bioproducts). Briefly, a 0.5% bottom agarose layer in complete culture medium was poured in the presence of either vehicle alone (0.5% DMSO) or A-419259 at twice the final desired concentration. After the bottom layered had hardened, the top layer was poured containing 1×10^4 Rat-2 cells in culture medium containing 0.3% agarose. Ten to 14 days later the colonies

were stained with MTT and quantitated using densitometry and colony counting software (BioRad QuantityOne).

2.5.6 Immunoprecipitation and Immunoblotting

The antibodies used in this study include anti-Hck polyclonal (N-30; Santa Cruz Biotechnology), anti-Hck monoclonal (Transduction Laboratories), anti-Src phosphospecific (Src pY-418; BioSource International), anti-phosphotyrosine (PY-99; Santa Cruz), anti-Actin (MAB1501; Chemicon), and anti-Stat5 (BD Transduction Laboratories).

To analyze Hck expression and phosphorylation in Rat-2 cells, 5 x 10^5 cells were plated in 100 mm dishes and treated with either A-419259 or vehicle control (0.5% DMSO). After incubation at 37 °C overnight, cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (96). Cell lysates were clarified by centrifugation at 16000g for 10 min at 4 °C, and protein concentrations were determined using either the Bradford or BCA assay (Pierce). Aliquots of total protein were heated directly in SDS sample buffer and separated by SDS-PAGE. To analyze expression and phosphorylation of Hck in K562 cells, 10^7 cells were collected by centrifugation, washed twice with PBS and lysed in ice-cold RIPA buffer and processed as above. For Hck or Stat5 immunoprecipitation, protein concentrations were first normalized in lysis buffer, followed by addition of 1 μg of anti-Hck or anti-Stat5 antibody and 25 μl of protein G-Sepharose (50% slurry; Amersham Pharmacia Biotech). Following incubation for 2 h at 4°C, immunoprecipitates were washed three times with 1.0 ml of RIPA buffer and heated in SDS sample buffer. Following SDS-PAGE, proteins were transferred to PVDF membranes for immunoblot analysis. Immunoreactive proteins were visualized with appropriate secondary antibody-alkaline phosphatase conjugates and NBT/BCIP colorimetric substrate (Sigma).

2.5.7 Retroviral transduction of leukemia cell lines

Wild-type and T338M mutant forms of Hck were subcloned into the retroviral expression vector pMSCV-IRES-neo (Clontech) between the MSCV promoter and IRES sequence. Retroviral stocks were produced from the resulting constructs by co-transfection of 293T cells with an amphotropic packaging vector as described above. K562 cells (2×10^5) were incubated with 5 ml of viral stock in the presence of 4 μg/ml polybrene, and centrifuged at 3000 rpm for 3 h at room temperature. After infection, cells were washed, returned to regular medium for 48 h and then put under G418 selection (800 μg/ml) for 14 days. At the end of the selection period, cells were maintained in medium with 400 μg/ml G418. Transformation of TF-1 cells with a Bcr-Abl retrovirus carrying a G418 resistance marker is described elsewhere (194). These cells were then infected with pMSCV-IRES-puro-based retroviruses carrying wild-type and T338M forms of Hck or the empty vector control as described above and selected with 2 μg/ml puromycin.

2.5.8 Proliferation assays

Proliferation was assessed using the CellTiter-Blue Cell Viability assay (Promega) according to the manufacturer‘s protocol. Fluorescence intensity was then measured using a Gemini XS microplate spectrofluorimeter (Molecular Devices), with the excitation wavelength set at 544 nm and emission at 590 nm. Data were analyzed with SoftMax Pro software (Molecular Devices). Each condition was assayed in triplicate and the results are presented as the mean fluorescence ± S.D.

2.5.9 Apoptosis assays

Apoptosis was measured using an anti-phosphatidylserine (PS) antibody conjugated to Alexa Fluor 488 (Upstate Biotechnology) and flow cytometry. Cells (10^5/ml) were treated with A-419259 or vehicle alone (0.5% DMSO) for 72 h at 37°C, centrifuged at 1000 rpm for 10 min, washed three times with ice-cold PBS and resuspended to 4 x 10^6 cells/ml in staining buffer (1% FBS in PBS). Aliquots (50 µl) were transferred to 96-well round bottom tissue culture plates and the anti-PS antibody was added to a final concentration of 0.21 µg/well. After 1 h incubation on ice, cells were washed three times in ice-cold PBS and analyzed using a FACSCalibur flow cytometer (Becton-Dickinson) and data were analyzed using CellQuest software.

Caspase activation was measured in $CD34^+$ cells, using the Apo-One Caspase-3/-7 assay (Promega) and the manufacturer's instructions. Fluorescence intensity was measured using a Gemini XS microplate spectrofluorometer (Molecular Devices), with the excitation wavelength set at 485 nm and emission at 520 nm. Data were analyzed with SoftMax Pro software (Molecular Devices). Each condition was assayed in triplicate and the results are presented as the mean fluorescence ± S.D.

2.5.10 Statistical analysis

Data obtained from multiple independent experiments are given as mean ± S.D. values. Statistical comparisons between two cell lines at individual drug concentrations were performed using two-tailed unpaired Student's t test (normal distribution, and unequal variance). For all analyses a $P< 0.02$ was considered statistically significant. Statistical analyses were performed in Excel.

2.5.11 Acknowledgements

This work was supported by grants from the National Institutes of Health (CA101828 and GM077629 to T.E.S.). The authors thank Dr. David Calderwood of the Abbott Bioresearch Center for the generous gift of the SFK inhibitor, A-419259.

3.0 EXPRESSION OF HCK IN CML CELLS INDUCES RESISTANCE TO IMATINIB IN A KINASE-DEPENDENT MANNER

3.1 ABSTRACT

Imatinib is the frontline therapy used to treat chronic myelogenous leukemia (CML). Although imatinib is remarkably efficient in CML patients in chronic phase, patients with accelerated or blast crisis CML, often develop resistance. Several recent studies performed on clinical specimens from imatinib-resistant patients with wild-type Bcr-Abl, found that the Src family kinase members Hck and Lyn are overexpressed or highly active, suggesting that Src kinases may play a role in imatinib resistance. To test whether Hck overexpression in CML cells induces resistance to imatinib in an Hck-kinase-dependent manner, we employed a chemical genetic method to generate an Hck mutant (Hck-T338A) that is sensitive to inhibition by a mutant – specific inhibitor, NaPP1. In vitro, Hck-T338A was 48 times more sensitive to NaPP1 than the wild-type kinase. In addition, the mutation was functionally silent and did not induce a loss or gain of function in a fibroblast transformation assay. Expression of wild-type Hck or the T338A mutant in K562 CML cells resulted in resistance to imatinib-induced apoptosis and inhibition of soft-agar colony formation. Treatment with the Hck-T338A-selective inhibitor NaPP1 restored sensitivity to imatinib in a NaPP1 concentration-dependent manner only in cells expressing the Hck-T338A mutant. In contrast, cells expressing wild-type Hck were not affected by NaPP1

addition, showing the selectivity of NaPP1 for the Hck-T338A mutant. This result demonstrates that Hck-induced imatinib resistance requires Hck kinase activity. Together, these data establish a direct cause and effect relationship between Hck overexpression and imatinib resistance in CML cells, and show that imatinib resistance requires Hck kinase function. Selective drug targeting of Hck may be of therapeutic benefit in imatinib-resistant CML patients with increased Hck expression or activity.

3.2 INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell disease with an incidence of approximately 5 cases per 100,000 people per year, and which progresses through three clinical phases. The initial chronic phase is characterized by a massive expansion of myeloid cells that retain their ability to undergo terminal differentiation. As the disease progresses, patients enter an accelerated phase followed by blast crisis characterized by differentiation arrest and accumulation of immature blast cells in bone marrow and blood. Accelerated and blast crisis phases frequently exhibit additional genetic abnormalities (377).

The cytogenic hallmark of chronic myelogenous leukemia (CML) is the Philadelphia chromosome, which arises upon a "head-to-tail" fusion of the *BCR* (breakpoint cluster region) locus on chromosome 22 with c-*ABL* proto-oncogene on chromosome 9. This translocation is present in 90% of CML patients and leads to the expression of Bcr-Abl, a chimerical protein of 210 kDa (142, 143) with abnormal cytoplasm localization and constitutive tyrosine kinase activity (378). This deregulated protein tyrosine kinase drives the pathogenesis of CML through the phosphorylation and activation of a broad range of downstream signaling pathways that

increase cell survival and promote unregulated cell cycle progression (379). These pathways include but are not limited to the mitogen-activated protein kinase/extracellular signal-regulating kinase cascade (MAPK/ERK), the nuclear factor kB (NF-kB) signaling pathway, the phosphtidylinositol 3'-kinase/Akt survival pathway, and activation of the signal transducers and activators of transcription (Stat) family (380-382).

In addition, Bcr-Abl has been shown to activate other non-receptor protein-tyrosine kinases, particularly members of the Src kinase family including Hck or Lyn (207). The importance of this interaction in CML pathogenesis is illustrated by numerous studies. Briefly, expression of a kinase-defective mutant of Hck blocked Bcr-Abl-induced transformation of DAGM myeloid leukemia cells to cytokine independence (209). In addition, Hck was shown to couple Bcr-Abl to Stat5 signaling and to be required for Bcr-Abl-induced transformation of 32Dcl3 murine myeloid cells (211). Furthermore, SFK-selective inhibitors block leukemia cell proliferation and induce apoptosis without affecting Philadelphia chromosome-negative myeloid cells (215). Moreover, work presented in Chapter 2 of this dissertation shows that Hck plays a non-redundant role in Bcr-Abl survival signaling in CML cells (216). Taken together, these reports indicate that SFKs act as essential mediators of Bcr-Abl-induced leukemogenesis.

Because Bcr-Abl plays a critical role in the initiation and maintenance of the CML phenotype, targeting its tyrosine kinase activity is the therapeutic strategy of choice. Imatinib mesylate (formerly known as STI-571) is a potent small molecule tyrosine kinase inhibitor relatively specific to Bcr-Abl that has become the frontline therapy for patients with CML (379, 383). Most CML patients in chronic phase achieve and maintain major cytogenetic responses and significant molecular responses (384, 385). However, despite its remarkable therapeutic effects, approximately 4% of chronic phase patients treated with imatinib develop drug

resistance per year. In addition, accelerated or blast crisis patients display higher rates of imatinib resistance (300, 302). Clinical resistance to imatinib can be grouped into several categories including Bcr-Abl mutation-dependent or Bcr-Abl mutation independent. The former category includes mutations in the Abl kinase domain of Bcr-Abl at residues that directly contact imatinib or at positions that can allosterically influence imatinib binding (204). The focus of this chapter is the mechanism of resistance in the latter category.

Overexpression of the myeloid SFKs Hck and Lyn has been associated with resistance to imatinib in the context of a wild-type Bcr-Abl. Selection of K562 CML cells for resistance to high-levels of imatinib resulted in cells with increased Lyn protein and activity levels (219). Exposure of these cells to imatinib resulted in an incomplete suppression of Bcr-Abl activity and was accompanied by persistent tyrosine phosphorylation of Bcr-Abl at Tyr-177, a known binding site for Grb2 that links Bcr-Abl to the Ras signaling cascade (210, 218). Examination of samples from patients upon imatinib failure found increased Hck and/or Lyn activity levels that were not affected by Bcr-Abl inhibition (219). In addition, no Bcr-Abl kinase domain mutations associated with imatinib resistance were present in these patients (219). Decreased Lyn expression using siRNA technology, or inhibition with dasatinib, a dual Abl/SFK inhibitor, induced apoptosis in cells from these imatinib-resistant patients (314). Collectively, these reports point to a role for both Hck and Lyn in imatinib resistance in the absence of Bcr-Abl mutations. While the role of Lyn has been addressed in some detail the contribution of Hck to this type of imatinib resistance is less well understood.

To address this question, we developed a system in which Hck activity could be suppressed in a highly specific manner. Typically, small molecule inhibitors are limited with regard to their specificity and selectivity for a particular kinase family. To circumvent this

specificity problem, we employed a chemical genetic method originally described by Shokat et al. [reviewed in (386)]. In this method, pyrazolo-pyrimidine 1 (PP1), a global SFK small molecule inhibitor was modified to obtain the more bulky, naphthyl-pyrazolo-pyrimidine 1 (NaPP1) that is far less potent towards SFKs. In addition, using site-directed mutagenesis, the ATP-binding site of a tyrosine kinase was engineered to become exclusively susceptible to inhibition by the bulkier NaPP1 molecule (300, 332, 348-350, 386). This "lock and key" approach allows rapid, reversible and unique inhibition of the activity of the desired kinase. For this approach to be useful, the mutation within the ATP-binding site has to be functionally silent and the modified kinase has to be used in a system that doesn't endogenously express the wild-type allele.

In this study, we used the chemical genetic principles described above to successfully engineer an Hck mutant (Hck-T338A) that is uniquely sensitive to NaPP1 inhibition. The validity of the mutant was confirmed in an in vitro assay using purified recombinant Hck and Hck-T338A and in a Rat-2 fibroblast transformation assay. To address the role of Hck over-expression in Bcr-Abl-mutation independent resistance to imatinib, we expressed wild-type Hck and the Hck-T338A mutant in the CML cell line K562, which does not express endogenous Hck. Expression of both Hck and Hck-T338A rendered K562 CML cells resistant to imatinib-induced inhibition of colony formation and apoptosis. This effect correlated with sustained phoshporylation of Bcr-Abl at several Tyr residues including Tyr-177, a residue that links Bcr-Abl to the Ras signaling pathway (210, 218). Furthermore, NaPP1 treatment restored imatinib sensitivity to cells expressing the Hck-T338A mutant but not the wild type kinase. This effect correlated with inhibition of Hck-T338A activity and loss of Bcr-Abl phosphorylation at Tyr-177. In contrast, NaPP1 did not have any effect on cells expressing the wild-type kinase or on

control cells. Taken together, these results establish a cause and effect relationship between Hck over-expression and imatinib resistance and identify Hck as a drug target in the treatment of imatinib-resistant CML where Hck is over-expressed.

3.3 RESULTS

3.3.1 Design of an inhibitor analog-sensitive Hck allele

Crystal structures of various non-receptor tyrosine kinases have shown that within the ATP-binding site lies a small, naturally occurring hydrophobic pocket. The access to this pocket is controlled by a large "gatekeeper residue", typically a Thr, Met, Leu or Phe, which sterically blocks the access of many small molecules to this pocket (349, 354). Based on this observation, numerous groups have successfully applied a kinase inhibitor-sensitization approach to a great number of protein kinase families (such as MAP kinase fus3p; CDK Cdc28; PAK Cla4; Abl; PI3K) by replacing the gatekeeper residue with a smaller residue such as Gly or Ala (295, 386, 387). In particular, replacement of the "gatekeeper residue" in v-Src (I338) to either Gly or Ala rendered it susceptible to inhibition by NaPP1 (388), suggesting this approach would work with Hck.

In this work, we applied this chemical genetic strategy to the development of an Hck kinase variant that is sensitive to inhibition by NaPP1. To do this, we replaced the gatekeeper residue [Thr338; numbering based on crystal structure of human c-Src (62)] by Ala. Figure 17A depicts the Hck crystal structure in complex with PP1 (PDB:1QCF) in which NaPP1 was overlaid onto the crystal coordinates of PP1. Analysis of this structure reveals potential steric

clash between the Thr338 and the naphthyl moiety of NaPP1. Replacement of T338 with Ala generates more space in the catalytic domain, allowing NaPP1 access to the hydrophobic pocket (Figure 17B). Based on the successful generation of analog-sensitive alleles in other Src kinases, we hypothesized that this mutation will render the kinase sensitive to NaPP1 without disrupting normal kinase activity or signaling.

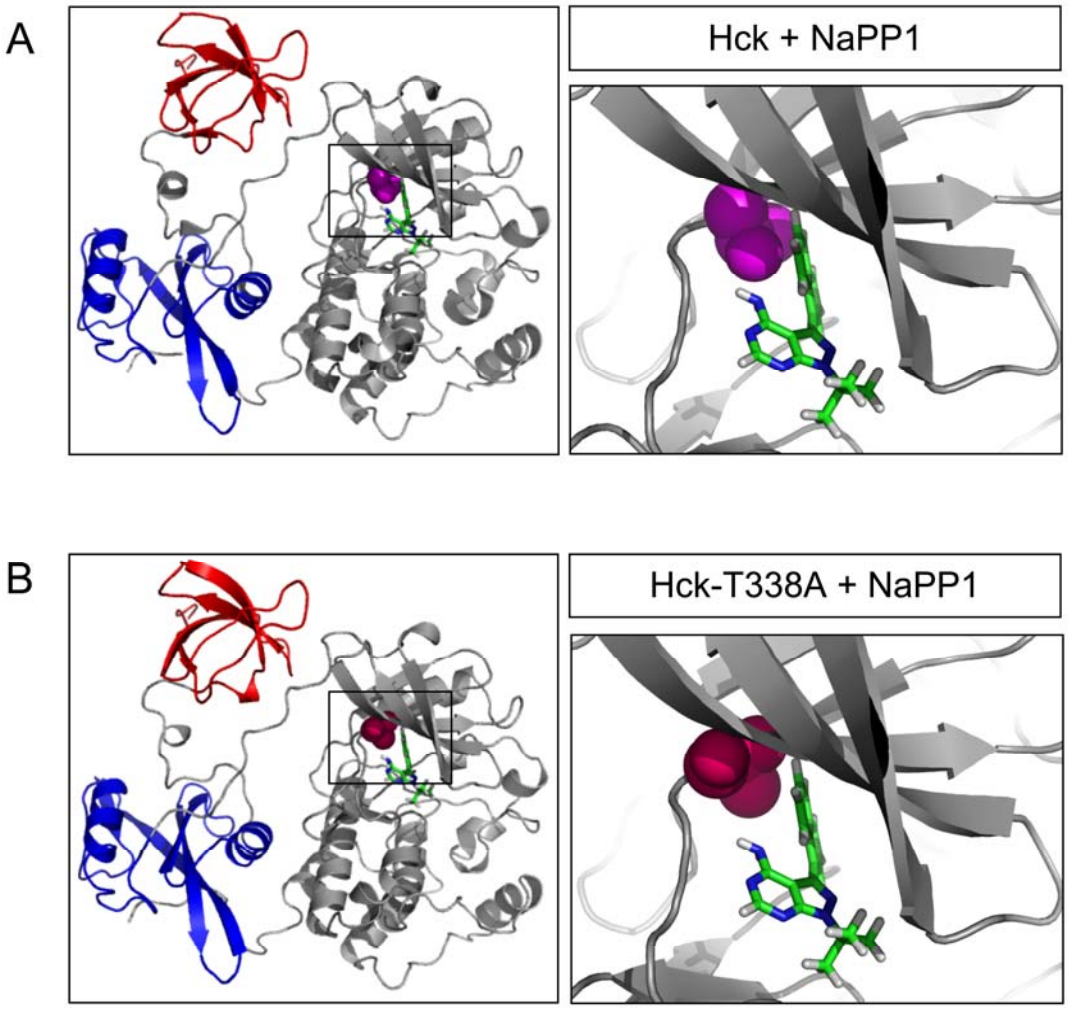

A
Hck + NaPP1
B
Hck-T338A + NaPP1
C
PP1
NaPP1
NH2
N
N
N
N
NH2
N
N
N
N

Figure 17: Generation of the NaPP1-sensitive Hck

(A) Orientation of NaPP1 in the ATP-binding site of Hck [based on the PDB: 1QCF of the crystal structure of Hck in complex with PP1; model created using PyMOL (Delano Scientific, LLC)]. The overall structure of Hck is shown in the left panel, with the SH3 domain in red, SH2 domain in blue, and the kinase domain in grey. The gatekeeper residue (Thr338) is highlighted in magenta. The spatial coordinates of the pyrazolo-pyrimidine ring of PP1 (not shown) were used to manually model the position of NaPP1 (shown as green sticks) within the ATP-binding site. The right panel shows a closer view of the NaPP1 position relative to the gatekeeper residue. This view shows that the naphthyl ring of NaPP1 clashes with the side chain of the gatekeeper residue, which may account for the lack of NaPP1 sensitivity of the wild type kinase. (B) The left panel represents Hck-T338A mutant expected to be sensitive to NaPP1. The T338A mutation was modeled in the Hck structure, and the Ala residue is highlighted in red. The right panel shows a close-up of NaPP1 orientation relative to the gatekeeper mutation. Note that the T338A mutation creates a space adjacent to the naphthyl moiety of NaPP1, which accounts for the NaPP1 sensitivity. (C) Chemical structures of the general SFK inhibitor PP1 and the modified analog, NaPP1.

3.3.2 Replacement of Thr338 with Ala renders Hck sensitive to NaPP1

To test whether the T338A mutation induces sensitivity to the bulky PP1 analog, NaPP1, the wild-type and T338A forms of Hck were expressed as recombinant proteins in Sf9 insect cells and purified to homogeneity. To facilitate the purification of these proteins in the downregulated conformation, we modified the natural C-terminal tail sequence, Y(527)QQQP, to Y(527)EEIP (here referred to as Hck-YEEI mutant). The YEEI modification promotes autphosphorylation of the tail Tyr527 in the absence of Csk, and increases affinity for the SH2 domain (63, 389). Thus, the YEEI tail variant of Hck does not require Csk to adopt the downregulated conformation, enabling high-yield purification from Sf9 cells without the need for Csk co-expression (63, 82). The recombinant purified Hck-YEEI and Hck-T338A-YEEI protein kinases were then assayed in vitro with a peptide substrate in the presence or absence of NaPP1. As shown in Figure 18A,

NaPP1 potently inhibited the Hck-T338A-YEEI mutant with an IC_{50} of 26.6 nM +/- 1.26 nM. In contrast, the NaPP1 IC_{50} for Hck-YEEI was almost 50-fold higher (1.3 μM +/- 0.39 μM). Importantly, NaPP1 did not inhibit a recombinant purified form of the c-Abl tyrosine kinase core consisting of the N-terminal cap (Ncap) region, the SH3 domain, the SH2 domain, and the kinase domain (referred to hereafter as "Ncap-c-abl").

One potential problem concerning the introduction of the gatekeeper mutation in the ATP-binding site of Hck is cross-sensitization to imatinib. To rule out this possibility, we also tested Hck-T338A sensitivity to imatinib in the in vitro kinase assay using the same set of purified recombinant kinases. As shown in Figure 18A, imatinib did not inhibit the activity of wild-type Hck or the Hck-T338A mutant, further supporting the validity of this approach. In contrast, imatinib inhibited the Ncap-c-Abl with an IC_{50} of 350 nM +/- 53 nM, consistent with previous work (390).

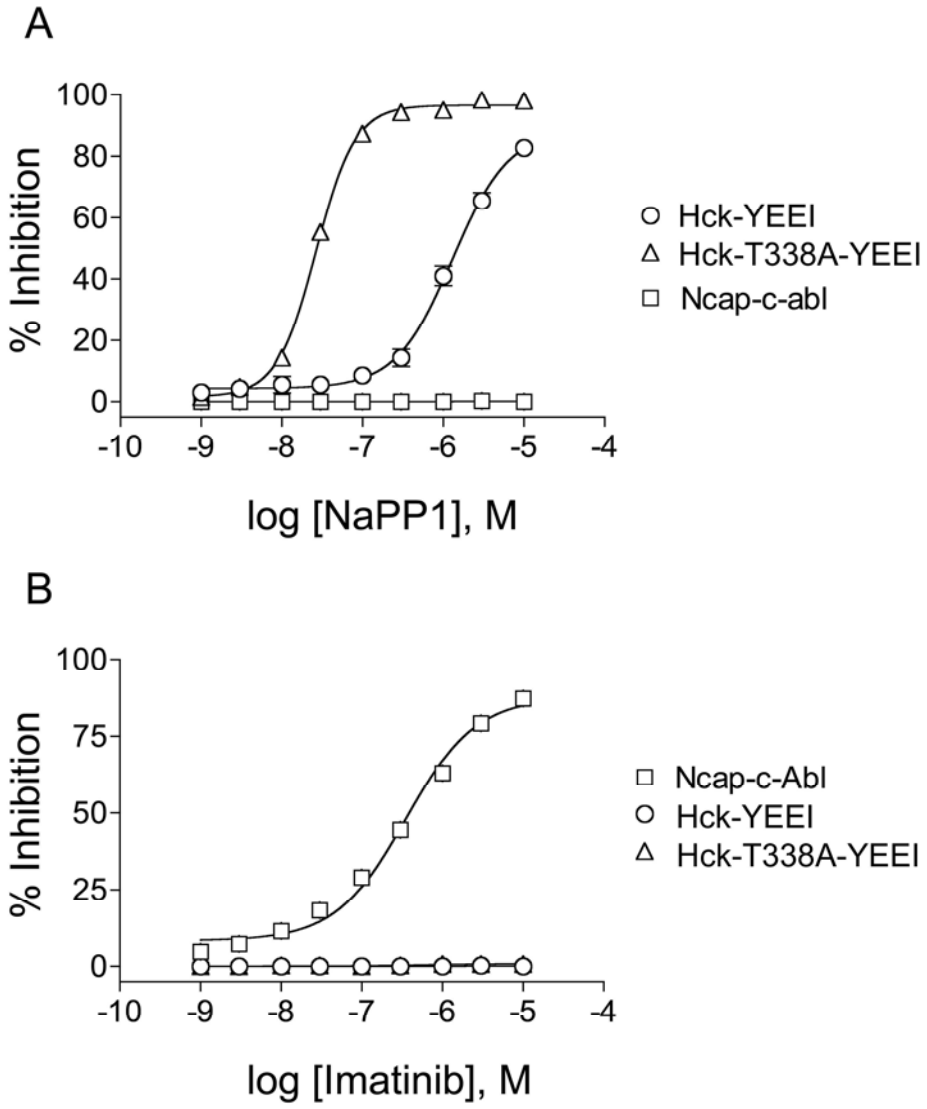


Figure 18: Hck-T338A mutant is sensitive to NaPP1 but not to imatinib in an in-vitro kinase assay

Recombinant wild type Hck-YEEI, Hck-T338A-YEEI, and Ncap-c-abl were purified to homogeneity from Sf9 insect cells. Kinase activity in the presence of NaPP1 (A) or imatinib (B) was assessed in vitro using a FRET-based assay with a peptide substrate. Representative experiments are shown and the percent of inhibition is expressed as mean ± S.D. from the results of four assay wells per condition. To obtain IC_{50} values, data were best fit using non-linear regression analysis. The entire experiment was repeated twice and produced comparable results.

