Novel targeted agents in Her-2 positive and triple negative breast cancer

A thesis submitted for the degree of M Sc

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I. Abstract

Novel targeted agents in Her-2 positive and triple negative breast cancer

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The development of Her-2 targeted therapies has improved the prognosis for patients with Her-2 positive breast cancer. However, not all Her-2 positive tumours respond to treatment with Her-2 antagonists. Triple negative cancers are resistant to hormone and Her-2 targeted therapies. This project focused on improving response in Her-2 overexpressing breast cancer and on developing effective targeted therapy strategies for triple negative breast cancer.

We tested a number of multi-target kinase inhibitors (imatinib, sunitinib, pazopanib and dasatinib) in Her-2 positive and triple negative breast cancer cell lines, alone and in combination with other agents.

Two of the Her-2 positive cell lines showed moderate sensitivity to sunitinib malate. Combined treatment with sunitinib and trastuzumab showed improved response compared to either drug alone, in the four Her-2 positive cell lines tested.

Dasatinib inhibited growth in 3 of the 5 triple negative but in only 1 of the 4 Her-2 positive cell lines tested. Based on response to the other multi-target kinase inhibitors, which have overlapping target specificities, and the Src,PP2, our results suggest that sensitivity to dasatinib in triple negative breast cancer is due to inhibition of ephrin type A receptors. Consistent with this hypothesis, neither Src expression nor phosphorylation predicted sensitivity to dasatinib, but high levels of Ephrin type A receptor 2 protein correlated with dasatinib sensitivity. High levels of caveolin 1 and caveolin 2 also correlated with dasatinib sensitivity in the panel of cell lines.

Dasatinib combined with cisplatin was synergistic in the three dasatinib-sensitive cell lines. Dasatinib, in combination with 5’-deoxy-5’-fluoruridine, displayed synergy or additivity. Moderate synergy was observed with docetaxel in two triple negative cell lines.

In conclusion, we have identified dasatinib with cisplatin as a rational combination for testing in triple-negative breast cancer, and have identified a panel of putative predictive biomarkers for dasatinib sensitivity (EphA2, CAV1 and CAV2).

II. List of common abbreviations

5’-DFUR: 5’-Deoxy-5’Fluorouridine

BRCA: Breast-ovarian Cancer

CI: Combination Index

EGFR: Epidermal Growth Factor Receptor

EphA receptor: Ephrin type A receptor

ER: Estrogen Receptor

HER-2: Human Epidermal growth factor Receptor -2

mTOR: mammalian Target of Rapamycin

PARP: Poly-ADP-Ribose Polymerase

PDGF: Platelet-Derived Growth Factor

PFS: Progression-Free Survival

PR: Progesterone Receptor

VEGF: Vascular Endothelial Growth Factor

Chapter 1. Literature Review

1.1 Introduction

Breast cancer is the most common form of non-skin cancer in women and the 5th cause of cancer death. Breast cancer is a remarkably heterogeneous disease characterized by aberrant signal transduction. During the last 20 years translational research has focused on isolating and interfering with signals responsible for breast cancer pathogenesis and progression. Some of these molecular drivers of carcinogenesis, like the estrogen receptor and the human epidermal growth factor receptor-2 (Her-2) pathways have been partially elucidated and targeted. However, many more still need to be defined.

These advances have shifted breast cancer classification from pathologic to molecular, allowing for more accurate prognostic and predictive information and rational drug development. The proof of principle for this approach was the discovery of Her-2 receptor and the development of trastuzumab.

1.2 Targeted therapies and breast cancer

Breast cancer was the first solid tumour where targeted therapy was applied. The discovery of estrogens and estrogen receptor signalling led to the development of hormone manipulation treatment which radically changed the survival and quality of life for women with hormone-sensitive breast cancer. Further molecular pathways involved in cancer growth and progression have been elucidated and moleculary targeted agents are being developed to block these signalling pathways. Targeted therapies tend to be less toxic compared to chemotherapy due to their selectivity and are often more effective, as in the case for trastuzumab in Her-2 positive breast cancer (1). Targeted therapies can

be broadly divided into 2 categories: monoclonal antibodies that block receptors or ligands extracellularly and small molecules which either inhibit intracellular protein kinases or other pathways relevant to cellular proliferation, growth and metastatic potential. The effectiveness of targeted therapies relies on the importance and uniqueness of the selected molecular pathway for tumour development and progression. Therefore, and since solid tumours including breast tumours are heterogenous, target selection is of paramount importance.

1.3 Molecular classification of breast cancer

Gene expression profiles have classified breast cancer into biologically and clinically meaningful subgroups (2) (3). These intrinsic subtypes consist of luminal, normal-like, HER-2 amplified, basal-like and most recently claudin-low tumours. Even within this classification important heterogeneity in clinical outcome exists.

In practice, since genomic methods of tumour classification are often not readily available, the use of simple immunohistochemical markers have proven useful but not perfect surrogates of the molecular subgroups which serve as prognostic and predictive markers for clinical use and clinical trial design (Table 1.1).

Table 1.1. Immunohistochemical subtypes of breast cancer (4).

Subtype	**Phenotype**
Luminal A	ER/PR +, HER-2 -, Ki-67<15-20%
Luminal B	ER/PR +, Ki-67>15-20%
Her-2 overexpressing	ER-, PR-, HER-2 +
Triple negative	ER-, PR-, HER-2 -

1.3.1 ER negative, PR negative, Her-2 normal (triple negative) breast cancer

Breast cancers that do not overexpress estogen receptors, progesterone receptors or Her-2 (triple negative) are an immunohistochemical surrogate of basal-like breast cancer. However, there is not a complete overlap between triple negative and basal-like tumours. Approximately 50-75% of triple negative tumours are basal-like, expressing the basal cytokeratins 5, 6 or 17. These tumours are highly proliferative, *p53* and RB1-protein deficient and often BRCA1 mutated (5). As gene expression studies evolve, further sub-classification is underway (3).

