Identification of Tumour Suppressor Genes whose loss mediates sensitivity to conventional chemotherapy and targeted therapeutics

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Abstract

Introduction: Progression to clinical trial is the ultimate goal of drug development. However the task of selecting the appropriate patient population is pivotal for the advancement of a drug from the preclinical to clinical setting. An innovative method of patient selection is to determine possible interactions between the drug being tested and known mutations in that particular type of cancer. Presently there are many small molecule inhibitors undergoing clinical trials which make use of this concept both, as single agents or in combination with other regimes. There is a wealth of literature surrounded the role of Src and Akt in tumour progression, invasion and metastasis and inhibitors of these kinases have become attractive drug targets in the treatment of cancer.

Aims: To identify tumour suppressor genes whose loss synergises with inhibition of Src and Akt. This would enable the identification of a patient population who would experience benefit from these drugs in clinical studies.

Methods: The effects of AZD0530 and MK2206 are investigated in HFF cells, reverse transfected with 178 siRNAs from a tumour suppressor library representative of all known tumour suppressor genes lost in cancer. Robust Z Scores were used to determine potential sensitive hits and functional analysis of such hits were carried out through DAVID- a bioinformatics functional analysis website.

Results: For HFF cells + siRNA treated with 5μM MK2206, 45 potential hits were identified as sensitive to this Akt Inhibitor, of which only 2 were targeted by multiple siRNAs. For HFF cells + siRNA treated with AZD0530, 61 potential hits were identified as sensitive to this Src Inhibitor, of which 6 were targeted by multiple siRNAs.

Discussion: This study was used to identify potential biomarkers of response to inhibitors of Akt and Src thus creating a patient population which would benefit from use of these single agents. From the screen treated with AZD0530, two tumour suppressor genes arose- ERCC-1 and FANCD2, which are components of the Fanconi anaemia pathway. Therefore loss or mutation of this pathway has the potential to be a biomarker of response to Src Inhibitors in the clinical setting.

Key Words: Tumour Suppressor Genes, AZD0530, MK2206.

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Abbreviations

ATM: Ataxia Telangiectasia mutated

BRCA1/2: Breast Cancer 1/2

BSA: Bovine serum albumin

DMSO: Dimethylsulphoxide

GM: Growth medium

HFF: Human foreskin fibroblasts

HR: Homologous recombination

IC: Inhibitory concentration

MAD: Median absolute deviation

NHEJ: Non-homologous end joining

PLK1: Polo-like kinase 1

PTEN: Phosphatase and tensin homolog

Rb: Retinoblastoma gene

siRNA: Short interfering ribonucleic acid

Chapter I: Introduction

Introduction

Cancer remains one of the leading causes of death worldwide. In 2010 157,250 people died from cancer in the UK. Numerous therapies are used in the treatment of cancer including chemotherapy, radiotherapy, targeted therapy, immunotherapy and gene therapy. Chemotherapy is at this present time the principal treatment modality, however its draw backs come in the form of quite toxic side effects such as nausea and immunosuppression attributed to the toxicity of the chemotherapeutic agent on the rapidly dividing normal tissues in the body (Druker 2002). The aim of cancer research has therefore shifted to identification of drugs which have less toxic side effects and are more effective, or the identification of traits which are tumour specific and can be exploited by targeted therapeutics (Chan, Sutphin et al. 2011).

Synthetic lethality is a form of cell killing. The concept of which is based on genetic studies in organisms such as yeast (Dobzhansky 1946). It is defined as a genetic interaction where the combination of mutations in two or more genes leads to cell death, if the mutation of either gene alone leaves the cell viable. By its very definition, cancer is a disease of DNA repair, caused by the acquisition of permanent genomic mutations. This is exhibited by malignancies which are caused by inherited mutations of DNA repair (Shaheen, Allen et al. 2011). Synthetic lethality can also be defined where the disruption of a gene in one pathway is non-lethal, as an alternative pathway can sufficiently maintain its processes. However, inhibitor of the alternative pathway in combination with disruption to the original pathway is incompatible with viability.

A group of genes commonly lost or inactivated are tumour suppressor genes, which can promote the initiation and progression of cancer through mediums such as cell proliferation, cell death and cell migration or invasion. Of late, evidence has emerged which suggests that tumour suppressor genes also play a significant role in the response of cancer to a variety of chemotherapeutic agents.

1.1 *Tumour Suppressor Genes*

Tumour suppressor genes are a group of genes which have emerged as significant mediators of response to chemotherapeutic agents. They are commonly dysregulated in hereditary cancer syndromes by mutations or genetic modifications. They are also responsible for the initiation and progression of all forms of cancer, therefore composing an essential class of signalling molecules within the cell. Important examples include p53, PTEN, Rb and BRCA1 and 2.

p53 is the most frequently mutated tumour suppressor gene in cancer. It is also very important in the regulation of various cellular functions including cell cycle progression, apoptosis, cell motility, DNA repair, genetic instability and cell metabolism by transcriptionally activating a number of cellular genes (Vousden, Prives 2009). p53 is able to efficiently inhibit cell proliferation, through its actions of blocking cell cycle progression and promoting apoptotic cell death. This would therefore be an essential mechanism for halting tumour cell growth and inhibiting cancer development. It has been widely reported from cell, animal and clinical studies that the status of p53 is associated with patient sensitivity to chemotherapeutic agents (Bertheau, Espie et al. 2008). It has been demonstrated that cells that possess mutant p53 are more resistant to a wide variety chemotherapeutic agents than those cells which possess wild-type p53. A study by Vasey, Jones and colleagues (Vasey, Jones et al. 1996) showed that inactivation of p53 is associated with reduced sensitivity to cisplatin in ovarian cancer but not to paclitaxel. In another study it was found that disruption of p53 rendered cells resistant to 5-fluorouracil in colorectal cancer, but sensitised the cells to doxorubicin (Bunz, Hwang et al. 1999). It has therefore been shown that the role of p53 in mediating a chemotherapeutic response is complex and depends on both the cellular context and the class of chemotherapeutic agent.

PTEN is a dual protein and lipid phosphate which is commonly mutated in many sporadic cancers (Li, Yen et al. 1997). Its tumour suppressor activity is dependent upon its lipid phosphatase activity. Loss of PTEN results in reduced dephosphorylation of phosphoinositide 3,4,5-triphosphate

(PIP_3), which allows phosphoinositide 3-kinase to phosphorylate PIP_2 , resulting in enhanced levels of PIP_3. This induction causes increased cell proliferation and cell migration, cell survival and cell size through activation of downstream proteins such as ATK. In a study by Nagata, Lan and colleagues (Nagata, Lan et al. 2004), it was shown that treatment of patients with HER-2 overexpressing breast cancer with Herceptin (Trastuzumab), increased PTEN membrane localisation and phosphate activity by reducing PTEN tyrosine phosphorylation via Src kinase inhibition. Src has important oncogenic functions, inactivating PTEN when bound to and activated by overexpressed ErbB2, a proto-oncogene which encodes for Her-2. Trastuzumab works in opposition by dissociating Src from ErbB2, inhibiting Src and therefore activating PTEN contributing to Trastuzumab anti-tumour function. However, down-regulation of PTEN resulted in Herceptin resistance in vitro and in vivo, demonstrated by xenografts in athymic nude mice (Nagata, Lan et al. 2004). PTEN down-regulation can be used as a biomarker in predicting low response to EGFR inhibitors such as cetuximab in the treatment of lung and colorectal carcinoma (Kokubo, Gemma et al. 2005).

The retinoblastoma gene (Rb) was originally identified in retinoblastoma (Friend, Bernards et al. 1986). It is a tumour suppressor gene which is mutated or functionally inactivated in the majority of malignancies (Zagorski, Knudsen et al. 2007). It has been shown to protect against tumourigenesis through the regulation of cellular senescence, differentiation, cell cycle progression, apoptosis and chromosomal integrity. It has also been shown that Rb status is indicative of predicting chemotherapeutic response. Human cancer cells have shown that multiple chemotherapeutic agents activate Rb causing cell cycle arrest and the activation of DNA repair pathways, resulting in cellular resistance (Bosco, Wang et al. 2007). On the other hand, the loss of Rb expression induced by RNA interference bypasses the RB-induced checkpoint response, thus sensitising cells to apoptosis induced by chemotherapeutic agents. One mechanism which has been proposed is that, in the absence of Rb, cells replicate unchecked. Replication of a damaged genome which was induced by chemotherapeutic agents results in an accumulation of double strand breaks and therefore genomic

instability (Bosco, Mayhew et al. 2004). Apoptosis is triggered when this DNA damage is irreparable thus preventing the replication of unstable cells. Clinical studies have also shown that loss of Rb increases sensitivity of tumours to chemotherapeutic agents, regardless of the class of drug or type of tumour. Zagorski, Knudsen and colleagues (Zagorski, Knudsen et al. 2007) demonstrated tumours which were xenografted into mice which contained RB knockdown lung cancer cell lines, regressed substantially when treated with cisplatin or 5-fluorouracil compared with tumours which expressed Rb. It is possible that Rb status could serve as an important biomarker in the prediction of chemotherapeutic response.

A further example of a tumour suppressor gene is BRCA1 and 2 (breast cancer susceptibility gene 1 and 2). BRCA1 is frequently mutated in inherited breast cancers. One of its most important roles is in the repair of DNS double strand breaks through homologous recombination- a process which will be discussed in further detail at a later point. BRCA1 and 2 encode proteins with many individual functions, but they share the responsibility in co-ordinating the response to DNA damage. BRCA1 is a key player in the regulation cell cycle checkpoint and DNA repair signalling, and BRCA2 directly translocates the DNA repair protein Rad51 to areas of DNA damage to allow repair to occur. BRCA1/2 therefore functions as a tumour suppressor gene through the regulation of transcription, cell cycle checkpoints and DNA repair (Deng 2006). The participation of BRCA1 in DNA repair evokes a strong theory for the role of BRCA1 in the response of DNA-damaging drugs.

Evidence has mounted in support of tumour suppressor genes playing a pivotal role in either enhancing or reducing the sensitivity of cancer cells to chemotherapeutic agents. Some tumour suppressor genes, such as BRCA1 have the capacity to be used as biomarkers in predicting the outcome of chemotherapeutic agents (Kennedy, D'Andrea 2006, Kennedy, Quinn et al. 2004), but this remains to be further studied in the context of large scale screening in clinical trials. It has also been suggested that single gene biomarkers are not sufficient in determine treatment outcome due to the complexity of cell signalling pathways.

1.2 *Exploring Tumour Suppressor Genes-Synthetic Lethality*

The concept of synthetic lethality originated from genetic studies in drosophila (Dobzhansky 1946) when the geneticist Theodosius Dobzhansky coined the term to illustrate the phenomenon where two genetic mutations, which occur individually can leave the cell viable, but are lethal if combined. This significantly affects function or signalling of a pathway when both genes are lost (Kaelin 2005). Loss of one gene can be tolerated by the cell if it is capable of using a redundant pathway to maintain function. Synthetic lethality can be induced if the gene from the redundant pathway is itself also deleted, or if the product of the pathway is inhibited. This is shown in the figure 1 below.

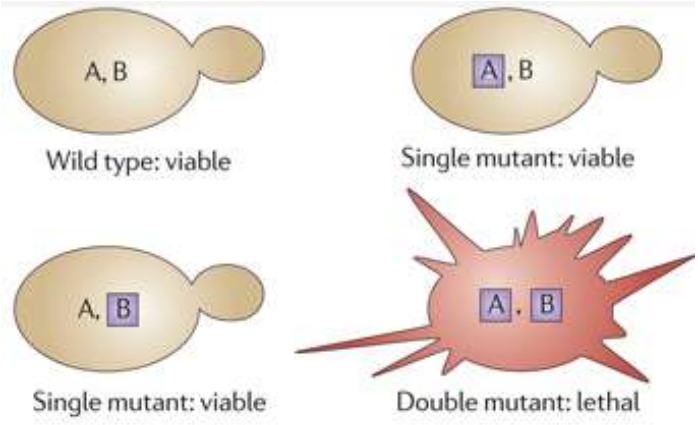

Figure 1. Genetic interaction between two genes which are synthetically lethal. Adapted from Chan, 2011.

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For tumour cells, both copies of the tumour suppressor genes will be deleted, whereas one normal copy is maintained in the healthy cells, it has been hypothesised that this would render synthetic lethality selective towards the tumour, whilst sparing the normal cells (Kaelin 2005).

