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# THE ROLE OF SRC FAMILY KINASES IN THE DEVELOPMENT AND PROGRESSION OF PROSTATE CANCER

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# ABSTRACT

Prostate cancer is the most common cancer in men and the second leading cause of cancer-related death in western world. Typically, the treatment of advanced and metastatic prostate cancer consists of castration therapy, which suppresses the development of the disease for 2 years in average. Virtually all patients, undergoing androgen deprivation therapy eventually develop castration-resistant prostate cancer. Currently, only taxane class of drugs has been proven to provide short survival advantage in patients with castration-resistant prostate cancer. This form of the disease is the cause of significant morbidity, resulting in long periods of gradual deterioration of patients' condition, pain related to local extension of the tumour and distant metastases, renal failure due to the invasion into the ureters etc.

Castration resistance allows prostate tumours to progress despite androgen deprivation. Several mechanisms have been described, outlining the nature of molecular pathways, employed by prostate cancer cells in order to proliferate and migrate in low androgen environment. Hormone-sensitive prostate cancer cells rely on androgens for their growth needs with androgens acting through the androgen receptor (AR). In castration-resistant prostate cancer AR can be activated by reduced concentrations of androgens, AR antagonists, protein kinases or bypassed altogether. Detailed knowledge of these processes should allow better understanding of molecular patterns, driving the progression of prostate cancer and, ultimately, could lead to the development of novel molecular targeted therapies.

Molecular pathways, implicated in the development of castration-resistant prostate cancer frequently show cross-talk, resulting in the ability of cancer cells to adapt to changing microenvironment. Inhibiting the proteins, facilitating these cross-talks provides an attractive targeting mechanism. Src family of non-receptor tyrosine kinases (SFK)

represent proteins involved in the development of various solid malignancies, including prostate cancer. These proteins are often found on the cross-roads of intracellular pathways, integrating molecular systems into complex signalling networks. SFK interact with receptor tyrosine kinases, G-protein coupled receptors, motility and adhesion factors and, thus, influence multiple cell functions. In prostate cancer, SFK have been demonstrated to form complexes with AR, activating AR by means of tyrosine phosphorylation.

SFK inhibitory compounds have been developed and are now in Phase II clinical trial in patients with castration-resistant prostate cancer. However, there is considerable lack of data regarding the role of SFK expression and activation in prostate cancer in clinical settings. In this thesis, we studied the role of SFK in prostate cancer using matched paired prostate cancer samples, taken from patients prior to castration therapy being administered and following the development of castration resistance. Using paired tissue specimens allows following molecular changes through the natural history of the disease and correlating these changes with various clinical parameters. We also conducted *in vitro* experiments, employing hormone-sensitive LNCaP cell line and its counterpart, castration-resistant LNCaP-SDM cell line, developed by gradual withdrawal of androgens from the culture medium.

Our main finding is that in a subgroup of prostate cancer patients, the increase in SFK activity in the transition of prostate cancer from hormone-sensitive to castration-resistant state is associated with significant decrease in survival ($p<0.0001$). Furthermore, the presence of bone metastases in patients with castration-resistant prostate cancer was associated with higher SFK activity in prostate tissue specimens. Our *in vitro* experiments have demonstrated that in prostate cancer the relationship between SFK and AR are important as androgen deprivation resulted in significant reduction in SFK activity. Using SFK inhibitor dasatinib, we have shown that in prostate cancer cell lines, SFK activity was

inhibited at low nanomolar concentrations. Inhibition of SFK activity was accompanied by the inhibition of downstream protein FAK at Src-specific phsophorylation site. Although the treatment with SFK inhibitor suppressed migration of both LNCaP and LNCaP-SDM cell lines, only proliferation of LNCaP-SDM cell was affected by dasatinib.

Taken together, our date suggests that SFK inhibitors may have a role in the treatment of castration-resistant prostate cancer. However, important considerations should be given to the molecular heterogeneity of prostate cancer in order to improve the outcomes of clinical trials and the response to treatment. There is considerable evidence that SFK inhibitors suppress prostate cancer cells migration and future studies will hopefully further clarify their role in cancer cells proliferation.

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# ABBREVIATIONS

*AR – androgen receptor*

*ARE – androgen response element*

*ASPC – androgen-sensitive prostate cancer*

*BCA – bicinchoninic acid*

*CRPC – castration-resistant prostate cancer*

*CSK – c-Src kinase*

*DAB – 3,3'-diaminobenzidine*

*DHT – dihydrotestosterone*

*DMSO – dimethyl sulfoxide*

*DOC-2/DAB 2 – differentially expressed in ovarian cancer-2/disabled 2*

*ECM – extra-cellular matrix*

*EGFR – epidermal growth factor receptor*

*ELISA – enzyme-linked immunosorbent assay*

*EMT – epithelial to mesenchimal transition*

*ERK – extracellular signal regulated kinase*

*FAK – focal adhesion kinase*

*FDA – Food and Drug Administration Agency*

*GDP – guanosine diphosphate*

*GnRH – gonadotropin-releasing hormone*

*GPCR – G-protein coupled receptor*

*GRP – gastrin-releasing peptide*

*GTP – guanosine triphosphate*

*HAT – histone acetyltransferase*

*HIF – hypoxia-inducible factor*

*HRP – horseradish peroxidase*

*HUVEC – human umbilical vein endothelial cells*

*ICCC – inter-class correlation coefficient*

*IGFR – insulin-like growth factor receptor*

*IHC – immunohistochemistry*

*IQR – inter-quartile range*

*LHRH – luteinising hormone releasing hormone*

*LREC – local research ethics committee*

*MAPK – mitogen-activated protein kinase*

*MMP – matrix metalloproteinase*

*MREC – multiple research ethics committee*

*NED – neuro-endocrine differentiation*

*NEP – neutral endopeptidase*

*NT – neurotensine*

*PI3K – phosphatidylinositol 3-kinase*

*PTEN – phosphatase and tensin homolog*

*PSA – prostate specific antigen*

*RANK – receptor activator of NF-kappaβ*

*RECIST – responce evaluation criteria in solid tumours*

*RIPA – radio-immune precipitation assay*

*RSV – Rous sarcoma virus*

*RT-PCR – reverse trascriptase polymerase chain reaction*

*SDM – steroid-depleted medium*

*SFK – Src family kinases*

*SH – Src homology*

*STAT - signal transducers and activator of transcription*

*TGF – transforming growth factor*

*TIMP – tissue inhibitor of metalloproteinases*

*TRUS – trans-rectal ultrasound*

*TURP – trans-urethral biopsy of prostate*

*VEGFR – vascular-endothelial growth factor receptor*

# INTRODUCTION

## 1.1 SFK in Cancer

### 1.1.1 Discovery and Evolution of Knowledge

Almost one hundred years passed since the discovery of a sarcoma virus by Peyton Rous at the Rockefeller Institute in New York. He studied tumours developed by chickens of Plymouth Rock breed, which on microscopic examination were found to be sarcomas. When the tumours were removed, homogenised and filtered, Rous was surprised to discover that if the filtrate was injected into another chicken of the same species, a similar tumour started to grow. Thus, Rous concluded that the malignancy was caused by a filterable agent and published the findings in the Journal of Experimental Medicine in 1911 (1). However, due to the controversy surrounding the issue of cancer being caused by a known agent it was not until 1950s when a similar strain of virus was found to induce sarcoma in mammals that intensive research on the topic resumed. Several decades later light was shed on the retroviral features of Rous Sarcoma Virus (RSV), as it was then called, and its genetic makeup. Finally, the transforming gene v-src, responsible for the malignant properties of RSV was identified and sequenced in the laboratory of Mike Bishop in late 1970s (2).

In 1969 Huebner and Todaro postulated that the cells of vertebrates contain viral genes or oncogenes that are under certain circumstances transformed and, when activated, could stimulate the cells to behave in a malignant fashion (3). However, it was later found that normal cellular genes when induced by viruses, could gain malignant potential and, therefore, they are termed ‘proto-oncogenes’. One of the first proto-oncogenes identified by Bishop and Varmus was c-src (4), cellular equivalent of v-src, this discovery earned them the Nobel Prize. The product of c-src gene, c-Src protein was found to possess

protein tyrosine kinase activity and, furthermore, c-Src itself was demonstrated to be phosphorylated at tyrosine residues (5). Two decades later and a considerable amount of work that followed gave insight into the complex modular structure of c-Src protein, which was shown to consist of several domains involved in various inter- and intramolecular interactions (6). It was discovered that c-Src can switch between inactive and active state depending on which of two tyrosine residues are phosphorylated, tyrosine $Y^{419}$, located on the carboxyl terminal of the protein or tyrosine $Y^{527}$ of the amino-terminal (7;8). Following identification of c-Src as a tyrosine kinase, multiple substrates and downstream signalling proteins were identified, including focal adhesion kinase (FAK) (9), mitogen-activated protein kinase (MAPK) (10), phosphatidylinositol 3-kinase (PI3K) (11) etc. As a result of multiple molecular interactions c-Src (later in the text referred to as Src) plays a role in multiple cellular functions that include proliferation, adhesion, migration and cell cycle progression (12).

### 1.1.2 Structure and Activation

Nine non-receptor protein kinases: Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn with similar structural features to Src, make up Src family kinases. Src, Fyn and Yes are ubiquitously expressed in all tissues whereas the expression of others may differ (13). So far, four SFK members have been implicated in the development of prostate cancer in its progression to hormone independence: Src, Lyn, Fyn and Fgr (14-17). Due to the similarity of the amino acid sequences and, as a result, cross-reactivity of antibodies, all family members are usually studied together and only now we are beginning to unravel the functional differences between them (18;19). Separate knockout of Src and Lyn by siRNA in PC3-derived prostate cancer cell lines revealed that these SFK members may play different roles in prostate cancer. While inhibition of Src affected primarily migratory

properties of prostate cancer cells, suppression of Lyn was responsible for inhibition of proliferation (18).

High resolution crystallographic analysis revealed complex 3-dimentional structure of Src protein, which is a prototype of other family members (20). Src consists of four domains (**Figure 1)**: a unique to each family member domain, two Src homology (SH) domains SH3 and SH2, kinase domain, containing tyrosine $Y^{419}$ (avian equivalent - tyrosine $Y^{416)}$ and carboxy-terminal negative regulatory domain, containing tyrosine $Y^{527}$, which is mutated in v-Src making it constitutively activated. In addition, the molecule contains amino-terminal myristoylation sequences necessary for the association of the protein with cellular membranes.

Several mechanisms of Src activation have been described. When tyrosine $Y^{527}$ of the negative regulatory domain is phosphorylated by c-Src kinase (Csk) the molecule is folded preventing its' association with substrates (21). Following tyrosine $Y^{527}$ dephosphorylation by protein tyrosine phosphatises, Src becomes autophosphorylated at tyrosine $Y^{419}$ allowing the protein to unfold and assume catalytically active conformation.

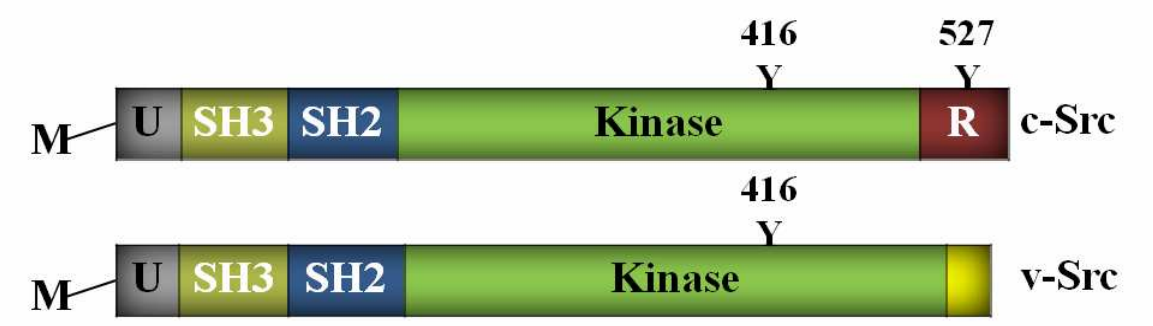



**Figure 1. The domain structure of c-Src and v-Src proteins. Adapted from Frame (13). For comparison, viral (v-Src) and cellular (c-Src) structures are presented. Src molecule is made up of several domains: U – unique to each family member domain with amino-terminal myristoylation sequences (M), Src homology 2 and 3 domains (SH2 and SH3), kinase domain with tyrosine $Y^{419}$ as an activation marker (aviav equivalent tyrosine $Y^{416}$) and negative regulatory domain (R), containing tyrosine $Y^{530}$ (avian equivalent tyrosine $Y^{527}$), which is absent in viral Src making it constitutively activated.**

Once Src unfolds, it becomes available for the interaction with various docking substrates that, in turn, can activate Src by intra-molecular displacement. Another mechanism of Src activation, called intracellular targeting, involves physical transfer of inactive Src from peri-nuclear region to the cell membrane, where it forms complexes with other proteins regulating various important cell functions including adhesion, invasion and migration (22). Finally, other mechanisms regulating Src function in cancers include increased expression, naturally occurring mutations (23) as well as post-translational events e.g. altered ubiquitin-dependent degradation (24).

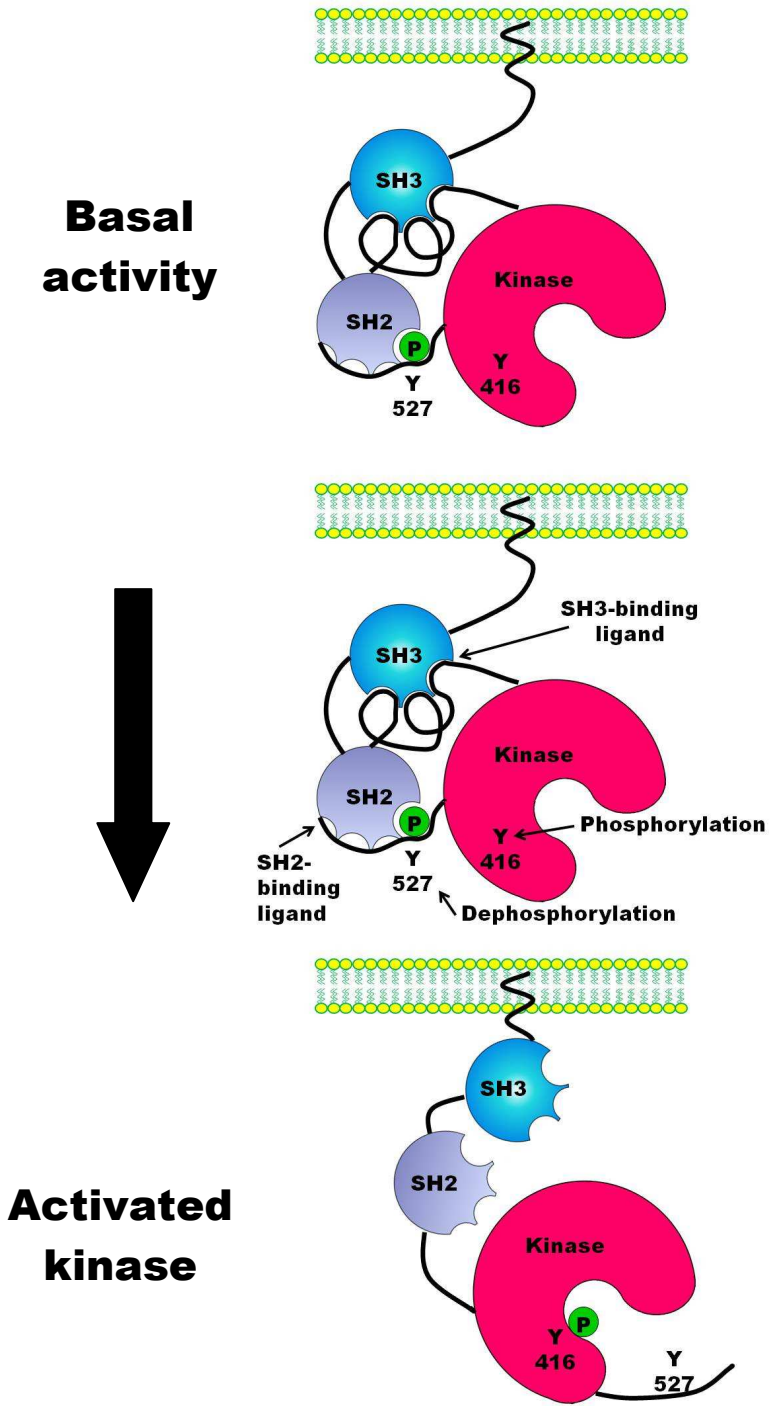



**Figure 2. Mechanism of Src activation. Adapted from Martin (25). In its basal state, Src molecule is kept folded by intramolecular constrains. Activation involves phosphorylation of tyrosine $Y^{416}$ in the kinase domain and dephosphorylation of tyrosine $Y^{527}$ in the negative regulatory domain, allowing the molecule to unfold and assume catalytically active conformation.**

### 1.1.3 Cellular Processes Controlled by SFK

Involvement of SFK in multiple intracellular molecular networks explains the role SFK play in various cell functions. Although considerable evidence implicating SFK in adhesion, migration and invasion exists, their role in proliferation is less well determined. Cell movement is a complex well coordinated process involving weakening of cell-cell bonds, formation of protrusions and their attachment to extracellular matrix (ECM) in the leading part of the cell, cytoskeletal reorganisation propagating the body of the cell forward and detachment of the cell at the rear end. Src is a key component in adherence junctions and focal adhesion complexes, regulating cells motility by disrupting cell-cell contacts and increasing focal adhesions turnover (26). Regulation of invasive properties of cancer cells by Src is achieved by inhibition of cadherins (proteins stabilising cell-cell junctions), increasing degradation of cadherin complexes and regulation of matrix metalloproteinases (MMP) (27;28).

Tumour growth depends on the balance between proliferation and apoptosis and SFK may play a role in both processes. Following stimulation by growth factors, Src becomes activated integrating signals into proliferative and anti-apoptotic cascades e.g. Ras/MAPK and PI3K/Akt. In addition, other important processes are influenced by Src, in particular induction of DNA synthesis and cell cycle progression. Thus, although the role of Src in cancer cell proliferation remains controversial, SFKs may regulate different stages of tumour growth in different tissues and the end result probably represents the cumulative effect of these processes (12).

## 1.2 SFK Molecular Interactions in Prostate Cancer

Prostate cancer is the most common malignancy in men living in the Western world and the second, after lung cancer, leading cause of cancer-related death. The number of new prostate cancer cases in the US in 2009 is projected to be in the region of 192,280, which will make up 25% of all new cancer cases (29). Approximately 27,360 prostate cancer patients will die in 2009 in the US, with only the number of deaths due to lung and bronchial cancer exceeding prostate cancer-related mortality. In the UK, 35,515 patients were diagnosed with prostate cancer in 2006 and in 2007 there were 10,239 deaths related to prostate cancer (30). Prostate cancer incidence has racial and geographical variations with men of african-american origin suffering from the highest rates of the disease, whereas east-asian men have the lowest incidence.

When prostate cancer is suspected on clinical examination or following prostate specific antigen (PSA) testing, the trans-rectal ultrasound scan (TRUS) biopsy of the prostate is performed and histological assessment of prostate tissue is conducted to confirm the diagnosis. The management depends on the individual patients' condition, co-morbidities as well as the grade and the stage of prostate cancer. Organ-confined disease can be treated with radical surgery or radiotherapy, whereas locally-advanced or metastatic disease is managed using androgen-deprivation therapy, which is also used in neo-adjuvant settings. Most of the patients, to whom the radical treatment is administered, are cured, although approximately a third develop prostate cancer relapse and, therefore, require androgen-deprivation therapy.

### 1.2.1 Castration Resistance in Prostate Cancer

Androgen-deprivation therapy has been the mainstay of locally advanced and metastatic prostate cancer treatment over the last five decades (31). The concept of this treatment is based on the fact that androgens play a crucial role in promoting proliferation and migration of prostate cancer cells while inhibiting apoptosis. Androgen-deprivation therapy can be administered in the form of surgical castration, gonadotropin-releasing hormone (GnRH) receptor agonists or androgen receptor (AR) antagonists. However, the effect of androgen ablation only lasts for 2 years in average, following which prostate cancer patients invariably develop castration resistant prostate cancer (CRPC). Currently, there are no effective therapies for this type of prostate cancer.

Several mechanisms, traditionally described as pathways, have been implicated in the development of CRPC (32). Prostate cancer cells may adapt to a low level androgen environment by increasing the expression of AR, increasing sensitivity of AR to androgens and stimulation of intracellular conversion of testosterone to a significantly more potent androgen dihydrotestosterone by 5α-reductase (33). This mechanism is frequently referred to as the hypersensitive pathway. AR could also become susceptible to activation by various non-androgen steroids and androgen antagonists (promiscuous pathway), non-steroidal molecules, such as growth factors (outlaw pathway) or prostate cancer cells may develop molecular cascades bypassing AR altogether (bypass pathway) (34-36). Finally, prostate tumours are thought to contain stem cells (previously known as lurker cells) with the potential to differentiate into heterogeneous population, frequently found in CRPC specimens, whilst maintaining self-renewal (37).

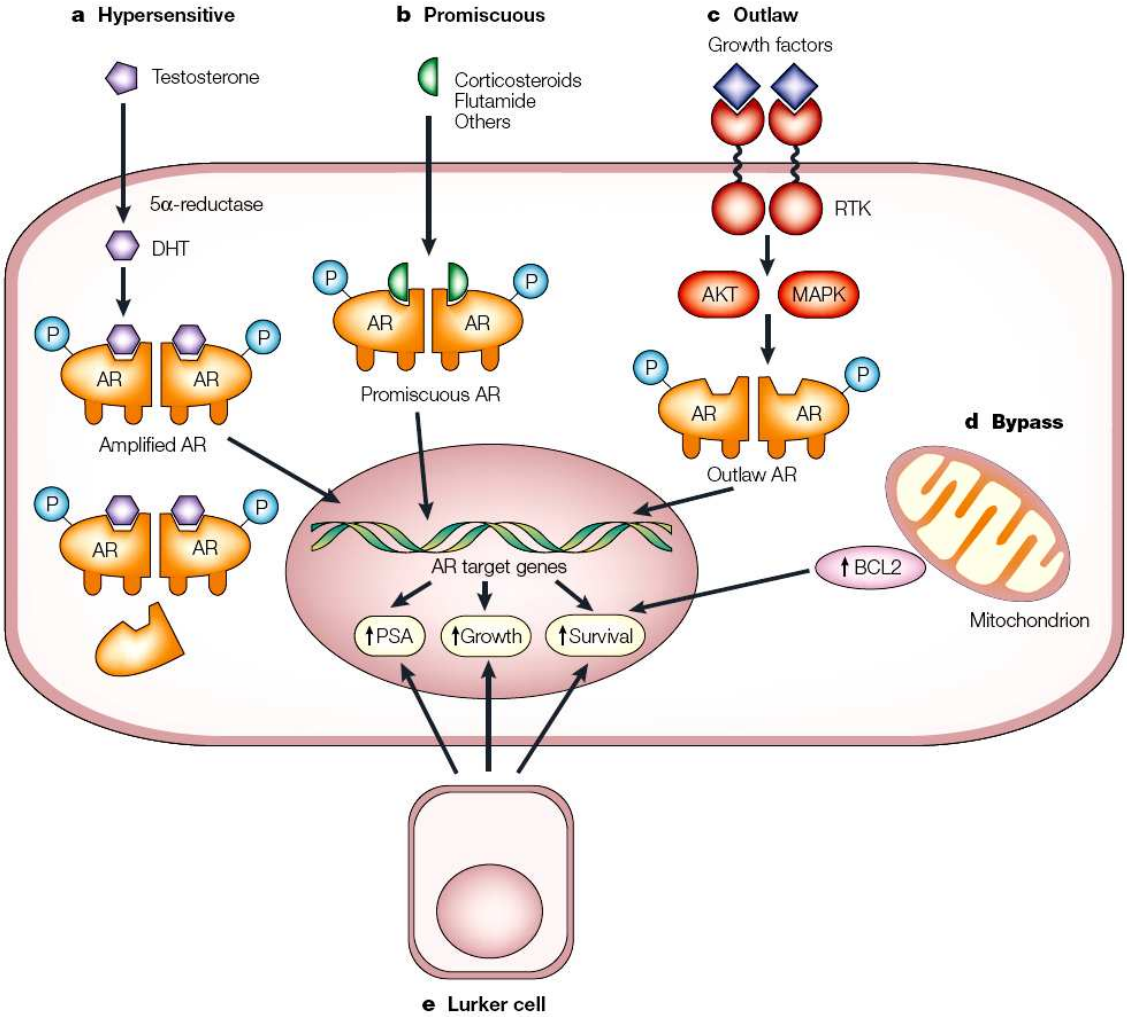



**Figure 3. Mechanisms involved in the development of CRPC. Adapted from Feldman et al. (32). Pathways involved in the development of CRPC are presented. Hypersensitive pathway – increase in sensitivity to low concentration of androgens by amplification of AR, promiscuous pathway – AR is stimulated by corticosteroids, anti-androgens etc., outlaw pathway – AR is activated via different other routes e.g. phosphorylation by tyrosine kinases and bypass pathway, where AR is bypassed altogether. Lurker cells represent a population of undifferentiated (stem) cells that are thought to develop into cancer, already adapted to low androgen environment.**

### 1.2.2 Interaction of Androgen Receptor with SFK

In prostate cancer AR signalling typically involves binding of androgens to the receptor located in the cytoplasm, dimerisation of the receptor, its translocation to the nucleus and activation of the transcriptional apparatus resulting in multiple biological events (38).

Although previously the role of the AR in prostate cancer had been regarded primarily as a ligand-dependent transcription factor, recently there has been much interest in studying non-genotropic properties of the receptor. AR could be integrated into the network of signalling pathways by forming physical association with tyrosine kinases, including Src, or responding to growth factors stimulation (39). Proline-rich sequences of AR have been shown to display affinity for SH3 domain of Src, so that the resulting complexes release Src intra-molecular constrains, activating the tyrosine kinase. Androgen stimulation acted as a trigger for the AR-Src complex formation, which was followed by activation of Src/Raf-1/Erk-2 pathway and, as a result, the increase in prostate cancer cells proliferation. Application of androgen antagonists, expression of Src lacking SH3 domain and treatment with Src inhibitor prevented androgen stimulated S-phase entry resulting in cell-cycle arrest (40).

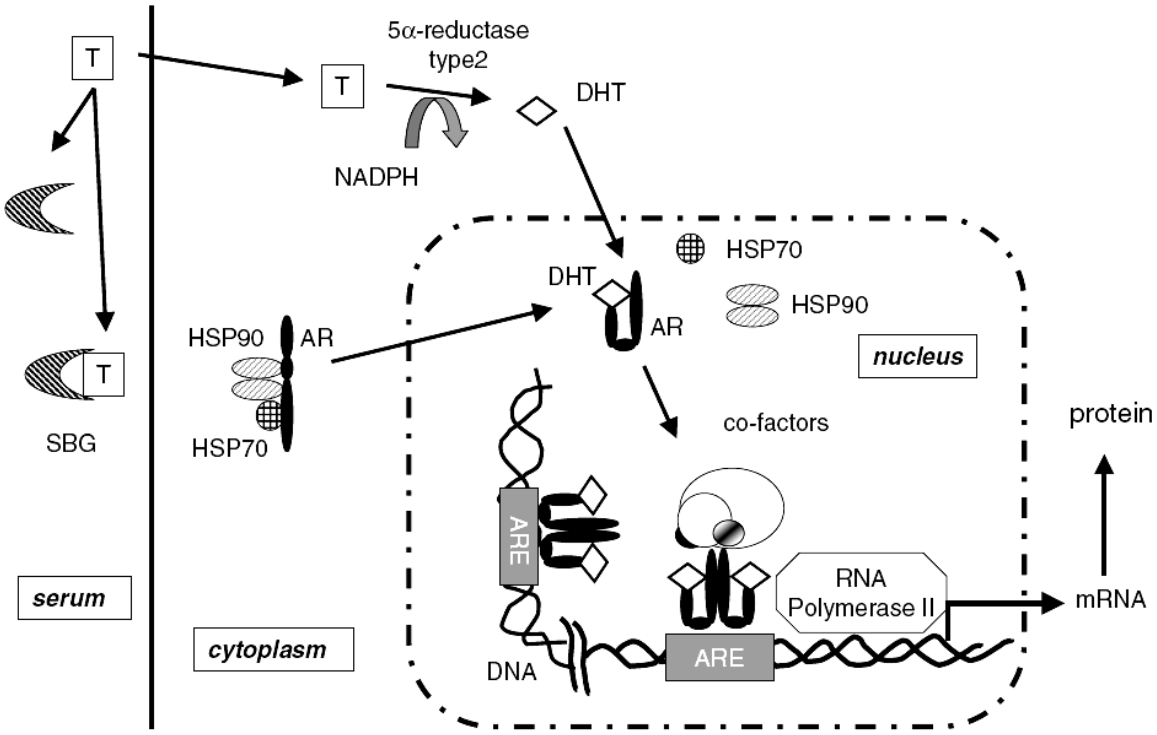



**Figure 4. Action of androgen receptor in prostate cancer. Adapted from Suzuki et al. (38). Testosterone, converted to DHT by 5α-reductase, stimulates AR located in cytoplasm in complexes with Hsp. Dimerised AR then translocates into the nucleus, where it form complexes with co-factors (ARA) in order to activate androgen responsive elements (ARE) regions of DNA. The end result – the increase in prostate cancer cells proliferation and production of various proteins, including PSA.**

Differentially expressed in ovarian cancer-2/disabled 2 (DOC-2/DAB 2) has originally been discovered in ovarian cancer as a candidate tumour suppressor gene (41). The protein contains proline-rich sequences interacting with Src and Fgr, SFK members, containing SH3 domain. This interaction was demonstrated to inhibit SFK activity due to conformational changes, locking SFK molecules in catalytically inactive conformation (42). There is inverse relationship between AR expression in normal prostatic epithelium and prostate cancer and DOC-2/DAB 2 expression. It is possible to increase proliferation of CRPC cells expressing AR by knocking out DOC-2/DAB 2 with siRNA. Tumour suppression properties of DOC-2/DAB 2 are thought to be due to the ability of this protein to disrupt AR/Src complex formation and inactivation of Erk and Akt. Non-genomic action of DOC-2/DAB 2 was further confirmed by the fact that AR transcriptional activity was not affected (43).

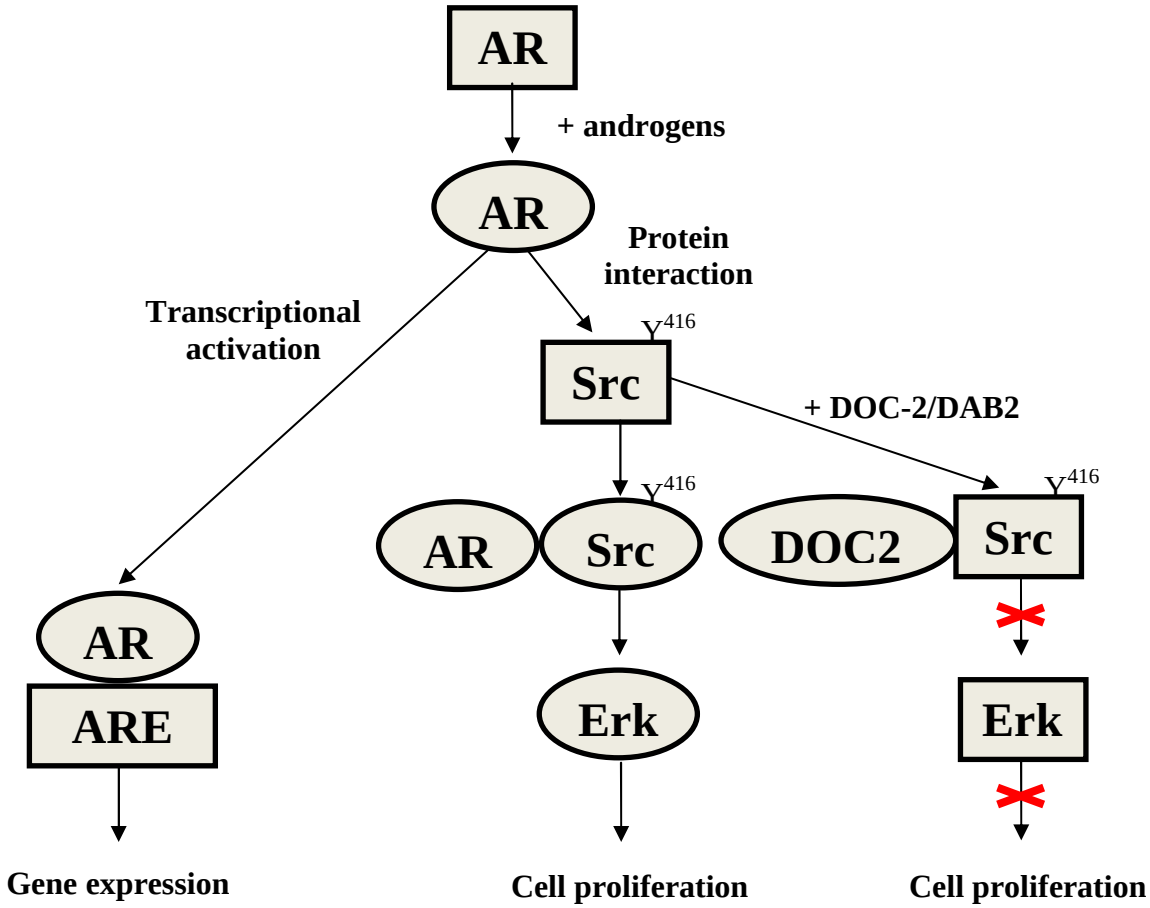



**Figure 5. DOC-2/DAB 2 prevents AR/Src complex formation. Adapted from Zhoul et al. (43). AR interacts with activated Src (pSrcY$^{416}$) and formation of AR/Src complexes leads to downstream Erk activation. DOC-2/DAB2 functions as a negative regulator by displacing AR from the complexes with Src, thus preventing Erk activation and, as a result, decreasing prostate cancer cells proliferation.**

Apart from androgens, growth factors and cytokines have the potential to activate SFK and stimulate AR-Src complex formation in prostate cancer cells. Epidermal growth factor (EGF) acting through epidermal growth factor receptor (EGFR) has been shown to activate Src in prostate cancer cells, experiencing acute androgen withdrawal by triggering AR-Src complex formation. Resulting DNA synthesis and cytoskeletal changes were abrogated by treatment with androgen antagonists, suggesting an important role of the relationship between AR and Src in prostate cancer cells biology (44;45). Androgen independent growth and cell migration, stimulated by Interleukin-8 (IL-8) signalling may involve trans-activation of AR by Src as both Src inhibitors and AR antagonists have been demonstrated to down-regulate these biological processes (46).

