Src Inhibitors as Potential Therapeutic Agents for Human Cancers

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Abstract: Selective inhibitors of the Src family of protein tyrosine kinases have been developed as therapeutic agents for human tumors, some of which are now in various stages of clinical trial. In this review, recently described novel small molecule ATP-competitive Src inhibitors are discussed, with an emphasis on their potential use as therapeutic inhibitors for advanced-stage malignancies.

Keywords: Src family kinase, Inhibitors, Adenocarcinoma.

INTRODUCTION

1n 1911, Peyton Rous isolated a retrovirus, later named Rous Sarcoma Virus, which harbored a gene that, when expressed was capable of inducing sarcomas in chickens and transforming chicken embryo fibroblasts in culture [1,2]. The oncogene responsible for cellular transformation was named v-*src*. More recently, v-*src* was identified as a constitutively active non-receptor protein tyrosine kinase with a normal cellular homolog (proto-oncogene), c-*src*. The ability of v-Src-v-*src* will be used to denote the gene, V-Src (or Src) to denote the p60 gene product) to induce cellular transformation suggested that if activated in humans, c-*src* might contribute to human carcinogenesis [3]. The discovery of Src and its ability to induce cellular transformation revolutionized the understanding of tumor signal transduction and malignant transformation. Although Src itself is rarely mutated in human cancers, Src activation in response to other genetic and epigenetic changes, such as increased expression and or activity of growth factor receptors, has been implicated as critical to deregulating diverse biologic properties including proliferation, angiogenesis, migration and apoptosis. As multiple growth factor receptors regulate these functions, and more than one are often overexpressed in tumor cells, targeting growth factor receptors themselves may be less effective than targeting common downstream mediators of their signals [4]. Thus, Src has emerged as a promising downstream candidate for cancer therapy, and targeting Src holds great promise in therapy of tumors at progressed stages, in which multiple genetic and epigenetic events have occurred. In this review, we will briefly describe Src structure and activation and subsequently discuss selective Src family kinase inhibitors, in current use in preclinical and/or clinical studies, and their potential implications for therapy in human cancers.

SRC FAMILY KINASES

Src family kinases (SFKs) comprise a subclass of membrane-associated non-receptor tyrosine kinases involved in a variety of cellular signal transduction pathways. Eleven Src family kinases have been reported, from various vertebrate species, including c-Src, c-Yes, Fyn, Lyn, Hck, Blk, Brk, Fgr, Frk, Srm, and Yrk [5]. Although most Src family members are expressed primarily in cells of hematopoietic origin, c-Src, c-Yes and Fyn display a more ubiquitous pattern of expression [6] with high levels in some epithelial tissues, platelets, and neurons. Src family kinases are activated in response to cellular signals that promote proliferation, survival, motility, and invasiveness, including activation of cytokine receptors, receptor protein tyrosine kinases, G-protein coupled receptors, and integrins [6]. Under basal conditions, Src is strictly regulated, favoring intracellular interactions that promote an inactive conformation that precludes the binding of ATP and substrates within the kinase domain active site, thereby preventing these proteins from participating in cellular signaling. However, a variety of physiologic stimuli lead to conformational changes in Src, activating tyrosine kinase activity and allowing for initiation of Src-mediated signaling events.

SRC FAMILY KINASE STRUCTURE AND REGULATION

Three Src family members, Src, Yes, and Fgr, have transforming counterparts encoded by retroviral oncogenes. While Yes, Lyn and other Src family members have been associated with various human malignancies, Src itself appears to be the most active as a kinase, and perhaps for that reason, most commonly associated with tumorigenesis/progression. Therefore, this review focuses primarily on Src itself, although the general principles discussed apply to all SFKs. The human Src gene encodes 536 amino acids. The structure of Src has been well characterized through mutational studies and structural models based on NMR and crystallographic data. [7]. All Src family kinases are compromised of six distinct functional domains [8]: the amino terminal 14 amino acids, including the membrane localization signal (Src Homology 4 (SH4) domain); a poorly conserved unique domain; an SH3 and SH2 domain; a protein tyrosine kinase domain (SH1 domain); and a short C-terminal regulatory sequence [9,10] (Fig. **1**).