Hartwell et al were the first group to outline the concept of using synthetic lethality screening to identify the efficacy of anti-cancer drugs. They investigated synthetic lethality between a panel of yeast strains and Food and Drug Administration approved cancer drugs, looking for drugs

which were specifically lethal to yeast that had gene defects which were analogous to tumour suppressor genes mutations (Hartwell, Szankasi et al. 1997). In their study they found a number of strains of yeast which had deleted genes, particularly sensitive to particular classes of drugs. One example of this was a yeast mutant for the DNA Post-Replication Repair pathway components Rad18 and Rad6 were most sensitive to cisplatin, a DNA cross-linked drug that any other the other strains of yeast mutation. They also found that yeast strains which were defective in double-stranded DNA break repair, had increased sensitivity to mitoxantrone- a topoisomerase II poison. This was further confirmed by a study by Dunstan, Ludlow and colleagues which found that 126 out of 85000 compounds were selectively toxic to yeast which was deficient in DNA double strand break repair, and that most of these compounds were inhibitors of topoisomerase I and II (Dunstan, Ludlow et al. 2002a, Dunstan, Ludlow et al. 2002b).

DNA Repair

It is understood that DNA mismatch repair pathway has an important role in predisposition to cancer and also in its response to therapy. Therefore it has been suggested that synthetic lethal strategies which target tumours that are MMR deficient could be used in a clinical setting. The concept of synthetic lethality is based on the reliance of cancer cells on DNA repair to maintain cell division. This means that by applying a mechanistic understanding of this DNA repair pathway, novel strategies could be transformed into the clinical scenario.

DNA is constantly being subjected to a range of insults which could ultimately lead to DNA mutations or altered cell behaviour. To allow continuous cell division, cells rely heavily on DNA repair pathways to mend DNA damage which occurs during replication (Ciccia, Elledge 2010). The choice of repair pathway is based upon the stage of the cell-cycle which the cell is in, and on the type of lesion which has occurred (Branzei, Foiani 2008). The regulation of DNA repair is usually dependent upon

the expression and stability of repair proteins, compaction level of chromatin and the availability of sister chromatids.

There are two major pathways which the cell utilises to repair double stranded breaks, which are hypothesised to be the most lethal damage if unrepaired. These are non-homologous end joining (NHEJ) and homologous recombination (HR).

Non-homologous end joining preferentially occurs in G_0/G_1, though is capable of occurring at any point, and works by directly ligating the free ends of the double stranded break (Sonoda, Hochegger et al. 2006). Non-homologous end joining has two sub-pathways- classic and alternative- the choice between which is regulated by 53BPI which promotes classic non-homologous end joining (Ward, Kim et al. 2006)(Ward, Kim et al. 2006) and PARP1 which promotes alternative non-homologous end joining (Wang, Wu et al. 2006b, Wang, Wu et al. 2006a). Alternative non-homologous end joining is much less accurate and can result in large deletions or translocations (Lieber 2008). However, any defects in proteins involved in classic non-homologous end joining, directs double strand breaks towards the less accurate alternative NHEJ pathway.

In comparison, homologous recombination can only occur after DNA replication in $S/G_2/M$ phases of the cell cycle. This pathway uses a homologous template to repair the double stranded break by using strand invasion of the homologous chromatid as a template from which DNA can be synthesised (Lieber 2008).

Homologous recombination also has both accurate and inaccurate sub-pathways. Within the accurate homologous repair pathway, genetic information is copied from templates of homologous DNA sequences. These are usually sister chromatids in S/G_2 phase cells (San Filippo, Sung et al. 2008). For double strand breaks within the S/G_2 phase, sister chromatids must be within close proximity to one another. Inaccurate homologous repair is referred to as single strand annealing,

where by extensive resection exposes complimentary sequences in linked direct repeats. It is always inaccurate as it deletes one of the repeats and the intervening sequence (Shaheen, Allen et al. 2011).

Case Studies of Synthetic Lethality

The first clinical trials which were performed with the aim of identifying the potential targets for novel chemotherapeutic agents was the study of poly(ADP-ribose) polymerase (PARP) inhibitors in BRCA1 and BRCA2 mutation carriers with advanced solid breast of ovarian tumours (Fong, Boss et al. 2009). PARPs are of a family of 17 enzymes which catalyse polymerisation and formation of highly negatively charged poly ADP ribose chains on target protein, thereby modifying their action, but only PARP1 and PARP2 are known to be involved in DNA repair (Ame, Spenlehauer et al. 2004). They work by maintaining genomic integrity, with particular responsibility for repair of single stranded DNA lesions and breaks, using the base excision repair pathway.

Replicating DNA relies on the repair of double stranded breaks to maintain cell replication and proliferation. However, patients who possess a mutant form of BRCA1 or 2, lose the ability to use that repair pathway to repair double stranded breaks. Therefore the cancer cells may become addicted another DNA repair pathway to remain viable; a DNA repair pathway other than the one which led to its initial mutation as this may be defective. Unrepaired single strand breaks in the DNA result in stalled replication forks and one of the main roles of homologous recombination is to repair such stalled forks. Therefore tumours with have impaired homologous recombination function due to impaired BRCA1 or BRCA2 function, could be sensitised to other inhibitors of enzymes which are involved in single strand break repair, such as PARP inhibitors (Ashworth 2008). It was discovered that inhibition of PARP resulted in synthetically lethal interactions with mutations in the BRCA1 or BRCA2 genes (Farmer, McCabe et al. 2005, Bryant, Schultz et al. 2005). Farmer, McCabe, Lord and colleagues suggested that the loss of PARP1 increases the incident of DNA lesions without directly altering the process of homologous recombination, presumably as the collapsed replication forks at

the site of the single strand breaks are no longer repaired. Stalling of the replication fork may subsequently lead to DNA double strand break formation. The tumour cell then resorts to alternative, error-prone DNA repair pathways which can result in chromatid instability, cell cycle arrest and eventually apoptosis (Ashworth 2008, Ashworth 2008).

Fong, Boss and colleagues carried out a Phase I clinical trial to analyse the pharmacodynamic and pharmacokinetic characteristics of olaparib (AZD2281) in patients who were carriers of the BRCA1 or BRCA2 mutation (Fong, Boss et al. 2009, Fong, Boss et al. 2009). This trial showed that olaparib did indeed inhibit PARP, had a substantial anti-tumour effect in patients who were carriers of the BRCA1/2 mutation and fewer adverse effects than conventional chemotherapy.

In two parallel phase II trials for the same drug, conducted by Tutt, Robson and colleagues (Tutt, Robson et al. 2010, Tutt, Robson et al. 2010, Audeh, Carmichael et al. 2010) to assess the efficacy, tolerability and safety of the drug, it was found that there was a positive proof of concept for PARP inhibition as a single agent therapy for patients with BRCA deficient breast cancers, and a favourable therapeutic index for patients with tumours that have a genetic loss of function of BRCA1/2 associated DNA repair.

1.3 *Finding Synthetic Lethality*

RNA interference is a sequence specific, post-transcriptional, gene-silencing process which is mediated by double stranded RNA molecules as shown below in figure 2 (Novina, Sharp 2004). RNAi is initiated by long stretches of double stranded RNA which are processed by Dicer- an enzyme which cuts long stretches of dsRNA into duplexes that have 19 paired nucleotides and two overhanging nucleotides at both 3’ ends. These duplexes are referred to as short interfering RNA (siRNA). Following this, the sense strand is unwound within RNA inducing silencing complex (RISC), a large protein complex which associates with dsRNA. The anti-sense strand of the siRNA directs RISC to the

target mRNA sequence where it anneals complementarily by Watson-Crick base pairing leading to inhibition of translation of the target protein. (Martinez, Tuschl 2004).

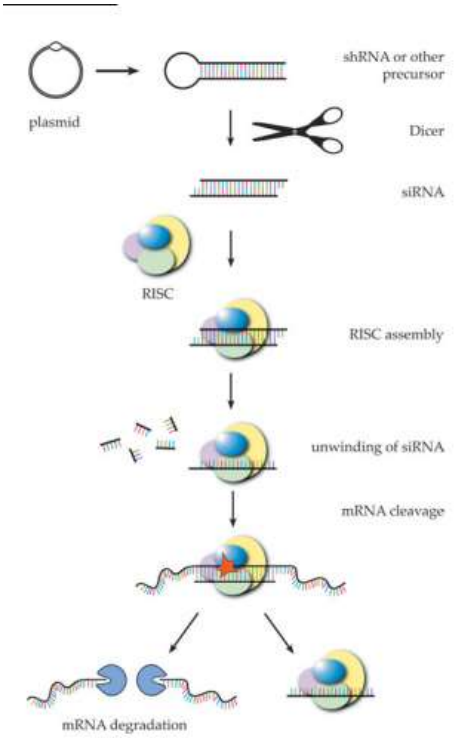


Figure 2. RNAi machinery creating siRNA from double stranded RNA molecules. Adapted from Cejka, Losert and Wacheck 2006.

RNAi takes advantage of the endogenous gene-silencing machinery and its leading application is in the study of gene knockdown in vitro. It provides a fast and reliable way to characterise a gene knockdown with excellent potency. Multiple in vitro siRNA studies have been carried out to evaluate the knockdown phenotype of oncogenes in cancer cells. One example is the effect of targeting the Bcr/Abl kinase Oncogene in Chronic Myeloid Leukaemia (Wilda, Fuchs et al. 2002). This study found that imatinib successfully inhibited Bcr/Abl signalling in Chronic Myeloid Leukaemia. It also found that silencing the Bcr/Abl protein by siRNA induced an apoptotic response which was comparable with that of imatinib. Experiments such as these showed the valuable role of siRNA-mediated knockdown for studying genetic impacts on human diseases. However, when conducting these experiments, the maximum mRNA knockdown which is able to be achieved must

also take into account the minimal amount of undesired effects seen also due to the complexities of the RNAi machinery.

RNAi in vitro has been used to investigate a number of areas of drug discovery such as target identification, target validation and compound identification and validation (Iorns, Lord et al. 2007). The aim of target identification is to distinguish key targets that are essential for the survival and behaviour of tumour cells but are largely redundant in normal cells. RNAi screens can be used to identify targets that exploit oncogene addiction, reverse the hallmarks of cancer, cause synthetic lethality, confer drug sensitisation and identify combination therapies. RNAi is also used for the purpose of demonstrating the clinical relevance of the targets which have been discovered. In addition, RNAi screens have the potential to improve the specificity of lead compounds and the final approved drug (Iorns et al. 2007).

McCaffery, Meuse and colleagues designed a study to determine whether siRNAs inhibit gene expression in vivo (McCaffrey, Meuse et al. 2002). In this study they injected a luciferase-expressing plasmid and a synthetically synthesised siRNA or a short-hairpin RNA-expressing plasmid into the livers of adult nude mice. The results of the study indicated that there was a specific, siRNA-mediated inhibition of luciferase expression in adult mice compared with the control. This study, like many others which followed it, suffered side effects with the application of systemic siRNA. In order to surpass this obstacle, administration of siRNAs into the airway epithelium was found to be particularly well suited for the uptake of RNAi-based drugs (Bitko, Musiyenko et al. 2005).

The first demonstration of RNAi efficacy in vivo was in a study by Fong, Lee and colleagues. This study demonstrated that a 99% knockdown of the Hepatitis B Virus core antigen in liver hepatocytes could be achieved by the expression of anti-hepatitis B virus short-hairpin RNA (Song, Lee et al. 2003). HIV was the first infectious agent targeted by RNAi. Synthetic and expressed siRNAs have been used to target a number of early and late encoded RNAs (Jacque, Triques et al. 2002).

Despite this success, in future clinical applications, targeting the virus directly represents a challenge, as high viral mutation rates will lead to escape mutations (Boden, Pusch et al. 2003). The use of RNAi also has its use in cancer therapeutics. It may be exploited to silence pathways which facilitate the effects of traditional cancer drugs, such as the targeting of multidrug resistance gene for resensitisation to chemotherapy (Nieth, Priebsch et al. 2003) or the silencing of double-strand break repair enzymes for enhanced radio- or chemotherapeutic effects (Collis, Swartz et al. 2003).

1.4 *Novel Therapeutics for testing in Synthetic Lethality Screens*

Modern chemotherapy is moving towards the use of selective inhibitors, with a particular interest in kinase inhibitors. These are designed to have specific effects in cancer with the advantage of reduced side effects in normal cells. Some examples of these agents are Src inhibitors, Wee1 inhibitors and AKT inhibitors.

Src Inhibitors

Src was the first proto-oncogene identified from the human genome. It encode for a non-receptor tyrosine kinase (RTK) (Puls, Eadens et al. 2011). Src has many regulatory functions in normal tissues such as participation in fibroblast cell division and cell-cell adhesion regulation via the modulation of integrins. Both overexpression and overactivation of Src can promote the development of cancer (Yeatman 2004). The Src protein is a member of the Src family kinases family, of which there are twelve members. Src's activity mainly occurs at the inner leaflet of the cell as membrane localisation is required for receptor-mediated signalling to occur. It is thought to facilitate motility and invasion of tumour cells by promoting endocytosis of cell-cell adhesions, mediating assembly and disassembly of focal adhesions and regulating expression of matrix metalloproteinases that contribute to the breakdown of the extracellular matrix (Zamir, Geiger 2001). Src is also believed to activate the signal transducer and activator of transcription (STAT)-3. This is an

important protein that mediates angiogenesis through vascular endothelial growth factor (Niu, Wright et al. 2002).

