Review

Molecular Therapeutics – Lessons from the Role of Src in Cellular Signalling

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Abstract. Src is the oldest and best studied protooncogene participating in normal cells in proliferation, maintenance of normal intercellular contacts and cell motility. Its discovery opened the way for fundamental discoveries on the cell cycle, cell growth and death, cell-cell signalling, cell morphology and motility, and cancer biology. Src is poorly transforming, consistent with a role as a normal cellular protooncogene that, when activated, might serve as an oncogene. Its activation promotes growth during the process of tumorigenesis by stimulating the proliferation of pre-cancerous cells and also regulates other activities such as adhesion and invasion during the later stages of tumour progression. Overexpression of the Src protein and the increase of its specific protein kinase activity have been observed in various human malignancies and linked to the development of cancer and tumour progression to distant metastases. These observations have led to the recent rediscovery of Src, a molecule that has been investigated during nearly a century and that shows promise as a new target for therapy of human cancer in the development of anticancer therapeutics. To determine the role of Src in human cancer, which is still not fully understood, molecular details of many pathways that intersect with Src are necessary to be uncovered. A detailed map of signalling pathways regulating signal transduction and signal integration induced by Src may identify location of different "checkpoints" for the therapeutic intervention of human diseases due to the altered activity of Src.

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All cellular signals that modulate or alter cell behaviour or function may be defined as signal transduction. To process the signals involved in extracellular, intracellular and intercellular communications, diverse signal transduction pathways are regulated in the cell, utilizing unique as well as overlapping sets of signalling molecules. The emerging map of the signalling pathways identifies a number of molecules that interact and interface with different signal transduction pathways to form a signalling network by integrating multiple receptors, signal transducers, signal amplifiers and second messengers. The observations that the multiple effector molecules can be activated by a single receptor and that the signals from different receptors can be integrated indicate that multiple signalling inputs are required for committing the cells to the critical pathways.

The signalling mechanisms that are present in the cell play a major role in all phases of its life. Cells respond to some types of signals by starting to proliferate, by initiating differentiation or by committing themselves to die. However, altered or asynchronous activation of some of these signals can commit cells to deregulations that may result in a different cell behaviour or function. Signalling needs to be precisely coordinated and integrated at all times; in particular, properly regulated differentiation signals are critical for preventing oncogenesis.

Protein phosphorylation is one of the most significant signal transduction mechanisms by which crucial intracellular processes are regulated. It is catalyzed by protein kinases that play key roles in regulating signal transduction in the context of multiple cellular processes and environments. They can be regulated by interacting proteins (activator proteins, inhibitor proteins), ligand binding, phosphorylation or autophosphorylation.

The protein kinase family is the second largest enzyme family and the fifth largest gene family in the human genome. There were identified more than 520 protein kinase genes in humans that correspond to about 1.7% of all human genes and 130 protein phosphatases,

indicating tight and reversible control of protein phosphorylation. The family of protein kinases includes 385 protein-serine/threonine kinases and more than 90 protein-tyrosine kinases. Of the 90 genes encoding tyrosine kinases, 58 encode transmembrane receptor protein-tyrosine kinases and 32 encode cytoplasmic non-receptor protein-tyrosine kinases distributed into 10 subfamilies (Blume-Jensen and Hunter, 2001).

Src protein

A critical role in transmitting the signals is played by the non-receptor tyrosine kinases belonging to the Src family of protein-tyrosine kinases. Src is the best characterized member of this large family of structurally related kinases that play important roles in cell differentiation, proliferation and survival, and in oncogenesis and apoptosis. Src is activated during the G_2/M transition and plays a role in cell adhesion, cell morphology and motility.

Src was discovered almost a century ago by the studies of Peyton Rous that led to the identification of the Rous sarcoma virus (RSV) transforming gene (v-*src*). Studies on RSV yielded some essentially important new and startling information: cellular transformation was caused by a single gene (*src*) that was found to be derived from a cellular gene (the protooncogene, c-*src*) and its protein product was a protein kinase catalyzing the phosphorylation of tyrosine. The term protooncogene was used to describe a new type of genes to distinguish these genes from the oncogenes and to emphasise that they are not themselves transforming unless mutated and/or overexpressed. The discovery that RSV contained a defined gene required for transformation opened up a new way of looking for genes that might be involved in cancer and led to the concept of cell transformation (Martin, 2004). The identification of the transforming gene of RSV as well as its protein product started an intensive research into the role of protooncogenes in cancer, which in 1989 earned M. J. Bishop and H. E. Varmus the Nobel Prize in Physiology or Medicine.