3.3.3 NaPP1 potently and selectively inhibits Hck-T338A in a fibroblast transformation assay

To determine whether the Hck-T338A mutant is sensitive to NaPP1 in a cell-based assay, we tested the ability of NaPP1 to reverse transformation of fibroblasts by an active form of Hck-T338A. For these experiments, we replaced the C-terminal Tyr-527 with a Phe residue (hereafter referred to as an YF mutation) in both the wild-type and the T338A mutant. This mutation was previously shown to prevent tail Tyr527 phosphorylation by Csk and SH2 domain engagement, resulting in a marked increase in the kinase activity and oncogenic transformation of fibroblasts (84-86). The resulting Hck-YF and Hck-T338A-YF mutants were expressed in Rat-2 fibroblasts and their transforming potential and sensitivity to NaPP1 was compared in a soft-agar colony formation assay. Fibroblasts expressing wild-type Hck, Hck-T338A, or the G418 selection marker were used as negative controls. As shown in Figure 19A, neither wild-type Hck nor the T338A mutant induced fibroblast transformation, demonstrating that Ala substitution at the gatekeeper position does not result in kinase activation. In contrast, both Hck-YF and Hck-T338A-YF had robust and comparable transforming activity, demonstrating that the T338A mutation does not interfere with the biological functions of the kinase in this system. Hck activity in each of the Rat-2 cell lines was assessed by anti-pTyr immunoblotting of lysates, and the results are presented in Figure 19B. Both Hck-YF and Hck-T338A-YF showed strong constitutive activity in this assay, demonstrating both autophosphorylation and phosphorylation of endogenous substrates such as pp40 (390). In contrast, Hck or Hck-T338A showed very little kinase activity, consistent with their lack of transforming function (Figure 19B).

We next assessed the specificity of NaPP1 for Hck-T338A-YF in this system. As shown in Figure 19A, NaPP1 blocked the transforming activity of Hck-T338A-YF with an IC_{50} of ~ 0.1

μM. This effect correlated with inhibition of Hck-T338A-YF activity as assessed by anti-pTyr immunoblotting (Figure 19B). Conversely, neither colony formation nor kinase activity were affected by NaPP1 in cells transformed by Hck-YF, even at concentrations as high as 1 μM (Figures 19A and B). These results establish that the T338A mutation is functionally silent and confers NaPP1 sensitivity on Hck in a cell-based assay.

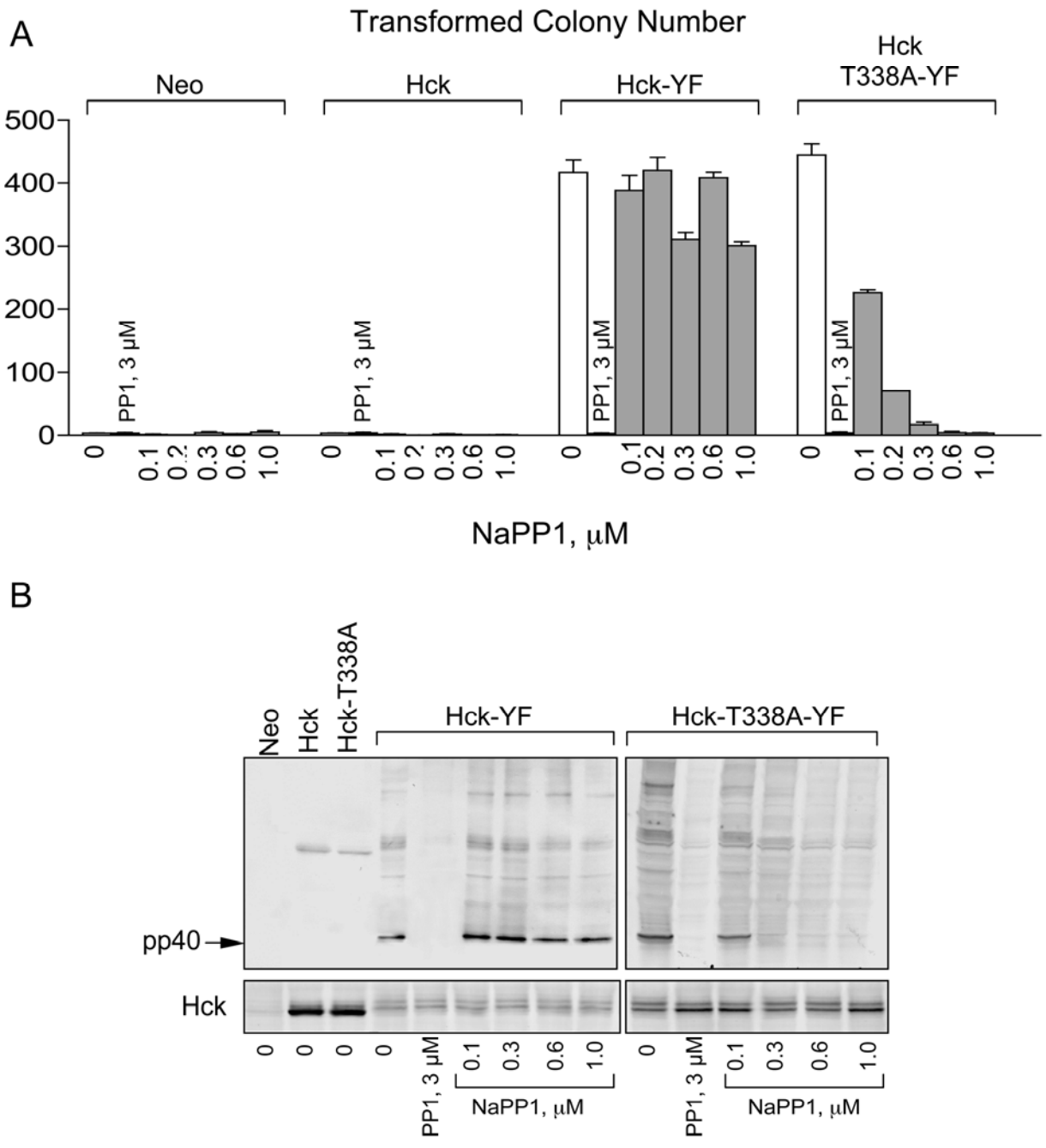

A
Transformed Colony Number
Neo
Hck
Hck-YF
Hck T338A-YF
500
400
300
200
100
0
PP1, 3 μM
0
0.1
0.2
0.3
0.6
1.0
NaPP1, μM
B
Neo
Hck
Hck-T338A
Hck-YF
Hck-T338A-YF
pp40
Hck
PP1, 3 μM
NaPP1, μM

Figure 19: Hck-T338A is biological active in fibroblasts and selectively sensitive to NaPP1

Rat-2 fibroblasts were infected with recombinant retroviruses carrying a neomycin selection marker (vector control), wild-type Hck, Hck-T338A, Hck-YF or Hck-T338A-YF and selected with G418. (A) The stable cell lines were plated in triplicate in soft-agar in the presence of the indicated concentrations of NaPP1. The general SFK inhibitor and parent compound PP1 (3 μM) was used as a positive control. Transformed colonies were stained with MTT after 10 to 14 days. The soft-agar plates were scanned and the number of colonies was determined using the BioRad imaging densitometry system and QuantityOne colony-counting software. Results from a representative experiment are shown as the mean number of colonies of three plates ± S.D. The experiment was repeated two times with similar results. (B) Control or Rat-2 fibroblasts transformed by Hck-YF or Hck-T338A-YF were plated overnight with the indicated concentrations of NaPP1. Lysates were probed with a general anti-phosphotyrosine antibody to determine the phosphorylation status of pp40, an endogenous substrate of Hck. As a loading control, Hck expression levels were determined in replicate blots using an anti-Hck antibody. One representative experiment is shown.

3.3.4 Over-expression of Hck and Hck-T338A protects K562 CML cells from imatinib-induced apoptosis and inhibition of colony formation

Previous studies have shown that blast crisis CML patients that develop resistance to imatinib display increased Hck and/or Lyn kinase expression levels or activities in the absence of mutation in Bcr-Abl (218, 219, 314). To determine whether Hck overexpression in CML cells is sufficient to induce imatinib resistance, Hck and Hck-T338A were expressed in K562 cells using recombinant retroviruses. Cells infected with a retrovirus carrying only the neomycin resistance marker served as a negative control. Following selection with G418, the K562-neo, K562-Hck and K562-Hck-T338A cell populations were treated with increasing concentrations of imatinib for 72 h. The percent of apoptotic cells were then determined by anti-phosphatidyl serine antibody staining and flow cytometry. As shown in Figure 20, imatinib induced apoptosis in a

dose-dependent manner in the K562-neo cell population, with apoptotic cells evident with as little as 0.3 μM imatinib. Strikingly, over-expression of wild-type Hck or Hck-T338A in K562 cells was sufficient to reverse the apoptotic effects of imatinib at both 0.3 and 1 μM ($p \leq 0.02$).

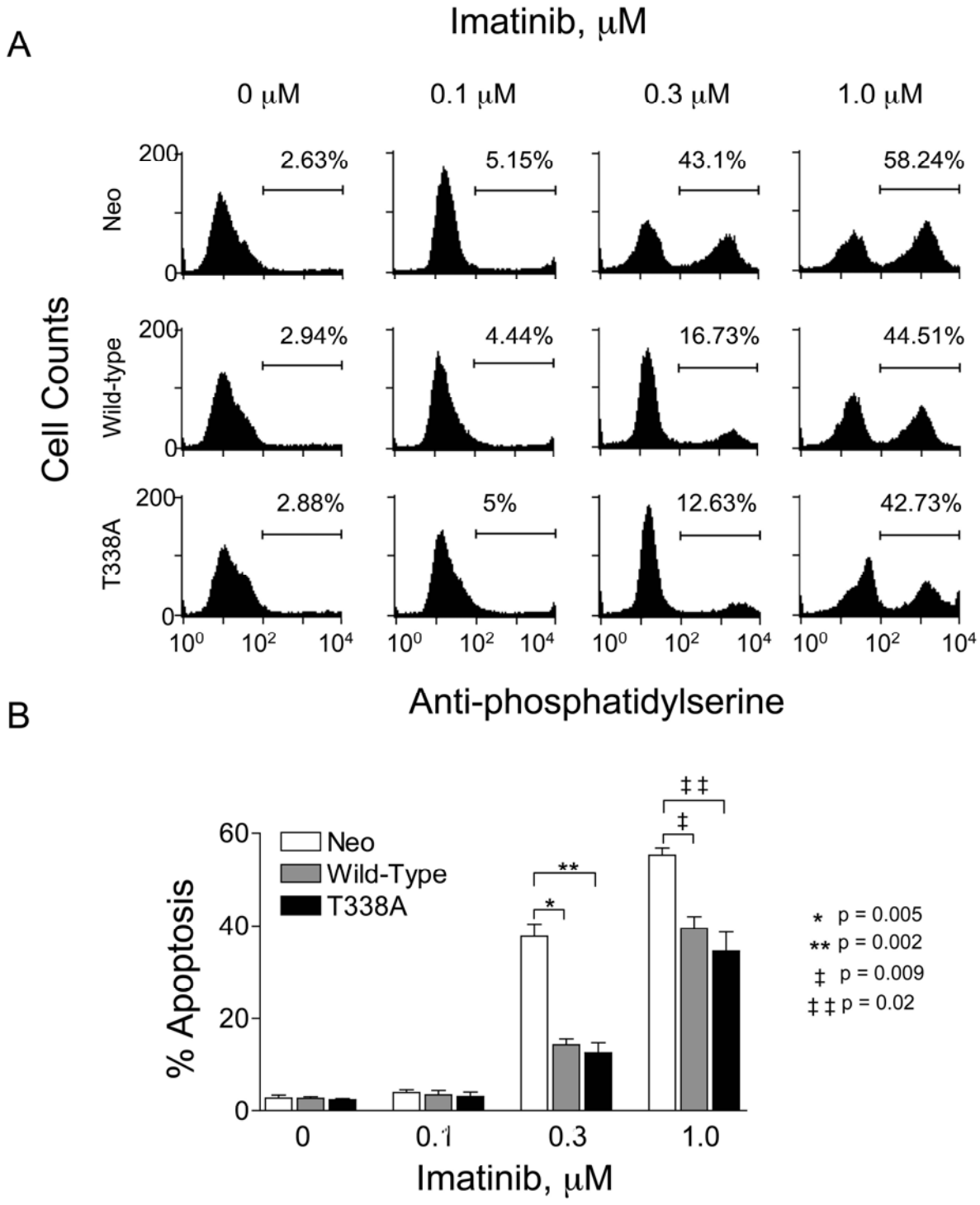

A
Imatinib, μM
0 μM
0.1 μM
0.3 μM
1.0 μM
Neo
Wild-type
T338A
2.63%
5.15%
43.1%
58.24%
2.94%
4.44%
16.73%
44.51%
2.88%
5%
12.63%
42.73%
Cell Counts
200
0
10^0
10^2
10^4
Anti-phosphatidylserine
B
% Apoptosis
60
40
20
0
Neo
Wild-Type
T338A
0
0.1
0.3
1.0
Imatinib, μM
* p = 0.005
** p = 0.002
‡ p = 0.009
‡‡ p = 0.02

Figure 20: Expression of wild-type Hck or Hck-T338A protects K562 cells from imatinib-induced apoptosis

K562-neo, K562-Hck and K562-Hck-T338A cell populations were incubated for 72 h in the absence or presence of the indicated concentrations of imatinib. Apoptotic cells were stained with an anti-phosphatidylserine-Alexa Fluor 488 conjugated antibody and the percentage of apoptotic cells was determined by flow cytometry. (A) Histograms from a representative flow cytometry experiment with the percentage of apoptotic cells shown above each plot. (B) Bar graph showing the average of three independent experiments ± S.D. The difference between K562-neo and K562-Hck or between K562-neo and K562-Hck-T338A was statistically significant as demonstrated by a two-tailed Student's *t*-test at 0.3 μM and at 1 μM imatinib ($p \leq 0.02$). No statistically significant difference was obtained between K562-Hck and K562-Hck-T338A at any of the imatinib concentrations tested.

As a second measure of biological activity, we next investigated whether expression of Hck in K562 cells reverses imatinib-induced inhibition of colony formation. To address this issue, K562-Hck, K562-Hck-T338A, and K562-neo cells were plated in soft-agar in the presence of the DMSO vehicle control (0 μM) or increasing concentrations of imatinib. As shown in Figure 21, K562 cells expressing wild-type Hck or Hck-T338A yielded a comparable number of colonies as the vector control cells when plated in soft-agar in the absence of imatinib. This suggests that expression of Hck or Hck-T338A alone does not enhance the basal level of K562 cell colony-forming activity. Imatinib induced a dose-dependent inhibition of colony formation in all three cell lines. However, cells expressing wild-type Hck or Hck-T338A displayed statistically significant resistance to imatinib-induced inhibition of colony formation when compared to control cells. More specifically, 0.03 μM imatinib reduced the number of K562-neo colonies by nearly 50%, while it did not have a statistically significant impact on cells expressing either form of Hck ($p \leq 0.01$). Furthermore, K562-Hck and K562-Hck-T338A were three times more resistant to 0.1 μM imatinib than K562-neo cells in this assay ($p \leq 0.01$). Taken together,

results from both the apoptosis and colony growth assays demonstrate that overexpression of Hck is sufficient to induce resistance to imatinib.

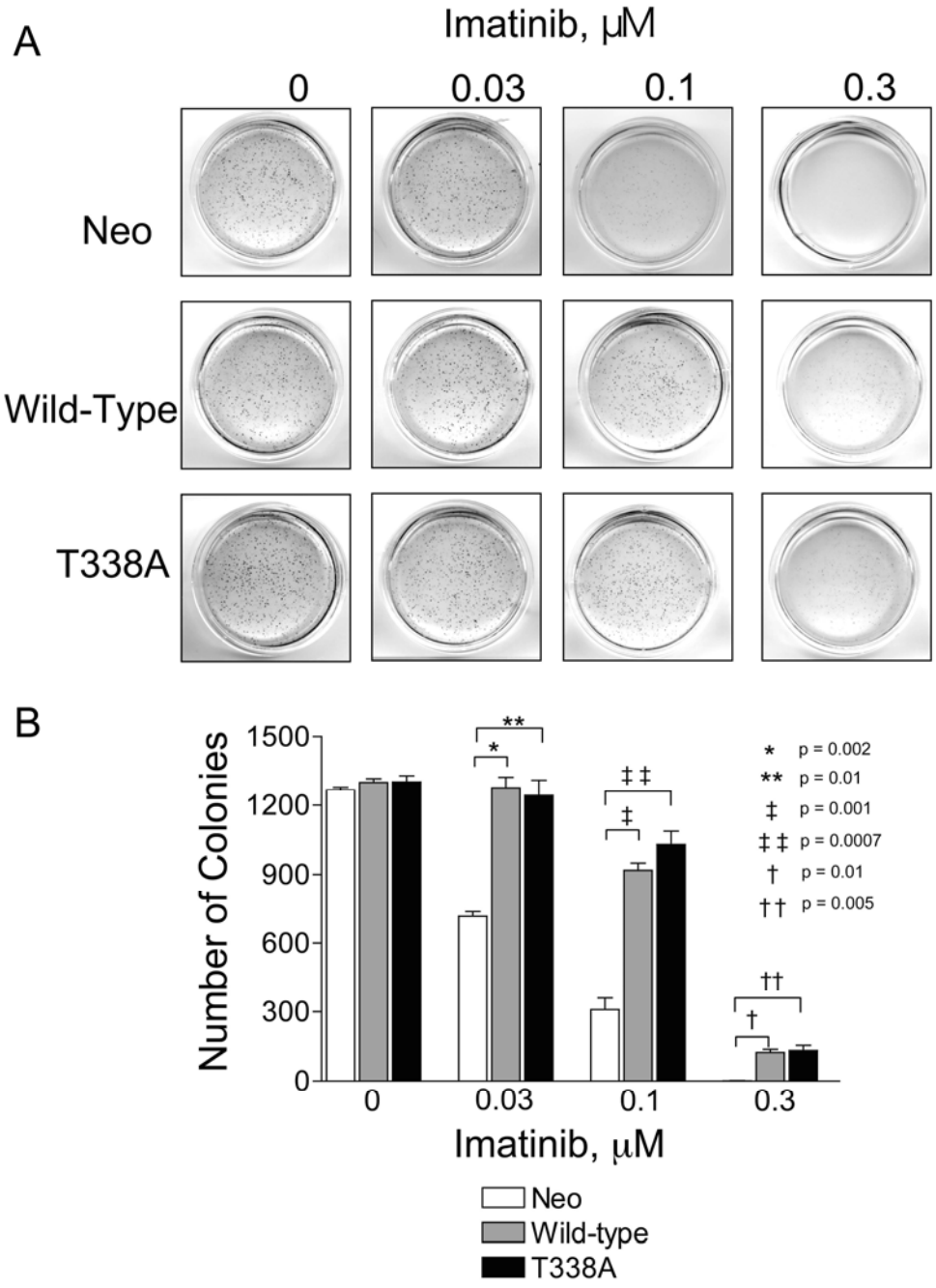

A
Imatinib, μM
0
0.03
0.1
0.3
Neo
Wild-Type
T338A
B
Number of Colonies
1500
1200
900
600
300
0
0
0.03
0.1
0.3
Imatinib, μM
Neo
Wild-type
T338A
* p = 0.002
** p = 0.01
‡ p = 0.001
‡‡ p = 0.0007
† p = 0.01
†† p = 0.005

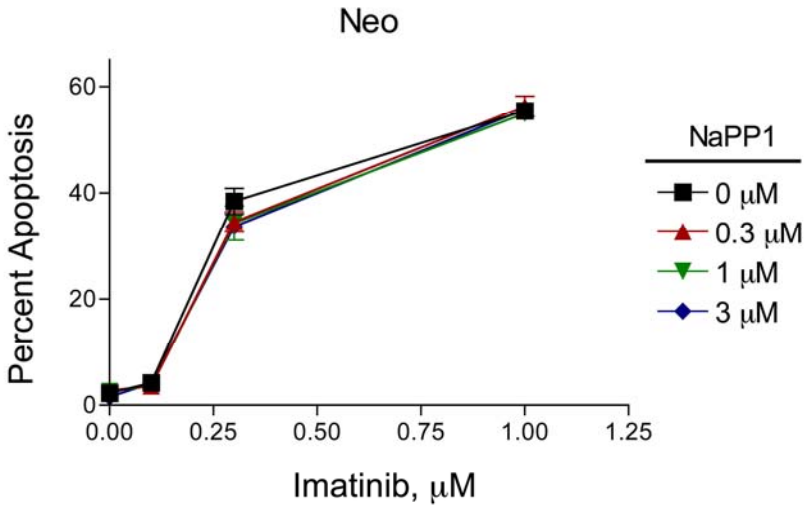
Figure 21: Expression of wild-type Hck or Hck-T338A protects K562 cells from imatinib-induced inhibition of soft-agar colony formation

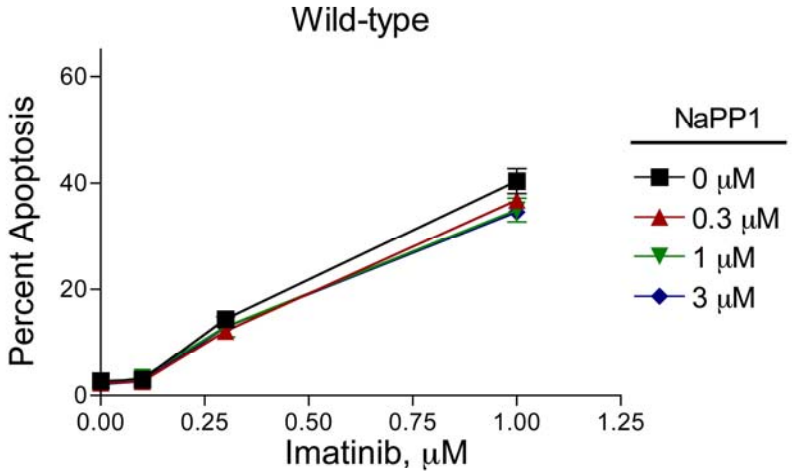
K562-neo, K562-Hck, and K562-Hck-T338A cells were plated in triplicate in soft-agar in the presence of the indicated concentrations of imatinib and incubated for 7-10 days to allow colony growth. At the end of the incubation period, the soft-agar plates were scanned. (A) Representative plates showing the soft-agar colony growth in the presence of imatinib. (B) Plates were imaged and colony counts were determined using the BioRad QuantityOne colony-counting software. The bargraph shows the mean of three independent experiments ± S.D. The difference between K562-neo and K562-Hck or between K562-neo and K562-Hck-T338A was statistically significant by two-tailed Student's *t*-test at 0.03 μM, 0.1 μM and 0.3 μM imatinib ($p \leq 0.01$). No statistically significant difference was obtained between K562-Hck and K562-Hck-T338A at any of the imatinib concentrations.

3.3.5 Resistance to imatinib-induced apoptosis and inhibition of colony growth is dependent on Hck kinase activity

To address the hypothesis that the resistance to imatinib requires Hck kinase activity, we tested whether specific inhibition of Hck-T338A with NaPP1 restored sensitivity to imatinib. For this experiment, K562-neo, K562-Hck and K562-Hck-T338A cells were incubated with imatinib over the same concentration range as in Figure 20 (0 to 1 μM) in the presence of escalating doses of NaPP1 (0 to 3 μM). After 72 h, cells were stained with an anti-phosphatidyl serine antibody and the percentage of apoptotic cells was determined by flow cytometry. The results presented in Figure 22 show the apoptotic response to imatinib plotted for each concentration of NaPP1. Similar to the results presented in Figure 20C, K562-Hck and K562-Hck-T338A cells displayed resistance to imatinib-induced apoptosis when compared to K562-neo control cells. Addition of NaPP1 to K562-neo cells as well as cells expressing wild-type Hck did not affect the level of apoptosis induced by imatinib, demonstrating that NaPP1 alone is not cytotoxic and does not

affect wild-type Hck at these concentrations. In contrast, addition of NaPP1 to K562-Hck-T338A cells induced a dose-dependent reversal of resistance to imatinib to a level similar to K562-neo control cells, suggesting that resistance to imatinib-induced apoptosis requires the kinase activity of Hck.