Castration resistant phenotype has been associated with constitutive activation of Src/MAPK pathway. This was demonstrated *in vitro* by the increased [(3)H]thymidine incorporation and the increased resistance to apoptosis. Furthermore, Src/MAPK activation in androgen-sensitive cells can be triggered by dihydrotestosterone (DHT), potent activator of AR (47). Therefore, AR could regulate transcription either following ligand binding, or by becoming involved in complexes with tyrosine kinases e.g. Src.

Tyrosine phosphorylation is thought to be an important mechanism of modulation of AR activity. Many biological processes involving AR, including cytoplasm to nuclear translocation and transcription, are regulated by tyrosine phosphorylation (34;48;49). Due to rapid and transient nature of this process, identification of AR phosphorylation sites specific to various tyrosine kinases has proved difficult. Guo et al. found significant correlation between AR and Src activation in human prostate tumours and proposed tyrosine $Y^{534}$ as a Src-specific phosphorylation site. These findings suggest that androgen deprivation therapy may result in Src activation by growth factors, which can then stimulate AR activity as a potential hormone escape mechanism (50).

### 1.2.3 G-protein coupled receptors and SFK

Guanosine phosphate binding protein coupled receptors (GPCR) have been implicated in the proliferation and survival of prostate cancer cells. Conventionally, the heptahelical receptor is made up of seven membrane-spanning regions. Following ligand binding, the receptor interacts with a heterotrimeric G-protein, consisting of three subunits: α, β and γ. This leads to α subunit, which carries guanosine diphosphate (GDP), to dissociate from the complex, followed by GDP becoming converted to GTP (51). Thus, the process creates second messengers that activate many intracellular signalling cascades.

Although it was previously thought that GPCRs were mainly involved in the short – term regulation of intermediary metabolism and neurotransmission, it has recently become known that these receptors can influence cell growth and differentiation in a variety of clinical conditions including benign prostatic hypertrophy and prostate cancer. SFK play key roles in integrating signals emanating from GPCRs into proliferative and anti-apototic molecular networks (52). The mechanisms of SFK-GPCR interaction are complex and include direct binding through SH2 and SH3 homology domains, association with heterotrimeric G-proteins and cross-talk with receptor protein kinases and focal adhesion complexes (53).

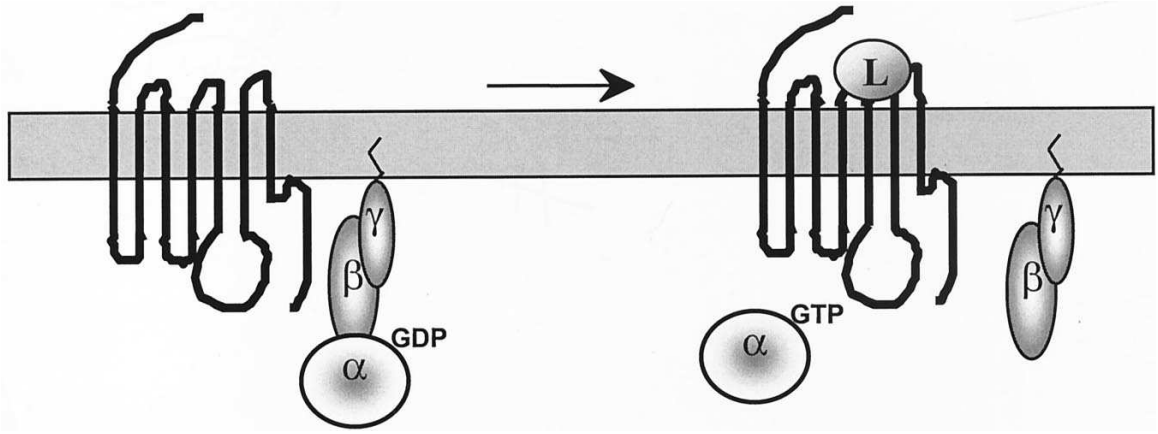



**Figure 6. Activation model of GPCRs. Adapted from Daaka (51). In inactive state, GPCRs form complexes with G-proteins, consisting of α, β and γ subunits. Ligand (L) binding leads to phosphorylation of GDP on α subunit to GTP and dissociation of α subunit from the complex.**

Formation of complexes between GPCRs and SFK as the mechanism of cooperation and mutual activation has been best described for β3 adrenoreceptor (54). This process is mediated through proline-rich regions in the third intacellular loop and carboxyl terminus of the receptor containing consensus sequences for SH3 domain of Src. Thus, complex formation is necessary for downstream MAPK signalling and point mutations, induced in these regions were shown to prevent Src co-immunoprecipitation and MAPK activation. Src may, in turn activate GPCRs by tyrosine phosphorylation and tyrosine $Y^{305}$ has been suggested as a Src-specific phosphorylation site on β2 adrenoreceptor (55).

Several lines of evidence indicate that Src can be activated directly by heterotrimeric G-proteins. Direct binding of Gα subunit to inactive Src phosphorylated at tyrosine $Y^{530}$ resulted in disruption of intra-molecular bonds and Src activation by intra-molecular displacement mechanism (56). Once again, this interaction appears to be mutual so that G-proteins are phosphorylated by Src, resulting in the increase of G-protein activity. Gα subunit became phosphorylated and hydrolysis of phosphatidyl inositol increased following stimulation of fibroblasts transformed by avian v-Src (constitutively active form of the protein) with endothelin-1. Furthermore, these processes were disrupted by application of Src inhibitor PP2 (57).

Stimulated GPCRs may produce downstream signals, resembling the signals emanating from receptor tyrosine kinases. This is due to GPCRs involvement in the extensive cross-talk with receptor tyrosine kinases and SFK may play a role in these processes. Although the exact nature of this interaction is unknown, several mechanisms have been described. One of them, called ectodomain shedding, involves the generation of growth factors from transmembrane precursors and their release into the extracellular milieu where they can interact with the receptors (58). Precursors of tyrosine kinases

agonists are insoluble and, therefore, undergo proteolysis by ADAM family of matrix metalloproteinases (MMPs), which are regulated by Src (59).

Focal adhesions represent protein complexes, consisting of transmembrane receptors integrins that interact with extracellular matrix and transmit signals through intracellular complexes, where SFK have an important role. Following integrin engagement with fibronectin, focal adhesion kinases are recruited to the membrane regions providing docking sites for downstream proteins, including Src, leading to the activation of ERK1/2 pathway (60). GPCRs interact with focal adhesions through activation of focal adhesion kinases FAK and Pyk2. This interaction is inhibited by the exposure to the proteins containing Arg-Gly-Asp sequences mimicking integrin ligands and by cytochalasin D that disrupts actin links (61). The exact nature of the interaction varies, depending on the cell type, although Src inhibitors prevented ERK1/2 activation and assembly of Ras activation complexes in all cases.

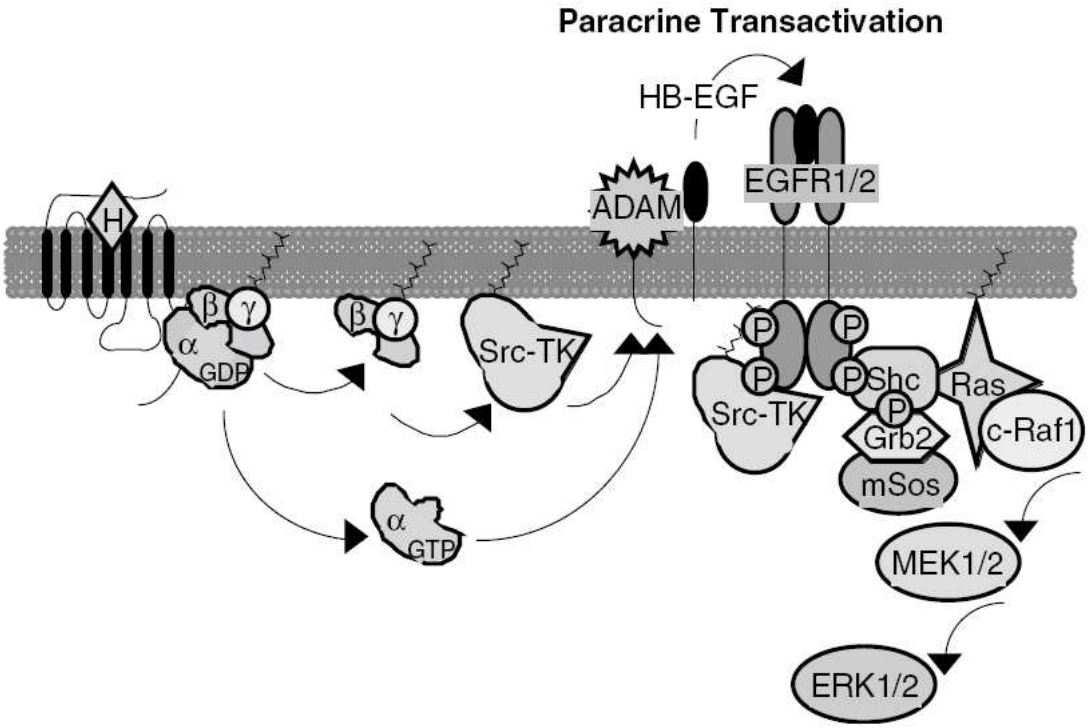



**Figure 7. GPCR transactivation by RTKs. Adapted from Luttrell et al. (53). Src, activated by G- proteins, stimulates ADAM family matrix-metalloproteinases that release growth factors from their precursors (e.g. EGF). EGF then binds to EGFR that phosphorylates multiple intracellular targets, including Src.**

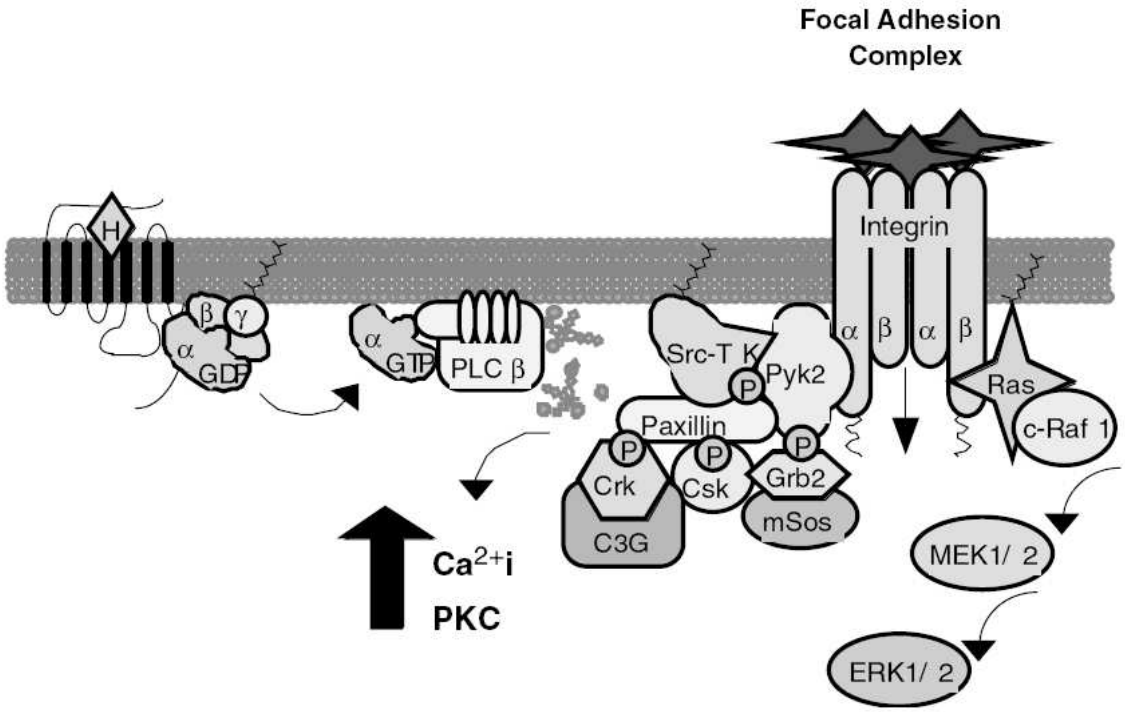



**Figure 8. GPCRs interaction with focal adhesions. Adapted from Luttrell et al. (53) . Ligand-bound GPCRs release α subunit of G-proteins that activates phospholipase Cβ (PLCβ). Resulting increase in intracellular $Ca^{2+}$ and activation of protein kinase C (PKC) leads to activation of Pyk2 or FAK in focal adhesion complexes that invariably involve Src.**

Neuroendocrine (NE) cells represent a type of epithelial cells found in normal prostatic tissue and in prostate cancer. They produce various substances that regulate growth, differentiation and survival of surrounding cells in autocrine and paracrine fashions. Prostate cancer cells acquire features of NE cells in a process called neuroendocrine differentiation (NED). NED has been linked with androgen deprivation therapy, progression of prostate cancer to the castration-resistant state and poor prognosis (62). Several factors, produced by NED cells including bombesin, neurotensin, serotonin etc. implicated in the progression of prostate cancer, act through GPCRs (63).

Bombesin, amphibian tetradecapeptide and its mammalian homolog gastrin-releasing peptide (GRP) are the most studied neuropeptides in prostate cancer. Significant proportion of patients with castration-resistant prostate cancer have elevated level of GRP in their serum (64). Prostate specimens containing invasive prostate cancers have been shown to overexpress receptors for bombesin/GRP whereas samples from areas with benign prostatic hyperplasia tended to have low level expression of these receptors (65). *In vitro* growth of PC3, castration-resistant prostate cancer cell line, stimulated by bombesin was inhibited by application of antibodies against GRP receptor, a member of GPCR superfamily (66). Ligand-bound GRP receptor is thought to trigger several transduction pathways that include Src. In PC3 cells, this process involves trans-activation of EGFR and $Ca^{2+}$ mobilisation, followed by Src and MAPK activation, eventually leading to stimulation of DNA synthesis (67). Src inhibitors PP1 and PP2 together with reduced level of intracellular $Ca^{2+}$ abolished EGFR transactivation and MAPK phosphorylation. In prostate cancer cells expressing AR, Src activated by bombesin may phosphorylate AR in androgen-independent manner, thus increasing its translocation to the nucleus and assembly of transcriptional complex involving c-Myc (68).

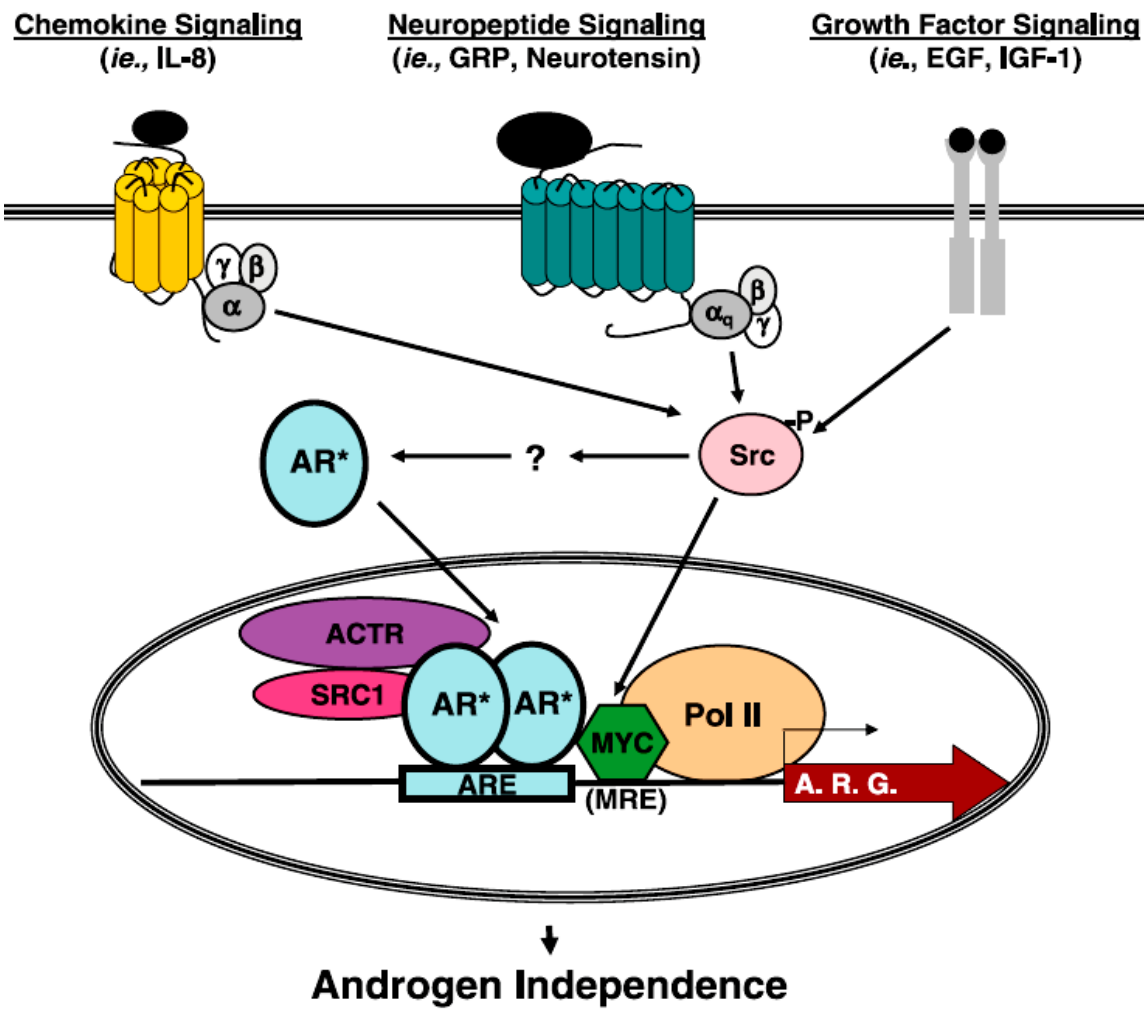



**Figure 9. AR activation and nuclear translocation triggered by Src. Adapted from Desai et al. (68). Src can be activated by neuropeptides, growth factors and chemotactik substances. Activated Src stimulates AR dimerisation and nuclear translocation, where AR acts as a transcription factor in complexes with steroid receptor co-factors SRC1 and ACTR/AIB1. c-Myc is activated directly by Src thereby decreasing apoptosis and increasing polymerase II (Pol II) expression.**

Bombesin and another neuropeptide neurotensin (NT) have been shown to promote survival and enhance migratory properties of androgen independent prostate cancer cells (69;70). In androgen depleted conditions, neuropeptides are able to circumvent normal AR activation pathway, trans-activating reporter genes by prostatic specific antigen (PSA) promoter and promoter of androgen responsive element (ARE). This effect was observed only in LNCaP cells expressing AR or PC3 cells that were transfected with a functional AR. Activated GPCRs transmit signals through the complexes involving SFK, FAK and Etk/BMX non-receptor tyrosin kinases. Furthermore, introduction of dominant negative mutant of Src kinase prevented AR activation and its translocation to the nucleus, suggesting this mechanism is Src-dependent (71).

As mentioned earlier, GPCRs exert influence on receptor tyrosin kinases through Src to promote growth of castration-resistant prostate cancer cells. Treatment of PC3 cells with conditioned culture medium from LNCaP-derived neuroendocrine cells, used as a source of NT, resulted in EGFR activation as measured by phosphorylation of Src-specific site at tyrosin $Y^{845}$ (72). EGFR trans-activation was Src dependent as application of catalytic inhibitors of both EGFR and Src abolished NT-stimulated DNA synthesis and proliferation of PC3 cells. Apart from MAPK, potential downstream proteins conducting the signals from EGFR trans-activated by Src included signal transducer and activator of transcription 5b (STAT5b) (72) and AR co-activator p300 with histone acetyltransferase (HAT) activity (73).

The role of aberrant AR activation by neuropeptides through SFK has recently been explored using *in vivo* model of LNCaP cells, trasfected with vector carrying GRP (74). Resulting LNCaP-GRP cell line exhibited castration-resistant properties and enhanced migratory abilities, linked with increased SFK activation. Orthotopic implantation of LNCaP-GRP cells in castrated nude mice with severe combined immunodeficiency

(SCID) produced primary tumour growth and metastases in lymph nodes. Re-cultured xenograft cells displayed higher Src activity and AR nuclear translocation in androgen depleted medium, compared to native LNCaP cells. Treatment with SFK inhibitor and Src knockout by siRNA reduced proliferation and migration *in vitro* and prevented formation of metastatic disease *in vivo* (75).

Neuropeptides are inactivated by neutral endopeptidase (NEP), a cell-surface enzyme implicated in the development of castration-resistant prostate cancer. NEP expression is lost in castratio-resistant PC3, DU145 and TSU-Pr1 prostate cancer cell lines, contributing to their invasive properties, in contrast to non-invasive androgen-sensitive LNCaP prostate cancer cell line that highly express NEP (76). Apart from functioning as a main degradation mechanism for neuropeptides, NEP has been linked with intracellular transduction pathways that influence cell proliferation, migration, invasion and apoptosis (77;78). Intriguingly, SFK members Src and Lyn are thought to play opposite roles in regulating these pathways. Activated GPCRs promoted complex formation between Src and FAK in focal adhesions, thus stimulating cells migration and invasion, whereas Lyn was shown to inhibit these cellular functions by acting as an intermediary between NEP and p85 subunit of PI3K, preventing association of PI3K and FAK (19).

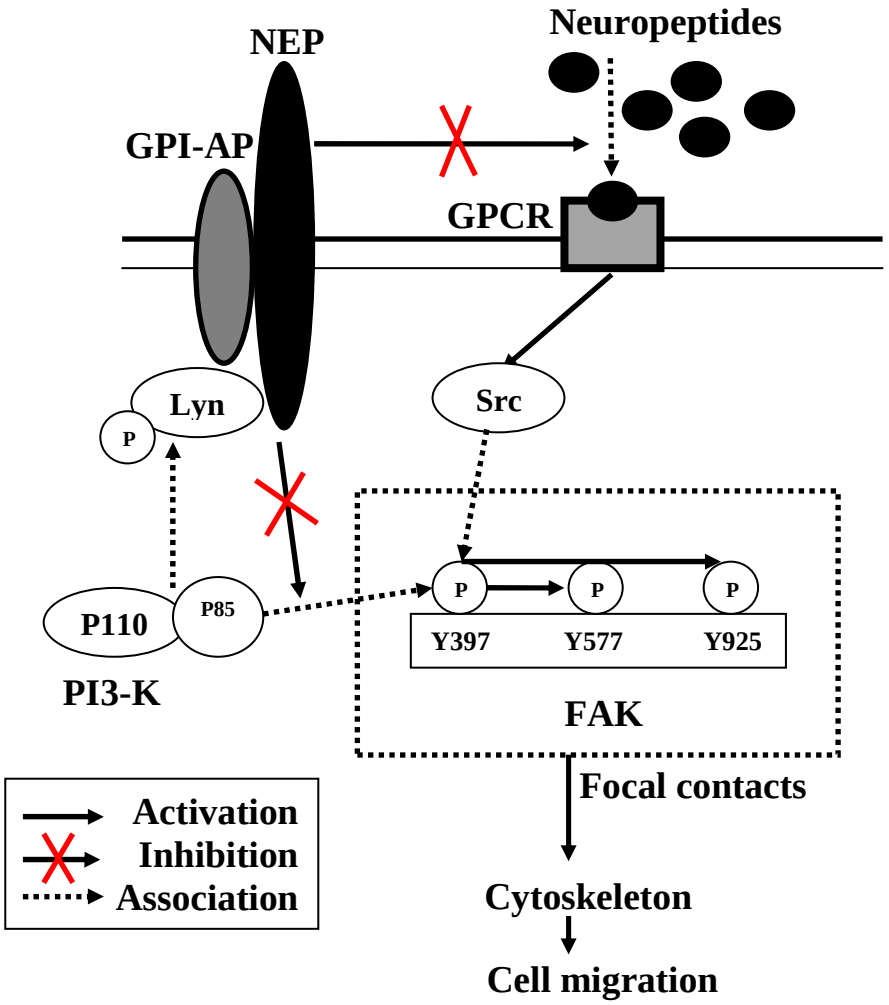



**Figure 10. Interaction of NEP with intracellular transduction pathways. Adapted from Sumimoto et al. (19). Neutral endopeptidase (NEP) associates with Lyn via glycosylphosphatidylinositol-anchored protein (GPI-AP). This leads to the interaction between Lyn and p85 subunit of PI3K, preventing association of PI3K with FAK. Src, activated by GPCRs, forms complexes with FAK via Src-specific phosphorylation site FAKY$^{397}$.**

### 1.2.4 Motility and Adhesion Factors

Oncogenic transformation of normal epithelium into cancer involves acquisition of highly motile phenotype, described as epithelial to mesenchymal transition (EMT). The changes allow cancer cells to break away from the main tumour mass, which is necessary for the local invasion and the development of metastatic disease. Microscopically, the cells gain the ability to produce amoeboid protrusions that interact with extracellular matrix (ECM) components at the same time destabilising cell-cell contacts (adherence junctions) eventually leading to cell detachment, migration or invasion (79). Molecular mechanisms involved in EMT involve multiple transduction pathways where SFK frequently play key roles (13).

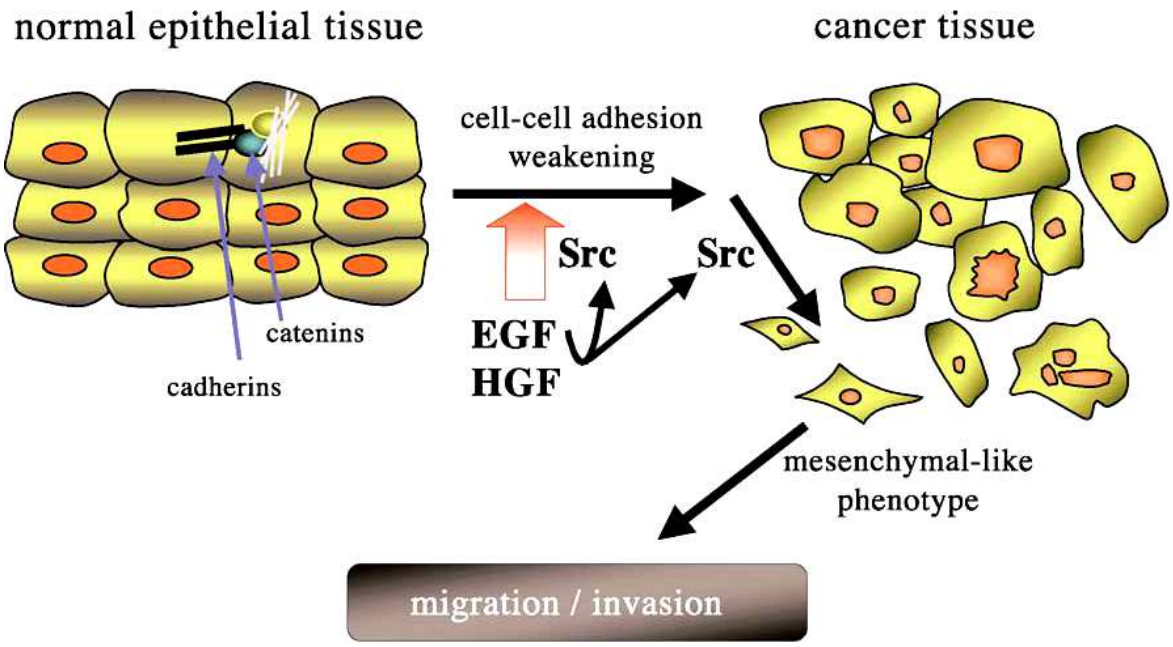



**Figure 11. Src plays a key role in EMT. Adapted from Frame (13). Activation of Src by various mechanisms, including growth factors (e.g. EGF, HGF), leads to the acquisition by the normal epithelial cells of highly motile phenotype, resulting in epithelial to mesenchymal transition (EMT).**

Adherence junctions are made up of cadherins (E-cadherin and N-cadherin), trans-membrane proteins that link the cells by their extracellular components, and catenins (α-, β-, γ-, δ- and p120catenin), intracellular proteins providing connections between cadherins and actin cytoskeleton. E-cadherin has been described as a suppressor of invasion due to its ability to produce stable adherence junctions (80), whereas N-cadherin is thought to be part of more dynamic cell-cell contacts and has been linked with aggressive cancer phenotype (81). Increased expression of N-cadherin together with reduced expression of E-cadherin, described as E-cadherin to N-cadherin (EN) switch, is one of the more prominent features of EMT in prostate cancer (82).

Activated Src destabilises adherens junctions through several mechanisms, including phosphorylation of β- and p120catenin that act as anchoring molecules between E-cadherin and actin, dissociation of E-cadherin and actin, prevention of E-cadherin translocation to the membrane and induction of E-cadherin ubiquitylation, recycling and degradation through activation of E3-ubiquitin ligases Cbl and Hakai (83). Phosphorylation of β-catenin by Src in PC3 cells has been shown to weaken adherence junctions by dissociation of β-catenin from E-cadherin, translocation of β-catenin to the cytoplasm and nucleus and stimulation of its transcriptional activity. Interestingly, expression of α-catenin restricted β-catenin to adherence junctions, thus stabilising cell-cell contacts, preventing β-catenin nuclear translocation and reducing proliferation. Furthermore, by changing its special conformation, α-catenin protected β-catenin from phosphorylation by Src (84).

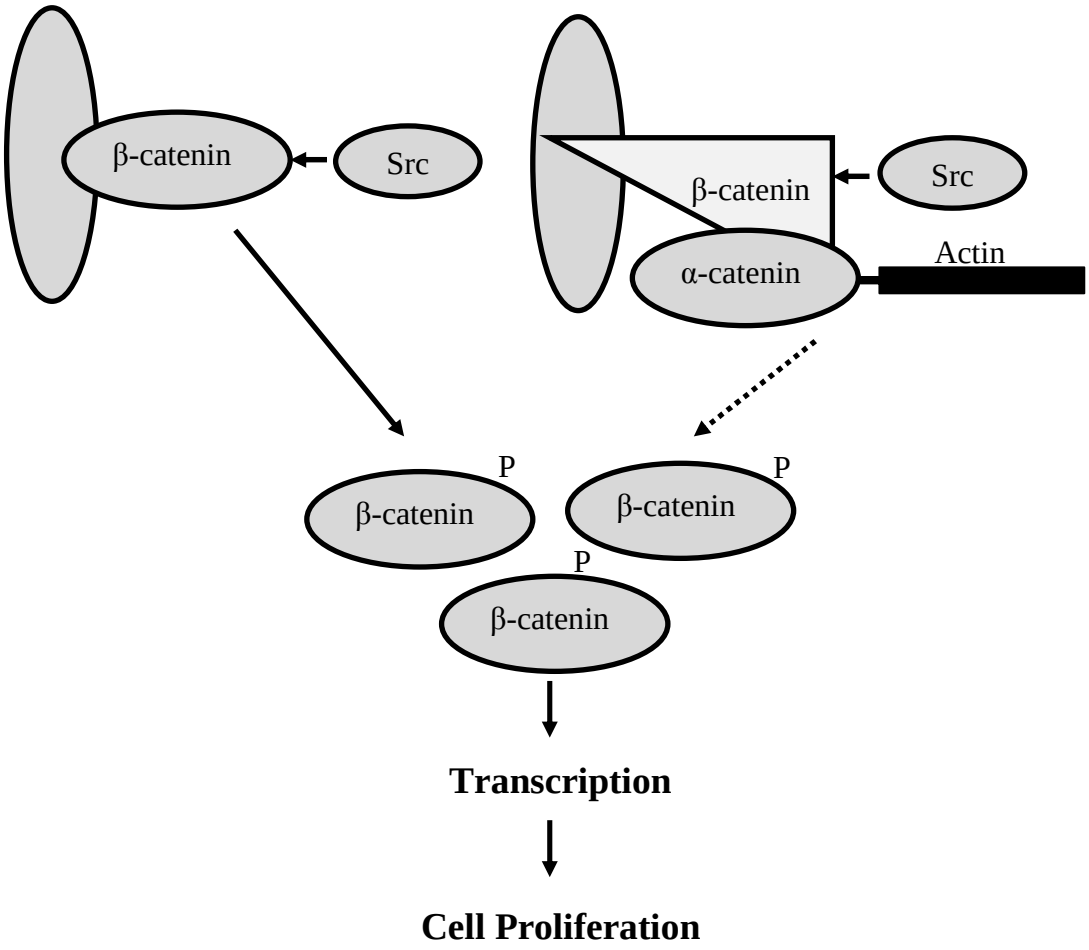



**Figure 12. α-catenin prevents Src phosphorylation of β-catenin. Adapted from Inge et al. (84). Activated Src releases βcatenin from adherence junctions. Translocation of β-catenin to the cytoplasm and nucleus results in the increase of its transcriptional activity. Overexpression of α-catenin stabilises β-catenin in adherence junctions.**

Focal adhesions represent complex structures providing cell-ECM contacts where more than 50 proteins co-localise and integrate. Facilitating cell movement requires a well orchestrated mechanism of focal adhesion assembly at the leading edge of the moving cell, accompanied by focal adhesion disassembly at the back and reorganisation of cytoskeleton (85). Integrins, trans-membrane proteins, are made up of various combinations of α and β subunits, each being sensitive to different ligands and having unique signalling properties. Integrins form clusters at the focal adhesions; they provide physical links between ECM and the structures of actin cytoskeleton and integrate extracellular signals into intracellular transduction cascades, regulating various oncogenic processes in prostate cancer (86). Expectedly, due to the necessity of coordination during cell movement, there is extensive cross-talk between pathways initiated by growth factor receptors, integrins and cadherins (85).