A great effort to understand the structure and function of the Src protein-tyrosine kinase was made in the end of the 20th century. It resulted in the solution of the structure of Src protein and in the understanding of its mode of regulation, Src substrates, Src-regulated signalling pathways and cellular functions, and finding that Src activity is elevated in many human epithelial cancers (Frame, 2002; Svoboda, 2000).

The Src protein is normally maintaned in an inactive state, but it can be activated transiently during cellular events such as mitosis, or constitutively by abnormal events such as mutation found in v-Src and in a variety of human malignancies such as colon, pancreatic, neural, ovarian, breast, lung and other common human cancers (Sovová et al., 1997; Irby et al., 1999; Bjorge et al., 2000; Summy and Gallick, 2003; Silva et al., 2004).

v-Src lacks the negative-regulatory C-terminal tail of human c-Src (Fig. 1A) and consequently shows high levels of activity and transforming ability (Penuel and Martin, 1999) and probably a different substrate specificity (Vojtěchová et al., 2004).

Src family of protein-tyrosine kinases

Studies on the Src protein came to the recognition that Src was a member of a large family of structurally related protein-tyrosine kinases, many of which were expressed predominantly in highly differentiated cell types. There are 11 members of the Src family kinases in humans. These include Blk, Brk, Fgr, Frc, Fyn, Hck, Lck, Lyn, Src, Srm, Yes; three of them (Src, Fyn, Yes) are expressed ubiquitously (Roskoski, 2004). Src is one that is most often implicated in human cancer.

While the origins of the Src family of protein-tyrosine kinases are nearly a century old, the understanding of their activity is recent.

The activity of Src family kinases is regulated in the cell by regulatory kinases and phosphatases that alter the state of phosphorylation of key tyrosine residues in their molecules and by protein-binding partners that stabilize the kinase in active or inactive conformation or localize the enzyme to specific subcellular or submembrane domains. The possible mechanisms through which the Src family can interact with different regulatory molecules and the different molecular motifs in Src protein that may assist in its interaction with other signalling proteins have been identified. However, although considerable structural information is available for the autoinhibited conformation of Src kinases, the molecular events involved in the activation of the Src family of tyrosine kinases and how these kinases assemble into active signalling complexes with substrates and regulators still remain to be fully resolved.

Members of the family exhibit a conserved domain organization which includes a myristoylated N-terminal segment (U), followed by SH3, SH2 domains, linker and tyrosine kinase domains and a short C-terminal tail (Fig. 1A). Src activity is regulated by intramolecular interactions controlled by tyrosine phosphorylation and by domains Src-SH2- and Src-SH3-mediated protein-protein interactions with sequences containing phophotyrosine and proline-rich motifs (Xu and Miller, 1996). Canonical mechanisms of phosphotyrosine recognition by the SH2 domain and proline-motif recognition by the SH3 domain was elucidated by crystallographic analysis (Fig. 1B), which revealed that these protein interaction motifs turn inward and lock the kinase in an inactive conformation via intramolecular interactions (Boggon and Eck, 2004).

Regulatory principles uncovered by the analysis of Src family kinases have proved to be surprisingly general among diverse modular signalling proteins and led to the development of the concept of modular protein interaction domains (Pawson, 2004).
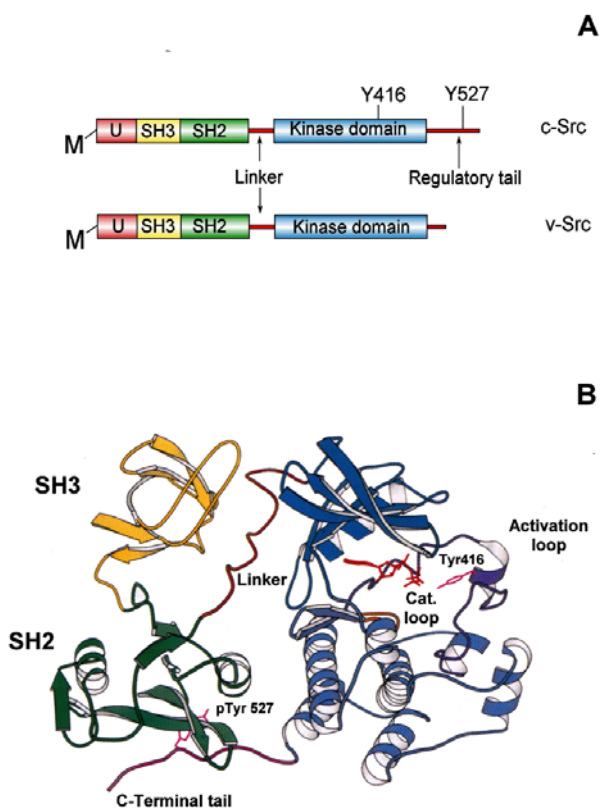
Fig. 1 (A). Domain structure of human c-Src and the protein product of v-*src* oncogene (v-Src). (B) Crystal structure of human c-Src (1FMK, Protein Data Bank)

