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Src signaling pathways in prostate cancer

Andreas Varkaris,

Department of Genitourinary Medical Oncology, The University of Texas, M.D. Anderson Cancer Center, Unit 1374 1155 Pressler St, Houston, TX 77030, USA

David H. Koch Center for Applied Research of Genitourinary Cancers, The University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA

Evangelismos General Hospital, University of Athens, Athens, Greece

Anastasia D. Katsiampoura,

Evangelismos General Hospital, University of Athens, Athens, Greece

John C. Araujo,

Department of Genitourinary Medical Oncology, The University of Texas, M.D. Anderson Cancer Center, Unit 1374 1155 Pressler St, Houston, TX 77030, USA

Gary E. Gallick, and

Department of Genitourinary Medical Oncology, The University of Texas, M.D. Anderson Cancer Center, Unit 1374 1155 Pressler St, Houston, TX 77030, USA

David H. Koch Center for Applied Research of Genitourinary Cancers, The University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA

Paul G. Corn

Department of Genitourinary Medical Oncology, The University of Texas, M.D. Anderson Cancer Center, Unit 1374 1155 Pressler St, Houston, TX 77030, USA

Andreas Varkaris: pcorn@mdanderson.org

Abstract

Knowledge of the molecular events that contribute to prostate cancer progression has created opportunities to develop novel therapy strategies. It is now well established that c-Src, a non-receptor tyrosine kinase, regulates a complex signaling network that drives the development of castrate-resistance and bone metastases, events that signal the lethal phenotype of advanced disease. Preclinical studies have established a role for c-Src and Src Family Kinases (SFKs) in proliferation, angiogenesis, invasion and bone metabolism, thus implicating Src signaling in both epithelial and stromal mechanisms of disease progression. A number of small molecule inhibitors of SFK now exist, many of which have demonstrated efficacy in preclinical models and several that have been tested in patients with metastatic castrate-resistant prostate cancer. These agents have demonstrated provocative clinic activity, particularly in modulating the bone microenvironment in a therapeutically favorable manner. Here, we review the discovery and basic

biology of c-Src and further discuss the role of SFK inhibitors in the treatment of advanced prostate cancer.

Keywords

Src; Prostate cancer; SFK inhibitors; Dasatinib

1 Introduction

In 1911, Peyton Rous described the virus that now bears his name (Rous sarcoma virus, RSV) as the causative agent of chicken sarcoma. In his report, Rous proposed for the first time that an infectious agent could induce the formation of a solid tumor [1]. His work was received with considerable skepticism and it was not until 1966, and the identification of additional tumor-inducing viruses, that he was awarded the Nobel Prize in Medicine. Sixty years after Rous's landmark discovery, the *v-src* gene was identified as the RSV gene responsible for malignant transformation [2]. In 1976, Stehelin *et al.* demonstrated that *v-src* had a normal cellular progenitor gene, named *c-src* [3]. At that time Bishop and Varmus used the term "proto-oncogene" to describe the cellular counterpart of the viral gene, the first of a long list of "proto-oncogenes" discovered in the following years. Bishop and Varmus received the Nobel Prize in Physiology and Medicine for this work. A year later, Collet *et al.* identified the v-Src protein, a 60-kDa phospho-protein with kinase activity [4]. In 1980, Hunter and Sefton demonstrated that Src kinase phosphorylates tyrosine residues in target proteins (the first time kinases were shown to phosphorylate this amino acid), and Src itself is also phosphorylated at tyrosine residues [5]. Thus, many discoveries in the field of oncogenesis are attributable to the discovery of Src, which then spurred numerous studies on other oncogenes and proto-oncogenes [6].

2 SFKs structure and regulation

The Src family kinases (SFKs) are comprised of nine structurally similar non-receptor protein tyrosine kinases (Src, Fyn, Lyn, Yes, Blk, Lck, Hck, Fgr, and Yrk) [7]. SFKs' amino acid composition consists of a conserved arrangement of four distinct peptide domains, named Src homology (SH) domains, as well as a regulatory sequence [8] (Fig. 1). The amino terminal SH4 domain facilitates (1) myristoylation (amide bond of myristoyl group with glycine residue of each of the SFKs), required for membrane attachment, regulation of kinase activity and intracellular stability; and (2) palmitoylation (covalent attachment of palmitic acid in cysteine residues of all the SFKs except Src and Blk) is required for membrane attachment and trafficking of several SFKs [9, 10]. The SH4 domain is followed by a region that is unique to each family member. SH3 and SH2 domains allow the association and interaction with adaptor and signaling proteins involved in the formation of complexes. Specifically, the SH3 domain leads to Src complexes with proteins having peptide sequences rich in proline and other hydrophobic amino acids [11–13]. These protein-protein interactions form a polyproline type II helix that associates with the hydrophobic sequence of SH3 domain. The SH2 domain has high affinity for phosphotyrosine-containing sequences (pTyr-Glu-Glu-Ile) [14]. Association of SH2 and peptides resembles a two-pronged plug (peptide) engaging a two-"holed" socket (SH2).