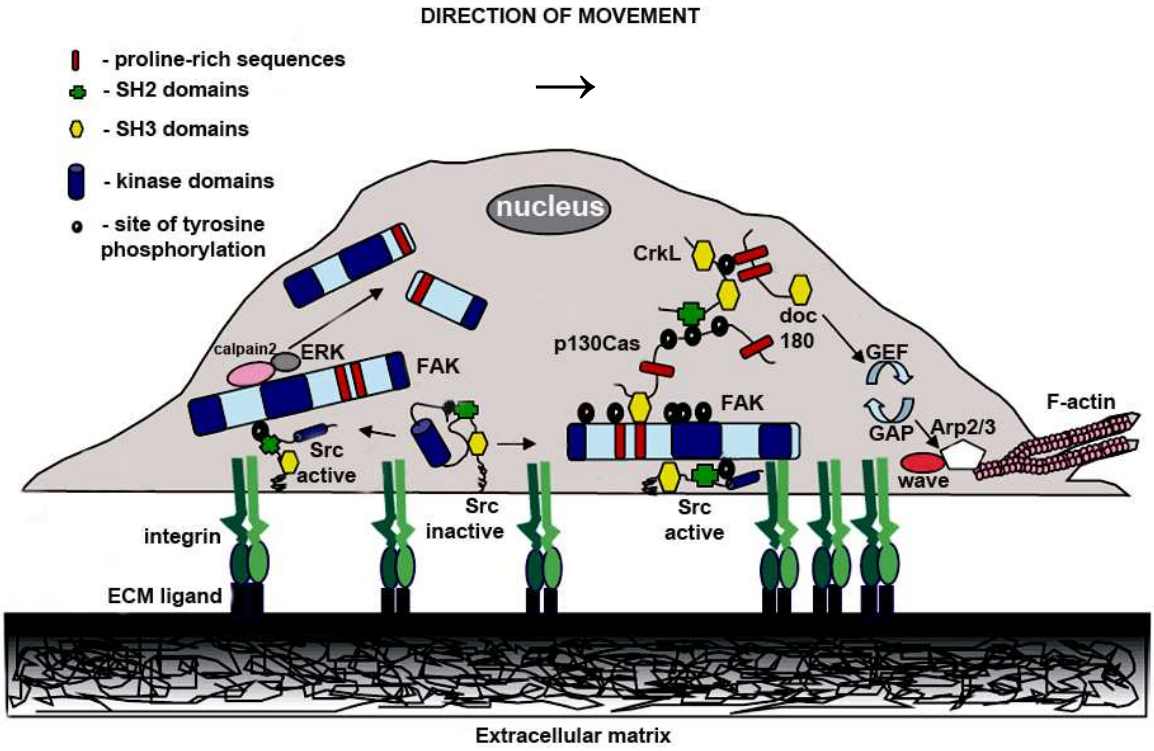



**Figure 13. Intracellular cascades regulating cell movement. Adapted from Playford et al. (85). Src regulates protrusion formation and focal adhesion disassembly, participating in biochemical reactions at the front and the rear of the cell. Activated Src in complexes with FAK phosphorylates p130$^{cas}$. This is followed by c-Crk/p130$^{cas}$ complex formation and activation of Rac and Cdc42 via translocation of Rac activator DOCK1. Activated Rac and Cdc42 promote the development of protrusion at the leading edge of the cell by WASP-dependent activation of Arp2/3. At the rear of the cell activated Src form complexes with FAK, Erk and calpain 2, leading to FAK cleavage and focal adhesion disasemblyfor the purpose of cell detachment ant retraction of the tail.**

Non-receptor tyrosine kinases FAK and SFK are at the heart of the system regulating cancer cells motility and EMT (87). Upon integrin engagement, FAK is recruited to the focal adhesions and rapidly becomes autophosphorylated at tyrosine $Y^{397}$, which is high affinity Src docking site. Src then induces tyrosine phosphorylation of large number of proteins, including FAK itself ($Y^{566}$, $Y^{577}$, $Y^{861}$ and $Y^{925}$), paxillin ($Y^{118}$), $p130^{cas}$ ($Y^{410}$) and other substrates (88). Apart from integrins, FAK can be activated by other factors, including GPCR ligands (bombesin and IL-8) (46;89) and epidermal growth factor receptor (EGFR) through the cross-talk with integrins (90). Activation and recruitment of FAK to focal adhesions was found to be essential for bombesin-unduced PC3 cells motility (89). Expression of FAK was found to be higher in invasive, highly tumourigenic PC3 and DU145 prostate cancer cell lines compared to relatively non-invasive LNCaP cells (91). Cell migration required activation of FAK/Src pathways as inhibiting FAK by infecting prostate cancer cells with adenovirus carrying focal adhesion kinase-related non-kinase (FRNK) or treatment by Src inhibitor PP2 significantly reduced migration. In LNCaP cells exposed to IL-8, activation of both FAK and Src was necessary to induce cell migration whereas Src activity was also required for androgen independent growth (46).

Proline-rich tyrosine kinase 2 (Pyk2) belongs to FAK family kinases; it is structurally and functionally related to FAK. Pyk2 becomes autophosphorylated at tyrosine $Y^{402}$, analogous to FAK tyrosine $Y^{397}$ docking site for Src, in response to variety of stimuli, including GPCR activation, integrin-ECM engagement and elevation of intracellular calcium (92). Although Pyk2 has been linked with various oncogenic processes, especially in the regulation of cell migration, its role in prostate cancer remains controversial. Sanzione et al. demonstrated that the expression of Pyk2 was highest in benign prostatic epithelium and it inversely correlated with prostate cancer grade (93). The same group then investigated the role of Pyk2 in proliferation of PC3 prostate cancer cell

line, concluding that Pyk2-promoted cell proliferation and loss of Pyk2 was accompanied by acquisition of neuron-like morphology, thus suggesting that the resulting NE-like cells then stimulated prostate cancer cell growth in a paracrine fashion (94). In AR-positive castratio-resistant prostate cancer cells, ErbB-2 (HER2) activated Pyk2 and, as a result, up-regulated adhesive properties of prostate cancer cells mainly via activation of MAPK thway(95). Although activation of Src was not required to stimulate cell adhesion, Pyk2 was shown to be autophosphorylated at tyrosine $Y^{402}$, facilitating interaction with Src and, possibly, regulating other cell functions.

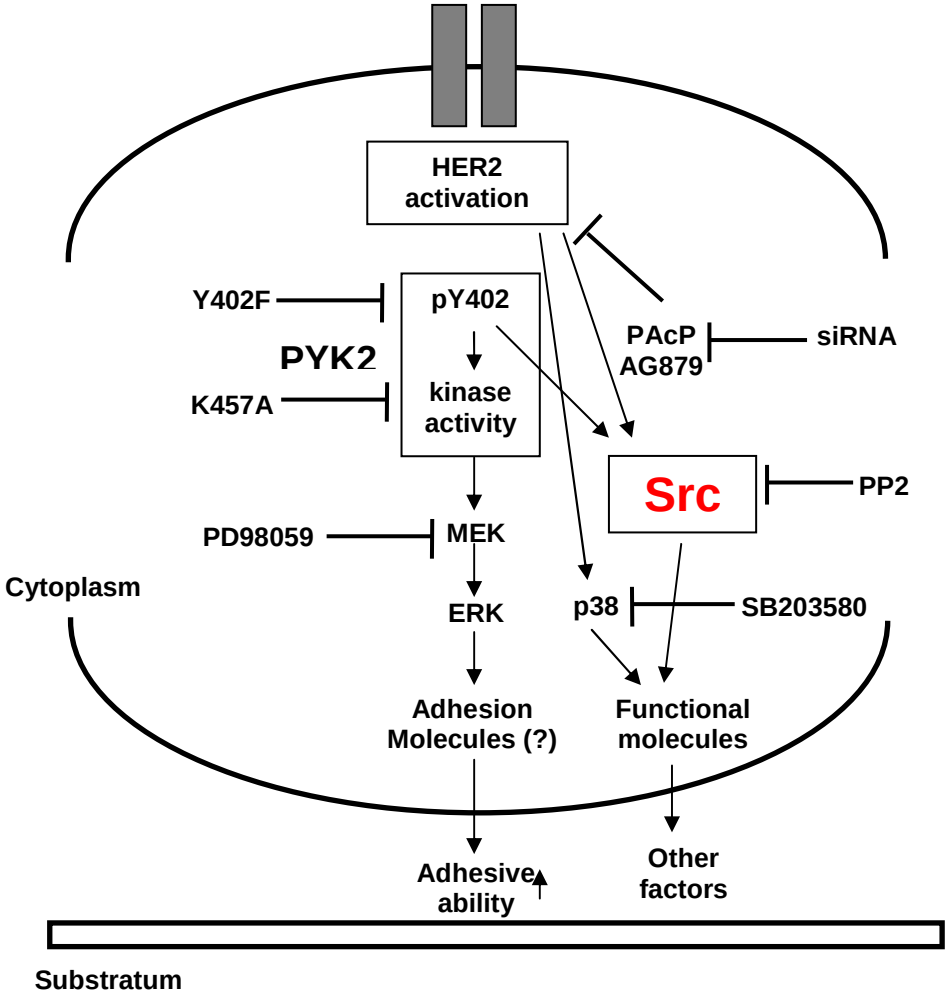



**Figure 14. Regulation of adhesive properties in AR positive castration-resistant prostate cancer cells by PYK2. Adapted from Yuan et al. (95). Constitutive activation of HER2 is thought to positively regulate adhesive ability of AR-positive prostate cancer cells via activation of Pyk2. This process involves MEK and Erk kinases. Phosphorylation of Pyk2 at tyrosine $Y^{402}$ leads to Src activation. Src with p38/MAPK regulate multiple downstream targets.**

Deregulation of Pyk2 in protein complexes facilitating focal adhesion turnover in prostate cancer cells has been compared to similar process in osteoclasts where Pyk2 is thought to functionally replace FAK (96). Dynamic coordination of focal adhesion assembly and disassembly is controlled by Src in association with other proteins, including focal adhesion kinases (FAK and Pyk2), members of paxillin family adaptor proteins and protein tyrosin phosphatases that dephosphorylate proteins, phosphorylated by tyrosine kinases. In PC3 prostate cancer cell line that has been originally derived from bone metastases of a patient with metastatic prostatic cancer, Src has been demonstrated to form complexes with Pyk2, leupaxin, adaptor protein functionally similar to paxillin, and protein tyrosin phosphatase-proline-, glutamate-, serine-, and threonine-rich sequence (PTP PEST) (97). Using siRNA approach, the authors showed that inhibition of leupaxin in PC3 cells resulted in reduced migration, whereas adenoviral-mediated overexpression of leupaxin was associated with activation of Pyk2 and formation of complexes with Src. Resulting increase in cell migration was linked with activation of enzyme Rho GTPase, regulating Rho family adaptor proteins, including Src substrate Rho GAP (98).

Ezrin is a member of ERM (ezrin, radixin and moesin) family adaptor proteins linking membrane molecules to actin cytoskeleton. Ezrin has been implicated in various oncogenic cellular processes including invasion, migration, survival and its deregulation is thought to be an important factor in the development of metastatic disease (99). Overexpression of ezrin in prostate cancer has been associated with adverse prognostic factors, such as high Gleason score and seminal vesicle invasion (100). Invasive properties of prostate cancer cells in response to androgens could be mediated by increased expression and phosphorylation of ezrin at threonin and tyrosin residues (101). While ROCK and PKCα kinases are responsible for threonin phosphorylation, Src is thought to be the main factor in tyrosin phosphorylation of ezrin at several residues, including $Y^{145}$

and $Y^{353}$. Phosphorylation is preceded by physical association between Src and ezrin, binding SH2 domain of Src to putative phosphorylated tyrosine $Y^{190}$ of ezrin (102). Application of Src inhibitor PP2 reduced ezrin phosphorylation and decreased invasive capacity of androgen stimulated prostate cancer cells (101).

Discovery of metastatic suppressor KAI1/CD82 has underlined the importance of Src signalling in prostate cancer cell motility. Being a member of transmembrane 4 superfamily (TM4SF), KAI1/CD82 transmits extracellular stimuli to intracellular signalling cascades, thereby regulating variety of cellular processes involved in metastatic spread, including adhesion, migration and cytoskeletal rearrangement (103). In prostate cancer, down-regulation of KAI1/CD82 expression was found to correlate with high Gleason grade and advanced clinical stage (104;105). Several studies have attempted to answer the question why the loss of KAI1/CD82 results in increased metastatic potential in prostate cancer. In PC3 prostate cancer cell line, expression of KAI1/CD82 resulted in reduction of integrin-dependent tans-activation of tyrosine kinase receptor for hepatocyte growth factor, scatter factor (HGF/SF) c-Met (106). As previously mentioned, cross-talk between integrins and growth factor receptors can initiate downstream activation of Src/FAK pathway with subsequent recruitment of various Src substrates, including $p130^{cas}$. Decreased phosphorylation level of Src, FAK at tyrosine $Y^{861}$, and $p130^{cas}$ upon KAI1/CD82 expression led to significant reduction in migration and invasion. Using DU-145 prostate cancer cell line that is known to express SFK member Lyn rather than Src, Zhang et al. reported that reduced activation of Lyn/FAK was not involved in KAI1/CD82 signalling despite inhibition of $p130^{cas}$-CrkII complex formation (107). However, overall expression levels of Lyn and FAK were found to be increased in KAI1/CD82-positive DU-145 cells probably due to a compensation mechanism.

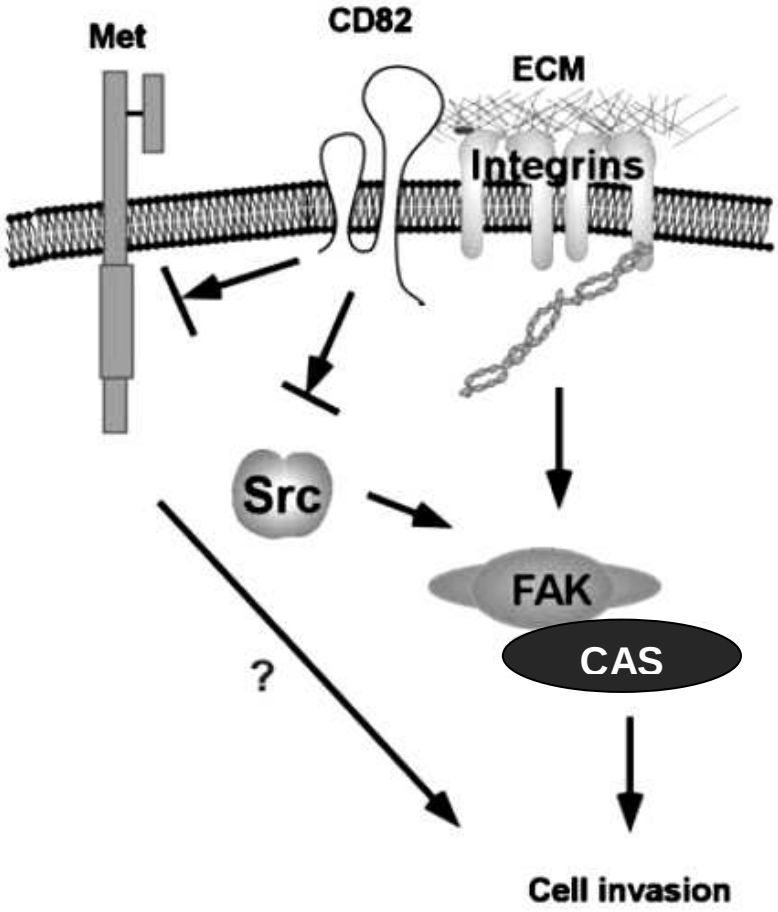



**Figure 15. The role of KAI1/CD82 in suppressing cell invasion. Adapted from Sridhar et al. (106). KAI1/CD82 limits integrin and ligand-induced activation of RTK c-Met. There is also independent suppression on Src, activated by integrins. Loss of KAI1/CD82 during late stage tumorigenesis results in increased c-Met and Src activation together with their downstream targets $p130^{cas}$ and FAK.**

## 1.3 SFK and RTK in Prostate Cancer

### 1.3.1 EGFR

Epidermal growth factor receptor belongs to the family of transmembrane proteins with tyrosine kinase activity consisting of four receptors: EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4). Protein expression studies have shown the correlation between EGFR expression and progression of prostate cancer to advanced disease and hormone independence (108-111), while the role of individual EGFR family members remain controversial (112). Although the exact nature of biological synergy between EGFR and SFK has not been identified, the cooperation appears to be an important factor contributing to aggressive tumour behaviour (113).

Ligand binding causes EGFR receptors to precipitate into homo- and hetero-dimers with other family members, followed by autophosphorylation at several tyrosine residues that serve as docking sites for various proteins, including SFK (114). EGFR/SFK complex formation leads to conformational rearrangements in Src protein thereby opening the catalytic domain for the interaction with downstream targets, among them EGFR itself (115). Within activation loop of EGFR catalytic domain, tyrosine $Y^{845}$ has been identified as Src-specific phosphorylation site and it is critical for the receptor function (116). EGFR phosphrylated at tyrosine $Y^{845}$ becomes a relay point, integrating various extracellular stimuli, including neuropeptides, cytokines, calcium, UV light, ionizing radiation etc. into complex intracellular molecular cascades (117-120).

Another important mechanism of cooperation between EGFR receptors and SFK is the regulation of receptor turnover by phosphorylation of the proteins responsible for endocytosis and ubiquitination, including clathrin, dynamnin and E3 ubiquitin ligase Cbl

(114). Ligand-bound membrane receptors are clustered to the clathrin-coated pits and internalised, which is then followed by dynamin-regulated detachment of endocytic vesicles from plasma membrane. Clathrin and dynamin, phosphorylated by SFK, are redistributed to the cell periphery, enhancing endosomal pool of the receptors, stimulating continuous receptor signalling from within the vesicles (121). Moreover, SFK-induced phosphorylation of Cbl, which is recruited to the activated EGFR in order to promote internalisation and degradation, results in Cbl deactivation, further increasing receptor availability for recycling (122).

Signals emanating from EGFR family receptors have been shown to activate AR and, as a result, promote proliferation and migration of prostate cancer cells. This is achieved by stimulation of androgen-independent AR transcriptional activity (123), increase of AR sensitivity to low concentrations of androgens (124), AR complex formation with intracellular signalling proteins (44) and direct AR phosphorylation (50).
SFK have been implicated in modulation of EGF-stimulated AR transcriptional activity and EGF-induced prostate cancer cell migration. Increase in AR transcriptional activity can be abrogated by selective SFK inhibitor SU6656 (50). The EGF-stimulated growth of C-81 prostate cancer cell line in androgen-depleted medium was suppressed by Src siRNA and AR siRNA, suggesting that proliferation of prostate cancer cells in androgen-depleted conditions is driven by EGF through Src signalling. In LNCaP prostate cancer cell line, EGF has been shown to trigger rapid Src/AR/ERβ complex assembly. Src-steroid receptor interaction is necessary for EGFR phosphorylation and can be inhibited by androgen antagonists (44;45).

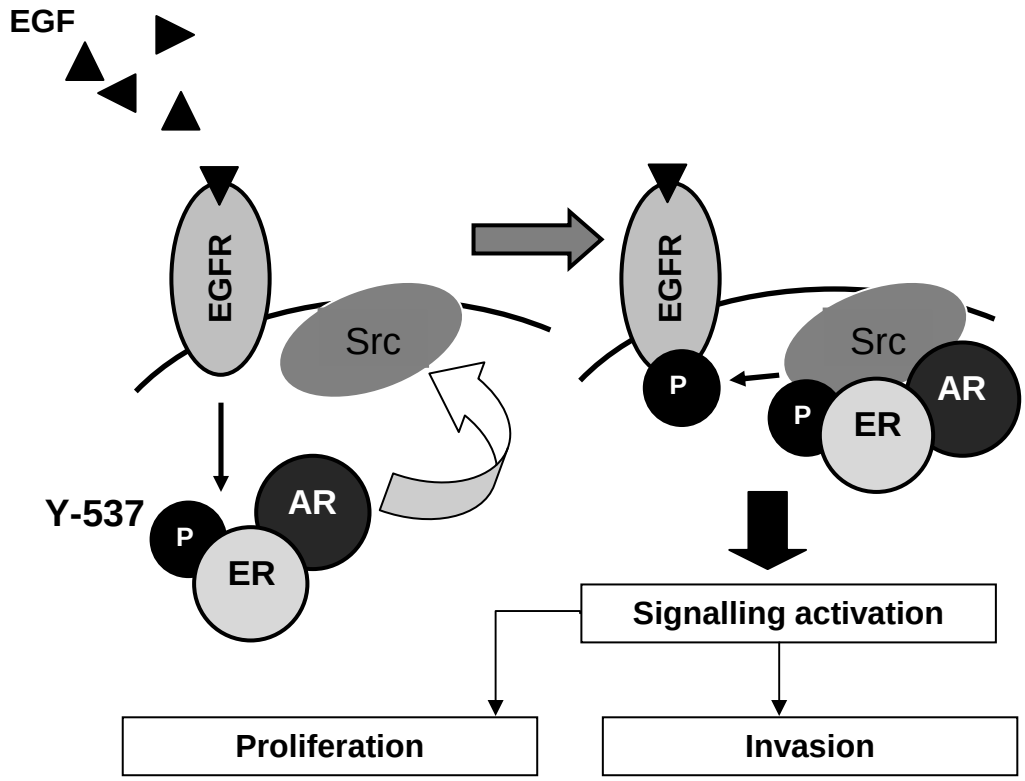



**Figure 16. Cross-talk between EGFR and steroid receptors mediated by Src. Adapted from Migliaccio et al. (125). Ligand-bound EGFR phosphorylates ER in complexes with AR at tyrosine $Y^{537}$. This creates a docking site for Src, which becomes activated by the release of intramolecular constrains. ER/AR/Src complex formation results in EGFR phosphorylation by Src, translating into further downstream signalling.**

### 1.3.2 IGFR

There is evidence that IGF family receptors play an important role in prostate carcinogenesis (126) and targeting IGF-related axis could potentially be beneficial in the management of castration-resistant prostate cancer (127). Two types of IGF receptors exist: IGF-1R and IGF-2R that bind two ligands, IGF-1 and IGF-2. IGF-1R represents a tetramer with higher affinity for IGF-1; it is made up of two α subunits that bind the ligand and two β subunits, possessing tyrosine kinase activity. Of two IGF receptors, IGF-1R is considered to be the main oncogenic factor as IGF-2R serves mainly as IGF-2 degradation mechanism, lacking tyrosine kinase domain. *In vitro* studies have demonstrated that IGF-1R downstream signalling via MAPK pathway may promote proliferation of prostate cancer cells whereas involvement of PI3K-Akt pathway is required to inhibit apoptosis (126).

Ligand-bound IGF-1R activates insulin receptor substrate protein (IRS), which displays multiple binding sites for SH2 homology domain-containing proteins, including SFK (12). Experiments using 3T3-L1 murine preadipocytes have shown that Src and Fyn mediate IGF-induced phosphorylation of adaptor protein Shc and MAPK activation (128). Introduction of dominant-negative Src abolished Shc and MAPK activation but not IRS, suggesting that SFK act upstream in relation to MAPK pathway. Evidence of SFK involvement in IGF-activated PI3K-Akt cascade has come from the studies of phosphatase and tensin homolog deleted on chromosome ten (PTEN) tumour suppressor that inhibits activation of Akt by IGF-1R. Stable overexpression of PTEN in PC-3 prostate cancer cells resulted in reduced IGF-1R synthesis, inhibition of proliferation and increased apoptosis (129). Rather than supressing PTEN enzymatic function, SFK seem to interfere with the

ability of PTEN to bind to the cellular membrane where its main activities take place (130).

Interaction between IGF system and steroid receptor signalling is thought to play an important role in prostatic carcinogenesis, although the precise mechanism of action is not completely understood (131;132). Androgens have been shown to up-regulate IGF-1R expression and IGF-1-induced Src/MAPK signalling in AR-positive prostate cancer cells (133). Interestingly, although AR requirement for this process was demonstrated by the lack of IGF-1R overexpression in AR-negative PC-3 cells, it did not depend on AR transcriptional activity. Furthermore, androgen-stimulated IGF-1R up-regulation was suppressed by both Src inhibitor PP2 and MEK-1 inhibitor PD98059. Activation of oestrogen receptor β (ERβ), known to form complexes with Src and AR in prostate cancer cells, can result in similar effect on IGF-1R expression and activation (134). Activation of ERβ by oestrogens increased IGF-1R sensitivity to IGF-1 and, as a result, stimulated downstream signalling via Src/MAPK and, to a lesser degree, PI3K-Akt cascades. The relationship between steroid hormones and IGF-1R appears to be mutual as IGF-1 has been shown to increase AR sensitivity to low concentration of androgens (135).

Castration-resistant prostate cancer cells are thought to rely on growth factors for their proliferative needs in androgen-depleted conditions and IGF signalling has been implicated in the development of CRPC (126). Krueckl et al. studied biological effects of IGF in LNCaP prostate cancer cells that are androgen sensitive and in the cell line C4-2, which was derived from parental LNCaP cells, capable of growing in androgen-depleted media (136). The authors concluded that under androgen-deprived conditions, the response to IGF stimulation was proportional to IGF-1R expression and that in LNCaP cell this effect was dependent on androgens, whereas C4-2 cells exhibited androgen-independent IGF-1R expression, activation and Akt signalling (137). IGF-1R can be trans-activated by nueropeptides, representing another potential mechanism of castration

resistance in prostate cancer. In TSU-Pr1 prostate cancer cell line, this process was mediated by Src and PI3K and inhibited by the expression of NEP (138).

### 1.3.3 VEGFR

Several growth factors, among them VEGF, promote angiogenesis, an important process required for the growth and spread of prostate cancer. VEGF system consists of VEGF family pro-angiogenic proteins (five main isoforms: A, B, C, D and E) and three types of trans-membrane receptors: VEGFR-1, VEGFR-2 and VEGFR-3. Biological responses resulting from VEGF signalling are mediated mainly through VEGFR-2 due to its high tyrosine kinase activity (139). VEGF family proteins and receptors have significant diagnostic, prognostic and therapeutic potential in prostate cancer and targeting VEGF with neutralising antibodies has been shown to reduce tumour progression in patients with prostate cancer and prevent formation of metastatic deposits (140).

The role SFK play in VEGF system is similar to the mutual relationship with other tyrosine kinase receptors. Downstream signals transmitted from ligand-bound VEGFR-2 can activate SFK, which in turn can lead to the increase in VEGF expression. Activated EGFR-2 undergoes autophosphorylation at several tyrosin residues, including $Y^{951}$ and $Y^{1212}$, creating consensus sequences for binding SH2 domain of Src (141;142). Gambogic acid, which is the main active substance in traditional Chinese medicine *Gamboge hanburyi*, has been shown to inhibit VEGFR-2 activation and downstream signalling *in vitro* and *in vivo* (143). Using human umbilical vein endothelial cells (HUVEC) and PC3 prostate cancer cell line, Yi et al. demonstrated that gambogic acid significantly inhibited angiogenesis, as indicated by new microvessel growth and tumour cell proliferation in PC3 xenografts in SCID mice. Furthermore, inhibition of VEGFR-2 phosphorylation by

gambogic acid was associated with reduced activation of Src and FAK, which correlated with inhibited proliferation and migration.

Although activated SFK do not seem to phosphorylate VEGFR-2 following physical association, unlike EGFR or IGFR, SFK are thought to up-regulate VEGF system by increasing VEGF protein expression. SFK-dependent pathways can be activated in response to various extracellular stimuli, including hypoxia or radiation that have been shown to compromise the effect of chemotherapy and radiotherapy in prostate cancer (144). Src, activated by hypoxia or radiation, has been demonstrated to phosphorylate signal transducer and activator of transcription proteins 3 (STAT3). Activated cytoplasmic STAT3 formed homodimers or heterodimers with STAT1 and translocated to the nucleus, binding to the target genes, thereby promoting transcription of various proteins, among them hypoxia inducible factor 1 alpha (HIF-1α). Up-regulation and stabilisation of HIF-1α by activated Src led to HIF-1α nuclear translocation, dimerisation with HIF-1β and physical association with transcription coactivators CREB binding protein/p300 (CDP/p300) and apurinic/apyrimidinic endonuclease 1/Redox effector factor 1 (APE1/Ref-1). Together these proteins formed transcription complex, binding to VEGF promoter and, thus, increasing VEGF expression (145).

SFK-induced VEGF expression in prostate cancer cells can be inhibited by soy isoflavones (146), adenoviral vector expressing the melanoma differentiation-associated gene-7 (Ad-mda7) (147) or overexpression of Src-suppressed C-kinase substrate (SSeCKS) (148). Soy isoflavones, the dietary components of soy-rich food, possibly contributing to the lower rate of prostate cancer in Asian population, have been shown to inhibit several protein tyrosine kinases, including Src (149). Pre-treatment with genistein, the most biologically active substance of isoflavones, can increase radiosensitivity of prostate cancer cells to radiotherapy, inhibiting molecular cascades stimulated by ionising radiation. Down-regulation of Src/STAT3/HIF-1α pathway by soy isoflavones in PC3 and

C4-2 castration-resistant prostate cancer cell lines, inhibited survival mechanisms and VEGF expression, which correlated with reduced expression of APE1/Ref-1and decreased DNA binding capacity of HIF-1α and NF-κB (146). In another experiment, treatment of LNCaP and DU-145 prostate cancer cells with Ad-mda7 inhibited Src kinase activity, which prevented STAT3 binding to VEGF promoter and resulted in reduced VEGF mRNA and protein level (147). Finally, tetracycline-stimulated re-expression of SSeCKS in MatLyLu prostate cancer cells prevented formation of lung metastases in nude mice by inhibiting VEGF expression and associated microvessel formation at metastatic sites, although the primary tumour growth was not significantly affected (148).

## 1.4 SFK Inhibitors

### 1.4.1 Development Strategies and Preclinical Studies

Recent advances in the research investigating the involvement of SFK in multiple signalling networks have led to the increased interest in the development of SFK inhibitors that can be used in the treatment of prostate cancer and other malignancies (150). Since SFK activation rather than total protein overexpression is considered to be the main driver of oncogenic processes, the development strategies have been focused on targeting specific activation mechanisms. The success so far has been achieved with the development of chemical compounds inhibiting catalytic activity of Src based on ATP competitive binding to the tyrosine kinase domain, inhibiting its enzymatic activity. The binding model was created according to X-ray crystallographic analysis of SFK members Src and Hck by determining spatial orientation of inhibitor molecule bound to the kinase domain (151). Progress in information technologies has now been used to increase the efficiency and accuracy of testing the chemicals that potentially could be used to inhibit SFK and other tyrosine kinases, based on complementing the traditional high-throughput screening with virtual analysis of computer-generated spatial images (152).