Src Family Kinases have been shown to be involved in numerous human cancers, with colon cancer being the type where this relationship has been most extensively studied. In an early study by Bolen, Veillette and colleagues, it was found that Src activity was elevated in colonic carcinoma cell lines (Bolen, Veillette et al. 1987). This was also identified in breast cancer, lung cancer, pancreatic cancer, gastric cancer, ovarian cancer and bladder cancer, to name but a few (Summy, Gallick 2003).

Therefore with the wealth of knowledge which has accumulated in support of the role of Src in tumour progression, invasion and metastasis, inhibitors are being developed to this non-receptor tyrosine kinase (Puls, Eadens et al. 2011). One example is this is Dasatinib (BMS-354825), an orally bioavailable, ATP-site competitive Src-Abl inhibitor which is approved for use in chronic myeloid leukaemia (CML) through its inhibition of the Bcr-Abl pathway and Philadelphia-chromosome positive acute lymphoblastic leukaemia. A recent study of 512 patients CML by Kantarjian, Shah and colleagues showed that dasatinib demonstrated higher rates of complete cytogenetic response, in a shorter time than imatinib as a first line treatment (Kantarjian, Shah et al. 2010). It also displayed a 325-fold greater potency for Bcr-Abl inhibition than imatinib. Another example is Saracatinib (AZD0530) which is also an orally active, highly selective small molecule, dual Src-Abl inhibitor. Its antitumour effects have been observed in various solid tumour cell lines, including breast, prostate and lung carcinoma. Preclinical breast cancer studies showed that saracatinib in combination with gefitinib, an EGFR inhibitor resulted in lower levels of Src (Hiscox, Morgan et al. 2006). Phase I trials have been conducted and in these it was ascertained that the maximum-tolerated dose was 175mg daily for advanced solid tumours (Baselga, Cervantes et al. 2010). Saracatinib has thus far had limited efficacy in phase II clinical trials in many solid tumour malignancies (Fury, Baxi et al. 2011). Many ongoing trials with Src inhibitors are investigating possible biomarkers of response which may be suggestive of a patient population which would benefit from this treatment.

Akt Inhibitors

AKT is a serine threonine kinase which comprises of a family of three different protein isoforms- AKT1, AKT2 and AKT3. It is a key regulator of phosphatidylinositide 3-kinase- AKT-mammalian target of rapamycin signalling (PIK3-AKT-mTOR) (Yap, Yan et al. 2011) with PIK3-dependent activation which results in increased cellular survival, proliferation, growth and metabolism (Engelman 2009). Hyperactivation of this pathway is a major driver of malignant progression but can occur through a number of different mechanisms, such as upstream stimulation by receptor tyrosine kinases, PIK3CA or AKT mutations and loss of phosphatase and tensin homolog (PTEN) function. In recent years, multiple targets have been developed to target critical components of this pathway. Several classes of small molecule AKT inhibitors have been developed with various potencies and specificities for the varying isoforms of AKT. There are both allosteric and ATP-site competitive forms of inhibitor, however ATP competitive forms have been reported to have a higher likelihood of off-target effects (DeFeo-Jones, Barnett et al. 2005). MK-2206 is an oral allosteric AKT inhibitor which inhibits phosphorylation at both Thr308 and Ser473 residues of AKT. It has in-vitro and in-vivo anti-tumour activity as a single agent and enhances the preclinical activity of conventional cytotoxics and other molecular targeted therapies (Hirai, Sootome et al. 2010).

1.5 *Rationale for Project*

It is evident that unique features of tumours can be exploited by targeted therapy. It is a field of research which is constantly expanding and the concept of synthetically lethal interactions appears to be a promising method to selectively kill tumour cells.

Within this project, the aim is to identify tumour suppressor genes, whose loss synergises with inhibitors of aforementioned kinases- Src and AKT. This will be undertaken with the use of a siRNA library which individually knocks down 178 tumour suppressor genes that are known to be disrupted in cancer. For example, if BRCA1 or BRCA2 knockdown enhanced the effect of the drug, it would be reasonable to recruit patients who have BRCA-associated breast or ovarian cancer for a clinical trial. Similarly, if knockdown of MSH2, MSH6 and MLH1 resulted in an enhanced response, it would be reasonable to recruit patients who have cancers such as colorectal cancer, ovarian cancer or endometrial cancer, where tumours possess mismatch repair defects.

This project aims to identify patient populations which would benefit from novel therapeutic agents in the clinical setting- either as a monotherapy or in combination with other agents due to the unique genetic makeup of the tumours that the therapeutics target. This would in theory reduce the number of patients receiving chemotherapeutic agents which they may not benefit from, thus reducing the extent of the side effects seen with current treatment.

Chapter II: Material and Methods

All materials are sourced from Sigma-Aldrick UK (Poole, Dorset) unless stated otherwise. AZD0530 (AstraZeneca, Wilmington, DE) is an orally active, highly selective, small-molecule dual Scr/Abl kinase inhibitor. It exerts its activity through ATP competitive and reversible inhibition of the target enzyme. MK2206 (Merck, US) is a potent non-ATP competitive allosteric Akt inhibitor.

2.0 *Human Cell Supply*

Human Foreskin Fibroblast cells (HFF) were obtained from ATCC and cultured in DMEM Growth Media (PAA Laboratories Gmbh, Austria). In addition, media was supplemented with 500µl Amphotericin-B and 10% Foetal Bovine Serum.

2.1 *Cell culture and Subcultivation*

All cells were cultured in a humidified incubator at 37˚C and 5% CO_2 (Sanyo Electric Co, Davidson and Hardy Ltd, UK) in T75 flasks (Nunc™, Roslike, Denmark). Medium was replaced aseptically every 2-3 days in a class II microbiological safety cabinet with modified laminar air flow, sprayed before and after use with 70% ethanol. Cells were sub-cultured every 4-5 days when ~70-90% confluent. After aspiration of medium, flasks were washed with sterile phosphate-buffered saline (PBS). This solution was aspirated and 3mls of Trypsin in PBS was added. Incubation for 2 minutes resulted in cell detachment. An equal volume of growth medium was added to neutralise trypsin. The resultant cell suspension was pipetted up and down to generate a single cell suspension and then divided between new culture flasks at a ratio of 1:2 or 1:3 according to cell density and rate of growth. Additional growth medium is added to bring the final volume to 20ml per 75cm^3 flask. Cells are placed in the humidified incubation.

2.2 Cell Density Optimisation

Cells were seeded onto 96 well plates at a concentration of 2000 cells per well and 4000 cells per well to assess confluency after 72 hours. This was carried out in normal growth media. It was found that 2000 cells per well was an appropriate concentration for further experimentation.

2.3 *Drug Optimisation*

In order to determine an IC 30 concentration of the SRC inhibitor and AKT inhibitor on the HFF cells, differing concentrations of each drug were plated onto triplicate wells within a 96 well plate onto previously adhered cells at 2000 cells/well.

The plates are returned to the incubator for a further 72 hours at which point a cell proliferation assay was performed.

2.4 *Cell Proliferation Assay*

Cell proliferation assays were carried out using the CellTitre Glo Luminiscent Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, the media in the wells was replaced with 100µl of normal growth media and allowed to equilibrate to room temperature for 30 minutes. 75µl of CellTitre Glo was then added to each well and the plate placed on a plate agitator for 2 minutes to shake and then left to stand for 10 minutes before being read.

The luminescent signal produced by the CellTitre Glo was then read using BioTek apparatus (BioTeck Instruments Inc, USA) and Gen 5.1.11 software and the results were used to produce a dose response curve to determine the inhibitory concentration of 30% of the cells. The dose response curve was drawn using graphpad-3. Data was plotted in the Prism Software and a line graph was created with error bars of n=3. From this software, the IC30 of the drug could be determined.

2.5 *Western Blot*

Sample buffer	4µl of 4x NuPage LDS Sample Buffer 1µl of 10x NuPage Sample Reducing Agent
Transfer buffer	50ml NuPage Transfer Buffer 200ml Methanol 750ml H_2O

2.5.1 *Protein Extraction*

Medium was aspirated and cells washed with ice-cold PBS. 200µl of RIPA buffer (1% v/v Triton X-100, 0.1% v/v sodium dodecyl sulphate and protease inhibitors in PBS) was then added. A cell scraper was then used to detach the cells from the surface of the plate. The resultant lysate was transferred into a 1.5ml eppendorf tube and placed on ice for 15 minutes. The lysate was subsequently centrifuged at 1400 rpm for 15 minutes at 4˚C. The supernatant containing the protein was then removed and added to a fresh eppendorf tube.

2.5.2 *Protein Quantification*

Protein standards were produced from a BCA Total Protein Assay Kit by serial dilution of albumin standards. A volume of 10µl of the first standard was put in row A1 and row B1 in duplicate and so forth for each of the standard in ascending order in a 96-well clear bottomed plate. The samples are then added to the plate in the same fashion starting at C1. 200µl of Protein Assay Reagent is then added to each well (50 parts of Reagent A to 1part of Reagent B). Incubate for 30 minutes at 37˚C. Using the Gen5 software, the plate is read and the protein quantified using a calibration graph.

2.5.3 Electrophoresis

Approximately 50µg of each protein is transferred to eppendorf tubes, to which RIPA (Radio Immuno Precipitation Assay) buffer and NuPage LDS sample buffer (Invitrogen Ltd, Paisley, UK) was added. The sample is then centrifuged for 10 seconds at 4˚C and placed on a heating block at 70˚C for 6 minutes. The samples were then placed on ice.

Using a 4-12% Bis-Tris Gel plate (Invitrogen Ltd, Paisley, UK), 8µl of SeeBlue Plus 2 Prestained standard (Invitrogen Ltd, Paisley, UK) is loaded into well 1. The following samples were loaded making note of their position in the gel using gel loading tips at a volume of 25µl. The running buffer is added to the outer and inner chambers of the bath. The gel is then run at 200V for 35 minutes.

2.5.4 *Transfer*

The proteins are then transferred onto transfer membrane. The transfer membrane must also be activated with methanol before being soaked in Transfer Buffer along with 6 sponges and filter paper. The transfer membrane is placed next to the gel and the two are sandwiched between sponges and filter paper in the fashion shown below in figure 3.

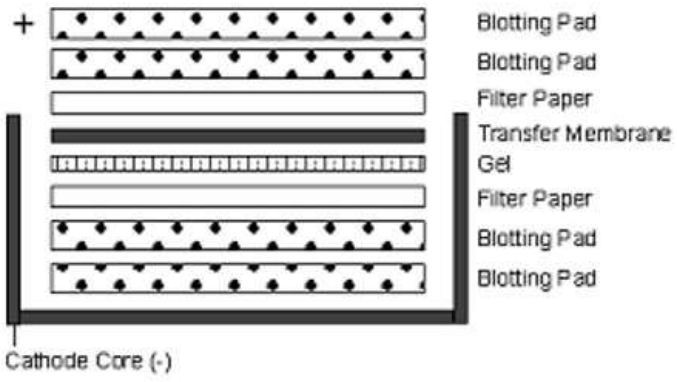


Figure 3. Set up of unit for protein transfer.

Transfer buffer was added to the bath and the electrophoresis power pack set for 90 minutes at 30V. Following this, the membrane must be blocked to prevent non-specific binding of antibodies in

future steps. Blocking solution is made up of 5% Marvel dried milk in Tris Buffered Saline (TBS) Tween.

10mls of blocking solution surrounds the membrane in a dish and is placed on a rocker for agitation (Stuart mini see saw rocker SSM4) for 60 minutes. The membrane is then washed twice for 5 seconds in TBS-T. A pre-determined dilution of 1:1000 of primary antibody was added to the membrane which is placed on a rocker in the cold room overnight. The following morning the membrane is rinsed 3 times in TBS-T for 5 minutes. A dilution of 1:2000 of secondary antibody was added to the dish, made up with 7.5mls of Marvel dried milk and 1.5µl of secondary antibody. Place on the rocker for 90 minutes.

After this, the membrane was washed 5 times for 5 minutes each in TBS-T. In order for the protein to be detected, a chemiluminiscent detection agent must be added to the membrane. 2mls of Novex ECL (Invitrogen) is added to the membrane placed on acetate for 5 minutes before the membrane is transferred onto fresh acetate in a cassette and brought to the dark room. Here a piece of autoradiography film is set on top of the membrane within the cassette and closed firmly. The film is developed using an automatic developer system machine (AFP Imaging Ltd).

2.6 *siRNA Transfection Optimisation and Method*

2.6.1 *Gene optimisation*

In order to determine suitable positive and negative controls for internal validation of the screen, 6µL of 0.2µM control siRNAs were placed in triplicate wells of a 96 well plate as shown in figure 4. A validated positive control was required and AllStars (Qiagen, USA) scrambled sequence was chosen. Likewise, a validated negative control was also required- PLK1 (Qiagen, USA). Suitable negative controls were also required for each of the drugs being tested and these are demonstrated in figure 4.

