Src Family Kinases: Potential Targets for the Treatment of Human Cancer and Leukemia

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Abstract: The inherited or acquired deregulation of protein kinase activity has been implicated in the pathogenesis of many human diseases, including cancer. Therefore, the inhibition of kinases has been proposed to be a promising strategy in the context of anti-cancer treatment. Many other kinases have been selected as drug discovery targets based on the prevalence of mutations, over-expression and unscheduled activation in human cancer. Of the various protein kinases chosen, Src family kinases are amongst the most extensively studied kinase oncogenes in academia and industry. This review focuses on our current understanding of the deregulation and role of Src family kinases in human cancer and leukemia. Recent data implicate the action of c-Src in cancer metastasis, mediated by up-regulation of various protease systems (calpain, uPA) as well as disruption of E-cadherin signalling. Moreover, novel roles of various Src family members in the development of human leukemia have been found. New insights into downstream signalling mechanisms, including the activation of STAT3, PDK1 and Akt, further corroborate the importance of Src family kinases in tumorigenesis and chemoresistance. Despite our rather clear understanding of Src family kinases as pro-oncogenes no Src family kinase inhibitor has entered a clinical trial so far. This review will discuss prerequisites to be fulfilled for clinically targeting c-Src and its homologues using small molecule drugs.

Key Words: Protein kinases, Src family kinases, small molecule kinase inhibitor, leukemogenesis, cancerogenesis

INTRODUCTION

Recent data analysis suggests that the human "kinome" comprises approximately 520 protein kinase genes encoded in our DNA [1, 2]. Amino acid alignments and crystal structure analyses of such kinases demonstrate that all of them share striking homology in the catalytic domain both at the primary sequence level and at the level of 3-dimensional topology and function [3]. This obvious homology led to the widespread belief that the development of ATP-competitive kinase inhibitors with sufficient specificity and safety for clinical application might be almost impossible. Despite these concerns, the recent approval of Gleevec (STI571, imatinib mesylat) for the treatment of chronic myelogenous leukemia (CML) has provided proof that inhibitors of protein kinases can be successfully transferred from preclinical research into clinical application and analyses of the binding mode of STI571 suggests that not only differences at single amino acid positions but also conformational differences of single amino acid side chains are sufficient to confer specificity to small molecule ATP-competitive kinase inhibitors (see below).

Driven by the success story of STI571, Iressa and other ATP-competitive compounds in clinical trials, the pharmaceutical industry is turning its attention towards other targets that might be of potential utility in the treatment of cancer and leukemia. These targets include receptor tyrosine kinases (RTKs), e.g. receptors of the EGF family, Tie-2, vascular endothelial growth factor (VEGF)-receptors and homo sapiens fms-related tyrosine kinase 3 (Flt3), as well as non-receptor kinases, including cyclin dependent kinases (CDK), protein kinase C (PKC), Akt or Raf. Amongst those, Src family kinases are amidst the most intensively studied kinases in academic as well as pharmaceutical research. Initially identified as the viral oncogene of the Rous Sarcoma Virus, v-Src turned out to be the viral relative to a family of 9 human Src family kinases (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes und Yrk), defined by their structural homology. Many of these fulfill important biological functions in a tissue specific context, but their hyperactivity in different tumor types also suggests a role in cancer and leukemia. Although no Src family specific kinase inhibitor has entered a clinical trial so far, recent research has shed new light on the regulation and function of Src family kinases in cancer and leukemia and many lines of evidence suggest a role of Src family kinases in the development and progression of human malignancies.

SRC FAMILY KINASES: NEW INSIGHTS ON AN OLD TARGET

Structure and Regulation of Src Kinases

Src kinases form a family of 9 non-receptor tyrosine kinases, all of which are expressed in a tissue specific manner. They closely resemble each other with regard to their overall structure and their mode of regulation [4]. All Src kinases consist of three functionally different Src-homology (SH) domains, called SH3, SH2 and SH1, the latter representing the kinase domain (Fig. **1A**). Both, the SH3 and the SH2 domain of Src kinases have been shown to

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mediate protein-protein interactions. It was shown that SH3 domains bind their partners through conserved proline rich (PXXP) motifs. In contrast, SH2 domains bind phosphorylated tyrosines embedded in conserved amino acid sequences. In addition, all Src family kinases contain a membrane targeting sequence (myristoylation site) and a non-conserved, unique region that differs significantly amongst different Src family kinases [4].

Many of the biological functions of Src kinases depend on their ability to interact with binding partners through SH3 and SH2 domain directed physical interactions. In addition, both domains have been implicated in regulating kinase activity through intramolecular binding mechanisms (Fig. **1B**). It was shown that the SH3 domain binds to a polyproline-type-II helix formed by the SH2-kinase-linker region [5-7]. The negative-regulatory function of the SH2 domain is mediated by binding to a phosphorylated tyrosine located within the C-terminal tail (Y527) [5, 6]. Several tyrosine kinases that phosphorylate this tyrosine residue specifically have been identified, including C-terminal Src kinase (CSK) and CSK homologous kinase (CHK), and their activity seems to be crucial for the negative regulation of the activity of various Src kinases [8-12]. Disruption of such regulatory intramolecular interactions by site directed mutagenesis led to deregulation of the kinase activity and released the transforming capacity of Src kinases [7, 13-17].

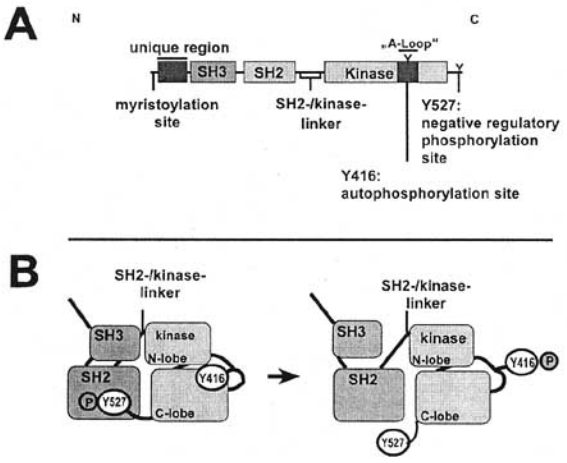


Fig. (1). Structure and regulation of Src family kinases.

Dysegulation of Src Kinases

Based on our understanding of the molecular network regulating Src kinase activity, several modes of hyper-activation of Src family kinases in cancer could be proposed. This includes binding of Src kinases to signalling molecules via SH3 and SH2 dependent interactions as well as point mutations disrupting their negative regulatory network. In support of this, it was shown that a subset of human colon cancer patients posses a mutant Src allele that lacks the negative-regulatory tyrosine phosphorylation site [18]. Mutations in other regions, such as the SH2-kinase linker might also be important, but have not been studied systematically. Briggs *et al.* could show that binding of the human immunodeficiency virus (HIV) Nef protein to the SH3 domain of Hck induces not only the kinase activity of Hck but also confers a transformed phenotype to Rat2 fibroblasts [19]. Growth factor receptors (Her2/Neu, PDGF, EGFR, c-Kit), adaptor proteins (c-Cbl) or other tyrosine kinases (focal adhesion kinase (FAK), Bcr-Abl) might be additional binding partners leading to Src family kinase activation through SH3 and SH2 domain displacement from its intramolecular negative regulatory interactions [20-28].

In addition, down-regulation of expression of CSK or up-regulation of a phosphatase dephosphorylating Src at the position Y527 could induce Src kinase activity. CSK protein expression and kinase activity appears to be reduced in hepatocellular carcinoma, paralleled by increased c-Src kinase activity [29]. Moreover, several phosphatases capable of dephosphorylating Src Y527 have been identified and increased phosphatase activity has been suggested to be a possible mechanism of c-Src activation in human breast cancer [30].