Neo
Percent Apoptosis
60
40
20
0
0.00
0.25
0.50
0.75
1.00
1.25
Imatinib, μM
NaPP1
0 μM
0.3 μM
1 μM
3 μM


Wild-type
Percent Apoptosis
60
40
20
0
0.00
0.25
0.50
0.75
1.00
1.25
Imatinib, μM
NaPP1
0 μM
0.3 μM
1 μM
3 μM

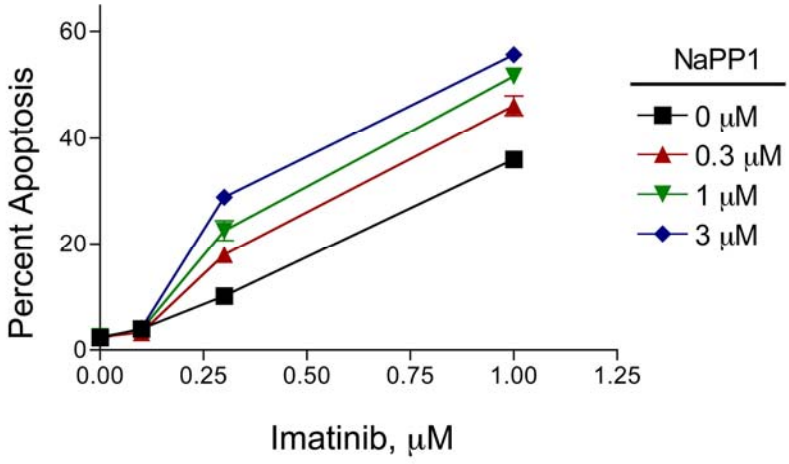

T338A
Percent Apoptosis
60
40
20
0
0.00
0.25
0.50
0.75
1.00
1.25
Imatinib, μM
NaPP1
0 μM
0.3 μM
1 μM
3 μM

Figure 22: Specific inhibition of Hck-T338A kinase activity using NaPP1 restores the apoptotic response to imatinib of K562-Hck-T338A cells

K562-neo, K562-Hck, and K562-Hck-T338A were incubated with the indicated combinations of imatinib and NaPP1 for 72 h. The concentration range for NaPP1 was from 0 to 3 μM, and for imatinib from 0 to 1 μM. Apoptotic cells were stained with an anti-phosphatidylserine-Alexa Fluor 488 conjugated antibody and the percentage of apoptotic cells was determined by flow cytometry. The response to imatinib was plotted for each individual NaPP1 concentration. Each point represents the means generated from three independent experiments ± S.D.

Next, we determined whether NaPP1 resensitizes K562-Hck-T338A cells to imatinib in the soft-agar colony assay. To address this issue, each K562 cell population was plated in soft agar with combinations of imatinib (0 to 0.3 μM) and NaPP1 (0 to 3 μM) and incubated for 7 days to allow colony formation. Imatinib-induced inhibition of colony formation was determined for each NaPP1 concentration, and the results are plotted in Figure 23. In addition, colony formation IC_{50} values for imatinib were determined by sigmoidal-dose response curve fitting. Imatinib inhibited K562-neo colony formation with an IC_{50} of 35.9 nM ± 6.8 nM in the absence of NaPP1, and this value was unaffected by NaPP1 treatment. Overexpression of wild-type Hck or Hck-T338A in K562 cells increased the IC_{50} value for the inhibition of colony formation by imatinib by a factor of four, to 135 nM ± 11 nM and 146 nM ± 17.7 nM, respectively. Similar to the K562-neo control cells, NaPP1 did not affect the inhibition of colony formation by imatinib in K562 cells expressing wild-type Hck, consistent with the lack of NaPP1 activity against the wild-type kinase. In contrast, addition of NaPP1 to K562-Hck-T338A cells induced a dose-dependent reversal of the inhibition of colony formation IC_{50} for imatinib, from 146 nM ± 17.7 nM to 32 nM ± 6.6 nM, a value close to that observed with the K562-neo control cell population.

These results demonstrate that selective inhibition of Hck-T338A kinase activity restores sensitivity to imatinib in terms of colony-forming activity as well.

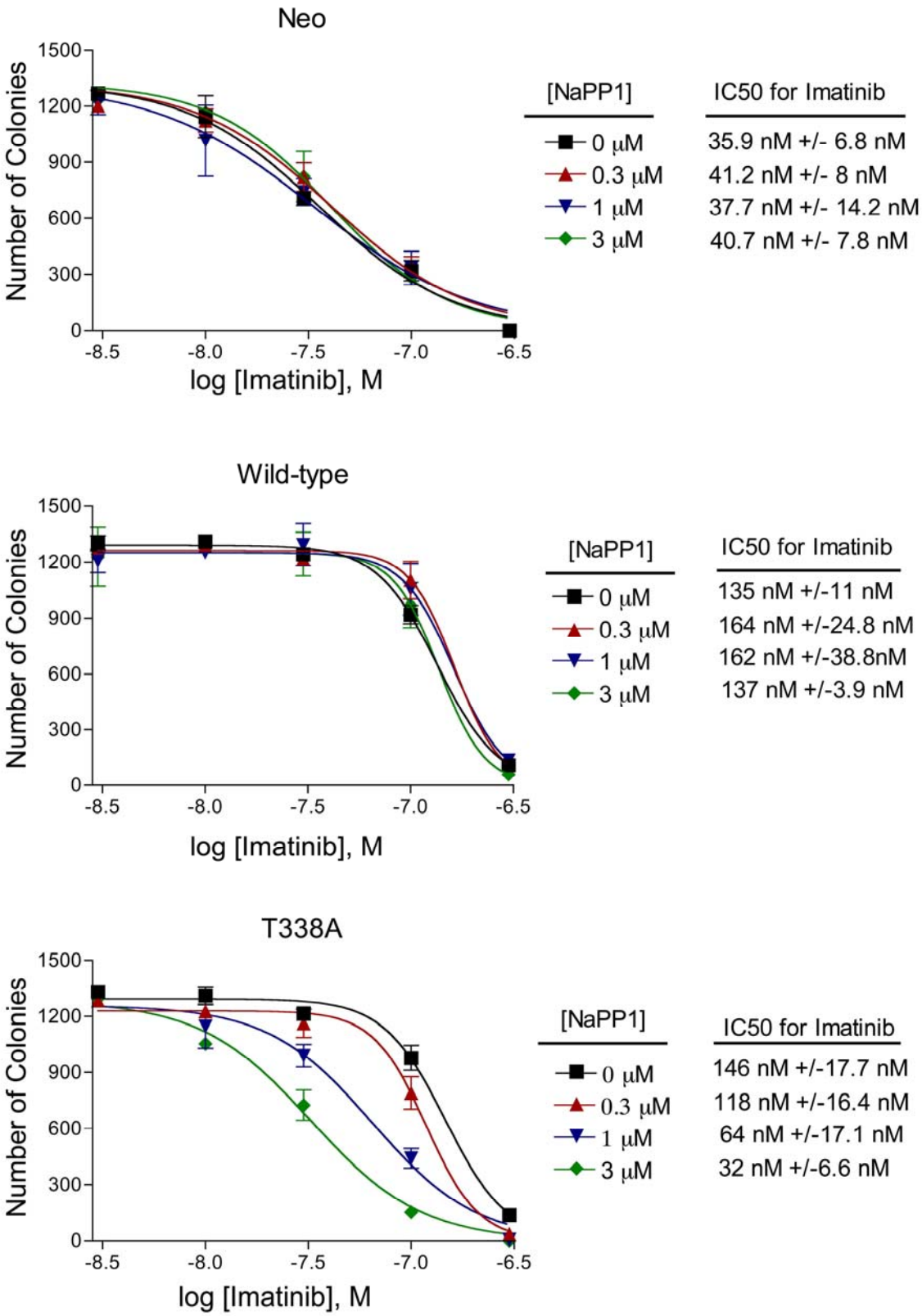

Neo
Number of Colonies
1500
1200
900
600
300
0
-8.5
-8.0
-7.5
-7.0
-6.5
log [Imatinib], M
[NaPP1]
0 μM
0.3 μM
1 μM
3 μM
IC50 for Imatinib
35.9 nM +/- 6.8 nM
41.2 nM +/- 8 nM
37.7 nM +/- 14.2 nM
40.7 nM +/- 7.8 nM
Wild-type
Number of Colonies
log [Imatinib], M
[NaPP1]
0 μM
0.3 μM
1 μM
3 μM
IC50 for Imatinib
135 nM +/-11 nM
164 nM +/-24.8 nM
162 nM +/-38.8nM
137 nM +/-3.9 nM
T338A
Number of Colonies
log [Imatinib], M
[NaPP1]
0 μM
0.3 μM
1 μM
3 μM
IC50 for Imatinib
146 nM +/-17.7 nM
118 nM +/-16.4 nM
64 nM +/-17.1 nM
32 nM +/-6.6 nM

Figure 23: Specific inhibition of Hck-T338A kinase activity using NaPP1 restores the sensitivity to imatinib of K562-Hck-T338A cells in soft-agar assay

K562-neo, K562-Hck, and K562-Hck-T338A cells were plated in triplicate in soft-agar in the presence of the indicated combinations of imatinib and NaPP1. Colonies were stained 10-14 days later, and were counted using scanned images of the plates and the BioRad QuantityOne colony-counting software. The entire experiment was repeated twice from independently derived cell populations and yielded similar results. A representative experiment is shown. Each point represents the average of three plates ± S.D. The response to imatinib at each NaPP1 concentration was determined by curve-fitting using non-linear regression analysis and IC_{50} determination. The IC_{50} values are displayed to the right of each plot.

To determine whether the reversal of imatinib sensitivity was associated with inhibition of Hck-T338A tyrosine kinase activity, Hck was immunoprecipitated from cells following exposure to the same imatinib concentrations used in the apoptosis and colony assays in combination with 3 μM NaPP1 (Figure 24). Immunoblot analysis using an antibody specific to phospho-Tyr416 in the activation loop of the active form of Hck was used to determine the activation status of Hck. The results show that 3 μM of NaPP1 completely inhibited the T338A mutant of Hck consistent with the complete reversal of imatinib sensitivity observed at this concentration. In contrast, NaPP1 did not inhibit the activity of wild-type Hck kinase. In addition, to confirm the specificity of NaPP1, we also immunoprecipitated Lyn and performed immunoblot analysis with anti-phospho-Tyr-416 antibody as a measure of Lyn activation. Figure 24B shows that 3 μM NaPP1 did not affect Lyn activation loop phosphorylation at any of the imatinib concentrations.

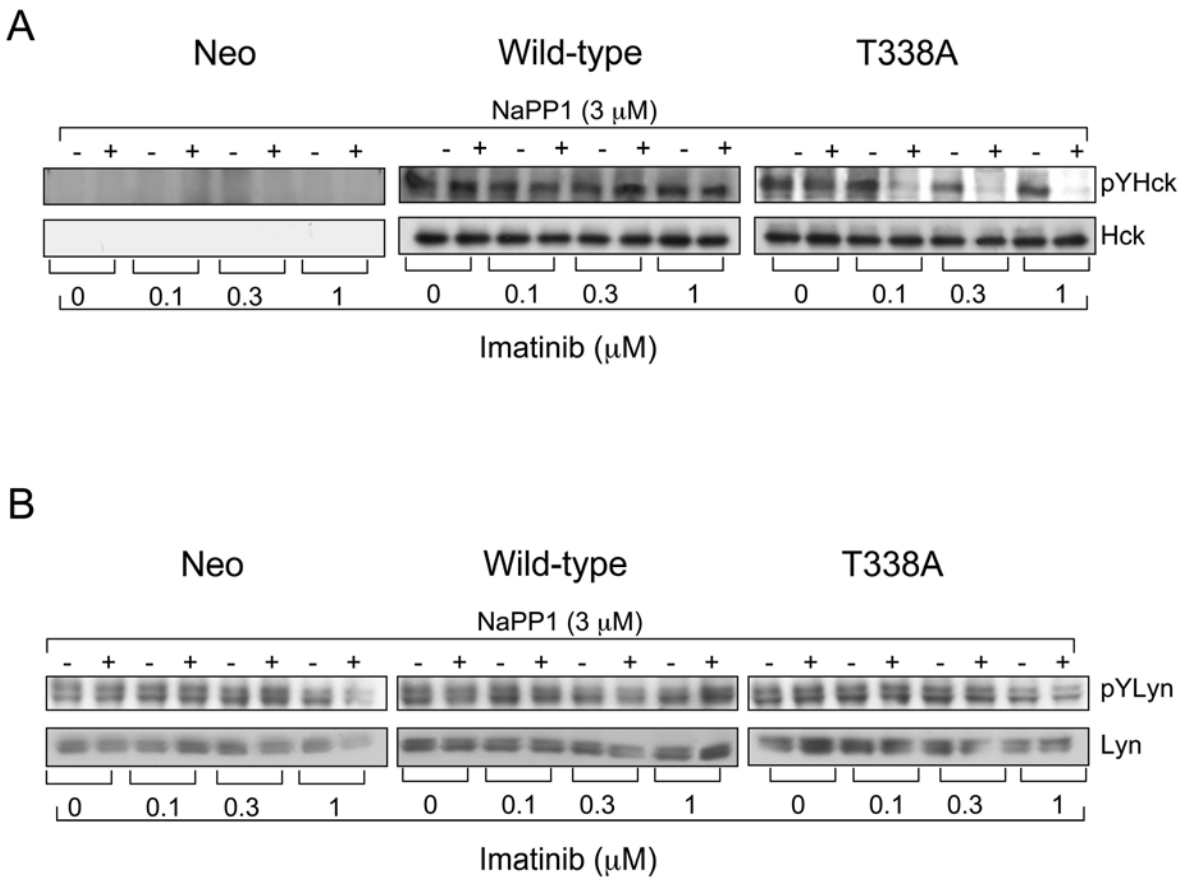


Figure 24: NaPP1 specifically inhibits Hck-T338A mutant in K562 cells

K562-neo, K562-Hck, and K562-Hck-T338A cells were plated in 0.5% FBS overnight and treated with the indicated concentrations of imatinib and NaPP1 for 5 hours. Hck (A) or Lyn (B) were immunoprecipitated from clarified lysates and immunoblotted with an anti-Src phosphospecific pY416 antibody that recognizes the tyrosine-phosphorylated activation loop of Hck and Lyn. Duplicate membranes were blotted for Hck and Lyn as a loading control. This experiment was repeated twice from independent cell lines, and representative blots are shown.

3.3.6 Hck or Hck-T338A overexpression in K562 cells is associated with increased phosphorylation of Bcr-Abl at multiple Tyr residues

Previous work from our laboratory and others has shown that SFKs trans-phosphorylate c-Abl or Bcr-Abl at various residues. This may in turn induce an active conformation and thus directly decrease sensitivity to imatinib, and may also increase the activity of some Bcr-Abl-dependent signaling pathways. For example, c-Src and Hck were shown to phosphorylate c-Abl at Tyr-412, and to induce imatinib resistance (367). Moreover, Hck and other SFKs were shown to phosphorylate Bcr-Abl at Tyr-89 in the SH3 domain and at Tyr-245 in the SH2:kinase linker region in vitro (194). Phosphorylation at both these residues was predicted to induce activation due to disruption of intra-molecular negative regulatory interaction between the SH3 domain and the SH2: kinase linker. However, subsequent dynamics analysis using hydrogen-deuterium exchange mass spectrometry suggests that phosphorylation of Tyr-89 but not Tyr-245 disrupts this interaction (195). Furthermore, Hck also phosphorylates Bcr-Abl in the Bcr-portion of the protein at Tyr-177, a residue that links Bcr-Abl to Ras activation and is required for leukemogenesis (229-232). In addition, work presented in Chapter 2 using Hck-T338M, a mutant resistant to the global SFK-inhibitor A-419259, suggests that Hck along with other Src kinases contributes to trans-phosphorylation of Bcr-Abl at Tyr-89, 245, and 177, but it does not affect Tyr-412 in K562 cells.

To test whether the resistance to imatinib observed in response to Hck overexpression correlates with phosphorylation of these Bcr-Abl Tyr residues, we performed immunoblotting with phosphospecific antibodies on K562-neo, K562-Hck, and K562-Hck-T338A cell lysates following treatment with a combination of imatinib and NaPP1. Figure 25 shows that in the absence of imatinib, the relative levels of phosphorylation at Tyr-89 and Tyr-245 increased in

response to the expression of either wild-type Hck or Hck-T338A. In addition, the blots from K562-neo control cells show phosphorylation at these residues is significantly inhibited at 0.1 μM imatinib, while K562-Hck and K562-T338A cells display an increased phosphorylation at 0.1 μM imatinib (in the case of pY245), and even at 0.3 μM imatinib in the case of pY89. Furthermore, addition of 3 μM NaPP1 inhibited the phosphorylation of these residues in cells expressing Hck-T338A when added with 0.1 μM imatinib (for pY245) or with 0.3 μM imatinib (for pY89). This suggests that the increase in phosphorylation at Tyr-89 and Tyr-245 is mediated by Hck and requires the Hck kinase activity. Although increased phosphorylation at Tyr-89 is expected to induce an increased activity of Bcr-Abl, this was not correlated with an increase in the phosphorylation of CrkL, a Bcr-Abl substrate. Therefore, the significance of Tyr-89 and Tyr-245 phosphorylation by Hck in the context of imatinib resistance remains unclear.

In addition to Tyr-89 and Tyr-245, we also determined how expression of Hck affects Tyr-177 phosphorylation in the Bcr-derived portion of the oncoprotein. As shown in Figure 25, Bcr-Abl from K562-Hck and K562-Hck-T338A cells demonstrate persistent phosphorylation at this residue even in the presence of 0.3 and 1 μM imatinib, while phosphorylation of Tyr-177 in control cells is completely inhibited at these imatinib concentrations. In addition, co-incubation with NaPP1 inhibits Bcr-Abl Tyr-177 phosphorylation in K562-Hck-T338A but not K562-Hck cells. Since phosphorylation of Tyr-177 creates a binding site for Grb2 and induces increased Ras signaling (210, 218), it is possible that Hck expression may restore Ras-dependent anti-apoptotic signaling that contributes to imatinib resistance.

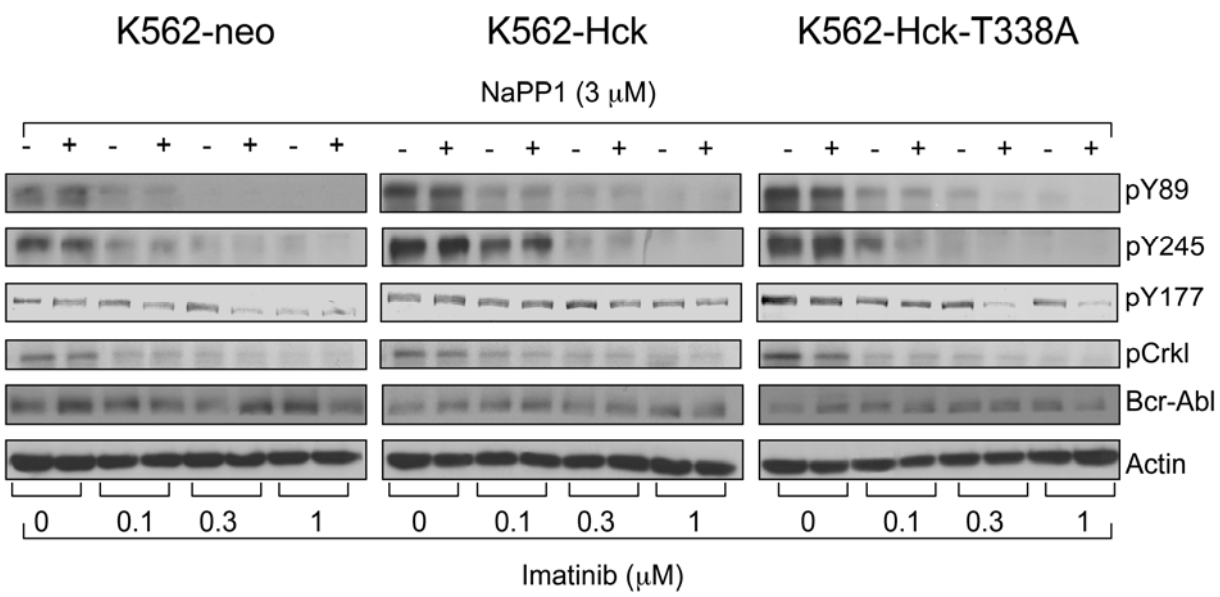


Figure 25: Wild-type Hck or Hck-T338A overexpression increases phosphorylation of Bcr-Abl at various Tyr residues in an Hck-kinase dependent manner.

K562-neo, K562-Hck, and K562-Hck-T338A cells were plated in 0.5% FBS overnight and treated with the indicated concentrations of imatinib and NaPP1 for 5 hours. Cell lysates were resolved on SDS-PAGE gels and immunoblotted with phosphospecific antibodies for Abl pY245 and pY89, Bcr pY177, and for CrkL pY207. As loading controls, duplicate blots were also probed with anti-actin and anti-Abl (for Bcr-Abl expression levels). The experiment was repeated twice from independent cell lines. Representative blots are shown.

3.4 DISCUSSION

Resistance to imatinib, the primary line of treatment for CML patients, has typically been associated with persistence or reactivation of Bcr-Abl signaling or due to selection for drug-resistant Bcr-Abl mutants. However, emerging evidence show that other mechanisms of imatinib

resistance in absence of Bcr-Abl mutations also have significant clinical importance. Consistent with a growing body of evidence implicating the Src family kinases in various phases of CML, recent evidence has linked Hck and Lyn overexpression or enhanced activation to imatinib resistance in the absence of Bcr-Abl mutations(218, 219, 314, 331). In this report, we investigated whether Hck overexpression is sufficient to induce resistance to imatinib and established a direct cause and effect relationship between Hck overexpression and resistance to imatinib in a CML cell line with wild-type Bcr-Abl.

To address this problem, we hypothesized that overexpression of Hck in CML cells would create resistance to imatinib. To be able to demonstrate that this imatinib resistance is due to the kinase activity of Hck, we employed a chemical genetic method to generate an Hck allele (Hck-T338A) that is uniquely targeted by NaPP1, a bulky analog of the global SFK inhibitor PP1. The advantage of this chemical genetic approach is that it allows for specific inhibition of Hck activity while leaving the Hck protein and its complexes intact. Thus data derived using this approach is more directly applicable to drug discovery than gene expression knockdown by siRNA or other approaches. Since the original description of this method by Shokat's group (388), this approach has been successfully applied to both serine/threonine and tyrosine kinases from diverse families (386).

As part of the Hck-T338A validation strategy, we first needed to establish that this inhibitor analog-sensitizing mutant did not result in a loss- or gain-of-function and that it was selectively sensitive to inhibition by NaPP1 but not affected by imatinib. To address these issues we used both in vitro and cell-based assays. We observed that the Hck-T338A mutant maintains its catalytic activity against an artificial substrate in an in-vitro kinase assay (Figure 18A). When expressed in fibroblasts as the active form Hck-T338A-YF, it induces Rat-2 fibroblast oncogenic

transformation. Moreover, as shown in Figure 19B, expression of Hck-T338A-YF induced phosphorylation of pp40, a transformation-related endogenous Hck substrate (95-97). Taken together, these results show that the T338A mutation is well tolerated and does not induce a loss of the catalytic activity of Hck.

Replacement of the corresponding gatekeeper residue with Ala in other receptor or non-receptor tyrosine kinases such as c-Abl, c-Src or PDGFRB were recently shown to have mild activating effects when expressed in the human embryonic kidney cell line 293T as shown by the increased tyrosine phosphorylation of total cellular protein (310). However, these mild activating effects were not sufficient to transform murine BaF3 myeloid cells to IL-3 independence (310). In the case of Hck, evidence presented in this report suggests that the replacement of the gatekeeper residue to Ala does not induce a gain-of-function in Hck. For example, expression of Hck-T338A in Rat-2 fibroblasts did not induce oncogenic transformation, while the active Hck-T338A-YF induced Rat-2 transformation at levels similar to those observed with Hck-YF with a wild-type kinase domain (Figure 19A). In addition, neither wild-type Hck nor Hck-T338A expression in Rat-2 fibroblasts induced tyrosine phosphorylation of total cellular lysates, as shown in Figure 19B. Lastly, K562-Hck-T338A cells did not form a higher number of colonies in soft-agar when compared to K562-Hck cells (Figure 19A). Although these data indicate that Hck-T338A does not exhibit gain-of-function activity, our experiments do not rule out the possibility that the gatekeeper mutation induces a gain-of-function undetectable by our end-point assays such as a change of substrate specificity.

We next evaluated the sensitivity of Hck-T338A to NaPP1, and found that this gatekeeper substitution made Hck almost 50 times more sensitive to NaPP1 than the wild-type kinase in vitro (Figure 18A). In addition, mutation of the gatekeeper residue to a less bulky

amino acid did not induce cross-sensitivity to imatinib (Figure 18B). Importantly, NaPP1 did not inhibit the recombinant Abl kinase core, consistent with the specificity of this compound for the Hck-T338A mutant (Figure 18A). Furthermore, NaPP1 inhibited Hck-T338A-YF-, but not Hck-YF-induced transformation of Rat2 fibroblasts (Figure 19A). Consistent with the inhibition of colony formation, NaPP1 inhibited Hck-T338A-YF activity, but not Hck-YF (Figure 19B).

Having validated the NaPP1-sensitive mutant, we next showed that Hck or Hck-T338A overexpression in CML cells is sufficient to cause increased survival and colony- forming activity in the presence of imatinib (Figures 20 and 21). Colony growth in soft agar was strongly affected, with the IC_{50} value for inhibition of colony growth increased by 4-fold upon overexpression of either form of Hck in K562 CML cells (Figure 23). The resistance to imatinib induced by Hck-T338A overexpression was completely reversed by NaPP1 in a concentration-dependent manner in cells expressing Hck-T338A, but not in cells expressing the wild-type Hck (Figure 22 and 23). The reversal of imatinib sensitivity in terms of survival and colony growth correlated with a complete inhibition of Hck-T338A activity by NaPP1 treatment, whereas wild-type Hck activity was unaffected (Figure 24). These results show that the imatinib resistance induced by Hck overexpression is specifically dependent upon Hck kinase activity.