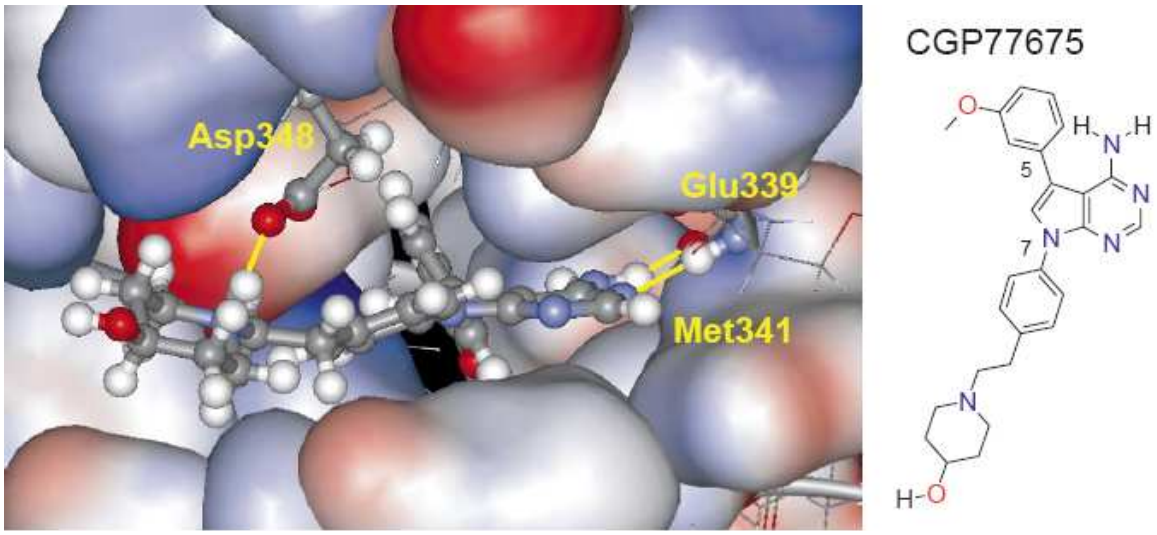



**Figure 17. Binding mode of Src inhibitor CGP77675 to the ATP binding pocket of Hck tyrosine kinase domain. Adapted from Suza et al. (151). The inhibitor is presented in ball-and-stick mode, whereas the aminoacids surrounding the ATP binding pocket are shown in surface mode. The amino acids, contributing to hydrogen bond interaction are shown in yellow. CGP77675 exhibits the characteristics of a typical tyrosine kinase inhibitor.**

At the time of writing, several catalytic Src inhibitors have progressed beyond the laboratory experiments and are currently undergoing the testing in clinical trials, among them dasatinib (BMS354825, Bristol Myers Squibb), bosutinib (SKI606, Wyeth) and AZD0530 (Astra-Zeneca). Dasatinib has emerged as a frontrunner; recently it has been given the US Food and Drug Administration Agency (FDA) approval for the treatment of adults with chronic myeloid leukaemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukaemia (Ph+ALL) with resistance to prior therapy. Dasatinib has shown effectiveness *in vitro* and *in vivo* using various cancer models, including lung cancer (153), cancer of pancreas (154), head and neck cancer (155) and human sarcoma (156).

*In vitro* experiments have demonstrated that dasatinib affected proliferation, adhesion, migration and invasion of various prostate cancer cell lines. Lombardo et al. reported inhibition of proliferation of PC3 cells by dasatinib with inhibitor concentration ($IC_{50}$) 9.4 nM, based on tetrazolium dye conversion test (157). Using LNCaP and DU-145 cell lines, Nam et al. showed that dasatinib inhibited adhesion, migration and invasion; this was associated with suppressed activity of SFK Src and Lyn (158). Interestingly, although downstream SFK/FAK/$p130^{cas}$ signalling pathway was also suppressed, the decrease in phosphorylation of STAT3, MAPK or Akt could not be demonstrated. PC3-derived cell lines PC-3MM2GL, which are AR-negative, and PC-3AR-A1cells, transfected with vector carrying functional AR, have been used to test the efficacy of dasatinib *in vivo* (18). Dasatinib treatment of mice implanted with androgen-sensitive PC-3AR-A1 as well as castration-resistant PC-3MM2GL cells resulted in significant suppression of both primary tumour growth and lymph node metastases.

AZD0530 is a highly selective orally available dual SFK/Abl inhibitor in clinical development for treatment of many solid tumours, including prostate cancer (159).

AZD0530 has shown anti-proliferative activity in vitro against PC3 prostate cancer cell line with $IC_{50}$ of 0.7 μM (160). Chang et al. has recently published a comprehensive study of molecular pathways involved in Src signalling in prostate cancer and immortalised normal prostate epithelial cell lines (161). The authors demonstrated that proliferation of DU-145 and PC3 prostate cancer cell lines was inhibited by AZD0530 due to down-regulation of cyclin D1, β-catenin, c-Myc and induction of G1/S cell cycle arrest. Interestingly, while in DU-145 cells cyclin D1 and c-Myc were regulated by Src/MAPK, PC3 cells utilised Src/Ras/Akt/GSK3β molecular pathway, although both cell lines did not display STAT3 involvement. As previously mentioned, cell migration was significantly inhibited by AZD0530 via Src/FAK/$p130^{cas}$ signalling. Administration of AZD0530 to SCID mice orthotopically implanted with DU-145 xenografts suppressed the tumour growth by 45% compared to controls. Finally, effectiveness of AZD0530 was demonstrated in neuropeptides-autocrine model of hormone-resistant prostate cancer, where the inhibitor suppressed Src-mediated AR transactivation by GRP (74).

Several other catalytic Src inhibitory compounds have shown effectiveness against prostate cancer in laboratory experiments. CGP76030 and CGP77675 (Novartis) pyrrolopyrimidine chemicals reduced proliferation, adhesion, migration and invasion of PC3 cell line, which was time- and concentration-dependent (16). This was the result of Src inhibition and down-regulation of the expression of MMP-9 and the tissue inhibitor of metalloproteinases 1 (TIMP-1). Pyrazolo[3,4-d]pyrimidines SI35 and SI40 were employed to study the role of Src activation in mediating EGF-induced migration using PC3 cell line model (162). These substances, structurally related to pyrazolo-pyrimidine-based PP1 and PP2 SFK inhibitors that have been extensively used *in vitro* (43;46;91), have proved effective in reducing growth factor-induced cell migration at concentrations corresponding to the inhibition of SFK activity. Although anti-proliferative activity was also shown,

much higher concentrations of Src inhibitors were required, suggesting possible non-specific mechanism of action.

Physical association between Src and its substrates provides an attractive targeting mechanism in the development of inhibitory compounds. Detailed structural analysis of Src docking sites has made it possible to produce short peptides that could be used to prevent the binding of substrates. Sequence-based peptide inhibitor KRX-123 has been designed to prevent the interaction between the substrates and HJ loop in the Lyn kinase domain, which in theory should improve the specificity of the inhibitor (15). *In vitro* and *in vivo* experiments using PC3 and DU-145 cell lines, conducted to establish the effectiveness of KRX-123 in prostate cancer, revealed inhibition of proliferation, induction of apoptosis and regression of tumour explants in nude mice. The inhibitor was shown to be Lyn-specific, inhibiting Lyn trans-phosphorylation in a dose-dependent manner with $IC_{50}$ of app.1µM, while phosphorylation of related SFK Lck and Fyn was not affected. Similar approach has been used to construct a short interfering peptide, mimicking AR proline-rich sequences, interacting with SH3 domain of Src (163). This 10 amino-acid long synthetic substance prevented AR/ER/Src complex assembly, affecting G1 to S cell cycle progression, cyclin D1 expression and DNA synthesis in AR-positive prostate cancer cells. The findings were further confirmed by in vivo experiments, where the inhibitor suppressed the growth of LNCaP xenografts in nude mice.

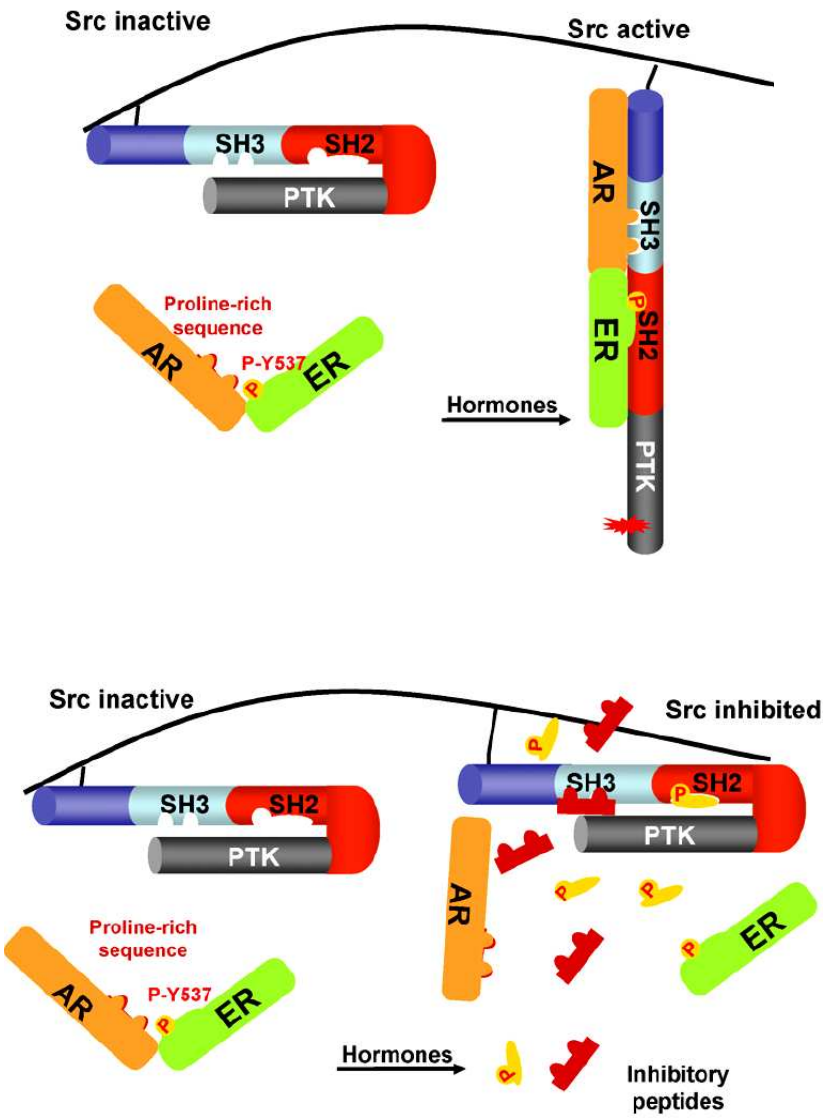



**Figure 18. Short peptides inhibit AR/ER/Src interaction. Adapted from Migliaccio et al. (164). In human prostate cancer cell androgens induce complex formation between ER/AR and Src. Src then becomes activated by the release of intramolecular constrains, followed by activation of downstream targets. ER/AR/Srs complexes could be disrupted by shirt peptides that mimic AR and ER docking sites on Src.**

### 1.4.2 Biomarkers of Sensitivity to SFK Inhibitors

Recent progress in the development of small molecule inhibitors has highlighted the importance of tailoring the treatment to the individual tumour profile due to significant heterogeneity of malignancies and difficulty predicting the therapeutic response. Biomarkers represent an important diagnostic and prognostic tool, used to test the likelihood of each particular individual with cancer responding to the inhibition of certain molecular pathway as well as monitor and correct the concentration of drugs to produce the desired biological effect. Gene microarray analysis has been used to identify *in vitro* and *in vivo* genomic signatures representing the genes up-regulated in the tumour cells responding to each particular therapeutic agent (165).

Identification and validation of AR genetic signature in prostate cancer patients, undergoing hormone deprivation therapy may allow more accurate estimation of individual hormonal status and intraprostatic DHT level (166). As expected, the application of AR signature revealed higher AR activity in untreated, hormone naïve tumours and lower AR activity in tumour samples, taken from the individuals undergoing hormone deprivation therapy and the patients with CRPC. More importantly, there was a significant correlation between reduced AR activity in CRPC samples and increased Src pathway activity, as defined by Src genetic signature (167), which was further supported by the increased predicted sensitivity to SFK inhibitor dasatinib.

Determining gene expression profiles in order to create genomic signatures has increasingly been used to separate patients' subsets according to the prognosis and the potential for targeting activated molecular pathways. Using large collection of human cancers, Bild et al. studied the patterns of gene expression and their clinical relevance with regards to disease outcomes (167). Several pathway deregulation signatures ware

identified, including Src, Ras and Myc signatures that were further confirmed by using specific SFK inhibitor SU6656. This Src signature was then applied to test the sensitivity of prostatic cell lines PC3 and MDAPCa-2b to the treatment with dasatinib *in vitro* and *in vivo* (168). While the proliferation of PC3 cells was suppressed by dasatinib, the treatment of MDAPCa-2b cells did not result in significant inhibition of proliferation. Genetic Src signature was then tested *in vivo* for the xenografts of these cell lines and was found to match perfectly with predicted anti-tumour activity.

As it is not always possible to obtain tumour tissue samples in clinical settings, the search for biomarkers has been concentrated on identifying the substances contained in the tumour and in the blood that would respond to the treatment in similar fashion. SFK inhibitors have the advantage in this respect as the peripheral blood mononuclear cells (PBMCs) contain abundant quantities of SFK and, therefore, provide sufficient read-outs of SFK activity (169). Activated form of Src pSrcY$^{419}$ has been proposed as a reliable biomarker of Src inhibition, allowing rigorous assessment of pharmacokinetic and pharmacodynamic properties of the drugs and their relationship with anti-tumour activity. Evaluation of pSrcY$^{419}$ inhibition by dasatinib in mice bearing PC3 xenografts revealed that similar inhibitor concentration and time were required to suppress Src activity in the tumour and in PBMCs. The effect of dasatinib on tumour growth was dependent on inhibition of Src activity, which was found to be similarly suppressed by dasatinib in tumour samples and in PBMCs (170).

### 1.4.3 SFK Inhibitors in Metastatic Bone Disease

Significant morbidity and mortality associated with bone metastases in advanced prostate cancer represent a serious clinical problem (171). Severe pain, pathological fractures, hypercalcaemia and spinal cord compression are the complications of metastatic

bone disease in prostate cancer, frequently limiting the lifespan of the patients. Propensity of prostate cancer cells to metastasise to the bones is well known and the relationship between tumour cells and bone microenvironment plays a pivotal role in this process (172). Although the introduction of chemotherapy, radiotherapy and bisphosphonates has improved the outcomes, it is the progress in our understanding of intricate molecular mechanisms, governing the processes involved in metastatic spread of tumours, provides the grounds for hope of cure and prevention (173).

The role of SFK signalling in normal bone physiology and metastatic bone disease was originally highlighted by the experiments with Src knockout mice that were surprisingly healthy apart from osteopetrosis and the lack of erupted teeth, due to deficient osteoclast function (174). The mice had high number of osteoclasts undergoing normal osteoclastogenesis, although mature cells failed to form ruffled membrane border necessary for bone resorption (175). At molecular level, SFK, activated by integrins in complexes with Pyk-2, phosphorylates various substrates, including Cbl and becomes involved in multiple cascades with receptor activator of nuclear factor κB (RANK) (176). Positive regulation of osteoclasts by SFK is complemented by reduced osteoblasts differentiation and increase in bone turnover (177).

In normal bone tissue, bone resorption by osteoclasts and new bone formation by osteoblasts exist in equilibrium, which is disrupted by tumour invasion. Resulting lesions could be osteoblastic or osteolytic in nature, depending on predominant activation of osteoblasts in former or osteoblasts in latter . Although bone metastases in prostate cancer are mostly osteoblastic, the initial stages of metastasis formation are accompanied by the increase in osteolytic activity (178). In addition, osteolytic processes occur in the background of skeletal metastases in prostate cancer. Once the tumour deposits become established in the bones, cancer cells release growth factors into the bone microenvironment, including transforming growth factor β (TGFβ), fibroblast growth

factor (FGF), IGF, PDGF etc. Growth factors stimulate Src-mediated osteoclast activity, leading to further bone destruction and release of biologically active substances from the bone matrix, stimulating the proliferation and migration of tumour cells (179). This positive feedback mechanism, frequently described as a ‘vicious circle’ of bone metastases, can be targeted by Src inhibitors due to the role Src plays in cancer, angiogenesis and bone metabolism (180).

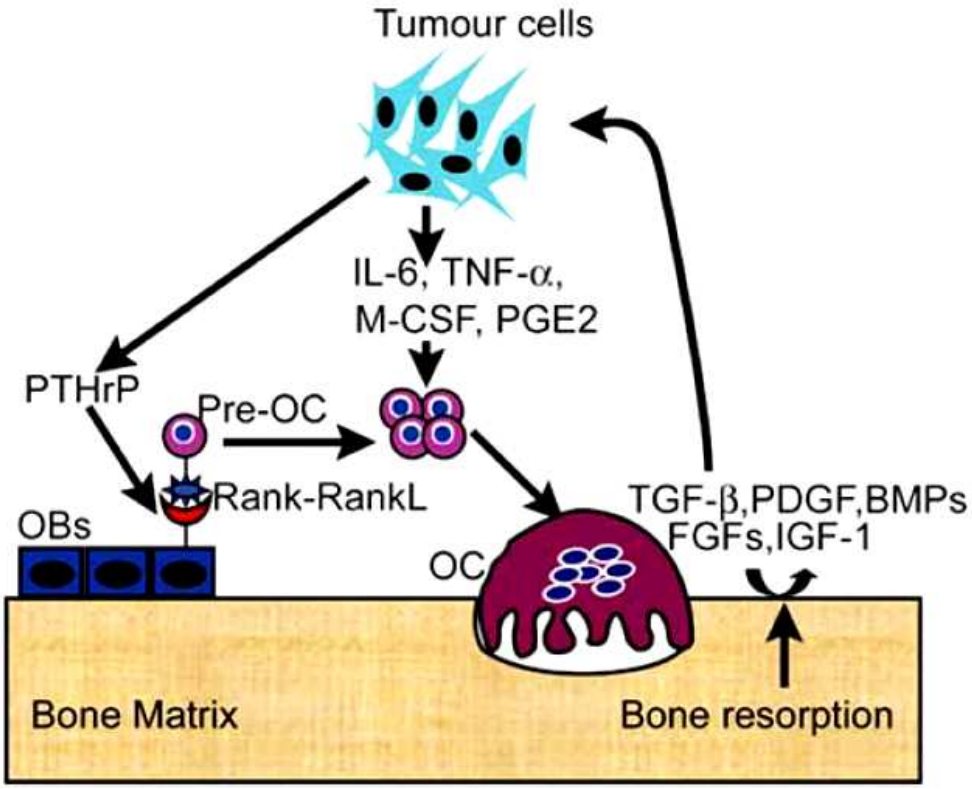



**Figure 19. 'Vicious circle' of bone metastases. Adapted from Rucci et al. (181). Tumour cells, established in bone marrow, produce various substances (IL-6, TNFα, M-CSF, PGE-2, PTHrP) which stimulated osteoclastogenesis (OC) directly or by increasing RANKL expression in osteoblasts (OB). Increase in osteoclasts formation leads to bone resorption followed by the release of growth factors from the bone matrix (BMPs, PDGF, IGF-I, FGF etc.) that stimulate tumour growth.**

Small molecule SFK inhibitors with anti-tumour activity have been studied to define whether they could be used for the treatment or prevention of metastatic bone disease. In pre-clinical experiments, dasatinib inhibited proliferation of osteoclasts in bone marrow cell culture and reduced bone resoption in animal model using thyro-parathyroidectomised (TPTX) male Sprague-Dawley rats (182). Using mouse fetal calvarial explants and isolated rabbit osteoclasts, de Vries et al. demonstrated that Src inhibitor AZD0530 suppressed osteoclasts activity and osteoclastogenesis, implying that SFK activity is essential for the initial phase of osteoclasts formation. Furthermore, the inhibitor prevented osteoclasts migration to the bone surface and disrupted formation of actin rings and resorption pits (183). CGP77675, pyrrolopyrimidine analogue that was used to inhibit proliferation and migration of bone metastasis-derived PC3 cell line (16), was shown to inhibit bone resorption in rat foetal long bone cultures and prevented bone loss in young ovariectomised rats (184). Purine-based compounds AP22161, AP22408 and AP23541 (ARIAD) represent a series of Src inhibitors, targeting SH2 domain and, thus, preventing protein-protein interaction. These chemicals incorporate a bisphosphonate group that confer bone-specific properties, achieving relative tissue selectivity (185-187).

### 1.4.4 SFK Inhibitors in Prostate Cancer Clinical Trials

Phase I and phase II clinical trials are now under way exploring potential efficacy of Src inhibitors dasatinib and AZD0530 in patients with castration-resistant prostate cancer. As a result of the FDA approval of dasatinib for the use in imatinib-resistant chronic myeloid leukaemia, the side effect and toxicity are best described for dasatinib. Reassuringly, the toxicity has been relatively mild, with pleural effusion accounted for the majority of patients who had to come off the phase I study in solid tumours (188).

Preliminary results for dasatinib and final results for AZD0530 as single agents in phase II trials in patients with castration-resistant prostate cancer have now been published. While the final results from phase II study of AZD0530 as a monotherapy did not show any clinical efficacy, the preliminary results from phase II study using dasatinib are more encouraging.

AZD0530 was tested in phase I clinical trials and was proven to well tolerated by healthy volunteers in doses up to 1000 mg. Further single dose escalation resulted in diarrhoea and vomiting, while the subjects taking lower doses over longer period experienced rash, flu-like symptoms, myalgia, arthralgia, headache, mild diarrhoea and raised creatinin (189). Another phase I study of AZD0530 in patients with cancer revealed acceptable tolerability with minimal side effect and positive response using FAK and paxillin phosphorylation as biomarkers. Paired tumour biopsies were obtained, before and after treatment that were stained by immunohistochemical method with antibodies against phosphorylated FAK and paxillin. Blind assessment of biopsy staining by pathology panel confirmed the modulation of phosphorylation and cellular localisation of selected biomarkers, consistent with AZD0530 therapy (190).

Despite promising pre-clinical data and the results of phase I studies, AZD0530 did not seem to be clinically effective in phase II clinical trial as a monotherapy in patients with hormone-resistant prostate cancer. 28 patients were recruited altogether, with 9 patients previously had taxane-based therapy and failed. PSA reduction greater than 30% was chosen as a primary end-point and the dose of 175mg of AZD0530 once daily was the treatment regimen. Although the majority of patients tolerated the treatment well, 5 cases of grade 3 toxicities were recorded, including liver toxicity, nausea, vomiting, lymphopenia and 1 patient unexpectedly died from various co-morbidities. Only 5 patients had transient PSA reduction with none achieving PSA response criteria. Based on strong

pre-clinical evidence, the authors concluded that further studies are warranted, perhaps as a combination therapy, or earlier stages of prostate cancer should be targeted (191).

Preliminary results from phase II trial of dasatinib as monotherapy in metastatic castratio-resistant prostate cancer are now available (192). The patients' cohort included 46 subjects with biochemical evidence of hormone relapse showing castrate levels of testosterone with no prior chemotherapy. Disease progress was measured using response evaluation criteria in solid tumours (RECIST) taking into account the composite of PSA levels and bone scan appearances. Urinary N-telopeptide estimation as a measure of bone metabolism, was determined on a 4 weekly basis. The disease stabilised with the treatment in 10(67%) patients out of 15, who could be evaluated using RECIST criteria. 27 patients had bone scan at 12 weeks, in 16(59%) patients metastatic disease was stable and in 1 patient it improved. PSA doubling time was better in 29(80%) patients out 36 evaluated and in 1 patient who had more than 2 PSA measurements, PSA declined from 19.3 to 3.3 ng/ml. Bone metabolism slowed in 21(57%) out of 37 evaluable patients as demonstrated by the decrease in urinary N-telopeptide by more than 35%. Several clinical trials are now under way to investigate the efficacy of dasatinib in CRPC in combination with prednisolone, docetaxel, VEGFR monoclonal antibody bavacizumab and as neo-adjuvant therapy with LHRH analogue therapy prior to radical prostatectomy (193).

## 1.5 Hypothesis and Statement of Aims

Src family kinases have been shown to play an important role in the development of prostate cancer and progression of prostate cancer to castration resistance. Multiple *in vitro* experiments have shown that SFK activation is necessary for the prostate cancer cells to migrate and invade. However, it has not been known whether the increase in SFK expression or activity in patients with prostate cancer has any effect on tumour growth or clinical parameters, including survival. As SFK inhibitors are now in Phase II clinical trials for the treatment of castration-resistant prostate cancer, translational studies of SFK in prostate cancer patients are urgently required.

We therefore hypothesised that SFK activation in the transition of prostate cancer from hormone-sensitive disease to castration-resistant state is associated with negative prognosis and stimulates the development of bone metastases.

**Project aims:**

1. To expand pre-existing database of paired prostate tumour samples, taken from the prostate cancer patients prior to androgen-deprivation therapy and following the development of castration resistance.
2. To select and optimise antibodies for the detection of SFK expression and activation in human prostate tumour samples, using paraffin-embedded sections.
3. To correlate SFK immunostaining with clinical parameters, including survival and the presence of bone metastases.
4. To characterise hormone-sensitive LNCaP and its counterpart LNCaP-SDM castration-resistant cell lines.

5. To investigate the effect of androgen deprivation on SFK activity in LNCaP and LNCaP-SDM cell lines.
6. To examine the application of SFK inhibitor dasatinib in proliferation and migration studies using prostate cancer cell lines.

# MATERIALS AND METHODS

## 2.1 Development of Clinical Database

Ethical approval was obtained for the retrospective recruitment of patients from Multiple Research Ethics Committee for Scotland (MREC) and, subsequently, applications were submitted to the Local Research Ethics Committees (LREC) for the site-specific assessment. Permission was granted to recruit by LREC NHS Greater Glasgow (Gartnavel General Hospital, Southern General Hospital, Western Infirmary and Royal Alexandra Hospital) and NHS Lanarkshire (Monklands Hospital, Hairmyres Hospital and Wishaw General Hospital). Patients' details were initially obtained from the local pathology departments at each site by performing pathology database searches, selecting prostate cancer patients undergoing palliative 'channel' trans-urethral resection of prostate (TURP). Medical records of selected patients were obtained and detailed analysis was performed according to the strict selection criteria to identify those who could be added to the existing database.

Each patient in the cohort was required to have prostate tissue samples taken prior to hormone deprivation therapy by means of TURP or trans-rectal ultrasound scan-guided (TRUS) biopsy of prostate and more than 50% fall in PSA as an indicator of response to androgen deprivation therapy. TRUS biopsies consisted of minimum 12 cores from both left and right prostatic lobes with minimum 4 cores taken from the transition zone. Subsequent, second line hormonal therapy was then administered to which the patient did not respond and PSA concentrations continued to rise above the PSA nadir to concentrations greater than 0.2ng/ml. The second prostate tissue sample was taken during 'channel' TURP, performed to relieve the symptoms of bladder outflow obstruction, after failure to respond to the second line hormonal therapy and following at least 2 sequential rises in PSA level above 0.2ng/ml, indicating biochemical relapse.

Clinical information, recorded from the case sheets, included the dates of diagnosis, biochemical relapse, death or last follow-up. Type of androgen deprivation therapy was noted, whether it was LHRH agonists or surgical orchidectomy. Depending on the results of the bone scan, the patients were considered to have locally advanced or metastatic disease and the results of repeat bone scan were considered for the patients who developed bone metastases whilst on treatment. Pathological data consisted of pathology database numbers, required to retrieve the tissue blocks, Gleason grade of hormone naive and hormone refractory tumours and the type of procedure used to harvest the tissue (TRUS biopsy or TURP). Information was also recorded on patients receiving radiotherapy with the intent of cure or for palliative purposes as well as secondary anti-androgen therapy was noted. The majority of patients died by the time the data was analysed, the details of death, including the date and causes of death were recorded from clinical case notes if available or by contacting the Registry Office. The patients who were alive at the time of analysis of data, the date of last follow-up (censored data) were used to calculate survival.

## 2.2 Immunohistochemistry

### 2.2.1 Preparation of Tissue Sections

Following the retrieval of formalin-fixed paraffin-embedded tissue blocks from pathology archives, each set was assigned a number for coding purposes and identification of patients was removed in order to make tissue slides anonymous. It was deemed necessary for this project to use full sections and not tissue microarrays due to the heterogeneous nature of prostate cancer. Prior to cutting tissue sections, the blocks were cooled on the tissue cooler to $-10^0$C. Using Leica RM 1235 microtome, 5 µm tissue sections were cut and applied onto silane-coated glass slides. The treatment of glass slides

with aminopropyltriethoxysilane was necessary to produce greater adherence of the tissue section to the glass. Silane coating was done by immersing the glass slides in acetone for 5 minutes, followed by exposure to 2% silane for 5 minutes. The excess of silane was removed by washing the slides in running water and after keeping the glass slides in tap water for 20 minutes, the slides were allowed to dry in the fume hood overnight.

### 2.2.2 Immunohistochemistry Principles and Protocol

Immunohistochemistry (IHC) is a tissue staining technique based on the binding of specific antibodies to antigens and detection of this binding in order to localise proteins in cells. IHC is widely employed in clinical practice and in basic science research to identify and localise differentially expressed proteins in biological tissues. Visualising of antigen-antibody reaction is accomplished by several methods, the most common being indirect immunoperoxidase technique. Initially, the antigen must be exposed to the primary antibody, which can be done by immersing tissue sections in citrate buffer under increased pressure conditions. Primary antibodies, which could be monoclonal or polyclonal, depending on whether they are raised from the single or several clones of plasma cells, are subsequently applied. The next stage involves reaction of primary antibody with biotinylated secondary antibody, labelled with enzyme horseraddish peroxidase (HRP). Finally, visualisation is accomplished upon addition of 3,3'-Diaminobenzidine (DAB), which reacts with HRP to produce an insoluble, brown coloured product.

Prostate tissue sections were dewaxed in xylene and rehydrated in graded alcohol. Antigen retrieval was performed under pressure for 5 min in citrate buffer (pH adjusted to 6.0). Endogenous peroxidase was inactivated in 0.3% hydrogen peroxide for 10 min and the slides were blocked using 1.5% horse serum. Incubation with primary antibody was performed in humidified chamber overnight at $4^0$C (anti-phospho $SrcY^{419}$ 1:250 and anti-phospho $SrcY^{530}$ 1:100, Cell Signalling Technologies, FGR 1:100, Abgent and Lyn 1:10, BD Biosciences) or for 1 hour at room temperature for total Src (1:1000, Cell Signalling Technologies). Tissue was then incubated in Envision solution (DAKO, UK) for 30 min and developed by application of 3,3'-diaminobenzidine (DAB) as a chromogen (DAKO, UK). Sections were counterstained with haematoxylin, dehydrated through graded alcohol and xylene and mounted in DPX. In each immunohistochemistry run LNCaP cell pellets and colon tissue is included as positive controls and an isotype matched antibody is used on colon and prostate samples to provide a negative control.

### 2.2.3 Histoscoring method

All immunostained tumour sections were quantified blindly by two independent observers, using a weighted histoscore method at high magnification (x400). Membrane and cytoplasm staining were scored separately. Specific staining in each tissue section was allocated 0 (no staining), 1 (weak intensity of staining), 2 (moderate intensity), or 3 (strong intensity). The final score (maximum 300) is calculated from the sum of:

$$(1 \times \%\ weak\ staining) + (2 \times \%\ moderate\ staining) + (3 \times \%\ intense\ staining)$$

## 2.3 Cell Culture

Prostate cancer cell lines LNCaP and LNCaP-SDM were a kind gift from Professor C Robson (Northern Institute for Cancer Research, Newcastle). LNCaP cells were routinely maintained in RPMI 1640 (Invitrogen, UK) containing phenol red and supplemented with 10% foetal calf serum (Invitrogen, UK), and 1% glutamine. LNCaP-SDM cells have been developed using parental LNCaP cells as a model of hormone resistant prostate cancer by gradual withdrawal of androgens from the medium. These cells were routinely cultured in RPMI 1640 supplemented with 1% glutamine and 10% charcoal-stripped foetal calf serum (Cambrex, UK) known to contain negligible amount of androgens (194).

Liquid nitrogen was used to cryopreserve the cells that were grown, split and frozen in order to use early passages for each experiment. To facilitate the detachment of the cells, routinely kept in 250 ml plastic flasks, after washing with PBS the cells were exposed to trypsin and then suspended in culture medium. Approximately $1x10^6$ cells were

placed in the centrifuge and pelleted at 1500 rpm for 10 minutes. The cell pellets re-suspended in freezing medium (10% dimethylsulfoxide, 20% foetal calf serum and 70% culture medium), placed in the freezer at -20$^0$C, and, after several hours, transferred into the liquid nitrogen, where the cells were kept until required. To raise the cell from frozen, the cryotubes containing the cells were retrieved from the liquid nitrogen and rapidly defrosted in water bath for 30 sec at +37$^0$C. Defrosted cell pellet was then transferred in to the flask, containing appropriate culture medium and placed in the incubator.

