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Targeting Tyrosine Kinases and Autophagy in Prostate Cancer

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Abstract Tyrosine kinases play significant roles in tumor progression and therapy resistance. Inhibitors of tyrosine kinases are on the forefront of targeted therapy. For prostate cancer, tyrosine kinases play an additional role in the development of castration-resistant disease state, the most troubling aspect of prostate carcinogenesis which presently defies any effective treatment. Among the 30 or so tyrosine kinases expressed in a typical prostate cancer cell, nearly one third of them have been implicated in prostate carcinogenesis. Interestingly, most of them channel signals through a trio of *non-receptor tyrosine kinases*, Src/Etk/FAK, referred here as Src tyrosine kinase complex. This complex has been shown to play a significant role in the aberrant activation of androgen receptor (AR) mediated by growth factors (e.g., epidermal growth factor (EGF)), cytokines (interleukin (IL)-6), chemokines (IL-8), and neurokines (gastrin-releasing peptide). These factors are induced and released from the prostate cancer to the stromal cells upon androgen withdrawal. The Src kinase complex has the ability to phosphorylate androgen receptor, resulting in the nuclear translocation and stabilization of un-liganded androgen receptor. Indeed, tyrosine kinase inhibitors targeting Src can inhibit androgen-independent growth of prostate cancer cells in vitro and in preclinical xenograft model. While effective in inducing growth arrest and inhibiting metastasis of castration-resistant tumors, Src inhibitors rarely induce a significant level of apoptosis. This is also reflected by the general ineffectiveness of tyrosine kinase inhibitors as monotherapy in clinical trials.

One of the underlying causes of apoptosis resistance is “autophagy,” which is induced by tyrosine kinase inhibitors and by androgen withdrawal. Autophagy is a self-digesting process to regenerate energy by removal of long-lived proteins and retired organelles to provide a survival mechanism to cells encountering stresses. Excessive autophagy, sometimes, could lead to type II programmed cell death. We demonstrated that autophagy blockade sensitizes prostate cancer cells toward Src tyrosine kinase inhibitor. Thus, a combination therapy based on Src tyrosine kinase inhibitor and autophagy modulator deserves further attention as a potential treatment for relapsed prostate cancer.

Keywords Androgen receptor · Tyrosine kinase · Prostate Cancer · Autophagy · Src

Tyrosine Kinases and Prostate Cancer

There is very strong evidence that tyrosine kinases (TKs) are involved in the growth and metastasis of prostate cancer (for example [1, 2]). TKs also play key roles in tumor sensitivity to radiation and chemical-induced apoptosis. There are a total of 90 human TKs, 58 receptor TKs and 32 non-receptor TKs. Based on our extensive profilings of CaP cells, we found that on average, about 30 to 40 tyrosine kinases are expressed in prostate cancer cells [3]. For example in LNCap, we identified approximately 22 receptor kinases (ErbB1, ErbB2, ErbB3, EphA1, EphB1, EphB2, EphB3, EphB4, Sky, Mer, Ddr1, Ddr2, PDGFRB, FGFR2, FGFR3, FGFR4, IGFR, MET, RON, RET, TrkA, VGFR) and 14 non-receptor tyrosine kinases (SRC, FYN, YES, LYN, BRK, FER, CSK, ETK, ABL, ARG, JAK1, TYK2, FAK, PYK2). This is likely an underestimate due to the sensitivity of the RT-PCR conditions used.

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Consistent with our profiling results, erbB/EGF-R, erbB2/HER2, erbB3/HER3, FGF-R, insulin-like growth factor Insulin-like Growth Factor (IGF)-R Met, Ron, Axl, Mer/Nyk, Kit, RET, and NGF-R have been implicated in prostate carcinogenesis [4–11]. For instance, EGF and IGF were shown to activate AR in the absence of androgen and induce androgen-independent growth of LNCaP cells via Src pathway [12, 13]. ErbB2/HER2 was reported to be overexpressed in androgen independent xenografts and confers androgen independence [14–17]. Its elevated expression in clinically localized prostate cancer identifies metastatic potential [8]. Likewise, erbB3 is found to be required for the growth [18] and malignant progression of prostate cancer [19]. ErbB3 was shown to signal through Src and Etk/BMX non-receptor tyrosine kinases [18]. We first recognized the diverse pathways and varied effects of erbB2/erbB3 signaling [20, 21] and found that they also play a role in channeling signals transmitted by cytokines such as IL-6. Earlier work also showed that Mer (also called Nyk) is overexpressed in prostate carcinomas and is able to activate Src and increase the expression of a host of cytokines and chemokines ([22] and unpublished work). Recent studies showed that erbB2/erbB3 as well as Mer signaled through Ack1 non-receptor tyrosine kinase to phosphorylate and activate AR [23]. Likewise, Ret overexpression is associated with high-grade PIN and prostate cancer [24], and was an activator of Src [25].

