Evaluation of novel anti-metastatic therapies.

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Abstract

The occurrence of metastasis from the primary tumour to distant organs is the primary cause of cancer mortality and therefore a highly attractive therapeutic target to improve cancer survival rates. Targeted anti-metastatic therapies hold the potential for lower cancer mortality rates, lower rates of reoccurrence and better quality of life for cancer patients.

Src is a non receptor tyrosine kinase, and the first recognized oncogene, which is known to play a role in metastasis. Increased activity of SFKs has been found in many human tumours, correlating with invasiveness and poor prognosis, and in experimental tumour cell lines, contributing to enhanced cell migration and invasion, adhesion independent growth, survival and proliferation.

Despite a body of evidence in pre-clinical trials demonstrating that inhibition of Src reduces the occurrence of cellular events associated with metastasis *in vitro,* and reduces tumour metastasis to distant organs in *in vivo* models, efficacy has not translated to phase II clinical trials.

One such recent failure has been a phase II trial with the Src inhibitor AZD0530 in recurrent advanced or metastatic soft tissue sarcoma. Here we investigate the anti-metastatic effects of Src inhibition in the HT1080 fibrosarcoma cell *in vitro* in order to elucidate further the effects of SFK inhibition in sarcoma cells laying the ground work for future *in vivo* work investigating potential compensatory mechanism overcoming SFK inhibition in sarcoma cells.

We found that SFK inhibition of HT1080 cells with AZD0530 inhibited cell migration and spreading but had no effect on cell polarization. AZD0530 treatment caused active paxillin (Tyr31-p) to relocalize from focal adhesions to the cytoplasm but had no effect on staining of active FAK (Tyr861-p). Paradoxically AZD0530 treatment led to increased phosphorylation of Src at the negative regulatory site Tyr 530. Results seen here provide evidence to warrant further elucidation of the effects of Src inhibition in HT1080 cells in order to elucidate potential combination therapies and biomarkers of tumour sensitivity to Src inhibition.

Introduction

2.0 Materials and Methods

3.0 Results

Abbreviations:

Src family kinases (SFKs), Phosphoinositide 3 kinase (PI3K), Signal transducers and activator of transcription (STAT; STAT3, STAT5), Mitogen activated protein kinase (MAPK), Focal adhesion kinase FAK, Extracellular matrix (ECM), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Guanine Exchange Factor (GEF), Jun N-terminal Kinase (JNK), Matrix Metalloprotease-9 (MMP-9), dominant negative FAK fragment (FRNK), Matrix metalloprotease-2 (MMP-2), Membrane type-1 matrix metalloprotease (MT1-MMP), Neural Wiskott-Aldrich syndrome protein (N-WASP), ADP ribosylation factor (Arf), Inhibitor of differentiation protein 1 (Id1), Epidermal growth factor receptor (EGFR), Vascular endothelial growth factor receptor (VEGFR), Platelet derived growth factor receptor (PDGFR), Fibroblast growth factor receptor (FGFR), Insulin like growth factor receptor 1 (IGFR-1), Transforming growth factor-β (TGF-β), Epithelial to mesenchymal transition (EMT) Growth factor receptor-bound protein-2 (Grb2), Son of sevenless (SOS), Interleukin-8 (IL-8), Hypoxia inducible factor-1α (HIF1α), Nuclear factor κB (NF-κB), Inhibitor κB-α (IκB-α), p130cas (crk-associated substrate), Janus kinase (JAK).

1.0 Introduction

1.1 Tumour metastasis

The occurrence of metastasis from the primary tumour to distant organs is the primary cause of cancer mortality (1). The metastatic process is therefore a highly attractive therapeutic target to improve cancer survival rates. Current methods to prevent the formation of metastases, such as hormone treatment, radiotherapy or chemotherapy, are poorly targeted to tumour cells. Metastatic cancer cells may become resistant to these treatments and toxicity occurs due to the non tumour cell specific nature of therapies. More finely targeted anti-metastatic therapies hold the potential for lower cancer mortality rates, lower rates of reoccurrence and better quality of life for cancer patients (2). Further investigation of the processes by which tumour cells develop a metastatic phenotype, and successfully form metastases, is crucial in order to exploit these pathways for therapeutic benefit.

The formation of metastases from the primary tumour is a multi-step process requiring multiple, appropriately timed changes in the tumour cell to complete the process and survive in distant organs. The tumour cell must first detach from its neighbouring cells and invade the basement membrane and interstitial matrix of the surrounding tissue. The next step is intravasation; invasion of the tumour cell into the blood or lymph circulatory systems. Tumour cells may become arrested at fine capillaries, facilitating extravasation from the circulatory system to the surrounding stroma. A small subpopulation of tumour cells will then divide to form micrometastases which will undergo extensive growth and vascularisation to form a secondary tumour (3).
This report will explore the involvement of the Src family kinases (SFKs) in the metastatic process by investigating the effects of the Src inhibitor in cultured tumour cell lines.

SFKs have been demonstrated as a promising therapeutic target by *in vitro* and *in vivo* studies of metastasis and have shown to be involved in signalling leading to cell proliferation, survival, migration, anchorage independent cell growth, angiogenesis and resistance to hypoxia. An understanding of the SFK mediated pathways involved is

crucial to highlight potential anti metastatic target proteins, including proteins that interact with SFKs and members of downstream signalling pathways, as well as pathways that may be targeted in combination with SFK inhibition.

1.2 Src family kinases

Src is a non receptor tyrosine kinase first identified as the 60kDa transforming protein carried by the Rous Sarcoma virus, and subsequently recognized to be the first tyrosine kinase and oncogene, with homologues in all vertebrate cells (4). There are eight members of the SFKs: c-Src, Fyn, Yes, Lyn, Lck, Hck, Fgr, Blk and Yrk. Src, Fyn and Yes are ubiquitously expressed. Other members of the SFKs show more restricted expression that varies widely between tissues (5). SFKs act in the regulation of integrin signalling, cell-cell adhesion, focal adhesion turnover, growth factor signalling and angiogenic signalling.

Overexpression / increased activity of SFKs has been found in many human tumours and cancer cell lines, including breast cancer (6), head and neck cancer (7), bladder (8), lung (9) and colon cancer (10). In many tumour types enhanced c-Src activity has been associated with tumour invasiveness and poor prognosis (7). Aberrant activation of Src in tumours leads to enhanced cell migration and invasion, adhesion independent growth, survival and, in some contexts, proliferation.

There are currently four Src inhibitors which are being developed for clinical use. Dasatinib, AZD0530 and bosutinib (SKI 606) are all low molecular weight agents which competitively inhibit ATP binding; they also inhibit Abl and Bcr-Abl, whereas KX01 inhibits binding of selective Src substrates (11).

1.3 Structure and Regulation of SFKs

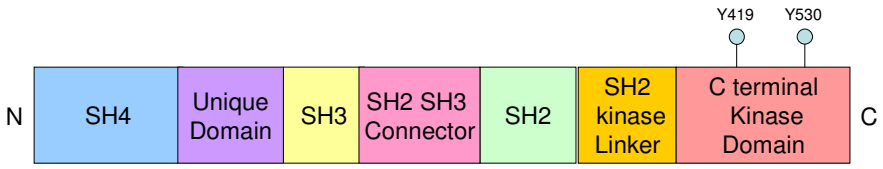


Figure 1.1

SH4) The N terminal region of SFKs contains a myristoylation sequence, additionally some SFK's are palmitoylated. These lipid modifications promote membrane localization. *Unique Domain*) 50-80 non conserved residues. *SH3*) Plays a major role in interacting with target proteins; binding to hydrophobic regions. Acts in regulation of kinase activity.*SH2*) Interacts with target proteins, binding to phosphotyrosine residues. Plays a role in regulation of kinase activity. *C terminal kinase domain*) Contains the catalytic site of the kinase and regulatory tyrosine residues Y419 and Y530 (33)

SFKs have two main regulatory tyrosines involved in regulation of its kinase activity. In the inactive conformation, phosphorylation of the regulatory tyrosine 530 (Tyr 530) in the C terminal kinase domain (See Fig.1.1) creates a phosphotyrosine residue to which the SH2 domain binds. This interaction is stabilised by an interaction of the SH3 domain with the SH2 kinase linker region. An alpha helix in the C terminal kinase region is orientated outwards, blocking catalytic activity. The activation loop of the kinase domain forms an alpha helix with the autophosphorylation site tyrosine 419 (Tyr419) facing inwards. In the active conformation, the kinase domain alpha helix is orientated facing inwards, the activation loop forms a conformation conducive to ATP and peptide binding. Autophosphorylation at Tyr419 stabilises the SFK in its active conformation. (12) The conserved inhibitory tyrosine residue Tyr530 is phosphorylated by the regulatory kinase, Csk (C terminal Src kinase). Csk is a negative regulator of SFKs that has been proposed to be upstream of SFK deregulation in some tumour types, for example in colon cancer cell lines Csk appears to be mislocalised causing aberrant SFK activity (13). Several protein tyrosine phosphatases have been shown to activate Src by dephosphorylation at Tyr530; including Phosphotyrosine Phosphatase 1B (PTP1B) which appears to be a mechanism of SFK activation in colon cancer cell lines (10).

1.4 SFKs role in survival and proliferation

SFK signalling can enhance survival and proliferation by signalling through Phosphoinositide 3 kinase (PI3K), Signal transducers and activator of transcription (STAT) and Mitogen activated protein kinase (MAPK) pathways; signalling cascades involved in regulating cellular growth, proliferation and survival.

Activation of FAK, a downstream target of active Src, triggers a variety of downstream signalling pathways, including the MAPK pathway and phosphoinositol-3 kinase (PI3K)/Akt survival pathway. c-Src phosphorylation of FAK Tyr925 in response to bound integrin, forms a binding site for the Grb2 SH2 domain, which leads to activation of the MAPK pathway (14).

Src can activate a form of PI3K composed of two subunits; p85 and p110, binding via its SH3 domain to a proline rich region of the p85 subunit of PI3K, or via binding of scaffolding proteins such as Cbl to the p85 subunit (15).

SFKs also interact with growth factor receptors to enhance downstream signalling pathways such as the MAPK pathway leading to cellular proliferation (16), (17).

The Signal transducer and activators of transcription (STAT) family are transcription factors which induce the upregulation of genes involved in survival and proliferation. STAT3 is a SFK target required for v-Src transformation. Activated STAT3 increases the expression of genes modulating survival and proliferation, including the anti-apoptotic Bcl-XL and the cell cycle regulator cyclin-D (18). SFKs can also activate STAT5 (19).

In some cancer types, for example prostate cancer, SFKs appear to play a role in initial tumourigenesis and primary tumour growth. In the prostate cancer cell line DUI45, Src mediates passage through the G1 cell cycle checkpoint by increasing β-catenin transcription, leading to induction of c-myc and cyclin-D (20). In the 7,12-dimethylbenz(α)anthracene (TPA) induced murine model of skin carcinogenesis Src appears to play a role in the early stages of tumourigenesis. Src was upregulated in response to TPA treatment. Src inhibition with AZD0530 reduced the TPA induced proliferation of keratinocytes, leading to a reduction in papilloma formation *in vivo* (21).

The ability of SFK signalling to induce proliferation appears to be highly variable between human cancer cell lines. The Src kinase inhibitor AZD0530 inhibited proliferation in some cell lines, including PC-3 prostate cancer cells, MDA MB 231 breast cancer cells and Swiss3T3. However, in other cancer cell lines, there was no effect on proliferation for example MCF-7 breast cancer cells and SKOV-3 ovarian cancer cells (22). The restriction of this anti-proliferative effect to certain tumour types only suggests the use of Src inhibitors will be most successful as part of combination therapies with cytotoxic agents and those that inhibit proliferation (11).

1.5.1 SFKs role in cell motility

Motile cells polarize toward the direction of movement with cell protrusions, lamellipodia and filopodia, at the leading edge of the cell and the retracting tail at the rear. Cell motility requires actin cytoskeleton rearrangements including polymerisation at cell protrusions and formation of actin bundles which provide the tension required to pull the cell body forward and retract the rear edge of the cell (22).

Cell motility also requires the remodelling of focal adhesions at structures such as lamellipodia and filopodia, as well as at the rear of the cell to allow release from the extracellular matrix (ECM); a mesh of protein and polysaccharide macromolecules which surround cells, and forward movement.

Src activity has also been shown to be required for cell motility in response to growth factors in NBTII bladder carcinoma cells. Cell motility in response to Fibroblast Growth Factor (FGF) and epidermal Growth Factor (EGF) was inhibited by expression of kinase defective Src, as measured by *in vitro* scratch wound assays (24).
Src is known to be involved in the regulation of both actin cytoskeleton rearrangements and the turnover of focal adhesions (53, 65, 55, 49).

1.5.2 Regulation of actin cytoskeleton rearrangements

SFKs play a role in the modulation of the Rho family of GTPases, a sub family of the Ras superfamily, including RhoA, Rac1 and Cdc42 which are important regulators of cell motility due to their ability to reorganize the actin cytoskeleton (14, 17, 25). RhoA

appears to be required for the formation of actin stress fibres, whilst Rac1 and Cdc42 stimulate the formation of lamellipodia and filopodia respectively (8, 26).

Src phosphorylation of p130 Crk associated substrate (p130cas) produces a binding site for Crk (27). The resulting p130cas/Crk/DOCK180 scaffold formation leads to activation of Rac1 via activation of Rho and its downstream effector mDia, and resulting lamellipodia formation (28). In fibroblasts assembly of the CAS/Crk/DOCK180 complex leads to the formation of the membrane ruffles which appear in the early stages of lamellipodia. Presently, it appears that this same mechanism also operates in epithelial cells, although this has not been fully elucidated (14). p130cas deficient fibroblasts show defects in cell motility, cell migration toward fibronectin and cell spreading which were rescued by the restoration of p130cas expression (29). Pancreatic carcinoma cells with *in vivo* metastatic properties showed a fourfold increase in migration on the ECM proteins fibronectin and vitronectin after transient expression of p130cas, which was associated with p130cas phosphorylation and binding of Crk (61). Paxillin is a downstream effector of active Src which is known to have a role in lamellipodia formation. Paxillin deficient cells displayed defects in lamellipodia formation and cell migration (31). Another major Src substrate, cortactin, has been shown to regulate Arp2/3 actin branching, involved in the formation of lamellipodia. Cell migration is inhibited *in vitro* by siRNA targeting cortactin, and in a cortactin deficient Drosphila model (32).

Src also binds and phosphorylates RhoGD12, a regulatory molecule which has been shown to suppress metastasis and that is frequently underexpressed in tumours. RhoGD12 inhibits the activation of GTPases by sequestering them to the cytoplasm and inhibiting Guanine Exchange Factor (GEF) activation. GEFs activate GTPases by stimulating the exchange of bound GDP for GTP. Where RhoGD12 expression is maintained it is thought that phosphorylation by Src at Tyr531 negatively regulates its tumour suppressor function. Bladder cancer cells expressing RhoGD12 mutated at the Src phosphorylation site showed a decreased propensity to metastasise to the lung when injected into nude mice (25).

In vitro studies have demonstrated the importance of Src activity in the formation of actin structures associated with forward cell movement. Swiss 3T3 cells expressing a kinase active mutant (Tyr527F Src) lacking the C terminal negative regulation site

resulted in the formation of continuous, uniform lamellipodia whereas expression of kinase deficient Src251 resulted in the formation of aberrant discontinuous structures (33). Expression of constitutively active Src in colon carcinoma cells leads to the formation of integrin dependent adhesions associated with cellular protrusions required for cell motility (34).

Knockout of the ubiquitously expressed SFKs: Src, Yes and Fyn (SYF), in mice led to severe developmental defects and was embryionic lethal at E9.5. SYF cells derived from mouse embryos displayed inhibition of migration in scratch wound assays. However, the activity of SFKs does not appear to be essential for the formation of cellular protrusions associated with motility; SYF cells formed extensions seen in motile cells, but could not utilise them for forward propulsion (35).

1.5.3 Focal adhesion turnover

The ability of SFKs to regulate cell motility appears to be due to their role in the turnover of focal adhesions.

Focal adhesions are the points at which the cell attaches to the ECM via integrin receptors, providing a link between the ECM, the actin cytoskeleton and downstream signalling pathways. Focal adhesions are continuously being disassembled and reassembled in response to integrin clustering upon ECM ligand binding. The turnover of focal adhesions, protein complexes involved in adhesion to the ECM, allows forward cell movement. SFK activity is required for the turnover of focal adhesions (35).

Focal adhesions are made up of signalling molecules; including SFKs, Focal adehesion kinase (FAK), paxillin, and various scaffolding proteins. FAK is a non receptor tyrosine kinase activated downstream of integrin receptors (see figure 1.2). Integrin receptor clustering results in FAK autophosphorylation at Tyr397 creating a phosphotyrosine residue for binding of SFKs via their SH2 domain (36). Src has been proposed to phosphorylate FAK at Tyr407, Tyr576, Tyr577, Tyr861 and Tyr925. Mutation of these tyrosine residues in colon carcinoma cells expressing constitutively active Src led to a decrease in the formation of motile cell protrusions; specifically a defect in the release of adhesion molecules at the rear edge of the cell which allow retraction of the trailing edge and forward cell movement. In this context, only the phosphorylation of Tyr925

was shown to be dependent on Src kinase activity whereas the phosphorylation of other tyrosine residues by Src was dependent on an intact SH2 domain as was Src:FAK localisation to the cell periphery (34). In murine fibroblasts, Src phosphorylation of FAK residues Tyr576 and Tyr577, appear to be required for full activation (37).

The FAK: Src complex acts as a potent signalling complex and plays a crucial role in focal adhesion turnover allowing for forward cell movement via phosphorylation of downstream effectors such as the scaffolding proteins p130cas and paxillin, which are required for focal adhesion turnover (33, 31, 4). Evidence from paxillin deficient cells suggests that paxillin also plays a role in FAK localisation to focal adhesions (31).

It appears that Src kinase activity is not essential for the formation of focal adhesions, but is critical for the dissociation of focal adhesions. SYF cells are able to form focal adhesions, despite a substantial reduction in the tyrosine phosphorylation of several focal adhesion components including paxillin, FAK and p130cas (34). Transformation of chicken embryonic fibroblasts with kinase inactive or myristylation defective temperature sensitive v-Src mutants demonstrated the requirement for the Src kinase activity and membrane association for phosphorylation of FAK, which precedes its dissociation from Src, FAK degradation and focal adhesion turnover. Expression of kinase deficient Src251 in v-Src transformed mouse embryonic fibroblasts displayed abnormally large focal adhesions associated with defective focal adhesion turnover (38).

RhoGTPases, known to be regulated by SFKs, are also involved in the remodelling of focal adhesions for cell motility. RhoA is required for the formation of focal adhesions located at the plasma membrane terminus of actin stress fibres (8). Both Rac1 and Cdc42 control the formation of focal complexes found in filopodia and lamellipodia which are distinct from focal adhesions but contain vinculin, paxillin and FAK (26). Src kinase activity is essential for the conversion of focal adhesions into the smaller focal complexes along the base of lamellipodia and filopodia, required for cell motility (33).