Src signalling and receptors

Src and Src family members of protein-tyrosine kinases play key roles in regulating signal transduction by a diverse set of cell surface receptors such as several receptor protein-tyrosine kinases, integrin receptors, G-protein-coupled receptors, antigen-coupled receptors, cytokine receptors and steroid hormone receptors.

The ability of Src-family kinases to mediate signalling from cell surface receptors is a function of their catalytic activity, location and interactions. Many molecular mechanisms have evolved to couple receptors with the cytoplasmic intracellular signalling network, which regulates many fundamental cellular processes including cell growth and death, differentiation, cell shape, migration, cell morphology, cell cycle, cell-cell signalling, and survival.

Receptor protein-tyrosine kinases

The Src-family kinases are involved in signalling from many receptor tyrosine kinases, including plateled-derived growth factor (PDGF), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), insulin-like growth factor-1 receptor (IGF-1R), hepatocyte growth factor receptor (HGFR), colony-stimulating factor-1 receptor (CSF-1R), stem cell factor receptor (NCFR) and others. It was shown that Src-family kinases are activated by these receptor tyrosine kinases and, in turn, Src-family kinases activate receptor tyrosine kinases in a variety of cell types (Bromann et al., 2004; Scapoli et al., 2004).

The Src-family kinases can promote mitogenic signalling from growth factor receptors in multiple ways including the participation in pathways required for DNA synthesis and control of gene expression, as well as controlling the turnover of cell surface receptors, modulating actin cytoskeleton rearrangements and promoting cell motility and survival.

Members of Src-family kinases associate with receptor tyrosine kinases via an interaction of their Src homology (SH2) domains with phosphorylated tyrosine of the activated receptors. However, the activation of Src-family kinases by receptor tyrosine kinases may be more complex than simple recruitment. For example, the tyrosine phosphatase Shp2 has its role in promoting Src-family kinase activation of growth factor receptors (Hakak et al., 2000) and the activation of Src by EGFR in transfected cells requires the small GTPases Ras and Ral (Goi et al., 2000).

Src signals to a variety of downstream effectors of EGFR, including STAT transcription factors. STAT (signal transducer activators of transcription) can be activated by growth factor receptors, particularly EGFR, as well as by Src-family kinases, particularly Src (Kazansky and Rosen, 2001). In many cases a differential activation of the STATs is mediated by these tyrosine kinases. This difference may provide potential for unique actions of STATs in cancers driven by growth factor receptors and Src kinase activation. In these cancers, STATs may play an important role in the tumorigenesis process (Silva et al., 2004). At least, full oncogenic activity of v-Src was dependent on STATs and STAT activation was required for transformation by v-*src* (Odajima et al., 2000).

G-protein-coupled receptors (GPCR)

Another family of receptors interacting with Src-family kinases are G-protein-coupled receptors (GPCR). They serve as ligand-activated guanine nucleotide exchange factors (GEF) or a class of heterotrimeric guanine nucleotide-binding (G) proteins. Ligand binding catalyses the conformation-dependent exchange of GTP for GDP on the Gα subunit of the heterotrimeric Gαβγ protein. This, in turn, leads to the dissociation of the GTP-bound Gα subunit from the Gβγ subunit heterodimer, rendering it free to regulate the activity of enzymatic effectors. Not only growth factor receptors, GPCR also exert control over cellular growth, proliferation and differentiation by stimulating tyrosine phosphorylation cascades (Fan et al., 2001).