## 2.4 Cell Count

### 2.4.1 Cell Count Method

Trypan blue exclusion method was used to count the cells exposed to DHT. 25 µl of cell suspension was mixed with the same amount of trypan blue (Sigma, UK) and the mixture was loaded into Neubauer haemocytometer. The cells were counted under light microscope (magnification x100) excluding non-viable cells stained blue. Total number of cells was calculated according to the following equation:

$$\frac{Number\ of\ cells\ per\ square}{4} = Total\ Number\ of\ Cells \times \frac{10^4}{ml}$$

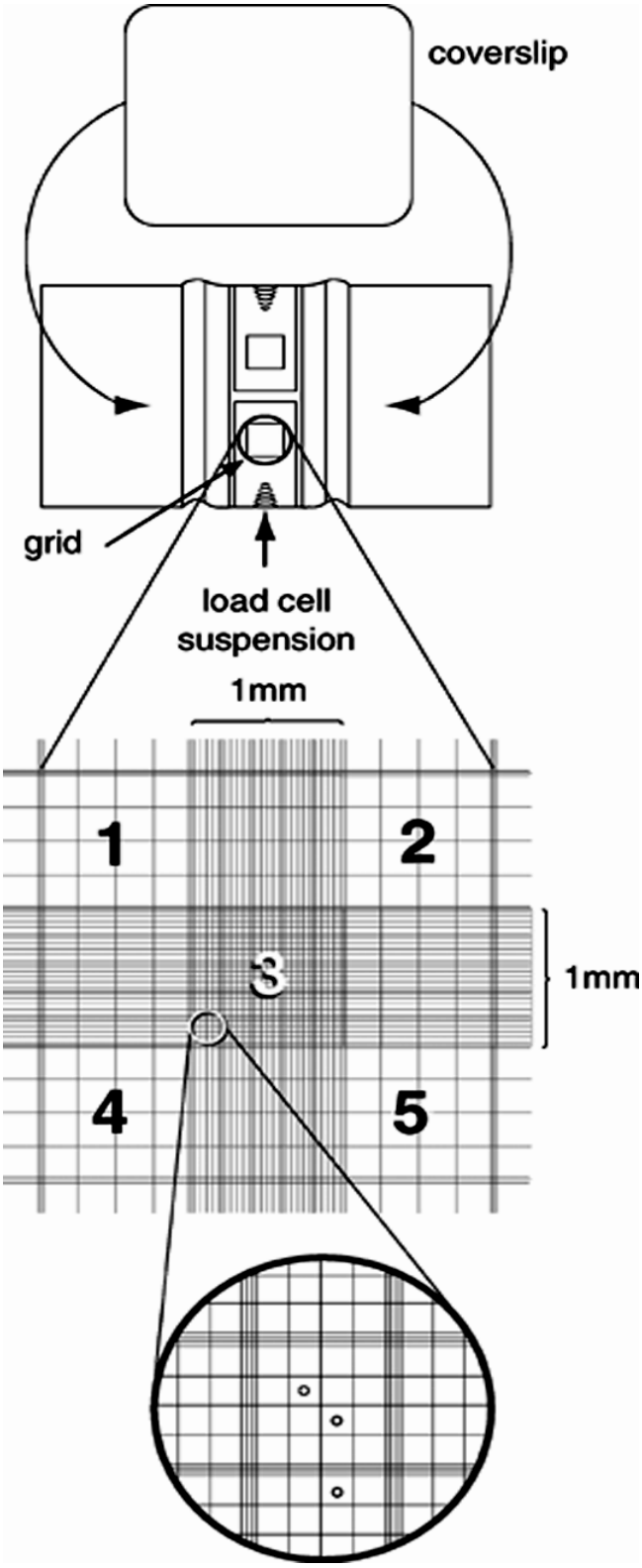



**Figure 1. Cell count using Neubauer haemocytometer. The cells counted in each of the 4 corne squares and the total number of the cells calculated using the equation above.**

### 2.4.2 DHT Stimulation

LNCaP and LNCaP-SDM cells were seeded in 24 well plates at concentration $5x10^4$ cells per well. 48 hours prior to seeding, full foetal calf serum was changed for LNCaP cells to charcoal-stripped foetal calf serum in order to eliminate endogenous steroids. LNCaP-SDM cells were kept in the medium containing charcoal-stripped foetal calf serum routinely. The cells were allowed to attach overnight and the following day each 4 wells were treated with the range of DHT concentrations (0.1 nM, 1 nM, 10 nM, 100 nM). Control wells for both cell lines contained the medium with either charcoal-stripped foetal calf serum or full foetal calf serum. The following 3 days, the cells were counted using trypan blue exclusion method and the final result was the average of 4 wells. The experiment was repeated 3 times.

## 2.5 Western Blot

### 2.5.1 Determination of Protein Concentration

Cells were grown in standard Petri dishes to the required density. The dishes were place on ice, the medium removed and the cells were washed in ice-cold PBS twice. Lysis buffer was then applied and the cells were removed using cell scrapers. The tubes containing cell lysates were kept in ice for further 30 minutes prior to spinning at 12000 rpm for 10 minutes in centrifuge at $+4^0$C. The supernatant was separated and used for further experiments.

Protein concentration was determined using bicinchoninic acid (BCA) protein assay prior to western blot to ensure equal protein loading. This method is based on the BCA

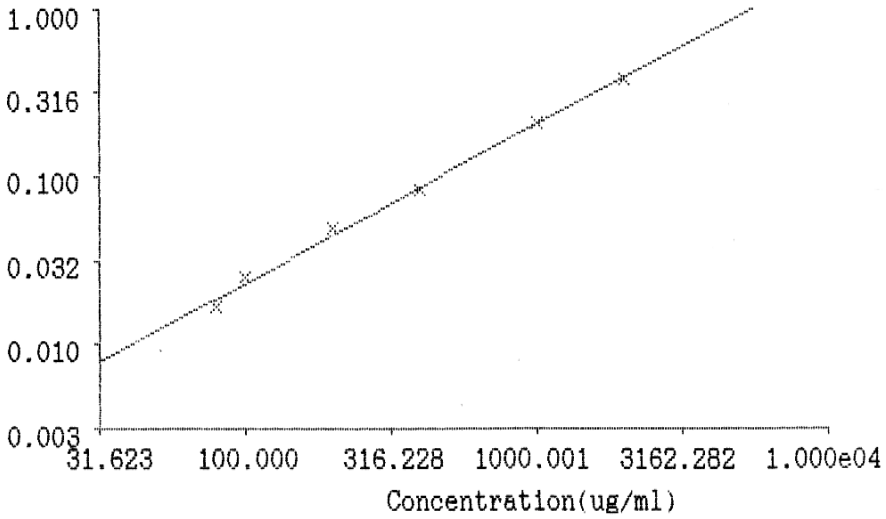
ability to react with complexes between coppers ions and proteins to produce a molybdenum/tungsten blue product. Briefly, when copper ions from copper sulphate ($CuSO_4$) are added to protein, $Cu^{2+}$ is reduced to cuprous cation ($Cu^{1+}$). BCA then reacts with $Cu^{1+}$ and the purple-coloured reaction product is formed by the chelation of two BCA molecules with one molecule of $Cu^{1+}$. BCA/$Cu^{1+}$ complex is water soluble and can be quantitatively read on a 96 plate reader with a filter set at 562 nm.

$$Cu^{2+} + protein \rightarrow Cu^{1+}$$

$$Cu^{1+} + 2\,BCA \rightarrow Cu^{1+}\,BCA\ complex\ (purple)$$

Protein standards were prepared using bovine serum albumin (BSA) in order to create standard curve, plotting relative light absorbance versus protein concentration. 96 plate reader was programmed to develop the standard curve and determine protein concentration.

O.D. versus Concentration




CURVE FITTING

LOG-LOG POWER CURVE FIT WITH TAILS
Y = -3.5187 +0.9419 X

R-SQR = 0.994

Trial #1

STANDARDS

| # | LABEL | LOC. | O.D. | CONC. | PRED.CONC. | %ERROR |
|---|---|---|---|---|---|---|
| 1 | | B5 | 0.016 | 80.000 | 68.946 | 13.818 |
| 2 | | B6 | 0.025 | 100.000 | 109.869 | 9.869 |
| 3 | | B7 | 0.049 | 200.000 | 222.930 | 11.465 |
| 4 | | B8 | 0.082 | 400.000 | 383.987 | 4.003 |
| 5 | | B9 | 0.206 | 1000.000 | 1018.373 | 1.837 |
| 6 | | B10 | 0.378 | 2000.000 | 1938.404 | 3.080 |

**Figure 2. Standard curve with protein concentrations**

### 2.5.2 Western Blot Protocol

Cell lysis was performed in radio-immune precipitation assay (RIPA) buffer (50 mM Tris pH7.6, 150 mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate and 1:100 Calbiochem protease inhibitor cocktail set 1) and protein concentration determined using BCA/$CuSO_4$ assay as described above. 40 µg of protein per well was resolved by 4-12% gradient Bis-Tris gel electrophoresis (Invitrogen, UK) alongside PageRuler pre-stained protein ladder (Fermentas, UK); proteins were transferred to nitrocellulose membranes (Millipore, UK). The membranes were blocked for 1 hour in 5% BSA in TTBS and probed with primary antibodies, diluted in 3% BSA/TTBS: anti-phospho $SrcY^{419}$ (1:10000), anti-total Src (1:10000 Cell Signaling Technologies, UK), anti-phospho $FAKY^{397}$ (1:10000), anti-phospho $FAK^{861}$ (1:10000 Biosource, Belgium), anti-total FAK (1:10000 BD Biosciences, UK), anti-phospho $PaxillinY^{118}$ (1:1000 Biosource , Belgium), anti-total Paxillin ( 1:10000 BD Biosciences, UK) at $4^0$C overnight. Membranes were then incubated with secondary antibodies (anti-rabbit 1:5000 or anti-mouse 1:5000, Cell Signalling Technologies) and visualized with ECL kit (Amersham, UK). Where necessary, the membranes were stripped by incubating with Re-Blot Plus stripping buffer (Chemicon, UK) before re-probing with other antibodies including anti-αTubulin (1:8000 Santa Cruz, USA) to confirm equal protein loading.

## 2.6 Steroid Exposure and Withdrawal

This experiment was conducted to determine the effect of exposure and withdrawal of androgens on SFK activity in prostate cancer cell lines. Charcoal-stripped foetal calf

serum, known to contain negligible concentration of androgens, at the same time providing the necessary growth factors, was used as a model of hormone deprivation. LNCaP and LNCaP-SDM cells were seeded in standard culture media at a density of $1x10^6$ per 9 cm dish and allowed to attach overnight. The following day, the cells were washed twice in PBS and the medium was changed. Each cell line was exposed to three types of media: serum-free medium, steroid depleted medium containing 10% charcoal-stripped serum and medium containing 10% full foetal calf serum. After 60 hours, which allows for the metabolism of intracellular steroid hormones, cell lysates were prepared and western blot analysis performed as described above. The membranes were probed with anti-phospho $SrcY^{419}$ (1:10000) and, after stripping, re-probed with anti-total Src (1:10000 Cell Signaling Technologies, UK) and anti-αTubulin (1:8000 Santa Cruz, USA) to confirm equal protein loading.

## 2.7 Src Inhibitor Exposure

LNCaP and LNCaP-SDM cells were seeded at a density of $1x10^6$ per 9 cm Petri dish and cultured using standard culture media until 80% confluent and then treated overnight with a range of dasatinib concentrations. Cell lysates were prepared and western blot analysis performed as described above. The membranes were probed with anti-phospho $SrcY^{419}$ (1:10000), anti-total Src (1:10000 Cell Signaling Technologies, UK), anti-phospho $FAKY^{397}$ (1:10000), anti-phospho $FAKY^{861}$ (1:10000 Biosourse, Belgium), anti-total FAK (1:10000 BD Biosciences, UK). Dasatinib was provided by Bristol Myers Squibb and made up as 10 mM stock in dimethyl sulfoxide (DMSO). Once the minimal concentration that inhibited Src activity was determined, the cells were treated with the range of dasatinib concentrations.

## 2.8 Immunoprecipitation

Immunoprecipitation method is based on the interaction between the antibody and the protein, antibody-protein complex formation, the separation of the complex by immunoglobulin (Ig), which reacts with the antibody and subsequent analysis by Western Blot. In this experiment, IgG agarose beads were used to provide solid phase support for immunoprecipitation. Cell lysates were prepared as described in previous sections and samples containing 500 µg of protein in 500 µl incubated with anti-total Lyn (1:50 BD Biosciences) or anti-total Src (1:100 Cancer Research UK) at $4^0$C overnight. Then, anti-mouse IgG agarose beads (Sigma, UK) were added to the samples (20 µl per sample) and further incubated for 1 hour at $4^0$C. Agarose beads were separated from supernatant by pelleting in centrifuge at 10000 rpm for 3 min and washed 3 times in lysis buffer. After adding sample buffer and loading dye, immune complexes, released by heating the samples at $95^0$C for 5 min, were analysed as per western blot protocol with anti-phospho $SrcY^{419}$ antibody (1:10000 Cell Signaling Technologies).

## 2.9 Migration Assay

### 2.9.1 Random Migration

To follow random migration, LNCaP and LNCaP-SDM cells were plated at a low density ($5x10^4$) on glass-bottomed 6-well plates (Iwaki, Japan), allowed to attach overnight and then treated with increasing concentrations of dasatinib, diluted in standard culture media. Migration was monitored by video time-lapse microscopy for 24 hours using a Zeiss Axiovert S100 microscope with x20 objective powered by AQM Advance software (Kinetic Imaging, UK). To quantify the ability of the cells to spread on glass surface and the effect of dasatinib on cell spreading, the protrusions produced by the cells in each field was counted and the number of protrusions per cell was plotted in a column graph.

### 2.9.2 Wound Healing

Wound healing assay was carried out to investigate the ability of prostate cancer cells to migrate into a denuded area in the presence or absence of dasatinib. LNCaP and LNCaP-SDM cells were plated at a density of $1x10^6$ cells per well of a glass-bottomed six-well plate (Iwaki, Japan). The following day, the wounds through the confluent cells monolayer were made using a fine pipette tip, three wound per well. The medium was then replaced with standard for each cell line culture medium containing increasing concentrations of dasatinib. The assay was performed over 24 hours with images taken from 3 fields per each well every 30 minutes using a Zeiss Axiovert S100 microscope at x20 magnification. Cell migration was measured by subtraction of the distance between the edge of the wound at time 0 and 24 hours using ImageJ software.

## 2.10 Proliferation Assay

Proliferation was assessed using the WST-1 assay (Chemicon, UK) as per manufactures instructions. The assay is based on the enzymatic cleavage of the tetrazolium salt WST-1 to a water-soluble formazan dye by cellular mitochondrial dehydrogenases. Overall activity of mitochondrial dehydrogenases increases proportionally to the number of viable cells. This leads to the increase in tetrazolium salt conversion to formazan dye, which correlates directly to the number of metabolically active cells. Following the addition of the reagent to the cell culture, the colour intensity changes over time and this can be measured by light absorbance using multiwell spectrophotometer at 450 nm. Plotting the absorbance level against time produces a parabolic curve, indicating that after a period of time a constant increase is reached. Measurements are taken at this point, although the cells could be incubated for longer if higher sensitivity is required.

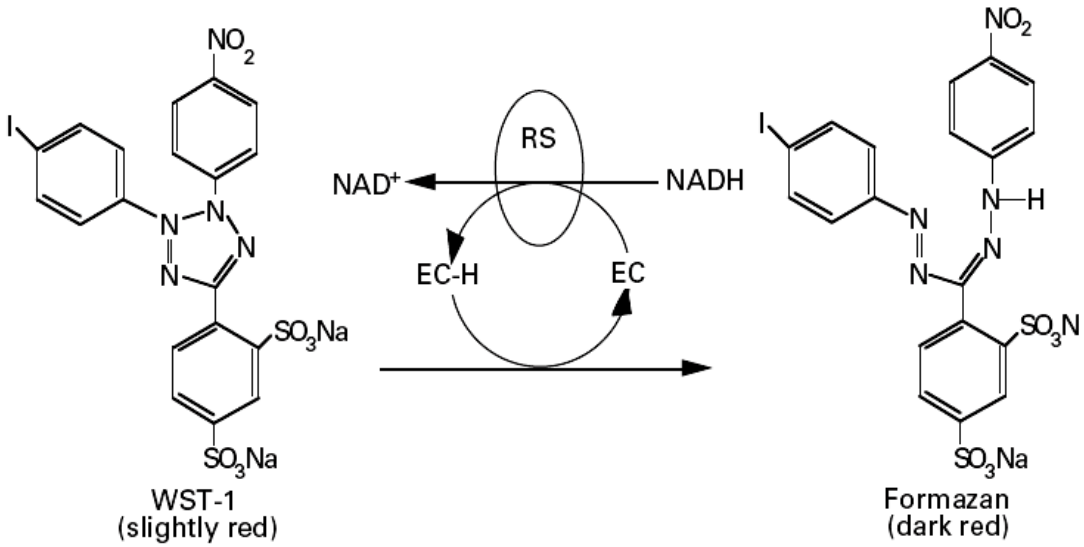



**Figure 3. Cleavage of WST-1 to formazan**

Cells were seeded in 96 well plates at a density $5x10^3$ cells per well in standard culture media, allowed to attach overnight and the following day treated with increasing concentrations of dasatinib, DMSO was added to the control well. The assay was performed at 48, 72 and 96 hours by adding 10 μL of WST-1 reagent prior diluted in Electro Coupling Solution (ECS) to each well. The optical absorbance level was measured after 2 hours incubation at $37^0$C using a 96 well microplate reader at 450 nm with reference wavelength 600 nm. The measurements were made from 4 readings in each condition and the experiment was repeated 3 times with consistent results.

To study the effect of androgens on proliferation in the presence of dasatinib, LNCaP and LNCaP-SDM cells were plated in standard culture media for 24 hours after which LNCaP cells were placed in the steroid depleted medium containing 10% charcoal stripped fetal calf serum and LNCaP-SDM cells in RPMI1640 supplemented with 10% full fetal calf serum. WST-1 assay was performed at 48, 72 and 96 hours as described above.

## 2.11 Statistical Analysis

Statistical analysis was performed using Microsoft Excel and the SPSS statistical package (version 15.0). Small changes in protein expression between paired ASPC and CRPC tumours could be due to random errors in the assessment of histoscores. To identify individual patients in whom there was strong evidence of a genuine rise or fall in protein expression, it was required that the change in expression exceeded a threshold equal to two standard deviations of the inter-observer difference for that protein. This threshold was chosen because, if there was in reality no difference in protein expression between ASPC and CRPC tumours in a given patient, there would be only a 5% probability of an apparent difference being observed that exceeded the threshold due to

random variation. This assumes that the random variation between two different observers assessing the same tumour is of a similar magnitude to the random variation that would affect a single observer assessing two different tumours with the same level of protein expression. Changes in protein expression in individual patients that exceeded this threshold were termed significant. Wilcoxon Signed Rank Tests were used to compare expression between ASPC and CRPC tumours. Survival analysis including time to relapse, time to death from relapse and overall survival was conducted using the Kaplan-Meier method and curves were compared with the log-rank test.

# RESULTS

## 3.1 Cohort Description

Clinical data, recorded for each patient (Table 1) included age (median 70, inter quartile range (IQR) 66-73), PSA at diagnosis (median 23.2 ng/ml, IQR 4.6-35.6), PSA at relapse (median 10 ng/ml, IQR 4-11), Gleason grade at diagnosis (median 7, IQR 4-9) and Gleason grade at relapse (median 8, IQR 6-10). All patients developed biochemical relapse (median time to relapse 2.53 years, IQR 1.57-4.43 years). At last follow-up, 34 patients had died of prostate cancer and 12 patients had died of other causes, median follow up was 8.3 years and inter quartile range was 5.2-9.1 years. Following diagnosis, 10 % (5/50) of patients underwent surgical orchidectomy and 90% (45/50) received LHRH analogue initially and then anti-androgen therapy in addition. Following biochemical relapse 64% (32/50) patients received radiotherapy, no patients in the current cohort received taxane therapy.

| Features | Median Value | Inter-Quartile Range (IQR) |
|---|---|---|
| **Age** | 70 years | 66-73 |
| **PSA at Diagnosis** | 23.2 ng/ml | 4.6-35.6 |
| **PSA at Relapse** | 10 ng/ml | 4-11 |
| **Gleason Grade at Diagnosis** | 7 | 4-9 |
| **Gleason Grade at Relapse** | 8 | 6-10 |
| **Time to Relapse** | 2.53 years | 1.57-4.43 |
| **Follow-up Time** | 8.3 years | 5.2-9.1 |

**Table 1. Main features of patients' cohort.**

**Table 2. Relationship between main features of the cohort and survival.**

| Features | Time to relapse, P value | Time to death from relapse, P value | Disease-specific survival, P value |
|---|---|---|---|
| **Age** | 0.809 | 0.137 | 0.434 |
| **PSA at Diagnosis** | 0.063 | 0.568 | 0.664 |
| **PSA at Relapse** | N/A | 0.953 | 0.045 |
| **Gleason Grade at Diagnosis** | 0.473 | 0.026 | 0.013 |
| **Gleason Grade at Relapse** | N/A | 0.890 | 0.781 |
| **Mets at Diagnosis** | 0.182 | 0.013 | 0.002 |
| **Mets at Relapse** | N/A | 0.001 | 0.012 |

In the table above, survival analysis presented according to the main features of the cohort. There was significant association between the presence of metastatic disease and survival. Gleason grade at diagnosis had an impact on time to death from relapse and disease-specific survival, although it did not influence time to relapse. Age and PSA at diagnosis did not demonstrate any relationship with survival characteristics and only PSA at relapse was associated with reduction in disease-specific survival.

## 3.2 Immunohistochemistry

In order to determine the level of Src, Lyn and Fgr expression and activation in the transition from ASPC to CRPC we used one hundred paraffin embedded prostate tissue sections taken from fifty patients with prostate cancer who underwent hormone-deprivation therapy and subsequently developed hormone relapse. Due to tissue heterogeneity, full prostate tissue sections were stained using antibodies against total Src, Lyn, Fgr and phospho $SrcY^{419}$ representing an active form of SFKs and phospho $SrcY^{527}$, a marker suggestive of inactive Src. The sections were double scored and ICCCs were calculated for each protein at cytoplasm and membrane. From the table below it is evident that ICCCs, determined for each protein, were consistently above level of 0.7, classed as excellent. Scatter graphs and Bland-Altman plots were used to confirm that there was no observer bias when the sections were immunohostoscored.

| **Cellular Locations** | **$pSrcY^{419}$** | **$pSrcY^{527}$** | **Total Src** | **Fgr** | **Lyn** |
|---|---|---|---|---|---|
| **Cytoplasm** | 0.94 | 0.89 | 0.88 | 0.91 | 0.88 |
| **Membrane** | 0.92 | 0.95 | 0.93 | 0.87 | 0.83 |
| **Nucleus** | N/A | N/A | N/A | 0.78 | 0.84 |

**Table 3. ICCC determined for each protein.**

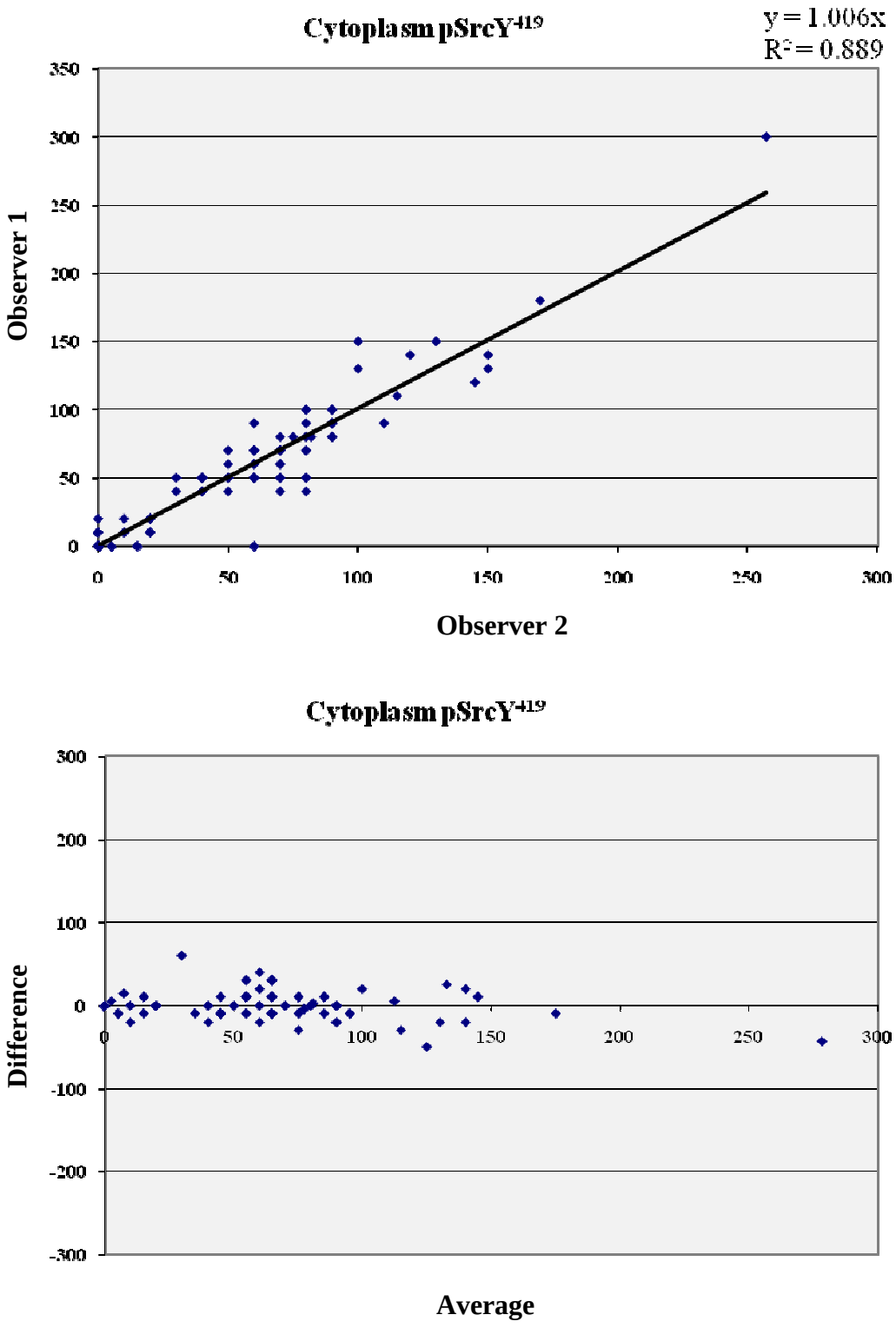



**Figure 1. Scatter graphs and Bland-Altman plots for cytoplasmic pSrcY$^{419}$ based on inter-observer variation. The range and distribution of scores confirmes appropriate inter-observer correlation.**

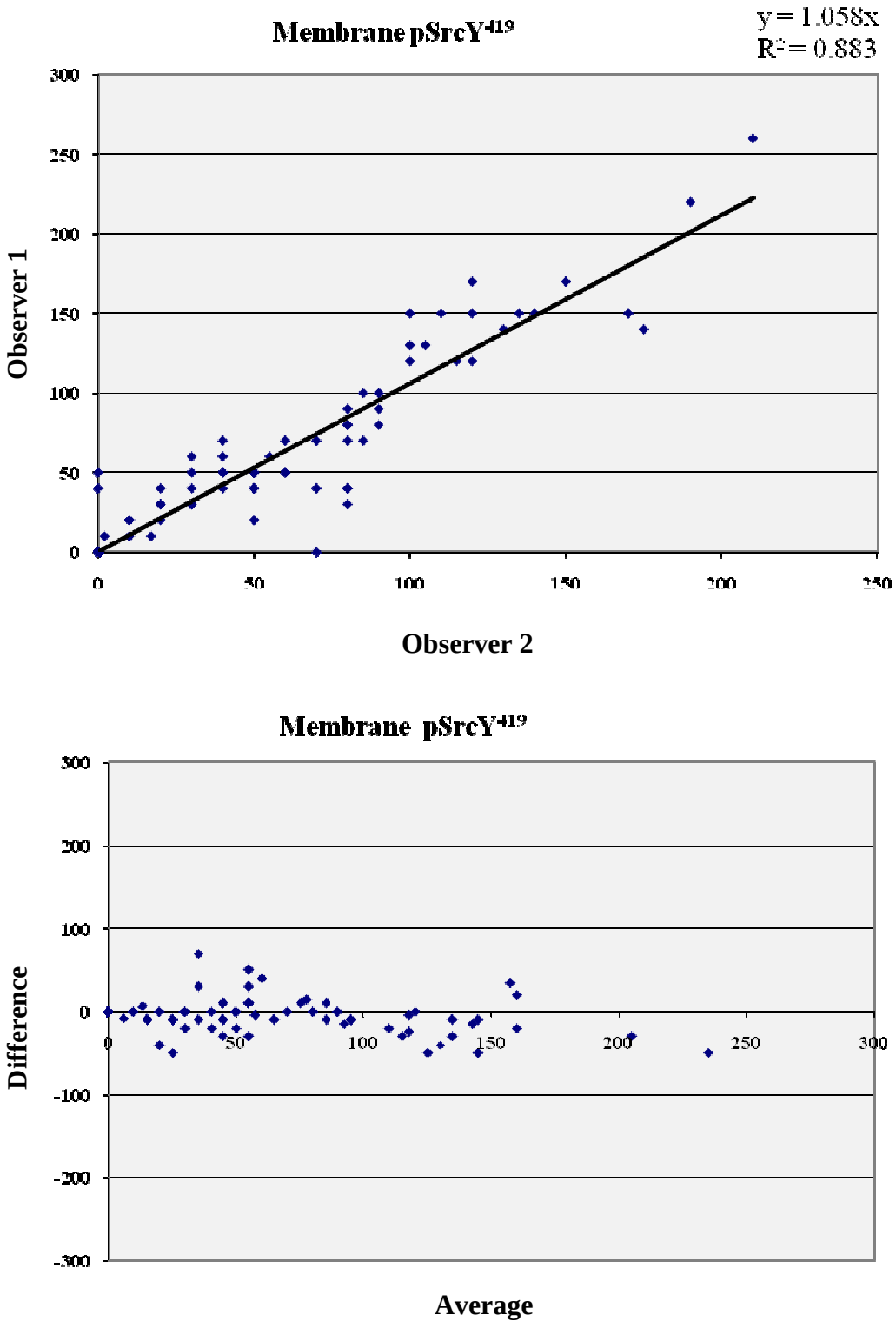



**Figure 2. Scatter graphs and Bland-Altman plots for membrane pSrcY$^{419}$ based on inter-observer variation. The range and distribution of scores confirmes appropriate inter-observer correlation.**

**Table 4. SFK protein expression profiles.**

| Antibody | Histoscores | | Number of Patients | | | P value |
|---|---|---|---|---|---|---|
| | ASPC | CRPC | Increase | Fall | No change | |
| **pSrcY$^{419}$** | | | | | | |
| cytoplasm | 45 (18.75-80) | 70(40-90) | 13(26%) | 3(6%) | 34(68%) | 0.035 |
| membrane | 20(0-60) | 50(0-90) | 14(28%) | 3(6%) | 33(66%) | 0.017 |
| **pSrcY$^{527}$** | | | | | | |
| cytoplasm | 80(50-112.5) | 50(20-90) | 6(12%) | 18(36%) | 26(52%) | 0.002 |
| membrane | 50(20-80) | 20(0-70) | 13(26%) | 21(42%) | 16(32%) | 0.101 |
| **Total Src** | | | | | | |
| cytoplasm | 130(100-180) | 100(80-126.3) | 4(8%) | 16(32%) | 30(60%) | 0.009 |
| membrane | 55(20-116.3) | 55(30-110) | 10(20%) | 13(26%) | 27(54%) | 0.926 |
| **Total Lyn** | | | | | | |
| cytoplasm | 28(5-70) | 50(20-80) | 17 (34%) | 8 (16%) | 25 (50%) | 0.094 |
| membrane | 38(10-80) | 70(35-95) | 25 (50%) | 6 (12%) | 19 (38%) | 0.005 |
| **Total Fgr** | | | | | | |
| cytoplasm | 65(39-100) | 50(30-78) | 8 (16%) | 15 (30%) | 27 (54%) | 0.104 |
| membrane | 95(69-112) | 90(63-108) | 8 (16%) | 14 (28%) | 28 (56%) | 0.567 |

The sequences surrounding Src autophosphorylation site tyrosine $Y^{419}$ are highly conserved in the majority of SFK and positive staining using this antibody represents autophosphorylation not only of Src but also other Src family members. There was a significant increase in overall pSrc$Y^{419}$ immunostaining in the transition from ASPC to CRPC, more intense cytoplasm and membrane staining was observed in CRPC samples compared to ASPC. In addition, although the majority of matched specimens on an individual patient basis showed no change in expression of phospho Src$Y^{419}$ in the transition from ASPC to CRPC, a subgroup of patients did have tumours that exhibited a rise in expression as determined by the weighted histoscore technique (Figure 1).