Phosphotyrosine occupies the first “hole”, whereas isoleucine the second SH2 “hole”. SH2 and SH3 domains further regulate the kinase activity through intramolecular interactions, and provide some specificity for protein-protein interactions of individual SFKs. The SH1 domain possesses the intrinsic tyrosine kinase activity of the molecule and is followed by a carboxy terminal regulatory domain [15].

The activity of the SFKs is regulated by both intramolecular and intermolecular interactions [16]. The principal mechanism of negative regulation is mediated through phosphorylation of a carboxy terminal tail tyrosine residue at Y530 for Src (analogous phosphorylation sites are found in all SFKs) [8, 17–19]. Phosphorylation of Y530 occurs by C-terminal Src kinase (Csk) and Csk homologous kinase, and induces a “closed” molecular conformation with low enzymatic activity (Fig. 1) [20, 21]. In this conformation, intramolecular interactions occur between the SH2 domain and the tyrosine-phosphorylated carboxyl terminal tail [21]. Further stabilization results from interaction of the SH3 domain with an SH2 and SH1 linker domain. As a result, the accessibility of the kinase catalytic site for ATP and substrates is limited. Activation of SFKs occurs through disruption of these intramolecular interactions. The dissociation of SH2-Y530 is a key step in the induction of an “open” molecular conformation that increases kinase activity (Fig. 1). The “open” conformation is facilitated by the formation of intermolecular interactions (complexes) between SH2 domain and tyrosine-phosphorylated proteins for which the SH2 domain has higher affinity than the carboxyl tail. As a result, the c-terminal phosphate becomes accessible to multiple phosphatases, including PTP- α , PTP- λ , SHP-1, SHP-2, and PTP1B that dephosphorylate Y530 and stabilize the “open” molecular conformation. In the active conformation the kinase domain binds to ATP and substrates, whereas SH2 and SH3 domains interact with downstream substrates, to facilitate signal transduction and regulation of the cytoskeleton. The importance of the intramolecular interaction in regulating Src activity is demonstrated by mutation of tyrosine 527 (in chicken c-Src) to phenylalanine, which leads to malignant transformation of tissue culture cells and is sufficient to induce tumors in immunocompromised mice [18]. SFK activity is increased in most solid tumors and some hematologic malignancies, often with further increases correlating with progressive stages of the diseases [22]. However, Src activating mutations are rarely found in human tumors, with the exception of a single report in colorectal cancer [23].

Alternatively, constitutive Src activation in human tumor cells frequently occurs secondary to alterations in one or more of its activators (Fig. 1). SFKs are downstream of multiple RTKs including PDGF receptor (PDGF-R), epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor (FGF-R), insulin-like growth factor-1 receptor (IGF-1R), hepatocyte growth factor/scatter factor receptor (HGF-R), colony-stimulatingfactor-1 receptor (CSF-1R), stem cell factor receptor (SCF-R), muscle specific kinase (MuSK), and others [24–32]. Overexpression of these RTKs cooperates and synergizes with SFKs to promote tumorigenesis, probably by disrupting the intramolecular interactions that hold SFKs in a closed configuration [33].

3 Expression of SFKs in prostate cancer

Normal prostate epithelium expresses six SFKs (Src, Lyn, Fyn, Yes, Hck and Fgr) and studies of human tumor specimens suggest that some SFKs are overexpressed during prostate cancer tumorigenesis and progression [34]. Goldenberg-Furmanov *et al.* reported that Lyn is extensively expressed in 95 % of primary PCa and 100 % of metastatic (lymph node and bone) specimens [35]. In this study, Lyn expression increased proportionally to the Gleason score in primary tumors, although direct comparison of Lyn expression in tumor *versus* normal tissue was not performed. Posadas *et al.* reported that the expression (not activity) of the majority of SFKs (Lyn, Src, Yes, Fgr, and Hck) is unchanged between normal and cancer prostate specimens. In contrast, this study reported that the SFK Fyn is greater than two-fold overexpressed in prostate tumors relative to normal tissue, although differences between metastatic and normal tissue were not observed (possibly due to the small number of metastatic samples examined) [36]. Tatarov *et al.* studied SFKs activation in matched cases of castrate-naïve (CN) and castrate-resistant (CR) prostate cancer tissues [37]. Intensity in the membrane or cytoplasm of phospho SFK (Y419) staining was assessed separately. Results of these investigators demonstrated a significant increase of overall SFK activity during the transition from CN to CR prostate tumors; although in the majority of matched samples there was no change. Membranous SFK activity correlated with the presence of bone metastasis in the subgroup of CR patients. Also, a subgroup of patients that had an increase in membranous SFK activity during the transition from CN to CR disease had shorter progression-free interval and overall survival. Thus, with the exception of Fyn, changes in expression of SFKs appear minimal; however, SFK activity increases during the development and in progressive stages of prostate cancer. Further, intracellular compartmentalization of activated SFKs might be useful as a marker of tumor metastatic potential and resistance to hormonal manipulations.