Hck-induced resistance to imatinib may occur through a feedback mechanism that induces an active conformation of Bcr-Abl tyrosine kinase domain and therefore imatinib resistance, or through Bcr-Abl-kinase independent mechanisms. Using phospho-specific antibodies, we present evidence that Hck expression induces a slight increase in phosphorylation of Tyr-89 and Tyr-245 in the Abl core, and that phosphorylation at these residues is reversible with NaPP1. Recent work has shown that phosphorylation at Tyr-89, which localizes to the RT-loop of the SH3 domain, disrupts its intra-molecular interaction with the SH2: kinase linker and

was predicted to promote an active conformation of the Abl core (195). Whether or not Hck-mediated phosphorylation of SH3 Tyr-89 in the context of Bcr-Abl contributes to the imatinib resistance observed here is not clear. The lack of increased phosphorylation of the Bcr-Abl substrate, CrkL, in the presence of Hck suggests that Tyr-89 phosphorylation does not markedly upregulate Bcr-Abl tyrosine kinase activity. In contrast to Tyr-89, our results also show an increase in Tyr-177 phosphorylation that is strongly reversed by NaPP1 in K562-Hck-T338A cells but not in cells expressing wild-type Hck. Since phosphorylation of Tyr-177 facilitates Ras signaling (210, 218), enhanced phosphorylation of this site may stimulate mitogenic and anti-apoptotic pathways downstream of Ras that contribute to increased growth and survival of Hck-expressing K562 cells in the presence of imatinib.

Although this study addressed the possible feedback effects of Hck on Bcr-Abl activity or signaling that may account for imatinib resistance, Bcr-Abl-independent mechanisms may play a role as well. For example, recent evidence shows that Bcr-Abl activates autocrine IGF-1 signaling through Hck and Stat5b (222). Thus, overexpression of Hck may induce an increase in autocrine IGF-1 signaling. In other experimental systems, IGF-1 was shown to have antiapoptotic effects due to ß-catenin stabilization (391). Therefore, it is possible that the Hck-induced imatinib resistance observed here may be due in part to an increase in IGF-1-induced anti-apoptotic effects. In addition, several SFKs have been shown to phosphorylate the cytoplasmic domain of Muc-1, a transmembrane glycoprotein overexpressed in CML. Phosphorylation of this Muc-1 region promotes the binding, stabilization, and increased nuclear targeting of ß-catenin (223, 224). Determination of whether Hck-mediated stabilization of β-catenin activation through either of these pathways leads to imatinib resistance will require further investigation.

Although significant, the degree of Hck-induced imatinib resistance reported in this study can be overcome by increasing the concentration of imatinib. In CML patients, therefore, therapeutically attainable concentrations imatinib (up to 5 μM) may override this resistance mechanism. However, if the Hck-induced imatinib resistance mechanism is independent of Bcr-Abl, then simply increasing the concentration of imatinib would not eliminate the resistant cell population. On the contrary, imatinib exposure may exert a selective pressure that could lead to the expansion of cells in which this resistance mechanism is predominant. Furthermore, in a clinical setting, patients may progress via clonal evolution and thus more than one molecule may be involved in imatinib resistance. The recent observation that both Lyn and Hck are activated in patients resistant to imatinib in the absence of Bcr-Abl mutations supports this hypothesis.

In addition, experiments in this study were performed in K562, a cell line derived from a CML patient in blast crisis. Since progression to blast crisis is accompanied by additional genetic abnormalities, it is possible that these cells are already equipped with the necessary changes in the signaling pathways that can support Hck-induced imatinib resistance. Whether or not Hck overexpression would have a similar effect in cells derived from patients in chronic phase is not clear. Future experiments will address this important possibility as well.

Given the occurrence of imatinib resistance, various second-generation tyrosine kinase inhibitors, such as nilotinib, have been developed to override this phenomenon. In an attempt to study the potential mechanisms of nilotinib-resistance, a recent report describes the generation of a nilotinib-resistant K562 cell line by exposure to gradually increasing concentrations of the drug (392). In this cell line, nilotinib resistance was linked to Lyn upregulation. In addition, an increase in Lyn mRNA expression was found in 2 out of 7 patients that developed resistance to nilotinib (392). This report suggests that SFK upregulation may represent a common mechanism

of resistance to Bcr-Abl inhibitors in addition to selection of drug-resistant Bcr-Abl kinase-domain mutants. This observation also suggests that patients developing imatinib resistance without Bcr-Abl mutations may also be cross-resistant to other Bcr-abl inhibitors. In this context, it would be interesting to determine whether Hck overexpression also induces resistance to nilotinib.

In sum, the results presented in this report demonstrate for the first time that overexpression of Hck is sufficient to confer imatinib resistance to CML cells derived from a blast crisis patient, and that this mode of resistance requires Hck kinase activity. In addition, using chemical genetic principles, we have created a cell-based model system that allows for specific and temporal inhibition of Hck kinase activity. Previously, such experiments were hindered by the lack of specific inhibitors for individual SFK members. Additional studies of the mechanisms by which Hck induces imatinib resistance will improve the understanding and therapy for advanced stage CML. Along these lines, inhibitors selective for Hck may be of benefit in imatinib–resistant CML.

3.5 MATERIALS AND METHODS

3.5.1 Cell Culture

Rat-2 fibroblasts were purchased from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin (Antibiotic-Antimycotic, Invitrogen). Sf9 insect cells were cultured in Grace's medium (Gibco) supplemented with 10% FBS and 50 μg/ml gentamicin

(Gibco). The K562 myeloid leukemia cell line, derived from a CML patient in blast crisis (373), was obtained from the ATCC and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), and Antibiotic-Antimycotic (Invitrogen).

3.5.2 Protein purification

Using site-directed mutagenesis, the Thr-338 to Ala (T338A) mutation was introduced into coding sequence of human p59 Hck-YEEI (QuikChange XL Site-directed Mutagenesis Kit, Stratagene) (86). The Hck-YEEI and Hck-T338A-YEEI constructs also contained N-terminal hexahistidine tags. The Ncap-c-Abl construct encompasses residues 1-531 of human c-Abl-1b with residues 15-56 deleted, and contains a C-terminal cleavage site for the tobacco etch virus (TEV) protease and a hexahistidine tag, both introduced by PCR. Hck-YEEI, Hck-T338A-YEEI and the Ncap-c-abl constructs were cloned into pVL1392 (BD Biosciences) and each plasmid was used to create high-titer recombinant baculovirus in Sf9 insect cells using Baculogold DNA and the manufacturer's protocol (BD Biosciences). For protein production, Sf9 cells (1 L) were grown in suspension in Grace's medium (Invitrogen) supplemented with 10% FBS and 50 µg/ml gentamycin. Sf9 cells were cultured to a density of 2×10^6 cells/ml and then infected with either Hck-YEEI or Hck-T338A-YEEI baculoviruses. For the Ncap-c-Abl construct, Sf9 cells were co-infected with Abl and YopH phosphatase baculoviruses at a multiplicity of infection of 10. YopH is a protein-tyrosine phosphatase that promotes a downregulated conformation of Ncap-c-Abl that permits high-yield purification from Sf9 cells (188). Sf9 cells were grown for 48 h, centrifuged, washed in PBS, and then the pellets were resuspended in buffer A [20 mM Tris-HCl (pH 8.3), 10% glycerol, and 5 mM ß - mercaptoethanol], lysed by sonication, and centrifuged at 16,000 rpm for 30 min. The recombinant Hck or Ncap-c-Abl proteins were purified from the

supernatant using a combination of ion exchange and affinity chromatography as originally described by Schindler et al. for Hck (63). Upon purification, the proteins were dialyzed against 20 mM Tris-HCl (pH 8.3) containing 100 mM NaCl and 3 mM DTT.

3.5.3 In vitro kinase assay

Kinase assays were performed using the FRET-based Z'-Lyte Src kinase assay kit and Tyr-2 peptide substrate according to the manufacturer's instructions (Invitrogen). All assays were performed in quadruplicate in low volume, non-binding 384-well plates (Corning). The assay was first optimized to determine the amount of each kinase and the incubation time necessary to phosphorylate 50-60% of the Tyr-2 peptide in the absence of inhibitor. Final ATP and Tyr-2 substrate concentrations were held constant at 50 μM and 2 μM, respectively. For inhibition experiments the kinases were pre-incubated with NaPP1 or imatinib in kinase assay buffer for 30 min, followed by incubation with ATP and Tyr-2 peptide for 1 h. Fluorescence was assessed on a Gemini XS microplate spectrofluorometer (Molecular Devices). IC_{50} values were calculated from the means of 4 wells and using a sigmoidal curve fit and Prism software (GraphPad Software, Inc).

3.5.4 Rat-2 fibroblasts transformation assays

Using site-directed mutagenesis, the T338A mutation was introduced in the active form of Hck (Hck-YF). The wild-type Hck, Hck-T338A, Hck-YF and Hck-T338A-YF constructs were subcloned into the retroviral vector pSRαMSVtkneo (375). The resulting constructs were used to generate high-titer retroviral stocks by co-transfection of 293T cells with an ecotropic packaging

vector. Control retroviruses were prepared using the parent pSRαMSVtkneo vector. Rat-2 fibroblasts were infected as follows: 2.5 x 10^4 Rat-2 cells were plated per well in 6-well plates and incubated with viral stocks in a final volume of 5 ml in the presence of polybrene (4 μg/ml final concentration). To enhance infection efficiency, the plates were centrifuged at 3,000 rpm for 4 h at 18 °C. Following infection, the virus was replaced with fresh medium. To obtain stable cell lines, G418 (800 μg/ml) selection was started 48 h after infection and continued for 14 days. At the end of the selection period, the G418 concentration was decreased at 400 μg/ml.

3.5.5 Retroviral transduction of K562 leukemia cells

Wild-type Hck and the Hck-T338A mutant were subcloned into the retroviral expression vector pMSCV-IRES-neo (Clontech) between the MSCV promoter and IRES sequence. Retroviral stocks were produced from the resulting constructs in 293T cells using an amphotropic packaging vector as described abovefor Rat-2 cells. K562 cells were plated in 6 well plates at 1 x 10^6 cells/ well in 5 ml of undiluted viral supernatant in the presence of 4 μg/ml polybrene, and centrifuged at 3000 rpm for 3 h at room temperature. After infection, cells were washed, returned to regular medium for 48 h and then placed under G418 selection (800 μg/ml) for 14 days. At the end of the selection period, cells were maintained in medium with 400 μg/ml G418.

3.5.6 Soft-agar colony assays

Soft-agar fibroblast transformation assays were performed in triplicate in 35 mm Petri dishes (Falcon) using Seaplaque Agarose (FMC Bioproducts). One ml of 0.5% bottom agarose in complete culture medium was poured in the presence of either vehicle (0.5% DMSO) or NaPP1

at twice the final desired concentration. After the hardening of the bottom layer, 1 x 10^4 Rat-2 cells were mixed in culture medium containing 0.3% agarose and 1 ml was poured onto the bottom layer. Seven to ten days later, the colonies were stained with MTT and the soft-agar plates were scanned and quantitated using colony counting software (BioRad QuantityOne).

K562 leukemia cells were plated in soft-agar as described above at a cell density of 2 x 10^3 cells/plate and in the absence or presence of imatinib or of combinations of NaPP1 and imatinib. Staining of the colonies with MTT was performed after 7 days of incubation. Colony counting was performed as described above.

3.5.7 Apoptosis assay

Apoptosis was determined by measuring cell-surface phosphatidyl serine (PS) using an Alexa Fluor 488-conjugated anti-PS antibody (Upstate Biotechnology) and flow cytometry. Cells (1 x 10^5/ml) were treated with vehicle alone (0.5% DMSO), imatinib or combinations of NaPP1 and imatinib for 72 h at 37°C. Following incubation, cells were centrifuged at 1000 rpm for 5 min, washed three times with ice-cold PBS and resuspended to 4 x 10^6 cells/ml in staining buffer (1% FBS in PBS). Aliquots (50 μl) were transferred to 96-well round bottom tissue culture plates, mixed with the anti-PS antibody (0.21 μg/well), and incubated on ice for 1 h. Cells were washed three times in ice-cold PBS and analyzed using a FACSCalibur flow cytometer (Becton-Dickinson) and CellQuest software.

3.5.8 Antibodies

The following antibodies were used in this study: anti-actin (MAB1501; Chemicon), anti-Hck polyclonal (N-30; Santa Cruz Biotechnology), anti-Hck monoclonal (Transduction Laboratories), anti-Lyn (Santa Cruz Biotechnology), anti-c-Abl (Calbiochem), anti-Src phosphospecific (Src pY-416; Upstate Biotechnology), anti-Bcr phospho-Tyr-177 (Cell Signaling), anti-c-Abl phospho-Tyr-89 (Cell Signaling), anti-c-Abl phospho-Tyr-245 (Cell Signaling), anti-CrkL phospho-Tyr-207 (Cell Signaling), and anti-phosphotyrosine (PY-99; Santa Cruz).

3.5.9 Immunoprecipitation and immunobloting

Hck expression and activation in Rat-2 fibroblasts was analyzed by plating 5 x 10^5 cells in 100 mm dishes in the presence of NaPP1 or vehicle control (0.5% DMSO). After incubation at 37 °C overnight, cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with the protease inhibitors aprotinin (25 μg/ml), leupeptin (25 μg/ml) and PMSF (1 mM) and the phosphatase inhibitors NaF (10 mM), and Na_3VO_4 (1 mM). Total protein concentration in clarified cell lysates was measured using the Bradford assay (Pierce). Aliquots of total protein were heated directly in SDS sample buffer and resolved by SDS-PAGE, transferred by PVDF membranes and blotted with either anti-Hck to detect Hck protein expression or anti-phosphotyrosine antibodies for the detection of phosphorylated pp40, an endogenous Hck substrate. To check the levels of Hck, Bcr-Abl or actin or to check the phospho-tyrosine content of Bcr-Abl using phospho-specific antibodies in K562 cells, 5 x 10^6 cells were incubated overnight in 0.5% FBS, and then treated for 5 h with vehicle

control (DMSO, 0.5%) or with imatinib and/or NaPP1. At the end of the incubation period, the cells were collected by centrifugation, washed twice with PBS and lysed in ice-cold RIPA buffer and processed as above. For Hck or Lyn immunoprecipitation, protein concentrations were first normalized in lysis buffer, followed by addition of 1 μg of anti-Hck or anti-Lyn antibody and 25 μl of protein G-Sepharose (50% slurry; GE Healthcare Life Sciences). Following incubation for 2 h at 4 °C, immunoprecipitates were washed three times with 1.0 ml of RIPA buffer and heated in SDS sample buffer. Following SDS-PAGE, proteins were transferred to PVDF membranes for immunoblot analysis. Immunoreactive proteins were visualized with appropriate secondary antibody-alkaline phosphatase conjugates and NBT/BCIP colorimetric substrate (Sigma).

3.5.10 Statistical analysis

Data obtained from multiple independent experiments are given as mean ± S.D. values. Statistical comparisons between two cell lines at individual drug concentrations were performed using a two-tailed unpaired Student's t-test (normal distribution and unequal variance). To protect against potential multiplicity problems, for all analyses a p value less than 0.02 was considered statistically significant. Statistical analyses were done using Microsoft Excel.

4.0 OVERALL DISCUSSION

4.1 SUMMARY OF FINDINGS AND SIGNIFICANCE

Prior to this study, extensive work from our laboratory and others showed that SFKs play an unquestionable role in CML pathogenesis as downstream targets and mediators of Bcr-Abl signaling. Typically, due to a lack of small molecule inhibitors specific to individual SFK members, these studies were performed using general SFK inhibitors such as PP1, or A-419259; using SFK dominant negative mutants; or using siRNA technology to selectively decrease the expression of SFK members (211, 215, 221). Although remarkably informative, as a group, these methods were not able to clearly delineate the relative contribution of individual SFKs to Bcr-Abl signaling, or to determine whether they have unique or overlapping functions. Therefore, in the first aim of my thesis, I set out to determine the individual contribution of Hck to Bcr-Abl signaling using chemical genetics.

In addition, in cell-based models of imatinib resistance obtained by prolonged exposure of CML cells to increasing concentrations of imatinib, resistance was shown to be associated with Lyn overexpression while no Bcr-Abl mutations or amplification was detected (219). Furthermore, analysis of clinical samples from CML patients with imatinib resistance but wild-type Bcr-Abl has shown that Lyn and/or Hck are highly activated, suggesting that both kinases may be involved in imatinib resistance (218, 219). Although Lyn's role in imatinib resistance has

been addressed in previous reports, the role of Hck is less understood. Given this lack of information, in the second aim of my thesis I addressed the role of Hck in Bcr-Abl-mutation-independent imatinib resistance.

4.1.1 Hck individual contribution to Bcr-Abl signaling

To directly define the individual contribution of Hck to Bcr-Abl signaling and pathogenesis, I used a chemical genetics method in which I introduced a point mutation at the gatekeeper position in the ATP-binding site of Hck to render it resistant to a general SFK inhibitor, A-419259. This mutant was designed based on the observation that variation at this position is an important structural determinant of kinase sensitivity to small molecule inhibitors (300, 332, 348-350). In addition, a close inspection of the crystal structure of Hck in complex with A-420983, an A-419259 analog suggested that replacement of the gatekeeper residue with Met, a bulky amino acid would induce a steric clash with the inhibitor molecule (Chapter 2). Thus, replacement of the gatekeeper residue Thr338 of Hck with Met resulted in a mutant resistant by more than 30-fold to the broad-spectrum SFK inhibitor, A-419259. In addition, I showed that, with the exception of a subtle decrease in the K_m for ATP, this mutation was silent in terms of kinase function and activity, a feature essential for the utility of this approach. Next, I showed that expression of the Hck-T338M mutant in CML cell lines has a moderate protective effect against the A-419259-induced inhibition of cell proliferation. This suggests that although Hck contributes to Bcr-Abl-induced cell growth, other Src kinases may play a role. Furthermore, I was able to show that Hck-T338M expression fully protects CML cells against the A-419259-induced apoptosis, and this effect correlated with a sustained Stat5 and Erk activation in the presence of the compound. This suggests that Hck plays a non-redundant role in mediating the

anti-apoptotic effects of Bcr-Abl. In addition, using phospho-specific antibodies against pTyr-177, pTyr-89, and pTyr-245 of Bcr-Abl, I showed that although Hck plays a role in phosphorylation of these residues, other Src kinases are likely to be involved as well. Finally, in an effort to show a role for SFKs in survival and proliferation of CML progenitor cells, I showed for the first time that A-419259 induces growth arrest and apoptosis in $CD34^+$ cells isolated from three CML patients, with potency equal to imatinib.

The significance of this study includes the following. (1) This is the first study to delineate the redundant and non-redundant functions of Hck in Bcr-Abl signaling. Based on this study, we can conclude that Hck has a non-redundant function in mediating the anti-apoptotic effects of Bcr-Abl, while it is likely to cooperate with other Src kinases in mediating the proliferative effects of Bcr-Abl, as well as phosphorylation of Bcr-Abl at residues important for oncogenic activity. (2) This study shows for the first time that inhibition of SFKs in primitive $CD34^+$ progenitor cells from CML patients induces apoptosis to an extent similar to imatinib, as measured by caspase activation. On a cautionary note, I was not able to correlate the level of caspase activation with an actual percentage of apoptotic cells in my study due to the small size of each sample. On this note, other laboratories reported that the apoptotic effects of imatinib in CML $CD34^+$ cells are limited to ~ 30 % (393). However, even a moderate apoptotic effect of SFK inhibitors on CML $CD34^+$ cells argues that SFK inhibitors used in combination with other anti-CML therapies may be beneficial for the elimination of CML progenitors. The fact that dasatinib, a dual Src/Abl inhibitor targets more primitive progenitors than imatinib supports this idea (217). In summary, this work validates Hck as a specific target for the development of apoptotic drugs for the treatment of CML and brings more supporting evidence for the role of SFKs in CML pathogenesis.

4.1.2 The role of Hck in Bcr-Abl-mutation-independent imatinib resistance

To establish whether Hck kinase activity is important in Bcr-Abl-mutation-independent resistance to imatinib, I adapted a second chemical genetic approach initially described by Shokat et al. for use with Hck [reviewed in (386)]. To do this, I introduced a space-creating mutation at the gatekeeper position (T338A) to render the kinase uniquely sensitive to NaPP1, a bulky analog of the global SFK inhibitor PP1. As expected, the T338A mutation induced sensitivity to NaPP1 while being silent in terms of Hck function and activity. Then, I expressed wild-type Hck and Hck-T338A mutant in CML cells and showed that it caused resistance to imatinib-induced apoptosis and inhibition of colony growth. Furthermore, sensitivity to imatinib was restored in cells expressing the Hck-T338A mutant upon the addition of NaPP1, demonstrating that imatinib resistance requires Hck kinase activity. In addition, data presented in this study suggests that Hck overexpression induced increased and sustained phosphorylation of Bcr-Abl at Tyr-89, Tyr-245 and Tyr-177, residues previously shown to be important for Bcr-Abl oncogenicity (194, 195). Phosphorylation at Tyr-89 in the SH3 domain of c-Abl was previously proposed to promote kinase activation due to the disengagement of the SH3 domain from the SH2:kinase linker (194, 195). However, I was not able to correlate sustained Tyr-89 and Tyr-245 phosphorylation in the presence of imatinib with enhanced Bcr-Abl activity as determined by the phosphorylation of Bcr-Abl substrate, CrkL. Sustained phosphorylation of Bcr-Abl Tyr-177 was also observed in the presence of imatinib following Hck overexpression, and this phosphorylation event was reversed by NaPP1 addition.

This study showed for the first time a direct cause and effect relationship between Hck overexpression and imatinib resistance in CML cells transformed by wild-type Bcr-Abl. In addition, this Hck-induced imatinib resistance required the kinase activity of Hck as

demonstrated using Hck-T338A and NaPP1. However, I was not able to clearly outline the mechanism of Hck-induced resistance to imatinib. Previous studies in our laboratory and others showed that Hck and other Src kinases such as Lyn cross-phosphorylate Bcr-Abl on residues important in oncogenesis such as Tyr-89, Tyr-245, and Tyr-177 (194, 195). The finding that Hck induces increased and sustained phosphorylation of Bcr-Abl at Tyr-89, Tyr-245 and Tyr-177 is consistent with these studies. However the relevance of Bcr-Abl phosphorylation to the mechanism by which Hck induces resistance to imatinib remains elusive, especially since no sustained of Bcr-Abl activity in the presence of imatinib was observed as a consequence of Hck overexpression. A recent study published by Wu and co-workers shows that in imatinib-resistant cells, Lyn activation mediates a sustained phosphorylation of Bcr-Abl at Tyr-177 (218), and raised the possibility that Lyn prevents full inactivation of Bcr-Abl signaling by retaining critical adaptor protein (Grb2) binding sites on Bcr-Abl (218). Our data shows a similar effect induced by Hck overexpression. Therefore, the observed imatinib resistance may be mediated by increased Bcr-Abl-dependent signaling in the absence of increased Bcr-Abl kinase activity.

Examination of phospho-CrkL blots from various imatinib-resistant patient samples which display increased levels of Lyn and/or Hck activities show a mixed response to imatinib (218, 314). More specifically, some patient samples show sustained phospho-CrkL levels in the presence of imatinib, while in other samples imatinib induces complete CrkL dephosphorylation(218, 314). This suggests that although the resistance to imatinib in all these patients is associated with increased SFK activity, the mechanisms may differ on a case-by-case basis.

The data presented in Chapter 2 show that Hck plays a non-redundant role in mediating the anti-apoptotic effects of Bcr-Abl. In addition, consistent with previous reports in our

laboratory (194, 195), these data suggest that although Hck phosphorylates Bcr-Abl at sites that might help to sustain an active conformation (Tyr-89, Tyr-245), other SFK also contribute to phosphorylation at these sites. Therefore, the moderate level of resistance observed upon Hck overexpression (Chapter 3) suggest that both Hck and Lyn activities are required to induce a level of resistance to imatinib observed in clinical samples. Furthermore, we can speculate that the mixed response to imatinib in terms of CrkL de-phosphorylation observed in clinical samples might be a consequence of the interplay between the relative protein or activity levels of Hck and Lyn.

4.2 UTILITY OF CHEMICAL GENETICS FOR TARGET VALIDATION STUDIES

Protein kinases are one of the largest groups of drug targets, considered to be the second most important after G-protein-coupled receptors (394). Traditionally, pharmacological target validation studies have been carried out using genetic approaches. However, these genetic approaches can be misleading and may fail to identify important targets due to some compensatory changes in related pathways. In addition, kinase-independent functions of genetically deleted proteins might complicate the interpretation of the results (395). In theory, an ideal alternative approach to target validation is the use of small molecule inhibitors to dissect signaling pathways. However, this approach is also problematic since most inhibitors act on multiple kinase targets due to the high degree of homology of the ATP-binding sites of tyrosine kinases. The work presented in this dissertation constitutes an example of how one can avoid these pitfalls by combining two conceptually opposite chemical genetic methods to tease out the involvement of putative druggable targets in a pathological process.

Chemical genetics approaches involve the use of small molecules (hence "chemical") in conjunction with mutations (hence "genetics") at a conserved hydrophobic residue (termed the "gatekeeper") located in the ATP-binding site of a kinase. Replacement of the gatekeeper residue of a kinase of interest with either a bulky or with a smaller amino acid serves as a selectivity filter with respect to the binding of ATP-analog inhibitors. For example, in the case of Hck, replacement of the gatekeeper residue (Thr-338) with Met precludes binding of the general SFK inhibitor A-419259. Conversely, replacement of Thr-338 with Ala induced unique sensitivity of Hck to NaPP1, a bulky analog of the SFK inhibitor PP1. Thus, on one hand, pairing the expression of Hck-T338M mutant with A-419259 in an appropriate cellular context allows the inhibition of all endogenously expressed wild-type SFKs without affecting the activity of Hck-T338M. On the other hand, pairing the expression of Hck-T338A with NaPP1 in an appropriate cellular context (that lacks the wild-type Hck) allows the highly specific inhibition of Hck-T338A activity while all the endogenously expressed wild-type SFKs are not affected. Both of these methods have been previously validated individually for use with other tyrosine kinases. However, when applied in combination to the study of one particular kinase of interest as shown in this thesis, they have the power to provide an increased understanding of the role of a particular kinase in a pathological process. This is why chemical genetics represents a highly valuable method for rational drug target validation studies, with applicability to many different tyrosine kinases and therefore, many different disease states.