Additionally, RhoGTPases modulate Src activity at focal adhesions by their ability to target Src to adhesion complexes at the cell periphery via actin stress fibre (8). Src may be targeted to focal adhesions or smaller adhesions at filopodia and lamellipodia. This fate is regulated by the balance of RhoGTPases within the cell (33, 28). The trafficking of Src to the cell periphery is dependent on the PI3K regulatory subunit p85, a subunit

known to be involved in Src activation of PI3K (as discussed in section 1.4) as well as an intact actin cytoskeleton, integrin engagement and the SFK SH3 domain, but may be independent of SFK kinase activity as shown by studies of temperature sensitive v-Src mutants (8).

1.6 Integrin signalling

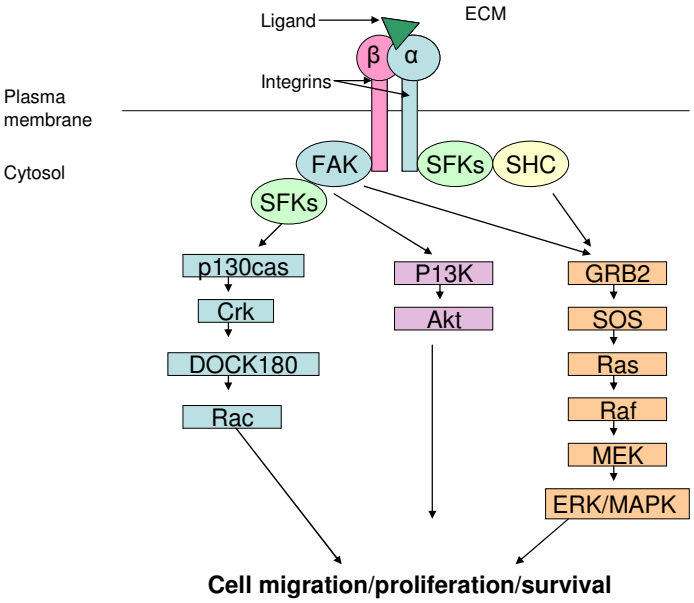


Figure 1.2. Integrin signalling via FAK/SFKs
Integrin clustering upon ECM ligand binding e.g. fibronectin, leads to the activation of FAK and formation of a binding site for SFKs. FAK is further activated by SFK. Signalling by the FAK:SFK complex leads to the formation of the p130cas/Crk/DOCK180 scaffold and activation of the PI3K/Akt and MAPK/Erk pathways, resulting in increased cell migration/proliferation and survival. SFKs can also activate the MAPK pathway downstream of integrin signalling by phosphorylation of the adaptor protein SHC, leading to Grb2 and SOS binding and downstream Ras/Raf/MEK/MAPK activation (4).

As discussed, the Src:FAK complex is activated downstream of integrin clustering. Src can also be activated downstream of integrin clustering, independently of FAK. In a cell model replicating platelets and osteoclasts, there was found to be a pool of Src bound via its SH3 domain to the β3 integrin cytoplasmic tail. This binding interrupts the intramolecular interactions that keep Src in an inactive conformation. Upon integrin clustering this pool of bound Src increases such that the trans-autophosphorylation of Tyr419 is sufficient to significantly increase overall Src activity (40). Downstream of this FAK independent integrin activation, Src phosphorylates the protein tyrosine kinase Syk leading to the activation of Rac and the formation of lamellipodia, see figure 1.2

(41).

During tumour progression, tumour cells switch their integrin expression to those that are associated with survival, proliferation and metastasis. Some of these pro-metastatic integrins signal via SFKs. α6β4 signalling is associated with a metastatic and invasive carcinoma cell phenotype and it is relocated from hemidesmosomes to filopodia and lamellipodia in metastatic carcinoma cells. It has been shown that α6β4 can signal through Src, as well as Akt and Nuclear Factor of Activated T-cells (NFAT), to increase expression of S100A4, a member of the S100 calcium binding family associated with poor prognosis and an invasive and metastatic phenotype (42). αvβ3 integrin signalling has also been associated with poor prognosis and has been shown to require binding of Src to the β3 cytoplasmic tail, Src activation, and recruitment of p130cas. Evidence suggests αvβ3 signalling via Src leads to enhanced anchorage independent cell growth, implying that it may allow tumour cells to survive in the circulation during metastasis. Treatment with the Src inhibitorDasatinib reduced the mass of metastases in nude mice injected with αvβ3 positive pancreatic tumour cells, as compared to αvβ3 negative pancreatic tumour cells (43).

1.7 Invasion

The process by which cells gain an invasive phenotype comprises of changes in gene expression, integrin expression and the release of proteases. The Src:FAK complex appears to be crucial for the development of an invasive phenotype. In FAK null cells v-Src transformation was able to rescue integrin mediated cell migration but not invasive capability. The formation of a p130cas/Crk/Dock180 scaffold appears to activate Rac and Jun N-terminal Kinase (JNK), leading to the increased expression of Matrix Metalloprotease-9 (MMP-9). V-Src transformed FAK null fibroblasts showed a decrease in MMP-9 mRNA levels. Their invasive phenotype could be restored by over expression of JNK, which elevated MMP-9 expression (44). Expression of a dominant negative FAK fragment (FRNK) in v-Src transformed 3T3 cells, inhibits formation of the Src:FAK complex, FAK phosphorylation at Tyr861 and Tyr925 and p130cas tyrosine phosphorylation, and leads to inhibition of invasion in matrigel invasion assays. FRNK was found to inhibit activation of Extracellular signal-regulated kinase (Erk) and JNK leading to a decrease in matrix metalloprotease-2 (MMP-2) mRNA levels and MMP-2 secretion. The invasive capacity of FRNK expressing cells could be restored by

MMP-2 over-expression. Subcutaneous injection of FRNK expressing v-Src transformed 3T3 cells in nude mice did not inhibit primary tumour growth but showed a significant reduction in the occurrence and extent of lung metastasis (44).
Src kinase has been shown to be essential for the formation of invadopodia; actin rich structures involved in degradation of the ECM at the leading edge of the cell. First identified in Src transformed cells, invadopodia are distinct from lamellipodia and filopodia in that they are enriched in proteases, such as Membrane type-1 matrix metalloprotease (MT1-MMP), MMP-2 and MMP-9 (46). There are several SFK target proteins which are involved in the formation of invadopodia.
The SFK substrate cortactin, a main component of invadopodia, plays a role in regulating actin branching and membrane trafficking of proteases by downstream activation of the Arp2/3 complex and Neural Wiskott-Aldrich syndrome protein (N-WASP) (32, 47). MDA MB 231 breast cancer cells expressing cortactin with mutations in sites of Src phosphorylation led to a significantly lower frequency of bone metastases in *in vivo* models (48). Cortactin over-expression occurs in many human tumours and correlates with poor prognosis (32). Src dependent phosphorylation of the ADP ribosylation factor (Arf) specific GTPase activating protein (GAP): ASAP1, which regulates actin polymerisation, is required for the formation of invadopodia (70). Inhibitor of differentiation protein 1 (Id1) is required for the formation of invadopodia in MDA MB 231 cells. Id1 regulates the expression of MMP-9 in a Src kinase dependent manner. The formation of invadopodia also involves reorganisation of the cytoskeleton which is regulated by RhoGTPases (46).

1.8 Anchorage independent growth

Anchorage independent cell growth is a common feature of tumour cells, allowing them to metastasise, survive in the circulation and colonise distant sites. Normal cells undergo anoikis, which is apoptosis induced upon detachment from the ECM. Anoikis is mediated by integrin and ligand independent growth factor receptor signalling leading to a change in balance of pro-apoptotic and pro-survival molecules. In normal cells detachment from the ECM leads to a reduction in Erk and P13K signalling and cell death by anoikis. Aberrant Src activation can lead to anchorage independent survival and cell growth via activation of Erk and PI3K (50).

The pro-metastatic integrin αvβ3 has also been shown to play a role in anchorage independent cell growth. αvβ3 expressing cells recruit and activate c-Src at the β3 cytoplasmic tail, leading to phosphorylation of p130cas and cell survival (43). p120ctn is essential for anchorage independent growth cell growth induced by oncogenic Src (51).

1.9 Growth factor signalling

SFKs interact bi-directionally with growth factor receptors including epidermal growth factor receptor (EGFR), Vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), Insulin like growth factor receptor 1 (IGFR-1), hepatocyte growth factor receptor, colony stimulating 1 receptor, stem cell factor receptor and muscle specific kinase, binding to the receptors via its SH2 domain (5). For example, Src, Fyn and Yes bind to sites of autophosphorylation on the PDGFR-β receptor and binding of PDGF has been shown to induce an increase in activity of bound SFKs (16, 17). Signalling via growth factor receptors triggers a plethora of signalling cascades, leading to increased proliferation, survival and growth factor stimulated migration. Src is involved in the aberrant signalling by growth factors during tumourigenesis. Oncogenic signalling by Transforming growth factor-β (TGF-β) has been shown to be dependent on Src and αvβ3 expression, leading to activation of MAPK pathway, invasion and epithelial to mesenchymal transition (EMT), see section 1.10 (52). SFKs can activate the MAPK pathway by phosphorylation and binding of the Shc adaptor protein, which recruits Growth factor receptor-bound protein-2 (Grb2) and Son of sevenless (SOS) and results in downstream activation of Ras and consequently the MAPK pathway (53).

1.10 Epithelial to Mesenchymal Transition (EMT)

The epithelial to mesenchymal transition (EMT) is a process which occurs during normal embryonic development, and is a common feature of tumour progression. It is characterised by transition from a polarised epithelial cell phenotype to a motile/mesenchymal phenotype. Downregulation of E-cadherin destabilises cell-cell adherens junctions and effects downstream pathways, (Fig 1.3.) leading to cell dissociation, acquisition of cell motility and cell scattering (54).

SFK activity has been shown to negatively regulate the stabilisation of E-cadherin containing cell-cell contacts in normal epithelial cells (55). In the majority of head and neck cancer cell lines and human tumour biopsies, levels of Tyr419 phosphorylated Src correlated with decreased E-cadherin expression and increased expression of the mesenchymal marker, vimentin. Increased Src activity was associated with poorly differentiated tumours and finger like invasive fronts. Inhibition of Src resulted in increased E-cadherin expression and inhibited migration in head and neck cancer cell lines (7).

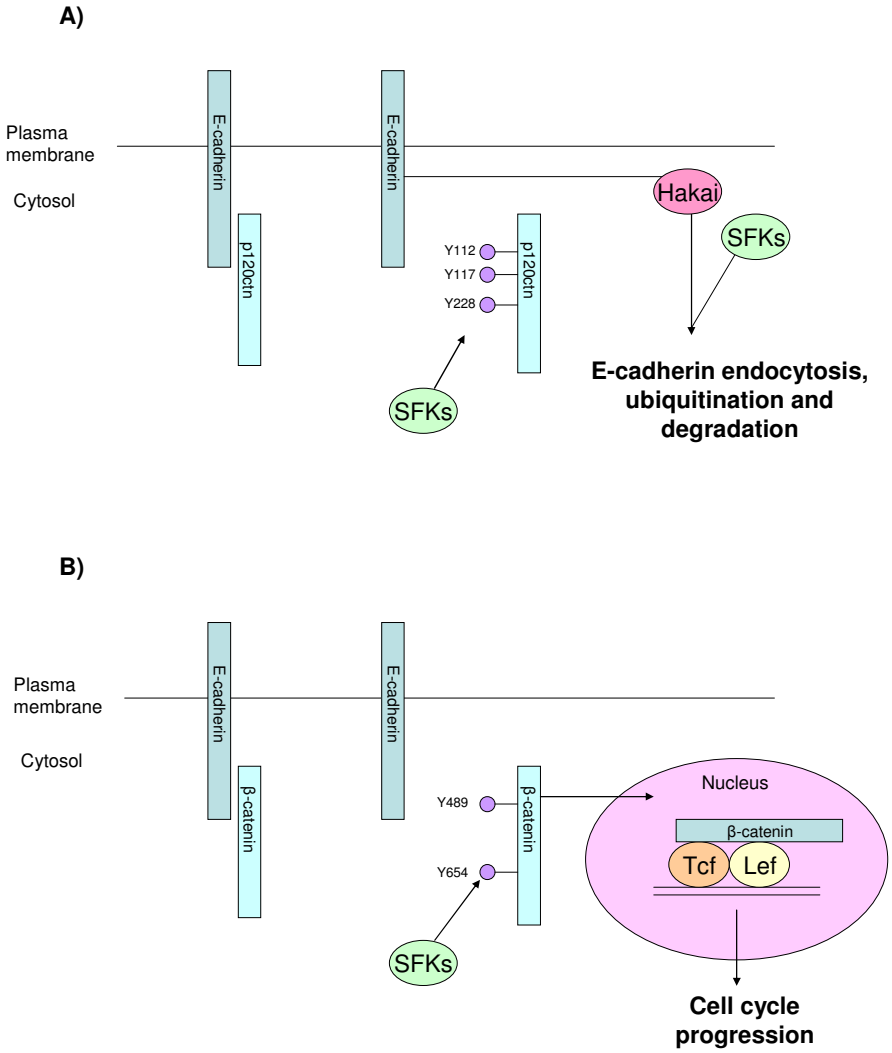


Figure 1.3. Adherens Junctions
Catenins p120ctn and β-catenin bind to the cytoplasmic tail of the cadherin molecules stabilising cell-cell adherens junctions and linking them to the actin cytoskeleton. A) p120ctn is phosphorylated on Y112, Y117 and Y228. The role of tyrosine phosphorylation by SFKs is unclear. Binding of p120ctn to E-cadherin may stabilise adherens junctions and prevent E-cadherin degradation. Destabilised E-cadherin may be targeted for degradation by Hakai, an E3 ubiquitin ligase, in a SFK dependent manner (54), (56), (57). B) Tyrosine phosphorylation of β-catenin at Y489 (by Abl) and Y654 (by SFKs) has been shown to disrupt binding to E-cadherin. Non cadherin bound β-catenin may translocate to the nucleus where it can bind to the transcription factors Tcf/Lef and enhance the expression of genes involved in cell cycle progression, for example in response to Wnt signalling (58), (57).

Downregulation of E-cadherin has been shown to be due to the targeting of E-cadherin to lysosomes. Expression of v-Src led to the phosphorylation of E-cadherin, likely to be a critical step in its ubiquitination and transport to the lysosome for

degradation. Expression of v-Src also activated two GTPases, Rab5 and Rab7, involved in transport to the lysosome (54). *In vitro* experiments show that this ubiquitination can be carried out by an E3 ubiquitin ligase, named Hakai. Expression of v-Src increased the interaction of Hakai with E-cadherin, and consequently E-cadherin ubiquitination. Overexpression of Hakai in Madin-Darby Canine Kidney (MDCK) epithelial cells lead to enhanced cell dissociation (56).

Src activity has been shown to be required for EMT in response to growth factors, in NBTII bladder carcinoma cells. Overexpression of c-Src led to the formation of a subpopulation of cells showing characteristics of the EMT including cell dissociation and vimentin expression (24).

Target proteins of the SFKs include β-catenin and p120-catenin (p120ctn) which stabilise cadherins at cell-cell junctions and link them to the actin cytoskeleton. It is thought that β-catenin has a main role in linkage to the actin cytoskeleton, whilst the primary action of p120-ctn is stabilisation of E-cadherin (51).

β-catenin has a dual role as a component of the cell adhesion/actin cytoskeleton network and as a transactivator for the TCF/LEF family of transcription factors with target genes, including cyclin D and c-myc, that are involved in cell cycle progression. Phosphorylation of β-catenin leads to dissociation from E-cadherin and destabilisation of cell-cell junctions. In Ls174T and DLD-1 colorectal cancer cells Src and β-catenin were physically associated. Inhibition of SFKs using bosutinib, or introduction of c-Src siRNAs, inhibited the phosphorylation of β-catenin, suggesting that Src is the primary activating kinase. There is evidence that Src activation of β- catenin diverts the protein from its function at cell-cell junctions to its nuclear function as a transactivator (58).

p120ctn is a major SFK substrate frequently downregulated or mislocalised in human tumours. p120ctn plays a role in the modulation of E-cadherin turnover and regulation of RhoGTPases. p120ctn is found mislocalised to the cytoplasm, and less frequently to the nucleus, in cells that have undergone EMT. There are several isoforms of p120ctn and EMT involves changes in expression of different p120ctn isoforms to those more conducive with an invasive phenotype. There are many regulatory phosphorylation sites on p120ctn and the role of SFK tyrosine phosphorylation of p120ctn is unclear. There is, as yet, no direct evidence that p120ctn is involved in Src mediated transformation (59), (60), (9).

1.11. 1 SFK signalling in angiogenesis

Angiogenesis is a critical step in tumour growth and metastasis, mediated by integrin and pro-angiogenic factors including vascular endothelial growth factor (VEGF) and Interleukin-8 (IL-8). Exposure to angiogenic factors leads to the formation of new blood vessels, which branch from existing vessels. An early event in angiogenesis is an increase in vascular permeability caused by dissociation of cells in the endothelium. Proteases such as matrix metalloproteases are produced which degrade the basal lamina. Endothelial cells are then stimulated to proliferate and migrate to form new blood vessels (61).

VEGF is an endothelial specific mitogen that induces vascular permeability and neovascularisation. VEGF is upregulated in many human tumours and has been associated with poor prognosis in colorectal cancer. There are three distinct VEGF receptors, (VEGF-R 1-3) expressed exclusively on endothelial cells. VEGFR-2 is the main mediator of angiogenesis, which interacts with PI3K/Akt to drive DNA synthesis and endothelial cell proliferation and differentiation (62).

SFKs have been shown to modulate VEGF both through the regulation of gene expression and interaction with the VEGF receptor. Over-expression of v-Src in a glioblastoma cell line increased activity from a VEGF promoter luciferase reporter construct (63). VEGF treatment of a colon carcinoma cell line enhanced cellular migration, but not proliferation and associated with the formation of a VEGFR/SFK complex, downstream phosphorylation of FAK, paxillin and P130cas. VEGF induced migration and proliferation were ablated by inhibition of SFKs or VEGF (62).

Chicken embryos infected with retroviral vectors encoding kinase deficient Src251 or Csk, which suppress multiple SFKs, demonstrated the requirement for SFKs in angiogenesis. In the same study Src knockout mice displayed a reduction in VEGF induced vascular permeability but no inhibition of angiogenesis. Src knockout mice received intradermal injections with an adenovirus expressing human VEGF cDNA in the ear. No difference in angiogenesis was evident, as measured by counting vascular branch points. Intravascular injection of Evans blue dye showed a complete blockage of vascular permeability in response to VEGF, as compared to heterogeneous Src +/- mice (64). Src and Yes knockout mice recipients of Lewis lung carcinoma cells showed a reduction in metastatic lesions in the lung. Injection of VEGF into Yes knockout mice resulted in reduced dissociation of the vascular endothelial cadherin β-catenin complex in lung epithelia as compared to wild type mice. These results suggest that members of the SFK family may be required for the increase in vascular permeability in response to

tumour growth, which facilitates the extravasation of tumour cells. Tumour growth was not inhibited in Src or Yes knockout mice, suggesting that angiogenesis was unaffected (65). In murine xenograft models using lymphoma cells, treatment with anti-VEGFR antibodies reduced tumour growth and vascularisation (66). Inhibition of Src in SKOV-3 ovarian carcinoma cells reduced VEGF mRNA levels and led to a reduction in vascular thickness and vessel branching in the resulting primary tumours when implanted in a nude mouse model (67).