Kit, usually found in hematopoietic stem cells, was shown to be aberrantly expressed as a truncated, activated receptor, which channels its effects by activating Src [26, 27]. IGF-1 receptor and Met receptor, as well as their ligands have been implicated in the development of advanced stages of CaP [27], although the expression of c-met is suppressed by AR [28, 29] and that of IGF-1R is augmented by androgen treatment. The ligand for Met, HGF, is involved in drug resistance [30] and metastasis [31] of CaP, with the former process involving Src signaling. The transcriptional activation of IGF-1R by androgen is via Src kinase [32]. These results suggest that different receptor TKs are implicated in different sets of CaPs and perhaps at various stages of the development. There is unlikely a common receptor TK target for all CaPs [33]. One interesting common thread of many of these receptor TKs is their ability to activate Src and its companion TKs, which will be the focus of our discussion below. While under normal conditions, c-Src activity is required for channeling multiple signals by these receptors and regulated by their ligands, elevated and constitutive Src activity, presumably due to the deregulation of the receptor activities, is a hallmark of end-stage CRPCs [34, 35].

Among non-receptor TKs, Src kinase complex, Src, Etk and FAK, as well as their respective family members figure prominently in CaPs and will be described separately in the

next section. Little is known about the functions of other families of non-receptor tyrosine kinases in prostate carcinogenesis. Ack was found to be able to directly phosphorylate AR and the Ack/AR complex is recruited to the ARE sites in the nucleus [36]. C-abl is generally not expressed or expressed at very low levels in various cell lines, based on our profile results, and virtually nothing is known about its role in CaP. Brk, a Src-related kinase, was shown to have different subnuclear localization in CaP cells vs normal counterpart, although its role in prostate carcinogenesis is not clear [37]. For a more detailed description on non-receptor TK, please see our recent review [1].

The Src/FAK/Etk Tyrosine Kinase Complex

Src is found to form a complex (or complexes) with another two TKs FAK and Etk. Src is known to interact with FAK through SH2–phosphor-Y397 interaction [38, 39] and we showed that Etk interacts with FAK through PH–FERM domain interaction [40]. Recent structural data revealed that FERM domain serves as an autoinhibitory domain of FAK. Interaction of FERM with other proteins (in this case Etk) would “open” up the FAK kinase domain and exposes Y397 for binding to Src, resulting in the activation of both Src and FAK. We showed Src phosphorylates and activates Etk [41]. These three kinases, thus, cross-activate one another, and signal received by one would enhance the others. That said, these kinases have their own signal pathways and cellular functions. Their individual connections to CaP are briefly summarized below.

Although the first oncogene identified, Src’s role in human malignancies has not been fully appreciated. This is likely due to the lack of frequent genetic mutations of this gene in human cancers, although rare mutation has been reported in colon cancer [42]. Src is ubiquitously expressed in all prostate cancer samples analyzed ([43], and unpublished data). Its activity is upregulated in hormone refractory CaP [35]. In addition to Src, two other Src family kinases, Fyn and Lyn, are also implicated in CaP progression [44, 45]. Inhibitors for Src family kinases, Dasatinib [35] and Saracatinib [46, 47], both arrest CaP cell growth and migration in vitro with the effect on metastasis more pronounced in vivo. Strong evidence suggests that Src is involved in invasion and metastasis especially bone metastasis [45, 48–50]. Src kinase channel signals through the conventional Ras/Raf/ERK and PI3K/Akt, but also activates Stat3, c-myc, and beta-catenin [51–53] and Id-1 [54]. The activation of beta-catenin and Id-1 by Src are relevant to invasion and metastasis [50, 54] so is its phosphorylation of FAK [55, 56]. Prostate cells have a propensity to undergo neuroendocrine differentiation in which Src pathway may be

