CHAPTER 2

Focal Adhesion Kinase (FAK) inhibition as a potential strategy for anticancer therapies

Running title: FAK: an anticancer drug target

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Published in *Drugs of the Future*, August 2009

ABSTRACT

Focal adhesion kinase (FAK) is a multidomain non-receptor tyrosine kinase that mediates growth factor and adhesion-derived cell signaling. FAK plays crucial roles in cell proliferation, survival, motility and invasion, hallmarks of cancer cells. Overexpression of FAK has been observed in diverse cancer types and is used as a marker for invasion and metastasis. Furthermore, in vivo animal studies demonstrate an involvement of FAK in tumor development and malignancy. Therefore, FAK is a potential target for drug discovery for anticancer therapies. In this review, we will first present FAK and its relation to cancer with the focus on target validation of FAK. Second the approaches to inhibit FAK as a potential drug target for therapeutic intervention in cancer treatment will be discussed.

1. Introduction

1.1 Role of FAK

Cellular interactions with extracellular matrix and growth factors play essential roles in tumor initiation, progression and metastasis. Focal adhesion kinase (FAK) is a 125kDa multidomain non-receptor tyrosine kinase that localizes mainly in the cytoplasm of cells. Upon cell adhesion on diverse extra-cellular-matrices and/or activation by growth factors, FAK is recruited to focal adhesions (FA), the closest contacts between the cell and the extra-cellular matrix, and mediates the focal adhesion signaling. The complex structure of FAK results in a broad range of protein-protein interactions with other tyrosine kinases, cytoskeletal and adaptor proteins that are part of the so-called adhesome[1]. FAK is shown to play important roles in tumor progression and metastasis through its regulation of cancer cell migration, invasion, anchorage-dependent cell proliferation and survival. Recently numerous *in vivo* studies demonstrate the role of FAK in tumor initiation and progression as well. In agreement with those experimental data, FAK is linked to human cancer mainly due to its overexpression and activation in a number of human tumors. Altogether these studies suggest that FAK is a potential target for drug discovery.

1.2 FAK structure and its regulation

FAK consists of several domains as shown in Fig. 1: The FERM domain (band 4.1-Ezrin-Radixin-Moesin homology) at the N-terminus negatively regulates the catalytic activity of FAK[2]. FERM interacts with integrins and growth factor receptors and through this domain, FAK also binds Arp2/3 complex to control actin assembly[3]. In the catalytic kinase domain, autophosphorylation at FAK Tyr397 residue recruits Src at focal adhesion site. Further, Src phosphorylates FAK at Tyr576 and Tyr577 which results in conformational change that enhances the catalytic kinase activity and hereby other residues and substrates are phosphorylated. PTyr861 increases the binding affinity of p130Cas to the proline-rich regions (PRRs) in FAK c-terminus and is crucial to sense mechanical force[4] and H-ras induced transformation[5]. The focal adhesion targeting (FAT) domain at the c-terminal region is responsible for FAK localization to FA[6] and spatially interacts with paxillin and talin. FAK PRR binds Src-homology-3 (SH3) domain-containing proteins such as p130Cas, the GTPase regulator associated with FAK (GRAF) and the Arf-GTPase-activating protein ASAP1. Phosphorylation of Tyr925 residue in the FAT domain promotes Grb2 binding to FAK which activates the MAPK pathway through FAK-Grb2-Ras-MEK1-ERK2. PTyr925 is also responsible for the cell survival function of FAK and mediates a MAPK-associated angiogenic switch during tumor progression[7,8]. PTyr407 has been recently reported to negatively regulate kinase activity and cell migration/invasion[9,10]. Most of tyrosine

phosphorylated residues of FAK are well studied while the role of serine phosphorylated residues (Ser722, Ser846, Ser910) still are poorly understood[11,12]. Recently, it has been shown that phosphorylation on Ser732 in endothelial cells plays a role in the regulation of centrosome during mitosis which may contribute to cell proliferation and angiogenesis[13]. Ser843 is known to be phosphorylated when FAs disassemble and cells detach from substratum probably via inhibition of PTyr397[14].