Finally, mis-regulation of Src kinase protein expression may lead to cellular transformation as a result of mRNA expression or perturbed proteasomal degradation. Indeed, several recent reports have shown that the expression level of Src kinases is influenced by their ubiqitination and subsequent degradation, which is dependent on the kinase activity [31-33]. As disregulation of proteasomal degradation of proteins is a common alteration in cancer, it is possible that c-Src and other family members are up-regulated due to an escape from self-guided degradation [34].

A ROLE FOR SRC KINASES IN HUMAN CANCER

Evidence from Cell Culture and Transgenic Model

Since its discovery in the early 80s much effort has been spent trying to identify the mechanisms and pathways leading to the transformation of cells by v-Src, the viral homologue of c-Src and founder of the family. It was shown that not only v-Src, but also gain-of function mutants of c-Src (and of other family members) are capable of transforming fibroblast cells and Src transformed cell lines were shown to illicit tumors in immunocomprised mice. Studies from fibroblasts and other cell types suggest a role of c-Src in almost every aspect of a cell's life, including mitogenesis, proliferation, survival, control of cellular adhesion and migration, all of which are deregulated during cancer progression. These data led to the investigation of a possible role of Src activation in human tumorigenesis. In support of this hypothesis, Webster *et al.* could show that transgenic expression of active Src in mammary glands of mice induces breast cancer [35]. Using v–Src kinase expressed under the control of a neuronal promotor element Weissenberger *et al.* were able to show that v-Src is capable of inducing low-grade astrocytomas that progressively evolve into hypervascularized glioblastomas [36].

Colon Cancer

Much evidence has been gathered implicating Src family kinases, above all c-Src itself, in the development of human cancer. The most compelling collection of data is available

on colon cancer. Several investigators could show that the specific activity of the non-receptor protein tyrosine kinase c-Src is increased 5 – 8 fold compared to normal mucosa in premalignant lesions as well as in the majority of colorectal adenocarcinomas, and that differences in c-Src activity correlate to tumor progression [37-39]. As one would expect, a further increase of specific c-Src activity is seen in cells derived from metastases versus cells from primary tumors [40]. The importance of these findings is emphasized by a recent report of Allgayer *et al.* showing that a two-fold and higher elevation of c-Src activity in tumor tissues (versus non-tumor controls) correlates with Dukes stage, tumor size and metastasis (pT and pN tumor classification), and an increase of u-PAR expression levels. Furthermore, elevated c-Src activity was associated with shorter overall survival and a decreased disease-free survival rate after curative resection, suggesting that c-Src activity is an independent indicator of poor clinical prognosis in all stages of human colon carcinoma [41].

While the analyses of primary tumor tissues suggested an involvement of elevated c-Src kinase activity in disease progression, a stringent genetic proof either from tissue culture or an animal model, is missing. Still, several recent reports further substantiate the notion that c-Src is a key player in colon cancer pathogenesis. Using a set of rat fibroblast cells with different "transformation indices" Malek *et al.* were able to identify a "Src transformation fingerprint" by gene expression analyses. The fingerprint showed a significant overlap with gene expression patterns identified in tissues from colon cancer patients which had advanced from adenomas to metastatic cancers [42]. Some of these genes give insight into a possible function of elevated c-Src activity in tumor progression. For example, elevated levels of cyclin G, cyclin D1 and Hsp90 as well as of Ubc12 and Ube2i have been found, suggesting a role of c-Src in deregulating cell cycle control. In addition, several genes implicated in regulating actin-based cell motility were found upregulated in Src-transformed fibroblasts and late stage colon cancer samples.

A possible role of c-Src in regulating cell motility and metastasis is evidenced by the suppression of tumor metastasis and invasion *in-vivo* of a murine colon adenocarinoma cell line by overexpression of Csk [43]. Furthermore, Allgayer *et al.* demonstrated that overexpression of a constitutively active mutant of c-Src in a human colon carcinoma cell line lead to increased expression of urokinase-type plasminogen activator receptor (uPAR) [44], a receptor that has been implicated in degradation of extracellular matrices and penetration of vessel walls by tumor cells during metastasis [45, 46]. uPAR forms a receptor for pro-uPA and uPA, which in turn is able to process Plasminogen to Plasmin [47, 48]. Similar to what has been shown for elevated c-Src activity, increases in tumor and serum levels of uPA, uPAR and Plasminogen activator inhibitor-1 (PAI-1) are associated with a worse prognosis in patients with colon cancer, establishing a link between two important prognostic markers [41, 49]. Characterization of the uPAR promotor showed that expression is controlled by both AP-1 and NFkB, suggesting that c-Src is a critical upstream regulator of these transcription factors in human colon cancer [47, 48].

In addition to the activation of uPAR expression, deregulation of E-Cadherin signalling by c-Src might be involved in colorectal cancer cell metastasis. Jones *et al.* found that elevated c-Src activity in two highly metastatic subclones of KM12C human colorectal cancer cells was associated with altered cell-matrix and cell cell-adhesion rather than increased proliferation [50]. Avizienyte *et al.* could show that overexpression of Src Y527F in KM12C cells led to reduced cell-cell contacts, paralleled by a failure of E-cadherin membrane recruitment [51]. Disorganization of E-cadherin-associated cell-cell contacts required a functional kinase domain and was dependent on specific integrin and focal adhesion kinase signalling. In support of this, Nam *et al.* could show that in a number of different human cancer cell lines (including colon, liver and breast cancer cell lines) disruption of E-cadherin signalling was reversed on treatment of cell with a Src kinase inhibitor, PP2 [52].

Finally, in support of a pro-metastatic function of c-Src in colon cancer, Irby *et al.* could show that overexpression of a constitutively active c-Src allele dramatically increased the invasiveness of a fibroblast cell line (3Y1) in a matrigel invasion assay and in an *in-vivo* model for experimental lung metastasis [18]. By using a Src specific kinases inhibitor and a dominant negative mutant of Src this could again be correlated to a reversible decrease of cell-cell-interactions [53].

The mechanisms of c-Src activation in human colon cancer remain obscure, they are probably multifactorial. Irby *et al.* reported an activating mutation abolishing the C-terminal, negative regulatory tyrosine to be present in 13% of patients with colon cancer but this data could not be confirmed by other groups [54]. Whether this was due to different preparation and detection methods, tumor stages or patient populations remains to be determined.

Other possible mechanisms include increased expression due to enhanced mRNA levels [55], defective proteasomal degradation [31, 33], down-regulation of negative regulatory proteins such as drs [56] as well as stimulation by growth factor receptors, above all ErbB2, EGF and c-Met [57].

Breast Cancer

Similar to the results observed in colon cancer, elevated Src kinase activity (up to 20-fold higher when compared to normal tissues) has been found in human mammary carcinoma tissues as well as in human mammary carcinoma cell lines [30, 58-61]. As with colon cancer, the causes for elevated c-Src activity in breast cancer seem to be multifactorial and data available in literature are conflicting.

A role for Src kinases in the development of mammary tumors was also suggested from two transgenic models. First, elevated c-Src kinase activity was reported in a mouse model based on the induction of mammary tumors by polyoma virus (PyV) middle T antigen, which forms a stable complex with c-Src. Interestingly, while mice transgenic for a mouse mammary tumor virus (MMTV)/PyV middle T antigen fusion gene developed rapidly progressive tumors, mice expressing the transgene in the absence of c-Src rarely developed mammary tumors [62]. Second, Muthuswamy *et al.* could show that in Her2/Neu-transgenic mice the resulting mammary tumors possessed six- to eightfold-higher

c-Src kinase activity than the adjacent epithelium [63]. Her2/Neu is over-expressed in a considerable subset of human breast cancer. The increase in c-Src tyrosine kinase activity was not due to an increase in the levels of c-Src expressed but rather to an increase of its specific activity. This observation was likely to be due to the specific interaction of c-Src with tyrosine-phosphorylated Neu and suggested that activation of c-Src during mammary tumorigenesis may occur through a direct interaction with activated Neu [23, 24]. Finally, both studies are in agreement with work published by Webster *et al.* showing that transgenic mice expressing a MMTV/activated c-src fusion gene developed mammary epithelial cell hyperplasias and mammary tumors [35].