An unexpected role has been found recently for the Src kinases in GPCR signalling. Several mechanisms, from the direct association of Src-family kinases with GPCR or receptor-associated proteins to the transacti-

vation of receptor tyrosine kinases and focal adhesion complexes by G-protein-mediated signals, permit GPCR to activate Src-family kinases (Ma and Huang 2002; Shajahan et al., 2004; Waters et al., 2005). Conversely, the activity of Src kinases plays a central role in controlling GPCR trafficking and their effect on cell proliferation and cytoskeletal rearrangement. It seems that Src-family kinases and GPCR are involved in multilayer forms of cross-talk that influence most cellular processes (Luttrell and Luttrell, 2004).

Steroid receptors

Evidence has emerged showing that both steroid hormones and growth factors stimulate proliferation of steroid-dependent tumour cells. Steroid receptors are ligand-activated transcription factors which activate target gene transcription upon ligand binding (Lange, 2004). In response to steroid ligands, the steroid receptors can be functionally linked to activation of Src or receptor tyrosine kinases. Recently, steroid hormones have been shown to rapidly activate intracellular signalling cascades by their binding to cognate cytoplasmic or membrane-associated receptors, and in some cases, steroid receptors interacted directly with Src (Boonyaratanakornkit et al., 2001) and other cytoplasmic signalling molecules, such as Shc, PI-3K and p130 Cas (Src-associated substrate) (Sakai et al., 1994; Cabodi et al., 2004). Cross-talk between growth factors and steroids in both the cytoplasm and nucleus may have a profound impact on complex biological processes such as cell growth and may play a significant role in the treatment of steroid-dependent cancers (Shupnik, 2004).

Cytoskeletal-linked events and Src

In regulation of cell-cell adhesion Src employs the function of β-catenin, γ-catenin, p120-catenin as well as E-cadherin, which are potential substrates of Src. p120-catenin was originally identified as a Src substrate in v-Src-transformed cells. This protein is now known to regulate cell-cell adhesion through its interaction with the cytoplasmic tail of classical and type II cadherins. New evidence indicates that p120-catenin regulates cadherin turnover at the cell surface, thereby controlling the amount of cadherin available for cell-cell adhesion. p120-catenin is supposed to be a regulator of cadherin abundance and activity, participating in regulation of the balance between adhesive and motile cellular phenotypes (Reynolds and Roczniak-Ferguson, 2004). The activities of RhoA, Rac and Cdc 42 are also modulated by p120-catenin, suggesting that, along with other Src substrates, p120-catenin contributes to the regulation of cytoskeletal dynamics (Clapper et al., 2004).

Src kinases target a number of molecules that contribute to the regulation of integrin-mediated signalling pathways, including focal adhesion kinase (FAK), paxillin and p130 Cas, proteins that are important for integrin signalling and are the substrates of Src (Brábek et al., 2004). Moreover, Src and integrin signalling is altered in human cancer. Studies of the oncogenic potential of several Src-family kinases demonstrated their effects on cell morphology, cell adhesion and motility and clearly indicated a role for Src in the regulation of cytoskeletal-linked events and in cancer (McLean et al., 2000; Frame et al., 2002).

Oncogenesis of Src and cell growth

The activity of Src is normally tightly controlled and regulated. Perturbation of Src signalling either by mutations or other alterations results in deregulated protein kinase activity and malignant transformation. Src tyrosine kinase activity is elevated in several types of human cancer and this has been attributed to both enhanced Src expression and increased specific activity.

The ability of oncogenic forms of Src family kinases to induce cell transformation early suggested a role for Src and its family members in regulating cell growth. Deregulated cell growth occurs as a result of pertubed signal transduction. Consequently, cancers may arise due to a critical balance between the rate of cell-cycle progression (cell division) and cell growth (cell mass) on the one hand, and programmed cell death (apoptosis) on the other (Blume-Jensen and Hunter, 2001).

Signal transduction pathways are deregulated in various ways in v-src-transformed cells. For example, v-Src transformation leads to a decrease in the expression of type 1 but not type 2 protein kinase A (Clinton and Roskoski, 1984), and important distinctions exist between the signalling pathways that modulate protein synthesis at the level of translational control in the non-transformed cells compared to RSV-transformed cells (Vojtěchová et al., 2003). Translation has an established role in cell growth. Until recently, however, little was known about the alterations in mRNA translation in cancer and about their role in the development and progression of cancer (De Benedetti and Harris, 1999; Ruggero and Pandolfi, 2003; Holland et al., 2004).

