C-Src and EGFR Inhibition

Subjects: Oncology

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The proto-oncogene c-Src is a non-receptor tyrosine kinase playing a key role in many cellular pathways, including cell survival, migration and proliferation. c-Src de-regulation has been observed in several cancer types, making it an appealing target for drug discovery efforts. Recent evidence emphasizes its crucial role not only in promoting oncogenic traits, but also in the acquisition and maintenance of cancer resistance to various chemotherapeutic or molecular target drugs. c-Src modulates epidermal growth factor receptor (EGFR) activation and amplifies its downstream oncogenic signals.

Keywords: Src kinase family ; c-Src inhibitors ; EGFR ; EGFR-TKIs ; drug resistance

1. Introduction

SRC is a representative member of nine-gene family of non-receptor tyrosine kinases (Src Family Kinases, SFKs) playing a key role in the modulation of several signaling pathways. As a cytoplasmic protein c-Src regulates cellular responses to external stimuli through interaction with multiple proteins [1] ocal-adhesion proteins, adaptor proteins and transcription factors are included in its complex network of interactions, which support c-Src role in the direct and indirect modulation of mitogenic signaling, cytoskeletal organization, angiogenesis, motility, cell cycle progression, survival and proliferation [2][3].

Structurally, c-Src consists of seven functional domains: 1) an N-terminal myristoylation sequence attached to a Src homology 4 (SH4) domain required for cellular membrane localization; 2) a unique domain, which provides unique functions and specificity to each SFK member, followed by 3) SH3 and 4) SH2 domains, important for protein–protein interaction and for the binding of phosphorylated tyrosine sites, respectively; 5) a linker region, involved in intramolecular binding to the SH3 domain; 6) a protein tyrosine-kinase region, also known as SH1 domain, representing the catalytic domain bearing the auto-phosphorylation site Tyrosine (Y) 419 and 7) a short C-terminal regulatory segment carrying an auto-inhibitory phosphorylation site, the Y530 [4][5]. Conformational changes in the molecular structure determine the activation and status of the c-Src protein. The phosphorylation of the C-terminal Y530 blocks the protein in a closed, inactive conformation, which masks the kinase domain, making it inaccessible to substrate proteins. This inhibitory phosphorylation at c-Src C-terminal region is fine-tuned by c-Terminal Src kinase (CSK). Conversely, c-Src activation occurs with the de-phosphorylation of the C-terminal site (i.e., by the protein tyrosine phosphatase 1B, PTP1B), which dissociates it from the SH2 domain, inducing c-Src in an open, active state. However, to fully obtain c-Src activation the Y419 auto-phosphorylation is required [6][7][8]. c-Src activation can be promoted also by CRK-associated substrate (CAS) and focal adhesion kinase (FAK) bindings to the c-Src SH2 and SH3 domains, leading in turn to the disruption of the inhibitory intramolecular interactions and allowing c-Src activation [9][10]. Likewise, activated growth-factor receptors can associate with the c-Src SH2 domain, prompting c-Src activation by a similar mechanism [11]. The intricate regulation of this pleiotropic protein increases the risk to alter c-Src levels and activity, events extensively studied in cancer. Although a truncated c-Src C-terminal region that exhibits constitutive catalytic activity was detected in small subsets of colon and endometrial cancers [12][13], the genetic mutations of c-Src represent a rare event in cancer development and progression. More commonly, increased expression and/or activity of wild type c-Src protein have been described in a number of human cancers, including lung, skin, colon, pancreatic, prostate, breast, ovarian, endometrial, and head and neck malignancies [14][15]. The effects of c-Src alteration in cancer tissues vary from motility and invasion to proliferation, apoptosis and angiogenesis [16][17], playing a critical role in the development of malignant phenotype. The c-Src activity can be modulated by protein kinases/phosphatases (i.e., the above mentioned CSK and PTP1B) regulating c-Src switch from inactive to active state, or can be boosted by alterations of its upstream or downstream partners. c-Src, indeed, interacts with several protein-tyrosine kinase receptors at the plasma membrane, producing a bi-directional flow of information: receptors affect c-Src activity and vice versa. Several studies defined the increased activity of c-Src as a result of the altered interactions with ligand-activated receptor tyrosine kinases, such as epidermal growth factor receptor

(EGFR) [18][19][20], platelet derived growth factor receptor (PDGFR) [21], fibroblast growth factor receptor (FGFR14–) [22], colony stimulating factor-1 receptor (CSF-1R) [23][24] , human epidermal growth factor 2 (HER2/neu) [19] and hepatocyte growth factor receptor (c-Met) [25].

Here, we describe the physical and functional interaction between c-Src and EGFR—both ubiquitously expressed and often over-expressed and/or mutated (i.e., EGFR) in cancer cells—and their involvement in cancer as well as in drug resistance. Particularly, we elucidate the role of c-Src in resistance to EGFR inhibitors and the current pre-clinical and clinical progresses of combined therapy of c-Src and EGFR inhibitors.