In addition to breast cancer, elevated Src activity has been reported in many other epithelial tumor entities, including pancreatic [64], lung [65], ovarian [66, 67], esophageal [68] and gastric cancer [69] as well as melanoma [70] and neuroblastoma [71, 72], further corroborating a role of these kinase(s) in tumorigenesis. Finally, it is noteworthy that several recent reports have implicated Src kinases in the control of VEGF-driven angiogenesis. This includes control of production of VEGF by tumor cells as well as control of VEGF-R regulated survival of endothelial cells. In support of this it was shown that overexpression of a kinase-inactive and dominant negative Src mutant in tumor-associated blood vessels blocked VEGF-induced angiogenesis and suppressed the growth of medullablastomas on the chicken chorioallantoic membrane [73].

THE ROLE OF SRC FAMILY KINASES IN MALIGNANT HEMATOPOIESIS

Insights from Cell Culture Models

Although artificial in nature, earliest evidence for a pro-leukemogenic potential of Src kinases came from the observation that v-Src, the viral homologue of c-Src and transforming oncogene of the Rous Sarcoma Virus, is able of inducing interleukin-3 (IL-3) independent growth to a variety of hematopoietic cell lines and substitutes for v-Abl in transformation of murine bone marrow lymphocytes [74-77]. Similar to v-Abl and Bcr-Abl, v-Src inhibits the granulocytic differentiation of an IL-3 dependent, myeloid progenitor cell line, 32D, in response to granulocyte-colony stimulating factor G-CSF stimulation [78]. It was shown that the ability of v-Src to transform hematopoietic cells is dependent on its SH3 and SH2 domains and v-Src induced transformation could be attributed at least in part to its ability to activate signal transducer and activator of transcription (STAT)-family proteins and induce c-myc expression [79]. It is noteworthy, however, that other than seen for transformation of fibroblasts, not all members of the Src family equal v-Src in their ability to transform hematopoietic cell lines [79].

Insights from Knockout, Transgenic and Bone Marrow Transplantation Studies

Studies using mice harboring targeted knockouts of various Src kinase genes have given insight into their important functions, especially in the development of the hematopoietic system. Lck- and Fyn-deficient mice display defects in the development of the T-lymphocyte compartment (reviewed in [80]). $Lyn^{-/-}$ mice show disturbed B cell development with a marked decrease in the number of mature peripheral B lymphocytes and a significant increase of lymphoblastoid cells [81]. In those mice B cell receptor (BCR) signalling is perturbed, displaying prolonged hyperactivation of several signalling molecules after receptor engagement [81]. The negative regulatory function of Lyn leading to defective BCR signalling might cause the lupus-like autoimmune disease that was observed in those mice [81].

$Src^{-/-}Hck^{-/-}$double knockout mice display an increase in extramedullar hematopoiesis [82]. This was attributed, however, to a loss of intramedullar hematopoiesis due to severe osteopetrosis rather than to a direct effect on myelopoiesis. $Hck^{-/-}Fgr^{-/-}$ as well as $Lyn^{-/-}Hck^{-/-}Fgr^{-/-}$ knockout mice were demonstrated to have defects in macrophage and granulocyte function, but no obvious quantitative impact was seen on myelopoiesis in young mice [83-85]. Surprisingly, however, a recent publication demonstrated that old cohorts of $Lyn^{-/-}$ mice are characteristic of splenomegaly, disseminated myelomonocytic cells and monocyte/macrophage tumors that could be transplanted to severe combined immunodeficiency disease (SCID) mice. In contrast, such tumors were not seen in wt mice or mice homozygous for a constitutively active mutant of Lyn [86]. This suggested a tumor suppressor function rather than a tumor promotor function of Lyn in myelopoiesis and was hypothesized to be due to a function of Lyn in signalling from growth inhibiting, ITIM (immunoreceptor tyrosine-based inhibitory motifs)-containing cell surface receptors such as PIR-B and SIRPα. In proof of this, the authors could show that recruitment of two phosphatases, SHP-1 and SHP-2, to PIR-B and SIRPα is defective in Lyn-/- mice. A negative regulatory function of Lyn in signal transduction has also been observed in other studies and it was shown that Src kinases are involved in receptor internalization, degradation and signal termination of a variety of growth factor receptors (e.g. EGFR, c-Kit, Flt3) [87-89].

In marked contrast to mice homozygous for a constitutively active mutant of Lyn, results from BlkY495F and LckY505F transgenic mice or from bone marrow transplantation experiments are in favor of a pro-leukemogenic role of Src kinases. For example, in one study the transgenic expression of BlkY495F in either the B lymphoid or T lymphoid compartment resulted in either B lymphoid tumors or clonal, thymic lymphomas [90]. Mice transgenic for LckY505F are characteristic of thymic tumorigenesis [91]. Finally, by means of bone marrow transduction/retransplantation experiments, Keller *et al.* showed that mice reconstituted with v-src-infected bone marrow cells rapidly developed a severe myeloproliferative disease reminiscent of the CML like disease induced by Bcr-Abl under similar conditions and characterized by splenomegaly, anemia, and a shift of hematopoiesis from the bone marrow to the spleen [92].

Src Family Kinase Signalling in the Development of Plasmocytoma

Much evidence points to a possible involvement of Src kinases in signal transduction initiated by hematopoietic cytokine and growth factor receptors, above all in leukemic

cells (reviewed in [93, 94]). Amongst these receptors is gp130, the β-chain of the receptor for interleukin-6 (IL-6). IL-6 is a pleiotropic cytokine and has been shown to be a crucial growth factor in the development of plasmocytoma [95]. Hallek *et al.* could show that IL-6 leads to the activation of several Src family kinases in plasmocytoma cells, including Lyn, Hck und Fyn [96]. Further analysis led to the identification of an "acidic" domain of gp130 as a binding region for Src kinases, which mediates proliferative signalling [97]. The deletion of this region resulted in a significant reduction of gp130 dependent cell proliferation and a disruption of growth factor-stimulated activation of mitogen-activated protein kinase-1/2 (Mapk-1/2; Erk1/2). Interestingly, signalling to STAT3 was not disrupted by this mutation. Similar results were obtained using a Src specific kinase inhibitor, PP2. These observations, however, are in contradiction to a recent paper suggesting that IL-6 independent activation of Src kinases by loss of CD45 expression is important for IL-6 to induce proliferation in human multiple myeloma cell lines and that neither signalling to Erk nor to STAT3 were dependent on an increase of Src kinase activity [98].

Src Family Kinase Signalling in the Pathogenesis of Bcr-Abl-Positive Leukemias

The most substantial compilation of data in support of an involvement of Src family kinases in the development of human leukemia is found with regard to their role in Philadelphia-Chromosome (Bcr-Abl)-positive malignancies. In 1996, Danhauser-Riedl *et al.* could show that the activity of several Src family kinases (e.g. Lyn and Hck) is increased in hematopoietic cells expressing Bcr-Abl [99]. Moreover, Bcr-Abl and various Src kinases were found in a physical complex both in Bcr-Abl positive cell lines and after overexpression of Bcr-Abl and various Src family kinases in Cos7 or insect cells [27, 28, 99]. Mutational analysis allowed for further insight into the regulation of Src kinases by Bcr-Abl. Using proteins consisting of isolated domains it was shown that the interaction of Src kinases and Bcr-Abl *in-vivo* might be mediated by multiple domains ([28] and own unpublished observations). The Hck SH3 and SH2 domains as well as the Bcr portion and the Abl SH3, SH2 domains and C-terminal tail were identified as potential interaction sites, but the relative contribution of each of these domains for the interaction of the full-length proteins is not clear. Most surprisingly, we found that inactivation of the Bcr-Abl tyrosine kinase by a targeted mutation of the ATP binding site did neither inhibit the complex formation with Src kinases nor their activation by Bcr-Abl [27]. As a consequence, STI571, an ATP-competitive inhibitor of Abl, does not reverse Src kinase activation by Bcr-Abl, despite the potential of STI571 to reduce Bcr-Abl mediated activation/phosphorylation of many other signalling molecules, including STAT5 and Jnk [100]. Even more surprising was the finding that inactivation of Hck even enhanced binding of Hck to Bcr-Abl and this increase in binding was mediated by the Hck SH3 domain ([27] and own unpublished data). These data suggest a binding mode that would prefer inactive Hck for binding through the Hck SH3 domain. By disruption of the intramolecular interaction of the SH3 domain with the SH2/kinase-linker region Hck kinase activity is increased, which in turn reduces Hck affinity and releases Hck from its binding site at Bcr-Abl [27].