4 Roles of SFKs in prostate cancer development and tumor growth

Lyn and Yes are essential for normal prostate development in mouse models [35]. At least three SFKs (Src, Fyn, and Lyn) have been demonstrated to be able to participate in prostate tumorigenesis in mouse models [38]. Cai *et al.* reported that individual SFKs have different functions in both paracrine (FGF 10-induced) and cell-autonomous transformation of prostate epithelium. Using a prostate regeneration system in mice, this group demonstrated that Src-knockdown abolishes the FGF10-induced transformation and AR overexpression, whereas loss of Fyn or Lyn has partial or no effect, respectively. In agreement with previous findings, constitutively active (mutated) c-Src has a higher potential to transform prostate epithelium, followed by constitutively active (mutated) Fyn and Lyn. Additionally, resultant tumors have different phenotypes. Prostate epithelial cells expressing Src Y529F develop poorly differentiated invasive adenocarcinoma without glandular pattern. In contrast, constitutive activation of Fyn (Y528F) promotes the formation of mPIN, whereas overexpression of LYN (Y508F) results in phenotypically normal prostate regeneration [39].

Not only do SFKs affect prostate tumorigenesis, they differentially affect proliferation and tumor growth of prostate cancer cell lines (Fig. 2). In cultured PC3MM2 tumor cells, Park *et*

al. demonstrated that stable Src-knockdown has little effect in cell proliferation but greatly inhibits migration and invasion, whereas Lyn directly affects proliferation [40].

Further evidence that one or more SFKs are important in prostate cancer proliferation has been derived from the use of multi-targeted inhibitors of the kinase activity of SFKs, several of which have been tested in clinical trials (see below). These kinase inhibitors universally inhibit prostate cancer cell proliferation *in vitro*. Park *et al.* demonstrated that dasatinib induces a 50 % reduction of PC3MM2 and LNCaP cell growth at doses which most SFKs are inhibited (100 nM) [40]. In agreement with these results, saracatinib, another inhibitor of SFK activity, leads to 50 % reduction of PC3 and DU145 cell growth at doses in which most SFKs are inhibited (6 μ M) [41]. Despite the anti-proliferative effect, inhibitors of SFK activity have only a minor cytotoxic effect in prostate cancer cell lines *in vitro*. *In vivo*, SFK inhibitors decrease proliferation of prostate cancer orthotopic tumors in immunocompromised mice. Dasatinib inhibits the PC3MM2-xenograft volume by 55 % compared to control after 25 days of treatment [40]. Similarly, saracatinib induces a 45 % reduction in tumor growth after 52 days of treatment [41]. The antitumor effect of SFK inhibitors is a result of combined anti-proliferative and pro-apoptotic effect. The pro-apoptotic action of SFK inhibitors *in vivo* is possibly mediated by inhibition of tumor vasculature, as SFKs have been shown to regulate pro-angiogenic molecules such as VEGF and IL-8 (Fig. 2) [42–44].

5 Src and AR crosstalk

PCa development and progression requires continued signaling through the AR [45]. Considerable recent work has implicated Src activation as an important mediator of AR signaling. Magliaccio *et al.* showed that ligand-induced activation of AR increases the autophosphorylation of Src, whereas culture of androgen sensitive LNCaP cells in charcoal strip media (androgen ablation conditions) dramatically reduces the autophosphorylation of SFKs [46]. Subsequent studies demonstrated that AR-induced Src activation promotes cell proliferation through phase G1 to S cell cycle progression [47]. Not surprisingly, dihydrotestosterone-induced proliferation of LNCaP and C4-2 cells (a metastatic variant of LNCaP cells) is inhibited by pharmacological SFK inhibition [48]. These studies established Src and its downstream targets as important mediators of the pro-proliferative effects of AR signaling. In addition to the *in vitro* studies, Cai *et al.* demonstrated the role of AR/Src interaction in prostate cancer tumorigenesis *in vivo*. Specifically, their studies showed that overexpression of both AR and Src in naïve prostate epithelium increases Src activity and induces the transformation to invasive adenocarcinoma. In contrast, Src overexpression alone is insufficient to promote tumorigenesis [39].

Persistent AR signaling despite low serum androgen levels is associated with the development of castration resistance [49, 50]. As is the case of castrate naïve tumors, in metastatic castrate-resistant human samples Src phosphorylation has been correlated with AR phosphorylation {Guo, 2006 12/id} [37]. Src activation induces direct (tyrosine) and indirect (serine/threonine) phosphorylation of AR [39, 51, 52]. An increasing number of molecules involved in ligand-independent phosphorylation of AR have been described, including growth factors (IGF-1 and EGF), neuropeptides (GRP and bombesin), interleukins

(IL-6 and IL-8) and saposins [51, 53–56] [57]. A common signaling intermediate activated in each of these pathways is Src, providing multiple mechanisms by which Src becomes activated and directly phosphorylates AR (Fig. 1). In addition to Src-induced AR phosphorylation, Asim *et al.* have shown that Src activation represses the interaction of co-repressors such as LCoR with AR, through inhibition of co-repressor-receptor interaction, and facilitates AR function in cell growth [58]. The effect of Src-induced AR activation is not completely understood. Several studies suggest that Src-induced AR phosphorylation promotes AR translocation, transactivation and binding to target genes. Overexpression of a kinase-inactive Src mutant decreases AR transactivation potential, whereas the constitutively active SrcY527F is sufficient to induce nuclear translocation of AR in the absence of androgens [52]. In agreement with previous findings, pharmacological inhibition of SFKs decreases ligand-independent, but not ligand-dependent AR transactivation and transcription of target genes [52, 54, 59]. Although the biological significance of AR phosphorylation is not completely understood, increasing evidence suggests that phosphorylation promotes the stabilization and the transcriptional activity (sensitivity) of AR in the presence of low androgen levels. These results were not assayed with *in vitro* cell growth assays and/or *in vivo* tumor growth measurements in the presence of low androgens. Thus, further preclinical studies are required to determine the importance of AR phosphorylation in prostate cancer progression to castrate resistance.