In general, triple negative breast cancers often share clinicopathologic characteristics. They are usually associated with a younger age, a high mitotic index and an adverse prognosis (6). Several studies have demonstrated chemosensitivity in this kind of tumour, especially the basal-like, yet the patients who relapse or whose disease progresses have a dismal prognosis and a short survival (triple negative paradox) (7) (8) (9). Although it represents the minority (10-25%depending on the demographics of the population) of breast cancer subtypes, due to its poor prognosis it accounts for a disproportionate number of breast cancer deaths.

The triple negativity results in a lack of targeted treatment opportunities. We therefore possess no “druggable” nuclear or surface receptor and in search of potential targets, sub-classification according to distinct characteristics should be helpful. Several studies have pointed out possible subgroups of triple negative tumours, including the HER-2 enriched, basal and claudin-low (3) (5).The HER-2 enriched gene expression subtype is a minor constituent of the triple negative group. These tumours are phenotypically triple negative despite having the HER-2-enriched gene expression signatures. Additionally, further classification is underway using the integrity of BRCA repair pathway (BRCA

mutations or BRCAness). These efforts have produced possible clinically relevant targets which are summarised in table 1.2.

Table 1.2 Current treatment strategies for targeting triple negative breast cancer in clinical trials.

Target	Drugs
EGFR	Cetuximab, erlotinib (10) (11)
VEGF(R)	Bevacizumab, ramucirumab, sorafenib, cediranib, apatinib (12)
DNA	Platinum and alkylators, PARP inhibitors (13) (14)
mTOR	Temsirolimus, everolimus
SRC	Dasatinib (15)

1.3.1.1 Possible targets for triple negative breast cancer

(i) Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor expression has been observed in 54% of basal breast cancer cell lines and has been independently associated with poor outcome (16) (17). In addition, EGFR mRNA has been detected more frequently and at higher levels in breast cancer tumour samples of basal subtype (4). Preclinical studies also showed EGFR overexpression in triple negative as compared to Her-2 positive cell lines. In the same study sensitivity to the small molecule EGFR inhibitor was tested. Although single agent EGFR inhibitor was ineffective, gefitinib enhanced sensitivity to taxane and platinum chemotherapy in the triple negative cell lines (18). Preliminary results of the BALI-1 clinical study suggested better response rates and progression free survival in patients

with metastatic triple negative breast cancer when EGFR targeting monoclonal antibody cetuximab was added to cisplatin chemotherapy (10) .

(ii) BRCA 1

Women who carry mutations in the BRCA-1 gene develop breast cancer which is often basal-like. There has been an increased interest in this subgroup, since it shares common characteristics with sporadic basal-like cancers, such as the triple negative phenotype, high grade, pushing margins, EGFR expression, high p53 mutation rate, X-chromosome inactivation pattern and sensitivity to DNA damage (19) (20). The BRCA 1 gene plays an important role in mediating the DNA damage response. As a result, BRCA-1-deficient tumours are particularly sensitive to DNA damaging agents, such as alkylators, platinum salts and radiation treatment. Unfortunately, in clinical practice we are still lacking clinical study data supporting the use of BRCA-1 pathway as an effective target for triple negative breast cancer.

(iii) Poly-ADP-ribose polymerase (PARP)

PARP may be another therapeutically meaningful target in patients with triple negative breast cancer. In the case of DNA damage, apart from BRCA-1 repair (homologous recombination), PARP-mediated base excision repair is a key mechanism. Inhibition of PARP in BRCA-1 deficient cells deprives cells of two vital repair mechanisms and leads to cell death (synthetic lethality) (21) (22). Conveniently, the non-cancer cells of BRCA-1 mutation carriers retain one functional allele of the BRCA-1 gene and can still repair DNA damage. Therefore, PARP inhibition is selective for cancer cells and generally has no toxicity to healthy cells. Additionally, inhibition of PARP can be better exploited in BRCA-1 mutation carriers due to synthetic lethality.

Results of two relevant clinical trials are available. Olaparib, a potent PARP inhibitor was used as a single agent in pretreated BRCA-1 or -2 mutation carriers with breast cancer. Remarkably there was a 41% response rate and a median PFS of 5.7 (4.6-7.4) months (23). On the other hand results of a phase III study using a weaker PARP inhibitor, iniparib, along with carboplatin and

gemcitabine chemotherapy in unselected triple negative metastatic breast cancer patients, were negative, although phase I and II results were very promising (24; 14).

(iv) Angiogenesis

Anti-angiogenic treatment, with the monoclonal antibody bevacizumab, has been tested extensively in unselected patients with metastatic breast cancer. It has produced positive results in terms of prolonging progression free survival but has failed to show an overall survival benefit. Subset analyses suggested that patients with triple negative breast cancer benefited as much as, if not more than, the average (12). Ongoing studies will provide data on effectiveness in triple negative breast cancer.

The small molecule antiangiogenic agent, sunitinib, which targets PDGFR-a and –b, VEGFR-1, -2, -3, c-KIT, FLT-3, CSF-1R and RET, has also been tested in clinical studies of unselected metastatic breast cancer patients, in combination with chemotherapy. Unfortunately, studies were prematurely interrupted due to lack of efficacy and toxicity.

(v) C-KIT

The growth factor receptor, c-KIT, is expressed in 30% of basal-like cancers (16). Inhibitors of c-KIT have shown modest activity in patients with metastatic breast cancer, yet there are no results to date in a c-KIT-mutated subpopulation and therefore the validity of c-KIT as a molecular target remains undetermined (25).