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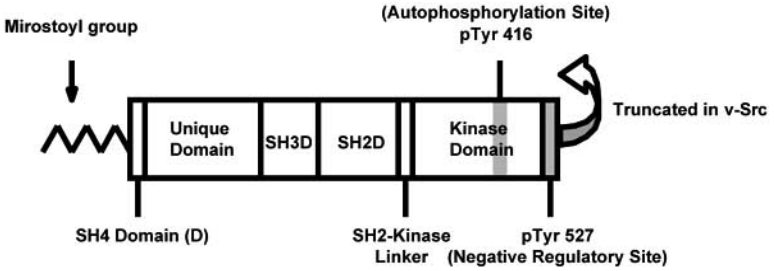


Fig. (1).

During synthesis of the Src protein, the N-terminal methionine is detached and the remaining N-terminal glycine is myristoylated through the action of N-myristoyl transferase. The remaining seven N-terminal amino acids are required for myristoylation of both c-Src and v-Src [11]. Myristoylation allows the anchoring of Src to the inner leaflet of the membrane and is required for Src-mediated cell transformation [9].

The SH3 domain, approximately 60 amino acid residues in length, is a modular domain with a well-conserved tertiary structure. SH3 domains bind to proline-rich sequences that can adopt a left-handed helical conformation. Src family kinase SH3 domains preferentially bind sequences with a P-X-X-P motif. SH2 domains, approximately 100 amino acid residues in length, are also modular domains involved in protein/protein interactions, binding to phospho-tyrosine containing motifs in a sequence-specific fashion. The optimal sequence motif recognized by Src family kinase SH2 domains is pYEEI. The SH3 and SH2 domains bind to cellular proteins harboring the appropriate target sequences, in many cases facilitating the phosphorylation of these proteins by Src family kinases and promoting the formation of multi-protein signaling complexes. However they also are integrally involved in negatively regulating kinase activity. In the inactive state, the Src SH2 domain binds to the phosphorylated form of Tyr 527 (note: the chicken amino acid sequence is used in these and the descriptions below to allow easy comparison to v-Src) in the C-terminal regulatory region, and the SH3 domain binds to a short linker sequence, which takes on a type-II left-handed helical structure similar to a polyproline helix, between the SH2 domain and the kinase domain when the kinase is inactive (Fig. **2**). The SH2/C-terminus and SH3/linker interactions stabilize the inactive conformation, and the unphosphorylated active site tyrosine, Tyr 416, is directed towards the active cleft site and buried (Fig. **2**). Under normal physiological conditions, 90-95% of Src is phosphorylated at Tyr 527 [12]. Phosphorylation of Tyr 527 is carried out by the non-receptor tyrosine kinase c-terminal Src kinase (Csk) [13] or its homolog Chk [14-17] resulting in a predominantly inactive state [7,18]. When Tyr 527 is dephosphorylated by cellular phosphatases, the c-terminal tail is released from the SH2 domain, facilitating a change in conformation to an open/active state (Fig. **2**). This change in conformation allows Src to autophosphorylate at Tyr 416, which results in stabilization of the active conformation and promotion of kinase activity [9,19] (Fig. **2**).

v-Src has been rendered transformation-competent due to a loss of the negative regulatory sequence at the carboxy-