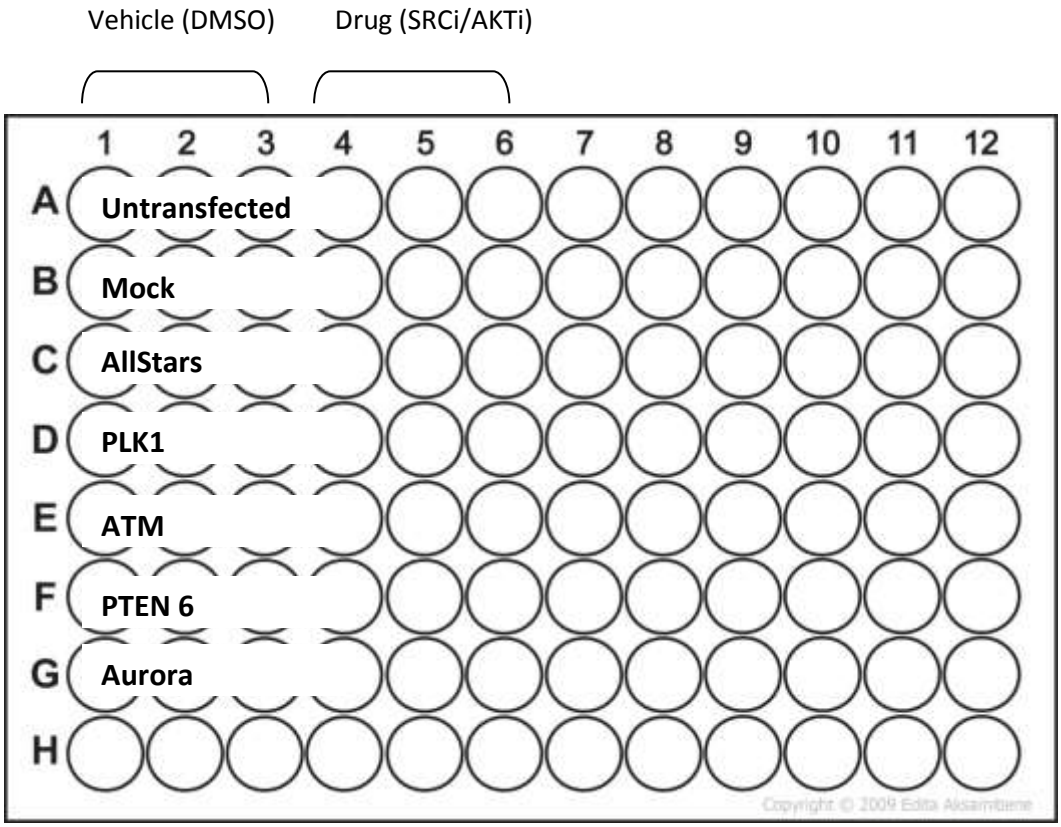


Figure 4. set up of siRNA positive and negative controls within 96 well plate

siRNAs were reverse transfected using Lipofectamine RNAiMax Reagent (Invitrogen) diluted in Opti-MEM media (Invitrogen) at a 1:1000 dilution as per the manufacturer's instructions. 48.5µl of this solution was added to each of the wells containing siRNA and left to complex for 20 minutes.

To each of the wells, HFF cells were added at a concentration of 2000 cells per well with thorough pipetting to ensure a homogenous mixture. The plate was returned to the incubator for 24 hours. The final concentration was approximately 10Nm.

After 24 hours, the original media was removed from the wells. Into the first three wells for each siRNA as depicted in figure 2.2, 100µl of a solution containing 10µM of DMSO in DMEM media was added. Into the following three wells, a solution containing 10µM AZD0530 was added. For

MK2206 the concentration of both drug and DMSO is 5µM. The plates are returned to the incubator for 72 hours, at which point a cell proliferation assay is performed as set out in section 2.4.

2.6.2 *siRNA Screen*

The siRNA library was stored at an initial concentration of 2µM and therefore required aliquoting to a working concentration of 0.2µM. To each well, 6µl of siRNA was reverse transfected using 48.5µl of the Lipofectamine RNAiMax Reagent and Opti-MEM solution as per the manufacturer's instructions. Within each of the 27 plates, rows 1 and 12 are left empty to facilitate the siRNA negative and siRNA positive controls for internal validation of the experiment. For this screen, AllStars (Qiagen) and Polo-Like Kinase 1 (PLK1) were used with PTEN 6, ATM and Aurora and were placed in the wells highlighted by the green box in figure 5. The plate was left to complex for 20 minutes. To each of the wells, HFF cells are added at a concentration of 2000 cells per 100µl with thorough pipetting to ensure a homogenous mixture. Each of the 27 plates was returned to the incubator for 24 hours.

After 24 hours, the original media was removed from each of the wells. For the first 9 replicate plates, 100µl of 10µM dimethylsulphoxide (DMSO) in DMEM media was added to each of the wells. For the second 9 replicate plates of the same siRNA, 10µM of AZD0530 in DMEM media was added and similarly for the third set of 9 replicate plates, 5µM of MK2206 replaced the original media. The plates are then returned to the incubator for a further 72 hours, after which, a cell proliferation assay is carried out on each of the plates, as outlined in section 2.4.

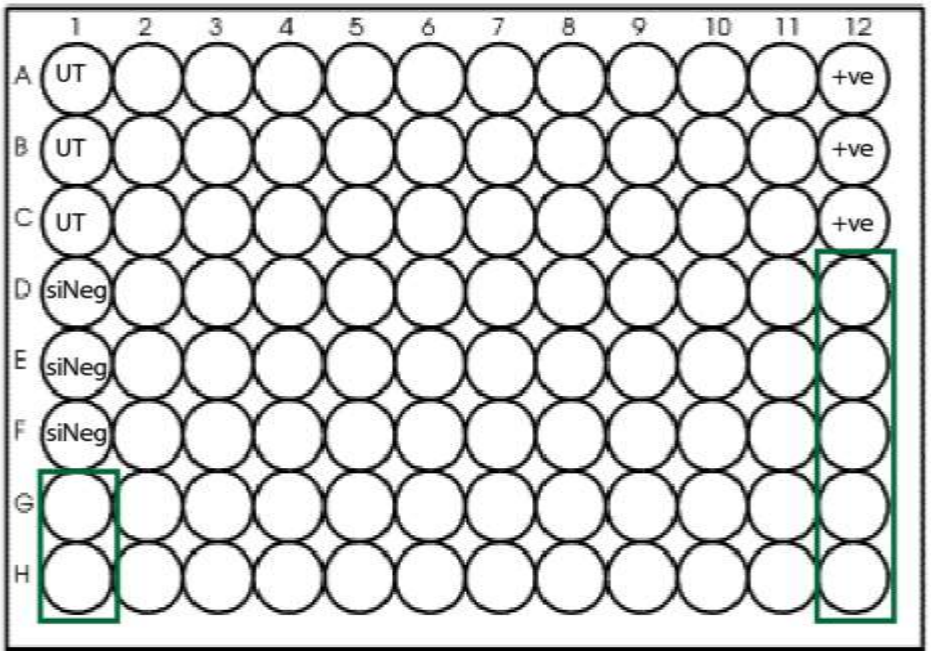


Figure 5. The layout of control siRNAs in rows 1 and 12 of the 96 well plate.

2.7 *Data Analysis*

Results were analysed in GraphPad Prism v.5 (GraphPad Software Inc, Ca, USA). Data was presented as mean ± SEM when appropriate with n indicating the number of measurements. A dose response curve for each of the drugs was drawn and thus the IC30 of each drug was determined from this.

The survival fraction of each of the cells was calculated by dividing the cell viability for each of the given drugs in turn by the cell viability of the vehicle treated cells. Statistical comparisons of the siRNA Screen was made using Robust Z-Scores from the data which was normalised to the overall average of Scrambled siRNA for all of the 27 plates. Initially the log 2 ratio for each of the siRNA was calculated. Potential hits were identified through the use of the Median Absolute Deviation of -1 or less for both sets of data. Data was eliminated from analysis if the mean growth of

the siRNA was less than 60% of the untransfected control well. These potential hits were plotted onto a scatter graph and genes which had more than one siRNA targeting them were noted.

Once the list of potential hits was determined, the gene list was inserted into DAVID; a bioinformatic functional analysis website, where functional annotation clustering was undertaken. This clustered the gene hits into the cellular processes, pathways and cellular compartments which they were involved in.

Chapter III: Results

3.1 *Results of Drug Optimisation*

As indicated in Figure 6, HFF cells at 2000cells/100µL were treated with 7 differing concentrations of AZD0530 for a 72 hour period before analysis with Cell Titre Glo Assay. These were 20uM, 10uM, 7.5uM, 5uM, 2.5uM, 1uM and 0.5uM of AZD0530. This was analysed at n=3 creating error bars and the concentration which produced an IC 30 was calculated using GraphPad Prism.

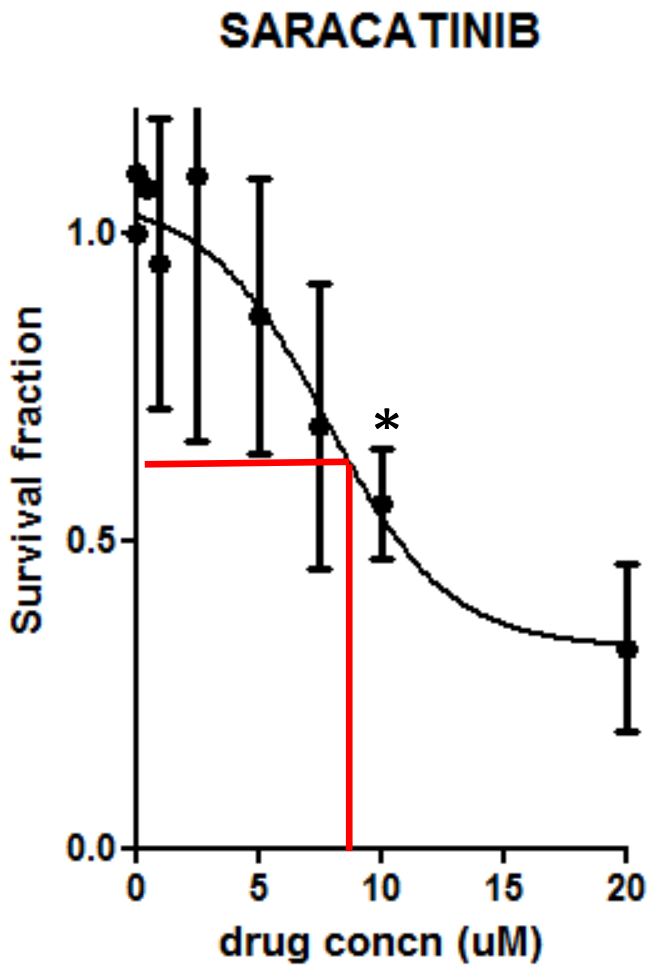


Figure 6. Survival fraction of HFF cells 72hrs following treatment with differing concentrations of AZD0530. * shows IC30 calculated at 9.41uM AZD0530.

To confirm the efficacy of the concentration found to produce an IC30 for the Src Inhibitor, a Western Blot was carried out to show that HFF cells treated with 10µM of AZD0530 produced a knockdown of protein expression, compared to an untreated sample and HFF cells treated with 10µM DMSO. This is shown in figure 7 below using Phospho-FAK (Tyr925) Antibody. The identity of the protein monomer was confirmed by the use of molecular weight standards. Beta-Actin was used as a loading control to show equal loading between each of the wells in the blot.

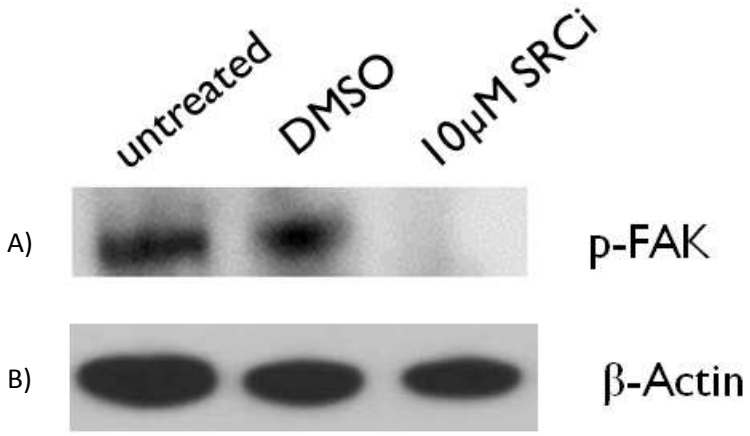


Figure 7. Immunoblot for 10uM AZD0530 A) Protein expression in HFF cells which are untreated, HFF cells treated with 10uM DMSO and 10uM AZD0530. B) β-Actin showing equal loading.