The crosstalk between the AR and Src is a potential target for the treatment of prostate cancer. In addition to AR targeting agents and SFKs tyrosine kinase inhibitors, several peptides that specifically interfere with AR proline rich with SH3 Src domain interaction have been developed. Preliminary studies have shown that these peptides successfully block both AR to Src and Src to AR activation. Furthermore, treatment with peptides greatly reduces the tumorigenic growth of LNCaP cells [60].

6 Src and metastasis

In prostate cancers, SFK activity is increased during progressive stages of the disease, with highest activity in bone metastasis. Activation of SFKs occurs primarily through “upstream” regulators that are aberrantly expressed in prostate tumor microenvironment. These may include growth factors that lead to Src activation such as HGF, Heregulin, IGF, cytokines such as IL-6, IL-8, IL-12, and bradykinin, bone effectors such as BMPs and RANKL, growth factor receptors such as MET, IGF-Rs, HER2/Neu, Axl; and ephrinA1, integrins and downstream mediators of integrin pathways such as FAK; activators of inside-out integrin activation such as talin; activators of outside in integrin activation such as TGFbeta, CD44, to illustrate only some of the molecules or pathways that directly lead to SFK activation [56, 59, 61–68]. Also, activation of Src by reactive oxygen species has been described [69]. All these factors are (1) aberrantly expressed in prostate cancer metastasis and (2) require Src activity to promote pro-metastatic properties of PCa. Regardless of the multiple mechanisms of Src activation, once Src is activated, it promotes numerous properties of metastasis including motility, migration, invasion, angiogenesis, and anoikis resistance (Fig. 2). The pro-metastatic effects of Src are mediated, in part through dynamic regulation of actin cytoskeleton structures; including cell-ECM adhesions, cell-cell adhesions and invadopodia [63, 70, 71].

Focal adhesions are integrin-dependent cell-ECM adhesion structures required for cell adhesion and traction [72]. During cellular migration, focal adhesions are formed at the leading edge of the cells, used as an anchor for cell traction and disassembled at the rear edge, and to allow cell movement over the ECM. The effect of Src on focal adhesions regulation is primarily mediated by direct interaction with integrins, RhoA and FAK [73]. Src-mediated phosphorylation of integrin subunits promotes activation of integrins and releases the attachment to the ECM. Detachment from ECM is essential for cell motility and migration. Src-mediated inactivation of RhoA interrupts the actin stress fibers assembly and thus reduces the stabilization of focal adhesions and promotes cell motility. Additionally, Src binds and form a complex with activated FAK Y397. Upon recruitment at FAK Y397 Src is activated and facilitates further phosphorylation of FAK at multiple sites (Y401, Y576, Y577, Y861, and Y925). Src/FAK complex interacts and phosphorylates multiple downstream signaling pathways including paxillin, p130^{cas} and PI3K and that are implicated in cell migration and invasion [74].

Adherens junctions are ephemeral cell-cell adhesions in which E cadherin/ β catenin complexes bridge the actin cytoskeleton of neighboring cells through hemophilic interactions [75]. Tyrosine phosphorylation of β catenin and Tiam 1 by Src and Src/FAK complex suppresses the association of β catenin with E-cadherin and disrupts the integrity of adherens junctions, a critical step in epithelial to mesenchymal transition [76–79].

Invadopodia are actin-rich dynamic membrane protrusions of cancer cells with high ECM adhesive and degradation capacity first identified in v-src cells [78]. Their functions are mediated by rapid formation of actin cytoskeleton adhesion structures and secretion of ECM degradation enzymes including metalloproteinases. Src promotes both these processes through phosphorylation of multiple proteins that contribute to invadopodia dynamics [80, 81]. Src-dependent phosphorylation of contracting and N-WASP induces actin polymerization as well as invadopodia maturation [82]. Also, activation of Tks4 and Tks5 promotes invadopodia formation and secretion of metalloproteinases [83, 84].