IL-8 induces vascular permeability in the early stages of angiogenesis via transactivation of VEGFR-2 by the IL-8 receptors CXCR1 and CXCR2. Src is required upstream of IL-8 induced VEGFR-2 transactivation. SFK inhibition with SU6656 blocked IL-8 induced VEGFR2 phophorylation, CXCR1/2:VEGFR2 receptor complex formation and increased vascular permeability in cultured endothelial cells (61). Src can also modulate IL-8 expression. Overexpression of v-Src increased IL-8 promoter activity. Src activation of a member of the STAT family, STAT-3 is found to be involved in IL-8 expression by endothelial cells in response to the phospholipid: 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (68).

The integrin complex αvβ5 has been shown to be required for angiogenesis *in vivo*. VEGF induced angiogenesis in rabbit corneas could be blocked by treatment with an antibody targeting $\alpha v\beta_5$ (69). VEGF induces the Src phosphorylation of FAK on Y861, leading to the formation of a FAK: $\alpha v\beta_5$ complex which induces angiogenesis. Src deficient mice show a reduction in the formation of FAK: $\alpha v\beta_5$ complex in response to VEGF and a reduction in vascular permeability (70).

1.11.2 SFKs and hypoxia

Hypoxia is a key feature of tumours caused by disorganised structure of tumour vasculature and increased distance between tumour cells and local blood vessels. Tumour hypoxia is associated with a negative prognosis and poor response to treatment (71, 72). Initially hypoxia is detrimental to the survival of tumour cells however it leads to selection of a hypoxia resistant subpopulation and induction of VEGF expression, which stimulates angiogenesis (73). Src activity is rapidly increased in response to hypoxia, in both normal cells and tumour cells and appears to play a role both in the induction of VEGF expression and tumour cell survival in hypoxia. In the adenocarcinoma cell line HT29, reduction of c-Src expression, using an antisense

vector, lead to a decrease in constitutive VEGF expression, a large decrease in hypoxia induced VEGF expression, and a reduction of *in vivo* tumour vascularisation (74). In pancreatic and prostate carcinoma cell lines exposure to cobalt chloride, which mimics hypoxia, led to VEGF expression that was shown to be dependent on Src activation. Hypoxia induced Src activation leads to the stabilisation of Hypoxia inducible factor-1α (HIF1α) and increased phosphorylation of STAT3 which bind to the VEGF promoter along with the co-factors CBP/p300 and REF-1/APE and induce VEGF expression (75). Src kinase is also found to be essential for the activation of the hypoxia signalling pathway by Notch, an extracellular ligand which promotes angiogenesis. Src kinase phosphorylates STAT3 which causes HIF1α stabilisation and transcription of target genes, including VEGF (76). Activation of Src and FAK occur as part of the tumour cell's response to hypoxia and may contribute toward tumour cell survival in hypoxic conditions. Mice bearing BxPC3 xenografts were exposed to air or 7% oxygen for 3 hours and tumour hypoxia increased from 14 to 29% respectively. Hypoxic regions showed a significant increase in total Src, Tyr419 phosphorylated Src and Tyr861 phosphorylated FAK, as compared to non hypoxic regions. There was a tendency towards accumulation of HIF1α at regions where activated Src and FAK had been located (77).

c-Src has been implicated in tumour cells ability to survive in hypoxic conditions via activation of Nuclear factor κB (NF-κB), a transcription factor which promotes cell survival, invasion, angiogenesis and glycolytic ATP production. After exposure to 2-5% oxygen heptoblastoma and neuroblastoma cell lines displayed an increase in c-Src Y416 phosphorylation, the murine equivalent of the human Tyr419 autophosphorylation site. This was shown to be dependent on mitochondrial ROS generation and which requires SFK Cys487 oxidation. Hypoxia induced c-Src activitation lead to phosphorylation of Inhibitor κB-α (IκB-α) and NF-κB, which was ameliorated by treatment with the Src inhibitor PP2 (78).

1.12 Osteoclast function

Homozygous Src KO mice show a reduction in bone resorption due to reduced osteoclast function (79). Src mediated osteoclast activity in patients with metastatic bone disease can result in a cycle of bone destruction. Growth factors are released into the bone microenvironment, stimulating SFK activity and so increasing osteoclast

function, leading to further bone destruction (80). A clone of MDA MB 231 cells with enhanced capacity for bone metastasis showed increased total Src and increased activated Src levels relative to parental MDA MB 231 cells. *In vivo*, cells transfected with c-Src carrying a mutation in the ATP binding site showed a reduction in size of bone metastases (81).

1.13 Resistance to cancer therapeutics

Enhanced c-Src activity has been found in Tamoxifen resistant, oestrogen receptor (ER) positive breast cancer cell lines and in metastatic tissue from recurrent hormone treated breast cancer. Proliferation of tamoxifen resistant cell lines was inhibited by SFK inhibition with PP2 suggesting that Src inhibitors may be useful in the treatment of tamoxifen resistant ER positive breast tumours (82).
Elevated p130cas expression has also been associated with Tamoxifen resistance in human cell lines and tumour biopsies. The mechanism by which p130cas promotes Tamoxifen resistance may be through Src activation and resulting phosphorylation of EGFR and STAT5, promoting growth and survival (19).

1.14.1 Preclinical studies with Src inhibitors

The availability of small molecule inhibitors of SFKs, and their low toxicity, has allowed for a large number of preclinical trials in a wide range of tumour types, both *in vitro* and *in vivo* (11). PD180970, SU6656/SU6566, PP2 and PP1 have been used in preclinical trials whereas Dasatinib, AZD0530 and bosutinib have been used in both preclinical and clinical trials (4).

1.14.2 *In vitro* studies

1.14.2.1 AZD0530

The Src kinase inhibitor AZD0530 inhibited migration in A549 lung cancer cells in a microdroplet assay and MDA MB 231 breast cancer cells in scratch wound assays. Collagen or EGF induced migration of NBT-II bladder cancer cells was inhibited by AZD0530. AZD0530 inhibited invasion of HT1080 human fibrosarcoma cells in a 3D collagen matrix, as measured by the proportion of cells invading beyond 60μm. Inhibition of migration and invasion was accompanied by reduced phosphorylation of

paxillin in a dose dependent manner (1-25μM) and relocalisation of paxillin from the cell membrane to the cytoplasm at 1μM. Cell scattering in response to EGF was completely inhibited by AZD0530 treatment in NBT-II cells. The effect of AZD0530 treatment on proliferation was found to be highly variable between human cancer cell lines (22). AZD0530 treatment of DU145 and PC3 prostate cancer cells inhibited migration on fibronectin in a Boyden chamber migration assay, in a dose dependent manner (0-2 μM). AZD0530 treatment decreased phosphorylation of p130cas Y410, paxillin Y118 and FAK Y576/577 (20).

1.14.2.2 Bosutinib

Bosutinib treatment of MDA MB 231 cells inhibited migration, in a 2D scratch assay. Bosutinib showed no affect on cell proliferation or survival in breast cancer cell lines. Treatment with bosutinib reduced phosphorylation of Src Y419, p130cas Y410 and FAK Y576/577 and Y925. There was no inhibition of STAT3 phosphorylation but STAT3 failed to localise to focal adhesions. Bosutinib treated cells showed an increase in membrane bound β-catenin, although total levels of the protein remained unchanged (83). Bosutinib also inhibited the migration of PC-3 and DU145 prostate cancer cell lines in in vitro scratch assays and in Boyden chamber matrigel invasion assay (86).

1.14.2.3 Dasatinib

Dasatinib reduced migration of PC-3 prostate cancer cells in a Boyden chamber migration assay. Dasatinib inhibited FAK Y861 and p130cas Y165 phosphorylation. Interestingly, in this study, Src and Lyn, two SFKs, implicated in prostate tumour growth and progression, appear to carry out distinct roles with Src primarily driving cellular proliferation and Lyn driving cell migration (84).

1.14.3 *In vivo*

1.14.3.1 AZD0530

Once day oral dosing of AZD0530 inhibited the growth of Swiss3T3 xenografts in nude mice. However, nude mice bearing human cancer cell xenografts treated with AZD0530 showed only a moderate inhibition of growth in 4/10 of the xenograft models, including mice bearing MDA MB 231 xenografts. This indicates that the growth inhibitory effects of AZD0530 are variable between human cancer cell types *in vivo*, as similarly

observed *in vitro*. AZD0530 treatment showed no effect on tumour vascularisation in mice bearing MDA-MB-231 xenografts. Once daily dosing with AZD0530 (10/ 25 or 50mg/kg) in nude mice bearing NBT-II xenografts resulted in a reduction in the number of mice from which tumour colonies could be grown from mesenteric lymph node extracts, in all dose groups. A decrease in FAK Y861 and paxillin Y31 phosphorylation was seen in the 50mg/kg dose group after 14-28 days of AZD0530 treatment. Interestingly a lag time of 7 days between cell implantation and the beginning of AZD0530 treatment had no effect on its anti-metastatic benefits (22). Nude mice bearing DU145 prostate cancer xenografts daily dosing with 25mg/kg AZD0530, beginning 2 days after tumour cell implantation, lead to a 45% reduction in primary tumour size (20). Treatment of breast cancer MDA MB 231 cells xenografts in immunocompromised mice with the Src inhibitor CGP76030 reduced the growth of primary tumour and the formation of lung metastasis and resulted in a delay in the appearance of bone metastasis. Xenografts created using MDA MB 231 cells transfected with dominant negative Src reduced the incidence of lung and bone metastasis when compared with non transfected MDA MB 231 cells and MDA MB 231 cells transfected with wild type Src (85).

1.14.3.2 Bosutinib

Bosutinib treatment reduced the frequency of the occurrence of skeletal metastases in mice bearing PC-3 xenografts (86).

1.14.3.37 Dasatinib

In murine PC-3 cell xenograft models, treatment with Dasatinib, beginning 2 days after implantation of tumour cells, led to an inhibition of tumour growth and the formation of iliac lymph node metastases, accompanied by a reduction in Tyr861 phosphorylated FAK. The ability of Dasatinib to inhibit metastasis was not due to reduced size of the primary tumour. This was demonstrated by a second experiment in which Dasatinib mice were sacrificed at a later time point than control group so there was no statistical difference in primary tumour weight at point of sacrifice 84). In nude mice transplantation of pancreatic tumour cells expressing reduced levels of c-Src due to expression of a stable vector encoding siRNA targeting c-Src, led to decreased tumour size and incidence of lymph node and liver metastasis (87).

1.15 Combination therapy

There is evidence that tumour cells may overcome SFK inhibition by upregulation of alternative signalling pathways. Prolonged SFK inhibition in head and neck squamous cell carcinoma cells led to an increase in Janus kinase (JAK):STAT3 binding and reactivation of STAT3. siRNA inhibition of JAK or STAT3 concurrently to SFK inhibition led to an increase in apoptosis (18). In lung cancer cells, Src inhibition with AZD0530 treatment longer than 1 hour led to an increase in levels of activated STAT3 and its activators JAK1-3. Activation of the JAK STAT has been shown to promote survival and proliferation. It was shown also that AZD0530 may sensitize the lung cancer cell lines used to pro-apoptotic signals, in this case irradiation, by inhibition of Akt (88). These results suggest that combined anti-JAK and SFK therapy maybe more successful than either therapy alone and may protect from the development of tumour cell resistance to SFK inhibition (18).

1.16 FAK as an anti-metastatic therapeutic target

FAK is a ubiquitously expressed protein tyrosine kinase with an essential role in cell migration, proliferation and survival as well as a role in promoting tumour metastasis and angiogenesis (52). Although v-Src FAK null fibroblasts show normal integrin mediated migration they show significant defects in *in vitro* invasion assays (44). FAK has been found to be upregulated in tumour cell lines and invasive human tumours (89). FAK overexpression has been shown to correlate with tumour invasiveness in breast and colon carcinomas (90). FAK has shown to be a promising therapeutic target in some preclinical studies. Expression of FRNK, a dominant negative FAK, in adenocarcinoma cells inhibited migration *in vitro* and reduced the formation of lung metastase *in vivo*. Expression of FRNK during the first five days after tail injection of tumour cells was sufficient to inhibit the formation of lung metastases whereas expression of FRNK 11 days post tumour cell injection was not, suggesting that FAK activity is important in the initial stages of the metastatic process (91).

There has been much less focus on FAK than Src as a potential target to inhibit tumour cells metastasise. Src and FAK act as a complex to synergistically modulate downstream effectors and so it would follow that inhibition of either protein would have the same consequence on cell signalling and metastatic capability. However, *in vitro* studies show that FAK retains some residual activity, even with complete Src inhibition,

due to autophosphorylation at Tyr397. In prostate cancer cell lines, paxillin phosphorylation was only inhibited at relatively high concentrations of AZD0530. This is thought to be because it is a Src:FAK complex substrate and whilst Src is strongly inhibited, FAK autophosphorylation at Tyr397 remains unchanged upon AZD0530 treatment (20).

Small molecule inhibitors of FAK, PF-562271 and PF-573228 have recently been developed and are being used to elucidate the role of FAK signalling in various tumour contexts. Treatment of 4T1 breast carcinoma cells with PF-562271 led to an inhibition of cellular migration and invasion *in vitro* and reduced primary tumour growth and lung metastases *in vivo* (92).

1.17 Summary and project aims

Inhibition of SFK activated pathways has shown great promise as an anti-metastatic therapy in *in vitro* and *in vivo* preclinical trials. Currently, SFK inhibitors Bosutinib, Dasatinib and AZD0530 have undergone phase I clinical trials and phase II trials are ongoing in a number of tumour types (22). However, mixed results from phase II trials with the SFK inhibitor AZD0530 have highlighted the need for further elucidation of the role of Src in tumour metastasis, and potential compensatory mechanisms at work which may contribute to clinical failure. Elucidation of the action of anti-metastatic therapeutic compounds can provide rationale for combination therapy with other compounds, including those that inhibit proliferation, induce apoptosis, anti-angiogenic compounds or compound that target growth factor receptors. Thorough characterisation of the mechanism of action of anti-metastatic compounds will support ongoing and future clinical trials by elucidating which tumour types and stages in tumour progression can be most successfully targeted.

- Elucidate the expression of active SFKs and their downstream targets in tumour cell lines *in vitro.*
- Carry out *in vitro* cell migration and invasion assays to look at the effect of Src inhibition with AZD0530 on tumour cell behaviours associated with metastasis, including:
    - Migration
    - Spreading
    - Polarization

- Provide rationale for further research *in vivo*, using nude mice recipients of tumour cell xenografts, which more closely resemble the tumour microenvironment within the human host and can be used to pre-clinically investigate efficacy of anti-metastatic compounds by measuring the extent of tumour cell invasion and metastasis to distant organs .

2.0 Materials and Methods

2.1 Cell lines and culture

Wild type and GFP expressing HT1080 human fibrosarcoma cells, MDA-MB-231 and MCF-7 breast cancer cells were routinely cultured in RPMI medium supplemented with 10% foetal calf serum and 2mM glutamine. Cell cultures were maintained at 37°C in a humidified 5% CO_2 environment.

2.2 Antibodies

Rabbit (polyclonal IgG) Anti-FAK (Tyr861-p) and rabbit (polyclonal) anti-paxillin (Tyr31-p) were a gift from Astra Zeneca (Alderly Edge, UK). Mouse (monoclonal) anti-Src (Tyr530 unphosphorylated) from Invitrogen. Mouse (monoclonal) anti- E-cadherin from abcam. Anti-G97 antibody kind gift from Dr Martin Lowe (University of Manchester).

2.3 Treatments

The src inhibitor, AZD0530 was supplied by Astra Zeneca. Drug was dissolved in Dimethyl sulfoxide (DMSO) and diluted in culture medium at a final concentration of 0.05% DMSO. Treatments were carried out with corresponding controls; 0.05% DMSO and culture medium only.

2.4 Cell lysis

Cells were allowed to seed overnight and treated with AZD0530 or DMSO control. Cell cultures were washed twice with ice cold phosphate buffered saline (PBS) and lysed in lysis buffer (50mM Tris-HCL pH 7.4, 120mM NaCl, 5mM Ethylenediaminetetraacetic acid (EDTA), 0.5% Tergitol-type NP-40 (NP40), 1mM Dithiothreitol (DTT), 1mM p-methylstyrene (PMST), 2mM Sodium orthovanadate (NaOv), 2mM Sodium fluoride (NaF), 20mM Boc-Gly-Pam Acid (BGP), 5mM sodium pyrophosphate (NaPPi) containing 1mg/ml protease inhibitor cocktail (Roche). Lysates were sonicated briefly, centrifuged (4°C, 10mins, 13000g) and supernatant retained. Protein concentration was determined using the Bradford assay; a solution of 50:1 bicinchoninic acid/copper II sulfate was added to to 10µl cell lyaste, incubated for 30minutes at 37°C. Absorbance was measured at 595nm using a microplate spectrophotometer (from Biotek) and cell lysate protein concentration was measured against a standard curve calculate from absorbance of bovine serum albumin (BSA) protein solutions of known concentrations.

2.5 Western blotting

40µg of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), a method used to separate proteins based on their size, using a 10% gel and transferred to a nitrocellulose membrane.

For Western blotting against phosphorylated proteins membranes were blocked in 5% milk Tris Buffered Saline 0.05% Tween20 (TBST). Membranes were incubated in primary antibody in 5% milk TBST overnight at 4°C and washed in TBST (3x5mins) and incubated in horseradish peroxidase (HRP) conjugated secondary antibody TBST for 1hour. Membranes were washed in TBS (2-3 hours).

For western blotting against non phosphorylated proteins, membranes were blocked in 5% milk Phosphate Buffered Saline 0.1% Tween20 (PBST) and incubated in primary antibody at the indicated concentrations in TBST for 2hours at room temperature. Blots were washed in PBST (3x15mins) and incubated in HRP conjugated secondary antibody in 5% milk PBST for 1hour. Membranes were washed in PBST (3x15mins). All membranes were visualised using ECL Western Blotting Detection reagents (Amersham) and exposure to X-ray film.

2.6 Immunofluorescence

Cells were cultured overnight onto glass coverslips in tissue culture plates. Treatment with AZD0530 was carried out as indicated. Cells were formalin fixed and blocked with 1% Bovine Serum Albumin (BSA) in PBS for 30mins before membrane permeabilisation with 0.1% TritonX100 for 7mins. Cells were washed in PBS (3x quick wash) and incubated in primary antibody in 1% BSA PBS as indicated for 1hour. Cells were washed in PBS (3x5mins). Incubation with appropriate secondary antibody; goat anti rabbit alexa 594 or 488 and goat anti mouse alexa 488 or 594 (Invitrogen) was carried out for 1 hour. Cells were further washed in PBS, treated with 4',6-Diamidino-2-phenylindole (DAPI) to stain nuclei, and mounted onto slides using DAKO mounting medium.

2.7 Cell proliferation assay

Cells were seeded overnight at 1000cells/well on a 96 well plate in 200ul growth media. Cells were treated with indicated AZD0530 concentrations/ DMSO for 24hours. After removal of treatment, cells were washed once with PBS and maintained in growth media for 96hours. Viable cells were measured by incubation with 0.5mg/ml (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4hours in the dark. MTT was removed and the product solubilised in 100ul DMSO. Absorbance was measured at 562nm using a spectrometer.