(vi) SRC

The SRC family kinases are non-receptor protein tyrosine kinases that have been extensively studied in cancer. Src is a protooncogene which appears to be a key messenger in important cellular pathways, including proliferation, differentiation, survival, motility and angiogenesis (26) (27). It can be activated either through ligand activation of cell surface receptors (e.g. EGFR) or by cytoplasmic proteins (e.g.focal adhesion kinase FAK, Crk-associated substrate CAS) or by dephosphorylation of tyrosine residue Y530 (28) (29). Activation of

SRC leads to an open conformation of the SRC protein and further downstream signalling as illustrated in figure 1.1. Increased EGFR, HER-2, PDGFR, VEGFR, ephrin, FAK signalling lead to elevated SRC activity (30) (31) (32). Preclinical studies have implicated SRC inhibition as a therapeutic option for triple negative breast cancer (33) (30) (34). Dasatinib, an orally active small molecule inhibitor of SRC-family and Abl kinases is approved for treating Philadelphia chromosome-positive leukemias. Additionally, dasatinib has been shown to inhibit invasion, growth and metastasis of breast cancer cell lines and particularly of basal-type. Among 39 human breast cancer cell lines tested, basal-type cell lines as well as cell lines that have undergone epithelial-to-mesenchymal transition (post-EMT) were highly sensitive to dasatinib. Additionally, a highly significant relationship between basal-type and post-EMT breast cancer cell lines and sensitivity to dasatinib was observed (33). Xenograft studies of dasatinib in athymicmice which were inoculated with the triple negative breast cancer cell lineMDA-MB-231 demonstrated antitumour activity (34). Results of a phase II clinical study of single agent dasatinib in unselected triple negative breast cancer patients showed limited activity but acceptable toxicity profile (15).

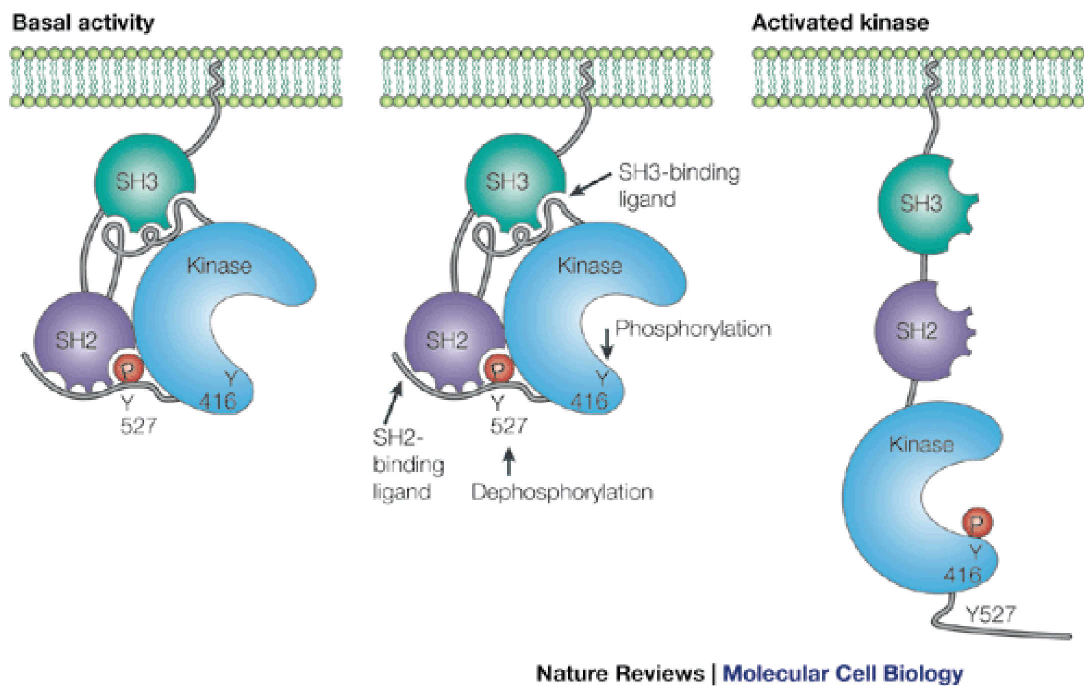

Figure 1.1.Basal conformation, mechanisms of activation and active conformation of SRC kinase (35).

(vii) PI3K-AKT-mTOR

Mammalian Target of Rapamycin (mTOR) is a downstream member of the phosphatidoinositide 3-kinases (PI3-K)/AKT kinase signalling pathway which can regulate cancer cell growth and cell cycle progression in malignant cells (36). mTOR, is a cell-signaling protein that regulates the response of tumour cells to nutrients and growth factors, as well as controlling tumour blood supply through effects on Vascular Endothelial Growth Factor, VEGF. The PI3K/Akt pathway is thought to be over activated in numerous cancer types. Over-activation of the PI3K/Akt kinase pathway is frequently associated with mutations in the phosphatase and tensin homologue (PTEN) gene, which is the second commonest mutation of a tumour suppressor gene. In preclinical and clinical studies chemotherapy and hormone therapy have proven to be more effective in the presence of a PI3-K/AKT/mTOR pathway inhibitor. Combined

treatment with an mTOR inhibitor and hormone treatment produced promising results in a phase 3 randomized study (37). Inhibition of this pathway in triple negative breast cancer is still under clinical investigation. Several phase I and II studies with combinations of everolimus or temsirolimus and other targeted agents (lapatinib, neratinib,etc) or chemotherapy (paclitaxel, carboplatin, etc) in triple negative breast cancer are ongoing.