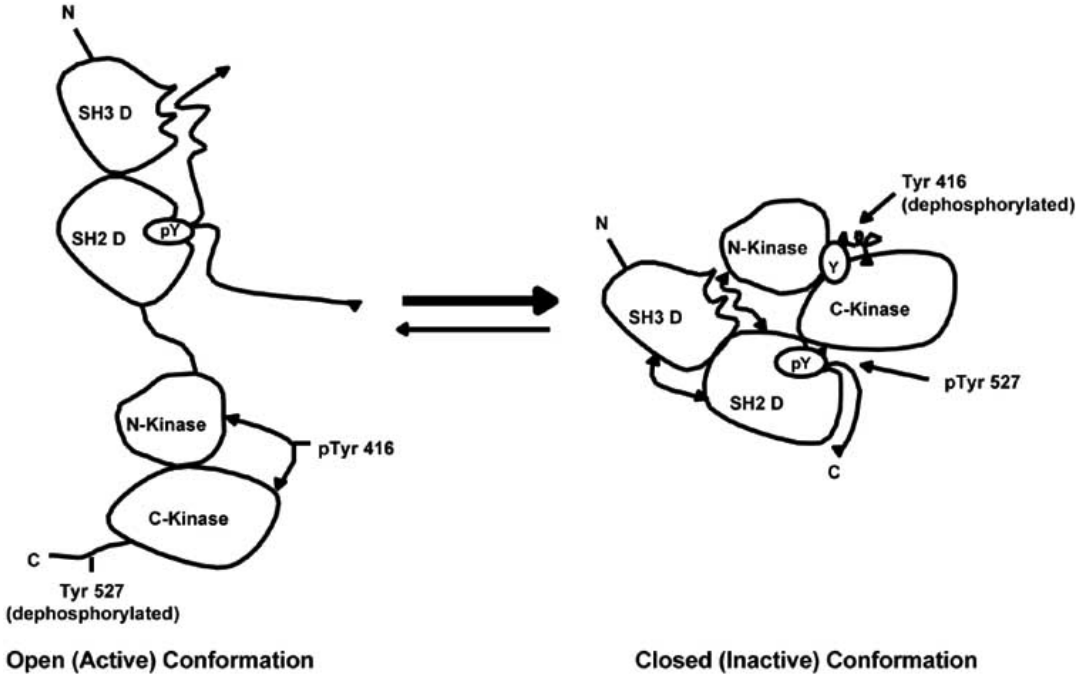


Fig. (2).

terminus which prevents regulation of the kinase and results in constitutive kinase activity, as well as additional mutations in the SH2 and SH3 domains that further increases the specific activity of the kinase [1,2]. Not surprisingly, carboxy-terminal tail mutations in c-Src that prevent binding to the SH2 domain also result in constitutive Src activity and a transformation-competent phenotype [20-22]. However, despite the fact that Src is frequently activated in human cancers, including human colon adenocarcinoma [23,24], breast cancer [25,26], pancreatic [27] and a host of other epithelial cancers, mutational activation of c-Src is rarely seen [3]. The augmentation of Src kinase activity occurs primarily through displacement of the intramolecular interactions that regulate kinase activity in normal physiological conditions, either through dephosphorylation of the negative regulatory site or intermolecular interactions with SH3/SH2 domain binding partners. In colon (and potentially other) cancers, increased transcription of Src is also observed [28].

ROLES OF SRC IN HUMAN TUMOR CELLS

Src family kinase activity correlates with malignant potential of colon and breast cancers [29-31] and activation is predictive of survival in colon cancer patients [32]. Although correlative, these studies are suggestive of a role for Src in progression of human cancers, and several recent *in vitro* and *in vivo* studies have helped define specific functions, mediated by Src, that are essential for the malignant phenotype. Src kinase activity has been implicated as important for several aspects of tumor progression, including proliferation, migration, invasion, angiogenesis, and chemoresistance [24,33-39]. In human breast cancer cells, Src activity has been demonstrated to be required for progression through mitosis [40]. In colon cancer cells, Src kinase activity is required for adhesion turnover and extracellular protease production associated with cell motility and invasion [41,42], as well as resistance to anoikis [24], resistance to oxaliplatin-induced apoptosis [43], and production of the pro-angiogenic protein VEGF [44]. Src kinase activity is also important for primary tumor growth and metastasis of colon tumor cells *in vivo* [45]. In pancreatic cancer, Src kinase activity is important for motility and invasiveness [34], expression of the pro-angiogenic proteins VEGF and IL-8 [38,39,46], and resistance to gemcitabine-induced apoptosis [33]. Src kinase activity has also been demonstrated to be important for angiogenesis, primary tumor growth, and metastatic spread of pancreatic cancer *in vivo* [47]. In human ovarian cancer, Src contributes to cell motility, anchorage-independent growth, paclitaxel resistance [48], VEGF expression and tumor growth *in vivo* [49].

Also important to the role of Src in promoting tumorigenesis in cancer cells is its function in endothelial cells. Src family members are signaling intermediates involved in promoting the biological functions of VEGF through its receptors on endothelial cells [50]. Recent studies using *src* -/- mice and Src inhibitors in a mouse colon cancer model have demonstrated that abolishing or reducing Src expression/activity decreases tumor cell extravasation, by decreasing the permeability of endothelial cells, and subsequently decreasing experimental metastases [51]. These results suggest that Src inhibitors might affect critical functions of both normal and tumor cells that contribute to tumor progression and metastasis, and have led to the development of many Src-selective inhibitors in recent years.