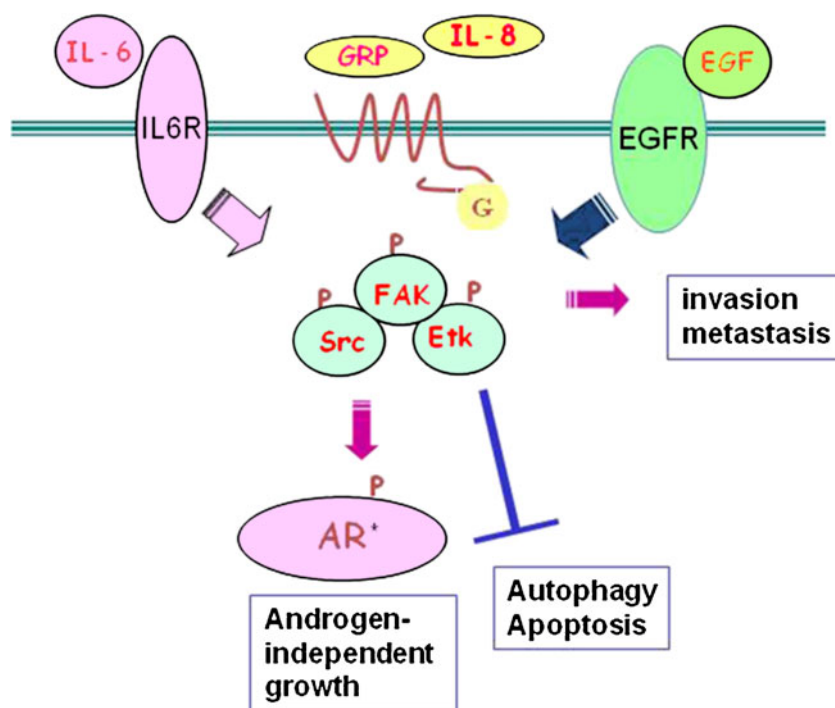
involved, as recent reports showed that RPTPalpha and PTP1B, phosphatases which activate Src, induce neuroendocrine differentiation [57, 58]. Probably, most relevant to the development of CRPC (castration-resistant prostate cancer) is Src's ability to activate androgen receptor and translocate AR into the nucleus [59–61] upon activation by neuropeptides [60, 62, 63] by chemokine IL-8 [64] and by EGF [44] (Fig. 1). Recently, it was shown that Src directly phosphorylates, stabilizes, and translocates AR, which is the major underlying mechanism whereby Src activates AR's transcriptional activity and how growth factors and cytokines activate AR under androgen-depleted conditions [23, 63, 65, 66]. While Src is able to activate AR and serves as a coactivator, Src is reciprocally activated by AR and serves as a downstream effector of AR signaling [67, 68]. The latter is usually referred to as the non-genomic action of androgen receptor. The above discussion illustrates the role of Src and family members in prostate carcinogenesis; they are signal integrators of many growth factors and appropriate targets for therapy.

FAK was originally identified as an Src substrate, with subsequent recognition of its role in focal adhesions upon integrin engagement. Src's association with and phosphorylation of FAK increases FAK's activity which then phosphorylates a host of proteins involved in cell adhesion and migration [38]. They include paxillin and p130cas [69]. FAK is involved in both the formation and dissolution of the focal adhesion plaques. FAK inhibitor inhibits the metastasis of PC3 in vivo, but does not affect its growth and survival [70]. Src

interactions with FAK depend on FAK's autophosphorylation of Y397, which is also the binding site for PI3K [71–74]. FAK is overexpressed, activated, and associated with paxillin in prostatic tissues from CaP patients with metastases [75, 76]. FAK is activated by neuropeptides and cell attachment, presumably via the RhoA pathway [77]. In the TRAMP mouse CaP model, FAK was shown to be required for androgen-independent formation of neuroendocrine carcinoma [78].

Etk belongs to the Btk family of TKs, typified by an N-terminal PH domain, followed by an SH3, SH2, and kinase domain. It was first reported as Bmx (X-linked bone marrow kinase) [79]. We independently cloned this kinase from the CWR22 CaP xenograft [80]. Etk is expressed in epithelial and endothelial cells, and hence its name. Our lab has carried out extensive structural and functional studies of this kinase in CaP [61, 81, 82]. The multidomain structure of Etk allows it to interact with a variety of signal molecules, including FAK, Src, RhoA, paxillin, STAT3, PAK1, PTPD1, VEGFR2, and p53. These molecules are known to be involved in migration, survival, and angiogenesis [83]. Through its PH domain, which binds PIP3, Etk is activated by PI3K [82], but in turn, it is a strong activator of PI3K pathway [84]. Etk's autokinase activity is further augmented by Src phosphorylation at Y566 (the Src 418 equivalent) [41]. We and others showed that Etk activation can be triggered by a variety of ligands, including IL-6 [82], bombesin [61], integrin [40], EGF [85], neuregulin (ErbB2/erbB3 activator) [18], and vascular