4.3 FUTURE DIRECTIONS

4.3.1 Investigate the role of Hck in Bcr-Abl signaling in Hematopoietic Stem Cells (HSC).

The cellular origin of CML begins with formation of the Ph^+ chromosome in the hematopoietic stem cell from where it is subsequently transmitted to all hematopoietic lineages. Multipotent hematopoietic progenitor cells are receptive to Bcr-Abl expression and generate an abnormal expansion of mature myeloid cells (396). Recent studies indicate that despite the impressive success of imatinib, CML patients have a rare but consistently detectable population of Bcr-Abl^+, $CD34^+$ HSC that are not efficiently killed by this drug (346). Dasatinib, a dual Abl/Src inhibitor was shown to inhibit Bcr-Abl in $CD34^+$ CML stem cells more effectively than imatinib, although not completely (217). However, the dasatinib activity spectrum comprises not only Bcr-Abl and Hck but also other SFKs including Fgr, Fyn, Lck, Lyn, and Yes (363) as well as the Kit, PDGFR, and Ephrin receptor tyrosine kinases (364). Many of these kinases have no demonstrated role in CML pathogenesis and therefore, their inhibition might be deleterious to the outcome of the disease or might contribute to side effects associated with long-term drug treatment. In addition, Owen Witte and co-workers showed that Bcr-Abl expression during in-vitro hematopoietic development of embryonic stem cells causes expansion of multipotent and myeloid progenitors (397). To study the involvement of Hck in early Bcr-Abl-induced HSC signaling, one could construct a Hck-T338A homozygous ES cell line and then perform an in vitro differentiation assay that allows Bcr-Abl expression (397). To test whether Hck plays a role in this Bcr-Abl-dependent abnormal expansion of hematopoietic progenitor cells, a two-step in vitro differentiation of ES-Hck-T338A cells in methylcellulose could be performed in the presence or absence of NaPP1. This procedure involves primary differentiation of ES cells into embryoid

bodies (EBs) followed by secondary plating in methylcellulose containing a cocktail of hematopoietic cytokines to form hematopoietic colonies. Bcr-Abl will be transfected in the EB – derived hematopoietic progenitors, and selection started at the time of methylcellulose plating. Hematopoietic progenitors present in the EBs will grow out into discrete hematopoietic colonies that are easily identified in the methylcellulose cultures. Quantitation of these colonies will allow a direct estimation of the number and type of hematopoietic progenitors that result upon Bcr-Abl expression and Hck-T338A inhibition by NaPP1. If Hck is required for the Bcr-Abl-induced abnormal expansion of multipotent and myeloid progenitors (395), then NaPP1 is expected to abolish these Bcr-Abl-dependent effects and induce normal hematopoietic differentiation in ES-Hck-T338A cells.

4.3.2 Further explore the relevance of SFK overexpression/activation in imatinib resistance

Data presented in this dissertation shows that Hck overexpression is sufficient to induce imatinib resistance. However, to further clarify the significance of SFKs in imatinib resistance, additional studies need to be undertaken.

First, although I showed that Hck overexpression is sufficient to induce imatinib resistance in cellular CML models, it is not clear whether Hck can sustain imatinib resistance in vivo. In a previous report, Wu and co-workers used an imatinib resistant K562 cell line overexpressing Lyn to generate a mouse xenograft CML model (314). Mice harboring tumors induced by these cells underwent an initial response to imatinib, however after continual therapy (8 days) tumors in imatinib-treated mice could not be distinguished from tumors in control mice (314). To develop evidence that Hck overexpression is sufficient to create imatinib resistance in

vivo, K562 cells expressing vector alone, wild-type Hck or the Hck-T338A mutant could be subcutaneously administrated in nude mice to generate solid tumors. Based on the results in Chapter 3 of this thesis, mice bearing tumors from K562-Hck or K562-Hck-T338A would be expected to show an initial response to imatinib, followed by a relapse. Furthermore, since in our cell-based experiments resistance to imatinib requires the kinase activity of Hck, administration of NaPP1 along with imatinib is anticipated to decrease the burden of the tumors originated from K562-Hck-T338A cells, due to a reversal of imatinib sensitivity.

Second, the mechanism of Hck-induced imatinib resistance should also be determined. Experiments performed in Chapter 3 suggest that Hck may induce resistance to imatinib by prompting a sustained activation of signaling pathways downstream of Tyr-177 of Bcr-Abl. To test this hypothesis, one could determine whether Hck expression induces a sustained Bcr-Abl/Gab2 association. In addition, the activation of signaling pathways downstream of phospho-Y177, such as the Ras/Erk and PI3K/Akt pathways should also be assessed.

Third, a recent study showed that resistance to nilotinib, a second-generation Bcr-Abl inhibitor is associated with an increased Lyn mRNA levels in CML patients(392). This suggests that in a clinical setting, various Bcr-Abl inhibitors may induce resistance through similar mechanisms and that patients may also display cross-resistance to various Bcr-Abl inhibitors. To determine whether Hck overexpression may induce cross-resistance to nilotinib, the same cell-based system described in Chapter 3 could be used. Thus, to test this hypothesis, one could compare the apoptosis or inhibition of colony growth in response to nilotinib in K562-neo control cells vs. K562-Hck and K562-Hck-T338 cells, and look for kinase-dependence of these effects using NaPP1 as I have successfully demonstrated with imatinib.

4.4 CLOSING REMARKS

Imatinib, the current first-line CML therapy, has demonstrated significant activity in all phases of the disease, due not only to its ability to inhibit Bcr-Abl, but also due to its promiscuous character (314). More specifically, the therapeutic effects of Bcr-Abl are in part associated with its ability to inhibit c-Kit along with Bcr-Abl (295). However, despite the high level of hematologic and cytogenetic responses in clinical trials, patients with advanced-stage CML often develop resistance. These growing levels of resistance to imatinib point to a need for new therapeutic targets. In addition, a recent survey conducted by The Chronic Myelogenous Leukemia Society of Canada (http://www.news-medical.net/?id=43908), showed that imatinib although generally deemed as well tolerated, induced persistent side effects such as nausea, fatigue, headaches, vomiting, edema, and myelosuppression. Moreover, imatinib has been recently shown to be cardiotoxic (http://www.fda.gov/medwatch/safety/2006/safety06.htm#Gleevec). Therefore, new therapies with an increased control on specificity are required since the incidence of side-effects may be associated with the promiscuous character of a drug. To design such dugs, it is important to have a better understanding of which signaling molecules are highly important in a disease process or in imatinib resistance. In this context, the work presented in this dissertation was aimed to refine the importance and role of Hck in Bcr-Abl signaling and imatinib resistance. It is my hope that similar work will be undertaken to evaluate the role of other individual signaling molecules in CML pathogenesis and resistance. This in turn will provide the necessary information for the development of new therapies not only effective in treating imatinib-resistant patients but also with minimal side effects. Recent advances in structure-based drug design methods are promising with regard to their ability to exploit

particularities of protein structures to target away the kinases not important in a pathological process (398).

BIBLIOGRAPHY

1 Rous,P. A sarcoma of the fowl transmissible by an agent separable from the tumor cell. *In*: Anonymous pp. 397-411. 1911.

2 Weiss,S.R., Varmus,H.E. and Bishop,J.M. The size and genetic composition of virus-specific RNAs in the cytoplasm of cells producing avian sarcoma-leukosis viruses, Cell, *12:* 983-992, 1977.

3 Wang,L.H., Duesberg,P.H., Kawai,S. and Hanafusa,H. Location of envelope-specific and sarcoma-specific oligonucleotides on RNA of Schmidt-Ruppin Rous sarcoma virus, Proc.Natl.Acad.Sci.U.S.A, *73:* 447-451, 1976.

4 Stehelin,D., Guntaka,R.V., Varmus,H.E. and Bishop,J.M. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses, J.Mol.Biol., *101:* 349-365, 1976.

5 Stehelin,D., Varmus,H.E., Bishop,J.M. and Vogt,P.K. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA, Nature, *260:* 170-173, 1976.

6 Chong,Y.P., Ia,K.K., Mulhern,T.D. and Cheng,H.C. Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases, Biochim.Biophys.Acta, *1754:* 210-220, 2005.

7 Parsons,S.J. and Parsons,J.T. Src family kinases, key regulators of signal transduction, Oncogene, *23:* 7906-7909, 2004.

8 Levinson,A.D., Oppermann,H., Levintow,L., Varmus,H.E. and Bishop,J.M. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein, Cell, *15:* 561-572, 1978.

9 Collett,M.S. and Erikson,R.L. Protein kinase activity associated with the avian sarcoma virus src gene product, Proc.Natl.Acad.Sci.U.S.A, *75:* 2021-2024, 1978.

10 Abram,C.L. and Courtneidge,S.A. Src family tyrosine kinases and growth factor signaling, Exp.Cell Res., *254:* 1-13, 2000.

11 Serfas,M.S. and Tyner,A.L. Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases, Oncol.Res., *13:* 409-419, 2003.

12 Thomas,S.M. and Brugge,J.S. Cellular functions regulated by Src family kinases, Annu.Rev.Cell Dev.Biol., *13:* 513-609, 1997.

13 Bolen,J.B. and Brugge,J.S. Leukocyte protein tyrosine kinases: potential targets for drug discovery, Annu.Rev.Immunol., *15:* 371-404, 1997.

14 Lock,P., Ralph,S., Stanley,E., Boulet,I., Ramsay,R. and Dunn,A.R. Two isoforms of murine hck, generated by utilization of alternative translational initiation codons, exhibit different patterns of subcellular localization, Mol.Cell Biol., *11:* 4363-4370, 1991.

15 Rider,L.G., Raben,N., Miller,L. and Jelsema,C. The cDNAs encoding two forms of the LYN protein tyrosine kinase are expressed in rat mast cells and human myeloid cells, Gene, *138:* 219-222, 1994.

16 Cooke,M.P. and Perlmutter,R.M. Expression of a novel form of the fyn proto-oncogene in hematopoietic cells, New Biol., *1:* 66-74, 1989.

17 Modafferi,E.F. and Black,D.L. Combinatorial control of a neuron-specific exon, RNA., *5:* 687-706, 1999.

18 Quintrell,N., Lebo,R., Varmus,H., Bishop,J.M., Pettenati,M.J., Le Beau,M.M., Diaz,M.O. and Rowley,J.D. Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells, Mol.Cell Biol., *7:* 2267-2275, 1987.

19 Holtzman,D.A., Cook,W.D. and Dunn,A.R. Isolation and sequence of a cDNA corresponding to a *src*-related gene expressed in murine haemopoietic cells, Proc.Natl.Acad.Sci.USA, *84:* 8325-8329, 1987.

20 Yamanashi,Y., Mori,S., Yoshida,M., Kishimoto,T., Inoue,K., Yamamoto,T. and Toyoshima,K. Selective expression of a protein-tyrosine kinase, p56lyn, in hematopoietic cells and association with production of human T-cell lymphotropic virus type I, Proc.Natl.Acad.Sci.U.S.A, *86:* 6538-6542, 1989.

21 Willman,C.L., Stewart,C.C., Longacre,T.L., Head,D.R., Habbersett,R., Ziegler,S.F. and Perlmutter,R.M. Expression of the c-fgr and hck protein-tyrosine kinases in acute myeloid leukemic blasts is associated with early commitment and differentiation events in the monocytic and granulocytic lineages, Blood, *77:* 726-734, 1991.

22 Golden,A. and Brugge,J.S. The Src Oncogene. *In*: AnonymousThe Oncogene Handbook, pp. 149-173. 1988.

23 Kawakami,Y., Furue,M. and Kawakami,T. Identification of fyn-encoded proteins in normal human blood cells, Oncogene, *4:* 389-391, 1989.

24 Luo,K.X. and Sefton,B.M. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site, Mol.Cell Biol., *10:* 5305-5313, 1990.

25 Dymecki,S.M., Niederhuber,J.E. and Desiderio,S.V. Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells, Science, *247:* 332-336, 1990.

26 Zhao,Y.H., Baker,H., Walaas,S.I. and Sudol,M. Localization of p62 c-yes protein in mammalian neural tissues, Oncogene, *6:* 1725-1733, 1991.

27 Boggon,T.J. and Eck,M.J. Structure and regulation of Src family kinases, Oncogene, *23:* 7918-7927, 2004.

28 Resh,M.D. Myristylation and palmitylation of Src family members: The fats of the matter, Cell, *76:* 411-413, 1994.

29 Resh,M.D. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, Biochim.Biophys.Acta, *1451:* 1-16, 1999.

30 Koegl,M., Zlatkine,P., Ley,S.C., Courtneidge,S.A. and Magee,A.I. Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif, Biochem.J., *303 (Pt 3):* 749-753, 1994.

31 Huang,Y. and Wange,R.L. T cell receptor signaling: beyond complex complexes, J.Biol.Chem., *279:* 28827-28830, 2004.

32 Brown,D.A. and London,E. Functions of lipid rafts in biological membranes, Annu.Rev.Cell Dev.Biol., *14:* 111-136, 1998.

33 Geahlen,R.L., Handley,M.D. and Harrison,M.L. Molecular interdiction of Src-family kinase signaling in hematopoietic cells, Oncogene, *23:* 8024-8032, 2004.

34 Galbiati,F., Guzzi,F., Magee,A.I., Milligan,G. and Parenti,M. Chemical inhibition of myristoylation of the G-protein Gi1 alpha by 2-hydroxymyristate does not interfere with its palmitoylation or membrane association. Evidence that palmitoylation, but not myristoylation, regulates membrane attachment, Biochem.J., *313 (Pt 3):* 717-720, 1996.

35 Paige,L.A., Zheng,G.Q., DeFrees,S.A., Cassady,J.M. and Geahlen,R.L. Metabolic activation of 2-substituted derivatives of myristic acid to form potent inhibitors of myristoyl CoA:protein N-myristoyltransferase, Biochemistry, *29:* 10566-10573, 1990.

36 Liang,X., Lu,Y., Wilkes,M., Neubert,T.A. and Resh,M.D. The N-terminal SH4 region of the Src family kinase Fyn is modified by methylation and heterogeneous fatty acylation: role in membrane targeting, cell adhesion, and spreading, J.Biol.Chem., *279:* 8133-8139, 2004.

37 Kim,P.W., Sun,Z.Y., Blacklow,S.C., Wagner,G. and Eck,M.J. A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8, Science, *301:* 1725-1728, 2003.

38 Johnson,T.M., Williamson,N.A., Scholz,G., Jaworowski,A., Wettenhall,R.E., Dunn,A.R. and Cheng,H.C. Modulation of the catalytic activity of the Src family tyrosine kinase Hck by autophosphorylation at a novel site in the unique domain, J.Biol.Chem., *275:* 33353-33364, 2000.

39 Luciano,F., Ricci,J.E. and Auberger,P. Cleavage of Fyn and Lyn in their N-terminal unique regions during induction of apoptosis: a new mechanism for Src kinase regulation, Oncogene, *20:* 4935-4941, 2001.

40 Shenoy,S., Choi,J.K., Bagrodia,S., Copeland,T.D., Maller,J.L. and Shalloway,D. Purified maturation promoting factor phosphorylates pp60c-src at the sites phosphorylated during fibroblast mitosis, Cell, *57:* 763-774, 1989.

41 Morgan,D.O., Kaplan,J.M., Bishop,J.M. and Varmus,H.E. Mitosis-specific phosphorylation of p60c-src by p34cdc2-associated protein kinase, Cell, *57:* 775-786, 1989.

42 Chackalaparampil,I. and Shalloway,D. Altered phosphorylation and activation of pp60c-src during fibroblast mitosis, Cell, *52:* 801-810, 1988.

43 Winkler,D.G., Park,I., Kim,T., Payne,N.S., Walsh,C.T., Strominger,J.L. and Shin,J. Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase $p56^{lck}$, Proc.Natl.Acad.Sci.USA, *90:* 5176-5180, 1993.

44 Kay,B.K., Williamson,M.P. and Sudol,M. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains, FASEB J., *14:* 231-241, 2000.

45 Ren,R., Mayer,B.J., Cicchetti,P. and Baltimore,D. Identification of a ten-amino acid proline-rich SH3 binding site, Science, *259:* 1157-1161, 1993.

46 Yu,H., Rosen,M.K., Sin,T.B., Seidel-Dugan,C., Brugge,J.S. and Schreiber,S.L. Solution structure of the SH3 domain of Src and identification of its ligand binding site, Science, *258:* 1665-1668, 1992.

47 Musacchio,A., Noble,M., Pauptit,R., Wierenga,R. and Saraste,M. Crystal structure of a Src-homology 3 (SH3) domain, Nature, *359:* 851-855, 1992.

48 Noble,M.E., Musacchio,A., Saraste,M., Courtneidge,S.A. and Wierenga,R.K. Crystal structure of the SH3 domain in human Fyn; comparison of the three-dimensional structures of SH3 domains in tyrosine kinases and spectrin, EMBO J., *12:* 2617-2624, 1993.

49 Briese,L. and Willbold,D. Structure determination of human Lck unique and SH3 domains by nuclear magnetic resonance spectroscopy, BMC.Struct.Biol., *3:* 3, 2003.

50 Feng,S., Kasahara,C., Rickles,R.J. and Schreiber,S.L. Specific interactions outside the proline-rich core of two classes of Src homology 3 ligands, Proc.Natl.Acad.Sci.U.S.A, *92:* 12408-12415, 1995.

51 Lim,W.A., Richards,F.M. and Fox,R.O. Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains, Nature, *372:* 375-379, 1994.

52 Feng,S., Chen,J.K., Yu,H., Simon,J.A. and Schreiber,S.L. Two binding orientations for peptides to the Src SH3 domain: Development of a general model for SH3-ligand interactions, Science, *266:* 1241-1247, 1994.

53 Weng,Z., Thomas,S.M., Rickles,R.J., Taylor,J.A., Brauer,A.W., Seidel-Dugan,C., Michael,W.M., Dreyfuss,G. and Brugge,J.S. Identification of Src, Fyn, and Lyn SH3-binding proteins: implications for a function of SH3 domains, Mol.Cell Biol., *14:* 4509-4521, 1994.

54 Briggs,S.D., Bryant,S.S., Jove,R., Sanderson,S.D. and Smithgall,T.E. The Ras GTPase-activating protein (GAP) is an SH3 domain-binding protein and substrate for the Src-related tyrosine kinase, Hck, J.Biol.Chem., *270:* 14718-14724, 1995.

55 Xu,W., Harrison,S.C. and Eck,M.J. Three-dimensional structure of the tyrosine kinase c-Src, Nature, *385:* 595-602, 1997.

56 Williams,J.C., Weijland,A., Gonfloni,S., Thompson,A., Courtneidge,S.A., Superti-Furga,G. and Wierenga,R.K. The 2.35 Å crystal structure of the inactivated form of chicken Src: A dynamic molecule mith multiple regulatory interactions, J.Mol.Biol., *274:* 757-775, 1997.

57 Kuriyan,J. and Cowburn,D. Modular peptide recognition domains in eukaryotic signaling, Annu.Rev.Biophys.Biomol.Struct., *26:* 259-288, 1997.

58 Welch,P.J. and Wang,J.Y.J. A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle, Cell, *75:* 779-790, 1993.

59 Waksman,G., Kominos,D., Robertson,S.C., Pant,N., Baltimore,D., Birge,R.B., Cowburn,D., Hanafusa,H., Mayer,B.J., Overduin,M. and . Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides, Nature, *358:* 646-653, 1992.

60 Songyang,Z., Shoelson,S.E., Chaudhuri,M., Gish,G., Pawson,T., Haser,W.G., King,F., Roberts,T., Ratnofsky,S., Lechleider,R.J., Neel,B.G., Birge,R.B., Fajardo,J.E., Chou,M.M., Hanafusa,H., Schaffhausen,B. and Cantley,L.C. SH2 domains recognize specific phosphopeptide sequences, Cell, *72:* 767-778, 1993.

61 Ayrapetov,M.K., Wang,Y.H., Lin,X., Gu,X., Parang,K. and Sun,G. Conformational basis for SH2-Tyr(P)527 binding in Src inactivation, J.Biol.Chem., *281:* 23776-23784, 2006.

62 Xu,W., Doshi,A., Lei,M., Eck,M.J. and Harrison,S.C. Crystal structures of c-Src reveal features of its autoinhibitory mechanism, Mol.Cell, *3:* 629-638, 1999.

63 Schindler,T., Sicheri,F., Pico,A., Gazit,A., Levitzki,A. and Kuriyan,J. Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor, Mol.Cell, *3:* 639-648, 1999.

64 Sicheri,F., Moarefi,I. and Kuriyan,J. Crystal structure of the Src family tyrosine kinase Hck, Nature, *385:* 602-609, 1997.

65 Young,M.A., Gonfloni,S., Superti-Furga,G., Roux,B. and Kuriyan,J. Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation, Cell, *105:* 115-126, 2001.

66 Harrison,S.C. Variation on an Src-like theme, Cell, *112:* 737-740, 2003.

67 Breitenlechner,C.B., Kairies,N.A., Honold,K., Scheiblich,S., Koll,H., Greiter,E., Koch,S., Schafer,W., Huber,R. and Engh,R.A. Crystal structures of active SRC kinase domain complexes, J.Mol.Biol., *353:* 222-231, 2005.

68 Hubbard,S.R., Wei,L., Ellis,L. and Hendrickson,W.A. Crystal structure of the tyrosine kinase domain of the human insulin receptor, Nature, *372:* 746-754, 1994.

69 Knighton,D.R., Zheng,J.H., Ten Eyck,L.F., Xuong,N.H., Taylor,S.S. and Sowadski,J.M. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase, Science, *253:* 414-420, 1991.

70 Hanks,S.K., Quinn,A.M. and Hunter,T. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domain, Science, *241:* 42-52, 1988.

71 Yamaguchi,H. and Hendrickson,W.A. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation, Nature, *384:* 484-489, 1996.

72 Gonfloni,S., Frischknecht,F., Way,M. and Superti-Furga,G. Leucine 255 of Src couples intramolecular interactions to inhibition of catalysis, Nat.Struct.Biol., *6:* 760-764, 1999.

73 Adams,J.A. Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model?, Biochemistry, *42:* 601-607, 2003.

74 Porter,M., Schindler,T., Kuriyan,J. and Miller,W.T. Reciprocal regulation of Hck activity by phosphorylation of Tyr(527) and Tyr(416). Effect of introducing a high affinity intramolecular SH2 ligand, J.Biol.Chem., *275:* 2721-2726, 2000.

75 Cowan-Jacob,S.W., Fendrich,G., Manley,P.W., Jahnke,W., Fabbro,D., Liebetanz,J. and Meyer,T. The Crystal Structure of a c-Src Complex in an Active Conformation Suggests Possible Steps in c-Src Activation, Structure.(Camb.), *13:* 861-871, 2005.

76 LaFevre-Bernt,M., Sicheri,F., Pico,A., Porter,M., Kuriyan,J. and Miller,W.T. Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue, J.Biol.Chem., *273:* 32129-32134, 1998.

77 Adams,J.A. Kinetic and catalytic mechanisms of protein kinases, Chem.Rev., *101:* 2271-2290, 2001.

78 Hubbard,S.R. and Till,J.H. Protein tyrosine kinase structure and function, Annu.Rev.Biochem., *69:* 373-398, 2000.

79 Taylor,S.S., Knighton,D.R., Zheng,J., Ten Eyck,L.F. and Sowadski,J.M. Structural framework for the protein kinase family, Annu.Rev.Cell Biol., *8:* 429-462, 1992.

80 Roskoski,R., Jr. Src protein-tyrosine kinase structure and regulation, Biochem.Biophys.Res.Commun., *324:* 1155-1164, 2004.

81 Brown,M.T. and Cooper,J.A. Regulation, substrates, and functions of Src, Biochim.Biophys.Acta, *1287:* 121-149, 1996.

82 Songyang,Z., Shoelson,S.E., Chaudhuri,M., Gish,G., Pawson,T., Haser,W.G., King,F., Roberts,T., Ratnofsky,S., Lechleider,R.J. and . SH2 domains recognize specific phosphopeptide sequences, Cell, *72:* 767-778, 1993.

83 Wang,D., Esselman,W.J. and Cole,P.A. Substrate conformational restriction and CD45-catalyzed dephosphorylation of tail tyrosine-phosphorylated Src protein, J.Biol.Chem., *277:* 40428-40433, 2002.

84 Reynolds,A.B., Vila,J., Lansing,T.J., Potts,W.M., Weber,M.J. and Parsons,J.T. Activation of the oncogenic potential of the avian cellular src protein by specific structural alteration of the carboxy terminus, EMBO J., *6:* 2359-2364, 1987.

85 Cartwright,C.A., Eckhart,W., Simon,S. and Kaplan,P.L. Cell transformation by pp60 c-src mutated in the carboxy-terminal regulatory domain, Cell, *49:* 83-91, 1987.

86 Lerner,E.C., Trible,R.P., Schiavone,A.P., Hochrein,J.M., Engen,J.R. and Smithgall,T.E. Activation of the Src Family Kinase Hck without SH3-Linker Release, J.Biol.Chem., *280:* 40832-40837, 2005.

87 Sun,G., Sharma,A.K. and Budde,R.J. Autophosphorylation of Src and Yes blocks their inactivation by Csk phosphorylation, Oncogene, *17:* 1587-1595, 1998.

88 Sun,G., Ramdas,L., Wang,W., Vinci,J., McMurray,J. and Budde,R.J. Effect of autophosphorylation on the catalytic and regulatory properties of protein tyrosine kinase Src, Arch.Biochem.Biophys., *397:* 11-17, 2002.