SRC INHIBITORS

PP1 And PP2

Inhibitors of SFK activity were first utilized over a decade ago. However, the earliest inhibitors, including genistein, Herbimycin A, and staurosporine, suffered from toxicity and poor selectivity. The pyrazolopyrimidines PP1 and PP2 (**1** and **2**- Fig. **3**) were the first small molecule inhibitors that could truly be classified as selective for Src family kinases and have remained the predominant small molecule inhibitors used for SFK inhibition in tissue culture. In human colon cancer cells, PP2-mediated Src kinase inhibition was demonstrated to activate the E-cadherin-mediated cell adhesion system, which is associated with suppression of metastasis [45]. Src inhibition with PP2 markedly reduced the rate of liver metastasis in a colon adenocarcinoma nude mouse model [45]. PP2 was recently used in ovarian cancer cells to demonstrate that Src activity is important for resistance to paclitaxel and cisplatinum-induced cytotoxicity [48]. In pancreatic cancer, PP1 was used to show the importance of Src kinase activity for collagen-mediated E-cadherin downregulation [52]. Ito *et al* utilized PP2 to illustrate the importance of Src for pancreatic cancer cell invasiveness [34]. Duxbury and co-workers demonstrated increased sensitivity to gemcitabine and a decrease in pancreatic tumor growth and metastasis in nude mice treated with PP2 [33]. PP2 was also utilized in pancreatic cancer cells to demonstrate the importance of Src activity for expression of the pro-angiogenic molecules VEGF and IL-8 [38,39,46].

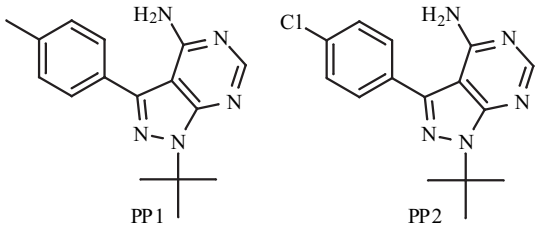


Fig. (3).

PP1 and PP2 inhibit SFK activity with IC_{50}s of approximately 5 nM *in vitro*, however, much higher concentrations are often necessary to achieve complete SFK inhibition in tissue culture systems [53]. When used at these concentrations, off-target kinases may also be inhibited, thus raising the question of whether results obtained through their use are truly due to inhibition of SFK activity specifically. These inhibitors are now rarely used in the absence of parallel studies involving dominant negatives, siRNA, or gene knockout studies [54].

NOVEL ATP-COMPETITIVE SRC FAMILY KINASE INHIBITORS

A recent surge of interest in Src family kinases as targets for rational drug design in the treatment of human cancers

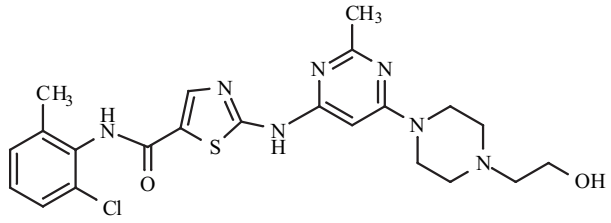
has led to the development of a new generation of ATP-competitive SFK inhibitors [55,56], improved in both potency and selectivity over PP1 and PP2. Some of these include the pyridopyrimidines PD-180970 and PD-166326 [57], the quinazoline AZM-475271 [58], the thiazole BMS-354825 [59], the purines AP23464 and AP23848 [60], and the 3-quinolinecarbonitrile SKI-606 [61]. Below, we will discuss the published data on the potency, selectivity, and *in vivo* efficacy of these key Src inhibitors in human cancers.

PD-180970 and PD-166326

PD-180970 and PD-166326 are novel pyridopyrimidine inhibitors of Src family kinases that have also been shown to have an inhibitory effect on Bcr/Abl kinase activity [62,63]. Due to their unfavorable pharmacokinetic profiles, these compounds are unlikely to be tested for clinical use [64].