(viii) MAPK

The mitogen-activated kinase (MAPK) pathways are major signal transduction routes that transfer and amplify messages from the cell surface to the nucleus. MAPK signaling pathways modulate many cellular events including: mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement and apoptosis, as well as cell differentiation (38). These pathways are organized in 3 kinase modules consisting of a MAP kinase, an activator of MAP kinase (MAP Kinase Kinase or MEK) and a MAP Kinase Kinase Kinase (MEK Kinase, MEKK, or MAPK Kinase Kinase). The Ras-Raf-MEK-ERK pathway has been implicated in uncontrolled cell proliferation and survival. This pathway is activated by a range of growth factor receptors, including epidermal growth factor receptor, platelet-derived growth factor receptor, type-1 insulin-like growth factor receptor, and fibroblast growth factor receptors. The pathway can also be activated by cytokines, steroid hormones, and several agonists that act via G-protein-coupled receptors. Growth factor stimulation leads to a signal transduction cascade, which involves sequential activation of a number of cytoplasmic and nuclear targets. MEK1/2 is a dual-specific kinase that is essential to the Ras-Raf-MEK-ERK pathway. All three Raf isoforms share Ras as a common upstream activator, whereas MEK is the only commonly accepted downstream substrate.MEK1/2 serves to amplify signals to ERK1/2 (also known as MAPK1/2) through phosphorylation of key tyrosine and threonine residues, thereby activating its catalytic activity. Activation of ERK1/2 via MEK1/2 is a critical step in growth factor signaling, as active ERK mediates a range of cellular effects including cell proliferation, migration and differentiation as well as promotion of epithelial to mesenchymal transition (39). This pathway

has been implicated with resistance to chemotherapy and adverse prognosis in patients with triple negative breast cancer (40) (41). Since there is often co-activation of the MAPK and AKT pathways, several phase I clinical trials are currently exploring the activity of dual inhibitors in patients with triple negative breast cancer (42).

1.3.2 Her-2/neu amplified/overexpressed breast cancer

The Her-2 amplified /overexpressed and ER/PR negative phenotype is a subgroup of breast cancer associated with a poor prognosis in the absence of Her-2 targeting treatment.

The Her system consists of several ligands including EGF, TGF, amphiregulin, neuregulins, etc and 4 receptor tyrosine kinases: EGFR, Her-2, Her-3 and Her-4 (43) (44). Activation and downstream signalling result in cell proliferation, differentiation, survival and gene transcription. Her-2/neu (cErb-B2) is a protooncogene which is located on chromosome 17q12. Her-2 possesses no known ligand. EGFR, Her-2 and Her-4 have an active kinase domain, whereas the Her-3 intracellular kinase domain is inactive. In response to ligand binding, receptors form homo- or heterodimers, their cytoplasmic tyrosine kinase is phosphorylated and numerous downstream signals are generated. Her-2 and Her-3 signals appear to be most potent (45). The best studied signalling pathways induced by the HER pathway are the phosphatidoinositol-3 kinase-Akt-mTOR pathway, the Ras-mitogen-activated protein kinase (Ras-MAPK) and the phospholipase Cγ-protein kinase C pathway (46).

Her-2 overexpression is usually due to gene amplification and results in an increased number of Her-2 molecules on the cell surface of cancer cells. This occurs in 15-30% of breast cancers and is of prognostic and predictive significance. Since 1987, several studies have correlated Her-2 amplification with shorter time to relapse, poorer overall survival and higher incidence of adverse histologic features, such as lymph node involvement, lymphovascular invasion and brain metastases (47). Overexpression of Her-2 has been shown

predictive of response to anthracyclines, resistance to hormonal treatment and response to anti-Her-2 treatment, including trastuzumab and lapatinib (48) (49) (50) (51) (52) (53).

1.3.2.1 Targeted therapy for Her-2 positive breast cancer

1.3.2.1.1 Monoclonal antibodies

Trastuzumab

Trastuzumab was the first humanized monoclonal antibody approved for breast cancer treatment. It binds to the extracellular domain of Her-2 preventing downstream signalling. The exact mechanism of action is still not fully elucidated. Several overlapping models have been proposed, including downregulation of Her-2, reduction of Her-2 signalling, inhibition of shedding of the Her-2 extracellular domain, induction of cell cycle arrest, induction of apoptosis, inhibition of angiogenesis and DNA repair and antibody-dependant cell-mediated cytotoxicity (ADCC) (46) (54).

In clinical trials, trastuzumab achieved response rates up to 15% as a single agent in pretreated patients with metastatic Her-2 positive breast cancer and 26-34 % in first line treatment (55) (56). In combination with chemotherapy it nearly doubled response rates, prolonged response duration and overall survival and reduced risk of death by 20%, compared to chemotherapy alone (1). Several studies support trastuzumab use even beyond disease progression and finally neoadjuvant and adjuvant trastuzumab for 1 year have been standard of care since 2006 in patients with early stage Her-2 positive breast cancer (57) (58).

Trastuzumab can be safely used concurrently with several chemotherapy agents but due to increased risk of cardiomyopathy, concurrent use with anthracyclines is not recommended (1).

Pertuzumab

Pertuzumab is a novel humanized monoclonal antibody which is currently studied in clinical trials. Unlike trastuzumab, pertuzumab binds to the dimerization domain of the Her-2 receptor and inhibits dimerization with other HER family receptors (59). Early phase II as well as phase III trial results suggest encouraging activity, in combination with trastuzumab and regulatory authority approval of this agent is awaited (60) (61).

T-DM1

This is a unique antibody-drug conjugate including trastuzumab, a linker and a potent cytotoxic agent, DM 1, which is a maytansine analog. The antibody binds to the Her-2 receptor, the receptor along with the T-DM1is internalised and the microtubule polymerization inhibitor is released in the cancer cell. A phase II study using this conjugate in heavily pretreated Her-2 positive metastatic breast cancer patients, resistant to other Her-2 targeting agents showed response rates of 32-35%, nearly double of those initially presented with trastuzumab (62). Early results of another randomized phase II study comparing T-DM 1 to combination docetaxel and trastuzumab showed similar response rates (63). Results of the phase III EMILIA study comparing T-DM 1 to the standard second-line combination lapatinib-capecitabine in patients with metastatic HER-2 positive breast cancer progressing after trastuzumab, demonstrated improved progression-free survival in patients receiving the antibody-drug conjugate (64).