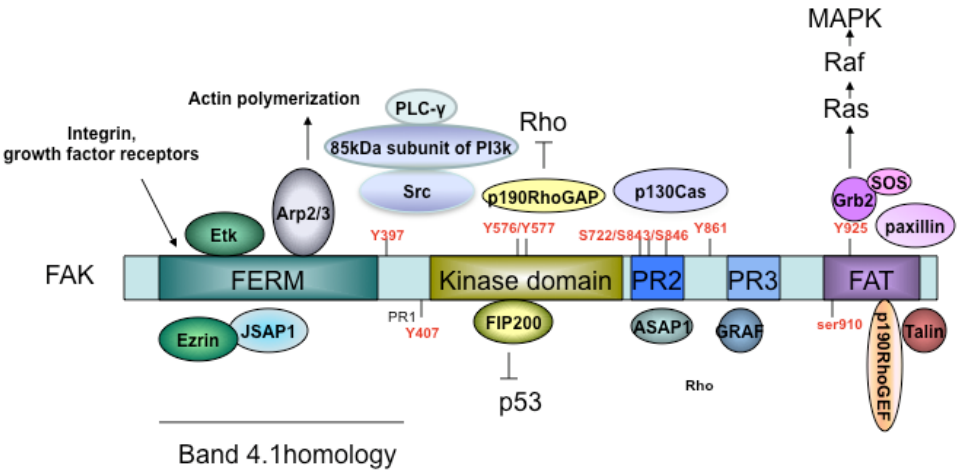

Figure 1: Focal adhesion kinase structural features and binding partners. The kinase domain of Focal adhesion kinase is flanked by the N-terminus that harbours the FERM domain, and by the C-terminus that consists, in addition to proline rich domains, of the FAT domain. The N-terminal domain has the Y397 autophosphorylation site which is also the target site for the different small molecule inhibitors. The kinase domain has the Y576/577 tyrosines, important for the catalytic activity of FAK. The C-terminal part of FAK has Y861 and Y925 tyrosines. Different proteins bind to these domains and are involved in cell proliferation, motility and survival signaling.

1.3 FAK in cellular processes

1.3.1 Processes related to tumor formation

FAK contribute to tumorigenesis through its promotion of cell survival and/or proliferation, hallmarks of cancer cells. FAK mediates survival signaling via for instance the PKB pro-survival pathway. FAK phosphorylation (PTyr397) is involved in doxorubicin-induced cell apoptosis in a Bcl-2 and caspase independent way. Indeed, inducible FRNK (FAK Related Non-Kinase) expression sensitizes cells to doxorubicin-induced apoptosis and inhibits doxorubicin-induced PKB activation[15]. FAK also regulates cell proliferation via up regulation of some cyclins, downstream of PKC, PI3K/AKT, MAPK/ERK pathways[16-18].The FERM domain

controls FAK function[19]. The deletion of FERM domain increases FAK phosphorylation and activity and affects cell cycle progression in CHO cells[2]. Besides its roles in focal adhesions, FAK is also playing a scaffolding role in the nucleus of the cells under cellular stress conditions. FAK facilitates p53 degradation via its FERM domain mediated nuclear localization and promotes cell proliferation and survival[19-21].

1.3.2 Processes related to tumor progression and metastasis

FAK controls focal adhesion assembly/disassembly at the leading edge of lamellipodia and disassembly at the rear of migrating cells. The role of FAK in cell motility is well documented (see reviews[6,12,22,23]). FAK interacts not only with integrins but also with growth factor receptors. EGFR as well as ErbB2 receptor signaling, also prognostic markers for cancer progression regulate FA turnover, cell migration and invasion through the Src-FAK pathway[24,25]. Cellular traction forces cause dynamic conformational changes in the FERM domain that are shown to be involved in cell spreading and motility[26]. A single residue Lys38 at the subdomain F1 is important for the kinase-inhibitory effect of FERM and the mutant K38A increases FAK phosphorylation level, cell cycle entry and cell migration[27]. The F2 subdomain of the FAK FERM domain and phosphorylation of PTyr1349 at c-Met are critical for c-Met binding and this interaction fully activates FAK activity and HGF-induced c-Met mediated cell invasion[28]. FRNK (FAK related non-kinase) lacks kinase activity and has competitively inhibitory effect on FAK at focal contacts. FRNK expression abolishes EGF or v-src–induced activation of downstream molecules (ERK, JNK, etc) and cell motility and prevents cell invasion via inhibiting MMPs secretion[29,30]. Inducible ectopic expression of FRNK sensitizes human embryonic kidney cell line to 5- fluorouracil and decreases haptotactic mobility[31]. Also, disruption of FAK with FRNK decreases cell attachment, motility and invasion in head and neck squamous cell carcinomas via down regulation of MMP-2 expression[32]. Introduction of the FAT domain which also competes the binding of endogenous FAK at focal adhesion sites, has also been reported to sufficiently inhibit cell invasion and sensitize cells to apoptotic stimuli[33]. The aggressive phenotype of prostate cancer cells depends on FAK expression which is regulated by ERK signaling[34]. In addition, FAK is also involved in tumor invasion and angiogenesis via the regulation of matrix metalloproteinase (MMPs) and vascular endothelial growth factor (VEGF) expression[8,29].