2.8 Scratch assays

Cells were seeded at high density on slides in tissue culture plates and allowed to reach 95-100% confluence in standard growth media. Several scratches were introduced per plate with a sterile pipette tip and cell cultures were treated with AZD0530 in low serum medium (0.04% FCS). Plates were formalin fixed at 0, 18 and 24hours and visualised using fluorescence microscopy on an Olympus widefield microscope at 4x magnification. The extent of migration was quantified using ImageJ software. Scratch closure was calculated using mean scratch size – measured from 10 fields of view per condition, with 30 measurements taken from each.

2.9 Cell spreading assay

Glass cover slips were soaked overnight in 5µg/ml fibronectin and washed once in PBS. Glass coverslips were blocked with 15 heat denatured BSA for 2hours.
Cultured cell monolayers were trypsinised to produce a suspension of dissociated cells. Cell suspensions were incubated for 10minutes in RPMI media at 37°C. 150,000 cells were plated per 3.5cm plate. Plated cells were incubated at 37°C and formalin fixed at 15 and 25minutes. Cells were stained with phalloidin and visualized using fluorescence microscopy on an Olympus widefield microscope at 10x magnification. Spread cells were judged to be those that possessed spindle like protrusions. 100 cells were assessed per condition. Three repeat experiments were performed.

2.10 Cell polarisation assay

Cells were seeded at high density on glass slides in tissue culture plates and allowed to reach 95-100% confluence in standard growth media. Several scratches were introduced per plate with a sterile pipette tip and cell cultures were treated with AZD0530 or DMSO control in low serum media (0.04% FCS). Plates were formalin fixed at 6hours. Immunofluorescent staining was carried out with the G97 anti-Golgi antibody and the nucleus was stained with DAPI. Immunofluorescence was visualised using fluorescence microscopy on an Olympus widefield microscope at 10x magnification. Polarisation was assessed by orientation of the Golgi apparatus for migration into the scratch. 100 cells per condition were assessed for polarisation, and three repeat experiments were performed.

2.11 Immunohistochemistry

A cryostat was used to cut 8μm sections from frozen HT1080 xenograft tissue which had previously been established in the hind leg muscle of nude mice. Tumour sections were fixed in ice cold acetone for 10mins. Sections were blocked in 10% horse serum in PBST for 10 mins, washed 2x 3mins in 0.1% BSA PBST and incubated with primary antibodies in 0.1% BSA PBST overnight at 4°C as indicated. Sections were washed in PBST (3x5mins) and incubated with secondary alexa fluor 594 or 488 antibodies (1:1000) in 0.1% BSA PBST for 1hour in the dark. Sections were then washed in PBS (3x 4mins) and coverslips were mounted onto slides using DAKO mounting media. Slides were visualised at 60x magnification.

3.0 Results

3.1 Investigating the expression of active Src, FAK and paxillin in tumour cell lines

3.1.1 Tumour cell lines express active Src

In order to investigate the expression of active Src in the human fibro-sarcoma cell line HT1080 and the breast carcinoma lines MDA-MB-231 and MCF-7, Western blotting was carried out with the Clone 28 antibody. Clone 28 is a monoclonal antibody which recognises a region adjacent to the Tyr530 in the C terminal regulatory domain and is selective for the active form of Src (Tyr530-unphosphorylated) (94).
Here we show that HT1080, MDA-MB-231 and MCF-7 cells express active Src (Tyr530-unphosphorylated). Western blotting produced two bands ~60KDa (Figure 3.1), the upper band has been shown to represent active Src (95).

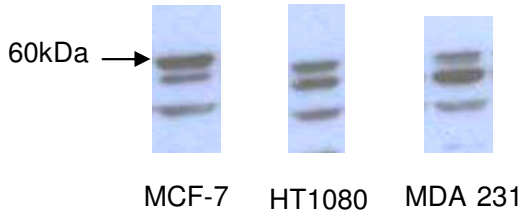
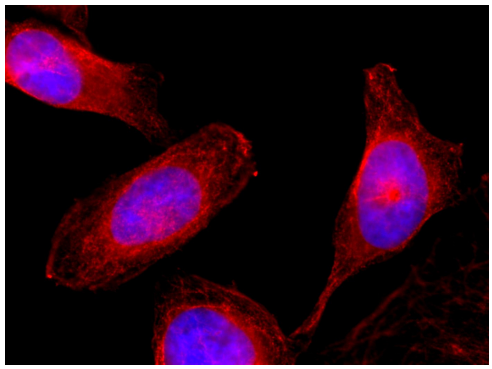


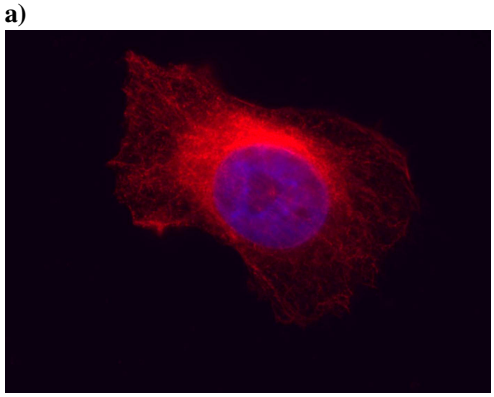
Figure 3.1. MDA-MB-231, MCF-7 and HT1080 cells express active Src. Western blotting with Clone 28 antibody on HT1080, MDA-MB-231 and MCF-7 cell lysates produced two bands ~60kDa, the upper band represents active Src (94). Lower band shows ß actin loading control (42kDa).

Immunofluorescent staining was also carried out to investigate the localisation of active Src (Tyr530 unphosphorylated). In all MDA-MB-231 and HT1080 tumour cells immunofluorescent staining with the Clone 28 antibody demonstrated that active Src was localised to the perinuclear region, associated with the actin cytoskeleton and at the cell periphery (Figure 3.2a, bi and ii), consistent with its localisation in normal rat 3Y1 fibroblasts (94). Active Src co-localised with β-tubulin in HT1080 cells, confirming localisation to the cytoskeleton (Figure 3.2d).

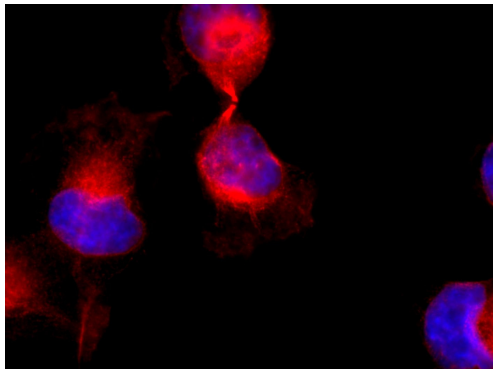
In HT1080 cells, expression of active Src at the cell periphery was restricted to structures associated with cell motility; strong staining was seen in spindle like protrusions associated with cell migration and cell-cell contact (Figure 3.2b, c and d). In MDA-MB-231 cells localisation of active Src around the edge of the cell was more restricted to the leading edge of lamellipodia (Figure 3.2a).
Staining of MCF-7 cells can be seen in figure 2.15 where active Src is strongly localized around the entire cell periphery; expression is not restricted to structures associated with forward cell movement, which cannot be seen in these non-motile cells (Figure 3.16).



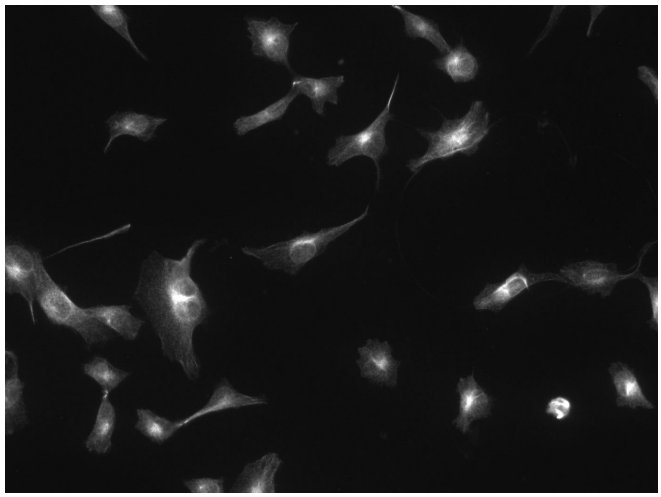
a)



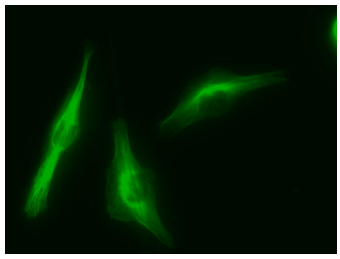
bi)



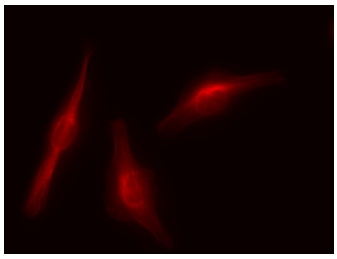
bii)



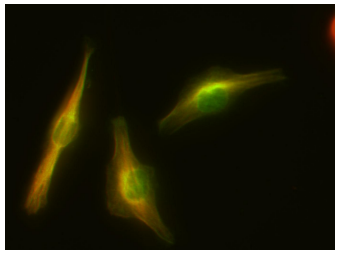
c)



di)



dii)



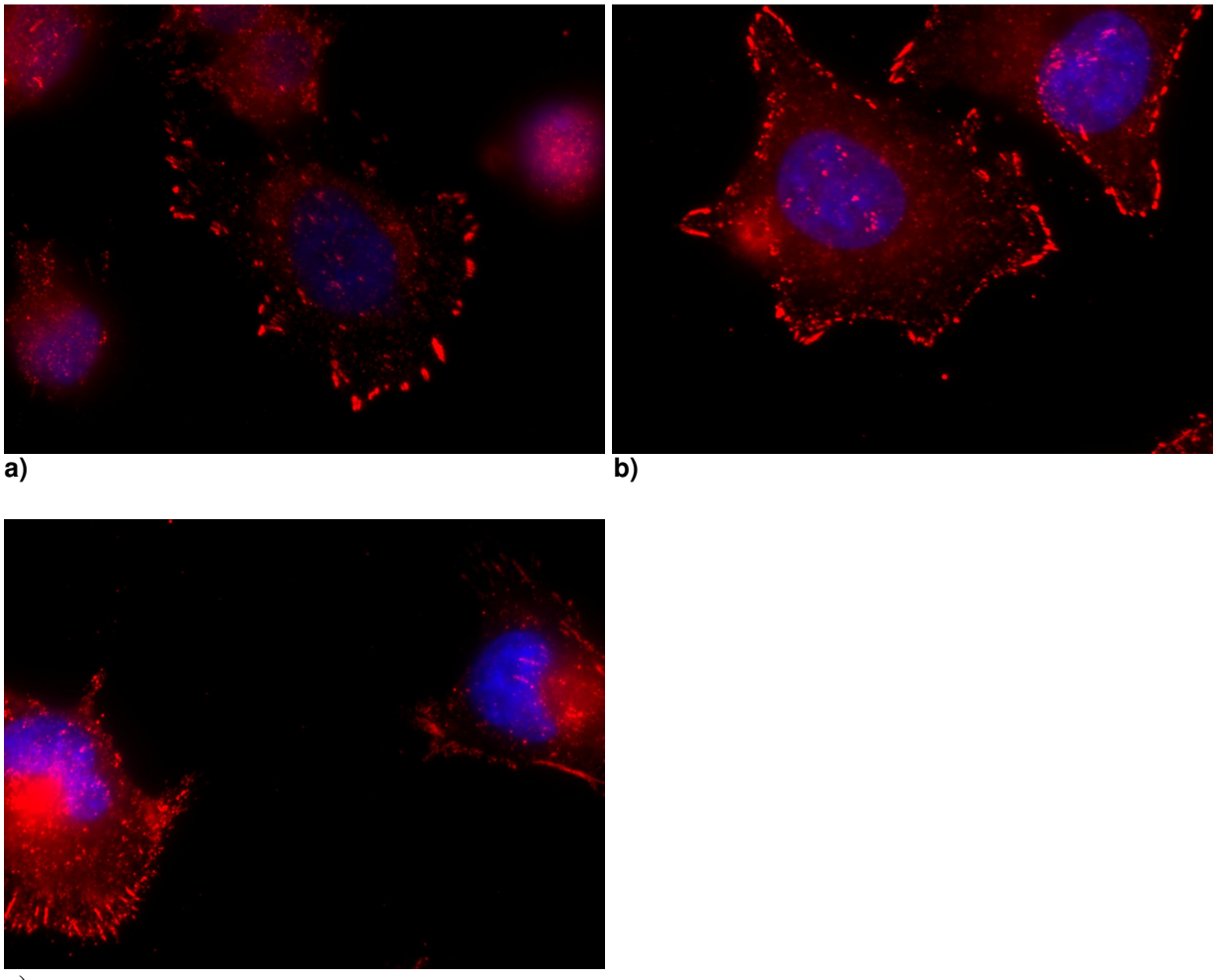
diii)

Figure 3.2. Localisation of active Src in MDA-MB-231, MCF-7 and HT1080 cells. (a-c) Immunocytochemistry with Clone 28 antibody (red), dapi (blue): **a)** MDA-MB-231, arrow shows localisation at lamellipodia b) MCF-7 (di and dii) HT1080. 100 x magnification **c)** Active Src is located at cell protrusions in HT1080 cells. 20 x magnification **(di-iii)** Active Src co-localizes with tubulin in HT1080 cells ei) Tubulin (green) eii) Clone 28 (red) eii) Tubulin and Clone 28. 40 x magnification.

3.1.2 Tumour cell lines express active Focal Adhesion Kinase

The expression of active FAK, a downstream target of Src, has been found to be upregulated in tumour cell lines and invasive human tumours (89). FAK overexpression has been shown to correlate with tumour invasiveness in breast and colon carcinomas (70). FAK expression was analysed in HT1080, MCF-7 and MDA-MB-231 cells by immunofluorescence with an antibody recognising the phosphorylated tyrosine residue 861(Tyr861) on FAK, a known site for activation by Src.

HT1080, MCF-7 and MDA-MB-231 cells express active FAK (Tyr861-phosphorylated). Active FAK was localised to distinct focal adhesion around the edge of the cell (Figure 3.3a-c).



a) b)

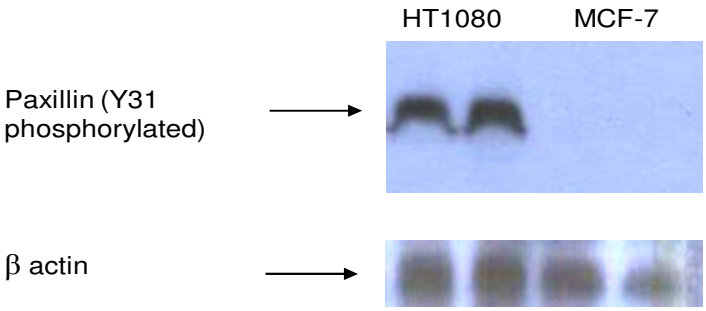
c)

Figure 3.3. HT1080, MCF-7 and MDA-MB-231 tumour cell lines express active FAK (Y861 phosphorylated) in focal adhesions. Immunocytochemistry to visualise FAK Y861-phosphorylated (red), and dapi (blue),100 x magnification a) HT1080 b) MCF-7 c) MDA-MB-231.

Motile HT1080 and MDA-MB-231 cells were visibly polarised for forward cell movement with increased size and frequency of active FAK containing focal adhesions at structures associated with forward cell movement, such as lamellipodia (Figure 3.3a and c).
Non-motile MCF-7 cells were not visibly polarised and active FAK containing focal adhesions were located consistently around the perimeter of the cell. There was increased size of active FAK containing focal adhesions at actin structures which are likely to be involved in cell spread and attachment (Figure 3.3b).

3.1.3 HT1080 cells express active paxillin

Paxillin is a downstream target of Src involved in focal adhesion turnover and cell motility. Activity of paxillin was investigated in the metastatic human fibrosarcoma cell line HT1080, and the non-invasive breast carcinoma cell line MCF-7, by Western blotting with an antibody recognising paxillin phosphorylated at tyrosine 31 (Tyr 31), a known site for activation by Src.
The metastatic tumour cell line HT1080 was found to express active paxillin whereas the non-invasive breast carcinoma cell line MCF-7, showed negligible expression. Western blotting with an antibody against active paxillin (Tyr31-phosphorylated), produced one band ~70kDa in lanes containing HT1080 lysates, this band could not be seen in lanes containing MCF-7 lysates (Figure 3.4a).
Immunofluorescent staining with an antibody recognising active paxillin (Tyr31 phosphorylated) was carried out to determine the localisation of active paxillin in HT1080 cells. Active paxillin was found to be localised to distinct focal adhesions at the cell periphery. HT1080 cells were visibly polarised for forward cell movement with active paxillin containing focal adhesions found most frequently at structures associated with cell motility at the front and rear of the cell (Figure 3.4b).



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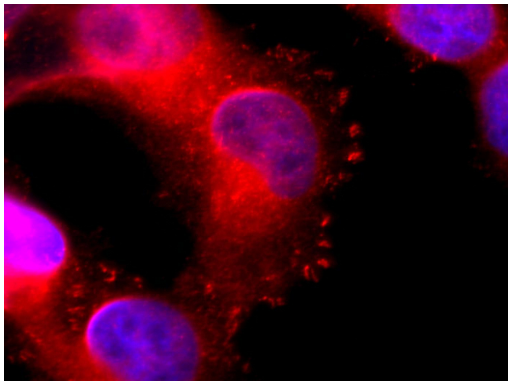


Figure 3.4. HT1080 cells express active paxillin (Y31 phosphorylated). a) Western blotting with anti paxillin Tyr31-phosporylated antibody produced one band ~70kDa in lanes containing HT1080 cell lysates which was absent in wells containing MCF-7 cell lysates **b)** Immunfluorescent staining of active paxillin (Tyr31-phosphorylated) (red), dapi (blue) shows that it is localised to focal adhesions in HT1080 cells.

3.2 Effect of AZD0530 treatment on tumour cell proliferation, migration, polarization and spreading.

3.2.1 Anti-proliferative effects of Src inhibition with AZD0530.

The anti-proliferative effects of AZD0530 have been previously suggested to be cell line specific (22). In order to assess the effect of AZD0530 inhibition of Src on HT1080 proliferation a tetrazolium dye based assay was carried out. MDA-MB-231 cultures were used as a control as AZD0530 treatment has been shown to have an anti-proliferative effect in this cell line (22). Absorbance at 450nm was used to measure the level of viable cells in MDA-MB-231 and HT1080 cell cultures incubated for 96 hours in AZD0530 (0.01-10μM), or DMSO control, in a 96 well plate.

Figure 3.5 shows the mean absorbance of wells treated with increasing concentrations of AZD0530 as a percentage absorbance of wells treated with DMSO control. Results

shown are the mean of three repeated MTT assays, each with three triplicate wells. AZD0530 treatment had an anti-proliferative effect on MDA-MB-231 breast carcinoma cells; with a half maximum inhibitory concentration (IC50) of ~ 4µM in the MTT proliferation assay. HT1080 cells were relatively refractory to the anti- proliferative effects of AZD0530; with an IC50 >10µM (Figure 3.5).