**ASPC** **CRPC**

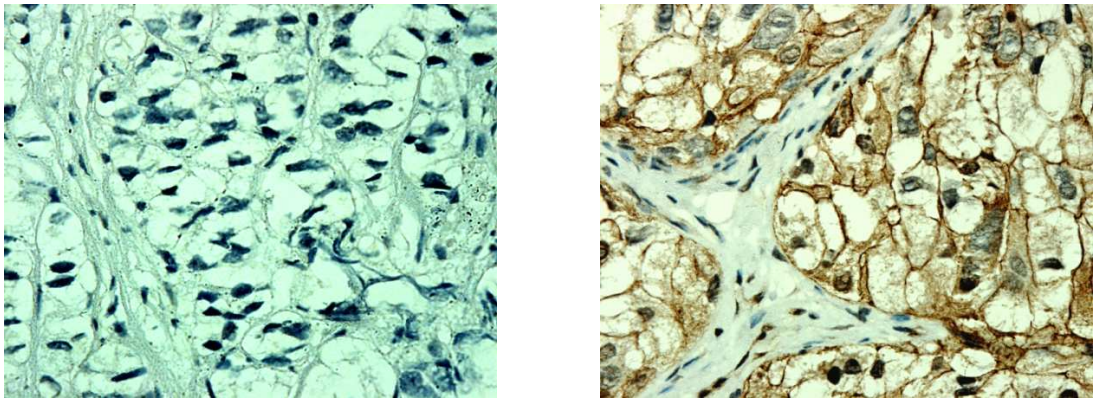


**Figure 3. Examples of pSrcY$^{419}$ immunostaining in specimens from a patient exhibiting an increase in Src activity in the transition from ASPC to CRPC. Magnification x400.**

Small changes in protein expression between paired ASPC and CRPC tumours could be due to random errors in the assessment of histoscores, therefore the mathematic method as described in the methods statistical analysis section was employed for selection of patients with genuine rises in expression. Changes in protein expression in individual patients that exceeded this threshold were termed an increase in expression. Using this method 13(26%) and 14(28%) patients were noted to have an increase in expression of activated SFKs (phospho SrcY$^{419}$) in the cytoplasm and membrane respectively (Table 2).

In addition, there was an increase in overall Lyn membrane immunostaining in the transition from ASPC to CRPC (Figure 1); more intense cytoplasm and membrane staining was observed in CRPC samples compared to ASPC (although only membrane staining reached significance). No change in expression was observed for Fgr and, interestingly, expression of total Src and phospho SrcY$^{527}$in the cytoplasm fell significantly after the patients developed hormone relapse whereas the membrane staining did not change.

### 3.2.1 SFK Expression and Clinical Parameters

Following the diagnosis of prostate cancer, the majority of the patients (46 or 92%) had bone scans, in 8 patients (16%) it was positive, implying the presence of bone metastases, and in 38(76%) it was negative. After the development of CRPC, the patients, who had positive bone scans at diagnosis and no bone scan after relapse were considered as having bone metastases as well as those who had negative bone scans at diagnosis and positive scans at relapse (26(52%)). The membranes in the tumour samples taken from patients with CRPC stained more intensely with the anti-phospho $SrcY^{419}$ antibody if there was evidence of bone metastases (P=0.011). No significant correlation was observed between the presence of bone metastases and cytoplasmic staining with antibodies against phospho $SrcY^{419}$, phospho $SrcY^{527}$, total Src, Fgr or Lyn.

The cohort was then further subdivided into groups according to Gleason grade at diagnosis and PSA value at relapse, important prognostic criteria in patients with prostate cancer. High or low PSA at relapse were not associated with IHC histoscores and only expression of cytoplasmic Lyn in the hormone refractory tumours was associated with Gleason grade (p=0.029).

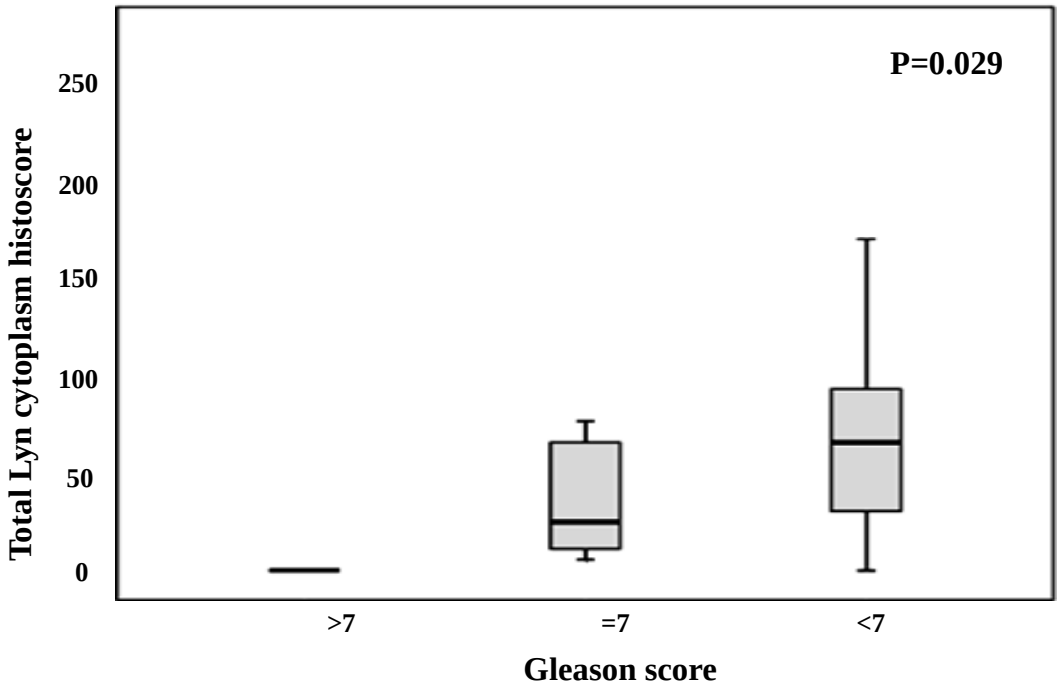



**Figure 4. Correlation of total Lyn histoscore with Gleason score in CRPC samples. There was a significant positive correlation between cytoplasmic total Lyn histoscores and Gleason grade of prostate cancer speciments.**

### 3.2.2 Survival Analysis

Use of paired prostate cancer specimens taken from patients prior to commencement of hormone manipulation therapy and after androgen escape, made it possible to correlate IHC scores of each individual patient with various survival parameters including time from diagnosis to biochemical relapse, time from relapse to death and overall survival. Statistical analysis revealed that a subset of patients, who had an increase in SFK activity (phospho $SrcY^{419}$ expression) at the membrane in the transition from ASPC to CRPC, had a significantly shorter time to relapse, median time from diagnosis to hormone escape in this subgroup of patients was 1.86(IQR 1.36-2.47) years compared to 2.98(IQR 1.93-4.63) years for those who had decreased membrane signal or no change (P=0.005).

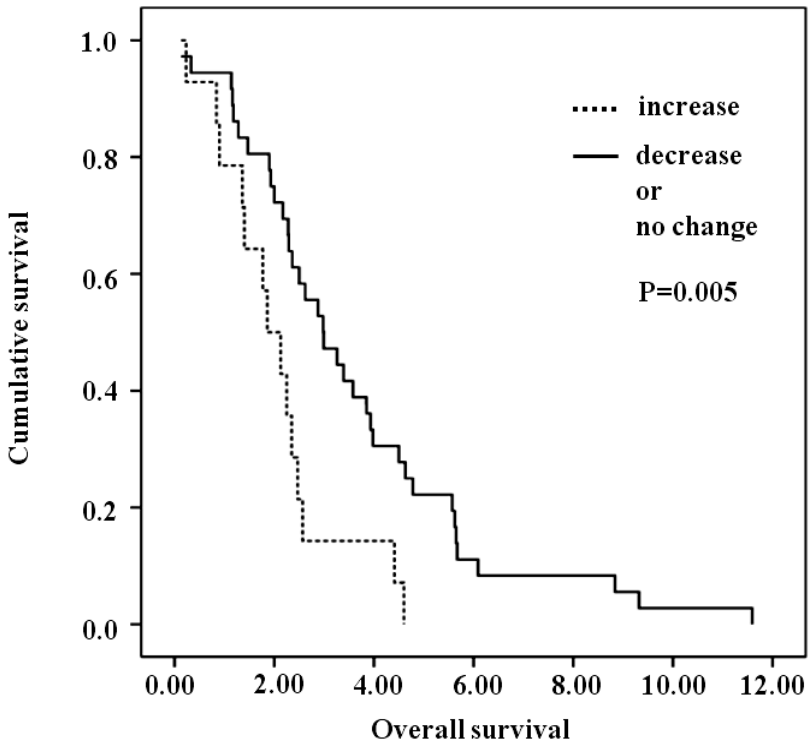



**Figure 5. Kaplan-Meier survival curve plotting the time from prostate cancer diagnosis to hormone relapse. The patients, who had an increase in Src activity developed hormone escape significantly earlier than those who had a decrease or no change in Src activation.**

Furthermore, the time from biochemical relapse to death was also reduced: 1.14 (IQR 0.58-2.15) years versus 1.87 (IQR 1.1-1.86) years (P=0.011).

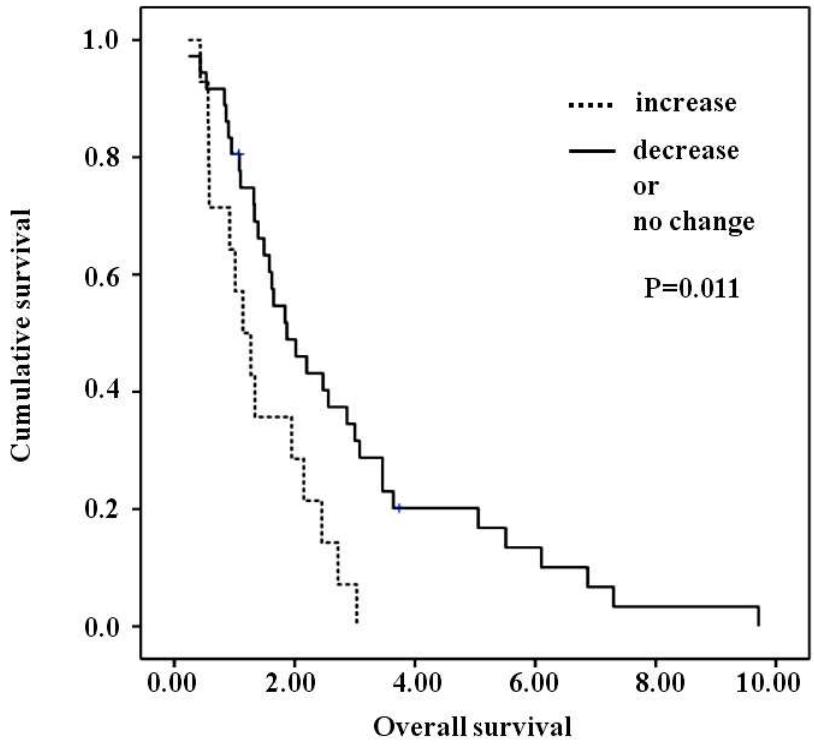



**Figure 6. Kaplan-Meier survival curve plotting the time from prostate cancer relapse to death. The patients, who had an increase in Src activity had significantly shorter time from hormone relapse to death than those who had a decrease or no change.**

Therefore, the combination of reduced time to relapse combined with reduced time to death following relapse resulted in a significant reduction in overall survival, median survival for those with an increase in membrane phospho $SrcY^{419}$ expression was 2.91(IQR 2.42-4.92) years compared to 6.33(IQR 4.15-7.27) years for those patients with tumours that had a decrease or no change in phospho $SrcY^{419}$ expression (P<0.0001).

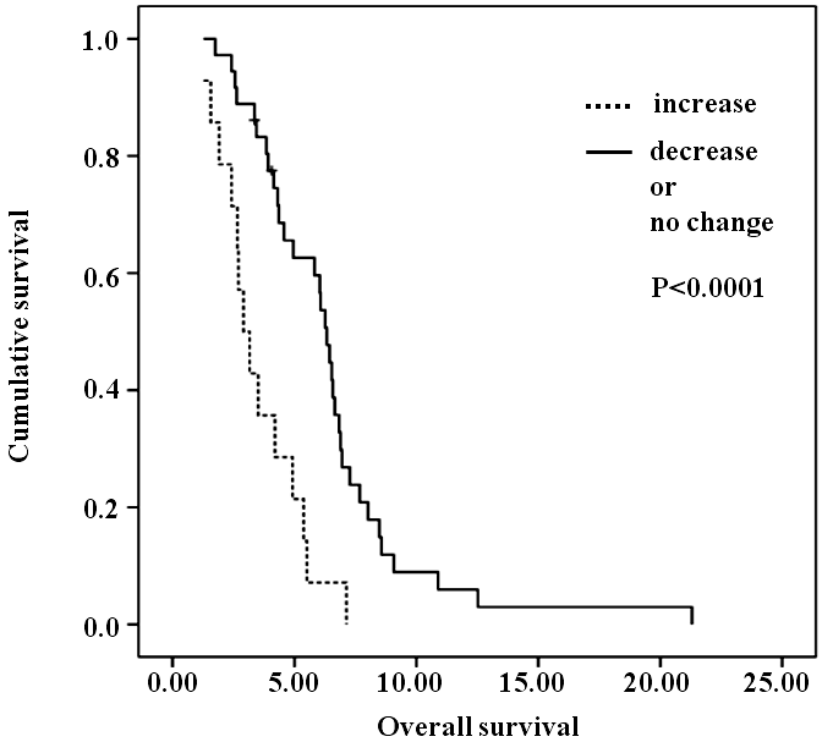



**Figure 7. Kaplan-Meier survival curve plotting the time from prostate cancer diagnosis to death. The patients, who had a decrease or no change in Src kinase activity lived significantly longer than those who had an increase in Src activity.**

However, when multivariate Cox regression analysis was performed for the patients with an increase in SFK activity taking into account the presence of metastases at relapse and Gleason score, SFK activity was noted to be independently significant only for the time from relapse to death (P=0.018) and overall survival (P=0.01).

No association with the time to relapse or survival was observed for Fgr, total Src and phospho $SrcY^{527}$, however an increase in membrane Lyn expression was associated with a shorter time to relapse (P=0.022). Median time from diagnosis to hormone escape in this subgroup of patients was 2.29(IQR 1.78-2.79) years compared to 3.2(2.45-3.99) years in those who had a decreased membrane signal or no change. This was also observed for an increase in cytoplasmic Lyn expression, median time from diagnosis to hormone escape in this subgroup of patients was 2.41(IQR 2.01-2.73) years compared to 3.2(IQR 1.98-4.45) years in those who had decreased membrane signal or no change (P=0.040).

However, multivariate Cox regression analysis demonstrated that increase in Lyn expression was not independently significant when the presence of metastases at relapse and Gleason score were considered. No association with time from biochemical relapse to death or overall survival were observed.

## 3.3 LNCaP and LNCaP-SDM Cell Lines

### 3.3.1 Androgen Stimulation

LNCaP cell line is an established model of hormone sensitive prostate cancer; whereas LNCaP-SDM cell line has been developed using parental LNCaP cells by gradual reduction of androgens in the medium, thus simulating androgen withdrawal during hormone deprivation therapy. In order to confirm androgen sensitivity of these cell lines, androgen stimulation experiment was conducted. Both cell lines were exposed to the medium containing charcoal stripped foetal calf serum, full foetal calf serum and a range of DHT concentration (0.1 nM, 1 nM, 10 nM, 100 nM). Cells were counted following 48, 72 and 96 hours (Figure 6).

Cell count showed that the cell line reacted differently to the DHT exposure. LNCaP cells displayed dose-dependent increase in the number of cells at 48 hours time point. At 72 and 96 hours intervals, this pattern continued with the exception of cells exposed to 100 nM of DHT the number of which did not increase, probably due to DHT toxicity. In contrast, the number LNCaP-SDM cells did not increase in response to DHT, there was slight decrease in cell numbers with increasing DHT concentration after 96 hours. Thus, it was concluded that LNCaP and LNCaP-SDM cells represent good *in-vitro* models of androgen sensitive and castration resistant prostate cancers respectively.

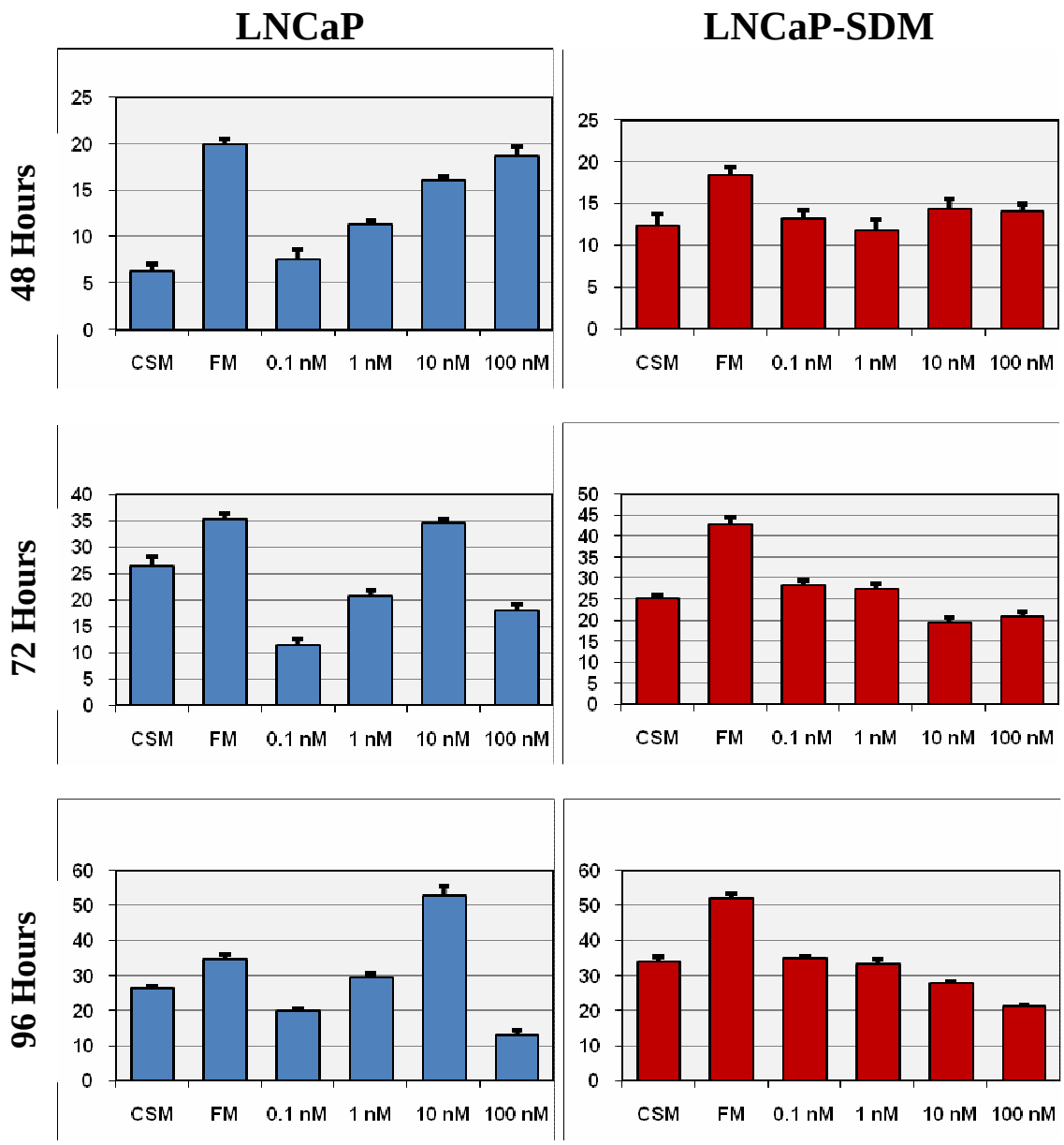



**Figure 8. LNCaP (left column) and LNCaP-SDM (right column) cells counted following the exposure to DHT. Medium containing charcoal stripped and full foetal calf sera used for controls. The cells were harvested after 48, 72 and 96 hours after the exposure to DHT. The column bars represent the relative numbers of the cells per condition. Error bars represent standard deviation from 4 counts.**

### 3.3.2 Steroid exposure and withdrawal

In order to assess the effect of androgen stimulation and withdrawal on Src kinase activity, both cell lines were exposed to various culture media including serum-free medium, medium containing dextran charcoal-stripped foetal calf serum (steroid depleted medium) and full foetal calf serum. SFK activity in LNCaP cells, experiencing acute androgen withdrawal in steroid depleted medium was reduced to the same degree as in the cells that were serum starved. In LNCaP-SDM cells, routinely cultured in charcoal-stripped serum, long-term androgen deprivation resulted in higher basal level of SFK activity compared to LNCaP cells in equivalent culture conditions. When deprived of growth factors in serum-free medium LNCaP-SDM cells displayed a further reduction in SFK activity, whereas treatment of these cells with full foetal calf serum containing physiological amount of androgens, increased SFK activity, although it did not reach the level observed in parental LNCaP cells.

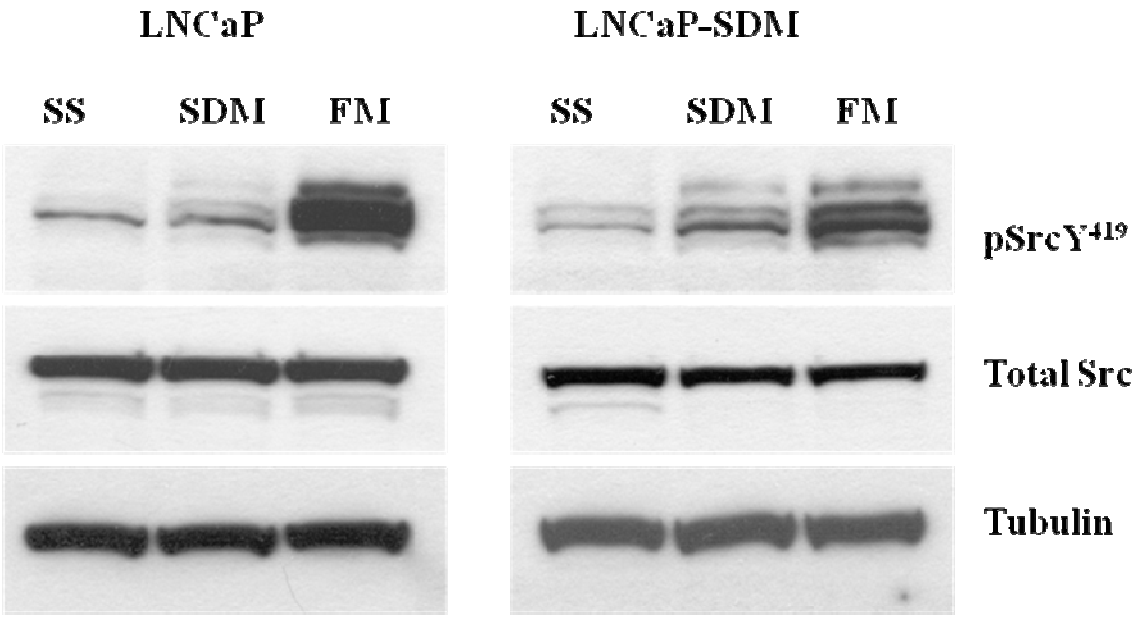



**Figure 9. Steroid exposure and withdrawal experiment. Both cell lines were exposed to various culture media, including serum starvation (SS), steroid depleted medium (SDM) and medium containing full foetal calf serum (FM). Src kinase activity was estimated by western blot analysis. The cells in full medium, containing physiological concentrations of androgens exhibited the highest Src activity, whereas hormonal deprivation as well as serum starvation dramatically reduced activity of Src kinase.**

## 3.4 Inhibition of SFK Activity *In Vitro*

### 3.4.1 Inhibition of SFK Activity and Downstream Markers

Dasatinib was used to investigate the effects of Src inhibition on LNCaP and LNCaP-SDM cells. Treatment of LNCaP and LNCaP-SDM cells with dasatinib resulted in a dose-dependent reduction of SFK activity using the autophosphorylation of Src at tyrosine $Y^{419}$ as a marker of activation. Complete inhibition of SFK activity in LNCaP cells was seen at 50 nM. SFK activity was also suppressed in LNCaP-SDM cells, although complete inhibition was achieved at slightly higher concentrations (75 nM). Interestingly, in LNCaP-SDM cells increasing the dose of dasatinib resulted in an increase in total Src.

FAK phosphorylation on tyrosine $Y^{861}$ (Src-dependent phosphorylation site) was also inhibited by treatment with dasatinib in both cell lines in a dose-dependent manner while the levels of phospho FAK$Y^{397}$ (Src-independent autophosphorylation site) and total FAK remained unchanged (Figure 10 and 11). We investigated whether inhibition of Src kinase activity would result in downstream inhibition of Paxillin, which is used in various studies as a biomarker of effectiveness of SFK inhibitor therapy. Our experiments have demonstrated that in both LNCaP and LNCaP-SDM cells there was no significant reduction in Src-dependent phosphorylation of pPaxillin$Y^{118}$ (Figure 12).

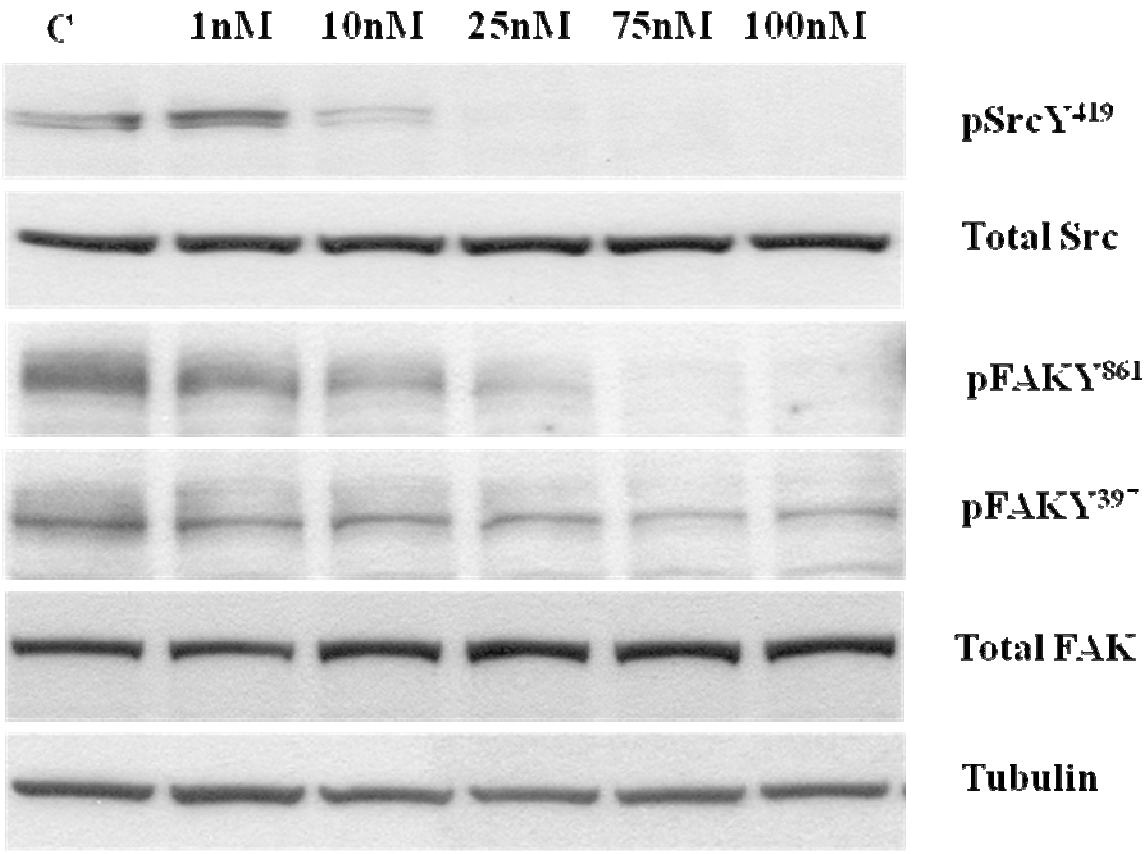



**Figure 10. LNCaP and cells were exposed to increasing concentrations of dasatinib. Western blot results confirmed Src suppression with 10 nM dasatinib and higher. Src-dependent phosphorylation site pFAKY$^{861}$ was also inhibited, whereas pFAKY$^{397}$, which is Src-independent was not affected.**

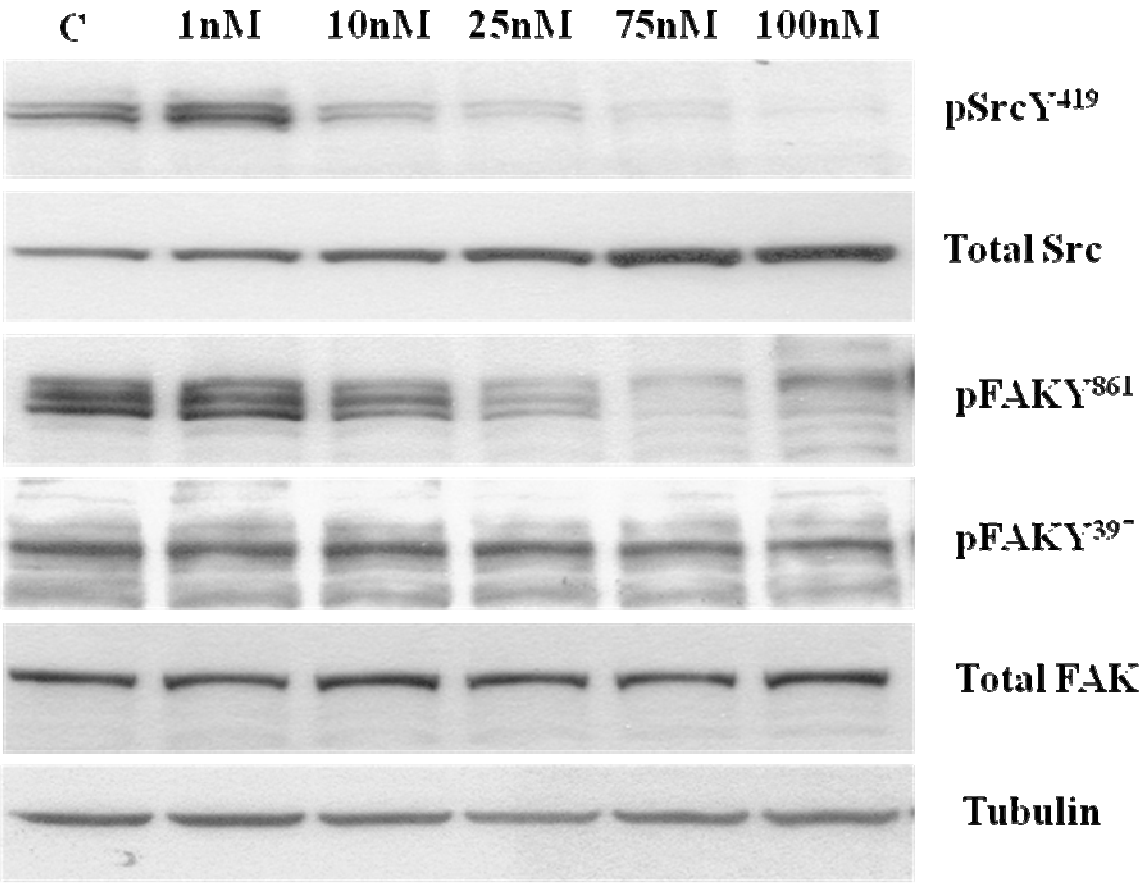



**Figure 11. LNCaP-SDM cells were exposed to increasing concentrations of dasatinib. Western blot results confirmed Src suppression with 25 nM dasatinib and higher. Src-dependent phosphorylation site pFAKY$^{861}$ was also inhibited, whereas pFAKY$^{397}$, which is Src-independent was not affected.**

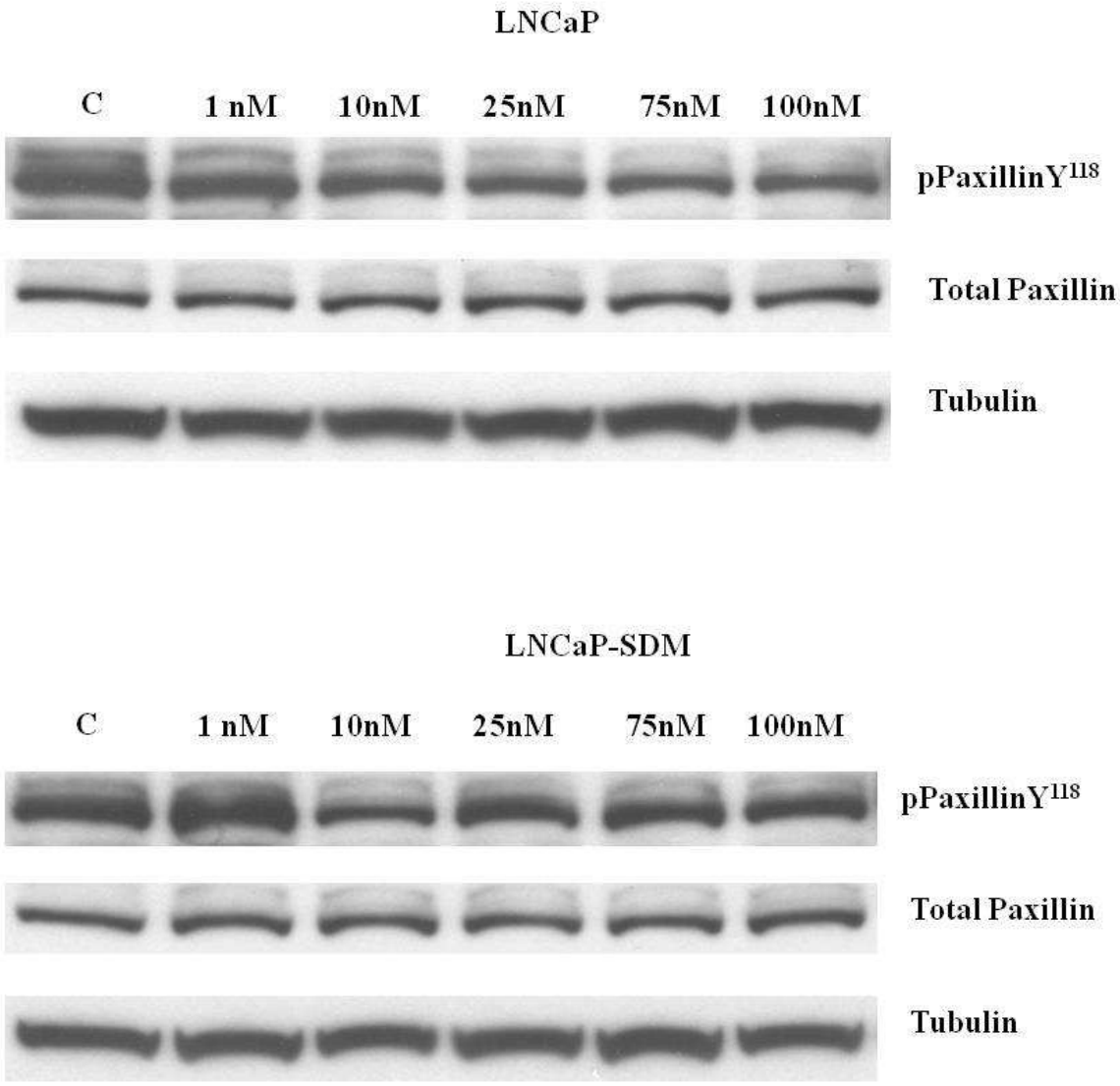



**Figure 12. LNCaP and LNCaP-SDM cells were treated with various concentration of dasatinib and Western blot performed to find out whether inhibition Src kinase activity would result in reduction of Src-dependent phosphorylation of PaxillinY$^{118}$. This experiment demonstrated that there was no inhibition of pPaxillinY$^{118}$ in both LNCaP and LNCaP-SDM cell lines.**

### 3.4.2 Study of Src and Lyn activity

Due to cross-reactivity of anti-phospho SrcY$^{419}$ antibody in the majority of SFK it was necessary to immunoprecipitate Src and Lyn to study the effect of dasatinib on kinase activity in individual family members. Therefore, initially it was necessary to extract each protein from the lysates of the LNCaP and LNCaP-SDM cells treated with dasatinib, by conjugating them with anti-total Src or Lyn antibodies. The extracted proteins were then processed using Western blot technique and the membranes probed with anti-phopsho SrcY$^{419}$ antibody that would detect both Src kinase and Lyn kinase activity. Dasatinib inhibited Lyn autophosphorylation in both LNCaP and LNCaP-SDM cells at equivalent concentrations (Fig. 3D). Higher concentrations were necessary to inhibit Src autophoshorylation in the LNCaP-SDM cells.