As shown in figure 8, HFF cells, also at 2000cells per 100µL were treated with differing 7 differing concentrations of MK2206 for a 72 hour period before analysis with Cell Titre Glo Proliferation Assay. These were 10uM, 7.5uM, 5uM, 2.5uM, 1uM, 0.5uM and 0.1uM of MK2206. This was also analysed at n=3, creating error bars and the concentration which produced an IC30 was also calculated using GraphPad Prism.

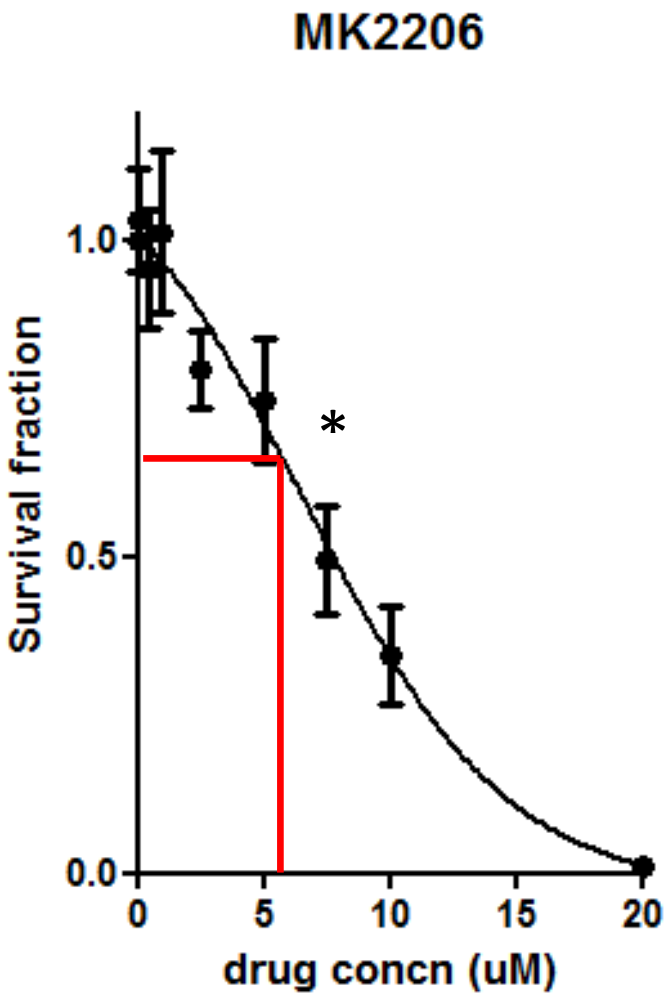


Figure 8. Survival fraction of HFF cells 72hrs following treatment with differing concentrations of MK2206. * refers to the IC30 calculated at 5.15uM MK2206

As with the Src inhibitor above, a Western Blot was also performed to show adequate protein knockdown with 5uM MK2206, compared with HFF cells which were untreated, and HFF cells which were treated with 5uM of DMSO. This is shown in figure 9 through the use of Phospho-GSK-3β (Ser9) antibody. The identity of the protein monomer was confirmed by the use of molecular weight standards. Beta-Actin was also used as a loading control to show equal loading between wells.

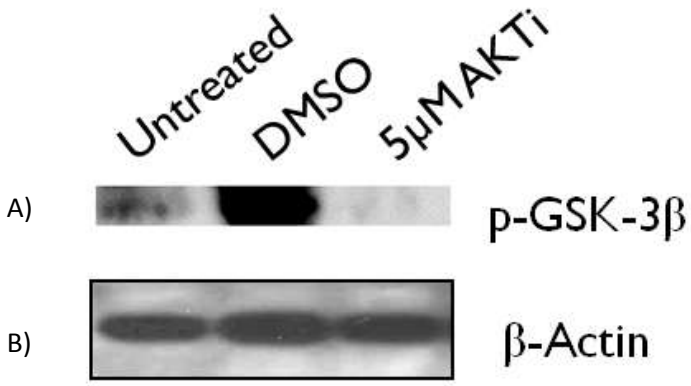


Figure 9. Immunoblot for 5uM MK2206 A) Protein expression in HFF cells which are untreated, HFF cells treated with 5μM DMSO and 5μM MK2206. B) β-Actin showing equal loading.

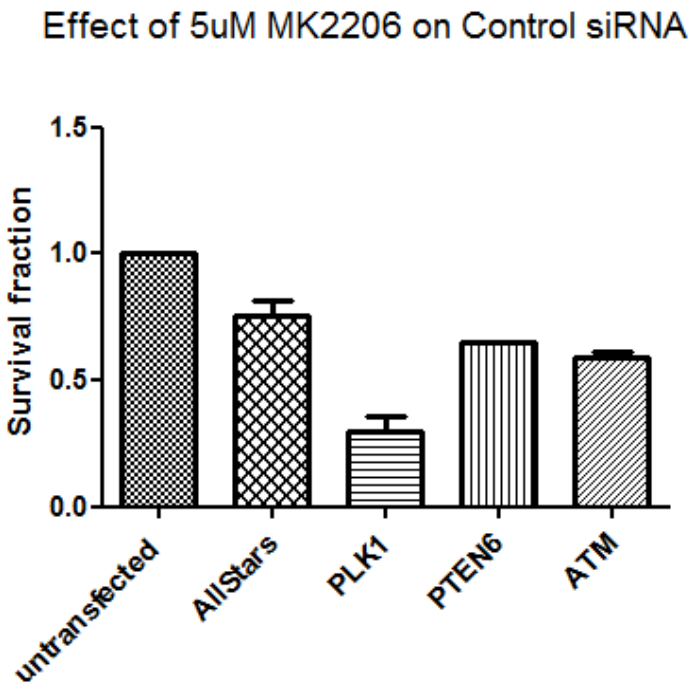
3.2 *High Throughput siRNA Screen*

3.2.1 *Screen Optimisation*

In order to assess reproducibility of the High Throughput Screen, it was imperative to determine control siRNAs for each of the drugs. A validated positive control, suitable for the entire screen was a scrambled siRNA such as AllStars Positive Control (Qiagen, USA), which was used to discount any changes to the gene expression profile which may have resulted from the siRNA delivery method. A validated negative control for the screen was PLK1 (Qiagen, USA), which was used to determine whether the experimental setup caused any non-specific effects. Similarly, negative controls must be determined for each of the drugs used in the screen. For the Akt inhibitor, these siRNA controls were found to be PTEN 6 and ATM, plotted in figure 10 at n=3, compared to the positive control, AllStars and negative control PLK1. The effect of cell survival was assessed in HFF cells treated with 5μM MK2206. Figure 10 shows that PLK1 has the desired effect with a survival fraction of 30%

For the Src Inhibitor, the siRNA control was found to be Aurora Kinase, which was also plotted at n=3, compared to AllStars and PLK1 in 10µM AZD0530.

A)



B)

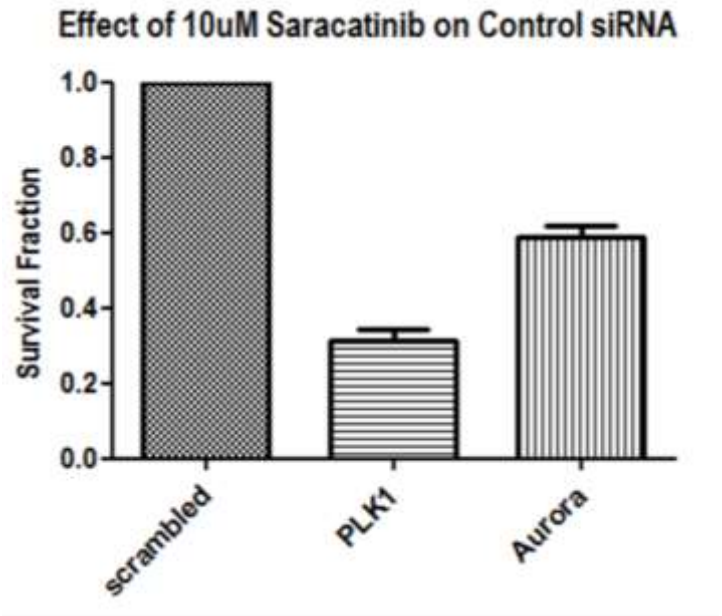
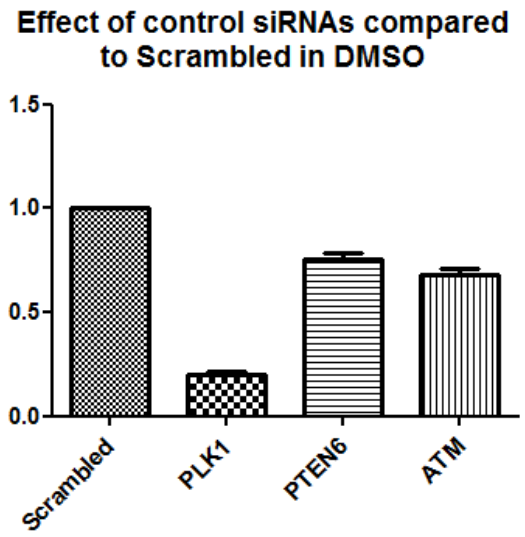


Figure 10. Survival Fraction of HFF cells treated with control siRNA over a 72 hr period in A) 5µM MK2206 and B) 10µM AZD0530

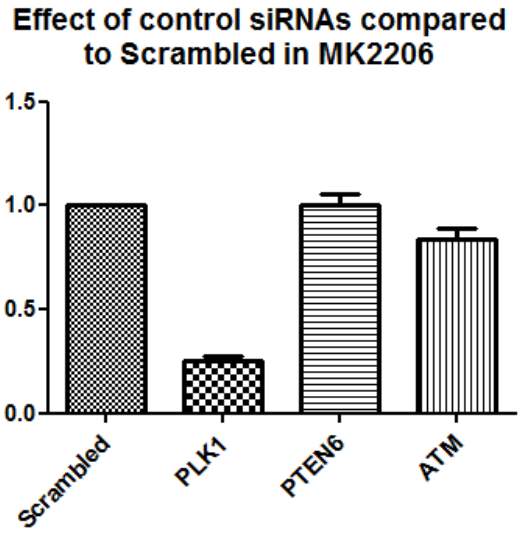
3.2.2 *Quality Control within Screen*

To determine whether the siRNA Screen was reproducible between the plates treated with DMSO, plates treated with 10µM AZD0530 and plates treated with 5µM MK2206. Initially the plates were analysed to show that the effect of the siRNA controls as discussed in section 3.2.1 were the same between each of the 9 plates. Error bars were plotted and for each of the controls it was found that there were all similar, with no siRNA controls having an extreme effect in any of the plates. This is shown in Figure 11 below.

A)



B)



C)

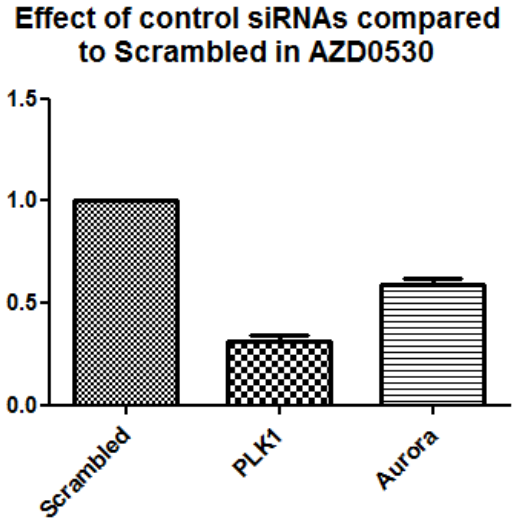
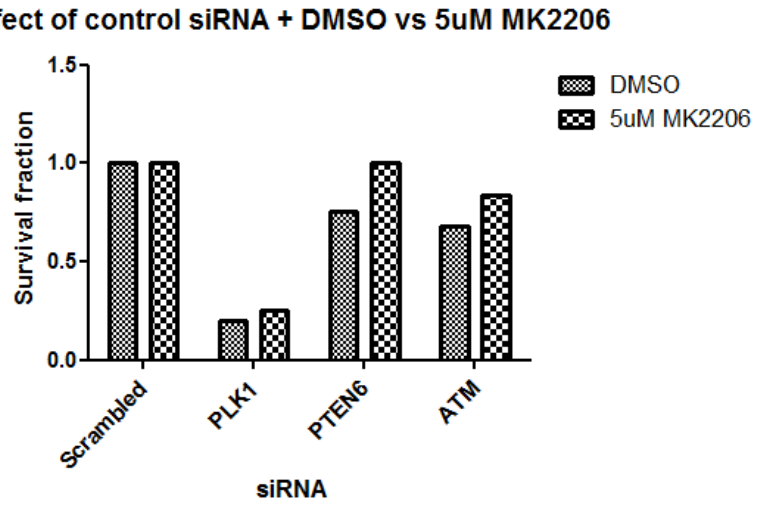


Figure 1. Effect of Control siRNAs compared to Allstars in A) HFF cells treated with DMSO, B) HFF cells treated with 5µM MK2206 and C) HFF cells treated with 10µM AZD0530.