In support of a *biological* function of the Src family kinase family in Bcr-Abl induced leukemia Lionberger *et al.* were able to show that expression of a kinase-inactive, dominant negative mutant of Hck greatly comprised the transforming potential of Bcr-Abl [28]. Work by Klejman *et al.* suggests that this is due to a role of Hck in the activation of STAT5. STAT5 phosphorylation and activity was considerably reduced in cells over-expressing kinase dead Hck or treated with a putatively Src-selective kinase inhibitor, PP2 [101]. However, the relevance of this finding is questioned by the observation that overexpression of wt Hck also considerably reduced the transformation of hematopoietic cells to cytokine independence [28]. Interestingly, Shishido *et al.* found that kinase-dead Src is able to compete with Abl for binding to c-Cbl, an important substrate of Bcr-Abl in leukemogenesis, and it is possible that the effect of kinase dead Hck described above is due to competition with Abl binding sites rather than a specific function of Src kinases in STAT5 signalling [102].

Potential Role of Src Kinases and Inhibitors in Gleevec-Resistance

Understanding the Binding Mode of STI571

While a proposed role of Src kinases in normal Bcr-Abl signalling still lacks a clear genetic proof, recent work rationalizes the use of Src kinase inhibitors to avoid or overcome STI571-resistance. STI571 has outstanding biochemical specificity. In addition to Abl, only three other kinases have been reported to be inhibited by STI571: the Abl-homologue Arg, stem cell factor receptor (c-Kit) and PDGF receptor (PDGF-R) [103, 104]. Recent structural as well as genetic analyses of the binding mode of STI571 to Abl have shed light on the keys to STI571's specificity [100, 105, 106]. STI571 not only blocks the ATP binding site, but, in addition, binds into a hydrophobic pocket and an allosteric inhibitor binding site adjacent to the ATP binding pocket (Fig. **2**). Amino acid alignments of various STI571-sensitive and insensitive kinases as well as comparisons of available protein kinase structures identified three amino acid positions, 315, 380 and 382, as critical gatekeepers controlling the access to this allosteric pocket. Despite the high homology of many amino acids within the kinase domain, amino acids found at positions 315 and 380 vary considerably amongst different protein kinases (Fig. **2B**). A careful analysis of the inhibitory profile of STI571 showed that most STI571 insensitive kinases have bulkier amino acids at these positions. It was shown that amino acids other than threonine at position 315 and alanine, cysteine or serine at position 380 are not compatible with STI571 binding to Abl [100, 106] and that mutation of position 315 (or position homologues thereto) in STI571-resistant tyrosine kinases, e.g. Flt-3, conferred STI571-sensitivity to the mutant [107].

Moreover, although Phe382 is highly conserved throughout the protein kinase family, its spatial orientation varies considerably. Interestingly, only an orientation similar to that in the inactive conformation of Abl is capable of opening the entrance of the allosteric pocket far enough to

allow STI571 binding (Fig. **2C** and **D**) [100, 105]. In Src kinases, which share high homology with Abl with regard to the amino acid sequence of the allosteric inhibitor binding pocket, Phe382 adopts an orientation which blocks STI571 binding to the allosteric pocket both in the active and the inactive conformation (Fig. **2C** and **D**) [5, 6, 108-110]. "Src-like" conformations of Phe382 are also found in inactive FGF receptor kinase and in Eph2B receptor kinase [111, 112] as well as in all active kinase structures published thus far [113].

STI571 Resistance

The efficacy and potency of STI571 in Bcr-Abl-positive leukemias was shown in several clinical trials. In 2001, Druker *et al.* could show that STI571 induces up to 98% of hematological remissions depending on the disease phenotype and stage, without causing any dose-limiting toxicity [114-116]. Subsequent Phase II and Phase III clinical trials corroborated the initial findings [117-119], but emerging data on direct resistances of patients towards STI571 significantly reduced the initial enthusiasm about STI571. It has been shown that patients with advanced Bcr-Abl positive leukemias rapidly relapse on STI571 based on a direct resistance of the cells to the compound. Further analysis of patient samples suggested that the mechanisms of resistance are diverse, including gene amplification, point mutations that interfere with STI571 binding, clonal evolution due to secondary mutations and the up-regulation of signalling molecules such as Btk [120-125].

In addition, Lyn and Hck, two Src family kinases, were shown to be upregulated in both an STI571 resistant cell line and in blasts of CML and ALL patients who relapsed on STI571 [126]. Donato *et al.* isolated a STI571-resistant K562 cell line that grew in the presence of STI571 despite preserved sensitivity of Bcr-Abl for the inhibitor. STI571-resistance could be correlated to an increased expression and activity of the Src family kinase Lyn, whereas Bcr-Abl expression was significantly lower as in STI571-sensitive K562 cells. As a result of increased dependence on Lyn activity a pronounced reaction of STI571-resistant K562 cells on addition of Lyn antisense RNA was observed. In the same article, the authors could show that both Lyn and Hck are upregulated in expression and hyperactive in blasts from patients that had relapsed on STI571 therapy.

Dualspecific Src/Abl Kinase Inhibitors – Binding Mode and Potency

These data in conjunction with a proposed role of Src kinases in Bcr-Abl signalling rationalized the use of Src family kinase inhibitors alone or in conjunction with Abl inhibitors as a treatment strategy for advanced Bcr-Abl-positive leukemias. Interestingly, the high homology of Abl and Src kinases in their ATP-binding site and the adjacent hydrophobic pocket accessed by STI571 [105] suggested that both kinases might be targeted by identical inhibitors. Only the different organization of the activation loop in Src kinases versus Abl, associated with a different availability of the allosteric STI571 binding site, keeps STI571 from binding to Src family members. In contrast, most Src inhibitors published thus far have considerable activity on Abl (and other kinases targeted by STI571, such as PDGF-R and c-Kit) and it was shown that such inhibitors, including PP1, PP2, CGP76030, SKI606, PD173955 and PD180970 inhibit not only Src kinases in a concentration dependent manner but also Bcr-Abl, which results in cell cycle arrest and apoptosis of various Bcr-Abl-positive cell lines tested [100, 127-131].

In an effort towards (1) understanding the binding mode of such inhibitors when compared to STI571 and to (2) dissecting the Abl-related from Src-related effects, we created a series of Bcr-Abl mutants that were resistant to both STI571 and two dual-specific Src-/Abl-kinase inhibitors, PP1 and CGP76030 [100]. This strategy was based on the successive elongation of the side chain of two of the three gatekeeping amino acids, 315 and 380, controlling the access to the allosteric inhibitor-binding pocket (see above and Fig. **2**). Although both positions are located within the ATP binding site or in close proximity thereof, the mutant proteins were equally active to Bcr-Abl wt and retained their full transforming potential in hematopoietic cell lines, provided that substitutions were made to amino acids present at identical positions in other kinases [100]. Interestingly, with only one exception (T315V), all mutants at position 315 rendering Bcr-Abl STI571 resistant also induced a resistance of Bcr-Abl to PP1 and CGP76030. In contrast, mutation of A380 to threonine did not impact on PP1 and CGP76030 binding to Bcr-Abl but significantly reduced the affinity of STI571 for Abl. These data suggested that STI571, PP1 and CGP76030 inhibited Bcr-Abl with overlapping binding modes, with PP1 and CGP76030 only occupying the ATP-binding site and hydrophobic pocket (Fig. **2G**). This hypothesis is in accordance with a recently published crystal structure analysis of Abl in complex with the Src-/Abl-kinase inhibitor PD173955 and modeling of CGP76030 binding to Abl, suggesting that "PP1"-like compounds inhibit both the active and inactive conformation of Abl (see Fig. **2G** and **E**) and [132]).