1.3.2.1.2 Small moleculekinase inhibitors

Lapatinib

Lapatinib is a quinazoline, a dual inhibitor of the phosphorylation of the tyrosine kinases of Her-2 and EGFR. It has produced activity in trastuzumab pretreated metastatic breast cancer patients as a single agent (65). In addition, the combination with capecitabine prolonged time to disease progression as compared to capecitabine alone (66). Recent phase III trial results confirm preclinical data supporting synergistic activity with trastuzumab (67).

As with most of the small molecule inhibitors lapatinib is orally administered and is generally well tolerated. The most frequent adverse events include diarrhea, skin rash and liver function test abnormalities.

Neratinib

Neratinib is a next generation orally available, 6,7-disubstituted-4-anilinoquinoline-3-carbonitrile inhibitor of the HER-2 receptor tyrosine kinase with potential antineoplastic activity. Neratinib binds to the HER-2 receptor irreversibly, thereby reducing autophosphorylation in cells, apparently by targeting a cysteine residue in the ATP-binding pocket of the receptor. Treatment of cells with this agent results in inhibition of downstream signal transduction events and cell cycle regulatory pathways; arrest at the G1-S (Gap 1/DNA synthesis)-phase transition of the cell division cycle; and ultimately decreased cellular proliferation. Neratinib also inhibits the epidermal growth factor receptor (EGFR) kinase and the proliferation of EGFR-dependent cells. Neratinib provides greater inhibition of HER2 signaling activity but does not increase HER2 protein levels as has been observed with lapatinib (68). Early results of several ongoing phase II have reported mixed results. Final results are awaited.

Other inhibitors

Heat shock protein 90 (Hsp90) has a role in maintaining Her-2 stability and regulates its function. Interaction of Hsp90 and the kinase domain of Her-2 is necessary for Her-2 dimerization and activation (69). Preclinical data with inhibitors of Hsp90 have shown activity (70). Other inhibitors affecting Her-2 protein are under development, including deacetylase inhibitors, vaccine immunotherapy etc (71).

1.3.3 Luminal breast cancer

ER and/or PR positive phenotypes are immunohistochemical surrogates of the luminal molecular signature. Luminal type breast cancers are the most prevalent subtype and bear variable clinical outcomes and responses to endocrine therapy

and chemotherapy. Further subclassification has divided this group into luminal A and luminal B, the first group having more favourable prognosis and greater hormone-sensitivity than the latter (2). Expression of the proliferation gene Ki-67 is helpful in discerning phenotypically these two groups; luminal A have a low Ki-67 score and luminal B a high Ki-67 score (72). Luminal B group also includes the ER/PR positive Her-2 enriched phenotype.

1.3.3.1 Targeted treatments for luminal-type breast cancer commonly used in clinical practice.

(i) Selective Estrogen Receptor Modulators (SERMs)

SERM's (tamoxifen, toremifene, raloxifene) bind to estrogen receptors, preventing estrogens from binding. In some tissues (i.e. breast) SERMs act as estrogen antagonists, in other tissues (i.e. uterus, bones) they act as estrogen agonists. Tamoxifen has been used for more than 30 years for the treatment of advanced and early hormone-receptor positive breast cancer (73).

(ii) Aromatase Inhibitors (AIs)

AIs block the activity of the enzyme aromatase which is responsible for the peripheral conversion of androgens to estrogens. AIs only have activity in postmenopausal women without ovarian estrogen production. These drugs have shown significant activity in the treatment of both advanced and early hormone-receptor positive breast cancer (74).

(i) Selective Estrogen Receptor Downregulator (SERD)

SERD fulvestrant is an antiestrogen. It irreversibly binds to the estrogen receptor and acts as an antagonist. Additionally, it prevents dimerisation and causes downregulation and destruction of the estrogen receptor. In the clinic it has been licenced for the treatment of hormone receptor positive advanced breast cancer after failure of other antiestrogen treatment in postmenopausal women (75).

(ii) Lutenizing Hormone Releasing Hormone (LHRH) analogs or antagonists

LHRH analogs (leuprolid, goserelin, triptorelin) ablate ovarian and consequently estrogen production in premenopausal women. These drugs are modelled after human hypothalamic gonadotropin-releasing hormone and are designed to interact with the GnRH receptor and modify the release of FSH and LH gonadotrophins by the pituitary gland. After an initial “flare” of FSH and LH secretion, gonadotrophin receptor is downregulated and FSH and LH levels drop. The absence of gonadotrophins cause a reversible ovarian ablation. LHRH analogues are indicated for the treatment of premenopausal hormone receptor positive early and advanced breast cancer (76). LHRH antagonists have also been developed in order to avoid the initial “flare phenomenon” and soon will be approved by regulatory authorities for clinical use in breast cancer.

1.4 Resistance to currently used targeted therapies

Both HER-2-targeting and endocrine manipulation treatment have improved considerably the outcomes of HER-2 positive and hormone receptor positive breast cancer respectively. Unfortunately, many patients present primary resistance or develop secondary resistance to targeting treatments. Resistance is complex because of the numerous downstream molecular signalling pathways along with their ability to cross talk. In the case of HER-2 positive breast cancer, pathways that have been implicated with resistance to trastuzumab and lapatinib include the PI3K-AKT-mTOR, the EGFR, the IGF-IR and other pathways (46).In hormone receptor-positive breast cancer the EGFR/HER-2, VEGFR, PI3K-AKT-mTOR pathways as well as epigenetic changes have been proposed as possible resistance mechanisms (37).Since targeted therapies may become inactive due to development of resistance through cross-talking escape pathways, combining targeted agents might be a strategy to prevent resistance.

1.5 Study Objectives

Both Her-2 positive (amplified or overexpressed) and triple negative breast cancer, in particular, are associated with poor prognosis (19) (2). The development of Her-2 targeted therapies, specifically trastuzumab and lapatinib, has improved the prognosis for patients with Her-2 positive breast cancer. However, not all Her-2 positive tumours respond to treatment with Her-2 antagonists, and acquired resistance frequently develops in patients who initially respond (77). Triple negative cancers are also resistant to hormone and Her-2 targeting therapies.