BMS-354825

BMS-354825 (dasatinib^{TM}; Bristol-Meyers Squibb; **1**, (Fig. **4**) is a thiazole based orally bioavailable ATP-competitive inhibitor of both Src and Bcr-Abl kinase activity. It is potent against c-Src, c-Yes, and Lyn, as well as wild type and mutant Abl kinases [59]. IC_{50} concentrations have been reported as 0.50, 0.50, 0.40, and <1.0 nM for c-Src, c-Yes, Lck and Abl kinase respectively. In patients with chronic myelogenous leukemia, the deregulation of the oncogenic protein tyrosine kinase Bcr-Abl has been effectively treated with the Bcr-Abl kinase inhibitor Imatinib mesylate, also known as Gleevec or STI571 [65,66]. Imatinib is a small-molecule inhibitor of Bcr/Abl tyrosine kinase, which does not affect Src kinase activity. Imatinib binds with greatest affinity to the ATP binding site of Abl only when the activation loop of the kinase is closed, stabilizing the protein in an inactive conformation [67]. Unfortunately, in a small but increasing subset of patients, imatinib-resistant CML has been observed, particularly in advanced disease. The resistance mechanism is in part due to mutations involving the Bcr-Abl kinase domain that result in activation loop instability and subsequent drug insensitivity or, less frequently, amplification of the genomic locus [68]. Another mechanism by which CML cells may acquire the gleevec-resistant phenotype is through up-regulation and/or activation of the SFK Lyn, which is insensitive to inhibition by Gleevec [69]. Fortunately, BMS-354825 not only inhibits Lyn and other SFKs, but also binds to the ATP-binding site of Abl regardless of the status of the activation loop (active or inactive). Thus, inhibitors such as BMS-354825, which inhibit SFKs and Abl family kinases, have provided a means by which imatinib-resistant CML may be overcome [62,63,70]. Recent studies of BMS-354825 have demonstrated a two-fold increased potency for Bcr-Abl inhibition, relative to Imatinib, as well as the ability to inhibit Imatinib-resistant Bcr-Abl mutants *in vitro* and *vivo* [59,71]. *In vivo*, BMS-354825 significantly prolongs survival in mice with Bcr/Abl-driven disease [59]. Oral dosing in a nude mouse model of imatinib-resistant, Bcr/Abl-dependent disease was 10 mg/kg twice daily, based on pharmacodynamic experiments evaluating phosphorylation of a Bcr/Abl substrate, Crkl, from isolated spleen specimens. BMS-354825 has also been demonstrated to inhibit proliferation of Bcr/Abl-positive bone marrow progenitor cells from patients with imatinib-sensitive and imatinib resistant CML [59] and has been shown to have anti-proliferative activity against solid tumor cell lines such as prostate, breast, and colon [71,72].



BMS-354825
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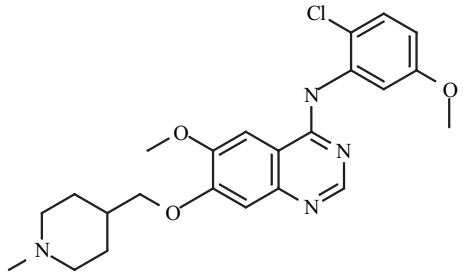


AZM475271
2

Fig. (4).

Recent preclinical trials in mice have demonstrated that established pancreatic tumors in an orthotopic model, treated with BMS-354825, showed significant reductions in primary tumor growth and incidence of metastases [47]. These results were supported by the use of pancreatic cancer cells selectively reduced in c-Src expression with siRNA, suggesting that Src activation contributes to pancreatic tumor progression in this model [47]. Due to its favorable pharmacokinetic profile, efficacy, and safety[71], BMS-354825 is currently being evaluated in Phase I/II clinical trials in CML patients with imatinib resistance[59], as well as solid tumors, including pancreatic cancer, at MD Anderson Cancer Center.

AZM475271 and AZD0530

AZM475271 (Astrazeneca; 2, (Fig. **4**) is a novel small molecule anilinoquinazoline inhibitor of Src family kinases. In previous reports, several new anilinoquinazolines, precursors of AZM475271, were discovered to be potent Src inhibitors [73]. The compounds displaying the best pharmacokinetics, including AZM475271, were evaluated *in vivo* and demonstrated more than 90% growth inhibition in a c-Src transformed 3T3 xenograft model [73]. Recently, AZM475271 was reported as the first orally bioavailable Src family kinase inhibitor used in an orthotopic nude mouse model for pancreatic cancer [58]. The IC_{50} concentrations of AZM475271 for Src family kinase members c-Src, c-Yes, and Lck, from *in vitro* cultured cells, were reported as 0.01, 0.08, 0.03 μM, respectively. Oral dosing of 50 mg/kg of AZM475271 resulted in plasma concentrations of 32.1 mmol/L (14,206 ng/ml), 20 mmol/L (8879 ng/ml), and 11.7 mmol/L (5187 ng/ml) at 2, 6, and 24 hours,