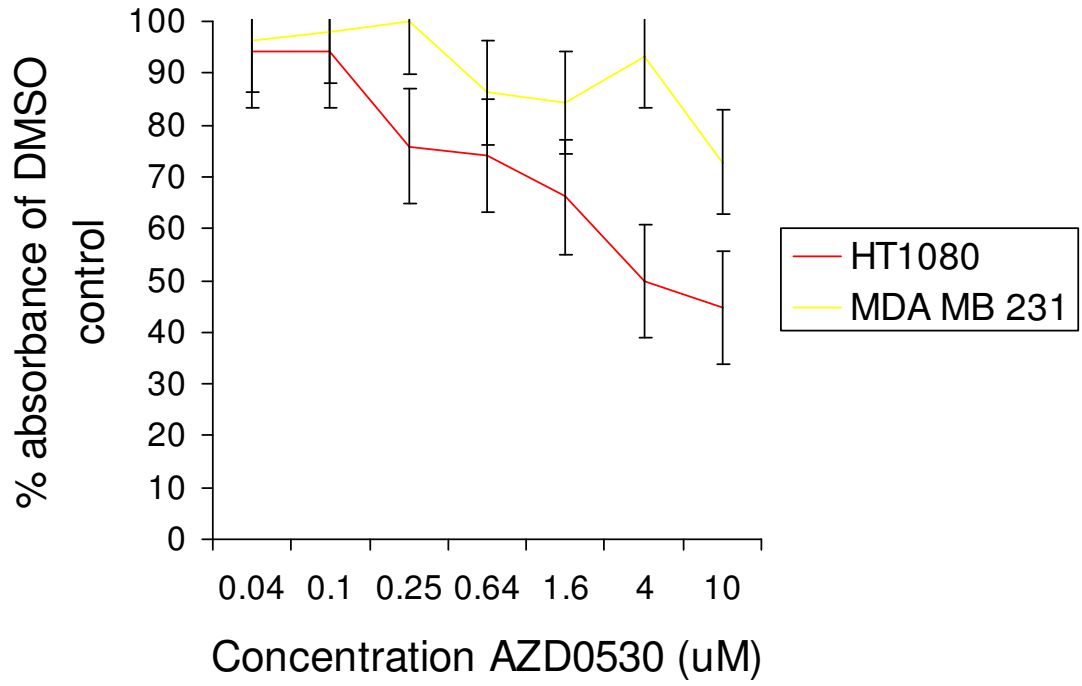


Figure 3.5. Effect of Src inhibition with AZD0530 on proliferation of MDA-MB-231 and HT1080 cell lines.

Cells were treated with AZD0530 (24hours; 0.01 - 10µM) and an MTT assay performed. Graph shows mean % Absorbance, as compared to DMSO control, versus AZD0530 concentration (µM). Means were obtained from three replicate experiments, each with three triplicate results. Error bars show SD.

3.2.2 AZD0530 inhibits the migration of HT1080 cells *in vitro*.

AZD0530 has been shown to inhibit cell migration in several tumour cell lines, and reduces metastasis in *in vivo* xenograft models (22, 20). Here we carried out 2D scratch assays on an uncoated surface as an initial assessment of the effect of Src inhibition with AZD0530 HT1080 cell migration.

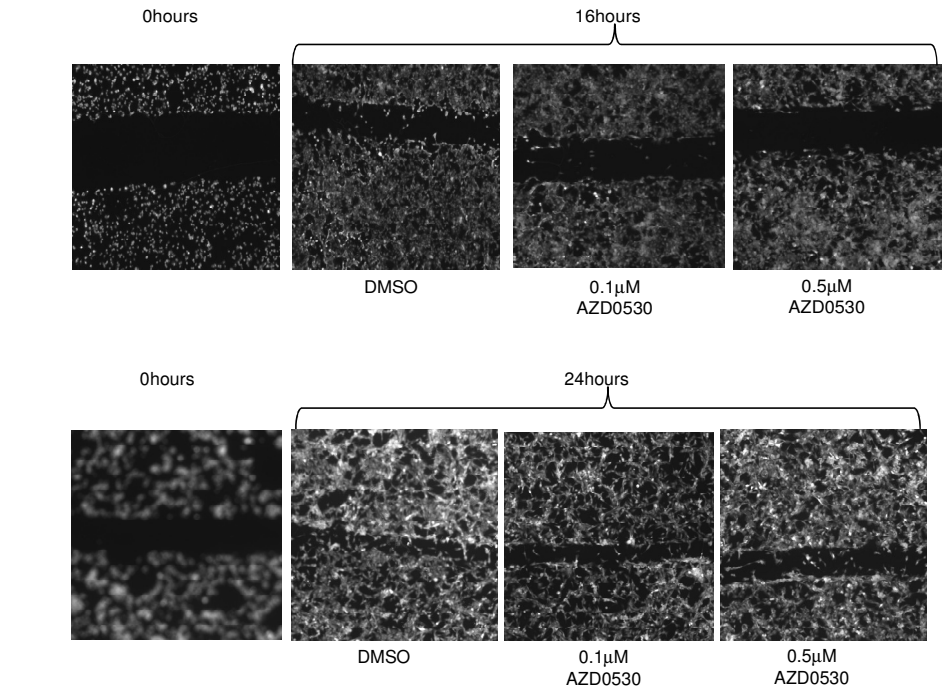
AZD0530 was shown to inhibit the 2D migration of HT1080 cells on an uncoated surface *in vitro* using a scratch assay (Figure 3.6a).

Treatment of confluent HT1080 monolayers with 0.1μM and 0.5μM AZD0530 significantly inhibited migration into the scratch at 16 hours and 24 hours, as compared to DMSO control (Figure 3.6 a and b). Scratch closure was measured as the mean scratch size at each time point minus mean scratch size at 0 hours. Mean scratch size was calculated from 10 fields of view over 3 scratches, with 30 measurments taken on each field of view. Statistical significance was calculated using the student T test.

At 16 hours, the extent to which HT1080 monolayers migrated into the scratch with 0.1μM AZD0530 treatment was 40% that of DMSO treated cells ($p=<0.05$), and with 0.5μM treatment was 38% that of DMSO treated cells ($p=<0.05$) (Figure 3.6bi).

At 24 hours, the extent to which HT1080 monolayers migrated into the scratch with 0.1μM AZD0530 treatment was 47% that of DMSO treated cells ($p=<0.05$), and with 0.5μM treatment was 36% that of DMSO treated cells ($p=<0.01$) (Figure 3.6bii).

There was no significant dose dependent effect on inhibition of HT1080 migration at 16 or 24 hours comparing the 0.1 and 0.5μM treatments used.



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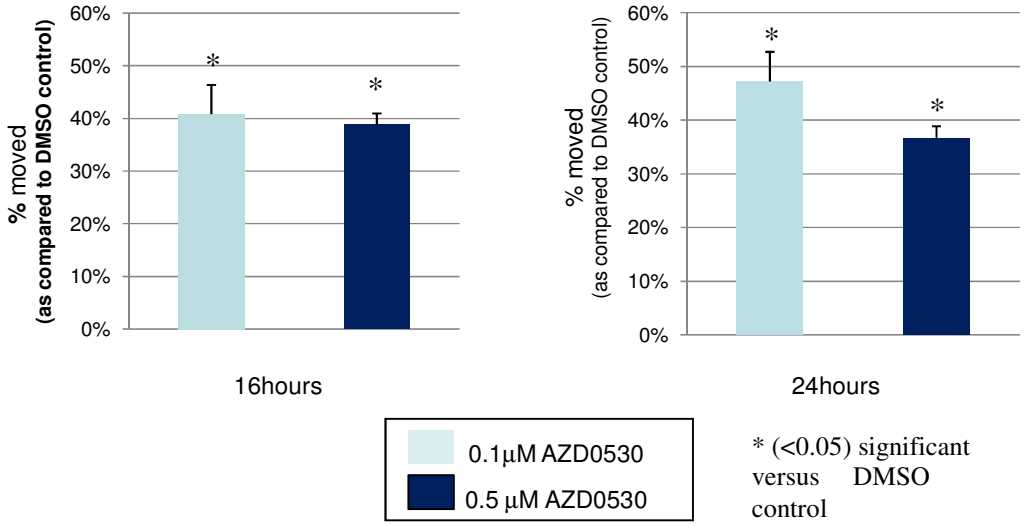


bi) **bii)**

Figure 3.6. AZD0530 treatment inhibits HT1080 migration in a scratch assay.

a) GFP expressing HT1080 cells were allowed to form a confluent monolayer and a physical scratch was introduced. Cell cultures were treated with DMSO control, 0.1µm or 0.5µm AZD0530 and fixed at 0hours, 16hours and 24hours. Images were captured at 4 x magnification and measured using ImageJ software **b)** Movement into the scratch as a % of DMSO control, with 0.1µm or 0.5µm AZD0530 treatment: bi) 16hours bii) 24hours. Data shown is the mean of three repeated experiments, each with three scratches and a total of 90 scratch width measurements per treatment condition. Statistical significance was calculated using the Student T test

3.2.3 Effect of AZD0530 treatment on HT1080 cell polarisation for migration

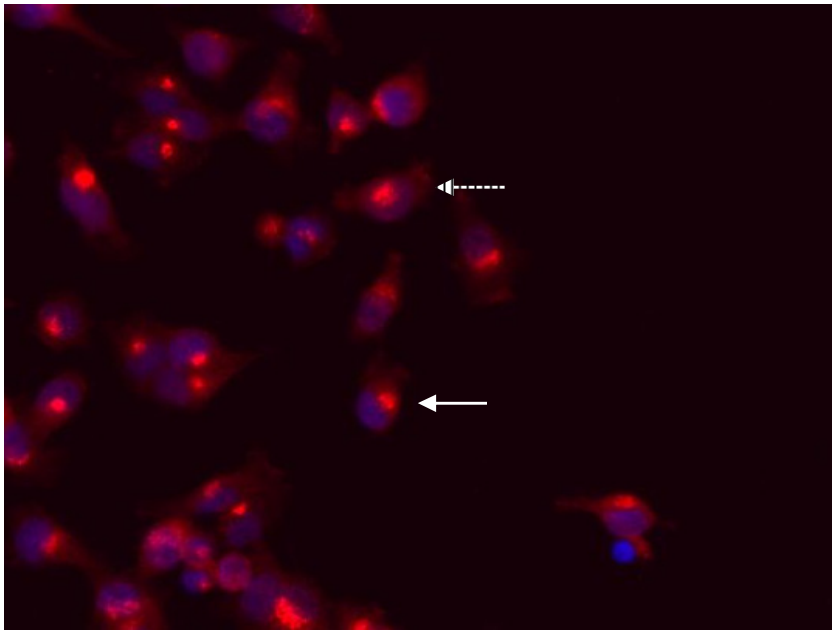
In order for cells to carry out forward cell movement they must undergo polarisation toward the direction of movement. This involves cytoskeletal rearrangements, remodelling of focal adhesions and reorientation of the golgi apparatus to the front of the cell (96). As Src has been shown to be involved in regulating cytoskeletal rearrangements and remodelling of focal adhesions the effect of AZD0530 inhibition of Src on polarisation of HT1080 cells was assessed.

Scratch assays were carried out in HT1080 monolayers in order to assess polarisation for forward movement into the scratch. Cell polarisation was assessed using immunofluorescent staining of the Golgi apparatus at 6hours. Polarised cells were defined as those with Golgi apparatus situated on the scratch side of the nucleus (Figure 3.7a, arrow shows cell orientated toward the scratch, dashed arrow shows cell not orientated towards the scratch).

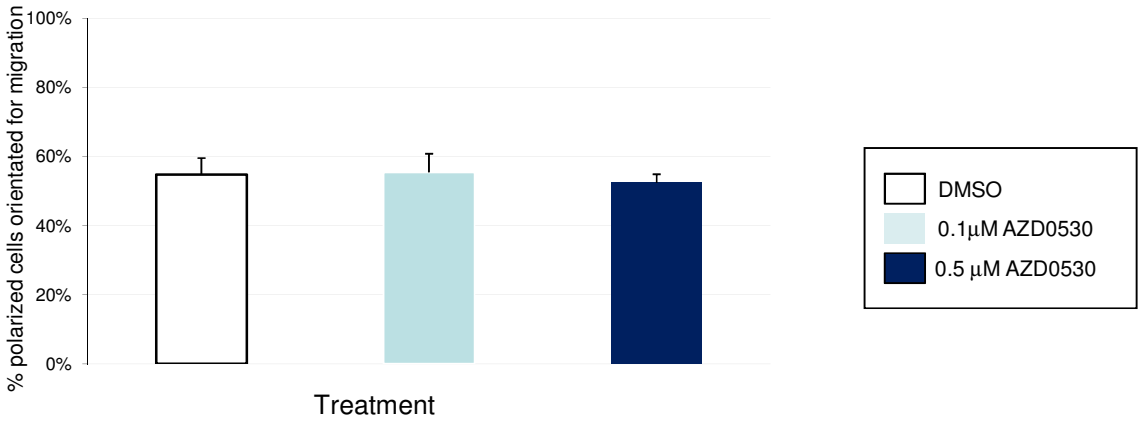
Src inhibition with 0.1µm or 0.5µm AZD0530 treatment had no significant effect on the percentage of polarised HT1080 cells which orientated for migration into the scratch, as compared to DMSO control (Figure 3.7).

At 6 hours, 54% of DMSO treated, 60% of 0.1µm AZD0530 treated, and 54% of 0.5µm AZD0530 treated HT1080 cells were orientated towards the scratch (Figure 3.7b).

These results would suggest that disruption of cell polarisation, as measured by orientation of the Golgi apparatus, is not the mechanism by which AZD0530 inhibits HT1080 cell migration.



a)



b)

Figure 3.7. Effect of AZD0530 treatment on HT1080 cell polarisation towards the scratch in a scratch wound assay a) A physical scratch was introduced into a HT1080 monolayer. Orientation of polarised cells was assessed by immunofluorescent staining for the Golgi apparatus with the antibody G97 (red), and dapi (blue); orientated cells were defined as having the Golgi apparatus located in the cytoplasm on the scratch side of the nucleus. Arrow = example of cell orientated toward the scratch, broken arrow = example of cell not orientated towards the scratch) **b)** Mean percentage of polarized cells orientated toward the scratch with DMSO, 0.1µm and 0.5µm AZD0530 treatment, at 6 hours. Calculated using 100+ cells from over 5 fields of view (60 x magnification) of three replicate experiments.

3.2.4 Effect of AZD0530 treatment on HT1080 cell spreading on fibronectin

Src and its downstream targets have been shown to be involved in cell spreading on ECM proteins. Cell spreading involves integrin binding to ECM proteins and promotes adhesion and migration of tumour cells to, thus contributing to the mechanisms of metastasis.

Trypsinised cells were allowed to spread on fibronectin and fixed at 15 and 25 minutes to assess the extent of cell spreading with 0.5μM AZD0530 treatment or DMSO control. Spread cells were defined as those that displayed spindle like protrusions associated with cell spreading (Figure 3.8b, white arrow = spread cell, red arrow = non spread cell).

0.5μM AZD0530 treatment inhibited HT1080 cell spreading on fibronectin at 15 minutes ($p=<0.05$) but not at 25 minutes. 92% of AZD0530 treated cells were spread on fibronectin at 15 minutes, as compared to 96% of DMSO treated cells (Figure 2.8ai). Conversely, at 25 minutes 5μM AZD0530 treatment there was no significant difference in the % of spread cells between DMSO and AZD0530 treated cells; 99% of AZD0530 treated cells were spread on fibronectin at 25 minutes, as compared to 97% of DMSO treated cells (Figure 3.8aii).

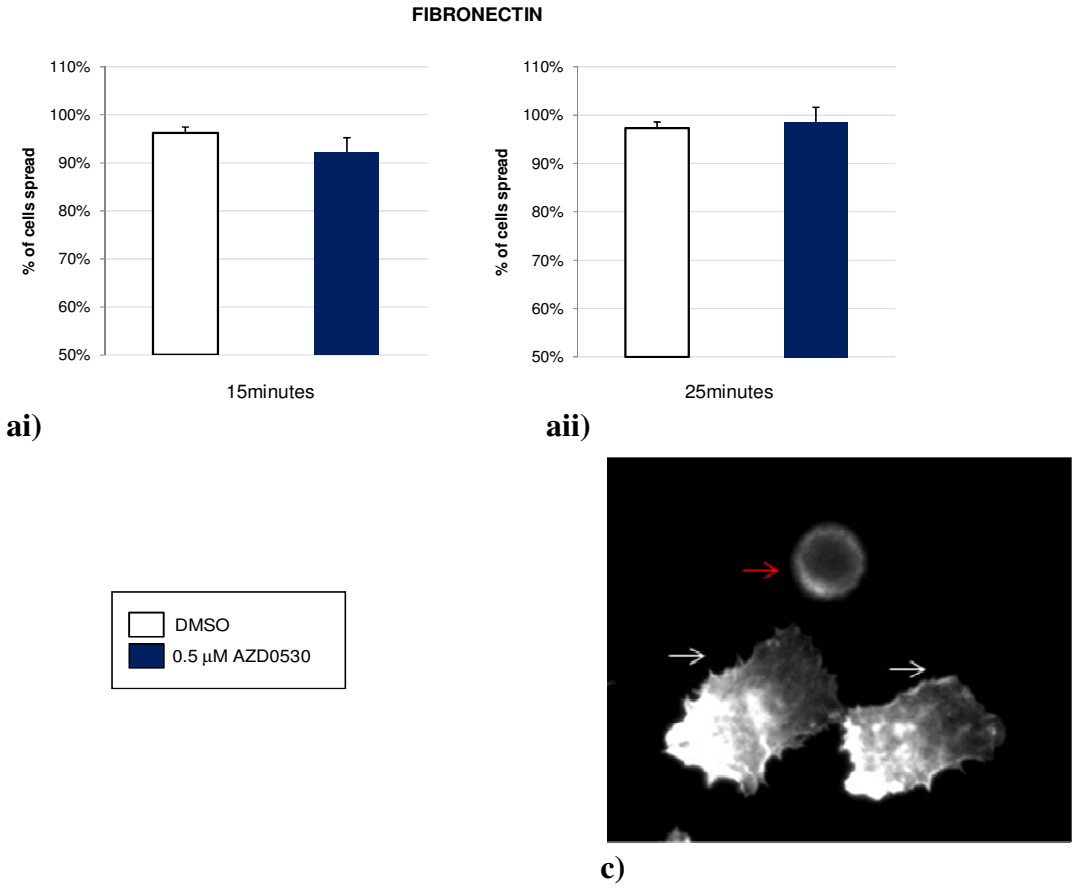


Figure 3.8. Effect of AZD0530 treatment on cell spreading of HT1080 cells on fibronectin. (a) Percentage HT1080 cells spread on fibronectin, with DMSO control or 0.5μM AZD0530 treatment ai) 15minutes aii) 25minutes **b)** HT1080 cells stained with phalloidin. Spread cells were classed as those with spindle like protrusions (white arrows) unspread cells were classed as those that lacked protrusions (red arrow).

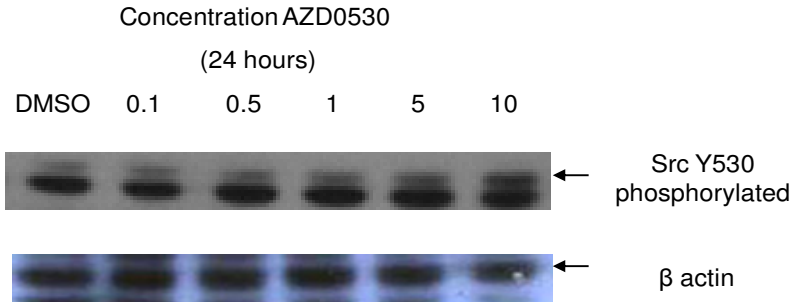
3.3 Effect of AZD0530 treatment on molecular markers of Src inhibition.