2. FAK and cancer

2.1 FAK increased expression in human cancers

In agreement with the experimental data, overexpression of FAK have been reported in a wide range of cancers (reviewed in[20,35-37]), such as breast[38-42], head and neck, cervical[38], colon[43,44], thyroid[45], prostate[46], liver[47,47,48], skin, lung, bone,

melanoma[49] and ovarian[50,51]. FAK apparently plays a distinct role depending on the tumor type and development stage. Similar observations have been obtained in cell-lines derived from tumor and normal tissue[52,53]. Altogether these studies show that FAK overexpression plays a role in tumor formation and metastasis and could be used as a prognostic marker[38,42,45,54,55].

2.2 Role of FAK in tumor development

Many recent studies using both xenograft mouse models and conditional KO mice have provided evidence that FAK signaling pathways can stimulate tumor initiation as well as tumor progression and metastasis through their regulation of cell migration, invasion, epithelial to mesenchymal transition and angiogenesis.

2.2.1 FAK in promoting tumor initiation

FAK knockout (KO) animals are lethal so to overcome the embryonic lethality, conditional tissue-specific FAK deletion *in vivo* have been developed with Cre recombinase (Cre) /loxP strategies, including myosin light chain 2a(MLC2v)-Cre/FAK^{flox} [56], Cre-ER(estrogen receptor)/ FAK^{flox} [15,57,58], mouse mammary tumor virus (MMTV)-Cre/ FAK^{flox} [59]. The first experimental proof using conditional FAK deletion in the epidermis demonstrated for the first time the role of FAK in skin tumorogenesis[60]. The intercross of FAK$^{flox/flox}$ mice with MMTV-Cre mice resulted in the deletion of FAK in mammary epithelial cells which disturbed the mammary gland development[61]. Also in our hands, complete deletion of FAK in both luminal and myoepithelial mammary epithelial cells disrupted mammary gland formation (van Miltenburg, *in press*). Using the polyomavirus middle T (PyVmT) transgenic breast cancer mouse model, conditional floxed *fak* was introduced. In the MMTV-Cre/PyVmT mice, the mammary epithelial-specific disruption of FAK altered the FAK– related signal cascades and retarded tumor initiation and progression as well as lung metastasis formation[62]. FAK deletion also reduced the population of mammary cancer stem/progenitor cell[63] and disrupted the transition of premalignant hyperplasias to carcinomas and subsequent metastasis[59]. FIP200 (FAK family-interacting protein of 200kDa, also named as retinoblastoma 1 (RB1-inducible coiled-coil 1) is identified as an inhibitor of FAK and Pyk2 by interaction with their kinase domains[64,65]. FIP200 inhibits the FAK/Pyk2 related cellular functions and interacts with RB1 and p53[66]. Large truncation deletion of the FIP200 gene has been observed in 20% of primary breast cancers and this indicates a possible role of FIP200 as a tumor suppressor[67].

2.2.2 FAK in promoting tumor progression and metastasis

Several studies in xenograft mouse models provide evidence that FAK is required in both tumor progression and metastasis. Overexpression of FAK in human malignant astrocytoma cells contributed to an increase in tumor volume due to enhanced cell proliferation[68]. Stable expression of FRNK results in a reduction of experimental lung metastases after tail vein injection of v-Src transformed NIH-3T3 fibroblasts[30] or B16-F10 mouse melanoma cells[69]. Recently, our group demonstrated that FRNK sensitizes rat breast cancer cell MTLn3 to doxorubicin *in vitro* and *in vivo* via inhibition of doxorubicin-induced PKB activation and down regulation of Fra-1 (a member of the activator protein-1 complex) ([15], *manuscript submitted*). Inducible expression of FRNK inhibits cell spreading and migration *in vitro* as well as primary tumor formation and the early phase of metastasis, but not the outgrowth of macro-metastasis[70]. In agreement with these results, by using FAK null or knockdown cells, others confirmed that the catalytic activity of FAK is required for metastatic breast cancer progression[8,71,72] and that FAK signaling is critical for ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion[73] and for Ras- and PI3K-dependent breast tumorigenesis in mice[74]. Pharmacological inhibition of FAK in wild type mice suppressed angiogenesis a key element in tumor progression and metastasis[58].