Some of the above-mentioned mutants, including one that was identified in STI571-resistant patients (T315I) were used for further analysis of the biology of STI571 versus Src-/Abl-kinase inhibitors. Interestingly, while such mutants rendered cells resistant to STI571 (about 40-fold less sensitive to STI571 when compared to Bcr-Abl wt), two dual-specific inhibitors, PP1 and CGP76030, retained their activity on Bcr-Abl-positive cells and considerably reduced their cell growth and survival even in the presence of active Bcr-Abl kinase. These data suggest that dual inhibition of Src and Abl could be superior to the specific inhibition of Abl alone as it might avoid selection of STI571 resistant mutants seen in many patients that relapsed on treatment with STI571.

While above strategy allowed a dissection of Abl and Src related effects of dual specific Src-/Abl-Inhibitors, it also gave insight into signalling pathways which are potentially mediated by Src kinases in Bcr-Abl-positive cells. In contradiction to data reported by Klejman *et al.* no influence of PP1 and CGP76030 was observed on phosphorylation of Y694/699 of STAT5 and STAT activity in cells expressing an inhibitor resistant Bcr-Abl allele, suggesting that Abl activates STAT5 without involvement of Src family kinase

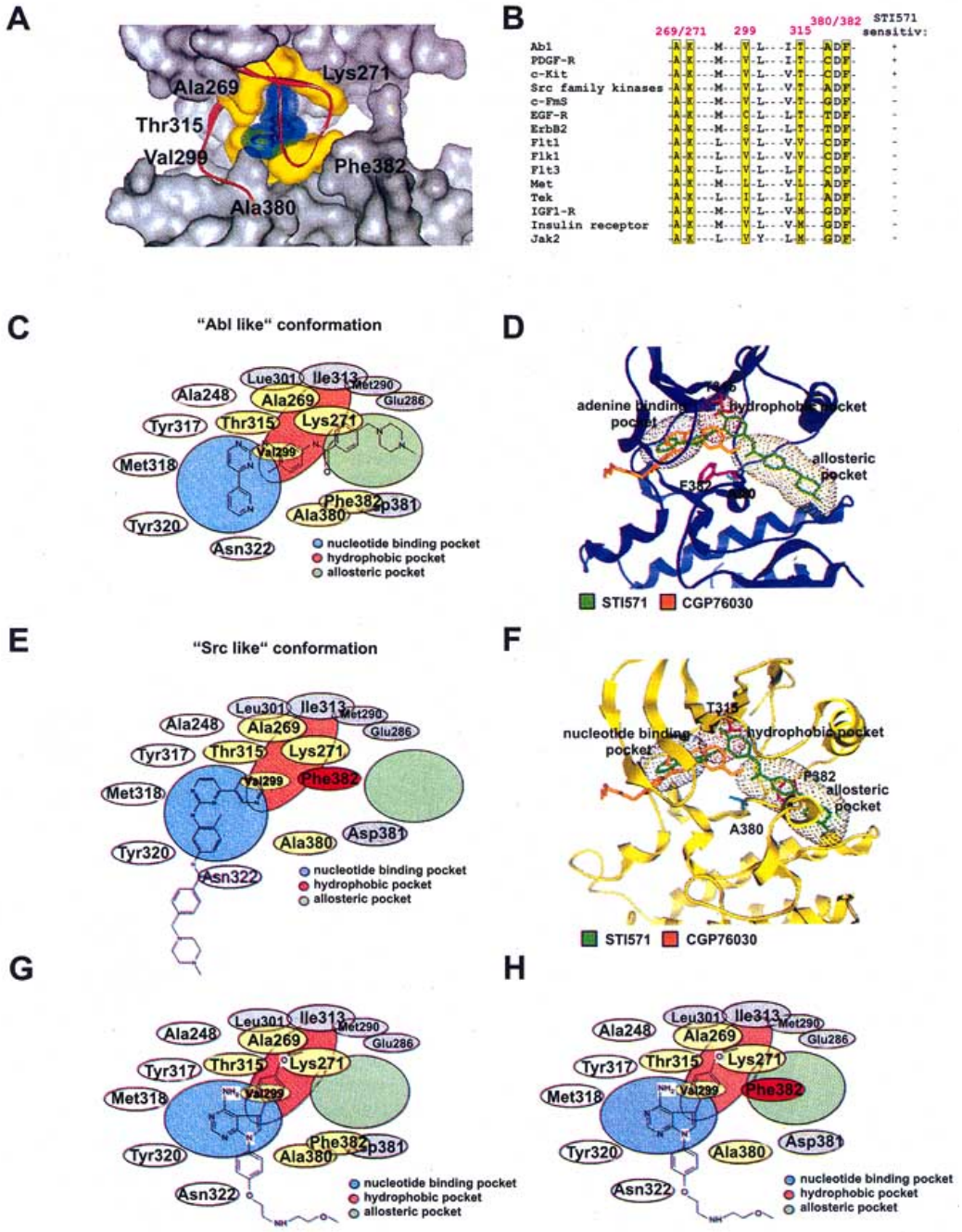


Fig. (2). Mechanims of binding of STI571 and CGP76030 to Abl.

signalling [100]. Similar observations were made for other pathways downstream of Abl, including the Erk1/2 and Jnk pathways ([100]) and own unpublished observations). In contrast, inhibition of Src kinases in cells expressing inhibitor resistant Bcr-Abl alleles was paralleled by inhibition of Akt and dephosphorylation of several Akt substrates, including Bad and Forkhead transcription factors ([100]) and own unpublished observations).

While progress is underway to evaluate the Src and Abl related potency of such inhibitors in animal models, recent structure analyses revealed a second feature of these inhibitors in favor of a clinical application. Nagar *et al.* could show that PD173955 binds Abl by a mechanism independent of the activation state of Abl and by a far less complex binding mode than observed for STI571. Based on this observation it was hypothesized that many Abl point mutations inducing STI571 resistance would still retain sensitivity to PD173955 or similar Src-/Abl-Inhibitors, a proposal that was recently proven by La Rosee *et al.* who tested the efficacy of PD180970, a compound with marked homology to PD173955, on BaF/B03 cells expressing a variety of different STI571 resistant mutants of Bcr-Abl [128]. In summary, although provocative in nature, these results suggest that the high specificity of STI571, based on its binding mode, increases the susceptibility for direct drug resistances due to mutation of the inhibitor binding site and proposes the use of less specific inhibitors with less complex binding modes given that their safety profile is sufficient for clinical applications. This might at least be advantageous for advanced stages of human leukemias and cancer for which a higher genetic instability is likely.

SIGNAL TRANSDUCTION PATHWAYS ELICITED BY SRC FAMILY KINASES IN TUMOR AND LEUKEMIC CELLS

Tremendous knowledge has been compiled on the various signal transduction pathways induced by Src kinases in various tumor cell lines, within different signalling systems and in Src transformed fibroblasts [133-136]. Summarizing all such pathways is beyond the scope of this review but several recent advances in our understanding of Src dependent signalling are worth mentioning.

First, Src kinases have been implicated in pathways controlling cell cycle progression and mitogenesis as well as survival through activation of STAT proteins. STAT signalling has been shown to be involved in mitogenic signalling downstream of receptor tyrosine kinases, cytokine receptors and G protein coupled receptors and seems to mediate induction of c-myc by c-Src [137-141]. STAT3 activation by v-Src was shown to be a crucial step in the transformation of fibroblasts as well as hematopoietic cells [79, 142-144]. Recent evidence also suggests that this pathway is important in carcinogenesis. Niu *et al.* could show that c-Src and STAT3 are constitutively active in a number of human melanoma cell lines and in melanoma tumor tissues. Inhibition of c-Src using two different small molecule kinase inhibitors, PD166285 and PD180970, blocked STAT3 activity, reduced expression of BCL-x_L and MCL-1, two anti-apoptotic STAT3 target genes, and induced apoptosis in melanoma cell lines [70]. Interestingly, comparable results have been found in breast cancer cell lines [145], suggesting a more general importance of this pathway in human cancer.