Fig. 1 Src kinase complex is an integrator of signals from growth factor (EGF), cytokine (IL-6) to chemokines (GRP and IL-8). Src, Etk, and FAK cross-activate one another. Src and Etk kinases activate AR by direct phosphorylation leading to androgen-independent growth; Src and Etk signals suppress autophagy and apoptosis providing survival factors and together with FAK, they promote invasion and metastasis. (GRP) gastrin-releasing peptide; (G) G protein, (AR*) aberrantly activated receptor in the absence of natural ligand



endothelial growth factor (VEGF) [86]. Upon activation by VEGF, Etk in turn augments the transcription of VEGF, providing a strong positive feedback loop during angiogenesis. Regarding cellular processes in CaP, Etk is involved in neuroendocrine differentiation, DNA-damage responses, hypoxia response, and the development of hormone refractory tumors. In most cases, it provides strong survival functions and protects CaP from thapsigargin and photodynamic therapy-induced apoptosis [87] and from p53-induced apoptosis through direct interaction with p53 [88], but under certain conditions (e.g., cleaved by Caspase), it can accelerate apoptosis [89], thus serving as a gatekeeper for cell survival. Etk was shown to be overexpressed in CaP and transgenic mice with prostate-specific expression of Etk developed PIN (prostatic intra-epithelial neoplasia) [81, 88]. In a recent report, Etk is shown to be overexpressed in hormone-resistant tumors and correlates well with the phosphorylation of AR at Y534 site, the phosphoacceptor of Src and Etk kinases (see below) [90]. Its potential role in the development of CRPC is shown by its ability to mediate the activation of androgen receptor by IL-6 [91] and its association, phosphorylation, and stabilization of androgen receptor in a manner similar to Src [90]. Etk is found to phosphorylate AR at Y534 and Y551/552, although not as potent as Src. The detailed mechanism of AR activation by Etk is not all identical to Src; for instance, Etk's association with AR requires phosphorylated tyrosine 551/552 of AR as anchor sites for the SH2 domain of Etk. By contrast, the association between Src and AR rely on the SH3/proline rich domains of Src and AR, respectively. In addition, the expression of Etk, but not Src, is in response of androgen deprivation, thus serving as a natural protector of prostate epithelial cells. That said, Etk is able to activate FAK and Src, and as such, the entire complex is involved in CRPC development. Both Etk and Src are important targets for therapeutic intervention.

Src Tyrosine Kinase Inhibitors and Prostate Cancer Therapy

There have been relatively few studies regarding the effects of Src family kinases inhibitors on CaP cell lines and xenografts. Dasatinib, a clinically approved thiazole and pyrimidine-based inhibitor, inhibits Src and Lyn TK at low nanomolar and effectively suppresses cell adhesion, invasion, and migration of DU145 [43]. This inhibition is accompanied by the decreased phosphorylation of FAK and p130cas, and decreased expression of MMP-9. Similarly, a pyrrolopyrimidine-based Src inhibitor inhibits the growth, adhesion, motility, and invasion of PC3 [92]. Another pyrazolo-pyrimidine-based Src inhibitor was shown to