Table 1. IC_{50}s of Src Kinase Activity of Different Src Family Kinase Inhibitors

Src Family Kinase Inhibitors	IC_{50}
PP1/PP2	5.0 nM (SFKs)
AP23464	≤ 1 nM (c-Src, Fyn, c-Yes)
AZM475271*	0.01, 0.08, and 0.03 μM (c-Src, c-Yes, and Lck respectively)
BMS-354825	0.50, 0.50, and 0.40 nM (c-Src, c-Yes, and Lck respectively)
SKI-606	1.2 nM (c-Src)

*, IC_{50} values for AZM475271 were determined from the concentrations required to inhibit activity of these kinases from cultured cells, and thus the values are higher than those from PP1/PP2, AP23464, BMS-354825, and SKI-606 which were obtained from direct treatment of purified kinase *in vitro*.

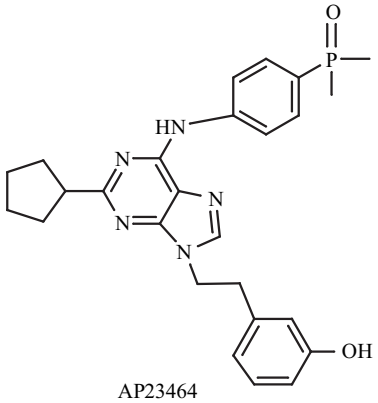
respectively [58]. Yezhelyev *et al.* demonstrated that inhibition of Src with AZM475271 resulted in significant inhibiton of primary pancreatic tumor growth (40%) and metastasis [58]. AZM475271 was effective as a single agent and showed a superior inhibitory effect on primary tumor growth (90%) when used in combination with a standard chemotherapeutic regimen [58]. The inhibitory effects of AZM475271 were attributed to a decrease in tumor cell proliferation, decreased tumor microvessel density, and increased apoptosis [58]. Although this is the first use of this drug in a solid tumor model, these results suggest that AZM47521, or closely related analogues, may be of efficacy in treating pancreatic adenocarcinomas. One such newer generation inhibitor, AZD 0530, shows limited toxicity in phase I trials, and will soon enter clinical trial for advanced-stage solid tumors.

AP23464

AP23464 (ARIAD Pharmaceuticals; **3**, (Fig. **5**), a potent and selective ATP-binding site inhibitor, was identified using structure-based design and focused synthetic libraries of 2,6,9-trisubstituted purine analogues [74]. It is potent against Src, Fyn, and Yes kinases and has also been shown to potently inhibit wild-type and mutant Abl kinases [74]. All of these kinases are inhibited by AP23464 with IC_{50} values of less than or equal to 1 nM *in vitro*. As with BMS-354825, studies with AP23464 have demonstrated antiproliferative activity and increased apoptosis against human CML cell lines and imatinib-resistant Bcr-Abl mutants [74]. It was also recently demonstrated that AP23464-treatment caused turnover of human colon cancer cell adhesions through blockade of the critical Src-FAK signaling axis [41]. *In vitro* preclinical trials carried out in our laboratory, using an analog of AP23464, have demonstrated excellent potency for inhibition of Src kinase activity in pancreatic cancer cells [38]. Inhibition of Src with an AP23464 analog reduced the production of diverse pro-angiogenic factors, including IL-8 and VEGF, and also abrogated angiogenesis in an *in vivo* assay. These results suggest possible mechanisms by which Src regulates tumor progression in pancreatic cancer. Recently, AP23464 and AP23848, an orally bioavailable analog of AP23464, were demonstrated to potently inhibit imatinib-resistant GIST tumors, which harbor a codon 816 mutation of Kit (D816V) and are unresponsive to imatinib therapy [60]. Unfortunately, the required oral dosage of AP23848 was 100 mg/kg three times daily, which proved to be poorly tolerated in mice due to toxicity. It is thus unlikely that either AP23464 or AP23848 will be suitable for clinical use. Compounds related to AP23848 are currently being developed to overcome this issue.