This project focussed on examining ways to improve response in Her-2 overexpressing breast cancer and on developing effective targeting therapeutic strategies for basal-like breast cancer.

It is increasingly recognised that molecularly targeted therapies are unlikely to be effective as single agents and must be evaluated in combination with other agents. As previously reported (78), strategies for optimising the use of novel targeted therapies should include: 1. Systematic pre-clinical assessment of targeted agents and combinations . 2. Correlative studies in both preclinical and clinical settings. 3. Development of reliable biomarkers for patient selection and/or measuring response.

In this project we :

- tested a number of multi-target kinase inhibitors in Her-2 positive and triple negative breast cancer cell lines
- We tested the most active multi-target kinase inhibitors in combination with other agents (targeted therapies and chemotherapy).
- We identified markers of response to specific multi-target kinase inhibitors.
- Novel pathway inhibitors were also tested in triple negative breast cancer cell lines in an attempt to identify potential therapeutic targets.

- We assessed the direct anti-tumour effects of a number of these multi-target kinase inhibitors, including imatinib, sunitinib, pazopanib and dasatinib (79).

- Previous studies have shown that triple negative breast cancer cells are more sensitive to dasatinib than luminal or Her-2 positive breast cancer cell lines (33). We compared sensitivity to dasatinib with sensitivity to other multi-target kinase inhibitors and investigated the specific targets responsible for sensitivity to dasatinib in triple negative breast cancer cells.

Chapter 2. Materials and Methods

2.1 Cell lines, cell culture and reagents

Five triple negative breast cancer cell lines (BT20, HCC1937, MDA-MB-231, MDA-MB-468, HCC-1143) and four *Her2* amplified cell lines (BT474, SKBR3, HCC-1419, JIMT-1) were obtained from the American Tissue Culture Collection, Manassas, VA. Cell lines were maintained in RPMI-1640 with 10% fetal bovine serum (BioWhittaker, Walkersville,MD), at 37ºC with 5% CO_2. HCC1937 medium was also supplemented with 1% sodium pyruvate (Sigma-Aldrich, Wicklow, Ireland). JIMT-1 cells were maintained in DMEM with 10% fetal bovine serum. Cell cultures were maintained and handled in aseptic conditions. Cell lines were subcultured twice a week at a ratio of 1:2 to 1:10 dependent on the cell line and on the confluency. Cell culture media was aspirated and the flask washed twice with 15ml phosphor buffered saline (PBS). Cells were then detached using 1ml trypsin-EDTA solution. Trypsin was neutralized using 20 ml of pre-warmed media. All cells were routinely tested for the presence of mycoplasma. In case of contamination, cell lines were destroyed and experiment repeated. The characteristics of the panel of cell lines tested are listed in table 2.1.

Table 2.1. Cell lines and characteristics (80).

Cell line	Sub-type	Characteristics
HCC-1419	Her-2 (+)	IDC, EGFR+, ER-, PR-, p53-
SKBR-3		IDC, Luminal, ER-, PR-, EGFR+, p53+
JIMT-1		Basal, ER-, PR-, EGFR+, CK5/6+
BT-474		IDC, Luminal, ER+,PR+,EGFR +,CK 5/6-,p53+
HCC-1954		IDC, ER-, EGFR+, CK 5/6-
BT-20	3ple (-)	IDC, Basal, EGFR+, CK 5/6 - ,p53++
MDA-MB-231		IDC, Mesenchymal, EGFR+, CK 5/6-, p53++
MDA-MB-468		Adenocarcinoma, EGFR+, p53+, PTEN loss
HCC-1143		Basal, p53+++
HCC-1937		IDC, Basal, EGFR+,CK 5/6 + ,p53-, BRCA1mut
MCF-7	Luminal	IDC, ER+, PR+, C-erbB2 normal
CAMA-1		

Docetaxel (Sanofi-Aventis) and cisplatin (Bristol Myers Squibb) were purchased from the Pharmacy Department, St Vincent's University Hospital, Dublin. Dasatinib, imatinib mesylate, sunitinib malate (Sequoia Research Products, Pangbourne, UK), pazopanib (GSK), U0126 (Promega), LY294002 (Calbiochem),5'-deoxy-5'-fluoruridine (5'-DFUR) and rapamycin(Sigma-Aldrich) were prepared as 10 mM stocks in DMSO and stored at -20ºC. The inhibitors tested and their corresponding targets are listed in table 2.2.

Table 2.2. Target specificities of the multi-target kinase inhibitors included in this study. Typical IC_{50} values needed to inhibit signalling through the corresponding targets.

Inhibitor	Targets	IC_{50} (nM)
Imatinib (Glivec™)	Bcr-Abl, PDGFR, c-Kit	100-600 (81)
Sunitinib (Sutent™)	VEGFR-1,-2,-3, PDGFR-a,-b, c-Kit, FLT3, CSF-1R, RET	2-80 (82)
Pazopanib (Votrient™)	VEGFR-1,-2,-3, PDGFR-a,-b, c-Kit	10-84 (83)
Dasatinib (Sprycel™)	Abl, PDGFR, c-Kit, Src, EphA receptors	0.6-0.8 (84)
Rapamycin	mTOR	<50 (85)
U0126	MAPK	58-72 (86)
LY294002	PI3K	1000-50000 (87)

2.2 Proliferation assays

Proliferation was measured using an acid phosphatase assay. Ninety six-well plates were seeded with 3-5 x 10^3 cells per well. Following overnight incubation, drug was added at the appropriate concentrations and incubated for 5 days. Medium was removed and cells washed once with phosphate buffered saline (PBS). One hundred µl of acid phosphatase substrate [7.25 mM p-nitrophenyl-phosphate (Sigma) in sodium acetate buffer, pH 5.5] was added to each well and incubated at 37ºC for 1 h. Fifty µl of 1 M NaOH was added to stop the reaction and the absorbance was read at 405 nM with 620 nM, as the reference wavelength (88).