The efficiency of the control siRNAs were then compared between drug and DMSO. It was found when 5µM DMSO was compared to 5µM MK2206, the survival fraction was greater than in the HFF cells treated with DMSO. This is shown below in Figure 12 and suggests that the control siRNAs were not having the desire effect within the screen. Similarly, when HFF cells treated with 10µM DMSO compared to HFF cells treated with 10µM ADZ0530, the survival fraction was greater in those which had been treated with the drug.

A)



B)

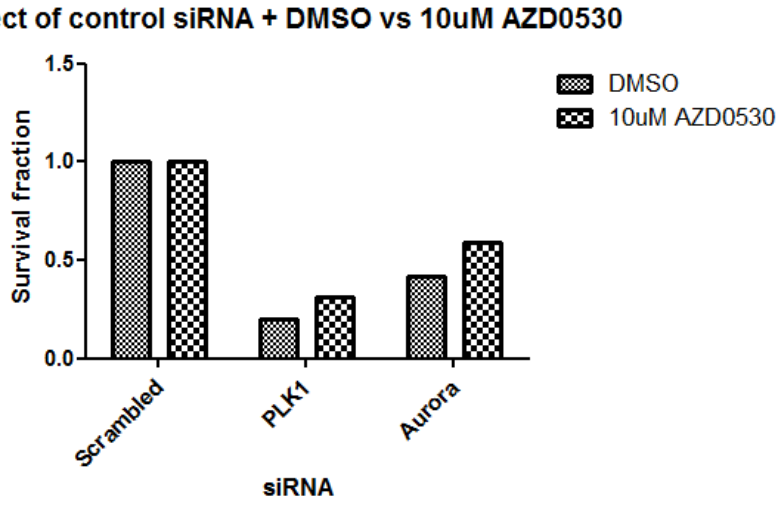
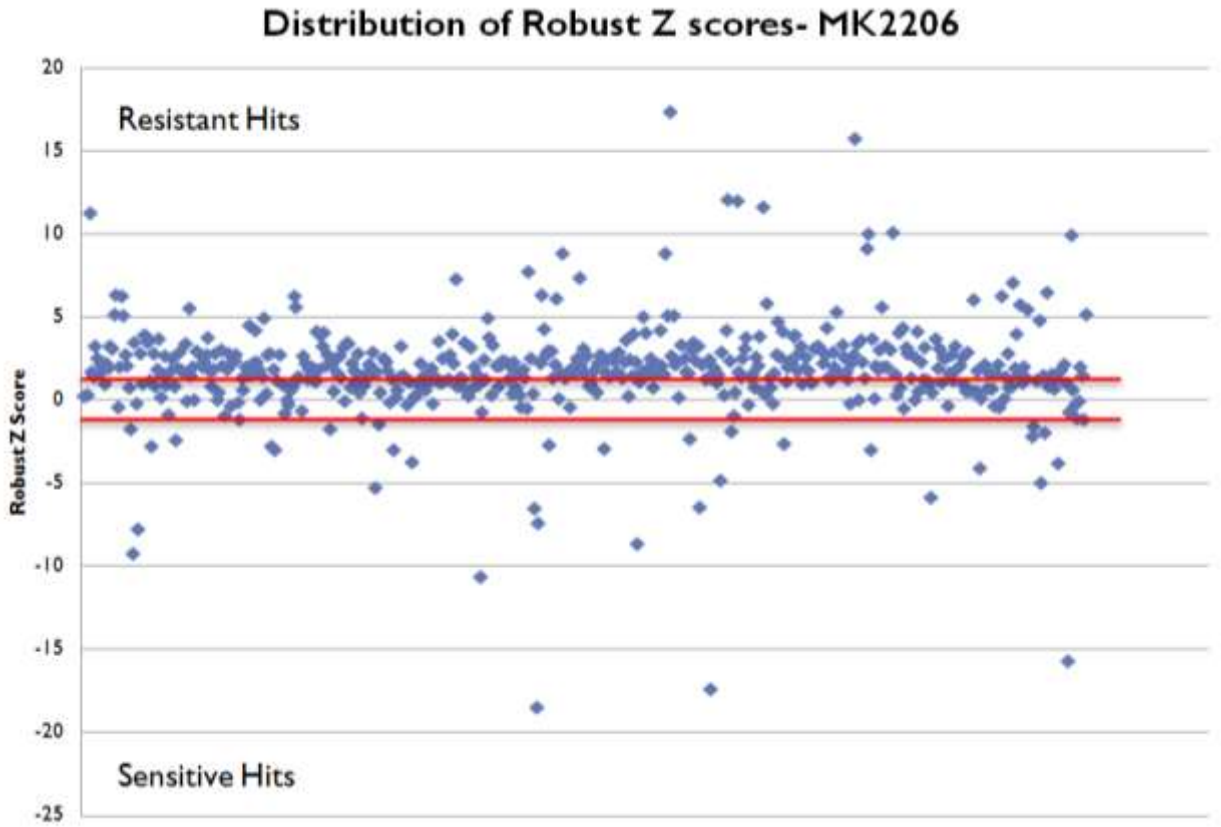


Figure 12. Effect of control siRNA on survival fraction of A) HFF cells treated with 5µM MK2206 compared to 5µM DMSO and B) HFF cells treated with 10µM AZD0530 compared to 10µM DMSO

3.2.3 *Result of siRNA Screen*

Following normalisation to scrambled, the data was used to determine Robust Z-Scores for each individual siRNA by dividing the median absolute deviation into the Log_2 Ratio. The robust Z scores were plotted on scatter graphs to show the distribution of the data, and visually represent which siRNA could be potential hits within the screen. This can be seen below in figure 13 for HFF cells treated with 5µM MK2206 and 10µM AZD0530. The data which lies above 0 are potential hits which show resistance to the Src Inhibitor or Akt Inhibitor. The data which lies below 0 are potential sensitive hits to each of the drugs.

A)



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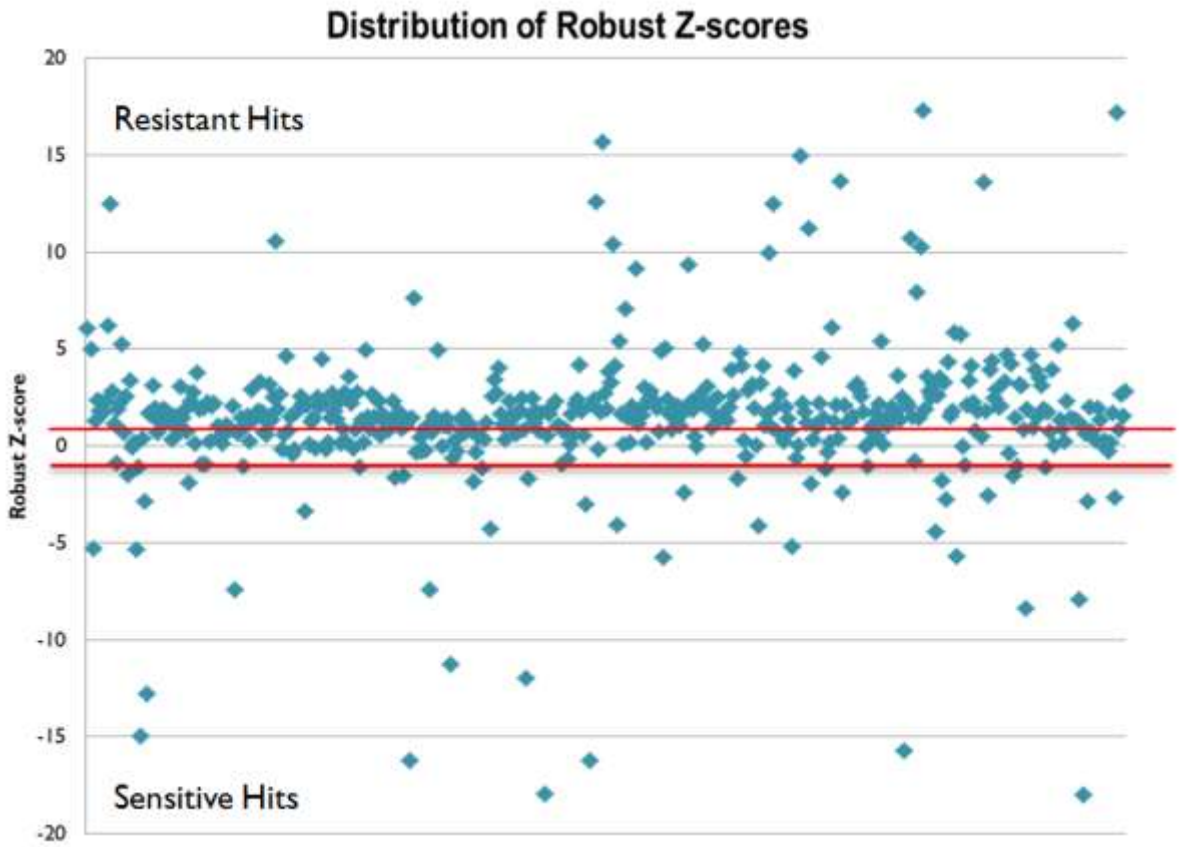
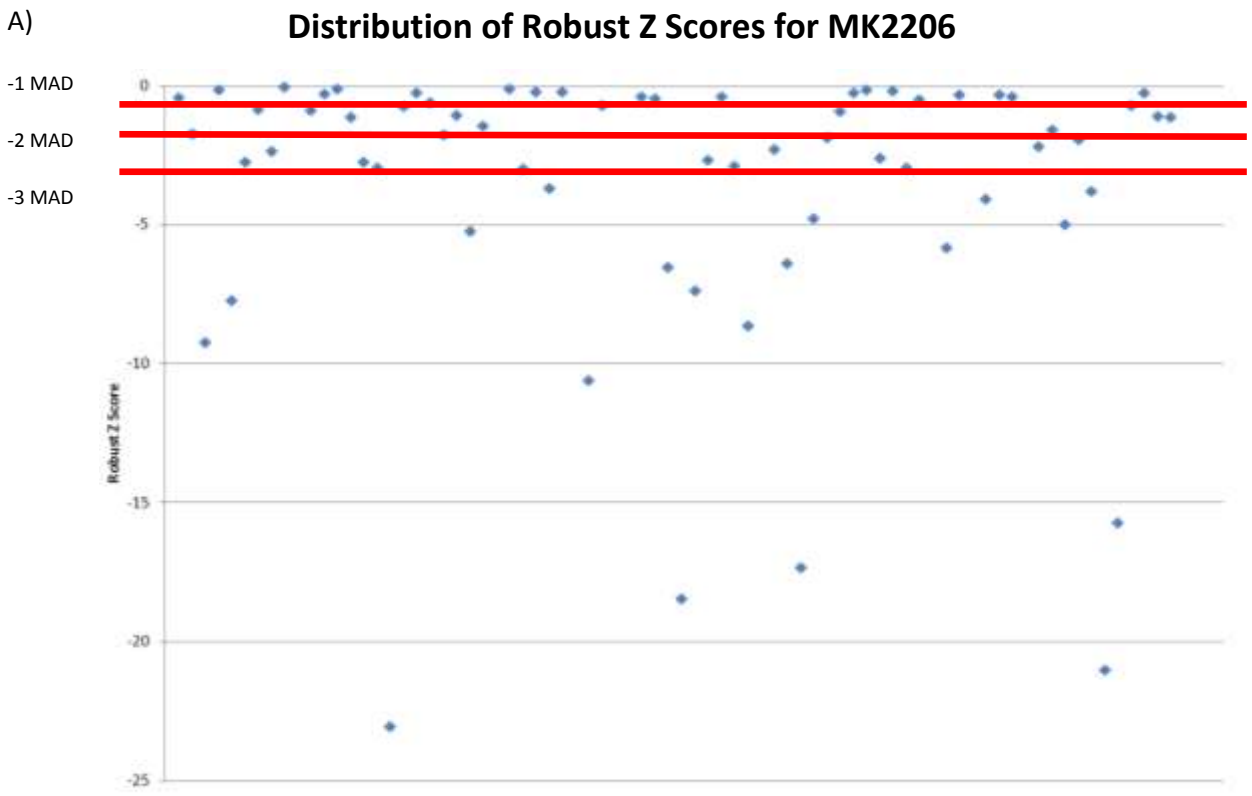


Figure 13. Distribution of Robust Z scores for A) HFF cells + siRNA treated with 5µM MK2206 and B) HFF cells + siRNA treated with 10µM AZD0530.

The Median absolute deviation was calculated, initially taking -3 as the cut off for potential hits. This was found to be too stringent for both the robust Z-Scores of the Src and Akt Inhibitor, therefore the cut off was set at -1 from MAD. This is shown below in figure 14.