As Src promotes numerous properties associated with metastatic potential, it would be expected that Src inhibition would inhibit metastasis (Fig. 2). This expectation is confirmed by several preclinical studies in multiple types of cancer, including prostate cancer [85]. Multi-targeted Src inhibitors including dasatinib, saracatinib and bosutinib inhibit in a dose-dependent pattern the motility (in scratch assays), migration and invasion (in modified Boyden chambers) of PC3, DU 145 androgen-independent and LNCaP androgen-dependent prostate cancer cell lines [40, 41, 86, 87]. Characteristically, at doses up to 100 nM dasatinib almost completely abolishes cell migration and invasion of PC3 and PC3MM2 cells [40, 67]. Furthermore, IGF-1 and HGF induction on prostate cancer cell migration is inhibited by dasatinib. Saracatinib (250 nM) induces a 40 and 80 % inhibition of migration of DU145 and PC3 cells, respectively. Similarly, bosutinib (1 μ M) induces a 55 % inhibition of invasion of DU145 and PC3 cells. In orthotopic mouse models, inhibition of SFKs with dasatinib significantly reduces the incidence of lymph node metastasis of PC3MM2 and PC3AR A1 derived tumors by 80 % after 35 days of treatment.

7 Src and bone metastasis

Prostate cancer has a propensity to metastasize to the axial skeleton and cause osteolysis and formation of woven bone (prostate cancer metastases are classified as osteoblastic based on radiographic appearance). Deregulation of bone remodeling by tumor cells initiates what has been classically termed the “vicious cycle”, in which interactions between the epithelial and tumor-associated bone stromal cells promote tumor growth and progression [88–90]. SFKs are extremely important in this vicious cycle, as they are critically involved in normal and pathological bone remodeling (Fig. 2) [91].

Bone homeostasis is elegantly regulated by interactions between bone stromal cells (including osteoclasts and osteoblasts) and extracellular matrix. The involvement of Src in bone homeostasis was first described by Soriano *et al.* who generated mice in which *c-src* is functionally deleted (*src*^{−/−} mice) [92]. These mice universally developed osteopetrosis, a rare syndrome in humans in which density and bone mass are increased, although the bones are fragile [92]. Osteopetrosis occurs because of decreased function of osteoclasts, the sole cell that induces bone resorption. Thus, not surprisingly, osteoclasts from *src*^{−/−} mice are phenotypically abnormal, and are unable to resorb bone. Src activity specifically affects ruffled borders of osteoclasts (necessary for bone resorption), through dynamic regulation of interactions of actin cytoskeleton and formation of podosomes. Podosomes are cell-ECM adhesion sites that consist of an actin core and actin-nucleation machinery. Src orchestrates the activity of the actin-nucleation machinery by interacting with a large number of its substrates, including FAK, Ezrin, HS1, P130^{cas}, Pyk2, AFAP-110, and ASAP-1 [70, 93]. Interactions between these proteins regulate the dynamic formation and disassembly of actin cytoskeletal complexes. The actin machinery facilitates the rapid polymerization and depolymerization of actin structures required for podosome formation and organization into rosettes, essential for osteoclast motility and invasion that leads to degradation of bone extracellular matrix. This degradation step is coupled to the deposition of abnormally woven bone by osteoblasts.

Marzia *et al.* reported that parallel to reduced bone resorption, bone formation is increased in *src*^{−/−} mice [94]. This observation suggested that Src is implicated in the activity of another bone stromal cell, the osteoblasts. Subsequent studies demonstrated that Src activation maintains osteoblasts in a less differentiated status. In agreement with the above findings, reduction of Src expression decreases osteoblast proliferation and increases differentiation. Lee *et al.* showed that reduction of Src activity by dasatinib (an SFK inhibitor that has been used in clinical trials for prostate cancer) also inhibits osteoblast proliferation and induces the differentiation of primary mouse osteoblasts [95]. Notably, differentiated osteoblasts secrete numerous pro-tumorigenic factors including osteonectin, which promotes prostate cancer cell invasion. Further, Dayyani *et al.* reported that osteoblast differentiation promotes the secretion of IGF-1, a growth factor implicated in PCa progression [96]. These results imply that the effect of Src inhibition in osteoblasts may promote the development of resistance to therapeutic agents and suggest the hypothesis that patients without osteoblast differentiation would respond longer to SFK inhibitors.

The mechanism of Src activation in stromal and epithelial cells in prostate cancer metastases is only partly understood. During the first step, tumor-bone interactions disrupt bone physiology. Osteoclast activation is a critical step in the initiation of epithelial-stromal interactions. Degradation of ECM releases a large number of growth factors and cytokines physiologically stored in ECM. Secreted factors bind to membrane receptors in both stromal and epithelial cells and induce Src activation [91, 97, 98]. Activation of Src enhances the vicious cycle of epithelial-stromal interactions by: (1) degradation of ECM by osteoclasts; (2) formation of new bone by osteoblasts; and (3) promotion of tumor growth and resistance to therapy. Thus, combined inhibition of both epithelial and stromal compartment by SFK inhibitors could have an additive effect against metastatic tumor growth. Not surprising, SFK inhibition by dasatinib reduces the growth of intratibial mouse xenografts of C4-2B Cap cells [99].