One of the signal transduction pathways that led to translational control of gene expression is a signalling pathway in which the central regulatory protein is mTOR (mammalian target of rapamycin). A macrolide antibiotic, rapamycin, specifically inhibits TOR, which results in reduced cell growth, a reduced rate of cell cycle progression and a reduced rate of proliferation.

The mTOR-dependent signalling pathway is activated during malignant transformation and cancer progression in many human cancers. mTOR is emerging as a central controller of cell growth and proliferation, which is mediated by its downstream targets implicated in a selective translational control of gene expression (Carrera, 2004). These are the repressor proteins 4E-BPs that regulate the activity of the „cap-binding
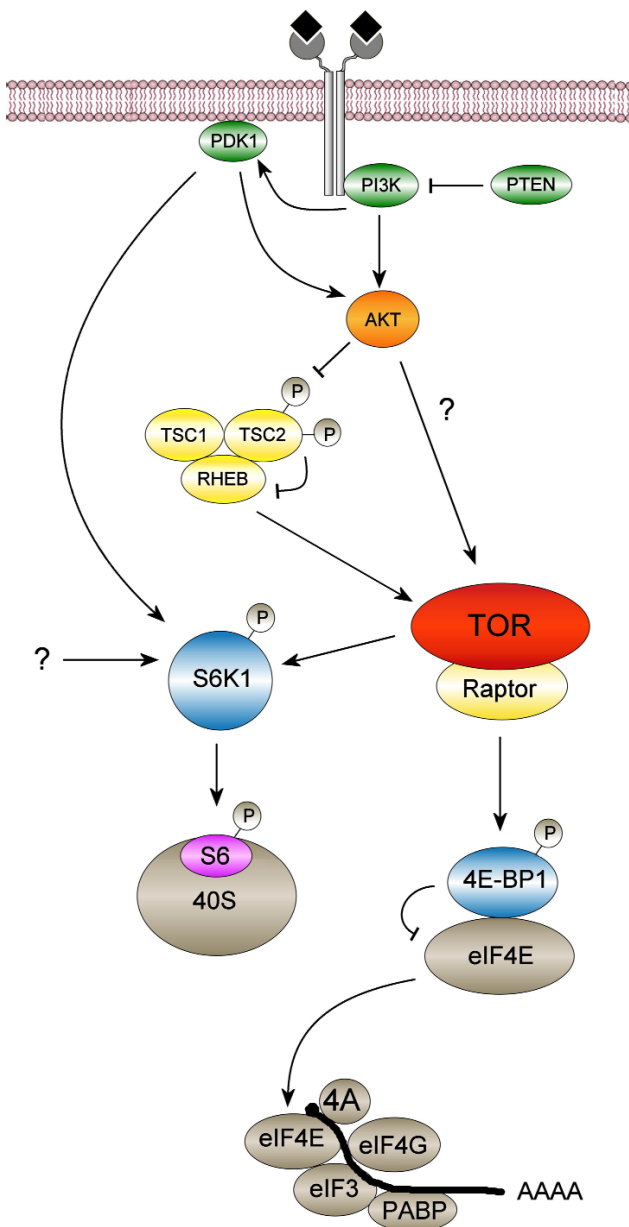
Fig. 2. Signalling pathway mTOR
Activation of mTOR is inhibited by the TSC protein complex (tuberous sclerosis protein 1 and tuberous sclerosis protein 2) and Rheb, which acts as a bridge between mTOR and TSC protein complex. Phosphorylation and thus inactivation of TSC by Akt (protein kinase B) releases mTOR from TSC inhibition to allow mTOR to regulate translation via phosphorylation of its target proteins, S6K 1 (ribosomal S6 protein kinase) and 4E-BP1 (initiation factor 4E-binding protein 1) that are recognized by Raptor. S6K 1 (p70 S6K) may also be activated by PDK (3-phosphoinositide-dependent protein kinase), which is regulated by PI-3K (phosphoinositide 3-kinase) controlled by phosphatase PTEN. Phosphorylation of ribosomal protein S6 catalyzed by S6K1 facilitates selective translation of a specific subset of 5'TOP mRNAs and phosphorylation of translational repressor 4E-BP1 releases initiation factor 4E (eIF4E) to allow the 5'cap-dependent initiation of translation.

protein", the initiation factor 4E (eIF4E), and S6 protein kinases (S6K) catalyzing phosphorylation of the ribosomal protein S6, a component of the 40S subunit of eukaryotic ribosomes (Dufner and Thomas, 1999) (Fig. 2).