3.3.1 Treatment of HT1080 cells with AZD0530 did not lead to a decrease in Src activity as measured by phosphorylation of tyrosine 530.

Western blotting was carried out to assess the activity of Src, as measured by Tyr530 phosphorylation status, in HT1080 cells treated with varying concentrations of the Src inhibitor AZD0530 or DMSO control.

Western blotting with the Clone 28 antibody demonstrated no reduction in the level of active Src (Tyr530 unphosphorylated) with AZD0530 treatment between 0.1 and 10µM, at 2, 4, 16hours (Figure 3.9a) or 24hours (Figure 3.9b). Phosphorylation status of Src on tyrosine 530 appears to be a poor marker of AZD0530 Src inhibition in HT1080 cells.

Interestingly, at high concentrations of AZD0530 (5µM and 10µM) there appeared to be an increase in levels of Tyr530-unphosphorylated Src, which can be seen clearly at 24hours (Figure 3.9b).



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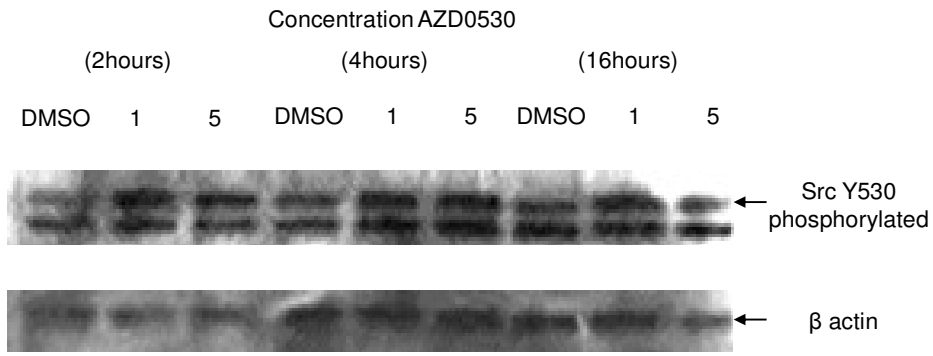


b)

Figure 3.9. AZD0530 treatment does not lead to a decrease in Src phosphorylated at Tyr530
Western blotting with Clone 28 antibody on HT1080 cell lysates, treated with AZD0530 or DMSO control, produced two bands ~60kDa, the upper band represents active Src (93) **a)** 0.1-10µM AZD0530 or DMSO control, 24hours **b)** 1-5µM AZD0530 or DMSO control, 2, 4 and 16hours

Active Src has been shown to be trafficked via the cytoskeleton to sites of action at the cell periphery (97). Immunfluorescent staining of active Src, as measured by phosphorylation status of Tyr530, was carried out in fixed HT1080 cultures to assess if →Src inhibition with AZD0530 leads to changes in localisation of active Src.
No alterations were seen in the localisation or intensity of immunofluorescent staining in cell cultures treated with AZD0530 (0.1and 0. 5µM) or DMSO control for 24hours. Active Src was found to be localised to the perinuclear region, actin cytoskeleton, and at the cell periphery, with strong staining of protusions associated with cell motility, both in HT1080 cells treated with DMSO control, or those treated with AZD0530 (Figure 3.10).

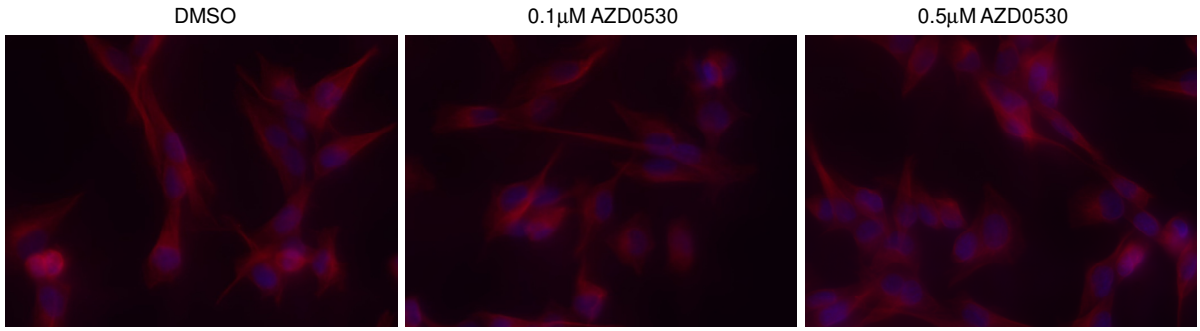
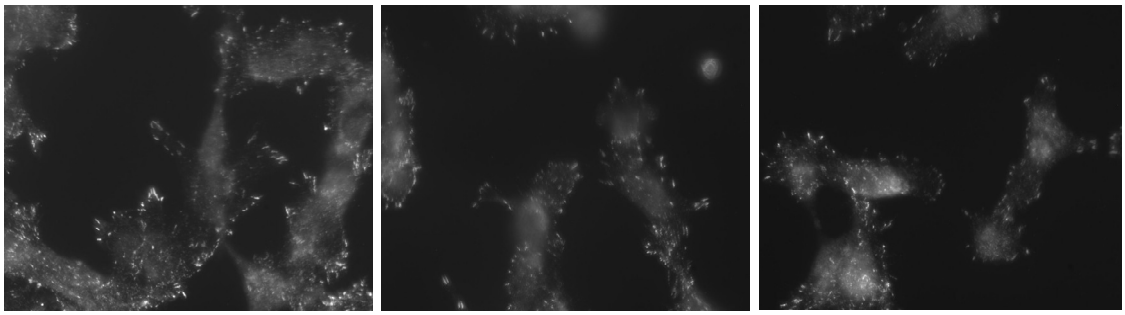


Figure 3.10. AZD0530 treatment does not alter the localisation of Src Tyr530-unphosphorylated in HT1080 cells. Cells were treated with AZD0530 or DMSO control for 24hours. Immunofluorescent staining of Src Tyr530-phosphorylated (red), and dapi (blue) 40 x magnification **a)** DMSO **b)** 0.1µM AZD0530 **c)** 0.5µM AZD0530

3.3.2 Src inhibition with AZD0530 in HT1080 cells had no effect on phosphorylation of FAK at tyrosine 861

FAK is phosphorylated by Src, leading to formation of the Src:FAK complex which activates downstream modulators of cell migration. Therefore we investigated the activity of FAK in HT1080 cells treated with the Src inhibitor AZD0530.
Immunofluorescent staining was carried out with an antibody specific for FAK phosphorylated at Tyr861, a known site for Src phosphorylation in fixed cultures of HT1080 cells treated with AZD0530 or DMSO control for 2hours, and 24hours. No evident change in staining of active FAK at focal adhesions was seen in HT1080 cells treated with AZD0530 (0.1-1µM) for 2 hours, as compared to DMSO control (Figure

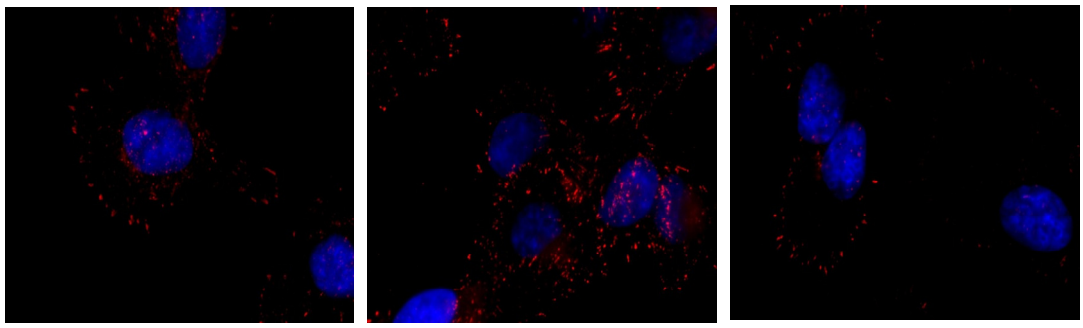
3.11)



a) **b)** **c)**

Figure 3.11. 2hour AZD0530 treatment has no effect on FAK Tyr861 phosphorylation. Cells were treated with AZD0530 or DMSO control for 24hours. Immunofluorescent staining of FAK Ty861-phosphorylated. 100 x magnification **a)** DMSO **b)** 0.1µM AZD0530 **c)** 1µM AZD0530

No changes were seen in the staining of active FAK (Tyr861 phosphorylated) in HT1080 cells treated with AZD0530 for 24 hours (0.1 – 0.5µM) (Figure 3.12), suggesting that decreased phosphorylation of FAK Tyr861 is not the mechanism underlying AZD0530 inhibition of HT1080 cell migration in the scratch assay, and that the phosphorylation status of FAK Tyr861 is a poor marker of AZD0530 Src inhibition in HT1080 cells.

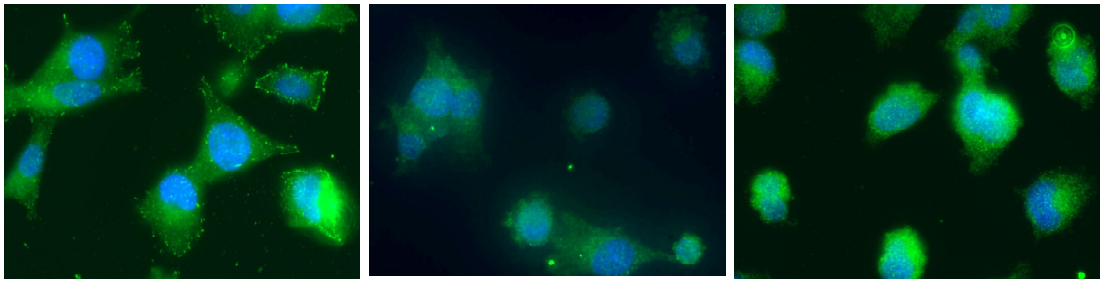


a) **b)** **c)**

Figure 3.12. 24hour AZD0530 treatment has no effect on FAK Tyr861 phosphorylation in HT1080 cells Cells were treated with AZD0530 or DMSO control for 2hours. Immunofluorescent staining of FAK Ty861-phosphorylated (red), and dapi (blue) as a counterstain for nuclei, 100 x magnification **a)** DMSO **b)** 0.1µM AZD0530 **c)** 0.5µM AZD0530.

3.3.3 Treatment of HT1080 cells with AZD0530 led to re-localisation of active paxillin from focal adhesions to the cytoplasm

Active paxillin is involved in focal adhesion turnover downstream of Src and FAK activation, allowing for forward cell migration.
Immunofluorescent staining of active paxillin was carried out to assess the affect of Src inhibition with AZD0530 on downstream paxillin localisation and activity. An antibody specific for paxillin phosphorylated at tyrosine 31 was used, as this is a known site for phoshporylation and actvation by Src.
HT1080cells treated with AZD0530 (1-5µM) for 2 hours displayed re-localisation of active paxillin (Tyr31 phosphorylated) from focal adhesions to the cytoplasm (Figure 3.13). Relocalisation of paxillin to the cytoplasm may negate its function at focal adhesions and could contribute to the anti-migratory effect of AZD0530 treatment on HT1080 cells.



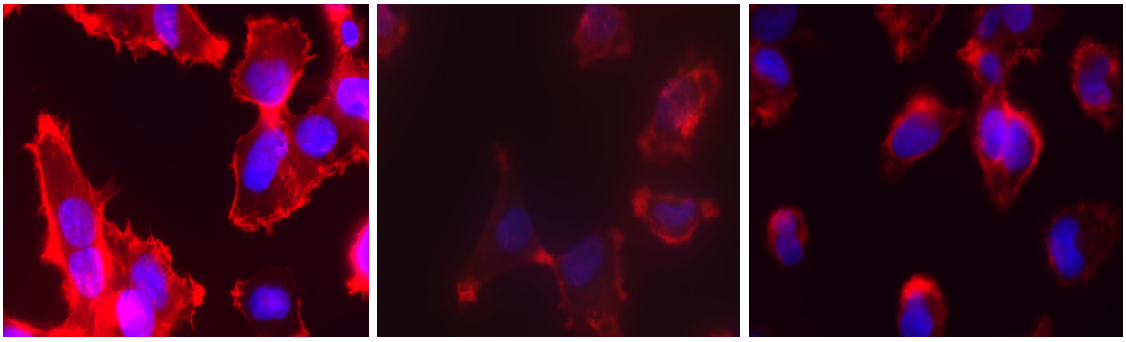
a) **b)** **c)**

Figure 3.13. AZD0530 treatment of HT1080 cells causes relocalisation of paxillin Tyr31phosphorylated from focal adhesions to the cytoplasm. Cells were treated with AZD0530 or DMSO control for 2hours. Immunofluorescent staining of paxillin Tyr31-phosphorylated (green), and dapi (blue), 100 x magnification **a)** DMSO **b)** 1µM AZD0530 **c)** 5µM AZD0530.

3.3.4 Effect of AZD0530 treatment on the actin cytoskeleton

Activity of the Src pathway has been shown to be involved in cytoskeletal rearrangements required for cell motility, via regulation of RhoGTPases (98). HT1080 cells display disorganisation of actin structures, with large membrane ruffles associated with the formation of lamellipodia, allowing for cell motility (99, 100). We investigated the effect of Src inhibition with AZD0530 on the actin cytoskeleton in HT1080 cells by phalloidin staining of F actin.

Treatment of HT1080 cells with 1 and 5µM AZD0530 for 2 hours reduced the occurrence of actin protrusions and membrane ruffling, associated with actin polymerisation, and also appeared to reduced cell spreading. This effect appeared to be dose dependant, with fewer actin protrusions in cells treated with 5µM AZD0530 seen than in cells treated with 1µM AZD0530 (Figure 3.14). Inhibition of cytoskeletal rearrangements allowing for cell spreading, polarisation and forward cell movement could represent one of the mechanisms by which Src inhibition reduces cell migration in HT1080 human fibrosarcoma cells.



a) b) c)

Figure 3.14. AZD0530 treatment effects actin cytoskeleton arrangement in HT1080 cells. HT1080 cells were treated with AZD0530 or DMSO control for 2hours. Fixed cells were stained with phalloidin (red) and dapi (blue) 100x magnification.
a) DMSO only **b)** 1µM AZD0530 **c)** 5µM AZD0530.

3.4 Investigating functional variability of active Src in the invasive MDA-MB-231 and non-invasive MCF-7 breast carcinoma cell lines

3.4.1 Src and FAK localisation in MDA-MB-231 and MCF-7 cells

Active Src and FAK are known to form a complex and activate downstream modulators of cell spreading and migration. To investigate whether Src is playing a differing role in metastatic and non metastatic cell lines we co-stained active Src and active FAK in the breast cancer cell lines MDA-MB-231 (metastatic) and MCF-7 (non metastatic), using immunofluorescent staining with antibodies specific for active Src (Tyr530 unphosphorylated) and active FAK (Tyr861 phosphorylated). In MCF-7 cells active Src was localised throughout the cytoplasm in a pattern consistent with association with the actin cytoskeleton, and at the cell periphery where active FAK containing focal

adhesions could be seen (Figure 3.15). In MDA-MB-231 cells active Src was localised throughout the cytoplasm in a pattern consistent with association with the actin cytoskeleton, however lower levels of active Src were seen at the cell periphery where active FAK containing focal adhesions were stained (Figure 3.16). This suggests that active Src is playing a differing function in non metastatic MCF-7 breast cancer cells than in metastatic MDA-MB-231 cells.

In both MDA-MB-231 and MCF-7 cell lines inhibition of Src with 0.5µM AZD0530 appeared to reduce the appearance of cellular protrusions associated with cell motility (Figure 3.15 and 16) , as seen in HT1080 cells (figure 3.14)

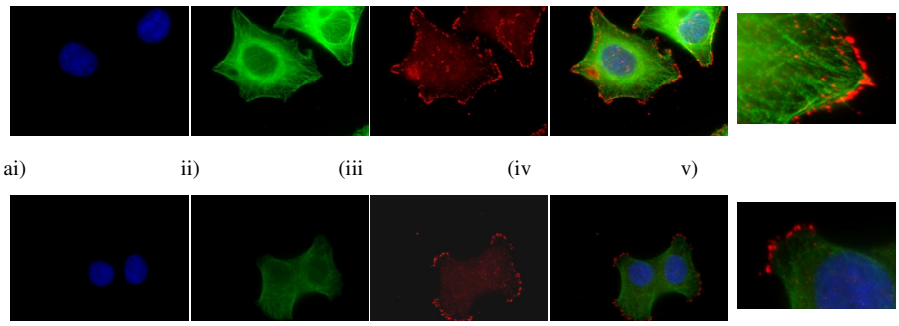


Figure 3.15. 24hour AZD0530 treatment has no effect on FAK Y861- phosphorylation localization at focal adhesions in MCF-7 cells MCF-7breast cancer cell lines were treated with AZD0530 or DMSO only control for 24hours Images were taken at 100x magnification **a)** DMSO control **b)** 0.5µM AZD0530. Immunofluorescent staining of **ii)** active SrcTyr530-unphosphorylated (green), **iii)** FAK Y861-phosphorylated (red), and **i)** dapi nuclear stain (blue), **iv)** composite images **v)** composite image, zoomed in on focal adhesions.

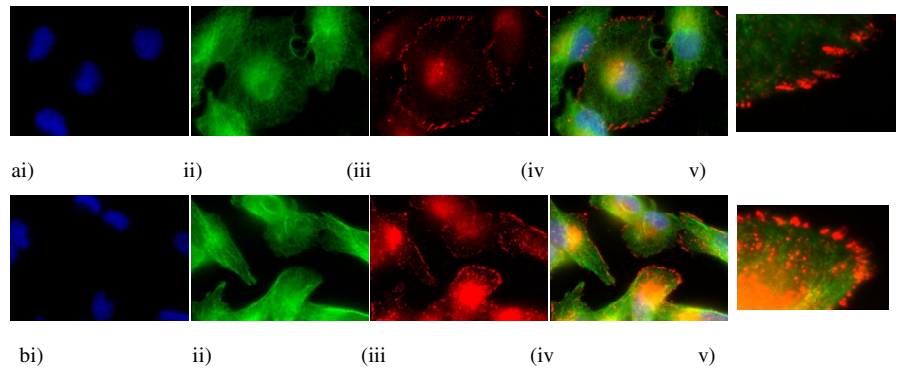


Figure 3.16. 24hour AZD0530 treatment has no effect on FAK Y861- phosphorylation localization at focal adhesions in MDA-MB-231 cells MDA-MB-231 breast cancer cell lines were treated with AZD0530 or DMSO only control for 24hours Images were taken at 100x magnification **a)** DMSO control **b)** 0.5µM AZD053. Immunofluorescent staining of **ii)** active Src Y530-unphosphorylated (green), **(iii** FAK Y861-phosphorylated (red), and **(i** dapi nuclear stain (blue), **(iv** composite images **v)** composite image, zoomed in on focal adhesions.

3.4.2 Effect of Src inhibition on focal adhesion morphology in MDA-MB-231 and MCF-7 tumour cell lines

Src is known to be involved in the turnover of focal adhesions, leading to forward cell movement. Src inhibition has been found by previous studies to lead to increased size of focal adhesions due to inhibition of focal adhesion turnover (101).
Src inhibition with AZD0530 in MDA-MB-231 and MCF-7 cells had no evident effect on the morphology or frequency of focal adhesions (Figure 3.15 and 16).