8 Src and tumor vasculature

Considerable work has elucidated mechanisms by which SFKs regulate pathologic processes in vascular biology, including angiogenesis and endothelial permeability (Fig. 2). Prostate cancer microenvironment is rich in pro-angiogenic factors including growth factors; VEGF-A, VEGF-C, PDGF, HGF, and IGF and cytokines; IL-8, IL-6. Activation of Src in tumor and tumor associated stromal cells promotes the secretion of VEGF and IL-8, through transcriptional up-regulation. Hypoxia induces the expression of pro-angiogenic factors by tumor and tumor associated stromal cells. Mykhopadhyay *et al.* first demonstrated that Src is essential for VEGF expression by attenuation of hypoxia induced up-regulation of VEGF in *src*^{-/-} fibroblasts [100]. Subsequent studies demonstrated that Src activation enhances the transcriptional activity of HIF-1 α and STAT3 that regulate VEGF expression [42]. Similarly, IL-8 is regulated by Src/STAT3 dependent mechanism [101].

In addition to the pro-angiogenic effects, activation of SFKs in endothelial cells increases vascular permeability; a process that is essential for tumor nutrition and metastasis [102]. SFKs promote structural changes through rearrangement of actin cytoskeleton and adherence junctions in endothelial cells that affect the vascular architecture and create intercellular gaps. Intercellular gaps are potential spaces that permit leakage of soluble factors from the circulation to the interstitial space. Additionally, cancer cells use this space to enter the circulation during hematogenous dissemination; intravasation and extravasation. In addition to paracellular transport, SFK-induced endothelial permeability is facilitated by transcellular transport [103]. Formation and internalization of caveolae requires the phosphorylation of caveolin-1 and dynamin-2 by SFKs. Caveolae transport through the cytoplasm and fuse with the basal membrane, releasing their macromolecule-rich contents into the interstitial space [104].

9 Clinical experience with inhibitors of SFK signaling

Given its important role in prostate cancer tumorigenesis, SFK inhibitors have been tested extensively in patients with advanced disease. To date, all SFK inhibitors are orally bioavailable small molecules divided into two general categories based on their mechanism of inhibition of Src family proteins: (1) ATP-site competitive inhibitors that directly inhibit

tyrosine kinase activity or (2) inhibitors of protein-protein interactions (SH2-, SH3-, or substrate-binding domain). While multiple SFK inhibitors are in early phase I development for both liquid and solid tumors and have been recently reviewed elsewhere, herein we will focus on only those agents with phase II and/or III published data in prostate cancer [105]. Three agents that have been tested in these settings include two kinase inhibitors BMSS354825 (dasatinib) and AZD0530 (saracatinib), and one inhibitor of protein-protein interactions (KX2-391).

As preclinical data demonstrate that SFK inhibitors target both prostate cancer epithelial cells and stromal cells (*e.g.*, osteoclasts, osteoblasts, endothelial cells) in the tumor microenvironment, it has been useful in clinical trials to incorporate biomarkers to distinguish these effects. For example, blood PSA levels measure modulation of cancer epithelial cells, while blood levels of bone-specific alkaline phosphatase (BAP) and urine levels of N-Telopeptides (uNTX) measure modulation of osteoblasts and osteoclasts, respectively (referred to as “bone turnover markers”). Analysis of these markers has helped elucidate whether SFK inhibitors principally target the epithelial and/or stromal compartment(s) and facilitated the design of rational therapy strategies combining Src inhibitors with other agents (*e.g.*, CYP17 inhibitors, cytotoxic chemotherapy).

9.1 Dasatinib

Dasatinib is the most clinically studied SFK family kinase inhibitor in prostate cancer. Dasatinib is an orally bioavailable, ATP-site competitive tyrosine kinase inhibitor that is FDA-approved for patients with chronic myelogenous leukemia or Philadelphia chromosome positive acute lymphoblastic leukemia failing first-line therapy with imatinib. As discussed previously in this review, an extensive body of preclinical evidence supports the ability of dasatinib to inhibit prostate tumor growth and metastases both *in vitro* and *in vivo*. In addition, preclinical studies have demonstrated the ability of dasatinib to inhibit osteoclast activity and bone resorption.

The safety, pharmacokinetics, and maximal tolerated dose (MTD) of dasatinib in patients with solid tumor malignancies were first established in a phase I study that did not report inclusion of any patients with prostate cancer [106]. Subsequently, two phase II studies were conducted testing the safety and activity of dasatinib in patients with metastatic castrate-resistant prostate cancer at different doses and schedules. In the first study (CA180085), chemotherapy-naïve patients with mCRPC received 70 or 100 mg orally twice a day [107]. Endpoints included changes in PSA, bone turnover markers, bone scan, and RECIST measurable disease. Progression was not based on PSA, but rather on tumor progression using RECIST or new lesion(s) on bone scan assessed every 12 weeks.

Of 47 patients treated, progression-free survival (PFS) was 43 % at week 12 and 19 % at week 24. Of 41 evaluable patients, 51 % patients achieved 40 % reduction in uNTX at week 12. Of 40 evaluable patients, 60 % had reduction in BAP at week 12. Dasatinib was reasonably well tolerated at these doses but grades 1–2 toxicities (fatigue, pleural effusions, nausea, diarrhea) occurred in >40 % of patients, making the drug difficult to take for prolonged periods of time without dose reductions or interruptions.