Second, a novel function of Src kinases in signalling of tumor cells might be concluded from several different publications: amplification of Akt signalling. Akt, a serine/threonine kinase downstream of PI3K, has been implicated in the development of cancer by controlling both survival and proliferation through phosphorylation of either serine or threonine residues embedded in a conserved amino acid motif (RXRXXS/T) (for review of Akt signalling see ([146, 147]). Some of the substrates of Akt include critical regulators of apoptotic cell death, e.g. p21, MDM2, Bad, caspase 9 and forkhead transcription factors. Based on the conserved phosphorylation motif more substrates are to be expected. Mechanisms of activation of Akt include amplification of the Akt1 and Akt2 genes itself or its upstream activator PI3K as well as inactivation of the PTEN tumor suppressor gene, a critical negative regulator of the Akt pathway. Hyperactivity of Akt and over-expression of Akt isoforms has been reported in ovarian, breast, prostrate and pancreatic cancers (for a detailed review on Akt in human cancer see [147, 148]).

Two recent reports demonstrated that Src kinases are critical regulators and amplifiers of Akt signalling downstream of PI3K (Fig. **3**). Chen *et al.* could show that Akt is phosphorylated on tyrosine upon stimulation of cells with EGF and induction of tyrosine phosphorylation correlated to an increase in Akt kinase activity [149]. Moreover, co-expression of Akt and constitutively active Src in Cos cells stimulated the activity of Akt and its phosphorylation at T308, again paralleled by increased tyrosine phosphorylation of Akt. Using site directed mutagenesis, the authors were able to map the phosphorylation sites to two tyrosines, Y315 and Y326, both located within its activation loop. Interestingly, Akt with a mutation of both Src phosphorylation sites to phenylalanine exhibited greatly reduced kinase activity and was shown to act as a dominant negative molecule, similar in activity to other kinase dead Akt mutants.

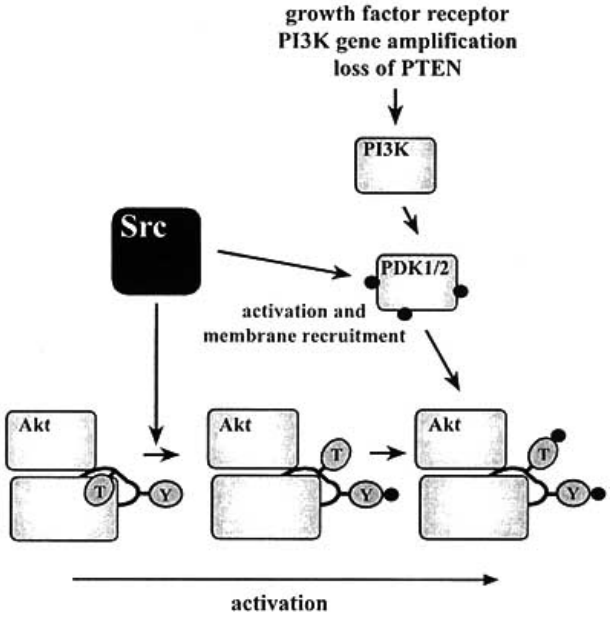


Fig. (3). Potential function of Src family kinases in PDK1/Akt signalling.

In addition, Park *et al.* could identify three tyrosine phosphorylation sites (Y9, Y373 and Y376) in PDK1, a kinase believed to act directly upstream of Akt and responsible for

phosphorylation of Akt at threonine 308, a step that is pivotal in activating Akt [150]. Using site-directed mutagenesis it was shown that phosphorylation on Tyr-373/376 is important for PDK1 activity as well as recruitment of PDK1 to the membrane. Both residues were shown to be substrates of the v-Src tyrosine kinase *in vitro*, and co-expression of v-Src led to the tyrosine phosphorylation and activation of PDK1.

Additional indirect support of above-mentioned observations comes from several other reports. Windham *et al.* demonstrated that an increase of Akt kinase activity and resistance of cells to apoptotic cell death paralleled increased Src activity in colon cancer cell lines. Akt activation could be reversed by addition of a Src kinase inhibitor, PD173955 [151]. Interestingly, in tissues from colon cancer patients the increase of Akt kinase activity is a common event, similar to what has been shown for Src, and both hyperactivation of Akt and Src, are likely to be early events in colon cancerogenesis [151]. Since Src kinases as well as Akt are over-expressed in many different types of cancer, future investigations should aim to further clarify a possible interaction between these two proto-oncogenes

NOVEL APPROACHES TO VALIDATE THE THERAPEUTIC POTENTIAL OF SRC KINASES AND THEIR INHIBITORS

The investigation of the role of Src family kinases in leukemia and cancer suffers from several problems. First, many studies are based on experiments in different cell lines, each presenting a different genetic background that might influence the corresponding results dramatically. The ability to grow permanently in cell culture is associated with mutations that do not necessarily reflect the mutations seen in different human tumor types. In support of this, Broome *et al.* showed that loss of p53 or overexpression of SV40 large T antigen, the latter of which is frequently used to immortalize cells, results in a loss of Src-dependence of mitogenic PDGF receptor signalling [152].

Methods used to study the role of Src kinases in cell lines and animal models include the generation of knockout mice, antisense mRNA strategies, microinjection of inhibitory antibodies or the overexpression of dominant negative Src alleles. Many of them are either technically difficult and time consuming (knockout, antibody microinjection), associated with unspecific toxicity (antisense RNA) or, as discussed for the overexpression of dominant negative Src kinase alleles, associated with a substantial risk of producing artificial results. As a matter of fact the hunt for a role of Src kinases in tumorigenesis has suffered immensely from a lack of appropriate tools to specifically address the role of different family members.

Indeed, mammalian cells usually express between two to five of the Src family kinases. Knocking out one Src kinase is often not sufficient to provoke a phenotypical change in a cell's behavior due to the fact that the remaining Src family members substitute for their deleted member's function. In some cases increased expression of the remaining members has been observed. This observation has significantly hampered the interpretation of Src kinase knockout data. On the other hand, generation of double and triple Src family kinase knockouts is difficult and has in some cases resulted in perinatal or early embryonic lethality ($Src^{-/-}Fyn^{-/-}$, $Src^{-/-}Yes^{-/-}$, $Src^{-/-}Fyn^{-/-}Yes^{-/-}$) [153], stressing the importance of these kinases in developmental processes. Down-regulation of Src family kinase expression by RNAi technologies might enable us to overcome this problem in the future, but the applicability of this method in mammalian systems, above all *in-vivo*, needs to be determined.

In addition to RNAi, traditional pharmacological and chemical genetic approaches should improve our abilities of characterizing the role of Src kinases in signal transduction, cell transformation and tumorigenesis. The use of small molecule kinase inhibitors to study Src kinase biology and signalling is an easy and reproducible method. Indeed, many results implicating Src kinases in carcinogenesis and other processes are based on the use of Src kinase inhibitors, but many of the compounds frequently used, e.g. PP1 and PP2, are not specific for Src family kinases and at least some of these compounds' effects might result from inhibition of other kinases. For example, although frequently used to investigate the role of Src kinases in EGFR and PDGFR signalling, PP1 and PP2 both have substantial direct activity on these receptors [154, 155]. Other kinases inhibited by PP1/PP2 include p38mapk, Btk, c-Kit, Csk and CK1 [130, 156]. Two recent publications suggest that this is a rather common occurrence when working with small molecule kinase inhibitors [157, 158], questioning not only results on Src signalling but also on the function of many other kinases obtained by using small molecule kinase inhibitors.