2.3 Combination assays and synergy analysis

The most active targeting agents were tested in combination with active chemotherapy in vitro. Fixed ratio combination assays were used in proliferation assays as described above and synergy analysis was performed as described in section G (statistical considerations).

2.4 Preparation of cell lysates

After 6-hours of dasatinib treatment, cells were washed with ice-cold PBS and lysed in 500 µl RIPA buffer containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate (Sigma). After 20-min incubation on ice, the lysate was passed through a 21-gauge needle and centrifuged at 16 000 x g for 5 min at 4ºC. Supernatants were collected and stored at -80ºC. Protein quantification was carried out using the bicinchoninic acid assay (Pierce Biotechnology).

2.5 Immunoblotting analysis

20-50 micrograms of protein in sample buffer was heated to 95ºC for 5 min, separated using 7.5% or 10% polyacrylamide gels (Lonza, Basel, Switzerland) and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% non-fat milk powder in phosphate-buffered saline (PBS)–0.1% Tween, at room temperature for 1 h. After overnight incubation at 4ºC with primary antibody in 5% blocking solution, three washes with 0.5% PBS–Tween were performed, followed by incubation at room temperature with secondary antibody in 5% blocking solution for 1 h. Following three washes with 0.5% PBS–Tween and one PBS wash, protein bands were detected using Luminol (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibodies used were mouse anti-alpha-tubulin (Sigma), mouse anti-EphA2 (Millipore), mouse anti-Src kinase (Upstate Cell Signaling Solutions), rabbit anti-phospho-Srcpy 419 (Biosource Europe), caveolin-1 (Millipore)and caveolin-2 (BD Biosciences) (Table 2.3).

Table 2.3. Antibodies and corresponding concentrations used for immunoblotting analysis.

Primary antibody	Source	Concentration (dilution)
α-tubulin	Mouse	1 µg/ml (1:1000)
EphA2	Mouse	1 µg/ml (1:1000)
Src (pp60^{src}Kinase)	Mouse	1 µg/ml (1:1000)
P-Src [pY^{418}]	Rabbit	2 µg/ml (1:500)
Caveolin-1	Rabbit	1 µg/ml (1:1000)
Caveolin-2	Rabbit	2 µg/ml (1:500)

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2.6 *In vivo* testing

In vivo testing was carried out in collaboration with Dr Annette Byrne, Royal College of Surgeons in Ireland. All animal experiments were licensed by the Department of Health and Children, Ireland and specific ethical protocols reviewed by the Animal Research Sub Committee (ARSC) at University College Dublin. All studies were in full compliance with EU guidelines.

For the toxicology study 8 groups of Balb-C mice (Harlan) (3 mice/group) were treated with fixed dose of dasatinib (15 mg/kg, by oral gavage,(po) 5 days/week) combined with escalating doses of cisplatin (1 mg/kg, 2 mg/kg, 3 mg/kg and 4 mg/kg, injected intraperitoneally (ip), once a week).

For the efficacy study, MDA-MB-231 cells (approximately $1x10^6$) were injected directly into the mammary fat pad of six-week-old female athymicBalb/C nude mice (Harlan). On day 13, 8 mice per group were randomly assigned to treatment with: (i) vehicle (50% propylene glycol) (group 1), (ii) dasatinib (15 mg/kg, po, 5 days/week)(group 2), (iii) cisplatin (4mg/kg, ip, once a week)(group 3), (iv) dasatinib and cisplatin (group 4) and (v) no treatment (group 5). Tumour diameter was serially measured with callipers, and tumour volumes calculated

using the formula: volume = width/2 x length/2. Treatment was continued for approximately 3 weeks.

2.7 Statistical considerations

IC_{50} values were calculated using CalcuSyn software (Biosoft, Cambridge, UK). For fixed ratio combination assays, combination indices (CIs) at the ED_{50} (effective dose of combination that inhibits 50% of growth) were determined using the Chou and Talalay equation (89), on CalcuSyn software (Biosoft, Cambridge, UK). CI < 0.9 implies synergy, CI 0.9-1.1 implies additivity and CI > 1.1 implies antagonism (Table 2.4) (90).

Table 2.4. Range of combinations index values which determine synergism or antagonism in drug combination studies analysed with the Combination Index Method.

Range of Combination Index (CI)	Symbol	Description
< 0.10	+ + + + +	Very strong synergism
0.10 – 0.30	+ + + +	Strong synergism
0.30 – 0.70	+ + +	Synergism
0.70 – 0.85	+ +	Moderate synergism
0.85 – 0.90	+	Slight synergism
0.90 – 1.10	±	Nearly additive
1.10 – 1.20	–	Slight antagonism
1.20 – 1.45	– –	Moderate antagonism
1.45 – 3.30	– – –	Antagonism
3.30 – 10.00	– – – –	Strong antagonism
> 10.00	– – – – –	Very strong antagonism

Chapter 3. Evaluation of multi-target kinase inhibitors in breast cancer cell lines

3.1 Introduction

Our aim was to screen a panel of multi-targeting kinases against a panel of breast cancer cell lines, including all subtypes. We focused on Her-2 amplified and triple negative cell lines in order to isolate the most active agent to select for combination testing. Additionally, in the search for new targets for triple negative breast cancer we tested inhibitors of the key signalling pathways, PI3-K/AKT/mTOR and MAPK.

The panel of cell lines tested included five Her-2 amplified, 5 triple negative and 2 ER/PR positive cell lines (table 2.1).