inhibit EGF-induced migration of PC3 [93]. A peptide inhibitor for Lyn inhibits the *in vitro* growth of DU145 and PC3 at low micromolar range. It reduces the tumor volume of *in vivo* DU145 explant in nude mice [44]. Thus, the Src family inhibitors appear to primarily affect migration and invasion of CaP cells and, to a lesser extent, growth. Our lab has been studying the efficacy of Saracatinib (AstraZeneca, formerly AZD0530) [94] and PP2 (Pfizer). The former is a drug under clinical development, whereas the latter is primarily a research tool. We found that the dual use of PP2 and Saracatinib will help us pinpoint the intended target better. In androgen-sensitive LNCaP cells, neuropeptides and EGF are able to activate Src, which in turn, translocates AR into the nucleus and activates AR target genes, resulting in androgen-independent growth of the cells [60, 62, 66]. Both PP2 and Saracatinib treatments reverse this course, resulting in the growth inhibition of these cells. Likewise, Dasatinib was also found to inhibit phosphorylation-activated AR and androgen-independent growth of CaP under various treatment conditions [23]. Saracatinib inhibits Src family kinases with an IC₅₀ at a high nanomolar range [46], but also targets c-abl with less potency. As a result, we initially chose DU145 for studies of signal attenuation, since this cell line expresses undetectable levels of c-abl and c-arg. To understand the general role of Src kinase in prostate cancer cell biology, the expression and activation were profiled in CWR22Rv1, DU145, LAPC-4, LNCaP, and PC3, as well as immortalized prostate epithelial cells such as RWPE-1. Saracatinib (and similarly PP2) was found to inhibit the growth of all cell lines with various IC₅₀s. The IC₅₀ for growth inhibition is around 6 μ M for LNCaP, CWR22Rv1, and DU145, but more than 16 μ M for RWPE-1. These results suggest that Src activation has oncogenic effects in both androgen receptor-dependent and independent CaPs, broadening the application of Src inhibitor as a treatment option for this disease. Both proliferation and migration of CaP cells are inhibited by the treatment, with little evidence of apoptosis, based on the appearance of sub-G1 fraction and caspase activation assays. These inhibitions were accompanied by reduced activation of the ERK1/2 pathway and reduced expression of β -catenin, c-myc, and cyclin D1, consistent with their being the downstream targets of Src and the arrest of growth at the G0/G1 phase. Likewise, the activation of cell motility factor such as FAK, p130CAS, and paxillin activation were inhibited. Using an *in vivo* androgen-independent xenograft model we developed, Saracatinib is found to be highly effective in inhibiting metastasis and to a much lesser extent, tumor growth [62]. It also exhibits efficacy in inhibiting bone metastasis of prostate cancer [49]. Despite these benefits, Src inhibitors do not seem to induce extensive killings of tumor cells, which compromise its efficacy as an anti-cancer agent. This, however, is not unique, as most of the tyrosine kinase

inhibitors failed as a monotherapeutic agent. The key question then is how to enhance the killing effects of the Src inhibitor specifically and the tyrosine kinase inhibitors in general.

Src Tyrosine Kinase Inhibitor and Autophagy

To search for the potential cause of apoptosis-resistance in Src inhibitor-treated cells, we looked for prosurvival signals still active or even induced after treatment. The most consistent observation is the induction by Src inhibitors of a high level of autophagy or macroautophagy, which is generally viewed as a survival mechanism for cells in response to stress (nutritional, genotoxic, hypoxia, etc.) [47]; however, when deregulated, excessive autophagy leads to the postulated type II programmed cell death. Modulating autophagy has recently emerged as a possible means to benefit cancer therapy [95]. Autophagy or macroautophagy is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. It begins with the formation of a double-membrane vesicle, or autophagosome, around a targeted region of the cell, separating the contents from the rest of the cytoplasm [96]. The resultant vesicle then fuses with a lysosome where the contents are degraded. A series of genes (ATG, autophagy genes), highly conserved from yeast to mammals, have been implicated in autophagic processes. For instance, autophagosome is formed by Atg5 and Atg12 conjugation, which then recruits microtubule-associated protein 1 light chain 3 (LC3), a mammalian homologue of Atg8. LC3 is cleaved by autophagin (Atg4) to produce the active cytosolic form LC3-I (18 kDa), which is then further activated by Atg7, transferred to Atg3, and posttranslationally modified into the membrane-bounded active form, LC3-II. The conversion of LC3 isoforms has been used as a biochemical index in autophagosome formation, and the number of autophagosomes (presented as cytoplasmic puncta) provides a semiquantitative measure of autophagy induction. A standard way of measuring autophagosome is to incorporate a GFP-LC3 construct into the cells to be studied. Upon autophagosome formation, the number of green dots represented by GFP-LC3-II embedded in autophagosome membrane provides a measure of the beginning step of autophagy. The cellular pathways that control the autophagic process are not well understood. The PTEN/PI3-kinase (Class I)/Akt/mTOR/S6K pathway have been strongly implicated as a negative regulator of autophagy. It is thought that S6K which regulates protein translation restricted the translation of some of the Atgs. Since Src/FAK tyrosine kinase complex associates with the p85 subunit of PI3 kinase and activates PI3 kinase, Src activation is expected to suppress autophagy and its