SKI-606

SKI-606 (Wyeth Research; **4** (Fig. **5**) is a novel small molecule 3-quinolinecarbonitrile originally described by Boschelli *et al* as a potent inhibitor of Src kinase activity [61]. SKI-606 was first described *in vitro* to revert Src-transformed fibroblasts to a non-transformed morphological appearance, inhibit colon cancer cell line HT29 colony formation in soft agar, and significantly inhibit HT29 tumor



AP23464
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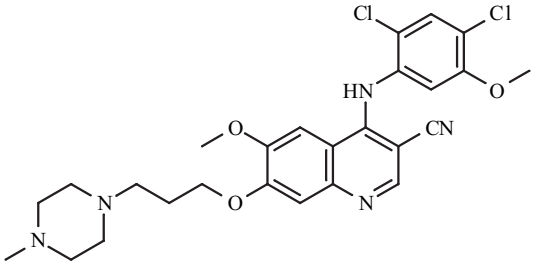

SK1-606
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Fig. (5).

growth in a xenograft model [61]. In these studies, IC_{50} concentrations are reported as 1.2 nM for Src enzymatic activity and 100 nM for inhibition of Src-dependent cell proliferation with limitations in oral bioavailability. Recently, SKI-606 has been demonstrated to have dual inhibitory activity against Src and Abl kinases activity with inhibitory effects on *in vitro* proliferation and in Bcr-Abl positive leukemic cell lines. *In vivo*, administration of oral or i.p. SKI-606 once a day resulted in a significant regression of K562 leukemic xenografts in nude mice without toxicity at doses as high as 150 mg/kg [75]. With initial studies demonstrating an effect on colon cancer tumor progression, recent oral bioavailability and successes in preclinical trials in CML, recent studies using colon tumor xenograft models with the formulated orally bioavailable SKI-606 caused substantial reductions in colon cancer tumor growth *in vivo* supporting the initial results by Boschelli *et al*, 2001 [76]. Analogues of SKI-606, with similar inhibitory effects *in vitro* and *vivo*, are currently being developed for their ability to have higher plasma levels of equivalent doses resulting in better efficacy in colon cancer xenograft models [77,78]. These results suggest a role for SKI-606 as a therapeutic agent for the treatment of colorectal cancer and SKI-606 is currently in clinical trials for the treatment of solid tumors [61,75,76].

FUTURE DIRECTIONS AND CONCLUSIONS

In summary, the studies discussed above utilizing Src kinase inhibitors highlight the critical roles that Src plays in human cancer promotion and progression. As a result of these studies, Src is rapidly emerging as a valid chemotherapeutic target. As mentioned above, BMS-354825 is currently in clinical trials for the treatment of CML, and trials are now being initiated for the treatment of solid tumors with Src family kinase inhibitors. However the question remains as to how Src family kinase inhibitors may be most effectively utilized for the treatment of solid tumors. Most primary solid tumors can be removed by surgical resection, with the majority of deaths resulting from subsequent or pre-existing metastases that are refractory to conventional chemotherapeutic strategies. The proven importance of Src kinase activity for cellular processes such as migration, invasion, protease production, anchorage independent growth, and angiogenesis, suggests that Src family kinase inhibitors may be most effective as anti-metastatic agents, helping to prevent the progression and metastatic dissemination of solid tumors. Thus Src inhibitors alone, or in combination with other targeted therapeutics that adversely affect cellular proliferation, may be effective in maintenance therapy, helping prevent or delay tumor progression and metastasis. In addition, the current data indicating the importance of Src activity for resistance to gemcitabine, paclitaxel, and cisplatinum suggest that, when used in combination with conventional chemotherapeutic agents, Src kinase inhibitors may facilitate apoptosis and cytotoxic death of normally chemoresistant solid tumors and metastases. Recent successes in pre-clinical *in vivo* model systems have buoyed the hopes that Src family kinase inhibitors may prove similarly effective in clinical trials for human solid tumors, some of which are currently underway. Nevertheless, Src has emerged as an attractive candidate molecule for the development of targeted therapies for human cancers, and it will be important to continue investigations of currently available and emerging Src family kinase inhibitors, both for their ability to illuminate the functions of Src in normal and cancerous cells and for their potential value as targeted therapeutics for the treatment of human cancers.

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