89 Baker,M., Gamble,J., Tooze,R., Higgins,D., Yang,F.T., O'Brien,P.C., Coleman,N., Pingel,S., Turner,M. and Alexander,D.R. Development of T-leukaemias in CD45 tyrosine phosphatase-deficient mutant lck mice, EMBO J., *19:* 4644-4654, 2000.

90 Chiang,G.G. and Sefton,B.M. Specific dephosphorylation of the Lck tyrosine protein kinase at Tyr-394 by the SHP-1 protein-tyrosine phosphatase, J.Biol.Chem., *276:* 23173-23178, 2001.

91 Cloutier,J.F. and Veillette,A. Association of inhibitory tyrosine protein kinase p50 csk with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells, EMBO J., *15:* 4909-4918, 1996.

92 Hermiston,M.L., Xu,Z. and Weiss,A. CD45: a critical regulator of signaling thresholds in immune cells, Annu.Rev.Immunol., *21:* 107-137, 2003.

93 van,V.C., Bukczynska,P.E., Puryer,M.A., Sadek,C.M., Shields,B.J., Tremblay,M.L. and Tiganis,T. Selective regulation of tumor necrosis factor-induced Erk signaling by Src family kinases and the T cell protein tyrosine phosphatase, Nat.Immunol., *6:* 253-260, 2005.

94 Ghose,R., Shekhtman,A., Goger,M.J., Ji,H. and Cowburn,D. A novel, specific interaction involving the Csk SH3 domain and its natural ligand, Nat.Struct.Biol., *8:* 998-1004, 2001.

95 Briggs,S.D., Sharkey,M., Stevenson,M. and Smithgall,T.E. SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1, J.Biol.Chem., *272:* 17899-17902, 1997.

96 Briggs,S.D. and Smithgall,T.E. SH2-kinase linker mutations release Hck tyrosine kinase and transforming activities in rat-2 fibroblasts, J.Biol.Chem., *274:* 26579-26583, 1999.

97 Moarefi,I., LaFevre-Bernt,M., Sicheri,F., Huse,M., Lee,C.-H., Kuriyan,J. and Miller,W.T. Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement, Nature, *385:* 650-653, 1997.

98 Miller,W.T. Determinants of substrate recognition in nonreceptor tyrosine kinases, Acc.Chem.Res., *36:* 393-400, 2003.

99 Parsons,J.T. and Parsons,S.J. Src family protein tyrosine kinases: Cooperating with growth factor and adhesion signaling pathways, Curr.Opin.Cell Biol., *9:* 187-192, 1997.

100 Lerner,E.C. and Smithgall,T.E. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo, Nat.Struct.Biol., *9:* 365-369, 2002.

101 Yadav,S.S. and Miller,W.T. Cooperative activation of Src family kinases by SH3 and SH2 ligands, Cancer Lett., *257:* 116-123, 2007.

102 Arold,S.T., Ulmer,T.S., Mulhern,T.D., Werner,J.M., Ladbury,J.E., Campbell,I.D. and Noble,M.E. The role of the Src homology 3-Src homology 2 interface in the regulation of Src kinases, J.Biol.Chem., *276:* 17199-17205, 2001.

103 Engen,J.R., Wales,T.E., Hochrein,J.M., Meyn,M.A., III, Banu,O.S., Bahar,I. and Smithgall,T.E. Structure and dynamic regulation of Src-family kinases, Cell Mol.Life Sci., *65:* 3058-3073, 2008.

104 Parsons,S.J. and Parsons,J.T. Src family kinases, key regulators of signal transduction, Oncogene, *23:* 7906-7909, 2004.

105 Erpel,T. and Courtneidge,S.A. Src family protein tyrosine kinases and cellular signal transduction pathways, Curr.Opin.Cell Biol., *7:* 176-182, 1995.

106 Summy,J.M. and Gallick,G.E. Src family kinases in tumor progression and metastasis, Cancer Metastasis Rev., *22:* 337-358, 2003.

107 Ogawa,M. Differentiation and proliferation of hematopoietic stem cells, Blood, *81:* 2844-2853, 1993.

108 Rane,S.G. and Reddy,E.P. JAKs, STATs and Src kinases in hematopoiesis, Oncogene, *21:* 3334-3358, 2002.

109 Kaczmarski,R.S. and Mufti,G.J. The cytokine receptor superfamily, Blood Rev., *5:* 193-203, 1991.

110 Weil,D., Power,M.A., Smith,S.I. and Li,C.L. Predominant expression of murine Bmx tyrosine kinase in the granulo-monocytic lineage, Blood, *90:* 4332-4340, 1997.

111 Anderson,S.M. and Jorgensen,B. Activation of *src*-related tyrosine kinases by IL-3, J.Immunol., *155:* 1660-1670, 1995.

112 Corey,S.J., Burkhardt,A.L., Bolen,J.B., Geahlen,R.L., Tkatch,L.S. and Tweardy,D.J. Granulocyte colony-stimulating factor receptor signaling involves the formation of a three-component complex with Lyn and Syk protein-tyrosine kinases, Proc.Natl.Acad.Sci.USA, *91:* 4683-4687, 1994.

113 Ihle,J.N., Witthuhn,B.A., Quelle,F.W., Yamamoto,K., Thierfelder,W.E., Kreider,B. and Silvennoinen,O. Signaling by the cytokine receptor superfamily: JAKs and STATs, Trends Biochem.Sci., *19:* 222-227, 1994.

114 Witthuhn,B.A., Quelle,F.W., Silvennoinen,O., Yi,T., Tang,B., Miura,O. and Ihle,J.N. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin, Cell, *74:* 227-236, 1993.

115 Torigoe,T., O'Connor,R., Santoli,D. and Reed,J.C. Interleukin-3 regulates the activity of the LYN protein-tyrosine kinase in myeloid-committed leukemic cell lines, Blood, *80:* 617-624, 1992.

116 Lowell,C.A., Soriano,P. and Varmus,H.E. Functional overlap in the *src* gene family: Inactivation of *hck* and *fgr* impairs natural immunity, Genes Dev., *8:* 387-398, 1994.

117 Stein,P.L., Lee,H.M., Rich,S. and Soriano,P. pp59 fyn mutant mice display differential signaling in thymocytes and peripheral T cells, Cell, *70:* 741-750, 1992.

118 Molina,T.J., Bachmann,M.F., Kundig,T.M., Zinkernagel,R.M. and Mak,T.W. Peripheral T cells in mice lacking p56lck do not express significant antiviral effector functions, J.Immunol., *151:* 699-706, 1993.

119 Tycko,B., Smith,S.D. and Sklar,J. Chromosomal translocations joining LCK and TCRB loci in human T cell leukemia, J.Exp.Med., *174:* 867-873, 1991.

120 Ernst,M., Gearing,D.P. and Dunn,A.R. Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells, EMBO J., *13:* 1574-1584, 1994.

121 Burton,E.A., Hunter,S., Wu,S.C. and Anderson,S.M. Binding of src-like kinases to the β-subunit of the interleukin-3 receptor, J.Biol.Chem., *272:* 16189-16195, 1997.

122 Appleby,M.W., Kerner,J.D., Chien,S., Maliszewski,C.R., Bondada,S., Perlmutter,R.M. and Bondadaa S [corrected to Bondada Involvement of p59fynT in interleukin-5 receptor signaling, J.Exp.Med., *182:* 811-820, 1995.

123 Li,B., Subleski,M., Fusaki,N., Yamamoto,T., Copeland,T., Princler,G.L., Kung,H. and Kamata,T. Catalytic activity of the mouse guanine nucleotide exchanger mSOS is activated by Fyn tyrosine protein kinase and the T-cell antigen receptor in T cells, Proc.Natl.Acad.Sci.U.S.A, *93:* 1001-1005, 1996.

124 Chen,H.C., Appeddu,P.A., Isoda,H. and Guan,J.L. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase, J.Biol.Chem., *271:* 26329-26334, 1996.

125 Cobb,B.S., Schaller,M.D., Leu,T.H. and Parsons,J.T. Stable association of pp60 src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK, Mol.Cell Biol., *14:* 147-155, 1994.

126 Nagai,K., Takata,M., Yamamura,H. and Kurosaki,T. Tyrosine phosphorylation of Shc is mediated through Lyn and Syk in B cell receptor signaling, J.Biol.Chem., *270:* 6824-6829, 1995.

127 Ptasznik,A., Traynor-Kaplan,A. and Bokoch,G.M. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase-Shc adapter protein signaling complexes, J.Biol.Chem., *270:* 19969-19973, 1995.

128 Hallek,M., Neumann,C., Schaffer,M., nhauser-Riedl,S., von,B.N., de,V.G., Druker,B.J., Yasukawa,K., Griffin,J.D. and Emmerich,B. Signal transduction of interleukin-6 involves tyrosine phosphorylation of multiple cytosolic proteins and activation of Src-family kinases Fyn, Hck, and Lyn in multiple myeloma cell lines, Exp.Hematol., *25:* 1367-1377, 1997.

129 Choi,S.H., Yamanashi,Y., Shiota,M., Takanashi,M., Hojo,I., Itoh,T., Watanabe,T., Yamamoto,T. and Mori,S. Expression of Lyn protein on human malignant lymphomas, Lab Invest, *69:* 736-742, 1993.

130 Abts,H., Jucker,M., Diehl,V. and Tesch,H. Human chronic lymphocytic leukemia cells regularly express mRNAs of the protooncogenes lck and c-fgr, Leuk.Res., *15:* 987-997, 1991.

131 Majolini,M.B., D'Elios,M.M., Galieni,P., Boncristiano,M., Lauria,F., Del,P.G., Telford,J.L. and Baldari,C.T. Expression of the T-cell-specific tyrosine kinase Lck in normal B-1 cells and in chronic lymphocytic leukemia B cells, Blood, *91:* 3390-3396, 1998.

132 Waddick,K.G., Chae,H.P., Tuel-Ahlgren,L., Jarvis,L.J., Dibridik,I., Myers,D.E. and Uckun,F.M. Engagement of the CD19 receptor on human B-lineage leukemia cells activates LCK tyrosine kinase and facilitates radiation-induced apoptosis, Radiation Research, *136:* 313-319, 1993.

133 Picard,C., Gabert,J., Olive,D. and Collette,Y. Altered splicing in hematological malignancies reveals a tissue-specific translational block of the Src-family tyrosine kinase fyn brain isoform expression, Leukemia, *18:* 1737-1739, 2004.

134 Kantarjian,H.M., Dixon,D., Keating,M.J., Talpaz,M., Walters,R.S., McCredie,K.B. and Freireich,E.J. Characteristics of accelerated disease in chronic myelogenous leukemia, Cancer, *61:* 1441-1446, 1988.

135 Sawyers,C.L. Chronic myeloid leukemia, N.Engl.J.Med., *340:* 1330-1340, 1999.

136 Jaffe,E.S.H.N.L.S.H.V.J.W. Chronic myeloproliferative diseases and Myelodysplastic/myeloproliferative diseases. *In*: AnonymousWorld Health Organization Classification of Tumours. Pathology and Genetics. Tumours of Haematopoietic and Lymphoid Tissues, pp. 17-52. 2001.

137 Wong,S. and Witte,O.N. The BCR-ABL story: bench to bedside and back, Annu.Rev.Immunol., *22:* 247-306, 2004.

138 Quintás-Cardama,A.C.J.E. Chronic myeloid leukemia: diagnosis and treatment, Mayo Clininic Proceedings, *81:* 973, 2006.

139 Sokal,J.E., Baccarani,M., Russo,D. and Tura,S. Staging and prognosis in chronic myelogenous leukemia, Semin.Hematol., *25:* 49-61, 1988.

140 Nowell,P.C. and Hungerford,D.A. A minute chromosome in human granulocytic leukemia, Science, *132:* 1497, 1960.

141 Rowley,J.D. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and G Giemsa staining, Nature, *243:* 290-293, 1973.

142 Shtivelman,E., Lifshitz,B., Gale,R.P. and Canaani,E. Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukemia, Nature, *315:* 550-554, 1985.

143 Groffen,J., Stephenson,J.R., Heisterkamp,N., de Klein,A., Bartram,C.R. and Grosfeld,G. Philadelphia chromosomal breakpoints are clustered within a limited region, *bcr*, on chromosome 22, Cell, *36:* 93-99, 1984.

144 Inokuchi and K. Chronic Myelogenous Leukemia: From Molecular Biology to Clinical Aspects and Novel Targeted Therapies, Journal of Nippon Medical School, *73:* 178, 2006.

145 Quackenbush,R.C., Reuther,G.W., Miller,J.P., Courtney,K.D., Pear,W.S. and Pendergast,A.M. Analysis of the biologic properties of p230 Bcr-Abl reveals unique and overlapping properties with the oncogenic p185 and p210 Bcr-Abl tyrosine kinases, Blood, *95:* 2913-2921, 2000.

146 Kurzrock,R., Shtalrid,M., Romero,P., Kloetzer,W.S., Talpas,M., Trujillo,J.M., Blick,M., Beran,M. and Gutterman,J.V. A novel c-*abl* protein product in a Philadelphia-positive acute lymphoblastic leukemia, Nature, *325:* 631-635, 1987.

147 Chan,L.C., Karhi,K.K., Rayter,S.I., Heisterkamp,N., Eridani,S., Powles,R., Lawler,S.D., Groffen,J., Foulkes,J.G., Greaves,M.F. and Wiedemann,L.M. A novel *abl* protein expressed in Philadelphia chromosome positive acute lymphoblastic leukemia, Nature, *325:* 635-637, 1987.

148 Okuda,K., Golub,T.R., Gilliland,D.G. and Griffin,J.D. p210BCR/ABL, p190BCR/ABL, and TEL/ABL activate similar signal transduction pathways in hematopoietic cell lines, Oncogene, *13:* 1147-1152, 1996.

149 Melo,J.V. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype, Blood, *88:* 2375-2384, 1996.

150 Inokuchi,K., Dan,K., Takatori,M., Takahuji,H., Uchida,N., Inami,M., Miyake,K., Honda,H., Hirai,H. and Shimada,T. Myeloproliferative disease in transgenic mice expressing P230 Bcr/Abl: longer disease latency, thrombocytosis, and mild leukocytosis, Blood, *102:* 320-323, 2003.

151 Preston,D.L., Kusumi,S., Tomonaga,M., Izumi,S., Ron,E., Kuramoto,A., Kamada,N., Dohy,H., Matsuo,T., Matsui,T. and . Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987, Radiat.Res., *137:* S68-S97, 1994.

152 Deininger,M.W., Bose,S., Gora-Tybor,J., Yan,X.H., Goldman,J.M. and Melo,J.V. Selective induction of leukemia-associated fusion genes by high-dose ionizing radiation, Cancer Res., *58:* 421-425, 1998.

153 Daley,G.Q., Van Etten,R.A. and Baltimore,D. Induction of chronic myelogenous leukemia in mice by the $p210^{bcr\text{-}abl}$ gene of the Philadelphia chromosome, Science, *247:* 824-830, 1990.

154 Honda,H., Oda,H., Suzuki,T., Takahashi,T., Witte,O.N., Ozawa,K., Ishikawa,T., Yazaki,Y. and Hirai,H. Development of acute lymphoblastic leukemia and myeloproliferative disorder in transgenic mice expressing p210bcr/abl: a novel transgenic model for human Ph1-positive leukemias, Blood, *91:* 2067-2075, 1998.

155 Fialkow,P.J., Jacobson,R.J. and Papayannopoulou,T. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage, Am.J.Med., *63:* 125-130, 1977.

156 Takahashi,N., Miura,I., Saitoh,K. and Miura,A.B. Lineage involvement of stem cells bearing the philadelphia chromosome in chronic myeloid leukemia in the chronic phase as shown by a combination of fluorescence-activated cell sorting and fluorescence in situ hybridization, Blood, *92:* 4758-4763, 1998.

157 Yilmaz,O.H., Valdez,R., Theisen,B.K., Guo,W., Ferguson,D.O., Wu,H. and Morrison,S.J. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells, Nature, *441:* 475-482, 2006.

158 Clarke,M.F. and Fuller,M. Stem cells and cancer: two faces of eve, Cell, *124:* 1111-1115, 2006.

159 Zou,G.M. Cancer stem cells in leukemia, recent advances, J.Cell Physiol, *213:* 440-444, 2007.

160 Holyoake,T.L., Jiang,X., Drummond,M.W., Eaves,A.C. and Eaves,C.J. Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia, Leukemia, *16:* 549-558, 2002.

161 de Fanritiis,P., Dowding,C., Bungey,J., Chase,A., Angus,G., Szydlo,R. and Goldman,J.M. Phenotypic characterization of Normal CD34-positive cells: only the most primitive CML progenitors include Ph-neg cells, Leukemia and Lymphoma, *11:* 51-61, 1993.

162 Coulombel,L., Kalousek,D.K., Eaves,C.J., Gupta,C.M. and Eaves,A.C. Long-term marrow culture reveals chromosomally normal hematopoietic progenitor cells in patients with Philadelphia chromosome-positive chronic myelogenous leukemia, N.Engl.J.Med., *308:* 1493-1498, 1983.

163 Grand,F.H., Marley,S.B., Chase,A., Titley,I., Healy,L., Spencer,A., Reiter,A., Goldman,J.M. and Gordon,M.Y. BCR/ABL-negative progenitors are enriched in the adherent fraction of CD34+ cells circulating in the blood of chronic phase chronic myeloid leukemia patients, Leukemia, *11:* 1486-1492, 1997.

164 Maguer-Satta,V., Petzer,A.L., Eaves,A.C. and Eaves,C.J. BCR-ABL expression in different subpopulations of functionally characterized Ph+ CD34+ cells from patients with chronic myeloid leukemia, Blood, *88:* 1796-1804, 1996.

165 Guyotat,D., Wahbi,K., Viallet,A., Piselli,S. and Campos,L. Selection of BCR/ABL-negative stem cells from marrow or blood of patients with chronic myeloid leukemia, Leukemia, *13:* 991-998, 1999.

166 Jaiswal,S., Traver,D., Miyamoto,T., Akashi,K., Lagasse,E. and Weissman,I.L. Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias, Proc.Natl.Acad.Sci.U.S.A, *100:* 10002-10007, 2003.

167 Jamieson,C.H., Ailles,L.E., Dylla,S.J., Muijtjens,M., Jones,C., Zehnder,J.L., Gotlib,J., Li,K., Manz,M.G., Keating,A., Sawyers,C.L. and Weissman,I.L. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML, N.Engl.J.Med., *351:* 657-667, 2004.

168 Michor,F. Chronic myeloid leukemia blast crisis arises from progenitors, Stem Cells, *25:* 1114-1118, 2007.

169 Hanks,S.K. Genomic analysis of the eukaryotic protein kinase superfamily: a perspective, Genome Biol., *4:* 111, 2003.

170 Abelson,H.T. and Rabstein,L.S. Lymphosarcoma: virus-induced thymic-independent disease in mice, Cancer Res., *30:* 2213-2222, 1970.

171 Shtivelman,E., Lifshitz,B., Gale,R.P., Roe,B.A. and Canaani,E. Alternative splicing of RNAs transcribed from the human abl gene and from the bcr-abl fused gene, Cell, *47:* 277-284, 1986.

172 Pendergast,A.M. The Abl family kinases: mechanisms of regulation and signaling, Adv.Cancer Res., *85:* 51-100, 2002.

173 Pluk,H., Dorey,K. and Superti-Furga,G. Autoinhibition of c-Abl, Cell, *108:* 247-259, 2002.

174 Van Etten,R.A. Cycling, stressed-out and nervous: cellular functions of c-Abl, Trends Cell Biol., *9:* 179-186, 1999.

175 Superti-Furga,G. and Courtneidge,S.A. Structure-function relationships in Src family and related protein tyrosine kinases, BioEssays, *17:* 321-330, 1995.

176 Smith,J.M. and Mayer,B.J. Abl: mechanisms of regulation and activation, Front Biosci., *7:* d31-d42, 2002.

177 Nagar,B., Hantschel,O., Young,M.A., Scheffzek,K., Veach,D., Bornmann,W., Clarkson,B., Superti-Furga,G. and Kuriyan,J. Structural basis for the autoinhibition of c-Abl tyrosine kinase, Cell, *112:* 859-871, 2003.

178 Hantschel,O. and Superti-Furga,G. Regulation of the c-Abl and Bcr-Abl tyrosine kinases, Nat.Rev.Mol.Cell Biol., *5:* 33-44, 2004.

179 Nagar,B., Bornmann,W.G., Pellicena,P., Schindler,T., Veach,D.R., Miller,W.T., Clarkson,B. and Kuriyan,J. Crystal Structures of the Kinase Domain of c-Abl in Complex with the Small Molecule Inhibitors PD173955 and Imatinib (STI-571), Cancer Res., *62:* 4236-4243, 2002.

180 Hochrein,J.M., Lerner,E.C., Schiavone,A.P., Smithgall,T.E. and Engen,J.R. An examination of dynamics crosstalk between SH2 and SH3 domains by hydrogen/deuterium exchange and mass spectrometry, Protein Sci., *15:* 65-73, 2006.

181 Barila,D. and Superti-Furga,G. An intramolecular SH3-domain interaction regulates c-Abl activity, Nat.Genet., *18:* 280-282, 1998.

182 Tanis,K.Q., Veach,D., Duewel,H.S., Bornmann,W.G. and Koleske,A.J. Two distinct phosphorylation pathways have additive effects on abl family kinase activation, Mol.Cell Biol., *23:* 3884-3896, 2003.

183 Brasher,B.B. and Van Etten,R.A. c-Abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines, J.Biol.Chem., *275:* 35631-35637, 2000.

184 Huse,M. and Kuriyan,J. The conformational plasticity of protein kinases, Cell, *109:* 275-282, 2002.

185 Wang,J.Y. Negative regulation of c-abl tyrosine kinase by its variable N-terminal amino acids, Oncogene Res., *3:* 293-298, 1988.

186 Hantschel,O., Nagar,B., Guettler,S., Kretzschmar,J., Dorey,K., Kuriyan,J. and Superti-Furga,G. A myristoyl/phosphotyrosine switch regulates c-Abl, Cell, *112:* 845-857, 2003.

187 Chen,S., Dumitrescu,T.P., Smithgall,T.E. and Engen,J.R. Abl N-terminal cap stabilization of SH3 domain dynamics, Biochemistry, *47:* 5795-5803, 2008.

188 Nagar,B., Hantschel,O., Seeliger,M., Davies,J.M., Weis,W.I., Superti-Furga,G. and Kuriyan,J. Organization of the SH3-SH2 unit in active and inactive forms of the c-Abl tyrosine kinase, Mol.Cell, *21:* 787-798, 2006.

189 Van Etten,R.A., Debnath,J., Zhou,H. and Casasnovas,J.M. Introduction of a loss-of-function point mutation from the SH3 region of the Caenorhabditis elegans sem-5 gene activates the transforming ability of c-abl in vivo and abolishes binding of proline-rich ligands in vitro, Oncogene, *10:* 1977-1988, 1995.

190 Schindler,T., Bornmann,W., Pellicena,P., Miller,W.T., Clarkson,B. and Kuriyan,J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase, Science, *289:* 1938-1942, 2000.

191 Dorey,K., Engen,J.R., Kretzschmar,J., Wilm,M., Neubauer,G., Schindler,T. and Superti-Furga,G. Phosphorylation and structure-based functional studies reveal a positive and a

negative role for the activation loop of the c-Abl tyrosine kinase, Oncogene, *20:* 8075-8084, 2001.

192 Plattner,R., Kadlec,L., DeMali,K.A., Kazlauskas,A. and Pendergast,A.M. c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF, Genes Dev., *13:* 2400-2411, 1999.

193 Brasher,B.B., Roumiantsev,S. and Van Etten,R.A. Mutational analysis of the regulatory function of the c-Abl Src homology 3 domain, Oncogene, *20:* 7744-7752, 2001.

194 Meyn,M.A., III, Wilson,M.B., Abdi,F.A., Fahey,N., Schiavone,A.P., Wu,J., Hochrein,J.M., Engen,J.R. and Smithgall,T.E. Src family kinases phosphorylate the Bcr-Abl SH3-SH2 region and modulate Bcr-Abl transforming activity, J.Biol.Chem., *281:* 30907-30916, 2006.

195 Chen,S., O'Reilly,L.P., Smithgall,T.E. and Engen,J.R. Tyrosine Phosphorylation in the SH3 Domain Disrupts Negative Regulatory Interactions within the c-Abl Kinase Core, J.Mol.Biol., *383:* 414-423, 2008.

196 Voncken,J.W., Kaartinen,V., Pattengale,P.K., Germeraad,W.T., Groffen,J. and Heisterkamp,N. BCR/ABL P210 and P190 cause distinct leukemia in transgenic mice, Blood, *86:* 4603-4611, 1995.

197 Hermans,A., Heisterkamp,N., von Lindern,M., van Baal,S., Meijer,D., van der Plas,D., Wiedemann,L.M., Groffen,J., Bootsma,D. and Grosveld,G. Unique fusion of *bcr* and c-*abl* genes in Philadelphia chromosome-positive acute lymphoblastic leukemia, Cell, *51:* 33-40, 1987.

198 Ghaffari,S., Daley,G.Q. and Lodish,H.F. Growth factor independence and BCR/ABL transformation: promise and pitfalls of murine model systems and assays, Leukemia, *13:* 1200-1206, 1999.

199 Zhang,X., Wong,R., Hao,S.X., Pear,W.S. and Ren,R. The SH2 domain of Bcr-Abl is not required to induce a murine myeloproliferative disease; however, SH2 signaling influences disease latency and phenotype, Blood, *97:* 277-287, 2001.

200 McWhirter,J.R. and Wang,J.Y. Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins, Mol.Cell Biol., *11:* 1553-1565, 1991.

201 Pendergast,A.M., Muller,A.J., Havlik,M.H., Maru,Y. and Witte,O.N. BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner, Cell, *66:* 161-171, 1991.