inhibition to cause autophagy. Indeed, Saracatinib or PP2 both inhibit the activation of PI3 kinase downstream effectors, AKT, mTOR, and S6K [47]. Although the AKT-mTOR/S6K pathway is likely to contribute to the suppression of autophagy, it may not be the only one. Src kinase is known to enhance intracellular energy resource in favor of survival and growth. For instance, Src activates AKT and PI3 kinase, which are capable of transporting GLUT4 to the plasma membrane, resulting in glucose influx [97, 98]. Recently, Src activation is also seen to be responsible for thrombin-induced p38MAPK-mediated activation of glucose transporter [99]. Likewise, Src is known to activate HIF-1alpha via the degradation of VHL, an E3 Ub ligase for HIF-1alpha [100], and HIF-1alpha transcriptionally activates GLUT1, also resulting in a rich supply of glucose [40]. Src inhibition may thus result in glucose depletion, a condition known to induce autophagy. In addition, Src may also function to provide an amino acid balance for the anaerobic glycolysis pathway by increasing the higher level of glutamine via c-myc, a target gene of Src [101]. Src inhibition would thus lead to an energy imbalance, another condition known to induce autophagy. Thus, Src inhibition may engender a nutritional starvation condition, leading to autophagy. Finally, as discussed above, Src inhibition severely compromises AR activity, which is another potential cause for autophagy. Li et al. previously showed that androgen depletion which attenuates AR activity results in autophagy [102]. The exact mechanism as to how Src inhibitor induces autophagy, deserves further attention. Our results that Src inhibitor induces autophagy may not be unique to this system. Recently, imatinib, an inhibitor of Abl/Src has also been found to induce a significant level of autophagy, which may account for its lower efficiency as a monotherapeutic agent [103].

Autophagy Blockade Enhances the Killing Effects of Src Inhibitors

Having demonstrated that Src inhibitors induced profound autophagy in prostate cancer cells, we asked whether autophagy may contribute to the apoptosis-resistance observed for tyrosine kinase inhibitors therapy. To test this thesis, we looked for inhibitors of the autophagy process and assessed its biological effects when used in combination with Saracatinib. The idea behind these experiments is that if autophagy is the barrier to apoptosis, autophagy inhibitor would enhance the Saracatinib-induced killing of prostate cancer cells. We chose both a genetic inhibitor, shRNA against Beclin, a critical ingredient of autophagy and a pharmacological inhibitor, chloroquine (CQ) for this study. Chloroquine is a lysotropic agent which disrupts

lysosomal functions by blocking autophagosome fusion and inability to degrade autophagosome and its cargo. Chloroquine is a well-tolerated, clinically approved antimalarial agent, increasing its prospect as an adjunctive therapy. Using shRNA against Beclin (Atg8) and CQ, we showed unambiguously that inhibition of autophagy dramatically enhanced the apoptosis-inducing effect of Src tyrosine kinase inhibitor. In vivo testing using PC3 xenograft confirmed a synergistic effect of Saracatinib and CQ on the inhibition of tumor growth and enhanced apoptosis in the tumor tissues. This finding suggests that autophagy modulator may serve as an effective adjunctive therapeutic agent to complement tyrosine kinase inhibitor [47].

Conclusion

Since the identification of the first tyrosine kinase profile in prostate cancer in 1996, numerous studies have implicated aberrant expression and activation of tyrosine kinases in the progression of prostate cancer, especially during the transition to a castration-resistant state. A common signal integrator of many of these tyrosine kinases is the Src tyrosine kinase complex, including Src, FAK, and Etk/BMX tyrosine kinases, which control growth, migration, survival, and tumor resistance. They are also involved in the phosphorylation and aberrant activation of androgen receptor in a ligand-independent manner. Targeting Src tyrosine kinase complex, thus represents a potential treatment option for castration-resistant prostate cancer, in conjunction with hormone therapy. As amply evident from recent exciting discoveries, tyrosine kinases are promising targets for cancer therapeutics and are on the frontline of targeted therapy; however, as monotherapy, tyrosine kinase inhibitors have not been found to be particularly effective. One possible reason is their propensity of inducing autophagy, which confers resistance to apoptosis. Several clinically approved drugs which block autophagy were shown to enhance cell killing, when used in combination with tyrosine kinase inhibitors. We look forward to the future development of more specific tyrosine kinase inhibitors as well as autophagy modulators and their applications to castration-resistant prostate cancers.

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