To ameliorate toxicity, protocol CA180085 was amended and an expansion cohort of 48 patients was treated with dasatinib at 100 mg daily [108]. This dose was considerably better tolerated and appeared to have similar efficacy to the prior regimens. PFS was 44 % at week 12 and 17 % at week 24. uNTX was reduced by 40 % from baseline in 22 (51 %) of 43 patients, and BAP was decreased in 26 (59 %) of 44 patients.

In considering the 95 reported patients with mCRPC treated with dasatinib monotherapy, only ~5 % achieved PSA responses of >50 %. In contrast, given the relatively greater response in bone markers, our group interpreted this data to suggest that dasatinib principally targets the stromal rather than the epithelial compartment in human patients. To build on this, we hypothesized that a rational therapy strategy would be to combine dasatinib with docetaxel, a cytotoxic agent with established efficacy in targeting the epithelial compartment in mCRPC [109]. To do this, our group conducted and published a Phase I/II study combining dasatinib and docetaxel chemotherapy in patients with mCRPC [110].

In the phase I portion of the study, 16 chemotherapy-naïve patients with mCRPC received dasatinib (50 to 120 mg by mouth daily) with docetaxel (60–75 mg/m² intravenously every 3 weeks) and the recommended phase II doses were dasatinib 100 mg once daily by mouth and docetaxel 75 mg/m² intravenously every 3 weeks. An additional 30 patients were treated in the phase II portion of the study. Efficacy endpoints included changes in PSA and bone markers, evaluation of measurable disease by RECIST, and evaluation of bone scans.

Durable PSA declines of >50 % occurred in 26 of 46 patients (57 %). Thirty-three of 38(87 %) and 26 of 34(76 %) patients had decreases in urinary N-telopeptide or bone-specific alkaline phosphatase levels, respectively. Of 30 patients with measurable disease, 18 (60 %) had a partial response and on bone scan, 14 patients (30 %) had a complete disappearance of all lesions. Adverse drug–drug interactions and maximum tolerated dose were not identified. The combination was generally well tolerated with <10 % grade 3 toxicities (fatigue, pleural effusion, anemia) and no grade 4 toxicities. Of 28 patients (61 %) who received single-agent dasatinib after docetaxel, 11 had stabilization of disease for 3 to 12 months after the last chemotherapy dose, with six patients still on dasatinib at the time of data capture.

Based on the high objective response rate, the favorable toxicity profile, and the observation that some patients experienced prolonged disease stabilization on dasatinib maintenance therapy, we next conducted a randomized phase III trial comparing docetaxel 75 mg/m² every 3 weeks plus prednisone 5 mg twice daily with dasatinib 100 mg or placebo once daily [111]. The primary endpoint was overall survival (OS) and secondary endpoints included PFS, objective response rate, time-to-first skeletal-related event (TFSRE), time-to-prostate-specific antigen progression, uNTx reduction, and pain reduction.

Patients with chemotherapy-naïve mCRPC were randomized 1:1 between the two arms (1,522 total with 760 receiving placebo and 762 receiving dasatinib). Dasatinib did not improve OS vs placebo (median 21.5 [95 % CI 20.3–22.8] vs 21.2 [95 % CI 20.0–23.4] months; hazard ratio [HR] 0.99 [95.5 % CI 0.87–1.13]; *p*=0.9009). Results for secondary efficacy endpoints and subgroup analyses were generally similar between the arms with the exception of TFSRE. Notably, median TFSRE was not reached for dasatinib and was 31.1

(95 % CI 28.8–not reached) months for placebo (HR 0.81 [95 % CI 0.64–1.02]). Most common grade 3/4 adverse events included diarrhea (placebo 3.6 %, dasatinib 7.6 %), fatigue (5.5 %, 8.1 %), and asthenia (3.0 %, 5.3 %).

9.2 Saracatinib

A Phase I dose escalation study of saracatinib in patients with solid tumors was recently published [112]. The maximum tolerated dose was 175 mg once by mouth each day. Saracatinib was well tolerated, with rare (<5 %) Grade 3 toxicities (diarrhea, anemia, asthenia, and neutropenia) and Grade 4 toxicities. Although no prostate cancer patients were studied, immunohistochemical analysis of paired tumor biopsies (baseline and on-treatment) revealed a reduction in expression of downstream Src targets consistent with inhibition of Src signaling activity. As there was activity of saracatinib in preclinical models of mouse prostate cancer, a phase II trial in patients with advanced metastatic castrate-resistant prostate cancer was performed [41, 113].

In this study, 28 patients with mCRPC were treated once daily with 175 mg of saracatinib. Patient characteristics included a mean age of 67 years and nine patients (32 %) had received prior docetaxel-based chemotherapy. The primary endpoint was PSA response rate defined as a reduction in PSA >30 %. The drug was well tolerated but only five patients evidenced reductions in PSA, and none met criteria for a PSA response. Changes in bone markers and analysis of intratumoral Src inhibition were not reported. Based on these data, saracatinib does not appear to have potential as monotherapy in patients with advanced disease.