2. c-Src and EGFR Physical and Functional Interaction

c-Src and EGFR have been shown to enhance pro-mitogenic signals upon epidermal growth factor (EGF) stimuli [26]. c-Src and activated EGFR cooperate to induce cell transformation and cancer development [27]. c-Src has been reported to bind to EGFR and phosphorylate tyrosine residues on its C-terminal domain, resulting in a variety of downstream effects. Particularly, c-Src-mediated EGFR activation involves tyrosine residues different from the auto-phosphorylation sites, including Y891, Y920, Y1101 and, most notably, Y845 [28][29][30][31]. Y845 is located within the catalytic domain of EGFR in a conserved position among all receptors and non-receptor tyrosine kinases that generally undergoes auto-phosphorylation to induce receptor catalytic activity. Nevertheless, Y845 phosphorylation of EGFR is mediated by c-Src and not by EGFR itself. Sato and colleagues hypothesized that EGF binding to EGFR triggers a conformational change in its kinase domain that allows Y845 to be accessible for c-Src recruitment, hence providing a docking site for physical interaction with either SH2 domain of c-Src itself and with other signaling molecules [32].

c-Src activation induced by EGFR ligands mediates the binding of phosphatidylinositol 3-kinase (PI3K) to EGFR, leading to AKT phosphorylation and, in turn, induction of survival and migration signaling pathways [33][34][35][36][37]. Additionally, although c-Src needs the contribution of other molecules to modulate the proliferative mitogen-activated protein kinase (MAPK) pathway, it has been demonstrated that it can enhance EGFR ligands-induced extracellular signal-regulated protein kinase 1/2 (ERK1/2) activation, in particular through phospholipase C γ-1 (PLCγ-1) or Raf-1 [38][39]. The significant role of c-Src in MAPK activation was further demonstrated using Src family inhibitor PP2, which partially prevented EGF-induced ERK1/2 activation [40].

These data suggested that pY845 requires several mediators for accomplishing the synergism between EGFR and c-Src, including for instance signal transducer and activator of transcription 5B (STAT5b), a transcription factor involved in mitogenesis [41], and cytochrome c oxidase subunit II (Cox II) [42], a mitochondrially encoded protein involved in oxidative phosphorylation and in cytochrome c release during apoptosis. In particular, pY845 is involved in both STAT5b regulation, responsible of EGF-induced cell proliferation and DNA synthesis [43], and in enhancing cell survival through Cox II.

c-Src is also engaged in EGFR activation by responding to extracellular stimuli other than EGFR ligands. c-Src, indeed, is required for EGFR trans-activation induced by multiple extracellular factors, such as G protein-coupled receptor ligands, steroids, cytokines, extracellular matrix proteins, ionizing radiation, ultraviolet light, and certain ions [44][45][46]. Moro and colleagues, for instance, elucidated the c-Src requirement for EGFR trans-activation following its association with integrins [47]. c-Src mediates the cross-talk between EGFR and other non-related membrane receptors and regulates the relative downstream effects through Y845 phosphorylation. It has been demonstrated that c-Src induces EGFR phosphorylation following G protein-coupled receptors (GPCR) activation. Src-specific inhibitors or the expression of mutated EGFR-Y845 reduced lysophosphatidic acid (LPA)-induced DNA synthesis [45], indicating that c-Src-induced phosphorylation of Y845 is crucial for the mitogenic response to both EGFR and GPCR (the LPA-receptor) [45]. c-Src modulation is also involved in the trans-activation of EGFR by endothelin receptor [48], transforming growth factor receptor (TGFR) [49], phorbol myristate acetate (PMA) receptor [50], Insulin receptor [51] and b2 adrenergic G-protein coupled receptor [52]. Finally, c-Src exhibited a key role in the internalization and degradation of EGFR. To promote internalization, c-Src modulates phosphorylation of clathrin and dynamin, involved in the formation of the coated pits embracing ligand-bound receptors and in the separation of the endocytic vesicles from the plasma membrane, respectively [53][54]. EGFR degradation, instead, is triggered by E3 ubiquitin-protein ligase Cbl (Cbl) ubiquitination, which promotes receptor endocytosis and degradation [55]. c-Src affects this process by promoting the ubiquitination and proteasomal degradation of Cbl, which in turn postpones EGFR degradation and down-regulation, thus inducing EGFR recycling to the plasma membrane and the recovery of its signaling [53].