One possible approach to validate a compounds target-specificity is the use of inhibitor-resistant mutant alleles of a kinase (Fig. **4A**). In support of this concept, Eyers *et al.* recently dissected p38/Sapk2a dependent and independent effects of a small molecule kinase inhibitor, SB 203580, by overexpression of a inhibitor-resistant p38/Sapk2a mutant that was generated by site directed mutagenesis based on a co-crystal of the inhibitor with the kinase [159]. Using a similar approach, we were able to generate inhibitor-resistant mutants of Bcr-Abl that allowed us to investigate Abl-dependent and independent effects of two dual-specific Src-/Abl kinase inhibitors, PP1 and CGP76030 [100]. In the future, a similar strategy in conjunction with improved specificity of Src kinase inhibitors might be used to define Src-specific effects of small molecule kinase inhibitors.

Recently, a second reciprocal chemical genetic strategy showed promise in investigating Src family kinase signalling (Fig. **4B**). As a reversal of above-mentioned method, Liu *et al.* generated Src family protein kinases with unnatural nucleotide specificity [160], a strategy that was later on shown to be applicable to many other protein kinases [161]. By opening the access to a hydrophobic pocket located adjacent to the ATP-binding site, Src kinase alleles were generated that were susceptible to inhibition by "bumped" inhibitors that were not accepted by any unmutated protein kinase. The inhibitor-sensitive Src alleles were also demonstrated to accept N6-modified nucleotide triphosphates thereby allowing the identification of direct kinase substrates. Using these highly specific inhibitors and nucleotide triphosphates in conjunction with the mutated kinase has resulted in identification of novel substrates as well as in elucidation of the function of various kinases in a temporally addressable

manner [161-165]. It is likely that the use of this method will allow further insight into the biological functions of Src family kinases in cell transformation and carcinogenesis as well as help to validate their potential as therapeutic targets.

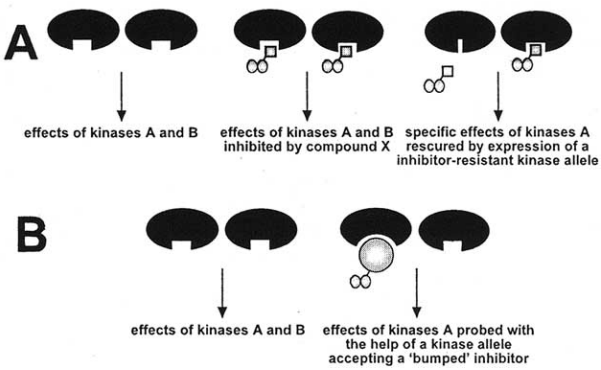


Fig. (4). Pharmacological and chemical genetic approaches to study the role of kinases and validate inhibitor specificity.

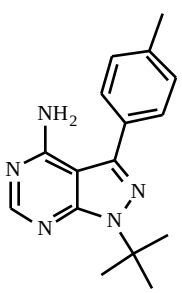
TOWARDS CLINICALLY TARGETING SRC FAMILY KINASES – A PERSPECTIVE

Stimulated by the role of Src kinases in cancer and leukemia as well as their function in bone metabolism and the regulation of the immune system, ATP-competitive Src kinase inhibitors based on different chemical scaffolds have been synthesized during the past years. Commonly used Src inhibitor scaffolds (see Fig. **5**) include pyrazolo[3,4-d]pyrimidines (e.g. PP1, PP2; Fig. **5** compounds 1 and 2) [154], pyrrolo[2,3-d]pyrimidines (e.g. CGP76030; Fig. **5** compound 4) [166-168], pyrido-pyrimidines (e.g. PD173955, PD180970; Fig. **5** compounds 5 and 6) [169, 170], quinolines and various derivatives thereof (e.g. SKI606; Fig. **5** compound 7) [171-176], anilinoquinazolines [177, 178], imidazoquinoxalines [179], indolinones (SU6656; Fig. **5** compound 8) [180] and hydroxyindoles [181]. Many of these scaffolds were first investigated as inhibitors of other protein kinases, such as EGFR and PDGFR. By varying different groups different affinity profiles of the various scaffolds could be generated, but usually this did not completely exclude activity on other kinases nor provide much selectivity within the Src family. In fact many of the Src kinase inhibitors published so far lack sufficient specificity desirable for clinical application or even pharmacological tool compounds. Only few inhibitors have been tested without provoking toxicity in animal models, and it needs to be determined if this toxicity results from inhibition of Src family kinase members or rather reflects the unsatisfactory specificity and safety profile of the specific compounds tested.

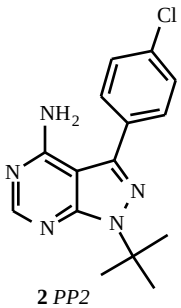
Recent crystal structure analysis has given insight into possible determinants of specificity of potential small molecule Src family kinase inhibitors. By co-crystallization of PP1/PP2 with either Hck or Lck, several investigators demonstrated that PP1 and PP2 both occupy the adenine binding site of Src family kinases and extend with their phenyl substituent into an adjacently located hydrophobic pocket (Fig. **6**) [110, 182]. While amino acids forming the pocket itself are highly conserved within the protein kinase family, two amino acid positions, 338 and 403, forming the entrance to the pocket are found to be highly variable. In many kinases these positions are represented by amino acids with longer side chains. Indeed, whereas all PP1 sensitive kinases, e.g. Src kinases, Abl, PDGF-R and c-kit, have a threonine at position 338, many PP1 insensitive kinases have longer or bulkier amino acids at the homologous position. In support of this concept, Liu *et al.* could show that v-Src, which bears a mutation of T338 to isoleucine, is greatly reduced in its sensitivity to PP1 and mutation of this position to even longer side chains, such as methionine or phenylalanine further increases resistance [156]. In contrast, mutation of this position to threonine, alanine or glycine yields Src kinase alleles with greatly increased PP1 sensitivity. Based on their structural similarity, one could imagine that similar results could be obtained for other Src family kinase inhibitors.

Importantly, comparisons of the active and inactive state of Src kinases found in various crystal structures suggested that the size and conformation of the pocket varies considerably between the active and inactive state [110]. While STI571 makes use of the structural features of this pocket in Abl in an almost optimal manner, PP1 leaves unfilled a considerable proportion of the pocket in the inactive state [105, 110]. In support of this, we could show that mutation of A380 in Abl and Hck (A403) to amino acids with longer side chains did not alter the sensitivity of Abl and Hck for PP1, whereas at least one mutation, A380T, rendered Abl STI571-resistant [100]. As a conclusion, the work by Schindler *et al.* suggested to concentrate medicinal chemistry effort towards the further optimization of the pocket binding parts of Src kinase inhibitors to improve their affinity and specificity. Indeed, replacing the methyl substituent of PP1 with a phenoxy moiety resulted in a compound that was at least 75-fold more active on Lck than PP1 itself and also possessed increased selectivity for Lck over KDR and Tie2, two receptor tyrosine kinases [183]. Interestingly, this modification also induced selectivity for Lck over c-Src suggesting that differences in the pocket conformation could be exploited for further enhancement of the inhibitor specificity.