9.3 KX2-391

KX2-391 is an oral, non-ATP competitive inhibitor that targets the peptide substrate-binding site of Src family kinases. Thus, this is an example of a peptidomimetic Src inhibitor. KX2-391 also inhibits tubulin polymerization (*via* an α,β -Tubulin heterodimer binding site) and has a wide spectrum of activity in cell lines and preclinical models of different solid tumors [114, 115]. A phase I study of KX2-391 in patients with advanced solid tumors was recently published [116]. Forty-four patients received oral KX2-391 and the maximum tolerated dose was 40 mg by mouth twice daily. Patients underwent restaging studies after two cycles (28 days/cycle) and were treated until disease progression, unacceptable toxicity or death. Five patients with mCRPC were treated and one demonstrated evidence of stable disease and remained on drug for 6 months. This patient's baseline PSA was 205 ng/ml and nadir PSA was 39 ng/ml. Markers of bone turnover were not assessed. The most common Grades 1 and 2 treatment-related toxicities were elevations in LFTs (27 %), fatigue (14 %), leukopenia (7 %), and nausea (14 %). Toxicities Grade 3 were rare and included elevations in LFTs (11 %) and leukopenia (11 %).

Based on these results, a phase II trial of KX2-391 was performed in 31 patients with chemotherapy-naïve, bone metastatic prostate cancer. The primary endpoint was the 24-week PFS, with PFS representing a composite endpoint defined as clinical progression, radiographic progression (on CT scan), or two new lesions on bone scan. Scans were performed every 12 weeks. Based on prior studies suggesting that no more than 30 % of

patients in this group would be free of disease at 24 weeks without treatment, a 50 % 24-week PFS with KX2-391 was predetermined to be a positive study.

Of the 31 patients enrolled, 26 were evaluable for the primary endpoint and 5 patients came off study for toxicity. Toxicities were predominantly grade 2 (fatigue, elevations in liver function tests, nausea, anorexia, constipation) and Grade 3–4 toxicities occurred in 10 % of patients (dehydration, bone pain, cytopenias, elevated LFTs). The 24-week PFS rate was 7.7 %, so the trial did not meet its primary endpoint of efficacy. PSA response rates of >30 % were rare (9.7 %), there were no objective radiographic responses, and 18 % (2/11) patients with unfavorable circulating tumor cells (>5) converted to favorable counts (<5) on therapy. Reductions in uNTX and BAP were observed in 32 and 10 % of patients, respectively. Based on these data, KX2-391 does not appear to have potential as monotherapy in patients with mCRPC.

10 Discussion and future directions

Over a century of elegant basic science led to establishment of Src as an important oncogene in human cancer and further provided the compelling rationale for SFK inhibition in human cancer. With this background, the clinical activity with SFK inhibitors in prostate cancer has arguably been disappointing to date. However, while SFK inhibitors will likely not be further developed as monotherapy or in combination with docetaxel in mCRPC, we believe that SFK inhibition will still prove to be clinically useful as part of an integrated therapy strategy in treating lethal disease. Inhibition of SFKs clearly has favorable effects on the bone stroma (even in patients concurrently receiving bisphosphonates) that underscore its potential to be combined with other novel epithelialtargeting agents.

Before successful combinations can be identified, however, several outstanding research imperatives remain. First, the optimal timing of Src inhibition in prostate cancer progression needs to be identified (*e.g.*, early versus late castrate-resistance). Put another way, we need to better define the biologic processes of prostate cancer progression that are specifically “src-driven”. Towards this goal, our group is presently conducting of a study maximal androgen depletion using an LHRH agonist plus abiraterone followed by randomization to combination therapy with either dasatinib or sunitinib, with crossover between targeted therapies at progression (ClinicalTrials.gov ID NCT01254864). Baseline and serial bone marrow biopsies on therapy are being collected with the goal to elucidate pathways of castration resistance that are Src-driven.

Second, molecular-pathologic biomarkers that predict response or resistance to Src inhibition need to be identified. The potential benefit to a subset of patients will be missed in large randomized trials without improved patient selection. We believe the many negative phase III trials combining docetaxel with other promising agents (other than Src kinase inhibitors) were similarly hampered by this problem.

Third, as suggested by the phase III docetaxel±dasatinib study, Src inhibitors may be most effective in delaying and/or preventing skeletal metastases from prostate cancer. As with bisphosphonates and RANKL inhibitors, this benefit could be tested as a primary endpoint

in subsequent clinical trials. Since bone is the preferred metastatic site of prostate cancer, this effect would significantly reduce the morbidity of advanced disease.

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Fig. 1. Src Activation. SFKs typically have four domains and their activity is regulated by different conformational states. SFKs are usually held in a “closed”, inactive form and transition to an “open”, active form upon stimulation from both extracellular and intracellular signals. Phosphorylation of Tyrosine 530 is a key molecular event associated with the closed conformation. SFKs are frequently activated when extracellular ligands associated with their cognate receptors. SFKs are also activated by androgen receptor signaling and during mitosis. Phosphorylation of Tyrosine 419 is a key molecular event associated with the open conformation. Activated Src can also activate AR, establishing crosstalk between these two growth promoting pathways

Fig. 2. Activation of SFKs and downstream targets stimulate signaling pathways that contribute to multiple biologic processes involved in prostate cancer progression including survival, proliferation, angiogenesis, and metastases