3. c-Src and EGFR Activation and Cooperation in Cancer Onset and Maintenance

After the discovery of c-Src and EGFR cooperation in cellular processes, many researchers focused on the understanding of how this interaction and its downstream signaling can deregulate cellular functions, pushing malignant cell transformation. Since the first studies, it has been suggested that the synergism between c-Src and EGFR contributes to a more aggressive phenotype in diverse tumors. Maa and collaborators verified that concomitant over-expression of c-Src and EGFR in murine fibroblasts led to a higher tumorigenic phenotype compared to cells over-expressing either the EGFR and c-Src alone [56]. Both c-Src and EGFR have been found co-overexpressed in several types of tumor, including glioblastomas and carcinomas of the colon, breast, and lung [57][58][59]. In lung cancer, c-Src over-expression is observed in 50–80% of non-small cell lung cancer (NSCLC) patients and is related to poor clinical outcome, which has increased the interest in using c-Src kinase inhibitors as therapeutic cancer agents [60][61]. EGFR over-expression and mutations, as well, play a key role in the carcinogenesis of NSCLC and frequently occur. Interestingly, Sonnweber and colleagues have shown that in a cohort of stage I NSCLC patients, the phosphorylation of Y845 on EGFR was a valuable prognostic factor —more than the incidence of the EGFRvIII mutation [62]. Lin and colleagues demonstrated that digoxin, a cardiac glycoside suggested as chemo-therapeutic agent, induced decrease of c-Src, EGFR and STAT3 activation and expression and, consequently, impaired cancer cell proliferation, migration and invasion [63]. Additionally, Lai and colleagues identified rhodomycin A as a promising compound for inhibiting c-Src activity in NSCLC. It also led to the decrease of Src-associated proteins, including EGFR, STAT3, and FAK. Interestingly, the inhibition of Src-related signaling pathways—such as PI3K, c-Jun N-terminal kinases (JNK), Paxillin, and p130cas—significantly inhibited in vitro and in vivo tumorigenicity of NSCLC cells [64], confirming the crucial role of c-Src and EGFR in these tumors. Finally, focusing on the role of tumor microenvironment in c-Src/EGFR regulation, Interleukin 10 (IL10) has been proposed as a cooperative agent in the oncogenic progression of lung cancer by increasing phosphorylation levels of EGFR and c-Src in a dose-dependent manner. IL10 induced Janus chinasi 1 (JAK1)/STAT3 activation through the recruitment of pSrc to pIL10 receptor, resulting in the up-regulation of EGFR expression. The latter event led to an increased transcription and mRNA stability of IL10 by EGFR itself, producing a positive feedback for EGFR over-expression that triggered lung cancer tumorigenesis [65].

In breast cancer, c-Src is over-expressed in ~70% of cases and, in the majority of them, is co-overexpressed with at least one member of EGFR family , suggesting their cooperation in promoting breast cancer development. Dimri and colleagues demonstrated that the concomitant over-expression of both EGFR and c-Src, but not of EGFR or c-Src alone, markedly cooperate to enhance breast cancer cells oncogenic properties, causing hyper-proliferation, aberrant three-dimensional acinar structures, increased migration and invasion, and anchorage-independent cell growth [66]. In 2011, Irwin and colleagues reported that EGFR and c-Src co-localized into lipid rafts in triple negative breast cancer cells, and that this co-localization prompted cell sensitivity to simultaneous treatment with EGFR and c-Src inhibitors. The authors described PI3K also associated with lipid rafts and that the inhibition of c-Src activity decreased AKT phosphorylation, suggesting c-Src regulation of PI3K/AKT survival signals within lipid rafts [67].

The involvement of c-Src in HER2-mediated cellular processes (such as anchorage-independent growth, motility, and survival) has been largely elucidated in breast cancer [68][69]. The over-expression of HER2 in mammary epithelial cells [70] or in HER2-expressing transgenic mouse systems [71] has been correlated with the activation of c-Src kinase, suggesting a functional interaction between c-Src and HER2 in breast cancer pathogenesis. Moreover, HER2 up-regulates c-Src protein levels by increasing its protein synthesis, activating the AKT/mTOR/4E-BP1 pathway, and stability inhibiting calpain-mediated Src protein degradation. The over-expression of c-Src, in turn, markedly enhances the ability of HER2 to promote invading and metastatic traits [72]. Successively, the pivotal role of c-Src upstream HER2 has been demonstrated by Ishizawar and colleagues, who found that the over-expression of c-Src enhances the formation and levels of HER2/HER3 heterocomplex, resulting in increased downstream signaling and biological functions (i.e., cellular motility and anchorage-independent growth) [73].

4. Current Status of c-Src Inhibitors and Their Effects in Drug Resistance

Although genetic mutations in SRC gene are not driver events in tumorigenesis, several studies documented its de-regulation in diverse cancer types, inducing alteration of many signaling pathways. For this reason, the development of selective c-Src inhibitors has become an attractive research topic [74].