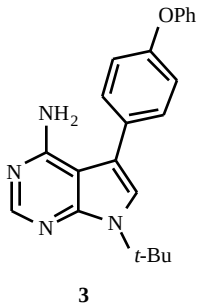
Despite 20 years of research on the role of Src kinases in human cancer and leukemia no Src kinase inhibitor has entered a clinical trial so far. While medicinal chemistry effort is ongoing, the validation of Src family kinase inhibitors as anticancer drugs suffers from several problems. For example, there is no mouse model (with the exception of PyV middle T antigen induced mammary gland tumors) for which a pivotal role of Src family kinases in tumor development and progression has been proven. Many studies used xenotransplantation models and tumor growth or shrinkage as the sole readout to validate the *in-vivo* potency of Src family kinase inhibitors. However, cells used in such studies must not necessarily grow Src dependent *in-vivo*, even if they do so *in-vitro*. Moreover, the growth of subcutaneously implanted tumors does by far not reflect all possible aspects of Src function in tumorigenesis. Many recent data suggest a role for c-Src in tumor spreading, metastasis and induction of angiogenesis rather than in tumor growth. Orthotopic transplantation models, although



1 *PP1*
c-Src 170nM
Lck 5nM
Abl 250nM
Zap70 > 10000nM
Jak2 > 10000nM
activity reported on:
PDGF-R, c-Kit, BTK, p38, CK1



2 *PP2*



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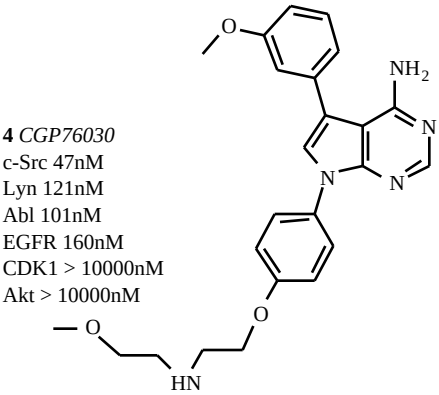
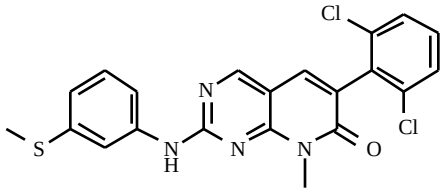
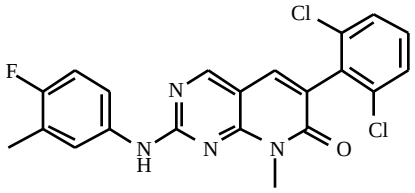


4 *CGP76030*
c-Src 47nM
Lyn 121nM
Abl 101nM
EGFR 160nM
CDK1 > 10000nM
Akt > 10000nM



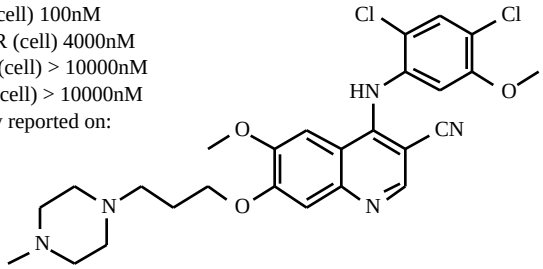
5 *PD173955*
c-Src 24.5nM
FGFR 1250 nM
PDGFR 1660 nM
activity reported on
Abl, c-Kit

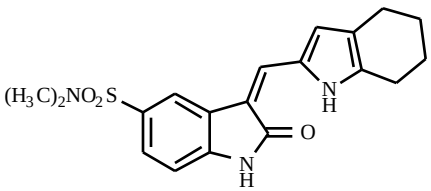


6 *PD180970*
c-Src 16.8 nM
FGFR 934 nM
PDGFR 1430 nM

activity reported on:
Abl, c-Kit

7 *SK1606*
c-Src 1.2nM
ErbB2 2600nM
cdk4 10% inhibition at 10µg/ml
c-Src (cell) 100nM
PDGFR (cell) 4000nM
FGFR (cell) > 10000nM
IGFR (cell) > 10000nM
activity reported on:
Abl





8 SU6656
c-Src 280nM
Lyn 130nM
Lck 6880nM
Abl 1740nM
PDGFR > 10000nM
CDK2 > 10000nM

Fig. (5). Chemical structures of selected Src and Src/Abl kinase inhibitors.

technically more difficult, could help to overcome this problem. Future effort should therefore not only aim at improving the specificity and pharmacokinetics of Src kinase inhibitors but also at finding more valuable and genetically clear systems for Src inhibitors to be tested in.

For a Src kinase inhibitor to enter a clinical trial several other prerequisites would need to be fulfilled. Although active in many different tumor tissues, Src kinases do not seem to be the 'leading' oncogene in any known tumor type. As a consequence, other than for Bcr-Abl, Flt3 or Her2/Neu, there is no clear patient collective to be recruited for a Src inhibitory therapy. Src kinases might be important in many different tumor types, but dependent on growth factor expression as well as secondary and tertiary mutations in

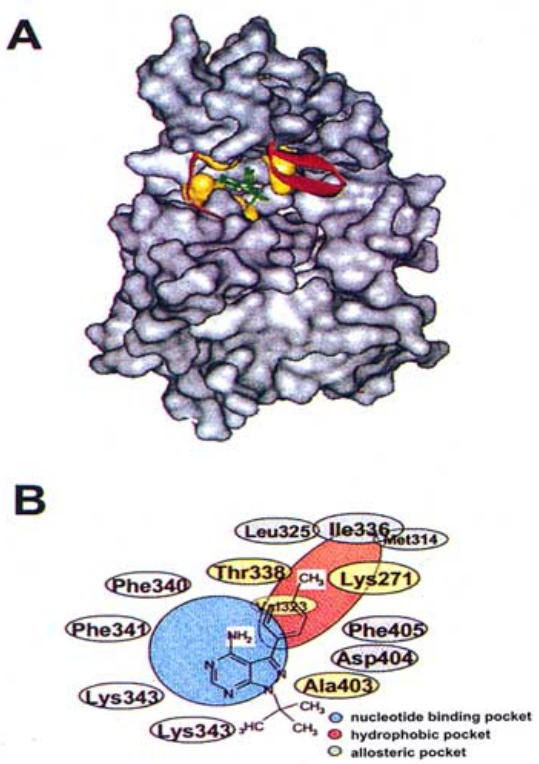


Fig. (6). Mechanism of binding of PP1 to Hck.

other signal transduction proteins, the treatment may be effective only in a subset of those patients, which needs to be identified since it is plausible that certain secondary mutations render tumors Src independent. In support of this, Broome *et al.* found that inactivation of p53 overcomes Src-dependence of mitogenic PDGF signalling [152]. In conclusion, surrogate markers need to be found that predict a possible dependence of tumors on Src kinase activity. This requires the identification of a Src kinase gene "fingerprint" in a variety of different tumor entities and a better understanding of the mechanisms rendering tumors Src independent. Finally, simple tools would be needed to determine the activity of Src family kinases (and other kinases) in tumor tissues in a quick and reliable way to accelerate the characterization of Src kinase activity in tumor tissues and to facilitate clinical decision-making.

Despite these challenges, targeting Src kinases seems to be attractive since their inhibition might influence several important characteristics of tumorigenesis and leukemia, e.g. growth, survival, spreading and migration metastasis and angiogenesis, at a time.

ABBREVIATIONS

ALL	=	Acute lymphoblastic leukemia
ATP	=	Adenosine triphosphate
Bcr	=	Breakpoint cluster region gene
BCR	=	B-cell receptor
CDK	=	Cyclin dependent kinase
CHK	=	CSK homologous kinase
CML	=	Chronic myeloid leukemia
CSK	=	C-terminal Src kinase
EGF(R)	=	Epidermal growth factor (receptor)
Flt3	=	Homo sapiens fms-related tyrosine kinase 3
G-CSF	=	Granulocyte-colony stimulating factor
HIV	=	Human immunodeficiency virus
IL-3	=	Interleukin-3
IL-6	=	Interleukin-6
ITIM	=	Immunoreceptor tyrosine-based inhibitory motif
MAPK	=	Mitogen-activated protein kinase
MMTV	=	Mouse mammary tumor virus
PAI-1	=	Plasminogen activator inhibitor
PDGF(R)	=	Platelete derived growth factor (receptor)
PKC	=	Protein Kinase C
PyV	=	Polyomavirus
RTK	=	Receptor tyrosine kinase
SCF	=	Stem cell factor
SCID	=	Severe combined immunodeficiency disease
SH	=	Src homology
STAT	=	Signal transducer and activator of transcription
VEGF(R)	=	Vascular endothelial growth factor (receptor)
uPA(R)	=	Urokinase-type plasminogen activator (receptor)

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