3. Strategies for FAK inhibition in cancer therapy

In vitro and *in vivo* studies as well as clinical studies support the fact that FAK is a potential target for cancer therapy. Currently, inhibition of FAK signaling is under investigation for its beneficial effects in cancer treatment. FAK signaling can be disturbed by affecting in practice either FAK expression or FAK activation.

3.1 Agents inhibiting FAK expression.

Interfering RNAs

FAK short interfering RNA (siRNA) shows high efficacy of gene expression downregulation in *in vitro* studies[75,76]. Inhibition of FAK with short hairpin RNAs (shRNAs) prevents FAK function in cell adhesion, migration and proliferation in the highly invasive human prostate cancer cell line PC3M and mouse breast cancer cell line 4T1 *in vitro* and suppresses tumor growth in heterotopic/ orthotopic mice models *in vivo*[77]. Moreover, knock down of FAK and family member Pyk2 extended the mouse survival period in orthotopic glioma xenografts[78]. However because of its fast degradation, it is difficult to deliver siRNA very efficiently *in vivo* questioning the clinical practicability of using siRNA. A modified polyethylenimine (PEI) gene carrier can be used to deliver FAK shRNA *in vitro* and *in vivo* to study the effect of FAK inhibition in melanoma tumor therapeutics[79].

Lately, a neutral lipid liposome, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) has been reported to introduce FAK siRNA successfully and silencing of FAK showed antitumor and antiangiogenic effects, especially it improved the chemosensitivity to docetaxel or cisplatin in an orthotopic ovarian carcinoma model[80]. This type of lipid liposome improved the intratumoral penetration, delivery efficacy and toxicity. If efficiency of specific RNAs delivery would be improved, the strategy to inhibit FAK expression is promising for cancer treatment.

3.2 (In)direct pharmacological modulation of FAK activity

3.2.1 Receptor tyrosine kinases inhibitors

FAK phosphorylates its downstream effectors so the use of kinase inhibitors consequently disturb signaling transduction. The EGF-receptor inhibitor Gefitinib ('Iressa') and the PTK inhibitor genistein are already used in the clinic. Gefitinib decreased FAK phosphorylation and inhibited the metastasis of oral squamous cancer cells to the lymph nodes[81]. Genistein increased the adhesion of prostate cancer cells by modulating FAK activity[82]. In addition, herbimycin A, also a PTK inhibitor decreased FAK phosphorylation and resulted in decreased migration of oral squamous cancer cells[83]. Herceptin which binds to the juxtamembrane region of the ErbB2 receptor is shown to inhibit Src and Fak activities thereby inhibiting focal adhesion turnover which magnifest by increased focal adhesion stability and reduced cell invasion[24]. SKI-606 (bosutinib), a novel Src kinase inhibitor inhibits phosphorylation of FAK and suppresses migration and invasion of human breast cancer cells[84]. Dasatinib (BMS-354825) inhibited migration and invasion in non-small cell lung cancer and head and neck squamous cell carcinoma. The effects on migration and invasion correlated with the inhibition of Src and downstream mediators of adhesion such as FAK, p130Cas and paxillin[85].

Inhibitors	*In vitro*/*In vivo* effects	References
PF-562,271 * (FAK/Pyk2)	Xenograft models and patients *(anti-angiogenesis, antitumor activity)* Phase 1 clinical trial (Pfizer, Inc. (PFE))	[88,90] http://clinicaltrials.gov/ct2/show/NCT00666926
TAE226 * (FAK/IGF-IR)	Breast cancer, glioblastomas, gastroinstestinal stromal tumor, ovarian carcinoma, esophageal adenocarcinoma *(cell proliferation/growth, cell cycle, cell migration, chemoresistance to docetaxel in vitro and in vivo)* Phase I clinical trial (Novartis AG (NVS))	[80,86,90,92,94,100]
PF-573,228 * (FAK)	Human tumor cell-lines *(cell migration)*	[87,98]
TAC544 * (FAK/Pyk2)	Animal study *(angiogenesis)*	[58]
1,2,4,5-Benzenetetraamine tetrahydrochloride * (FAK)	Breast cancer *(cell adhesion, tumor regression)*	[101]
PF-04554878 # (FAK/Pyk2)	non-hematologic malignancies Phase 1 clinical trial (Pfizer, Inc. (PFE))	http://clinicaltrials.gov/ct2/show/NCT00787033