Nowadays, five c-Src ATP competitive multikinase inhibitors are FDA-approved for their use in several cancer types or currently tested in clinical trials, most for the treatment of hematological malignancies, such as chronic myelogenous leukemia or acute lymphoblastic leukemia. A detailed summary of the main targets and clinical applications of c-Src

inhibitors—bosutinib, dasatinib, ponatinib, vandetanib, and saracatinib—is reported in Table 1. In this review, we mainly describe the synergistic effect of c-Src inhibitors in combination with EGFR inhibitors and/or with chemotherapeutic drugs on solid tumors, so that we have focused our attention on dasatinib, saracatinib and bosutinib, typically tested in lung, pancreatic, colorectal and breast cancer (Table 1).

Table 1. c-Src ATP competitive inhibitors.

Drugs	Molecular Targets	Clinical Applications
Bosutinib	BCR-Abl, c-Src, Lyn, Hck, Kit, PDGFR	CML, ALL + clinical trials for breast cancer, glioblastoma
Dasatinib	BCR-Abl, SFKs, Arg, c-KIT, EGFR, PDGFR, DDR1, DDR2, c-FMS, ephrin receptors, TEK, BTK, EphA2	CML + clinical trials for ALL, breast, colorectal, endometrial, head and neck, ovarian, and small cell lung cancers, glioblastoma, melanoma, and NSCLC
Ponatinib	BCR-Abl, SFKs, VEGFR, PDGFR, FGFR, Eph, Kit, RET, Tie2, Flt3	CML, ALL + clinical trials for endometrial, GIST, hepatic biliary, small cell lung, and thyroid cancers
Vandetanib	RET, SFKs, EGFR, VEGFRs, Brk, Tie2, EphR	medullary thyroid carcinoma
Saracatinib (**AZD0530**)	c-Src, BCR-Abl	Clinical trial for SCLC, NSCLC, colorectal, gastric, ovarian and metastatic osteosarcoma

CML = chronic myelogenous leukemia; ALL = acute lymphoblastic leukemia; GIST = gastrointestinal stromal tumor; SCLC = small cell lung cancer; NSCLC = non-small cell lung cancer.

Despite the anti-tumoral effects of c-Src inhibitors reported in pre-clinical studies [75][76][77][78] the controversial outcomes of recent clinical trials highlighted the need to identify novel predictive biomarkers of tumor response to c-Src inhibitors. The high expression of Estrogen Receptor (ERα) and HER2 were firstly reported as favorable indicators for the use of c-Src inhibitors in breast cancer cell lines [75][76]. Recently, it has been reported that triple negative breast cancer (TNBC) cells also showed sensitivity to c-Src inhibitors [79]. Lou and colleagues reported that c-Src inhibitors were able to prevent TNBC invasive ability by determining a significant decrease in vimentin expression. Thus, authors suggested vimentin as a predictive biomarker to stratify breast cancer patients in clinical trials testing c-Src inhibitors [79].

Beyond its well documented effect on tumor growth and invasion, c-Src has also a crucial role in the acquisition and maintenance of resistance to many chemotherapeutic drugs, thus encouraging the concurrent use of c-Src inhibitors in combination with different cytotoxic agents. In NSCLC, it was demonstrated that vinorelbine resistant cells showed a hyper-activation of focal adhesion pathways, including SFKs and Protein Kinase B (PKB or AKT), and that the treatment with SFKs inhibitor saracatinib increased tumor cell sensitivity to vinorelbine [80]. In colorectal cancer (CRC) dasatinib treatment is able to re-sensitize cells to oxaliplatin and fluoropyrimidines, that are among the most reliable therapies for both early and late stage CRCs. Perez and colleagues showed that high levels of phosphorylated Src on Tyr419 increased resistance to oxaliplatin, but not to 5-fluorouracil. Further, dasatinib rescued this effect and the combination with oxaliplatin inhibited tumor growth, in patient derived xenografts (PDXs) from human CRC liver metastasis [81]. Similarly, the highly potent pan-SFK inhibitor A-770041 and/or short hairpin RNAs (shRNAs) against SRC mRNA reduced c-Src expression in osteosarcoma cell lines and enhanced their sensitivity to doxorubicin or paclitaxel [82].

c-Src kinase has been shown to modulate the efficacy of linsitinib, an Insulin-like Growth Factor Receptor 1 (IGF-1R) inhibitor. Min and colleagues reported that in NSCLC cells expressing high levels of pSrc, linsitinib had a slight effect on c-Src, EGFR and AKT kinase activity, followed by a rapid Src-dependent EGFR activation. In contrast, low pSrc levels were

associated with higher sensitivity to linsitinib. The combined treatment of linsitinib with dasatinib successfully abrogated IGF-1R, AKT and c-Src activation and affected cell proliferation, anchorage independent colony formation and increased apoptosis in NSCLC cells and had anti-proliferative effects also *in vivo* [83].

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