In RSV-transformed cells, the enhanced expression and activity of the v-Src oncoprotein correlated with increased levels of protein synthesis. Phosphorylation of 4E-BP1, ribosomal protein S6 and its physiological protein kinase, p70 S6K, were found highly upregulated in RSV-transformed cells and inhibited by the mTOR specific inhibitor, rapamycin (Tuháčková et al., 1999). Cell transformation by v-*src* oncogene thus appears to be dependent on targets that control translation through the signalling pathway of mTOR. The activation of the mTOR signalling pathway by Src may thus promote selective expression of proteins that are involved in the malignant process induced by v-*src* transformation. However, it is not yet quite clear which signalling pathways are important for Src transformation.

The fundamental principle of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells. Indeed, increasing evidence supports mTOR as a selective target for cancer therapy with possible direct clinical applications in human diseases (Hidalgo and Rowinsky, 2000). Rapamycin synthetic analogues, such as CCL-779 and RAD001, are now under intensive examination as potent anti-tumour agents, currently being tested in Phase I-III oncology clinical trials for efficiency against a variety of human tumours, as single agents and in combination with chemotherapy.

Src protein as a therapeutic target

Src has just recently become a target for drug therapy. Although Src has been linked for the development and progression of cancer for many years (Irby et al., 1999; Bjorge et al., 2000; Frame 2002; Windham et al., 2002; Summy and Gallick, 2003; Silva 2004), there has recently been renewed interest in this protein as a novel therapeutic target for treatment of cancer (Yeatman, 2004). Src minimal deletion phenotype and the ubiquitous overexpression of Src in cancer has indicated that Src inhibition, which seems to be growth-inhibitory, might not have significant toxicity.

Whereas several inhibitors of Src have been available for *in vitro* experimentation to modulate Src kinase activity and function (PP2, SU11333, CGP77675), compounds that are highly specific for Src and are stable *in vivo* have been developed only recently. Drug discovery efforts have led to the development of several Src inhibitors for potential use as anticancer therapeutics for the first time in studies of this molecule in human cancer. They are now entering early Phase I oncology clinical trials or are in pre-clinical trials with efforts to obtain Src inhibitory compounds that target the Src kinase domain (SKI-606, SU6656, AP-23464 and AP-23451).

Pharmacological inhibition of Src activity by tyrosine kinase inhibitor PD162531 resulted in reduced rate of cell motility in epithelial cells that suggests effectiveness of Src kinase inhibitors in the treatment of potentially invasive tumors (Owens et al., 2000).

However, Src inhibition by targeting the kinase domain might only be partially effective. Src-neutralizing antibodies and inhibitor SU6656 also inhibits receptor-stimulated DNA synthesis (Roche et al., 1995; Blake et al., 2000). Inhibitors of the Src-family kinases, PP1 and PP2, are also potent inhibitors of a number of receptor tyrosine kinases and PP2 was shown to block the tyrosine phosphorylation of Raf-1 and induced the Ras-independent activation of Raf-1 kinase (Lee et al., 2004).

The effects of Src inhibitors on the metastatic growth and survival of cancers driven by EGFR and related growth factor receptors may be significant in cancers that also exhibit elevated expression of Src. In many human cancers the increased expression and activity of Src proteins kinases and Src in particular, is accompanied with the overexpression of EGFR and related growth factor receptors. Selective EGFR tyrosine kinase inhibitor ZD1839 (gefitinib, or Iressa), an inhibitor used for EGFR gene-targeted therapy (Gambacorti-Passerini, 2004), which is in late phase trials for advanced small-cell lung cancer, probably might be used in combination with Src inhibitors. Recent data indicate that targeting Src and FAK simultaneously may be very effective in promoting apoptosis of colon cancer cells (Golubovskaya et al., 2003), which promises a potential for combined therapy strategies.

An excellent tool for studies of the role of Src activation in multistage carcinogenesis may represent transgenic mice inducibly expressing an activated form of the human Src (Matsumoto et al., 2004).

Despite the progress that has been made in targeting Src for cancer treatment, much remains to be learned about Src contribution to cancer initiation, progression, and metastasis. What are the molecular mechanisms that are implicated in perturbation of signal transduction and signal integration by which Src deregulates critical cellular processes and how these mechanisms participate in cancer development *in vivo*?

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