202 McWhirter,J.R., Galasso,D.L. and Wang,J.Y.J. A coiled-coil oligomerization domain of *bcr* is essential for the transforming function of *bcr-abl* oncoproteins, Mol.Cell.Biol., *13:* 7587-7595, 1993.

203 Zhao,X., Ghaffari,S., Lodish,H., Malashkevich,V.N. and Kim,P.S. Structure of the Bcr-Abl oncoprotein oligomerization domain, Nat.Struct.Biol., *9:* 117-120, 2002.

204 Azam,M., Latek,R.R. and Daley,G.Q. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL, Cell, *112:* 831-843, 2003.

205 Mayer,B.J. and Baltimore,D. Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase, Mol.Cell.Biol., *14:* 2883-2894, 1994.

206 Van Etten,R.A. Oncogenic signaling: new insights and controversies from chronic myeloid leukemia, J.Exp.Med., *204:* 461-465, 2007.

207 Danhauser-Riedl,S., Warmuth,M., Druker,B.J., Emmerich,B. and Hallek,M. Activation of Src kinases p53/56^{lyn} and p59^{hck} by p210$^{bcr/abl}$ in myeloid cells, Cancer Res., *56:* 3589-3596, 1996.

208 Stanglmaier,M., Warmuth,M., Kleinlein,I., Reis,S. and Hallek,M. The interaction of the Bcr-Abl tyrosine kinase with the Src kinase Hck is mediated by multiple binding domains, Leukemia, *17:* 283-289, 2003.

209 Lionberger,J.M., Wilson,M.B. and Smithgall,T.E. Transformation of Myeloid Leukemia Cells to Cytokine Independence by Bcr-Abl Is Suppressed by Kinase-defective Hck, J.Biol.Chem., *275:* 18581-18585, 2000.

210 Warmuth,M., Bergmann,M., Priess,A., Hauslmann,K., Emmerich,B. and Hallek,M. The Src family kinase Hck interacts with Bcr-Abl by a kinase- independent mechanism and phosphorylates the Grb2-binding site of Bcr, J.Biol.Chem., *272:* 33260-33270, 1997.

211 Klejman,A., Schreiner,S.J., Nieborowska-Skorska,M., Slupianek,A., Wilson,M.B., Smithgall,T.E. and Skorski,T. The Src family kinase Hck couples Bcr-Abl to Stat5 activation in myeloid cells, EMBO J., *21:* 5766-5774, 2002.

212 Ptasznik,A., Urbanowska,E., Chinta,S., Costa,M.A., Katz,B.A., Stanislaus,M.A., Demir,G., Linnekin,D., Pan,Z.K. and Gewirtz,A.M. Crosstalk between BCR/ABL oncoprotein and CXCR4 signaling through a Src family kinase in human leukemia cells, J.Exp.Med., *196:* 667-678, 2002.

213 Geay,J.F., Buet,D., Zhang,Y., Foudi,A., Jarrier,P., Berthebaud,M., Turhan,A.G., Vainchenker,W. and Louache,F. p210 BCR-ABL inhibits SDF-1 chemotactic response via alteration of CXCR4 signaling and down-regulation of CXCR4 expression, Cancer Res., *65:* 2676-2683, 2005.

214 az-Blanco,E., Bruns,I., Neumann,F., Fischer,J.C., Graef,T., Rosskopf,M., Brors,B., Pechtel,S., Bork,S., Koch,A., Baer,A., Rohr,U.P., Kobbe,G., Haeseler,A., Gattermann,N., Haas,R. and Kronenwett,R. Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with CML in chronic phase, Leukemia, *21:* 494-504, 2007.

215 Wilson,M.B., Schreiner,S.J., Choi,H.-J., Kamens,J.S. and Smithgall,T.E. Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis, Oncogene, *21:* 8075-8088, 2002.

216 Pene-Dumitrescu,T., Peterson,L.F., Donato,N.J. and Smithgall,T.E. An inhibitor-resistant mutant of Hck protects CML cells against the antiproliferative and apoptotic effects of the broad-spectrum Src family kinase inhibitor A-419259, Oncogene, *27:* 7055-7069, 2008.

217 Copland,M., Hamilton,A., Elrick,L.J., Baird,J.W., Allan,E.K., Jordanides,N., Barow,M., Mountford,J.C. and Holyoake,T.L. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction, Blood, *107:* 4532-4539, 2006.

218 Wu,J., Meng,F., Lu,H., Kong,L., Bornmann,W., Peng,Z., Talpaz,M. and Donato,N.J. Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-Cbl protein stability in imatinib-resistant chronic myelogenous leukemia cells, Blood, *111:* 3821-3829, 2008.

219 Donato,N.J., Wu,J.Y., Stapley,J., Gallick,G., Lin,H., Arlinghaus,R. and Talpaz,M. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571, Blood, *101:* 690-698, 2003.

220 Hu,Y., Swerdlow,S., Duffy,T.M., Weinmann,R., Lee,F.Y. and Li,S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice, Proc.Natl.Acad.Sci.U.S.A, *103:* 16870-16875, 2006.

221 Ptasznik,A., Nakata,Y., Kalota,A., Emerson,S.G. and Gewirtz,A.M. Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells, Nat.Med., *10:* 1187-1189, 2004.

222 Lakshmikuttyamma,A., Pastural,E., Takahashi,N., Sawada,K., Sheridan,D.P., DeCoteau,J.F. and Geyer,C.R. Bcr-Abl induces autocrine IGF-1 signaling, Oncogene, *27:* 3831-3844, 2008.

223 Kawano,T., Ito,M., Raina,D., Wu,Z., Rosenblatt,J., Avigan,D., Stone,R. and Kufe,D. MUC1 oncoprotein regulates Bcr-Abl stability and pathogenesis in chronic myelogenous leukemia cells, Cancer Res., *67:* 11576-11584, 2007.

224 Li,Y., Kuwahara,H., Ren,J., Wen,G. and Kufe,D. The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 β and β-catenin, J.Biol.Chem., *276:* 6061-6064, 2001.

225 Hu,Y., Liu,Y., Pelletier,S., Buchdunger,E., Warmuth,M., Fabbro,D., Hallek,M., Van Etten,R.A. and Li,S. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia, Nat.Genet., *36:* 453-461, 2004.

226 McCubrey,J.A., Steelman,L.S., Chappell,W.H., Abrams,S.L., Wong,E.W., Chang,F., Lehmann,B., Terrian,D.M., Milella,M., Tafuri,A., Stivala,F., Libra,M., Basecke,J., Evangelisti,C., Martelli,A.M. and Franklin,R.A. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance, Biochim.Biophys.Acta, *1773:* 1263-1284, 2007.

227 Steelman,L.S., Pohnert,S.C., Shelton,J.G., Franklin,R.A., Bertrand,F.E. and McCubrey,J.A. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis, Leukemia, *18:* 189-218, 2004.

228 Chang,F., Steelman,L.S., Lee,J.T., Shelton,J.G., Navolanic,P.M., Blalock,W.L., Franklin,R.A. and McCubrey,J.A. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention, Leukemia, *17:* 1263-1293, 2003.

229 Pendergast,A.M., Quilliam,L.A., Cripe,L.D., Bassing,C.H., Dai,Z., Li,N., Batzer,A., Rabun,K.M., Der,C.J., Schlessinger,J. and Gishizky,M.L. *BCR-ABL*-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein, Cell, *75:* 175-185, 1993.

230 Goga,A., McLaughlin,J., Afar,D.E.H., Saffran,D.C. and Witte,O.N. Alternative signals to RAS for hematopoietic transformation by the BCR-ABL oncogene, Cell, *82:* 981-988, 1995.

231 Puil,L., Liu,J., Gish,G., Mbamalu,G., Bowtell,D., Pelicci,P.G., Arlinghaus,R. and Pawson,T. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway, EMBO J., *13:* 764-773, 1994.

232 Cortez,D., Stoica,G., Pierce,J.H. and Pendergast,A.M. The BCR-ABL tyrosine kinase inhibits apoptosis by activating a Ras-dependent signaling pathway, Oncogene, *13:* 2589-2594, 1996.

233 Million,R.P. and Van Etten,R.A. The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase, Blood, *96:* 664-670, 2000.

234 Zhang,X., Subrahmanyam,R., Wong,R., Gross,A.W. and Ren,R. The NH(2)-terminal coiled-coil domain and tyrosine 177 play important roles in induction of a myeloproliferative disease in mice by Bcr-Abl, Mol.Cell Biol., *21:* 840-853, 2001.

235 Pendergast,A.-M. Bcr/Abl protein domain function and signaling. *In*: AnonymousChronic Myeloid Leukemia: Biology and Treatment, pp. 19-39. 2001.

236 Pelicci,G., Lanfrancone,L., Salcini,A.E., Romano,A., Mele,S., Grazia,B.M., Segatto,O., Di Fiore,P.P. and Pelicci,P.G. Constitutive phosphorylation of Shc proteins in human tumors, Oncogene, *11:* 899-907, 1995.

237 Cortez,D., Kadlec,L. and Pendergast,A.M. Structural and signaling requirements for BCR-ABL-mediated transformation and inhibition of apoptosis, Mol.Cell.Biol., *15:* 5531-5541, 1995.

238 Cortez,D., Kadlec,L. and Pendergast,A.M. Structural and signaling requirements for BCR-ABL-mediated transformation and inhibition of apoptosis, Mol.Cell Biol., *15:* 5531-5541, 1995.

239 Nosaka,Y., Arai,A., Miyasaka,N. and Miura,O. CrkL mediates Ras-dependent activation of the Raf/ERK pathway through the guanine nucleotide exchange factor C3G in hematopoietic cells stimulated with erythropoietin or interleukin-3, J.Biol.Chem., *274:* 30154-30162, 1999.

240 Oda,T., Heaney,C., Hagopian,J.R., Okuda,K., Griffin,J.D. and Druker,B.J. Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia, J.Biol.Chem., *269:* 22925-22928, 1994.

241 Darnell Jr.,J.E. STATs and gene regulation, Science, *277:* 1630-1635, 1997.

242 Leaman,D.W., Leung,S., Li,X.X. and Stark,G.R. Regulation of STAT-dependent pathways by growth factors and cytokines, FASEB J., *10:* 1578-1588, 1996.

243 Ihle,J.N., Witthuhn,B.A., Quelle,F.W., Yamamoto,K. and Silvennoinen,O. Signaling through the hematopoietic cytokine receptors, Annu.Rev.Immunol., *13:* 369-398, 1995.

244 Coppo,P., Dusanter-Fourt,I., Millot,G., Nogueira,M.M., Dugray,A., Bonnet,M.L., Mitjavila-Garcia,M.T., Le,P.D., Guilhot,F., Vainchenker,W., Sainteny,F. and Turhan,A.G. Constitutive and specific activation of STAT3 by BCR-ABL in embryonic stem cells, Oncogene, *22:* 4102-4110, 2003.

245 Carlesso,N., Frank,D.A. and Griffin,J.D. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl, J.Exp.Med., *183:* 811-820, 1996.

246 Shuai,K., Halpern,J., ten Hoeve,J., Rao,X.P. and Sawyers,C.L. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia, Oncogene, *13:* 247-254, 1996.

247 Durbin,J.E., Hackenmiller,R., Simon,M.C. and Levy,D.E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease, Cell, *84:* 443-450, 1996.

248 Nieborowska-Skorska,M., Wasik,M.A., Slupianek,A., Salomoni,P., Kitamura,T., Calabretta,B. and Skorski,T. Signal transducer and activator of transcription (STAT)5 activation by BCR/ABL is dependent on intact Src homology (SH)3 and SH2 domains of BCR/ABL and is required for leukemogenesis, J.Exp.Med., *189:* 1229-1242, 1999.

249 Nakamura,Y., Yujiri,T., Nawata,R., Tagami,K. and Tanizawa,Y. MEK kinase 1 is essential for Bcr-Abl-induced STAT3 and self-renewal activity in embryonic stem cells, Oncogene, *24:* 7592-7598, 2005.

250 Sillaber,C., Gesbert,F., Frank,D.A., Sattler,M. and Griffin,J.D. STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells, Blood, *95:* 2118-2125, 2000.

251 Gutierrez-Castellanos,S., Cruz,M., Rabelo,L., Godinez,R., Reyes-Maldonado,E. and Riebeling-Navarro,C. Differences in BCL-X(L) expression and STAT5 phosphorylation in chronic myeloid leukaemia patients, Eur.J.Haematol., *72:* 231-238, 2004.

252 Ilaria,R.L., Jr. and Van Etten,R.A. P210 and $P190^{BCR/ABL}$ induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members, J.Biol.Chem., *271:* 31704-31710, 1996.

253 Ward,A.C., Touw,I. and Yoshimura,A. The Jak-Stat pathway in normal and perturbed hematopoiesis, Blood, *95:* 19-29, 2000.

254 Xie,S., Wang,Y., Liu,J., Sun,T., Wilson,M.B., Smithgall,T.E. and Arlinghaus,R.B. Involvement of Jak2 tyrosine phosphorylation in Bcr-Abl transformation, Oncogene, *20:* 6188-6195, 2001.

255 Samanta,A.K., Lin,H., Sun,T., Kantarjian,H. and Arlinghaus,R.B. Janus kinase 2: a critical target in chronic myelogenous leukemia, Cancer Res., *66:* 6468-6472, 2006.

256 Sawyers,C.L., Callahan,W. and Witte,O.N. Dominant negative MYC blocks transformation by ABL oncogenes, Cell, *70:* 901-910, 1992.

257 Varticovski,L., Daley,G.Q., Jackson,P., Baltimore,D. and Cantley,L.C. Activation of phosphatidylinositol 3-kinase in cells expressing abl oncogene variants, Mol.Cell Biol., *11:* 1107-1113, 1991.

258 Skorski,T., Kanakaraj,P., Nieborowska-Skorska,M., Ratajczak,M.Z., Wen,S.C., Zon,G., Gewirtz,A.M., Perussia,B. and Calabretta,B. Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells, Blood, *86:* 726-736, 1995.

259 Sattler,M., Mohi,M.G., Pride,Y.B., Quinnan,L.R., Malouf,N.A., Podar,K., Gesbert,F., Iwasaki,H., Li,S., Van Etten,R.A., Gu,H., Griffin,J.D. and Neel,B.G. Critical role for Gab2 in transformation by BCR/ABL, Cancer Cell, *1:* 479-492, 2002.

260 Sattler,M., Mohi,M.G., Pride,Y.B., Quinnan,L.R., Malouf,N.A., Podar,K., Gesbert,F., Iwasaki,H., Li,S., Van Etten,R.A., Gu,H., Griffin,J.D. and Neel,B.G. Critical role for Gab2 in transformation by BCR/ABL, Cancer Cell, *1:* 479-492, 2002.

261 Sattler,M., Salgia,R., Okuda,K., Uemura,N., Durstin,M.A., Pisick,E., Xu,G., Li,J.L., Prasad,K.V. and Griffin,J.D. The proto-oncogene product p120CBL and the adaptor

proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway, Oncogene, *12:* 839-846, 1996.

262 Jain,S.K., Langdon,W.Y. and Varticovski,L. Tyrosine phosphorylation of p120cbl in BCR/abl transformed hematopoietic cells mediates enhanced association with phosphatidylinositol 3-kinase, Oncogene, *14:* 2217-2228, 1997.

263 Skorski,T., Bellacosa,A., Nieborowska-Skorska,M.N., Majewski,M., Martinez,R., Choi,J.K., Trotta,R., Wlodarski,P., Perrotti,D., Chan,T.O., Wasik,M.A., Tsichlis,P.N. and Calabretta,B. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3K/Akt-dependent pathway, EMBO J., *16:* 6151-6161, 1997.

264 Vivanco,I. and Sawyers,C.L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer, Nat.Rev.Cancer, *2:* 489-501, 2002.

265 Salomoni,P., Condorelli,F., Sweeney,S.M. and Calabretta,B. Versatility of BCR/ABL-expressing leukemic cells in circumventing proapoptotic BAD effects, Blood, *96:* 676-684, 2000.

266 Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J. and Greenberg,M.E. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor, Cell, *96:* 857-868, 1999.

267 Mayo,L.D. and Donner,D.B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus, Proc.Natl.Acad.Sci.U.S.A, *98:* 11598-11603, 2001.

268 Zhou,B.P., Liao,Y., Xia,W., Zou,Y., Spohn,B. and Hung,M.C. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation, Nat.Cell Biol., *3:* 973-982, 2001.

269 Ozes,O.N., Mayo,L.D., Gustin,J.A., Pfeffer,S.R., Pfeffer,L.M. and Donner,D.B. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase, Nature, *401:* 82-85, 1999.

270 Silverman,N. and Maniatis,T. NF-kappaB signaling pathways in mammalian and insect innate immunity, Genes Dev., *15:* 2321-2342, 2001.

271 Nishikori,M. Classical and alternative NF-kB activation pathways and their roles in Lymphoid malignancies, Journal of Clinical and Experimental Hematopathology, *45:* 15-24, 2005.

272 Rieger,K., Loddenkemper,C., Maul,J., Fietz,T., Wolff,D., Terpe,H., Steiner,B., Berg,E., Miehlke,S., Bornhauser,M., Schneider,T., Zeitz,M., Stein,H., Thiel,E., Duchmann,R. and Uharek,L. Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD, Blood, *107:* 1717-1723, 2006.

273 Sattler,M., Verma,S., Shrikhande,G., Byrne,C.H., Pride,Y.B., Winkler,T., Greenfield,E.A., Salgia,R. and Griffin,J.D. The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells, J.Biol.Chem., *275:* 24273-24278, 2000.

274 Reya,T., Duncan,A.W., Ailles,L., Domen,J., Scherer,D.C., Willert,K., Hintz,L., Nusse,R. and Weissman,I.L. A role for Wnt signalling in self-renewal of haematopoietic stem cells, Nature, *423:* 409-414, 2003.

275 Liu,C., Li,Y., Semenov,M., Han,C., Baeg,G.H., Tan,Y., Zhang,Z., Lin,X. and He,X. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism, Cell, *108:* 837-847, 2002.

276 Klymkowsky,M.W. beta-catenin and its regulatory network, Hum.Pathol., *36:* 225-227, 2005.

277 Kikuchi,A. Regulation of beta-catenin signaling in the Wnt pathway, Biochem.Biophys.Res.Commun., *268:* 243-248, 2000.

278 Staal,F.J. and Clevers,H.C. WNT signalling and haematopoiesis: a WNT-WNT situation, Nat.Rev.Immunol., *5:* 21-30, 2005.

279 Coluccia,A.M., Vacca,A., Dunach,M., Mologni,L., Redaelli,S., Bustos,V.H., Benati,D., Pinna,L.A. and Gambacorti-Passerini,C. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation, EMBO J., *26:* 1456-1466, 2007.

280 Thannickal,V.J. and Fanburg,B.L. Reactive oxygen species in cell signaling, Am.J.Physiol Lung Cell Mol.Physiol, *279:* L1005-L1028, 2000.

281 Holmes,T., O'Brien,T.A., Knight,R., Lindeman,R., Shen,S., Song,E., Symonds,G. and Dolnikov,A. Glycogen synthase kinase-3beta inhibition preserves hematopoietic stem cell activity and inhibits leukemic cell growth, Stem Cells, *26:* 1288-1297, 2008.

282 Hu,Y., Chen,Y., Douglas,L. and Li,S. beta-Catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia, Leukemia, 2008.

283 Savona,M. and Talpaz,M. Getting to the stem of chronic myeloid leukaemia, Nat.Rev.Cancer, *8:* 341-350, 2008.

284 Druker,B.J., O'Brien,S.G., Cortes,J. and Radich,J. Chronic myelogenous leukemia, Hematology.Am.Soc.Hematol.Educ.Program., 111-135, 2002.

285 Salesse,S. and Verfaillie,C.M. BCR/ABL: from molecular mechanisms of leukemia induction to treatment of chronic myelogenous leukemia, Oncogene, *21:* 8547-8559, 2002.

286 Talpaz,M., Kantarjian,H.M., McCredie,K., Trujillo,J.M., Keating,M.J. and Gutterman,J.U. Hematologic remission and cytogenetic improvement induced by recombinant human interferon alpha A in chronic myelogenous leukemia, N.Engl.J.Med., *314:* 1065-1069, 1986.

287 Darnell,J.E., Jr., Kerr,I.M. and Stark,G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins, Science, *264:* 1415-1421, 1994.

288 Druker,B.J., Tamura,S., Buchdunger,E., Ohno,S., Segal,G.M., Fanning,S., Zimmermann,J. and Lydon,N.B. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells, Nat.Med., *2:* 561-566, 1996.

289 Kasper,B., Fruehauf,S., Schiedlmeier,B., Buchdunger,E., Ho,A.D. and Zeller,W.J. Favorable therapeutic index of a p210(BCR-ABL)-specific tyrosine kinase inhibitor; activity on lineage-committed and primitive chronic myelogenous leukemia progenitors, Cancer Chemother.Pharmacol., *44:* 433-438, 1999.

290 Dewar,A.L., Cambareri,A.C., Zannettino,A.C., Miller,B.L., Doherty,K.V., Hughes,T.P. and Lyons,A.B. Macrophage colony-stimulating factor receptor c-fms is a novel target of imatinib, Blood, *105:* 3127-3132, 2005.

291 Heinrich,M.C., Griffith,D.J., Druker,B.J., Wait,C.L., Ott,K.A. and Zigler,A.J. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor, Blood, *96:* 925-932, 2000.

292 Okuda,K., Weisberg,E., Gilliland,D.G. and Griffin,J.D. ARG tyrosine kinase activity is inhibited by STI571, Blood, *97:* 2440-2448, 2001.

293 Buchdunger,E., Matter,A. and Druker,B.J. Bcr-Abl inhibition as a modality of CML therapeutics, Biochim.Biophys.Acta, *1551:* M11-M18, 2001.

294 Druker,B.J. and Lydon,N.B. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia, J.Clin.Invest, *105:* 3-7, 2000.

295 Wong,S., McLaughlin,J., Cheng,D., Zhang,C., Shokat,K.M. and Witte,O.N. Sole BCR-ABL inhibition is insufficient to eliminate all myeloproliferative disorder cell populations, Proc.Natl.Acad.Sci.U.S.A, *101:* 17456-17461, 2004.

296 Druker,B.J., Talpaz,M., Resta,D.J., Peng,B., Buchdunger,E., Ford,J.M., Lydon,N.B., Kantarjian,H., Capdeville,R., Ohno-Jones,S. and Sawyers,C.L. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia, N.Engl.J.Med., *344:* 1031-1037, 2001.

297 Druker,B.J., Sawyers,C.L., Capdeville,R., Ford,J.M., Baccarani,M. and Goldman,J.M. Chronic myelogenous leukemia, Hematology.Am.Soc.Hematol.Educ.Program., 87-112, 2001.

298 O'Brien,S.G., Guilhot,F., Larson,R.A., Gathmann,I., Baccarani,M., Cervantes,F., Cornelissen,J.J., Fischer,T., Hochhaus,A., Hughes,T., Lechner,K., Nielsen,J.L., Rousselot,P., Reiffers,J., Saglio,G., Shepherd,J., Simonsson,B., Gratwohl,A., Goldman,J.M., Kantarjian,H., Taylor,K., Verhoef,G., Bolton,A.E., Capdeville,R. and Druker,B.J. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia, N.Engl.J.Med., *348:* 994-1004, 2003.

299 Gambacorti-Passerini,C.B., Gunby,R.H., Piazza,R., Galietta,A., Rostagno,R. and Scapozza,L. Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias, Lancet Oncol., *4:* 75-85, 2003.

300 Gorre,M.E., Mohammed,M., Ellwood,K., Hsu,N., Paquette,R., Rao,P.N. and Sawyers,C.L. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification, Science, *293:* 876-880, 2001.

301 von,B.N., Schneller,F., Peschel,C. and Duyster,J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study, Lancet, *359:* 487-491, 2002.

302 Hochhaus,A., Kreil,S., Corbin,A.S., La,R.P., Muller,M.C., Lahaye,T., Hanfstein,B., Schoch,C., Cross,N.C., Berger,U., Gschaidmeier,H., Druker,B.J. and Hehlmann,R. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy, Leukemia, *16:* 2190-2196, 2002.

303 Shah,N.P., Nicoll,J.M., Nagar,B., Gorre,M.E., Paquette,R.L., Kuriyan,J. and Sawyers,C.L. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia, Cancer Cell, *2:* 117-125, 2002.

304 Deininger,M.W. Basic science going clinical: molecularly targeted therapy of chronic myelogenous leukemia, J.Cancer Res.Clin.Oncol., *130:* 59-72, 2004.

305 Weisberg,E., Manley,P.W., Cowan-Jacob,S.W., Hochhaus,A. and Griffin,J.D. Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia, Nat.Rev.Cancer, *7:* 345-356, 2007.

306 Roche-Lestienne,C., Soenen-Cornu,V., Grardel-Duflos,N., Lai,J.L., Philippe,N., Facon,T., Fenaux,P. and Preudhomme,C. Several types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment, Blood, *100:* 1014-1018, 2002.

307 Griswold,I.J., MacPartlin,M., Bumm,T., Goss,V.L., O'Hare,T., Lee,K.A., Corbin,A.S., Stoffregen,E.P., Smith,C., Johnson,K., Moseson,E.M., Wood,L.J., Polakiewicz,R.D., Druker,B.J. and Deininger,M.W. Kinase domain mutants of Bcr-Abl exhibit altered transformation potency, kinase activity, and substrate utilization, irrespective of sensitivity to imatinib, Mol.Cell Biol., *26:* 6082-6093, 2006.

308 Skaggs,B.J., Gorre,M.E., Ryvkin,A., Burgess,M.R., Xie,Y., Han,Y., Komisopoulou,E., Brown,L.M., Loo,J.A., Landaw,E.M., Sawyers,C.L. and Graeber,T.G. Phosphorylation of the ATP-binding loop directs oncogenicity of drug-resistant BCR-ABL mutants, Proc.Natl.Acad.Sci.U.S.A, *103:* 19466-19471, 2006.