3.5 Characterisation of E-cadherin expression in tumour cell lines

Transition from a polarised epithelial cell type to a motile mesenchymal cell type occurs as part of carcinoma progression, and promotes tumour metastasis. This epithelial to mesenchymal transition (EMT) is accompanied by loss of E-cadherin cell-cell junctions, leading to cell dissociation and motility. Src is known to regulate E-cadherin expression and inhibition of Src has previously been shown to decrease expression of E-cadherin (7, 54).
We characterised E-cadherin expression in the breast carcinoma cell lines MDA-MB-231 and MCF-7 to enable further investigation into the role of Src in these cells. As expected, the metastatic breast carcinoma cell line MDA-MB-231 had undergone EMT and had negligible remaining expression of E-cadherin, whereas the non-metastatic breast carcinoma cell line MCF-7 has retained expression of E-cadherin. Western blotting with an anti E-cadherin antibody, one band was seen ~100kDa in lanes containing MCF-7 lysates whereas no bands were seen in lanes containing MDA-MB-231 lysates (Figure 3.17).
Sarcomas, such as the human fibrosarcoma cell line HT1080, are mesenchymal in origin and are known to lack E-cadherin expression even before malignant transformation. HT1080 cells known to express the pro-invasive cadherin molecules: cadherin11 and N-cadherin respectively and this should be investigated further (70). The lack of E-cadherin expression in HT1080 cells was confirmed here using Western blotting with an anti-E-cadherin antibody (Figure 3.17).

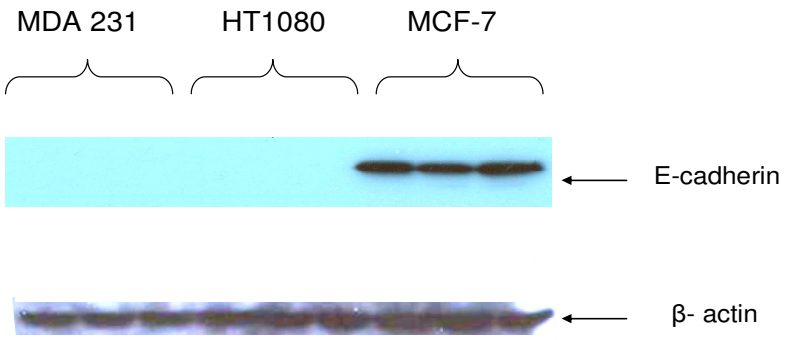


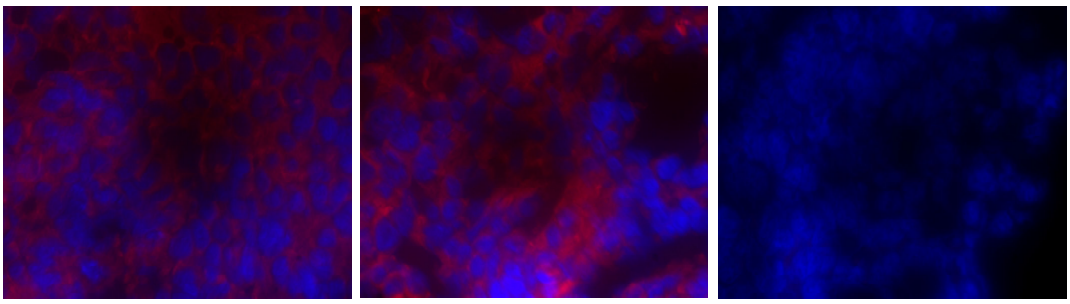
Figure 3.17. MCF-7 cells retain expression of E-cadherin. Western blotting with anti E-cadherin antibody produced 1 band ~100kDa in lanes containing MCF-7 cell lysates. No bands were seen in lanes containing MDA-MB-231 and HT1080 tumour cell lysates. Each lane contains 40µg protein, β actin was used as a loading control.

3.6 Translation into *in vivo* models

In order to validate inhibition of Src as a potential anti-metastatic target in HT1080 cells the expression of molecules associated with Src activation were investigated in HT1080 tumour xenografts.

3.6.1 HT1080 tumour xenografts express active paxillin

HT1080 xenografts express active paxillin (Tyr31 phosphorylated) as demonstrated by immunohistochemical staining of fixed cryostat sections of HT1080 xenografts grown in the hind limbs of nude mice (Figure 3.18). As it has been demonstrated that AZD0530 can affect the localisation of active paxillin *in vitro*, this could represent an *in vivo* marker of AZD0530 Src inhibition. It should be investigated whether the same relocalisation of paxillin (Tyr31 phosphorylated) from focal adhesions to the cytoplasm can be detected *in vivo*.

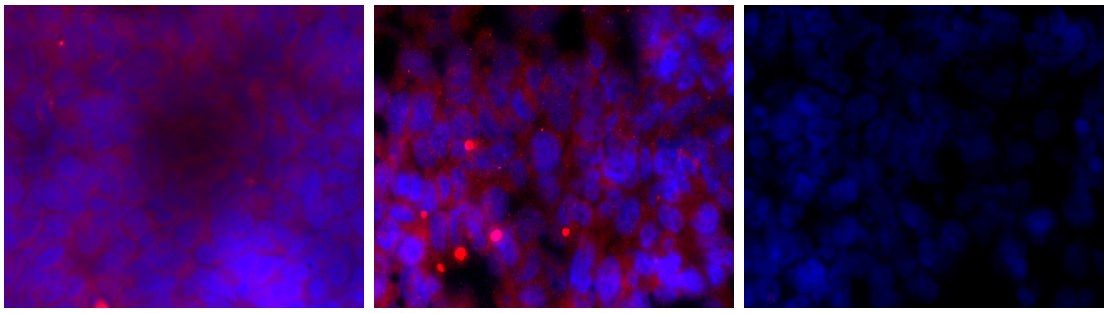


a) b) c)

Figure 3.18. HT1080 tumour xenografts express active paxillin (Tyr31-phosphphorylated).
Immunohistochemistry on 8µM cryostat sections of HT1080 xenograft tissue from two separate nude mouse models **(a and b)** Fixed sections from two HT1080 xenografts stained with an anti paxillin Tyr31-phosphorylated antibody(red), and hoescht (blue) **c)** negative control; stained with secondary antibody only.

3.6.2 HT1080 tumour xenografts express active FAK

HT1080 xenografts express active FAK (Tyr861 phosphorylated) as demonstrated by immunohistochemical staining of fixed cryostat sections of HT1080 xenografts grown in the hind limbs of nude mice (Figure 3.19). It should be investigated whether AZD0530 treatment *in vivo* has any effect on the expression of active FAK in HT1080 xenograft tissue.



a) b) c)

Figure 3.19. HT1080 tumour xenografts express active FAK (Tyr861-phosphorylated).
Immunohistochemistry on 8µM cryostat sections of HT1080 xenograft tissue from two separate nude mouse models **(a and b)** Fixed sections from two HT1080 xenografts stained with an anti FAK Tyr861-phosphorylated antibody(red), and hoescht (blue) **c)** negative control; stained with secondary antibody only.

4.0 Discussion

Numerous *in vitro* and *in vivo* pre-clinical investigations of the anti-migratory effects of Src inhibition have been carried out on tumour cell lines. However, mixed results from recent clinical trials suggest that the mechanism by which AZD0530 inhibits cell migration may be more complex than currently understood. The failure of a recent phase II studies to show to show any benefit of AZD0530 treatment in recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) or castration resistant prostate cancer suggest that it is unlikely to be used successfully as a single agent and on going clinical trails are mainly focused around the use of AZD0530 in combination with chemotherapeutic agents (102, 103).

The failure of results from pre-clinical studies showing an anti-metastatic effect of AZD0530 to translate into clinical efficacy highlights the need for further investigation into the role of Src and its interplay with other factors driving tumour progression. A deeper understanding of the role of Src in tumour growth, invasion and metastasis may provide rationale for the combination of AZD0530 with other targeted agents or chemotherapy and elucidate patient specific biomarkers to guide effective use.

A large body of the presented work focused on the effects of AZD0530 treatment on HT1080 cells, a human fibrosarcoma cell line. Early results from an on-going phase II trial in recurrent or metastatic soft tissue sarcoma, including fibrosarcoma, have indicated that whilst the drug can be used safely, no confirmed responses have been seen. Further clinical studies in this tumour type are likely to involve use in combination and investigation in tumours with target gene expression (104).

Whilst pre-clinical studies have demonstrated that AZD0530 consistently blocks the invasion and metastasis of tumour cells *in vivo* pre-clinical models, there is a variable tumour type specific affect on growth and proliferation of tumour cells, providing a rationale that it should be used in combination with anti-proliferative agents in many tumour types. For that reason the results laid out here focus mainly on investigating the anti-metastatic effect of AZD0530 on tumour cells.

4.1 Expression of Src and downstream modulators of metastasis

HT1080 cells were found to be expressing active Src, as shown by Western blotting with an antibody specific for Src Tyr530-unphosphorylated. However levels of active Src appeared to be lower than in MDA-MB-231 and MCF-7 cells, which are known to overexpress active Src (105). Levels of active Src should be quantified fully in HT1080 cell lines using Densitometry on Western blots, with a control tumour cell lines such as SW480, which is known to express low levels of active Src (106).

Localisation of active Src were measured by immunocytochemistry in the metastatic cell lines; HT1080 and MDA-MB-231, and the non invasive breast cancer cell line MCF-7 (Figure 2a-c).

In all cell lines active Src was found to be localised strongly to the perinuclear region, throughout the cytoplasm in a pattern consistent with localisation around the actin cytoskeleton, and at the cell periphery. Localisation of Src in HT1080, MDA-MB-231 and MCF-7 tumour cells is consistent with its localisation in Swiss 3T3 fibroblasts (105), keratinocytes (93) and mouse embryonic fibroblasts, and MCF-7 (107), where Src is seen localised to the perinuclear region, throughout the cytoplasm and at the cell periphery. It is thought that Src becomes activated in the perinuclear region of the cell and is then trafficked to its site of action at the cell periphery via the actin cytoskeleton, modulated by small GTPases (108, 107).

Association with the cytoskeleton was confirmed in HT1080 cells by co-localisation of active Src with β-tubulin (Figure 2e), a structural component of the actin cytoskeleton. Interestingly in the motile cell lines HT1080 and MDA-MB-231 active Src at the cell periphery was selectively localised to structures involved in cell motility such as at the tip of lamellipodia, suggesting it is playing a role in cell motility. This is consistent with evidence that active Src is trafficked along newly formed actin protrusions to the cell periphery where it plays a role in the turnover of focal adhesions allowing for forward cell movement (38, 97).

In the non motile MCF-7 cell lines active Src was found located around the whole perimeter of the cell periphery, suggesting it is serving a differing function in these cells.

In HT1080, MDA-MB-231 and MCF-7 cells we found that active Src, when measured by tyrosine 530 phosphorylation status, was largely localised to the perinuclear region, with smaller amounts at the cell periphery. Previous studies looking at the localisation of active Src as measured by phosphorylation status of the autoregulatory site Tyr416

have found there to be a concentration gradient across the cytoplasm with active Src being largely localised to the cell periphery (107, 109). These findings suggest that Tyr530 phosphorylation may be an early event in Src activation and relocalisation to sites of action at the cell periphery. This is in agreement with studies demonstrating that there is a transient increase in Src activity, by dephosphorylation at Tyr530, which causes active Src to relocalise along microtubules to newly formed focal adhesions (97).

HT1080, MDA-MB-231 and MCF-7 cells express active FAK in distinct focal adhesions at the cell periphery, as measured by immunofluorescent staining with an antibody specific for FAK phosphorylated at tyrosine 861 – a known site for Src activation.

The motile cell lines MDA-MB-231 and HT1080 were visibly polarised and active FAK containing focal adhesions were found at increased frequency at the tips of structures such as lamellipodia, associated with forward cell movement. This is consistent with a role for active FAK in integrin signalling and focal adhesion turnover, required for cell motility (109, 111).

Non-motile MCF-7 cells were not visibly polarised; however protrusions which appeared to be associated with cell spreading could be seen around the entire cell perimeter. Focal adhesions were localised around the entire cell periphery but active FAK containing focal adhesions were larger at the tip of protrusions associated with cell spreading. These results are consistent with a role for active FAK in cell spreading and attachment (39).

Bioimaging software such as Metavue® should be used to quantify the variability of size and frequency of active FAK containing focal adhesions in HT1080, MDA-MB-231 and MCF-7 cells. Furthermore Western blotting should be carried out, and quantified using densitometry, to assess the total expression of active FAK in each cell line.

The motile breast cancer cell line MDA-MB-231 and the non motile breast cancer cell line MCF-7 were co-stained for active Src and FAK.

MCF-7 cells displayed active Src staining throughout the cytoplasm, in a pattern consistent with association with the actin cytoskeleton, and at the cell periphery where active FAK could were stained in distinct focal adhesions. In MDA-MB-231 cells, active Src was localized throughout the cytoplasm, again in a pattern suggestive of

association with the actin cytoskeleton, but was found at lower levels at the cell periphery, where active FAK containing focal adhesions could be found.
These results suggest that active Src is playing a differing function in these two cell types. In non motile cells, active Src could be playing a role in cell attachment to the ECM, via its function at integrin receptors, or playing a role at cadherin containing cell-cell junctions.
The expression of active Src at the cell periphery in motile MDA-MB-231 cells was restricted to structures associated with forward cell movement, where there was also found to be a high frequency of active FAK containing focal adhesions, active Src may be involved in the turnover of focal adhesions in this cell line.
The function of Src in motile and non motile cells should be investigated further using a larger panel of metastatic and non metastatic cell lines.

HT1080 cells were found to express active paxillin, phosphorylated at tyrosine 31 - a known site for activation by Src, located in distinct focal adhesion complexes at the cell periphery, most frequently located at structures associated with cell motility at the front and the rear of the cell (Figure 3.13). This is consistent for the established role of paxillin in focal adhesion turnover downstream from Src (31).
Bioimaging software such as Metavue® should be used to robustly analyse the increased frequency of paxillin containing focal adhesions at structures such as lamellipodia, associated with cell motility.

Interestingly, higher levels of active paxillin were found in the metastatic tumour cell line HT1080, whereas negligible activity was seen in the non-invasive MCF-7 tumour cell line. In a number of tumour types expression of active paxillin has been found to correlate with the metastatic potential of tumour cells, therefore a lack of constitutive paxillin activity may contribute to the less invasive nature of MCF-7 cells. (112, 113)
However, this should be investigated further before conclusions can be made as it is unexpected that paxillin would be inactive in MCF-7 cells which have been found to express constitutively active Src, it could be that in this cell type, Src activity does not lead to phosphorylation of paxillin specifically at tyrosine residue 31. The phosphorylation of alternative regulatory tyrosine residues on paxillin, such as Tyr118, should be investigated in MCF-7 cells.

4.2 Effects of AZD0530 treatment

The effect if Src inhibition on cellular proliferation of tumour cells *in vitro* has been found to be cell line specific. AZD0530 inhibits proliferation in a number of cell lines, including LoVo and SW480 colon carcinoma cells, the breast carcinoma line MDA-MB-231 and NIH 3T3 fibroblasts expressing constitutively active Src (22).
Results from an MTT proliferation assay in MDA-MB-231 and HT1080 cells confirm that AZD0530 has an anti-proliferative effect in MDA-MB-231 tumour cells (22), but shows no significant inhibition of proliferation in HT1080 cells (IC50 >10μM). These results suggest that HT1080 proliferation is independent of Src activity. HT1080 cells are known to possess a mutated, constitutively active, N-ras protein which may drive proliferation in this cell line (99,100). Src has recently been found to be involved in proliferation of MDA-MB-231 cells; a known anti-proliferative agent, honokiol, was found to inhibit proliferation via downregulation of Src/ EGFR signalling and Akt, so it is unsurprising that AZD0530 has an anti-proliferative effect in this tumour cell line (135). It is important to note that cell line specific presence or absence of an *in vitro* anti-proliferative effect of AZD0530 does not necessarily translate to its inhibition of growth of tumour xenografts in *in vivo* models (22).
The lack of an anti-proliferative effect of AZD0530 on HT1080 cell lines does not negate the potential of Src as an anti-metastatic target in this cell line but does provide rationale that it is likely to be most clinically successful when combined with therapies which inhibit tumour cell proliferation, in order to control growth of the primary tumour.

In HT1080 cells, treatment with AZD0530 (0.1-0.5μM) led to an inhibition of cell migration at 16 and 24 hours in an *in vitro* 2D scratch assay. The effect was not found to be dose dependent.
AZD0530 has been found to inhibit migration of MDA-MB-231 cells in a scratch assay at concentrations between 0.01 – 0.5μM, in a dose dependent manner (22). AZD0530 has also been shown to inhibit migration in the murine fibrosarcoma cell line KHT (114) and Src inhibition with Dasatinib inhibits migration of human sarcoma cell lines (115).

Although scratch assays are a convenient and inexpensive way of measuring 2D cell migration, it can often be challenging to create clear defined scratches which are a consistent width. This can lead to variability in results; reflected in confidence intervals reaching close to 0.05 in these experiments. It is likely that there is a dose dependent effect of AZD0530 but the scratch assay method used here lacked the sensitivity to detect this.
A more accurate 2D assay was attempted using the Oris™ cell migration assay (Amsbio), consisting of a 96 well plate in which GFP expressing HT1080 cells were seeded into a monolayer around a stopper. The stopper was removed and migration of the cells into the resulting clear area could be recorded at different time points by measuring cell fluorescence. We attempted to optimise the protocol for use in HT1080 cells to compare cell migration between cells treated with AZD0530 at varying concentrations, or DMSO control. However this 96well plate 2D assay was technically difficult and expensive and no consistent results were obtained.

Additionally, scratch assays poorly represent the microenvironment in which a tumour cell migrates under physiological conditions. The scratch assays shown here were carried out on an uncoated surface, whereas tumour cell typically migrate through a 3D matrix of ECM proteins such as fibronectin and collagen, often in response to chemotactic stimuli. An improved methodology is to examine the invasion of cell through a 3D matrix of ECM proteins, which more accurately represents the movement of tumour cells through stromal tissue which is required for tumour metastasis. This is typically carried out using a method frequently referred to as a Boyden chamber assay or 3D invasion assay. Tumour cells are placed in an upper transwell chamber and migrate through a medium, such as collagen, to the lower transwell chamber, in response to chemotactic stimuli. AZD0530 has been shown to inhibit invasion and migration of HT1080 cells through a 3D collagen matrix in a dose dependent manner (127). Furthermore AZD0530 inhibited migration of DU145 and PC3 prostate cancer cell lines in a Boyden chamber assay, in a dose dependent manner (20).

In order to elucidate the mechanism by which AZD0530 inhibition of Src in HT1080 cells lead to a reduction in migration, activity of Src and its downstream targets was investigated.