A)



B)

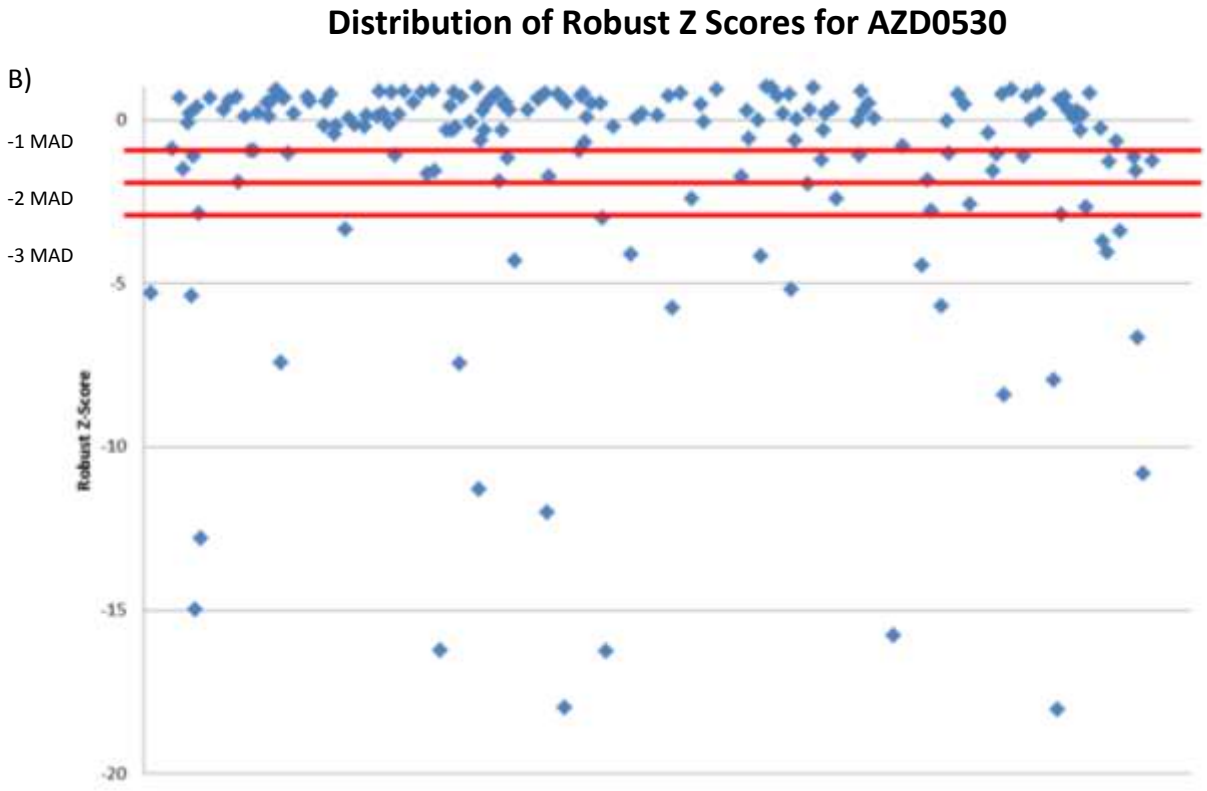
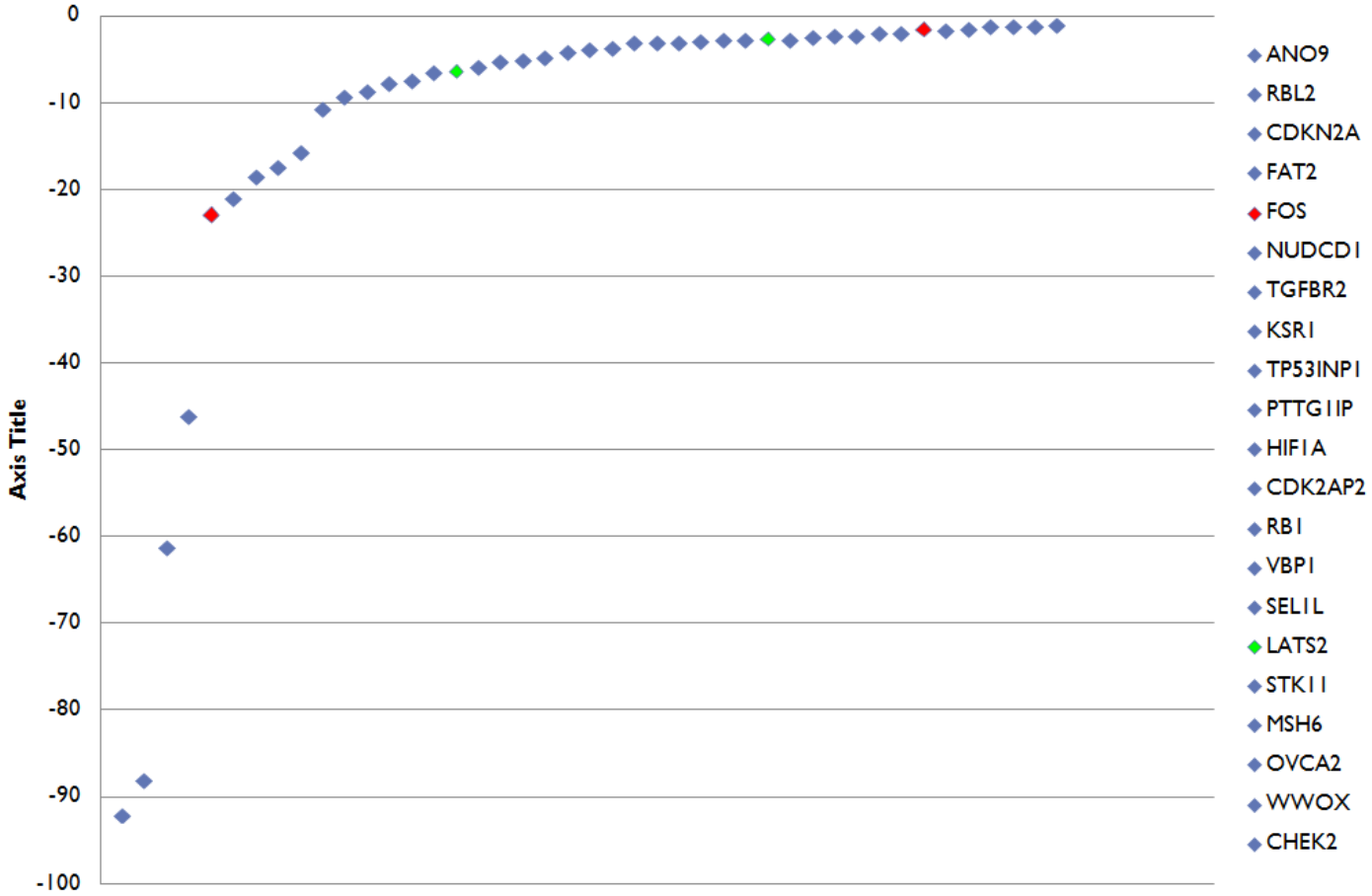


Figure 14. Distribution of Robust Z Scores of A) HFF cells treated with 5µM MK2206 with Median Absolute Deviations and B) HFF cells treated with 10µM AZD0530 with Median Absolute Deviations.

Taking –3 from MAD as the cut-off for potential hit sensitive hits in the screen, those siRNAs which fell below this point were plotted on a scatter graph. Figure 15 shows the distribution of potential hits from HFF cells treated with MK2206 and HFF cells treated with AZD0530. For those cells treated with 5µM MK2206, potentials hits are plotted, and those which have been highlighted in green and red are genes which have been targeted multiple times. For those cells treated with 10µM, more genes were targeted multiple times; therefore the distribution is only representative of those tumour suppressor genes which have been 'hit' by more than one siRNA for that specific gene.

A) Distribution of Robust Z-Score for Potential Hits- MK2206



B) Distribution of Robust Z-Score for Potential Hits- AZD0530

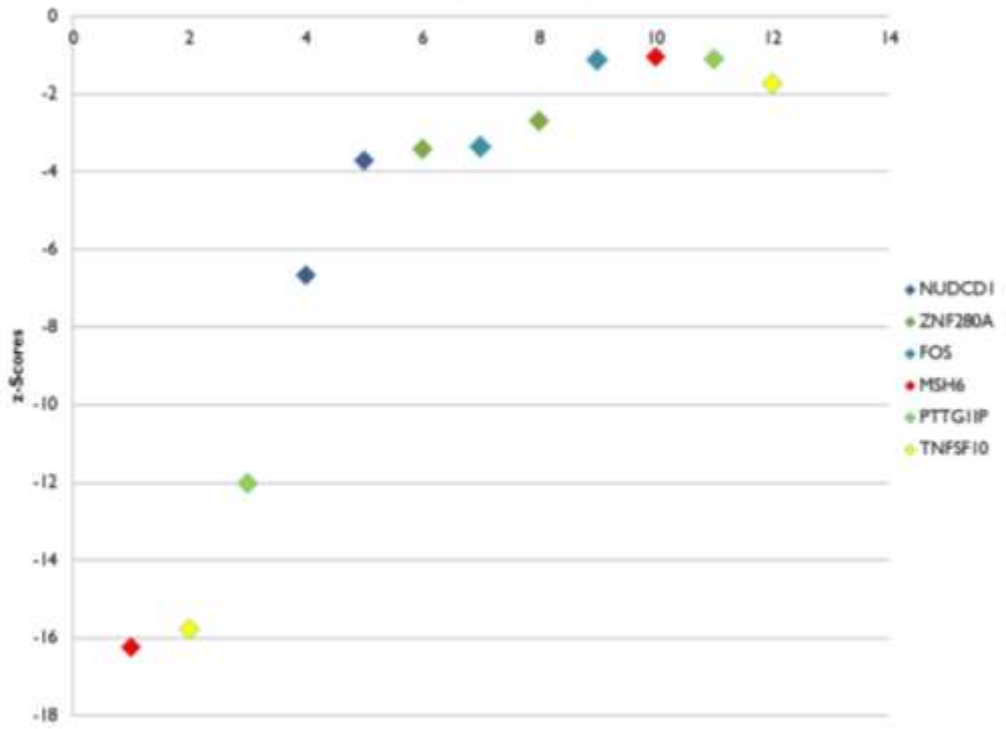
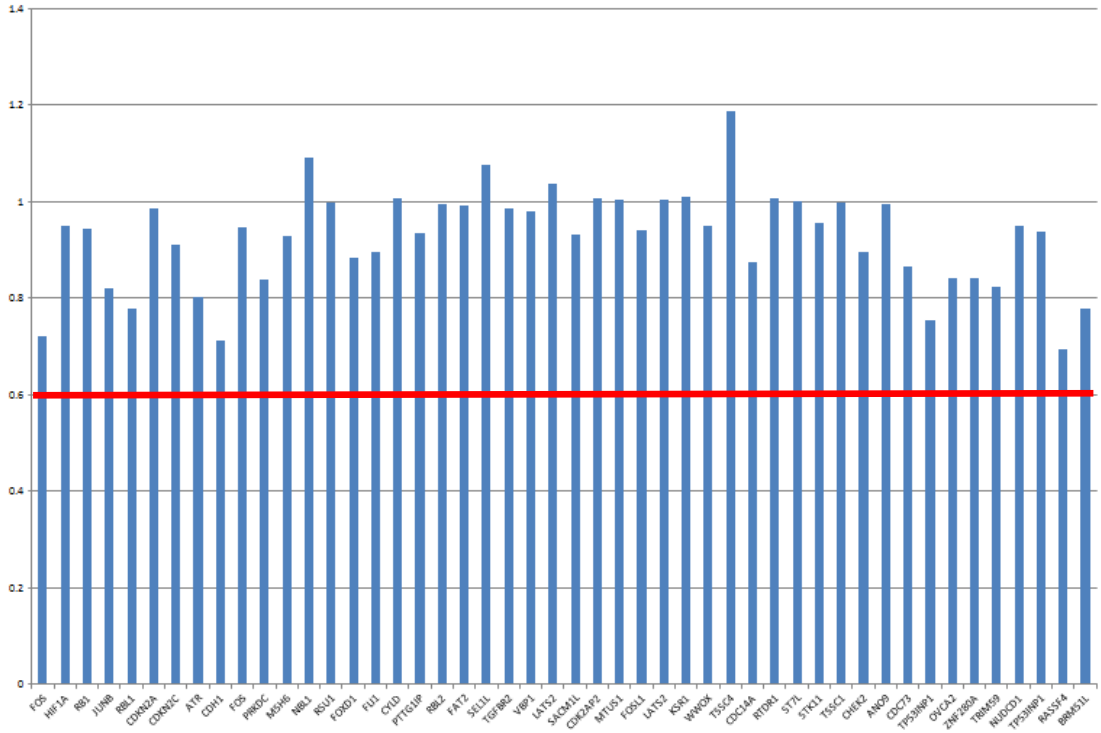


Figure 15. Distribution of Robust Z-Scores for Potential Hits for A) HFF cells + siRNA treated with 5µM MK2206 and B) HFF cells + siRNA treated with 10µM AZD0530

To ensure that none of the data which was found to be a potential hit, was influenced by siRNAs which were toxic, they effect of growth of that specific siRNA in DMSO was analysed. This is shown in figure 16 where those potential hits which were -1 MAD were plotted from the results of the siRNA treated in DMSO. Those which had an effect on growth of greater than 40% would have been excluded from further analysis, but this did not occur and none of the siRNAs were deemed to be toxic for either of the drugs.