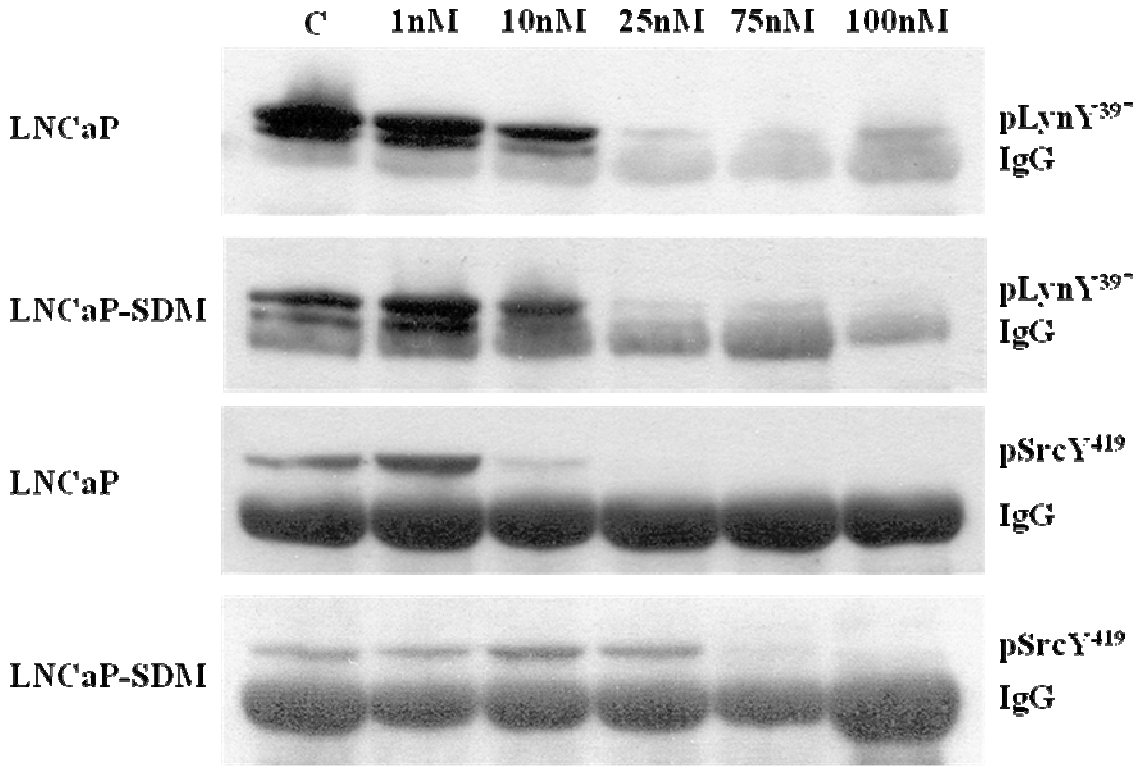



**Figure 13. Inhibition of Src and Lyn activity studied separately by immunoprecipitation due to cross-reactivity of anti-pSrcY$^{419}$ anitboby in SFK. Src and Lyn were initially extracted from the lysates of LNCaP and LNCaP-SDM cells exposed to dasatinib in different concentrations, with anti-total Src and Lyn antibodies and then processed using Western blot technique. The membranes were then probed with anti-pSrcY$^{419}$ antibody that detected both Src and Lyn kinase activity.**

## 3.5 Inhibition of Cell Migration by Dasatinib

### 3.5.1 Protrusion Dynamics

Dasatinib treatment of prostate cancer cells altered microscopic appearances of the cells, resulting in cell rounding and the loss of cell protrusions. The changes, observed using light microscope, were dose dependent and correlated with the results of western blot, showing inhibition of Src and FAK activity. Interestingly, morphological changes in LNCaP cells were more pronounced when compared to LNCaP-SDM cells.The dramatic changes in cells shape could not be attributed to dasatinib toxicity as the cells, esp. LNCaP continued to proliferate at the same rate in each condition.

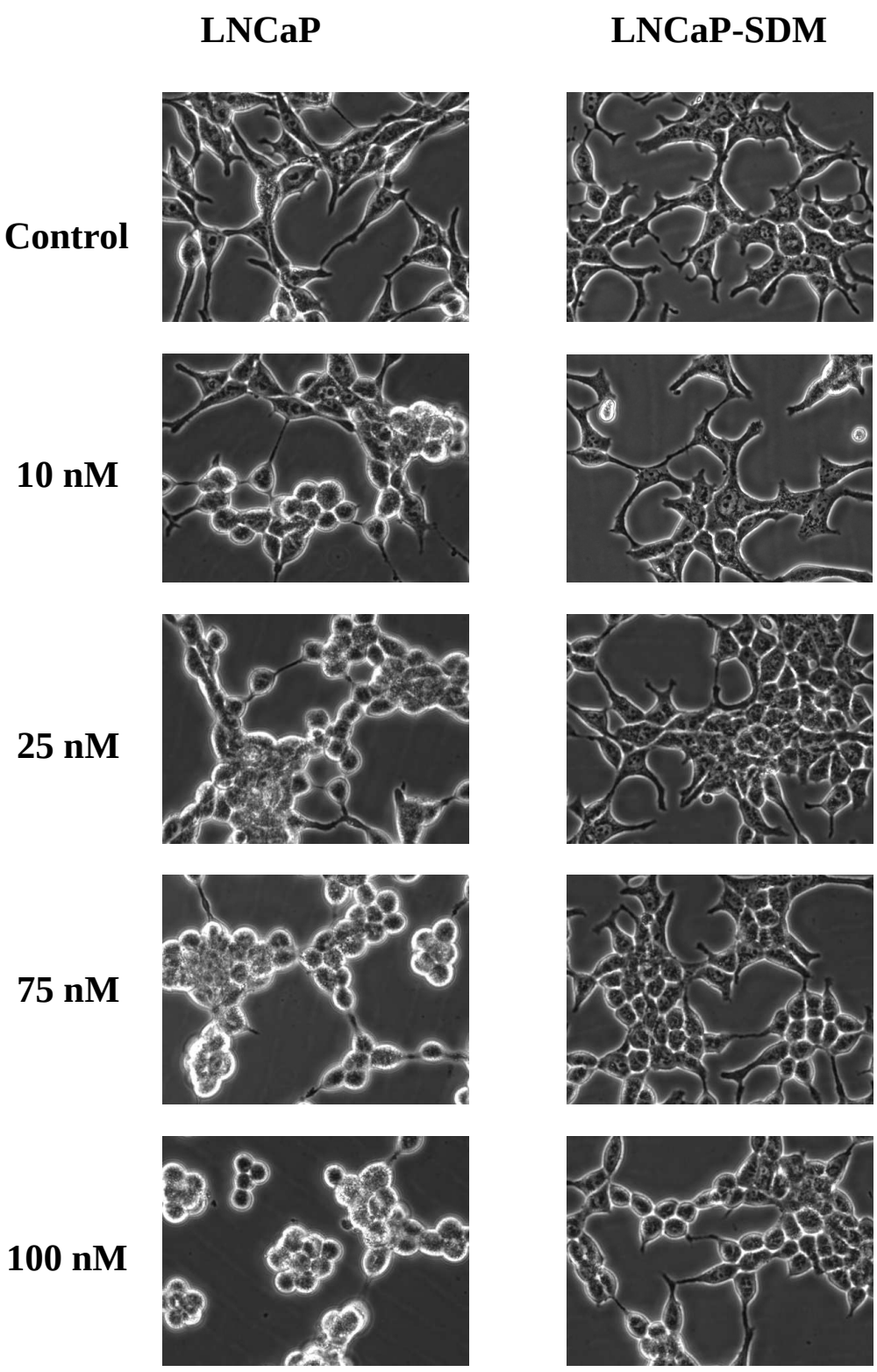



**Figure 14. Morphological changes in LNCaP and LNCaP-SDM cells observed after dasatinib treatment overnight in standard culture media using increasing concentrations of the inhibitor. There was dramatic loss of cells shape with the appearences corresponding to the changes in SFK activity.**

Microphotographs in figure 13 reveal alteration in the shapes of cells treated with dasatinib, resulting in cell rounding and loss of protrusions. Development of cell protrusions is an important phase in cell migration and proliferation. Protrusions serve as propulsion mechanism, allowing the cells to penetrate through extracellular milieu and, in case of the cells growing in the laboratory, attach and spread on the underlying surface as well as develop cell-cell contacts. Preventing the cells from developing protrusions, dasatinib inhibited cell spreading. This was observed and quantified over period of 24 hours using time lapse microscopy.

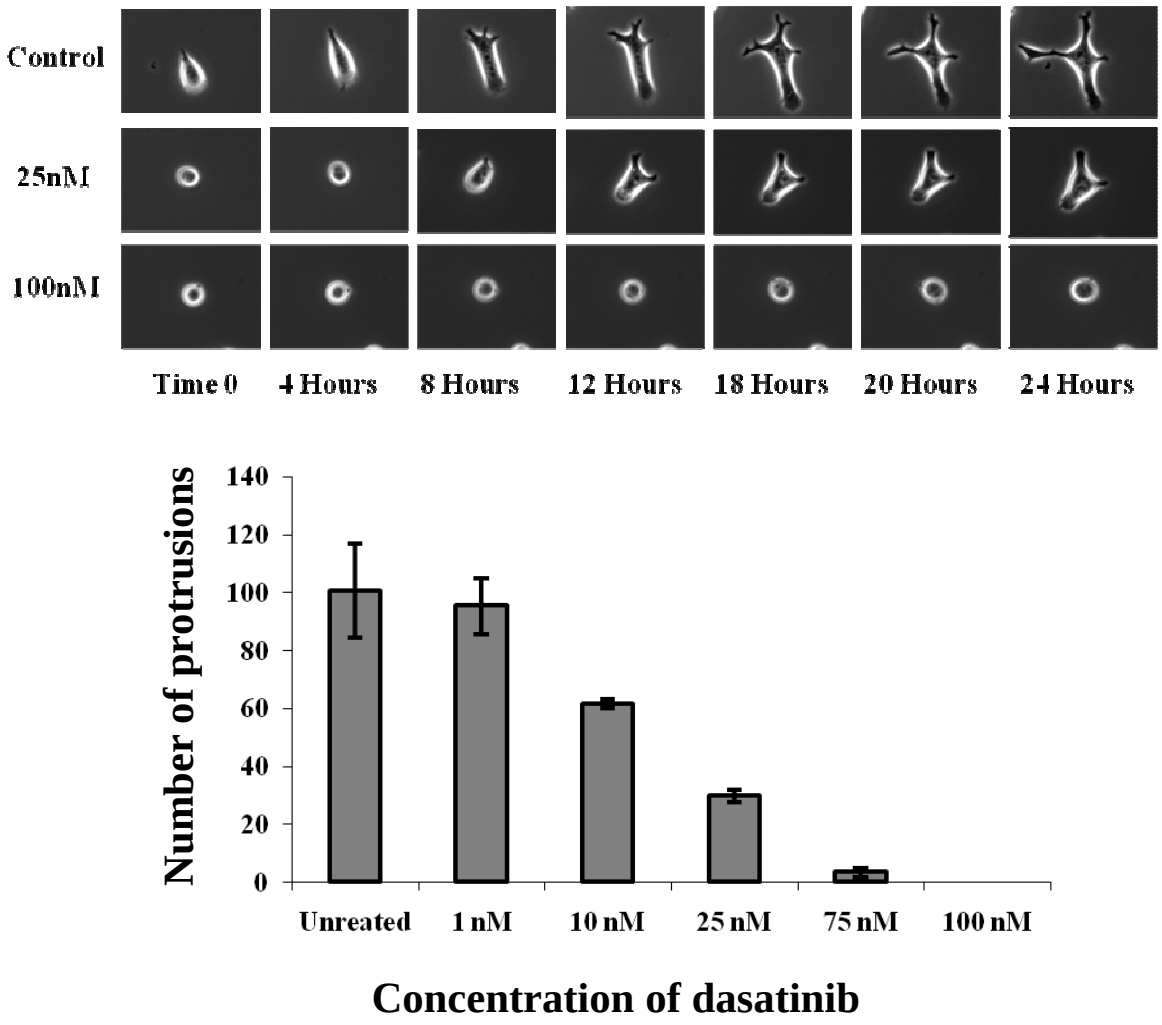



**Figure 15. Protrusion formation in LNCaP-SDM cells treated with dasatinib. LNCaP-SDM cells ceded on glass surface and treated with increasing consentrations of dasatinib. Protrusion count was performed for every cell in the field and the final number was the average number of protrusion per cell. Protrusions count result represents the average of 3 fields in each condition.**

### 3.5.2 Wound healing

Time lapse video microscopy was employed to investigate the effect of dasatinib on LNCaP and LNCaP-SDM cell migration into a denuded area using a wound healing assay. The cells were ceded and the wound were made through a confluent monolayer, 3 wound per field. The results represent an average of 3 measurements per each condition and the experiment repeated 3 times with consistent results.

Both, LNCaP and LNCaP-SDM cells exhibited a similar dose-dependent reduction in migration when treated with increasing concentrations of dasatinib for 48 hours (Figure 15). Column graphs, representing quatification analysis are included (Figure 16). The measurements reflect the difference in the distance between the edges of wounds at the beginning of the experiment and at the end. Thus, the difference was greater for the cells that were untreated or treated with low concentrations of dasatinib as they travelled more, while the difference between the edges of wounds for the cells treated with high concentrations of dasatinib was minimal.

LNCaP cells cultured in full medium, containing foetal calf serum were noted to have long protrusions, whereas the protrusions, produced by LNCaP-SDM cells when kept in androgen depleted medium, rarely exceeded the length of the body of the cell. Interestingly, when cultured in the medium containing physiological concentrations of androgens (foetal calf serum), the number of LNCaP-SDM cells forming long protrusions significantly increased.

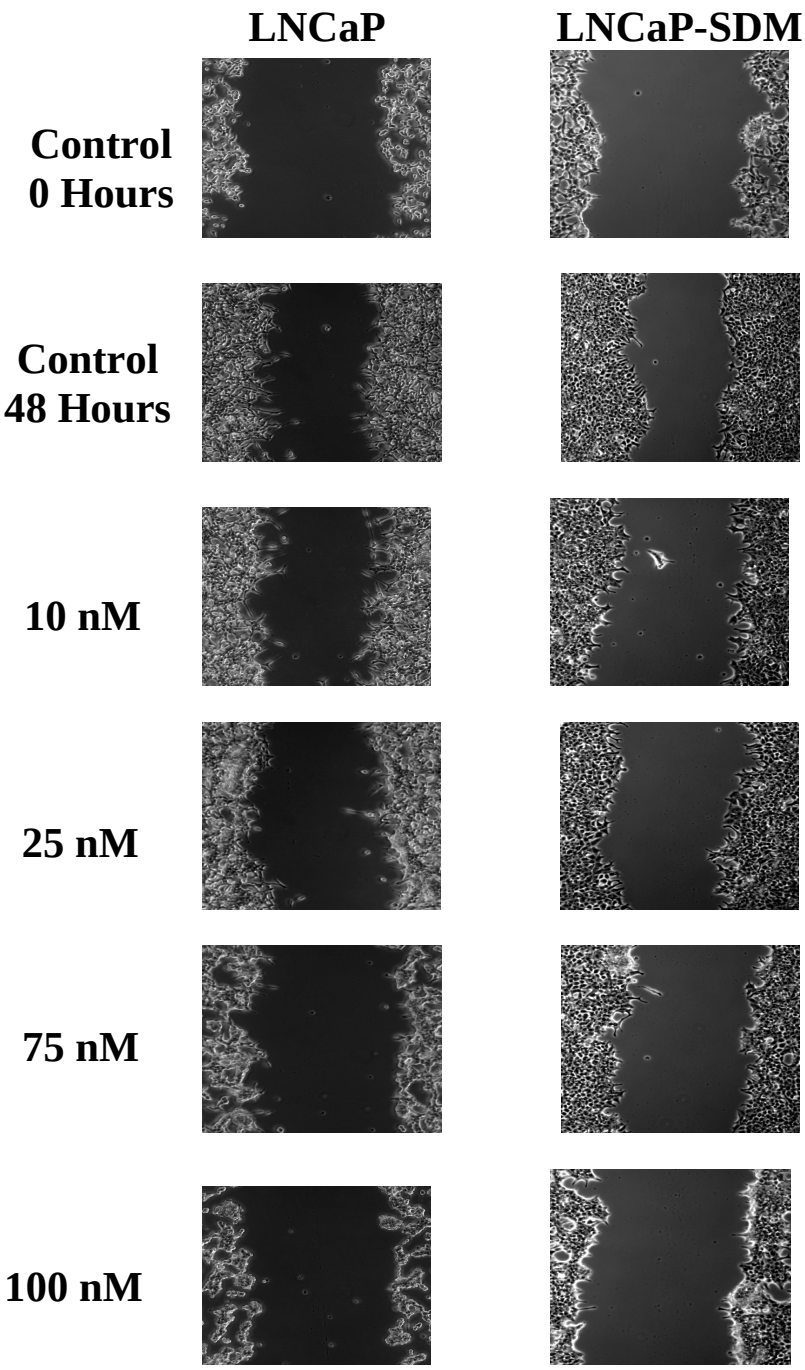



**Figure 16. Migration of prostate cancer cells into denuded areas. LNCaP and LNCaP-SDM cells were treated with dasatinib and the wound were made through the confluent monolayer. The cells then migrated into the denuded space as represented by the control at the beginning of the experiment and after 48 hours. Microphotographs of treated cells at the end of the experiment are presented.**

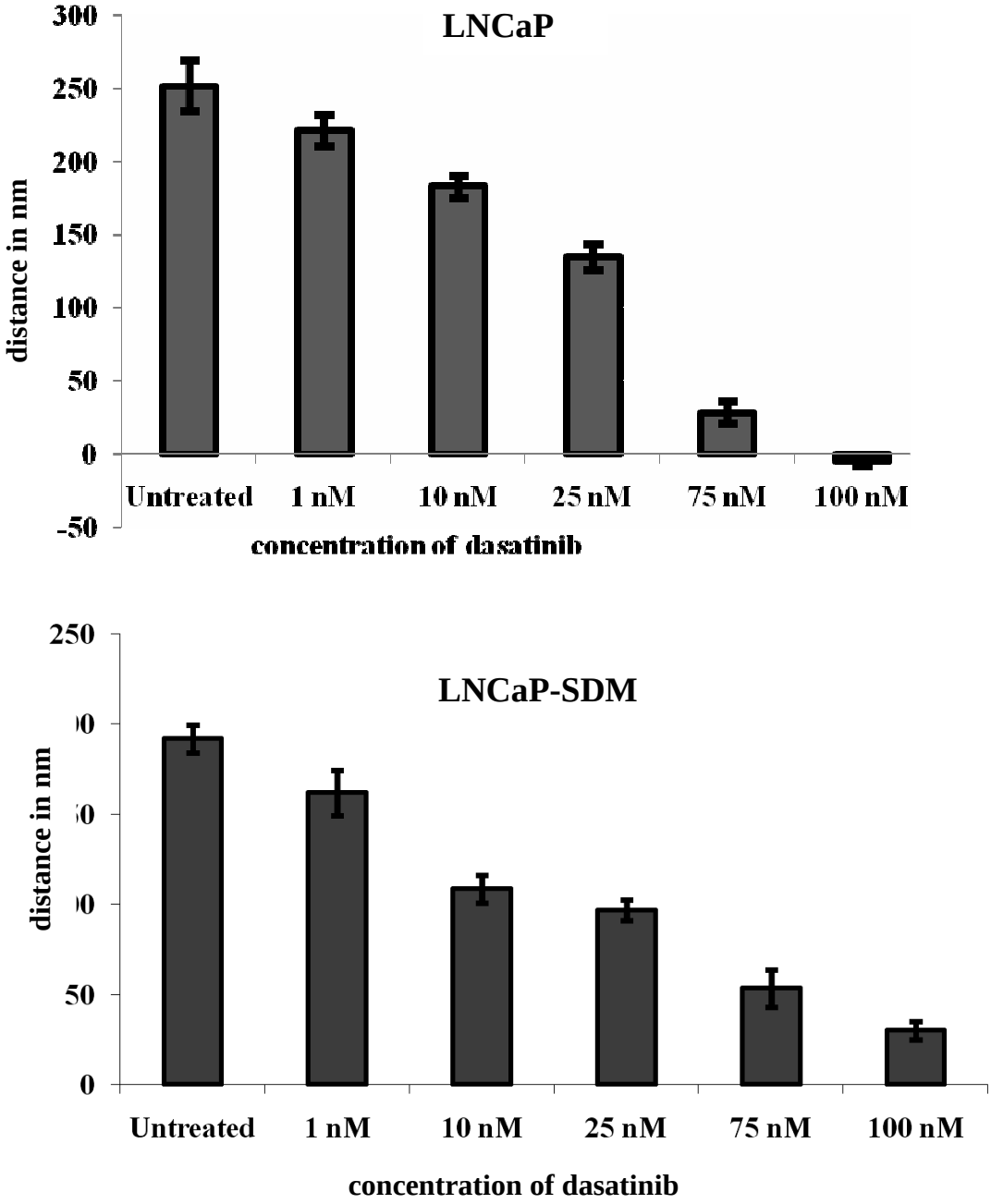



**Figure 17. Quantitative assessment of wound healing assay. Column bars represent the difference between the distance between wound edges at the beginning of the experiment and after 48 hours. The cells in control wells migrated further than the cells treated with dasatinib. Error bars represent standard deviation from 3 measurements in each condition.**

## 3.6 Inhibition of Cell Proliferation by Dasatinib

Growth of hormone sensitive LNCaP cells is driven mostly by androgens while hormone refractory LNCaP-SDM cells are thought to depend on growth factors transmitting oncogenic signals via tyrosine kinases. Treatment with dasatinib inhibited proliferation of hormone-refractory LNCaP-SDM cells in a dose-dependent manner (IC50 500 nM) at concentrations corresponding to inhibition of SFK activity. Proliferation of hormone sensitive LNCaP cells was not significantly inhibited by dasatinib even at concentrations up to 10 µM. Interestingly, introduction of androgens to the steroid depleted medium, used for routine culture of LNCaP-SDM cells, did not rescue proliferation suppressed by dasatinib. In addition, treatment of LNCaP cells experiencing acute androgen withdrawal in steroid depleted medium did result in further inhibition of proliferation although this was observed at concentrations of dasatinib higher than 10 µM and, therefore, this effect was thought to be non-specific.

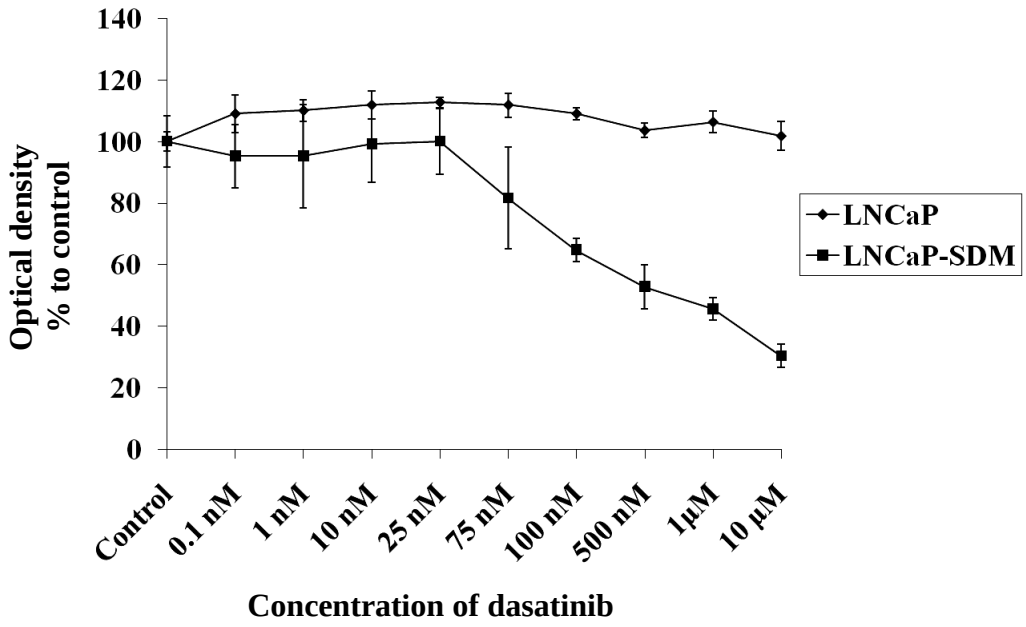



**Figure 18. Effect of dasatinib on cell proliferation as determined by WST-1 assay. LNCaP and LNCaP-SDM cells were treated with a range of dasatinib concentrations. This graph represents the results of WST-1 assay performed at 96 hour time point. Optical absorbance level is proportional to the number of metabolically active cells and presented here as the percentage of the control, with the control being 100%. There was no significant reduction in proliferation of LNCaP cells whereas the proliferation of LNCaP-SDM cells was significantly reduced with dasatinib concentrations 75 nM and higher. Error bars represent the standard deviation from 4 measurements in each condition.**

# DISCUSSION

# Introduction

Prostate cancer is the most common cancer in men and second leading cause of cancer-related deaths in the western world (29). Patients with locally advanced or metastatic prostate cancer are mostly prescribed castration therapy in the form of LHRH agonists, anti-androgens or surgical orchidectomy. Inevitably, despite the treatment, the disease progresses towards hormone resistant state, leaving the patients and the clinicians few viable options. Palliative methods usually consist of pain relief by means of analgesia and palliative radiotherapy to the areas of painful bone metastases, bisphosphonates and radioisotopes. Application of systemic chemotherapy in patients with hormone refractory prostate cancer has proved disappointing, only taxane-based therapy has shown clinically significant improvement in survival, palliation of symptoms and improvement in quality of life (195).

Urgent requirement for further research into molecular mechanisms underlying the development of CRPC has been highlighted by the lack of progress in the treatment of the disease. In a recent systematic review of clinical trials investigating the effect of chemotherapy in prostate cancer, published by Cochrane Collaboration Project (196), the authors recommended further research in molecular and genetic complexities of the disease as a way to improve the outcomes. Numerous controversies, resulting in difficulty interpreting and comparing the trials, stem from heterogeneity in patient populations as well as individual cases. Therefore, stratification of patients into well defined groups by selecting individuals according to positive biomarkers, for example, is considered important in order to increase the likelihood of successful treatment (197).

Malignancies are characterised by up-regulation of multiple signalling pathways, allowing the cancer cells to evolve and adapt to constantly changing microenvironment. Defining key elements, driving intracellular signalling can potentially result in the

development of inhibiting strategies, although it is unlikely that single agents would have ‘magic bullet’-like impact. Combining the agents, used to target multiple molecular cascades or using novel small molecule inhibitors with conventional cytotoxic drugs and hormone deprivation therapy could be a way forward (150). Although multiple potential targets have been identified, certain molecules, including SFK, are more promising in terms of inhibiting potential. This is due to convenient position of SFK on the map of signalling pathways, being involved in the extensive cross-talk and regulating multiple intracellular events.

## 4.1 Correlation of SFK Activity with Clinical Parameters

### 4.1.1 Immunohistochemistry

Despite exponential growth in number of laboratory tests available to clinicians over the last 50 years, histopathology remains the gold standard when it comes to decision making in surgical oncology and pathology. Interpretation of histological data is based primarily on tissue morphology and, therefore, inherently it is a subjective process. In order to add objectivity, immunohistochemistry (IHC) has initially been used to complement morphological appearances of the tissue with molecular information. More recently, IHC has become standard in predicting and evaluating response to novel molecular based targeted therapies (198). In breast cancer, for example, IHC is used to determine ER status in order to select the patients who should receive tamoxifen. IHC has been crucial in discovery that HER-2 status could predict resistance to systemic therapies and HER-2 positivity is associated with increased mortality and higher rate of recurrence (199). Moreover, targeting HER-2 with humanised monoclonal antibodies, such as Trastuzumab (Herceptin; Genentech, CA) or small molecule dual HER-1/HER-2 inhibitor Lapatinib (Tykerb, GlaxoSmithKline, PA) improves outcomes in context of metastatic disease and adjuvant therapy (200;201).

Since the discovery of IHC by Coons and Jones, who established immunofluorescent method to detect bacteria, the technique has become standard in diagnostic pathology and experimental biology. In our laboratory, IHC has been employed extensively to study cancer cell signalling using paraffin-embedded tissue sections (112;202;203). The technique typically starts with antigen retrieval with the goal to unmask the antigen, hidden by formalin cross-links. Although several methods of antigen retrieval exist, for the

purpose of SFK staining, when optimising the procedure it was established that it is best performed in pressure cooker with tissue slides immersed in citrate buffer. The next step involved application of primary antibody and it was determined that best results were achieved when exposure to antibody for total Src was limited to 1 hour, whereas it was necessary to incubate the tissue section with phospho-specific antibodies overnight. This followed by exposure to secondary antibody, depending on species in which primary antibody was raised. It was possible to achieve consistent immunostaining with the range of colours allowing necessary quantification.

Molecular quantification methods have been in existence for several decades. These techniques include reverse transcriptase polymerase chain reaction (RT-PCR) for quantification of nucleic acids, and antibody-based methods for protein quantification, e.g. enzyme-linked immunosorbent assay (ELISA). A major disadvantage of these methods is the requirement for tissue destruction in order to quantify specific biomolecule, with complete loss of valuable spatial information. In case of SFK, it was essential to differentiate cytoplasmic from membrane immunostaining due to the fact that inactive SFK are positioned mostly in peri-nuclear area, whereas SFK activation is associated with the transfer of the proteins through the cytoplasm to the membrane. In fact, most important findings were made when analysing membrane immunostaining.