We investigated the effect of AZD0530 treatment on the phosphorylation status of Src at the negative regulatory site Tyr530 which is phosphorylated by the negative regulatory protein Csk, leaving Src in a closed, inactive conformation (12).
No increase in phosphorylation at the negative regulatory Tyr530 could be seen with AZD0530 treatment for 2, 4, 16 or 24 hours. Inhibition of Src activity by AZD0530 in HT1080 cells appears to be independent of phosphorylation of the regulatory tyrosine 530. This is consistent with the fact that AZD0530 competitively binds the ATP-binding site, which is held in a conformation conducive for ATP binding by dephosphorylation of tyrosine 530 (12). Strongly supporting this hypothesis is the fact that AZD0530 binds inactive Src 10x more weakly than active Src (22).
No change was seen in the localisation of Src (Tyr530 unphosphorylated) at 24hours suggesting that AZD0530 Src inhibition does not lead to changes in the trafficking of Src to sites of action.
Interestingly at higher concentrations (5μM and 10μM) there appeared to be an increase in levels of Tyr530 unphosphorylated Src, which could be seen clearly at 24 hours. Whilst this is surprising, given that AZD0530 is a known Src inhibitor, the same effect has been seen previously in NIH 3T3 cells treated with pyrazole pyrimidine - type Src inhibitors (PP1 and PP2) when using phosphorylation status at tyrosine 530 to assess Src inhibition with AZD0530 (116). It has been postulated that this effect is caused by off target AZD0530 inhibit of Csk, the negative regulator of Src which negatively regulates Src activity by phosphorylation at Tyr 530 (94). It has been shown that AZD0530 has an inhibitory effect on Csk at high concentrations (IC50 >1000nm) (22). The effect of AZD0530 on Src phosphorylation status at the negative regulatory site Tyr530 seen here is consistent with previous studies and confirms it to be a poor marker of Src inhibition with AZD0530. There have been few studies looking at the effect of treatment with Src inhibitors on phosphorylation of Src at tyrosine 530, studies have typically focused on the Src regulatory auto-phosphorylation site, tyrosine 419 (83, 101). However, phosphorylation at tyrosine 419 has also shown to be an inconsistent marker of Src inhibition with AZD0530 (22). Therefore downstream targets of active Src should be investigated as potential markers of Src inhibition by AZD0530 in HT1080 cells.

The activity of the FAK was measured in HT1080 cells treated with the Src inhibitor AZD0530. FAK is known to be a key component of pathways leading to cell motility;

fibroblasts from FAK deficient mice show a decreased rate of spreading and migration, and reduced turnover of focal adhesions (39). Phosphorylation between Src and FAK leads to the formation of a transient FAK: Src complex which recruits and phosphorylates downstream modulators of cell motility events, such as p130CAS (p130 Crk-associated substrate) and paxillin. Src has been proposed to activate FAK by phosphorylation at regulatory tyrosine residues within the kinase domain activation loop (Tyr576 and Tyr577) and in the C terminal domain (Tyr861 and Tyr925) (27). Inhibition of cell migration caused by treatment with Src inhibitors has been shown to be accompanied by reduced FAK phosphorylation in various tumour cell lines (84, 2, 83).

Tyr861 phosphorylated FAK was chosen as a marker of FAK activity as Tyr 861 is a known site for Src phosphorylation and Src inhibition has been shown to cause inhibition of FAK Tyr861 phosphorylation in previous studies (22, 84). Additionally, FAK is strongly phosphorylated at Tyr861 in HT1080 cells and has been shown to be activated downstream from integrin receptors, involved in cell adhesion and motility (110).

There was no reduction in FAK Tyr861 staining with AZD0530 treatment at 2hours or 24hours at concentrations which inhibited HT1080 cell migration in the 2D scratch assay (0.1-1μM). This was unexpected as the role of FAK as a major target of active Src is well established. However, the precise mechanism of Src regulation of FAK has not been fully elucidated and may be cell line specific. For example, in the colon carcinoma cells it was found that only phosphorylation of Tyr925 was dependant on Src kinase activity (34). In murine fibroblasts FAK tyrosine residues Tyr576 and Tyr577 were found to be critical for activation by Src (37) and in PC3 prostate cancer cells inhibition of Src with Dasatinib lead to a reduction in FAK phosphorylation at Tyr861 (84).

From the results shown here, phosphorylation of FAK at Tyr861 does not appear to be strictly dependent on Src activity in HT1080 cells. The phosphorylation status of alternative regulatory tyrosine residues within FAK should be investigated to elucidate the role of FAK activity in the reduced HT1080 migration seen in 2D scratch assays with AZD0530 treatment as it is likely that inhibition of FAK is occurring downstream of Src inhibition with AZD0530.

Cells with defective Src:FAK signalling have been found to have reduced dissociation of focal adhesion components and display abnormally large focal adhesions (39, 31). The presence of active Src at focal adhesions has been shown to be required for converting larger focal adhesions at the cell periphery into smaller focal adhesions on the leading edge of lamellipodia (38). Therefore it could be expected that Src inhibition may lead to effects on focal adhesions frequency and morphology.
In previous studies, a motile variant of MCF-7 tumour cells; Tamoxifen-resistant MCF-7 cells treated with 0.1μM AZD0530 were found to have increased size of focal adhesions, thought to be due to reduced focal adhesion turnover (101).
Inhibition of Src with AZD0530 has no evident effect on the morphology of focal adhesions in MDA-MB-231 and MCF-7 cells in our initial experiments. Here active FAK (Tyr861 phosphorylated) was used as a marker of focal adhesions, as there was found to be no effect of AZD0530 treatment on FAK phosphorylation at this site. These preliminary experiments should be followed up with similar experiments using a more robust marker of focal adhesions such as vinculin, and a large number of images should be analysed using bioimaging software, to detect an effect of Src inhibition with AZD0530 on the frequency and morphology of focal adhesions.

Paxillin is a downstream substrate of the Src:FAK complex, activated and recruited into the complex by phosphorylation at two tyrosine residues, Tyr31 and Tyr118. Once active, paxillin provides a platform for the recruitment of downstream modulators of cell contractibility and focal adhesion disassembly, such as paxillin kinase linker (PKL) (27, 39, 31). Paxillin null fibroblasts have been shown to have defects in focal adhesion signalling and cell migration (29). Src inhibition with AZD0530 inhbition of Src has been shown to lead to reduced phosphorylation of paxillin phosphorylation in several tumour cell lines (21, 20).
In HT1080 cells active paxillin, as measured by staining of paxillin Tyr31-phosphorylated, was found to relocalise from focal adhesions to the cytoplasm in HT1080 cells treated with AZD0530 >1μM. The same effect has been seen previously; NBT-II bladder cancer cells treated with 1μM AZD0530 active paxillin, as measured by Tyr118 phosphorylation, was found to relocalise from focal adhesions at the cell periphery to the cytoplasm (22).

In normal cells (MDCK) and NBT-II bladder cancer cells localisation of paxillin to focal adhesions has been found to be dependent on the Crk adaptor protein which binds phosphorylated paxillin through its SH2 domain. Targeting of paxillin to focal adhesions by Crk requires the Rho GTPase Rac1 (117, 118, 119). Reduced paxillin phosphorylation downstream of Src inhibition with AZD0530 may cause dissociation of paxillin from focal adhesions by abrogating Crk binding.
It should be noted however that the role of Crk in paxillin localisation to focal adhesions has not been fully elucidated, in normal fibroblasts, paxillin was found to localise to focal adhesions in the absence of a Crk binding site but was found to be dependent on LIM3; one of four zinc finger domains present in the carboxy terminal of paxillin (120). Reduced phosphorylation of paxillin downstream of Src inhibition with AZD0530 in HT1080 cell should be confirmed using Western blotting and quantification using densitometry.

In HT1080 cells phalloidin staining demonstrated the presence of large membrane ruffles associated with lamellipodia formation and forward cell movement, in agreement with the literature (121, 100). HT1080 cells treated with AZD0530 (≥1μM) displayed reduced membrane ruffling and actin protrusions associated with cell motility and also appeared to reduce cell spreading. As expected a reduction of cellular protrusions associated with forward cell movement was also seen in MDA-MB-231 and MCF-7 cells upon Src inhibition with AZD0530.
The formation of cellular protrusions associated with forward cell movement has been associated with the expression of constitutively active Src in colon carcinoma cells (34). Src signalling has been shown to be involved in the cytoskeletal reorganisation required for the formation of membrane ruffles and lamellipodia occurring during cell motility events (14).
A major downstream target of Src, paxillin, is implicated in the rearrangement of the actin cytoskeleton leading to cell migration and spreading. Active paxillin acts as a molecular adaptor at focal adhesions, providing binding sites for an array of molecules, including structural proteins such as vinculin which bind the actin cytoskeleton, and signalling proteins which regulate Rho GTPases mediated changes in the actin cytoskeleton (98). Paxillin kinase linker binding to phosphorylated paxillin at focal adhesions has been found to be crucial for the Rac dependant actin cytoskeleton rearrangements that allow for cell spreading and motility (132). Therefore it is

unsurprising that reduced cell spreading and a reduction in the formation of actin structures associated with forward cell movement accompany paxillin relocalization from focal adhesions to the cytoplasm.

The downstream effector of Src signalling P130CAS leads to the formation of the p130CAS/Crk/DOCK180 complex and reorganisation of the actin cytoskeleton via regulation of GTPases such as Rac1 (14). P130CAS phosphorylation is found to be inhibited by AZD0530 treatment in DU145 and PC3 prostate cancer cell lines, and in HNSCC cell lines (20, 123). It should be investigated whether p130CAS activity is reduced downstream of AZD0530 inhibition in HT1080 cells as this may represent a mechanism by which AZD0530 affects cytoskeleton rearrangements and associated cell motility events such as the formation of lamellipodia.

The activity of cortactin, a downstream substrate of Src is known to have a role in the formation of lamellipodia, though its exact role is unclear. AZD0530 treatment in HNSCC cells was found to reduce phsophorylation of cortactin at the specific Src regulatory site Tyr421, and inhibited invasion in a Boyden chamber transwell assay (123). The phosphorylation status of cortactin should be investigated in HT1080 cells treated with AZD0530 to see if reduced activity may contribute to the drug effects on cytoskeletal rearrangements.

To investigate further the effects of Src inhibition in HT1080 cells on cell spreading we investigated the proportion of spread cells on the extracellular matrix proteins fibronectin and collagen. Spread cells were defined as those possessing actin-based cellular protrusions associated with cell spreading and motility. Cell spreading is regulated by Src downstream of integrin adhesion to extracellular matrix proteins; integrin clustering leads to the autophosphorylation of FAK and formation of the Src:FAK complex which activate downstream modulators such as paxillin which regulate rearrangements of the actin cytoskeleton involved in cell spreading and motility (27).

AZD0530 significantly inhibited HT1080 cell spreading on fibronectin at 15minutes but not at 25minutes. This is consistent with previous studies demonstrating a role for Src in the early events of fibroblast spreading on fibronectin. Kaplan et al. showed that Src deficient fibroblasts display a decreased rate of spreading on fibronectin at 10minutes, but not at 25minutes, which could be rescued by expression of wild type Src. Src deficient fibroblasts showed no defects in initial attachment to fibronectin. Interestingly

the rescue of cell spreading on fibronectin by c-Src expression was independent of kinase activity but required intact SH2 and SH3 domains (97). This suggests that AZD0530 may be inhibiting cell spread in HT1080 cells through a mechanism that is independent of inhibition of the kinase function, for instance by abrogating binding of Src to downstream targets through its SH2 and SH3 domains.

It was also found that there is a transient activation of Src by dephosphorylation at regulatory Tyr530 during the early stages of fibroblast adhesion to fibronectin, accompanied by Src relocalisation to focal adhesions (97). The effect of AZD0530 treatment on the localisation and levels of Src Tyr530-phoshporylated should be investigated at these early stages of cellular adhesion to fibronectin as this may be a mechanism by which AZD0530 inhibits cell spreading. Overexpression of Csk, a negative regulator of Src which phosphorylates Src Tyr530 was found to reduce the rate of cell spreading of fibronectin; expression of a kinase deficient mutant of Csk in astrocytes increased tyrosine phosphorylation of paxillin and FAK, and enhanced cell spreading on fibronectin (124). P130CAS deficient fibroblasts displayed normal attachment of cells to fibronectin but decreased rates of spreading on fibronectin, at 15minutes 70% of P130CAS re-expressing cells had flattened, as compared to 20% of P130 deficient cells (30). Paxillin has been found to have an important role in cell spreading and motility on fibronectin (29, 122)

AZD0530 has also been shown to inhibit cell spreading in tamoxifen resistant MCF-7 breast cancer cells, which have increased motility compared to non invasive MCF-7 cells In this study AZD0530 treatment was found to be associated with reduced cell motility in a Boyden chamber assay, and increased size of focal adhesions, as a result of decreased focal adhesion turnover (99).

Cell dissociation assays should be carried out to confirm that in HT1080 cells Src inhibition has no effect on the initial attachment of cells to fibronectin (97, 30).

Rho GTPases are known to regulate directional movement, both in a scratch wound assay towards a chemotactic signal, via reorganisation of the actin cytoskeleton and the Golgi apparatus (96). Src is known to interact with the Rho GTPases to regulate actin cytoskeleton rearrangement and remodelling of focal adhesions at structures such as lamellipodia and filopodia required for directional movement (38). Here we looked at polarisation of HT1080 cells towards the wound in a scratch assay to assess if Src inhibition led to defects in polarisation toward the scratch, which could account for the

inhibition of migration into the scratch by AZD0530. Polarization was judged from the orientation of the Golgi apparatus towards the direction of forward cell movement (125, 96). In these experiments, Src inhibition of HT1080 cells did not lead to an inhibition of cell polarization towards the wound.

This is somewhat surprising as Src / FAK dependant phosphorylation of paxillin kinase linker (PKL) has found to be required for polarisation of fibroblasts towards a wound in a scratch assay. However, Src was found to contribute to, but not be solely responsible for, the tyrosine phosphorylation of PKL and the contribution of FAK was not investigated (126). Both PKL and FAK deficient cells are unable to polarise their Golgi apparatus towards a wound in a scratch assay, suggesting that they play a key role (125, 96). The FERM domain of FAK has been shown to be required for cell polarization in a scratch wound assay, which binds RACK1 and allows for cell polarization (126). Based on the results here Src activity appears not be required for polarization of cells towards a scratch, as measured by orientation of the Golgi apparatus.

In carcinoma cells, Src activity has been shown to contribute to the destabilisation of E-cadherin containing cell-cell junctions, contributing to the development of an invasive phenotype. Inhibition of Src has shown to lead to increased expression of E-cadherin in HNSCC cell lines (7, 55). E-cadherin is widely considered to be a tumour suppressor; however loss of expression does not always correlate with invasiveness (128). Here we have showed that the non-invasive breast carcinoma cell line MCF-7 has retained E-cadherin expression, whilst the invasive breast cancer cell line MDA-MB-231 has lost E-cadherin expression. Therefore, they represent cell lines where the effect of AZD0530 on E-cadherin expression and its subsequent effect on invasive potential of these tumour cells can be studied. Expression of N-cadherin or cadherin-11, members of the same cadherin family, correlates positively with carcinoma cell invasiveness, through cooperation with the fibroblast growth factor receptor (FGFR). Forced expression of N-cadherin leads to an invasive phenotype even in carcinoma cells that retain E-cadherin expression (128). Sarcoma cell lines, such as HT1080 cells, are mesenchymal in origin and as such do not express E-cadherin. Instead HT1080 cells are known to express another member of the same family; N-cadherin, which has been shown to confer metastatic potential. Decreased expression of N-cadherin led to a reduction in metastasis of HT1080 tumour cells *in vitro* and *in vivo.* HT1080 N-cadherin knockout models led to inhibition of migration in scratch assays, an increase in actin stress fibres and

reduced appearance of lamellipodia (129, 130). There is evidence to suggest that Src activity leads to the destabilisation of N-cadherin containing cell-cell junctions, as it does for E-cadherin. SW480 cells expressing a Src mutant with increased kinase activity led to the phosphorylation of N-cadherin and its translocation from the cell surface to the cytoplasm, accompanied by a 7 fold reduction in homotypic cell adhesion (131). Conversely, the Src inhibitor PP2 led caused a decrease in N-cadherin phosphorylation in WM239 melanoma cells (132). Src inhibition with AZD0530 in HT1080 cells could lead to the maintenance of N-cadherin at the cell surface, which has been shown to contribute to the metastatic potential of these cell lines. This could represent a significant compensatory mechanism and should be investigated further.

4.3 Conclusions and future directions

Src inhibition with AZD0530 inhibits cell spreading and migration in HT1080 cells, but shows no effect on cell proliferation. Further investigation should be carried out to determine if these *in vitro* effects of AZD0530 translate into anti-metastatic effects in *in vivo* in HT1080 xenograft models in orthotopic mice, with particular focus on the use of combination therapies.

Src inhibition with AZD0530 has previously shown to inhibit *in vivo* proliferation of DU145 prostate cancer cells and MDA-MB-231 cells and reduced lymph node metastasis of NBT-II bladder cancer cells (22, 20). In NBT-II bladder cancer xenografts, treatment with AZD0530 was accompanied by a reduction in FAK Tyr861 and paxillin Tyr31 phosphorylation (22).

In order to move forward into *in vivo* xenograft models immunohistochemistry was used to investigate the expression of active Src, FAK and paxillin in untreated HT1080 xenograft tissue. It was found the HT1080 xenografts, grown in nude orthotopic mice, express active FAK Tyr861-phosphorylated and active paxillin Tyr31-phosphorylated. Further in vivo studies should be carried out to indentify if there is an effect of AZD0530 treatment in nude mice bearing HT1080 xenografts. The effect on growth of the primary tumour, and tumour metastasis to distant organs should be investigated, as well as the effect of AZD0530 treatment on tumour vascularisation. On a molecular level, the tumour xenograft, and metastatic tumour cells, should be analysed for expression and activity of downstream modulators of Src including FAK, paxillin, P130CAS, cortactin and PKL. It should be investigated whether FAKTyr861 remains a

poor marker of Src inhibition in HT1080 cells *in vivo*, as shown here *in vitro* and whether there is a similar relocalisation of paxillin.

The effect of AZD0530 treatment on molecules which regulate SFKs, such as the Csk, and Phosphotyrosine Phosphatase 1B, as well as signalling pathways which have cross talk with SFKs, such as growth factor receptor signalling, should be investigated to any highlight potential compensatory mechanisms bought about by Src inhibition with AZD0530. As discussed the expression of pro-invasive Cadherin molecules should be investigated as theoretically this could represent a significant compensatory mechanism to inhibition of the SFK pathway.

Lessons from previous phase II clinical trials, where inadequate efficacy has been obtained when AZD0530 is used as a mono-therapy, mean that the drive should be to test potential combination therapies.

A recent study in oestrogen receptor (ER) expressing breast cancer cells showed that treatment with AZD0530 alone led to resistance to the anti-proliferative effects of AZD0530 and up regulation of the ER. Resistance to AZD0530 was found to be associated with marked activation of the MTOR pathway. Treatment with AZD0530 in combination with ER blocking drugs led to inhibition of tumour cell proliferation both *in vitro* and *in vivo* (133). A similar study in ER positive breast cancer cells showed that the MAPK and PI3K pathways could both represent bypass pathways leading to AZD0530 resistance (134).

AZD0530 has been found to lower the resistance of lung tumour cells to radiotherapy, raising the possibility that it could be used in combination with radiotherapy (88).

Moving forward pre-clinical studies should be carried out *in vitro* and *in vivo* to test potential therapeutic combinations which may enhance the anti-metastatic effect of Src inhibition and overcome compensatory mechanisms which may be causing failure in clinical trials.

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