309 Soverini,S., Martinelli,G., Rosti,G., Bassi,S., Amabile,M., Poerio,A., Giannini,B., Trabacchi,E., Castagnetti,F., Testoni,N., Luatti,S., de,V.A., Cilloni,D., Izzo,B., Fava,M., Abruzzese,E., Alberti,D., Pane,F., Saglio,G. and Baccarani,M. ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chronic Myeloid Leukemia, J.Clin.Oncol., *23:* 4100-4109, 2005.

310 Azam,M., Seeliger,M.A., Gray,N.S., Kuriyan,J. and Daley,G.Q. Activation of tyrosine kinases by mutation of the gatekeeper threonine, Nat.Struct.Mol.Biol., *15:* 1109-1118, 2008.

311 Nicolini,F.E., Corm,S., Le,Q.H., Sorel,N., Hayette,S., Bories,D., Leguay,T., Roy,L., Giraudier,S., Tulliez,M., Facon,T., Mahon,F.X., Cayuela,J.M., Rousselot,P., Michallet,M., Preudhomme,C., Guilhot,F. and Roche-Lestienne,C. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP), Leukemia, *20:* 1061-1066, 2006.

312 Gambacorti-Passerini,C., Zucchetti,M., Russo,D., Frapolli,R., Verga,M., Bungaro,S., Tornaghi,L., Rossi,F., Pioltelli,P., Pogliani,E., Alberti,D., Corneo,G. and D'Incalci,M. Alpha1 acid glycoprotein binds to imatinib (STI571) and substantially alters its pharmacokinetics in chronic myeloid leukemia patients, Clin.Cancer Res., *9:* 625-632, 2003.

313 Mahon,F.X., Belloc,F., Lagarde,V., Chollet,C., Moreau-Gaudry,F., Reiffers,J., Goldman,J.M. and Melo,J.V. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models, Blood, *101:* 2368-2373, 2003.

314 Wu,J., Meng,F., Kong,L.Y., Peng,Z., Ying,Y., Bornmann,W.G., Darnay,B.G., Lamothe,B., Sun,H., Talpaz,M. and Donato,N.J. Association between imatinib-resistant BCR-ABL mutation-negative leukemia and persistent activation of LYN kinase, J.Natl.Cancer Inst., *100:* 926-939, 2008.

315 Breccia,M., Diverio,D., Pane,F., Nanni,M., Russo,E., Biondo,F., Frustaci,A., Gentilini,F. and Alimena,G. Discontinuation of imatinib therapy after achievement of complete molecular response in a Ph(+) CML patient treated while in long lasting complete cytogenetic remission (CCR) induced by interferon, Leuk.Res., *30:* 1577-1579, 2006.

316 Rousselot,P., Huguet,F., Rea,D., Legros,L., Cayuela,J.M., Maarek,O., Blanchet,O., Marit,G., Gluckman,E., Reiffers,J., Gardembas,M. and Mahon,F.X. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years, Blood, *109:* 58-60, 2007.

317 White,D.L., Saunders,V.A., Dang,P., Engler,J., Zannettino,A.C., Cambareri,A.C., Quinn,S.R., Manley,P.W. and Hughes,T.P. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib, Blood, *108:* 697-704, 2006.

318 O'Hare,T., Walters,D.K., Stoffregen,E.P., Jia,T., Manley,P.W., Mestan,J., Cowan-Jacob,S.W., Lee,F.Y., Heinrich,M.C., Deininger,M.W. and Druker,B.J. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants, Cancer Res., *65:* 4500-4505, 2005.

319 Weisberg,E., Manley,P., Mestan,J., Cowan-Jacob,S., Ray,A. and Griffin,J.D. AMN107 (nilotinib): a novel and selective inhibitor of BCR-ABL, Br.J.Cancer, *94:* 1765-1769, 2006.

320 Adrian,F.J., Ding,Q., Sim,T., Velentza,A., Sloan,C., Liu,Y., Zhang,G., Hur,W., Ding,S., Manley,P., Mestan,J., Fabbro,D. and Gray,N.S. Allosteric inhibitors of Bcr-abl-dependent cell proliferation, Nat.Chem.Biol., *2:* 95-102, 2006.

321 Giles,F.J., Cortes,J., Jones,D., Bergstrom,D., Kantarjian,H. and Freedman,S.J. MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation, Blood, *109:* 500-502, 2007.

322 Lombardo,L.J., Lee,F.Y., Chen,P., Norris,D., Barrish,J.C., Behnia,K., Castaneda,S., Cornelius,L.A., Das,J., Doweyko,A.M., Fairchild,C., Hunt,J.T., Inigo,I., Johnston,K., Kamath,A., Kan,D., Klei,H., Marathe,P., Pang,S., Peterson,R., Pitt,S., Schieven,G.L., Schmidt,R.J., Tokarski,J., Wen,M.L., Wityak,J. and Borzilleri,R.M. Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays, J.Med.Chem., *47:* 6658-6661, 2004.

323 Shah,N.P., Tran,C., Lee,F.Y., Chen,P., Norris,D. and Sawyers,C.L. Overriding imatinib resistance with a novel ABL kinase inhibitor, Science, *305:* 399-401, 2004.

324 Burgess,M.R., Skaggs,B.J., Shah,N.P., Lee,F.Y. and Sawyers,C.L. Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance, Proc.Natl.Acad.Sci.U.S.A, *102:* 3395-3400, 2005.

325 Bartholomeusz,G.A., Talpaz,M., Kapuria,V., Kong,L.Y., Wang,S., Estrov,Z., Priebe,W., Wu,J. and Donato,N.J. Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells, Blood, *109:* 3470-3478, 2007.

326 Copland,M., Pellicano,F., Richmond,L., Allan,E.K., Hamilton,A., Lee,F.Y., Weinmann,R. and Holyoake,T.L. BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergizes with tyrosine kinase inhibitors, Blood, *111:* 2843-2853, 2008.

327 Kessler,J.H., Bres-Vloemans,S.A., van Veelen,P.A., de,R.A., Huijbers,I.J., Camps,M., Mulder,A., Offringa,R., Drijfhout,J.W., Leeksma,O.C., Ossendorp,F. and Melief,C.J.

BCR-ABL fusion regions as a source of multiple leukemia-specific CD8+ T-cell epitopes, Leukemia, *20:* 1738-1750, 2006.

328 Martin,G.S. The road to Src, Oncogene, *23:* 7910-7917, 2004.

329 Fojo,T. Commentary: Novel therapies for cancer: why dirty might be better, Oncologist., *13:* 277-283, 2008.

330 Dai,Y., Rahmani,M., Corey,S.J., Dent,P. and Grant,S. A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2, J.Biol.Chem., *279:* 34227-34239, 2004.

331 Donato,N.J., Wu,J.Y., Stapley,J., Lin,H., Arlinghaus,R., Aggarwal,B.B., Shishodia,S., Albitar,M., Hayes,K., Kantarjian,H. and Talpaz,M. Imatinib mesylate resistance through BCR-ABL independence in chronic myelogenous leukemia, Cancer Res., *64:* 672-677, 2004.

332 Bishop,A.C., Ubersax,J.A., Petsch,D.T., Matheos,D.P., Gray,N.S., Blethrow,J., Shimizu,E., Tsien,J.Z., Schultz,P.G., Rose,M.D., Wood,J.L., Morgan,D.O. and Shokat,K.M. A chemical switch for inhibitor-sensitive alleles of any protein kinase, Nature, *407:* 395-401, 2000.

333 Ben-Neriah,Y., Daley,G.Q., Mes-Masson,A.M., Witte,O.N. and Baltimore,D. The chronic myelogenous leukemia-specific p210 protein is the product of the *bcr/abl* hybrid gene, Science, *233:* 212-214, 1986.

334 Van Etten,R.A., Jackson,P. and Baltimore,D. The mouse type IV c-*abl* gene product is a nuclear protein, and activation of its transforming ability is associated with cytoplasmic localization, Cell, *58:* 669-678, 1989.

335 Lugo,T.G. and Witte,O.N. The BCR-ABL oncogene transforms Rat-1 cells and cooperates with v-*myc*, Mol.Cell.Biol., *9:* 1263-1270, 1989.

336 McLaughlin,J., Chianese,E. and Witte,O.N. In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome, Proc.Natl.Acad.Sci.U.S.A, *84:* 6558-6562, 1987.

337 Ren,R. The molecular mechanism of chronic myelogenous leukemia and its therapeutic implications: studies in a murine model, Oncogene, *21:* 8629-8642, 2002.

338 Kelliher,M.A., McLaughlin,J., Witte,O.N. and Rosenberg,N. Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL, Proc.Natl.Acad.Sci.U.S.A, *87:* 6649-6653, 1990.

339 Gesbert,F. and Griffin,J.D. Bcr/Abl activates transcription of the Bcl-X gene through STAT5, Blood, *96:* 2269-2276, 2000.

340 de Groot,R.P., Raaijmakers,J.A., Lammers,J.W. and Koenderman,L. STAT5-Dependent CyclinD1 and Bcl-xL expression in Bcr-Abl-transformed cells, Mol.Cell Biol.Res.Commun., *3:* 299-305, 2000.

341 Harrison-Findik,D., Susa,M. and Varticovski,L. Association of phosphatidylinositol 3-kinase with SHC in chronic myelogeneous leukemia cells, Oncogene, *10:* 1385-1391, 1995.

342 Raffel,G.D., Parmar,K. and Rosenberg,N. In vivo association of v-Abl with Shc mediated by a non-phosphotyrosine-dependent SH2 interaction, J.Biol.Chem., *271:* 4640-4645, 1996.

343 Neshat,M.S., Raitano,A.B., Wang,H.G., Reed,J.C. and Sawyers,C.L. The survival function of the Bcr-Abl oncogene is mediated by Bad-dependent and -independent pathways: roles for phosphatidylinositol 3-kinase and Raf, Mol.Cell Biol., *20:* 1179-1186, 2000.

344 Smith,K.M., Yacobi,R. and Van Etten,R.A. Autoinhibition of Bcr-Abl through its SH3 domain, Mol.Cell, *12:* 27-37, 2003.

345 Kantarjian,H., Sawyers,C., Hochhaus,A., Guilhot,F., Schiffer,C., Gambacorti-Passerini,C., Niederwieser,D., Resta,D., Capdeville,R., Zoellner,U., Talpaz,M., Druker,B., Goldman,J., O'Brien,S.G., Russell,N., Fischer,T., Ottmann,O., Cony-Makhoul,P., Facon,T., Stone,R., Miller,C., Tallman,M., Brown,R., Schuster,M., Loughran,T., Gratwohl,A., Mandelli,F., Saglio,G., Lazzarino,M., Russo,D., Baccarani,M. and Morra,E. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia, N.Engl.J.Med., *346:* 645-652, 2002.

346 Bhatia,R., Holtz,M., Niu,N., Gray,R., Snyder,D.S., Sawyers,C.L., Arber,D.A., Slovak,M.L. and Forman,S.J. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment, Blood, *101:* 4701-4707, 2003.

347 Liu,Y., Shah,K., Yang,F., Witucki,L. and Shokat,K.M. A molecular gate which controls unnatural ATP analogue recognition by the tyrosine kinase v-Src, Bioorg.Med.Chem., *6:* 1219-1226, 1998.

348 Blencke,S., Ullrich,A. and Daub,H. Mutation of threonine 766 in the epidermal growth factor receptor reveals a hotspot for resistance formation against selective tyrosine kinase inhibitors, J.Biol.Chem., *278:* 15435-15440, 2003.

349 Blencke,S., Zech,B., Engkvist,O., Greff,Z., Orfi,L., Horvath,Z., Keri,G., Ullrich,A. and Daub,H. Characterization of a conserved structural determinant controlling protein kinase sensitivity to selective inhibitors, Chem.Biol., *11:* 691-701, 2004.

350 Eyers,P.A., van,d., I, Quinlan,R.A., Goedert,M. and Cohen,P. Use of a drug-resistant mutant of stress-activated protein kinase 2a/p38 to validate the in vivo specificity of SB 203580, FEBS Lett., *451:* 191-196, 1999.

351 Tamborini,E., Bonadiman,L., Greco,A., Albertini,V., Negri,T., Gronchi,A., Bertulli,R., Colecchia,M., Casali,P.G., Pierotti,M.A. and Pilotti,S. A new mutation in the KIT ATP pocket causes acquired resistance to imatinib in a gastrointestinal stromal tumor patient, Gastroenterology, *127:* 294-299, 2004.

352 Cools,J., DeAngelo,D.J., Gotlib,J., Stover,E.H., Legare,R.D., Cortes,J., Kutok,J., Clark,J., Galinsky,I., Griffin,J.D., Cross,N.C., Tefferi,A., Malone,J., Alam,R., Schrier,S.L., Schmid,J., Rose,M., Vandenberghe,P., Verhoef,G., Boogaerts,M., Wlodarska,I., Kantarjian,H., Marynen,P., Coutre,S.E., Stone,R. and Gilliland,D.G. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome, N.Engl.J.Med., *348:* 1201-1214, 2003.

353 Kobayashi,S., Boggon,T.J., Dayaram,T., Janne,P.A., Kocher,O., Meyerson,M., Johnson,B.E., Eck,M.J., Tenen,D.G. and Halmos,B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib, N.Engl.J.Med., *352:* 786-792, 2005.

354 Liu,Y., Bishop,A., Witucki,L., Kraybill,B., Shimizu,E., Tsien,J., Ubersax,J., Blethrow,J., Morgan,D.O. and Shokat,K.M. Structural basis for selective inhibition of Src family kinases by PP1, Chem.Biol., *6:* 671-678, 1999.

355 Porter,M., Schindler,T., Kuriyan,J. and Miller,W.T. Reciprocal regulation of Hck activity by phosphorylation of Tyr(527) and Tyr(416). Effect of introducing a high affinity intramolecular SH2 ligand, J.Biol.Chem., *275:* 2721-2726, 2000.

356 Lerner,E.C. and Smithgall,T.E. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo, Nat.Struct.Biol., *9:* 365-369, 2002.

357 Schreiner,S.J., Schiavone,A.P. and Smithgall,T.E. Activation of Stat3 by the Src family kinase Hck requires a functional SH3 domain, J.Biol.Chem., *277:* 45680-45687, 2002.

358 Kitamura,T., Tange,T., Terasawa,T., Chiba,S., Kuwaki,T., Miyagawa,K., Piao,Y.-F., Miyazono,K., Urabe,A. and Takaku,F. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3 or erythropoietin, J.Cell.Physiol., *140:* 323-334, 1989.

359 Nakajima,A., Tauchi,T. and Ohyashiki,K. ABL-specific tyrosine kinase inhibitor, STI571 in vitro, affects Ph-positive acute lymphoblastic leukemia and chronic myelogenous leukemia in blastic crisis, Leukemia, *15:* 989-990, 2001.

360 Kantarjian,H.M., Giles,F., Quintas-Cardama,A. and Cortes,J. Important therapeutic targets in chronic myelogenous leukemia, Clin.Cancer Res., *13:* 1089-1097, 2007.

361 Olivieri,A. and Manzione,L. Dasatinib: a new step in molecular target therapy, Ann.Oncol., *18 Suppl 6:* vi42-vi46, 2007.

362 Martinelli,G., Soverini,S., Rosti,G. and Baccarani,M. Dual tyrosine kinase inhibitors in chronic myeloid leukemia, Leukemia, *19:* 1872-1879, 2005.

363 Das,J., Chen,P., Norris,D., Padmanabha,R., Lin,J., Moquin,R.V., Shen,Z., Cook,L.S., Doweyko,A.M., Pitt,S., Pang,S., Shen,D.R., Fang,Q., de Fex,H.F., McIntyre,K.W., Shuster,D.J., Gillooly,K.M., Behnia,K., Schieven,G.L., Wityak,J. and Barrish,J.C. 2-aminothiazole as a novel kinase inhibitor template. Structure-activity relationship studies toward the discovery of N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl)]-2-methyl-4-pyrimidinyl]amino)]-1,3-thiazole-5-carboxamide (dasatinib, BMS-354825) as a potent pan-Src kinase inhibitor, J.Med.Chem., *49:* 6819-6832, 2006.

364 Melnick,J.S., Janes,J., Kim,S., Chang,J.Y., Sipes,D.G., Gunderson,D., Jarnes,L., Matzen,J.T., Garcia,M.E., Hood,T.L., Beigi,R., Xia,G., Harig,R.A., Asatryan,H., Yan,S.F., Zhou,Y., Gu,X.J., Saadat,A., Zhou,V., King,F.J., Shaw,C.M., Su,A.I., Downs,R., Gray,N.S., Schultz,P.G., Warmuth,M. and Caldwell,J.S. An efficient rapid system for profiling the cellular activities of molecular libraries, Proc.Natl.Acad.Sci.U.S.A, *103:* 3153-3158, 2006.

365 Pao,W., Miller,V.A., Politi,K.A., Riely,G.J., Somwar,R., Zakowski,M.F., Kris,M.G. and Varmus,H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain, PLoS.Med., *2:* e73, 2005.

366 Hoover,R.R., Gerlach,M.J., Koh,E.Y. and Daley,G.Q. Cooperative and redundant effects of STAT5 and Ras signaling in BCR/ABL transformed hematopoietic cells, Oncogene, *20:* 5826-5835, 2001.

367 Furstoss,O., Dorey,K., Simon,V., Barila,D., Superti-Furga,G. and Roche,S. c-Abl is an effector of Src for growth factor-induced c-myc expression and DNA synthesis, EMBO J., *21:* 514-524, 2002.

368 Calderwood,D.J., Johnston,D.N., Munschauer,R. and Rafferty,P. Pyrrolo[2,3-d]pyrimidines containing diverse N-7 substituents as potent inhibitors of Lck, Bioorg.Med.Chem.Lett., *12:* 1683-1686, 2002.

369 Arnold,L.D., Calderwood,D.J., Dixon,R.W., Johnston,D.N., Kamens,J.S., Munschauer,R., Rafferty,P. and Ratnofsky,S.E. Pyrrolo[2,3-d]pyrimidines containing an extended 5-substituent as potent and selective inhibitors of lck I, Bioorganic & Medicinal Chemistry Letters, *10:* 2167-2170, 2000.

370 Holtz,M.S., Forman,S.J. and Bhatia,R. Nonproliferating CML CD34+ progenitors are resistant to apoptosis induced by a wide range of proapoptotic stimuli, Leukemia, *19:* 1034-1041, 2005.

371 Cortes,J., O'Brien,S. and Kantarjian,H. Discontinuation of imatinib therapy after achieving a molecular response, Blood, *104:* 2204-2205, 2004.

372 Mauro,M.J., Druker,B.J. and Maziarz,R.T. Divergent clinical outcome in two CML patients who discontinued imatinib therapy after achieving a molecular remission, Leuk.Res., *28 Suppl 1:* S71-S73, 2004.

373 Lozzio,B.B., Lozzio,C.B., Bamberger,E.G. and Feliu,A.S. A multipotential leukemia cell line (K-562) of human origin, Proc.Soc.Exp.Biol.Med., *166:* 546-550, 1981.

374 Trible,R.P., Emert-Sedlak,L. and Smithgall,T.E. HIV-1 Nef selectively activates SRC family kinases HCK, LYN, and c-SRC through direct SH3 domain interaction, J.Biol.Chem., *281:* 27029-27038, 2006.

375 Muller,A.J., Young,J.C., Pendergast,A.M., Pondel,M., Landau,R.N., Littman,D.R. and Witte,O.N. *BCR* first exon sequences specifically activate the *BCR/ABL* tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias, Mol.Cell.Biol., *11:* 1785-1792, 1991.

376 Pear,W.S., Nolan,G.P., Scott,M.L. and Baltimore,D. Production of high-titer helper-free retroviruses by transient transfection, Proc.Natl.Acad.Sci.USA, *90:* 8392-8396, 1993.

377 Deininger,M.W., Goldman,J.M. and Melo,J.V. The molecular biology of chronic myeloid leukemia, Blood, *96:* 3343-3356, 2000.

378 Raitano,A.B., Whang,Y.E. and Sawyers,C.L. Signal transduction by wild-type and leukemogenic Abl proteins, Biochim.Biophys.Acta, *1333:* F201-F216, 1997.

379 Faderl,S., Talpaz,M., Estrov,Z., O'Brien,S., Kurzrock,R. and Kantarjian,H.M. The biology of chronic myeloid leukemia, N.Engl.J.Med., *341:* 164-172, 1999.

380 Kirchner,D., Duyster,J., Ottmann,O., Schmid,R.M., Bergmann,L. and Munzert,G. Mechanisms of Bcr-Abl-mediated NF-kappaB/Rel activation, Exp.Hematol., *31:* 504-511, 2003.

381 Danial,N.N. and Rothman,P. JAK-STAT signaling activated by Abl oncogenes, Oncogene, *19:* 2523-2531, 2000.

382 Voss,J., Posern,G., Hannemann,J.R., Wiedemann,L.M., Turhan,A.G., Poirel,H., Bernard,O.A., Adermann,K., Kardinal,C. and Feller,S.M. The leukaemic oncoproteins Bcr-Abl and Tel-Abl (ETV6/Abl) have altered substrate preferences and activate similar intracellular signalling pathways, Oncogene, *19:* 1684-1690, 2000.

383 Goldman,J.M. and Melo,J.V. Chronic myeloid leukemia--advances in biology and new approaches to treatment, N.Engl.J.Med., *349:* 1451-1464, 2003.

384 Hochhaus,A., Druker,B., Sawyers,C., Guilhot,F., Schiffer,C.A., Cortes,J., Niederwieser,D.W., Gambacorti-Passerini,C., Stone,R.M., Goldman,J., Fischer,T., O'Brien,S.G., Reiffers,J.J., Mone,M., Krahnke,T., Talpaz,M. and Kantarjian,H.M. Favorable long-term follow-up results over 6 years for response, survival, and safety with imatinib mesylate therapy in chronic-phase chronic myeloid leukemia after failure of interferon-alpha treatment, Blood, *111:* 1039-1043, 2008.

385 Druker,B.J., Guilhot,F., O'Brien,S.G., Gathmann,I., Kantarjian,H., Gattermann,N., Deininger,M.W., Silver,R.T., Goldman,J.M., Stone,R.M., Cervantes,F., Hochhaus,A.,

Powell,B.L., Gabrilove,J.L., Rousselot,P., Reiffers,J., Cornelissen,J.J., Hughes,T., Agis,H., Fischer,T., Verhoef,G., Shepherd,J., Saglio,G., Gratwohl,A., Nielsen,J.L., Radich,J.P., Simonsson,B., Taylor,K., Baccarani,M., So,C., Letvak,L. and Larson,R.A. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia, N.Engl.J.Med., *355:* 2408-2417, 2006.

386 Bishop,A.C., Buzko,O. and Shokat,K.M. Magic bullets for protein kinases, Trends Cell Biol., *11:* 167-172, 2001.

387 Alaimo,P.J., Knight,Z.A. and Shokat,K.M. Targeting the gatekeeper residue in phosphoinositide 3-kinases, Bioorg.Med.Chem., *13:* 2825-2836, 2005.

388 Bishop,A.C., Shah,K., Liu,Y., Witucki,L., Kung,C. and Shokat,K.M. Design of allele-specific inhibitors to probe protein kinase signaling, Curr.Biol., *8:* 257-266, 1998.

389 MacAuley,A., Okada,M., Nada,S., Nakagawa,H. and Cooper,J.A. Phosphorylation of Src mutants at Tyr 527 in fibroblasts does not correlate with in vitro phosphorylation by CSK, Oncogene, *8:* 117-124, 1993.

390 Rix,U., Hantschel,O., Durnberger,G., Remsing Rix,L.L., Planyavsky,M., Fernbach,N.V., Kaupe,I., Bennett,K.L., Valent,P., Colinge,J., Kocher,T. and Superti-Furga,G. Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets, Blood, *110:* 4055-4063, 2007.

391 Satyamoorthy,K., Li,G., Vaidya,B., Patel,D. and Herlyn,M. Insulin-like growth factor-1 induces survival and growth of biologically early melanoma cells through both the mitogen-activated protein kinase and beta-catenin pathways, Cancer Res., *61:* 7318-7324, 2001.

392 Mahon,F.X., Hayette,S., Lagarde,V., Belloc,F., Turcq,B., Nicolini,F., Belanger,C., Manley,P.W., Leroy,C., Etienne,G., Roche,S. and Pasquet,J.M. Evidence that resistance to nilotinib may be due to BCR-ABL, Pgp, or Src kinase overexpression, Cancer Res., *68:* 9809-9816, 2008.

393 Holtz,M.S., Slovak,M.L., Zhang,F., Sawyers,C.L., Forman,S.J. and Bhatia,R. Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation, Blood, *99:* 3792-3800, 2002.

394 Vlahovic,G. and Crawford,J. Activation of tyrosine kinases in cancer, Oncologist., *8:* 531-538, 2003.

395 Fan,Q.W. and Weiss,W.A. Chemical genetic approaches to the development of cancer therapeutics, Curr.Opin.Genet.Dev., *16:* 85-91, 2006.

396 Kabarowski,J.H. and Witte,O.N. Consequences of BCR-ABL expression within the hematopoietic stem cell in chronic myeloid leukemia, Stem Cells, *18:* 399-408, 2000.

397 Wong,S., McLaughlin,J., Cheng,D. and Witte,O.N. Cell context-specific effects of the BCR-ABL oncogene monitored in hematopoietic progenitors, Blood, *101:* 4088-4097, 2003.

398 Zhang,X., Crespo,A. and Fernandez,A. Turning promiscuous kinase inhibitors into safer drugs, Trends Biotechnol., *26:* 295-301, 2008.