Interpretation of IHC results has been the subject of extensive debates, particularly following introduction of IHC-based tests into routine medical practice. The need to standardise IHC reading has been highlighted recently, when it was found that in up to 20% of HER-2 tests, performed in breast cancer patients may be inaccurate, with resulting clinical implications for the treatment and prognosis (204). Inter-observer variability, therefore, represents a major issue affecting the accuracy of results with clinical and scientific implications. Although IHC is interpreted subjectively by pathologists or experimental biologists, it is possible to produce an objective and continuous system or

scale to measure protein expression. The technique of weighted histoscore, employed in this study, provides the possibility to quantify protein expression by means of continuous variable (205).

Regardless of tumour type or size, if the tissue sections are scored by two independent appropriately trained observers, it is possible to achieve consistency and reproducibility, reducing the need for dual scoring down to 10% of samples without sacrificing the accuracy. The quality and standards of inter-observer variation can be determined by interclass correlation coefficient (ICCC), which is expressed as the variance between subjects relative to the sum of the variance components for subjects, observers and random error (205). ICCC represents a step further from HSCORE (206) and Allred (207) criteria that attempted initially to produce more continuous scale than binary positive-negative end point or a 3- to 4-point scale. While the lower acceptable threshold is a matter of debate, the ICCC level of >0.9 is considered to be excellent (205). In this study, it was possible to achieve ICCC score higher than 0.9, producing sufficient quality of data interpretation.

### 4.1.2 Tissue Sampling

Another possible issue affecting variability of IHC results relates to tissue heterogeneity, stemming from processing methods (fixation methods, extent of fixation and validation of reagents) as well as true biological heterogeneity. While the former received much attention in the literature, very little is known about the latter. It is important to consider that prostatic adenocarcinoma represents a heterogeneous group of neoplasms with broad spectrum of pathologic and molecular characteristics and variable natural history of the disease (208). Therefore, when tracking the changes in the tissue throughout the course of the disease, it is preferable to study the samples that are related to

each other as closely as possible. As there is significant geographical and racial variation in the rates and aggressiveness of prostate cancers (29), the individuals should ideally be of the same race and living in the same area. Although it is not always possible, the series of tissue samples should be from the same individuals and this should contribute to the reduction of variability.

The subjects, recruited for this study were all Caucasian males from Central Scotland region. Fifty patients were selected according to stringent criteria and, therefore, only one patient was deemed suitable out of twenty considered . The selected patients received no prior treatment and following the diagnosis, were given castration therapy, resulting in biochemical response as represented by a significant reduction in PSA. Thus, the tissue samples taken prior to the treatment being administered could be considered as hormone naïve. The patients had regular PSA measurements allowing to determine when they reached PSA nadir and when PSA levels started to rise, the patient were considered as having hormone relapse. The second tissue sample was taken following hormone relapse when the patients developed bladder outflow obstruction and underwent channel TURP. This second tissue sample, therefore, represented castration-resistant prostate cancer. Accordingly, having two sets of prostatic tissue samples, taken from the same individual prior to castration therapy and following hormone relapse, made it possible to track the changes in SFK protein expression and activation in each individual.

While having matched paired tissue samples provided an advantage in comparing the protein expression and activation before and after hormone relapse, by including the patients all of whom underwent channel TURP may have introduced selection bias. At the time of writing there were no publications, studying patients with CRPC undergoing channel TURP. Sehgal et al. reported 42 patients with advanced prostate cancer requiring channel TURP, although the status of hormone sensitivity was not commented upon (209). The authors noted that the patients requiring channel TURP had higher Gleason grade than

the patients, who presented with acute urinary retention and had successful trial of voiding. In another study of 19 patients with advanced prostate cancer, 67 % had high grade (Gleason grade 8-10) prostate cancer, whereas only 22.7% of prostate cancer patients, undergoing TURP for bladder outflow obstruction, prior to diagnosis being established, had high grade disease (210). It is also possible to argue that the patients, who had operative interventions may have had less co-morbidities, than those, who developed symptoms of bladder outflow obstruction and were treated conservatively, with indwelling catheter for example. At present, prostate biopsy is not performed routinely in patients with CRPC and, therefore, channel TURP represents the only source of prostate tissue in patients with CRPC.

### 4.1.3 Analysis of SFK Immunostaining

Analysing matched paired prostate cancer tissue samples, taken from patients prior to hormone deprivation therapy being administered and after hormone relapse, made it possible to correlate molecular changes, occurring in the tissue as the patients developed hormone resistance, with various clinical parameters. Despite the fact that a wealth of information on SFK status *in vitro* in prostate cancer cell lines is available and the commencement of clinical trials of SFK inhibitors in CRPC, there are no studies of SFK expression and activation in clinical samples in relation to clinical data, including survival. Goldenberg-Furmanov et al. reported immunohistochemical analysis of total Lyn expression in prostatic epithelium in human prostate cancer samples and in prostate cancer metastases (15). The authors noted that Lyn immunostaining in prostate cancer cells was at least as intense as in adjacent normal prostatic epithelium. In addition, when prostate cancers were showing two Gleason grade patterns, Lyn expression tended to be higher in

less differentiated regions. Another SFK member Fyn was also found to be overexpressed in prostate cancer as compared to normal prostatic epithelium (17).

Although the expression of total SFK proteins in cancer is probably important, it is SFK activation that is thought to be a key element in oncogenesis (13). Phosphorylation of tyrosine $Y^{419}$ of Src kinase domain and its equivalent in other family members is regarded as a reliable marker of SFK activation and, therefore, application of phospho-specific antibodies can be used to define SFK activation status in human prostate cancer tissue (211). Paronetto et al. described the use of anti-phospho $SrcY^{419}$ antibody to stain prostatic tissue and found that in 4 patients with advanced prostate cancer (Gleason grade 7-9), membrane immunostaining of cancer cells was more intense than that of normal prostatic epithelium. In another publication on the role of AR phosphorylation in prostate cancer, $pSrcY^{419}$ immunostaining was performed using prostate cancer tissue microarrays (50). The prostate cancer tissue taken from the patients with hormone-refractory disease stained more intensely, when compared to hormone-naïve tumours and benign prostatic hyperplasia.

In this study, IHC was performed using antibodies against total Src, total Lyn, total Fgr, phospho $SrcY^{419}$ and phospho $SrcY^{530}$ (marker of Src inactivation). $SrcY^{419}$ autophosphorylation site is highly conserved in all SFK members and, therefore, it is not possible to say activation of which SFK member is prevalent in stained tissue due to cross-reactivity. In the future, commercially produced antibodies could be used to distinguish the role of activation of different SFK members. SFK immunostaining was almost exclusively confined to prostate cancer epithelium with very little staining of stroma cells. Comparing the staining of pre-treatment prostate cancer tissue to the tissue taken following hormone relapse, subgroups of patients were identified, in whom there was a significant change. In a subgroup of 14 patients (24%), there was a significant increase membrane anti-phospho $SrcY^{419}$ immunostaining ($p=0.017$), in the transition from ASPC to CRPC. Survival

analysis showed that these patients had shorter tome to relapse (p=0.005), shorter time to death from relapse (p=0.011), which resulted in shorter overall survival (p<0.0001). This is the first study to demonstrate the correlation between the increase in membrane pSrcY$^{419}$ immunostaining and survival in prostate cancer patients.

Separating cancer patients according to positive biomarkers is becoming common in oncology, where the treatment is increasingly tailored according to individual tumour profile. The patients with advanced breast cancer are now routinely tested for HER-2 status of their breast tumours to identify those who are likely to benefit from HER-2 targeted therapy. It is thought that approximately 20-30% of patients with advanced breast cancer are found HER-2 positive and receive HER-2 inhibitor trastuzimab (198). As SFK inhibitors are now in clinical trials for CRPC, it may be beneficial to test prostate cancer patients in order to identify those with the increase in SFK activity in prostatic epithelium. Defining this subgroup may increase the quality of statistical data (enrichment method), which ultimately could be translated into clinical practice.

Much attention in literature has been given to the role of Src in normal function of bone cells and bone re-modelling in metastatic disease (179). Preponderance of metastases in prostate cancer to the bones is well known and represents a major source of morbidity. Targeting key molecules, participating in formation and development of bone metastases in prostate cancer may provide clinicians with novel therapies that could be used at different stages, including localised prostate cancer in neo-adjuvant context as well as progressive and advanced stage, where limiting growth of metastatic deposits may help to palliate the symptoms (180). We found that in patients with CRPC, prostate tumour samples taken from those who had metastatic disease stained more intensely with anti-phospho SrcY$^{419}$ antibody than those who did not have evidence of bone metastases (p=0.011). This is probably due to up-regulation in SFK activity leading to more aggressive and motile phenotype, promoting detachment of cancer cells and metastatic

spread. Thus, selecting the patients at an earlier stage may provide the opportunity to target SFK activation and potentially prevent or reduce the chance of developing secondary bone tumours.

## 4.2 Cell Line Model of Castration Resistance

Attempts to establish *in vitro* cultures of prostatic carcinoma have been ongoing since early 20$^{th}$ century. First attempts were successful in so far as the development of primary tissue cultures that only lasted short term. It was not until 1970s when Fraley et al. described a prostatic cell line, although it was later found to be cross-contaminant of HeLa cells (212). Over the following years, establishment of stable continuous cell lines has proved particularly difficult in prostate cancer. To date, there are over 30 cell lines used in research settings and the number continues to grow although, most *in vitro* studies employed the three earliest established prostate cancer cell lines: LNCaP (213), PC-3 (214) and DU-145 (215). Interestingly, all three cell lines have been derived from metastatic sites: LNCaP from lymph node, PC-3 from bone and DU-145 from brain. While LNCaP cell line is considered to be hormone responsive and, therefore, represents a model of ASPC, PC-3 and DU-145 are androgen independent. Thus, PC-3 and DU-145 cell lines have been used in laboratory experiments as *in vitro* models of castration resistance.

Potential drawback of the use of PC-3 and DU-145 cell lines in studies of molecular mechanisms of the development of CRPC in general and the role of SFK in prostate cancer in particular, is the fact that none of these cell lines express AR (216). Increasingly, it is thought that AR plays a major role in the progression of prostate cancer to castration resistance. Studies of clinical tumour samples have shown that the majority of CRPC samples express functional AR and that the tissue concentrations of PSA and other androgen-responsive genes increase in the setting of castration-resistant tumour growth (217). In context of SFK signalling in prostate cancer, AR has been demonstrated to create complexes with Src, activating Src-dependent pathways and stimulating growth of CRPC (40). It is possible to transfect PC-3 cells with AR in order to investigate the action of anti-

androgens on various cell functions, although taking a hormone sensitive cell line and attempting to develop castration resistance by withdrawing the androgens would probably produce more physiologically relevant results.

Recently, *in vitro* studies and in animal experiments, scientists have increasingly been using derivatives of LNCaP cell line expressing functional AR with the ability to progress in androgen depleted conditions. Thalmann et al. described the development of C4-2 cell line by inoculating LNCaP cells into male athymic nude mice, castrating the mice and maintaining the resultant cell line in the castrated host (218). This model was mimicking closely the natural history of prostate cancer with the ability to develop both lymph node and bone metastases *in vivo* and, therefore, was suggested for further studies on molecular mechanisms of CRPC. This cell line was later found to overexpress Src protein and the treatment with Src inhibitor PP2 was demonstrated to increase apoptosis (219). Furthermore, inhibiting Src resulted in reduced AR transactivation and subsequent recruitment to AR target genes, reduced growth and invasion. Another related cell line C4-2B was used to produce metastatic animal model of prostate cancer by injecting the cells into tibiae of SCID mice. Application of SFK inhibitor dasatinib alone or in combination with docetaxel was effective in lowering sacrifice serum PSA and increasing bone mineral density in the tumour sites (220).

In this study, we used LNCaP-SDM cell line, a variant of LNCaP cell line that was developed by gradual withdrawal of androgens from culture medium and maintained in the medium supplemented by charcoal stripped foetal calf serum, containing significantly reduced concentration of androgens. Androgen stimulation experiment demonstrated that LNCaP cells displayed dose-dependent reaction to DHT stimulation, whereas LNCaP-SDM cells did not increase proliferation when DHT was added to steroid-depleted medium. There was increase in proliferation of LNCaP cells with the concentrations of DHT up to 10 nM, although the cells stimulated by 100 nM DHT demonstrated reduced

growth probably due to DHT toxicity. Therefore, LNCaP-SDM cell line, developed by mimicking androgen withdrawal during hormone deprivation therapy, represented a good model of castration-resistant prostate cancer.

We then investigated whether androgen deprivation would affect SFK activity by placing the cells in various media with or without androgens. Androgen withdrawal led to dramatic reduction in SFK activity in both cell lines, especially in androgen-sensitive LNCaP cells. This is probably due SFK in the presence of androgens being activated by AR via complex formation and SFK intramolecular displacement. Although this effect was more pronounced in LNCaP cells, androgen stimulation also led to the increase in SFK activity in LNCaP-SDM cell line, suggesting continuous importance of AR signalling in CRPC and cross-talk with SFK. In LNCaP cells, significant reduction in SFK activity when being placed in androgen-depleted medium, equalled to serum starvation further underlying the importance of AR signalling for SFK-dependent pathways.

## 4.3 Role of SFK Activation in Prostate Cancer

### 4.3.1 Biomarkers of SFK Activation

With increasing use of SFK inhibitors in clinical practice, it is imperative that reliable biomarkers of SFK activation are developed. Phospho-specific antibodies, raised against activated enzymes have proved essential when studying molecular signalling pathways. These antibodies can be applied in clinical settings in order to determine the concentrations of inhibitors necessary to achieve the desired clinical effect. This is especially important in context of SFK inhibitors, given that it is Src activation rather than total expression, which is thought to drive oncogenic signalling (13). Identification of Src autophosphorylation site tyrosine $Y^{419}$ provided a reliable read-out of Src activity due to correlation of its level with kinase activity of the enzyme (169). Upon Src activation, multiple downstream substrates become phosphorylated and, therefore, these could be also used as biomarkers. Among them, Src-dependent phosphorylation sites on paxillin phosphorylated at tyrosine $Y^{118}$, FAK phosphorylated at tyrosine $Y^{861}$ and $p130^{cas}$ phosphorylated at tyrosine $Y^{410}$ have been previously suggested (158;169).

In this study, we used anti-phospho $SrcY^{419}$ as a biomarker of Src activity in clinical samples. *In vitro* experiments have demonstrated that Src kinase activity was reduced in dose-dependent manner, when LNCaP and LNCaP-SDM cells were exposed to SFK inhibitor dasatinib. As expected, dasatinib treatment led to the decrease in Src-dependent phosphorylation of FAK at tyrosine $Y^{861}$ and autophosphorylation of FAK at tyrosine $Y^{397}$, which is Src-independent, did not change. Therefore, both $pSrcY^{419}$ and $pFAKY^{861}$ could potentially represent biomarkers of SFK inhibition. Dose-dependent inhibition of Src and FAK activity was further correlated with microscopic appearances of the cells as dasatinib

induced dramatic morphological changes, resulting in protrusion withdrawal and cell rounding. Interestingly, our experiments showed that phosphorylation of paxillin at tyrosine $Y^{118}$ was not affected by dasatinib and, therefore, the role of paxillin activation in prostate cancer would need to be further elucidated.

Phospho-sequences surrounding Src tyrosine $Y^{419}$ and its equivalents in other SFK are very similar and, therefore, it is not possible to distinguish between various SFK members, unless SFK members are investigated separately. This could be done in both tissue cultures and clinical samples, although important spacial information would be lost due to the necessity of tissue destruction. It has previously been suggested that various SFK members may have different roles in the development of prostate cancer and its progression to castration resistance. Knockout of Src by siRNA revealed that Src suppression impacted mostly on cell migration, whereas inhibition of Lyn resulted in the reduction of proliferation (18). Dasatinib has previously been demonstrated to inhibit both Src and Lyn using *in vitro* kinase assay (158). Immunoprecipitation experiments, performed in this study have shown that kinase activity of both Src and Lyn were inhibited by dasatinib. Presently, it is not known which SFK member is responsible for the functional outcome of SFK suppression as this data will only be available when phospho-specific antibodies are developed against each SFK member.

### 4.3.2 SFK Role in Cell Proliferation

Despite considerable evidence implicating SFK in cellular proliferation, questions remain whether SFK activation or elevated expression has any effect on the actual tumour growth in solid malignancies. In studies involving colon and bladder cancer cell lines, increased expression of constitutively active or kinase-defective mutants of Src did not affect cell proliferation (221;222), whereas other studies demonstrated inhibition of

proliferation, when expression of Src was reduced by antisense approach (223). In prostate cancer, the proliferation of hormone-sensitive tumours is mostly driven by androgens and, therefore, the focus of investigations on the role of SFK in cellular proliferation has been on castration-resistant tumours. Extensive cross-talk of SFK with growth factor receptors and GPCRs may imply certain SFK involvement in proliferative cascades, although it is not clear whether SFK expression or activation is essential in stimulating CRPC growth.

Conflicting results from the studies based on molecular manipulation stem from the lack of specificity small molecule inhibitors often display. PP1 and PP2 for example, pyrazolopyrimidine-based competitive inhibitors of ATP binding have been extensively used in tissue culture, including prostate cancer cell lines, to study the cellular features of Src inhibition (224). Pyrazolo-pyrimidine compounds have been applied in proliferation and migration studies in order to elicit the effect of Src inhibition in prostate cancer cells stimulated with various growth factors (43;46;91). These chemicals are proven, however, to inhibit other tyrosine kinases including PDGF, stem cells factor receptor c-Kit, tyrosine kinase Ret etc. with an *in vitro* $IC_{50}$ below the concentration used in these experiments (225-227). Recently developed SI35 and SI40 pyrazolo(3, 4-d) pyrimidines were effective in reducing EGF stimulated PC-3 cells proliferation at concentrations 5 μM and higher, whereas much lower doses of the inhibitors were used to elicit the reduction in cell adhesion and migration (162).

Dasatinib, SFK inhibitor used in this study, has been demonstrated to inhibit proliferation of PC-3 cells at low nanomolar concentrations (157). Park et al. showed that dasatinib significantly inhibited proliferation of LNCaP and a highly tumorigenic variant of PC-3 cells at concentrations above 100 nM (18). As the concentration of dasatinib required to inhibit Src kinase activity was 10 nM and Lyn activity 100 nM, the authors concluded that inhibition of proliferation was primarily due to inhibition of Lyn. However, at concentrations 100 nM and above, dasatinib is known to inhibit multiple targets and,

therefore, this effect could be non-specific (157). Although we are in agreement with Park et al. that Src kinase activity in LNCaP cells is inhibited by dasatinib at concentration 10 nM and above, we did not find that the suppression of Lyn kinase required higher concentrations of the inhibitor. Furthermore, no evidence of the inhibition of LNCaP cell proliferation was demonstrated, whereas proliferation of LNCaP-SDM cells was inhibited by dasatinib at concentrations corresponding to the suppressed SFK activity.

Suppression of proliferation by SFK inhibitors at concentrations correlating to the inhibition of SFK activity have only been shown in a small subset of prostate cancer cell lines (157;220). Similarly, in vitro studies in other solid tumours, including colon cancer, head and neck cancer, non-small cell lung cancer, the majority of cell lines were insensitive to the treatment with dasatinib at concentrations required to inhibit Src activity. Moreover, dasatinib at higher concentrations has been shown to inhibit proliferation of $SYF^{-/-}$ cells, lacking the expression of all main Src family members, confirming the possibility of non-specific action (169). Thus, it appears that certain cell lines involve SFK in their proliferative signalling pathways, whereas others employ different mechanisms. Inhibition of proliferation of LNCaP-SDM cells, demonstrated in this study is probably due to the higher dependence of castration-resistant tumours on tyrosine kinases, whereas hormone-sensitive LNCaP cells mostly rely on androgens for their growth.

### 4.3.3 SFK Role in Cell Migration

Unlike the role of SFK in proliferation that is still debatable, there is consensus among scientists that SFK are essential in promoting motility of cancer cells (13). SFK activation influences cancerogenesis from early stages in the process involving normal epithelial cells acquiring highly motile phenotype (EMT) and later, during the course of the disease stimulating local invasion and metastatic spread. In prostate cancer, SFK play a

major role in cell motility in both androgen-sensitive and castration-resistant disease. In this study, SFK inhibitor dasatinib suppressed migration of LNCaP and LNCaP-SDM cell lines using concentrations required to inhibit SFK activity. Dasatinib treatment resulted in major changes in the shape of the cells with cell rounding and loss of protrusions. When the cells were placed on a flat surface, dasatinib affected cell spreading in a dose-dependent manner.

Regulation of the motility of cancer cells requires activation of Src in complexes with FAK and subsequent recruitment of multiple signalling proteins (87). Extracellular signals, emanating from integrins and receptor tyrosine kinases stimulate Src/FAK complex formation, which is an essential element in the regulation of focal adhesions turn-over and changes in tumour cells leading to EMT. Inhibiting Src kinase activity may prevents FAK phosphorylation and downstream signalling, disrupting protein complexes clustering in focal adhesions and adherens junctions. *In vitro* experiments have consistently shown that SFK inhibitors reduce Src-dependent phosphorylation of FAK on tyrosine pFAKY$^{861}$, while phosphorylation of Src-independent pFAKY$^{397}$ is not affected (18;158). Our data has also confirmed that treatment of LNCaP and LNCaP-SDM cells with small nanomolar concentrations of dasatinib reduced pFAKY$^{861}$ phosphorylation in dose-dependent manner.

Inhibition of Src and downstream proteins may result in dramatic changes in cancer cells' behaviour. Recchia et al. demonstrated that PC-3 cells treated with Src inhibitor were unable to spread on flat surface. This was due to inability by cancer cells to produce amoeboid protrusions necessary for subsequent migration (16). Using time-lapse microscopy, we showed that the cells treated with small doses of dasatinib developed fewer protrusions when seeded on glass surface as compared to controls, whereas the cells treated with higher doses did not form protrusions at all. Dasatinib treatment also resulted in the cells that had already spread to become rounded although, as expected the cell-cell

contacts were maintained. Interestingly, treatment of androgen-sensitive LNCaP cells resulted in more profound changes, while castration-resistant LNCaP-SDM cells maintained their shape better.

Inability of prostate cancer cells to produce protrusions following dasatinib treatment resulted in limited migratory capacity as confirmed by wound healing assay in this study. This is due to the cells that unable to develop protrusions and, therefore, lacking the power of propulsion that propagate the body of the cells in the direction of movement. SFK inhibitors also affect invasive properties of the cells due to downregualtion of MMP-9 activity, which is FAK-dependent. MMP-9 and TIMP-1 and -2 are necessary for degradation of protein constituencies of extra-cellular matrix, allowing cancer cells to invade neighbouring structures (16). Importantly, the concentrations of SFK inhibitors used to elicit inhibition of migration have consistently been found much lower than concentrations, required to suppress proliferation and, generally, correlate well with inhibition of SFK activity probably suggesting this effect is Src-specific (162).

## 4.4 SFK Inhibitors in Clinical Trials and Practice

Development of molecular biology and our knowledge of how cancers behave stimulated the discovery of multiple potential targeting agents, many of which have never progressed beyond clinical trials. Despite unprecedented investment into the field, there has been meagre progress with only several drugs officially approved for the use in solid malignancies. Delay in adjusting the design of clinical trials to the complexities of new molecular targeted agents has been recently highlighted as a potential reason why so many substances that have been found promising in pre-clinical stages, have not been demonstrated to be effective in clinical trials (228).

Historically, histological diagnosis with staging has been the principal factor guiding the clinicians and the researchers in providing specific treatment or defining the eligibility for the clinical trials. This is despite the fact that the many cancers that histologically seem equal, possess different genetic and molecular make-up and have varying natural history. In prostate cancer, the implications of biological heterogeneity have led to the search of new approaches to the management of the disease, and yet the molecular differences are still not taken into account when selecting the patients for the clinical trials. In a letter to the editor in the Journal of Clinical Oncology, Michael Castro has written that the previous researchers' habit of presuming homogeneity in the sample group represented the simpleton's error from the vantage point of today's molecular biology (228). The author went further to question whether it is ethical to treat the patients, enrolling into the clinical trial with the inhibitors of the enzymes their cancers do not express.

Molecular heterogeneity implies that in some cases only a small subset of cancers would be sensitive to a specific targeted therapy. Therefore, inevitably the treatment might fail to show the efficacy in a conventional randomised clinical trial simply due to the

diversity of the studied population. As a result, the promising drug might never find its way into the clinical practice, denying the patients with the sensitive malignancy a potentially beneficial treatment. In an elegant statistical experiment, Betensky et al. have demonstrated that in genetically different groups of patients entering a hypothetical clinical trial comparing chemo-radiotherapy with radiotherapy alone, failure to take into account the genetic heterogeneity will in most cases result in the phase III trial being underpowered and too small (229). This is possibly due to several reasons, including enrichment of phase II trials with treatment-sensitive individuals, followed by dilution or reversal of positive effect in responders by non-responders.

In the present study, we identified a subgroup of 28% of patients in whom there was significant association between SFK activation and overall survival ($p<0.0001$). Using $pSrcY^{419}$ as a biomarker, identifying these patients may prove beneficial in patients' selection for specific targeting with SFK inhibitors. Obtaining the cancer tissue represents potential difficulty as the patients with CRPC do not routinely undergo prostatic biopsy, unless they develop the symptoms and signs of bladder outflow obstruction and the tissue is harvested by means of channel TURP. However, contemporary methods increasingly incorporate the prostate biopsy into management protocols in order to determine the progression of the disease, e.g. during active surveillance. Thus, in the future, the development of molecular targeted therapies may necessitate the biopsy of the prostate in CRPC patients with a view to identify molecular and genetic changes, guiding the treatment and defining the prognosis.

Several SFK inhibitors are now in phase I and phase II clinical trials for the patients with CRPC (155;188-192). Traditionally, there has been an assumption that the dose of cytotoxic drug is proportional to their anti-tumour activity and, therefore, in order to get maximum benefit in phase I trials the dose is escalated until it reached the level causing an acceptable toxicity. However, with new biological agents, this may not be necessary as the

aim of the treatment is to inhibit the specific enzyme. Indeed, the concentrations of dasatinib, SFK inhibitor used in this study, required to completely inhibit Src kinase activity was in the region of 10-25 nM, and this has also been demonstrated by others (18;158). Pharmacodynamics of the drug can be monitored by measuring the concentration of the inhibitor in blood and by studying the level of $pSrcY^{419}$ in peripheral blood cells as a surrogate target tissue that has been shown to correlate with intra-tumour Src activity (170).

It has been suggested that the design of phase II trials of molecular targeted agents should be altered. Enrichment of phase II trials may need to be more robust, selecting groups of patients who are likely to respond and, perhaps, carrying this further into phase III trials (230). Important considerations would have to be given to the mechanism of action of particular drugs when selecting response criteria. In the recently reported results of phase I/II clinical trials of dasatinib alone and in combination with docetaxel in patients with CRPC, the researchers used PSA response as a main endpoint, although PSA level mostly reflects the bulk of the disease, which may not necessarily be affected by the drugs (231;232). Given that it is not clear whether SFK inhibitors in general and dasatinib in particular would reduce prostate cancer cell proliferation and, therefore, actual tumour growth, more weight perhaps should be given to the appearances of new metastatic deposits. We have demonstrated that $pSrcY^{419}$ immunostaining in CRPC specimens correlated with the presence of bone metastatic disease. It would of interest to look into the development of metastases in other organs, i.e. lymph nodes, liver etc.

## 4.5 Conclusion

There is considerable evidence implicating SFK in the development of prostate cancer in its progression to castration resistance. However, translational studies investigating the significance of increased SFK expression and activation in clinical samples are lacking. To date this is first study to address the question whether existing *in vitro* data and the results of animal experiments would be relevant in clinical settings. It appears that in a subgroup of prostate cancer patients, SFK activation is associated with reduced survival and the presence of bone metastases correlated with increased SFK activity. Although the SFK role in tumour cell growth is debatable, in our experiments the treatment of castration-resistant cell line LNCaP-SDM with dasatinib resulted in inhibition of proliferation. Moreover, both androgen-sensitive LNCaP and castration-resistant LNCaP-SDM cell lines exhibited reduced migration upon exposure to dasatinib, implying potential use of the inhibitor as an anti-metastatic agent.

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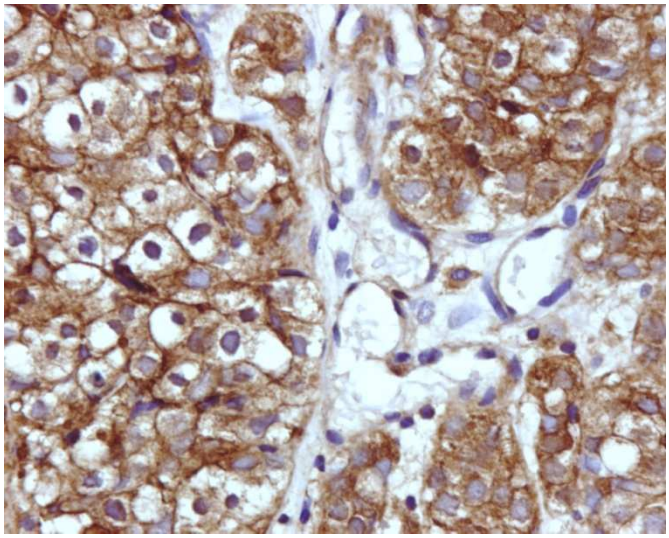
# APPENDICES

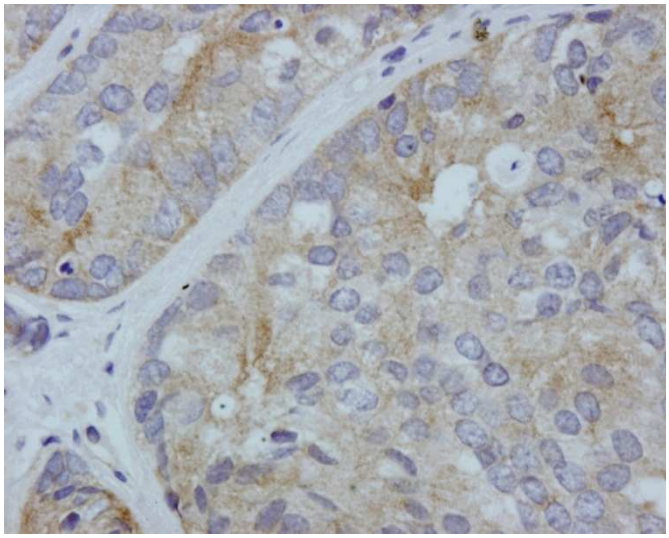
# APPENDIX 1

**Examples of immunostaining for total Src, pSrcY$^{527}$, total Lyn and total Fgr**

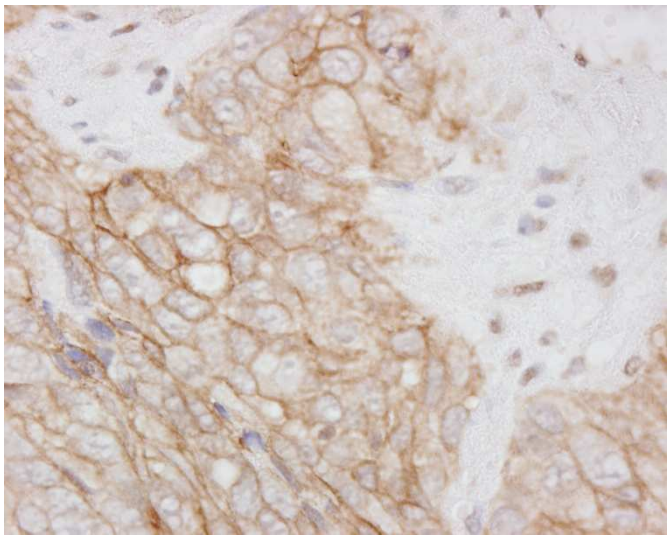
**total Src**



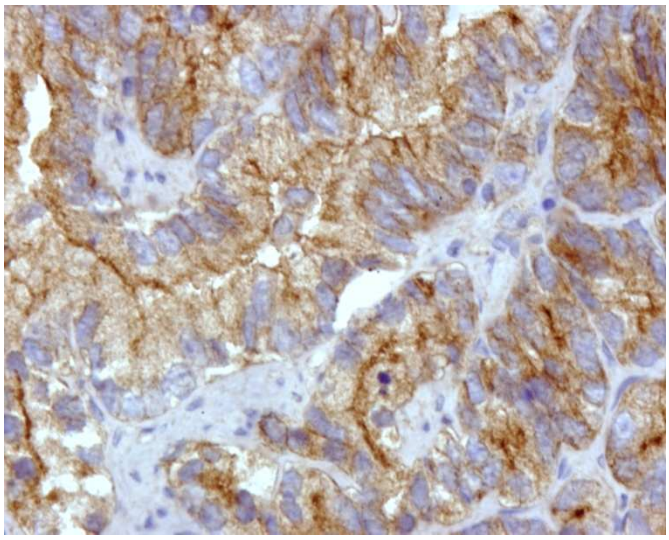
**pSrcY$^{527}$**



**total Lyn**



**total Fgr**



# APPENDIX 2

**Full reprint of published articles**