Effect of siRNA + DMSO on growth: potential hits for MK2206



Effect of siRNA + DMSO on growth: potential hits for AZD0530

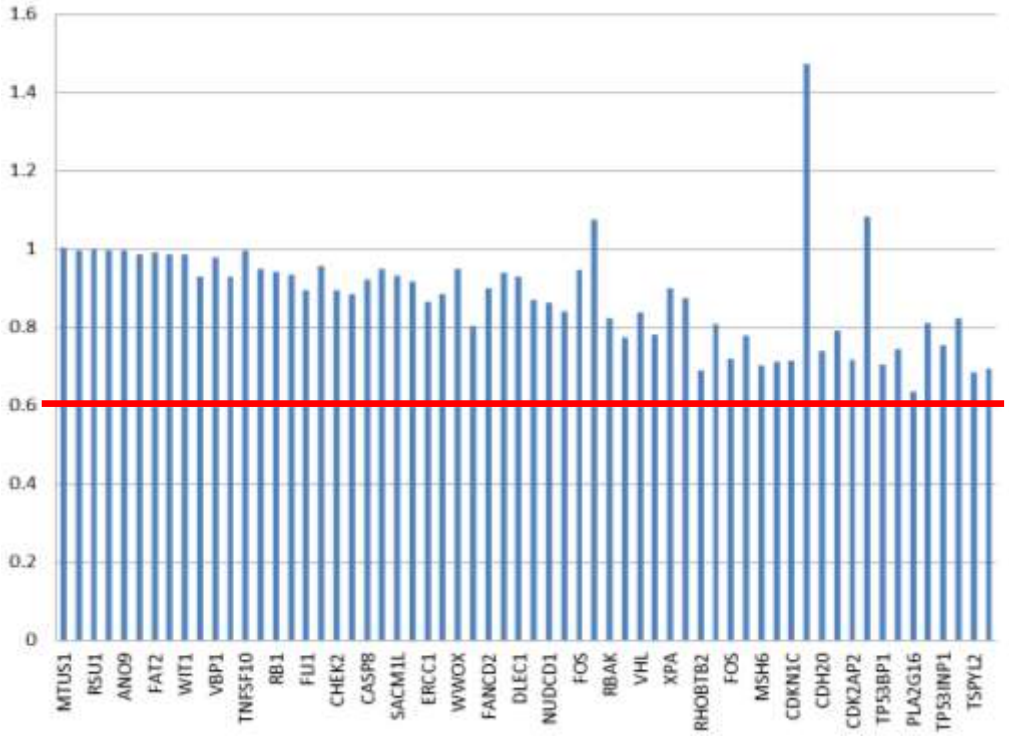


Figure 16. Effect of siRNA + DMSO on growth in A) genes which are potential hits in MK2206 and B) genes which are potential hits in AZD0530. The red line depicts the cut off for toxic siRNA

For those siRNAs which were treated with MK2206, there were 45 'on-target' hits, however only two of these had two or more individual siRNAs targeting the same gene sensitised to MK2206. These were FBH murine osteosarcoma viral oncogene homolog (FOS) and Large tumour suppressor, homolog 2 (LATS2).

For those siRNAs which were treated with AZD0530, there were 61 'on-target' hits of which 6 tumour suppressor genes had two or more individual siRNAs targeting them. These were NudC domain containing 1 (NUDCD1), Zinc finger protein 280A (ZNF280A), FBH murine osteosarcoma viral oncogene homolog (FOS), MutS homolog 6 (MSH6), Pituitary transforming gene 11P (PTTG11P) and Tumour necrosis factor (ligand) superfamily, member 10.

A full list of the genes identified in each of the screens can be found in Appendix B.

3.3 *Functional Analysis*

Once all the potential tumour suppressor genes, which were shown to be sensitive to the Src or Akt Inhibitor were determined from the screen, were noted, the gene list was exported to DAVID; a Bioinformatic Functional Analysis Website. This website clustered the genetic hits into the cellular processes, pathways and compartments which they were involved in.

For the tumour suppressor genes which were found to be sensitive to MK2206, there were several pathways which the genes were involved in. This included regulation of apoptosis, cell cycle checkpoint, regulation of transcription and pathways in cancer.

DAVID analysis for those tumour suppressor genes which were found to be sensitive to AZD0530, there were also similar pathways between the genes involved. This included response to stress, cell cycle regulation, regulation of apoptosis and regulation of transcription.

Chapter IV: Discussion

Chemotherapeutic agents remain the mainstay of treatment in cancer. Standard chemotherapeutic agents were discovered on the basis that they were capable of killing rapidly dividing cells, however, this attributed to many adverse effects such as hair loss, nausea and immunosuppression due to their toxicity. Therefore, the aim of modern research has turned its focus to identifying targeted therapies which would be more effective and cause fewer adverse effects by traits that are specific to the tumour or to the patient.

There is a wealth of literature surrounding the role of Src and Akt in tumour progression, invasion and metastasis. Therefore these kinases have become an attractive target for drug development {{224 Puls,L.N. 2011}}. Different classes of inhibitors for both kinases have been developed and are currently undergoing early phase I and phase II clinical trials. The results thus far have been somewhat disappointing when these inhibitors have been administered as single agents {{250 Pal,S.K. 2010}}. However, some patients have shown an excellent response to these drugs. Pharmaceutical industries therefore wish to determine biomarkers which determine sensitivity to each of these drugs, as a patient population could be identified in which benefit would be seen.

This study was designed to identify such biomarkers of sensitivity to two novel therapeutic agents- a Src Inhibitor; AZD0530 (AstraZeneca) and an Akt Inhibitor; MK2206 (Merck). The concentration of each of these drugs was optimised for the screen to produce an Inhibitory Concentration (IC) of 30%. The IC30 was found to be 10µM for the AZD0530 and 5µM for MK2206. This concentration showed that the drug was having an effect on the HFF cells, but any further effect caused by sensitivity of the drug to any of the tumour suppressor genes within the library would be evident in the screen. Optimisation of cell number was also carried out and it was found that 2000cells/100µL produced an adequate confluency for the screen.

The aim of the high throughput siRNA screen was to identify genes which are important in a pathway context and thus can provide a better understanding of the processes and biology behind

the interactions between the pathways and the chemotherapeutic agents which appear to sensitise these tumour suppressor genes.

For those HFF cells + siRNA which were treated with MK2206, it was found that 45 hits were potentially 'on-target' inferring sensitivity to this Akt Inhibitor. A full list of these genes can be found in the appendix. In order to determine which genes were sensitive, robust Z-scores were found and the median absolute deviation was used to separate the resistant hits from the sensitive ones. Of note, those genes which were found to be potential hits within the gene, only 2 were targeted by multiple siRNAs. The screen in itself was not too stringent as the cut off for resistant hits was set at -1 MAD. Therefore functional analysis was carried out on any potential hits at this value, even if they had only been targeted once. It is likely that those genes which were targeted by multiple siRNAs would be more likely to be sensitive to MK2206 than those which were only targeted once.

One such tumour suppressor gene which was found to be sensitive to MK2206 was cyclin dependent kinase inhibitor-2A (CDKN-2A). This is also known as P16 and plays an important role in cell cycle regulation. The progression of cells from G1 to S phase is under the control of the p16-Rb pathway {{251 Rayess,H. 2012}}. Current literature suggests that this gene is mutated or lost in approximately 20% of solid tumours {{252 Negrini,S. 2010}}. The results of the high throughput screen are suggestive that MK2206 may interfere with cell cycle progression in those cells which are deficient in the G1 checkpoint due to mutation or loss of this tumour suppressor gene. Therefore this could be used as a biomarker of response to Akt Inhibitors in patients who are deficient in this gene.

For those HFF cells + siRNA which were treated with AZD0530, it was found that 61 hits were potentially 'on-target' inferring sensitivity to this Src Inhibitor. A full list of these genes can also be found in the appendix. As above, robust Z scores were found and median absolute deviation was used to separate the resistant hits from the sensitive hits. As in the screen for cells treated with

MK2206, the cut off for potential hits was set a 1 MAD from the median, lessening the stringency of the screen. Within these 61 tumour suppressor genes, 6 were targeted by multiple siRNAs. It is likely that the 6 tumour suppressor genes, targeted by multiple siRNAs would be more likely to be sensitive to AZD0530 than those which have only been targeted by one siRNA.

Two tumour suppressor genes in particular were significant within the screen for those cells + siRNA treated with AZD0530. Both of these genes are known to be associated with the Fanconi anaemia pathway; a pathway essential for the repair of DNA interstrand cross links {{253 Kim,H. 2012}}. Recent studies have demonstrated how the Fanconi Anaemia pathway coordinates three critical DNA repair processes including homologous recombination. Loss or mutation of this pathway predisposes individuals to cancer {{3 Kennedy,R.D. 2006}}. ERCC-1 and FANCD2 are components of the Fanconi Anaemia pathway. Current literature suggests that this pathway is known to be lost in up to 20% solid tumours {{254 D'Andrea,A.D. 2010}}. Within this study, it was found that both ERCC-1 and FANCD2 were sensitive to AZD0530, suggesting that loss or mutation of this pathway could act as a potential biomarker for the response of patients to Src Inhibitors.

To determine the efficiency of the screen, internal validation was carried out within the screen itself. To ensure that the control siRNAs were behaving in the same fashion in each of the plates, the survival fraction of the cells was compared with those treated with the same substance- DMSO, 10µM AZD0530 or 5µM MK2206. Survival fractions were found to be the same amongst all of the plates and the controls could therefore be deemed to be efficient. However, it was found when the survival fraction of the HFF cells + control siRNAs were compared between those treated with the DMSO and those treated with either the Src or Akt Inhibitor, the results weren't what was expected. Within the plates treated with 10µM AZD0530 and 5µM MK2206, the survival fraction of the control siRNAs were greater than those in the corresponding plates treated with DMSO. In order to compensate for this, functional analysis of the screen was carried out using DAVID (a

bioinformatic functional analysis website) to determine the cellular pathways, processes and compartments of the tumour suppressor genes which sensitised to Src and Akt inhibitors.

Future Directions

It is imperative that those tumour suppressor genes which have been identified as potential hits from this screen are validated in other cell lines. This would be achieved through the knock down of those tumour suppressor genes in individual cell lines. For example, by knocking down the expression of FANCD2, the effect of 10µM AZD0530 could be analysed and compared to the effect of the drug elucidated in the screen.

Once those biomarkers which have arisen from the screen are validated in isogenic cell lines, further investigation into the pathways they are associated with, should be carried out in tumour material. Within this research lab, tumour material from University College London is due in the near future, from which Src expression in ovarian cancer will be investigated alongside experimentation of the effect of AZD0530 and Paclitaxel in platinum resistant ovarian cancer. Similar measures should be taken with those genes identified through the screen treated with 5µM MK2206.

Although it was not within the realms of this project, it was acknowledged from analysis of the screen that many tumour suppressor genes appeared to exhibit resistance to AZD0530 and MK2206. Further analysis of these potential hits for resistance should be carried out, using isogenic cell lines as, although it does not identify a patient population who would see benefit from these single agents, it would eliminate a patient population who shouldn't be treated with these drugs as they are resistant.

Chapter V: Conclusions

Identification of Tumour Suppressor Genes whose loss mediates sensitivity to conventional chemotherapy and targeted therapeutics

There is evidence that tumour suppressor genes can enhance the sensitivity of cancer cells to chemotherapeutic agents and thus may be used as biomarkers in predicting the response to these agents in cancer patients. However, the complexity of cell signalling processes suggests that, in order to provide a more reliable predictor for response to chemotherapy, it is crucial that we must understand the contribution of each player in the context of one another. This will allow for the progression of a therapeutic strategy where targeted therapeutics provides benefit to patient populations who are sensitised to these therapies.

In this study, the losses of a number of tumour suppressor genes have been found to be suggestive of synergy with AZD0530 or MK2206. Of note, ERCC1 and FANCD2, who interact within the Fanconi Anaemia pathway, may provide potential biomarkers to patient response to Src Inhibitors for patients who are deficient in this pathway. Further examination of this hypothesis is required in isogenic cell lines and tumour material to enable patient populations to benefit from these potential biomarkers of response.

Chapter VI: Appendices

Appendix A

HFF cell line

Designation	HFF-1
Biosafety Level	1
Growth Properties	Adherent
Organism	*Homo Sapiens*
Morphology	Fibroblast
Source	Tissue: skin, foreskin Cell type: fibroblast
Gender	Male
Age	Newborn

Chapter VII: References

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