Table 1: FAK inhibitors in cancer research.
*: ATP analogs that inhibit FAK kinase activity
#: unknown inhibitory mechanism

3.2.2 *Small molecule inhibitors*

Several FAK inhibitors have been developed by pharmaceutical companies (see Table 1). Most promising inhibitors are PF-562,271 (Pfizer, Inc.) and TAE226 (Novartis AG). They are ATP analogs and effectively inhibit the kinase activity of FAK. Treatment of cells with these inhibitors results in a decrease in PTyr397 phosphorylation which is associated with the inhibition of cell migration[86,87]. PF-562,271 has a dual effect on FAK and Pyk2 with an IC_{50} in nano-molar scale *in vitro*[88]. *In vivo*, maximal inhibition of pFAK (78%) is obtained 1 hour after a 33 mg/kg p.o. (per. oral). dose in tumor-bearing mice and with a single dose, the inhibition (>50% inhibition of FAK phosphorylation) lasts above 4 hours. Furthermore, dose-dependent tumor growth inhibition and regression were observed in a broad range of human subcutaneous xenograft models, including prostate, breast, pancreas, colon, lung and glioblastoma with no observation of weight loss, morbidity or death. The *in vivo* inhibitory mechanisms of PF-562,271 rely on anoikis/ apoptosis and reduction of micro vascular density[88]. Recently, it has been shown that this compound inhibits MDA-MB-231 tumor growth in the bone without altering normal bone formation in a rat bone model of human cancer[89]. Initial Phase 1 data on PF-562,271 conducted by Pfizer in patients with different types of cancer revealed that the compound seems to be performing well[90]. TAE226 is another dual inhibitor specific for both FAK and insulin-like

growth factor I receptor (IGF-IR) so the direct effect due to FAK inhibition cannot be really determined. Nevertheless, it is a novel bis-anilino pyrimidine inhibitor that is reported to efficiently inhibit FAK signaling, arrest tumor growth and invasion and prolong the life of mice with glioma or ovarian tumor implants[86,91,92]. TAE226 induces an intermediate conformation of the kinase activation loop[93] which inhibits the phosphorylation of FAK and downstream molecules AKT and ERK. TAE226 decreases also cell proliferation, adhesion, migration and invasion in glioma cell[86]. It has significant action in ovarian carcinoma and inhibits PTyr397, PTyr861 and cell growth in time and dose-dependent ways. Moreover, it shows a synergic effect on docetaxel-mediated cell growth inhibition, reduction of tumor burden and prolonged survival in tumor-bearing mice[91]. TAE226 is also promising in the therapy of imatinib-resistant gastrointestinal stromal tumor[94]. A phase 1 clinical trial study of TAE226 seems to be underway by Novartis while Pfizer initiated in November 2008 a phase 1 clinical study with another pharmaceutical inhibitor PF-04554878 in patients with advanced non-hematologic malignancies. The primary mechanisms of action of FAK inhibitors *in vivo* are apoptosis/anoikis, anti-angiogenesis and reduced invasion, however the role of specific FAK inhibitors *in vivo* and their possible long term side effects should be investigated in the future.

4. Conclusions and prospective research

FAK, a central protein in focal adhesion sites, regulates various cellular processes, including cell proliferation, survival, migration and invasion which are crucial steps in tumorigenesis and metastasis formation. The implications of FAK overexpression in cancer suggest that FAK inhibition is a potential target for anticancer therapy. More and more evidence shows that FAK inhibition alone and in combination with other traditional therapies are promising therapeutics in cancer treatment[76,95-99]. This review summarizes the latest studies and findings about genetic, functional, pharmaceutical inhibition of FAK. Future work should focus on the development and clinical trials of new inhibitors, as well as modulation of target-specific delivery.

Acknowledgments

We thank all members in the Division of Toxicology for their helpful discussions. This work was financially supported by grants from the Dutch Cancer Society (UL 2006-3538 and UL 2007-3860).

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