3.2 Sensitivity to multi-target kinase inhibitors

Imatinib mesylate (Glivec™) is a small molecule inhibitor of Bcr-Abl, PDGFR and c-kit. It is licensed for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumours. Typical IC_{50} values range between 100-600nM (81).The cell lines tested showed little sensitivity to imatinib with IC_{50}values over 3 μM (91)(Table 3.1).

Table 3.1. Sensitivity to multi-target kinase inhibitors.

Cell Line		IC_{50} values ± SD (μM)			
		Imatinib	Sunitinib	Pazopanib	Dasatinib
HCC-1419	HER-2	32.3±3.7	8.4±2.9	13.9±4.0	4.7±0.7
SKBR-3		14.9±2.3	5.4±0.9	18.2±2.2	2.1±0.5
JIMT-1		23.4±4.1	7.3±1.9	11.2±1.3	0.1±0.01
BT-474		17.7±3.6	5.0±1.3	11.6±1.1	5.8±0.8
BT-20	3ple (-)	32.5±3.6	9.3±2.5	12.5±1.0	2.5±0.6
MDA-MB-231		23.6±2.0	6.7±1.4	9.6±4.1	0.04±0.01
HCC-1937		27.3±2.0	9.1±1.8	13.0±1.8	0.13±0.07
HCC-1143		ND[a]	ND	ND	0.12±0.02
MDA-MB-468		ND	ND	ND	>5[b]
MCF-7	Luminal	24.5±3.2	15.5±0.3	7.7±0.4	ND
CAMA-1		10.5±3.4	20.2±3.0	9.2±0.9	ND

[a]ND= not determined, [b]An IC_{50} value was not determined as up to 5 μM dasatinib did not achieve 50% inhibition of growth.

Pazopanib (Votrient™) is a novel tyrosine kinase inhibitor of VEGFR-1, -2, -3, PDGFR-a, -b, and c-kit. It was recently licensed for the treatment of renal cell carcinoma and is being tested on clinical trials for breast and other forms of cancer. Typical IC_{50} values range between 10-84nM (83). All cell lines tested were resistant to pazopanib with IC_{50} values greater than 7 μM (Table 3.1).

In addition to the targets of pazopanib, sunitinib (Sutent™) also inhibits FLT3, CSF-1R and RET. It is currently licensed for the treatment of renal cell carcinoma and gastrointestinal stromal tumours. It was also tested in clinical trials for unselected patients with breast cancer, with no success. Typical IC_{50} values range between 2-80nM (82).Although the IC_{50} values found were in the µM range, the Her2 positive and triple negative cell lines showed greater sensitivity to sunitinib than the two luminal cell lines, and the IC_{50} values were consistently lower than for either imatinib or pazopanib in these cell lines (Table 3.1).

Dasatinib (Sprycel™) is a novel multi-target inhibitor of Abl, PDGFR, c-Kit, Src, EphA receptors (30). It is currently licensed for the treatment of imatinib-resistant chronic myeloid leukemia and is being tested in clinical trials for breast, prostate and other solid tumours. Typical IC_{50} values range between 0.6-0.8nM (91). Among the Her-2 positive cell lines, JIMT-1 showed marked sensitivity to dasatinib (Table 3.1). Three of the 5 triple negative cell lines (MDA-MB-231, HCC1937 and HCC1143) showed marked sensitivity to dasatinib (Table 3.1). BT20 cells were also significantly more sensitive to dasatinib than the other multi-target kinase inhibitors. The MDA-MB-468 cells showed resistance to dasatinib as only 34.8±6.7% of cell growth was inhibited at concentration of 800nM of dasatinib. The two non-overlapping targets between dasatinib and the other multi-target kinase inhibitors (imatinib, sunitinib, pazopanib), Src and EphA2, may contribute to the observed sensitivity to dasatinib.

Sensitivity to the Src kinase selective inhibitor (Src IC_{50}= 100 nM), PP2, was also assessed in the triple negative cell lines but none of the cell lines showed sensitivity to PP2 at concentrations up to 5 µM.

3.3 Cell signalling inhibitors in triple negative breast cancer cell lines

Rapamycin (Sirolimus) is an antibiotic product of Streptomyces hygroscopicus. It binds to the cytosolic protein *FK-binding protein 12* (FKBP12). The sirolimus-FKBP12 complex inhibits the mammalian target of rapamycin (mTOR) pathway by directly binding the mTOR Complex1 (mTORC1). Rapamycin inhibits mTOR signaling with IC_{50}<50 nM (85). Additionally, it has been shown to directly inhibit mTOR kinase activity with IC_{50} values of 1.74 μM independently of FKBP12, implying direct antitumour activity and profound translational repression at the high yet clinically relevant concentrations (92). Three of the 4 triple negative breast cancer cell lines showed marked sensitivity to rapamycin with IC_{50} values in the low nanomolar range (30-35 nM) (Table 5). However, the mesenchymal MDA-MB-231 cells were resistant to clinically relevant concentrations (Table 3.2).

Table 3.2. Sensitivity of triple negative cell lines to novel inhibitors.

Cell lines	**IC_{50} values ± SD**		
	Rapamycin	U0126	LY294002
HCC-1143	32.4 ± 8.5 nM	14.0 ± 2.3μM	9.3 ± 1.8μM
HCC-1937	30.4 ± 1.0 nM	18.2 ± 2.0μM	10.4 ± 2.0μM
MDA-MB-468	35.2±4.7nM	6.3± 0.2μM	8.2 ± 1.4μM
MDA-MB-231	>2.0 μM[a]	14.2 ± 3.2μM	10.7 ± 1.8μM

[a]An IC_{50} value was not determined as up to 2 μM rapamycin did not achieve 50% inhibition of growth.

LY 294002 [2-(4-Morpholinyl)-8-phenyl-4 H-1-benzopyran-4-one] is a potent and specific cell-permeable inhibitor of PI 3-kinases with IC_{50} values in the 1–50 μM

range (87). LY 294002 competitively inhibits ATP binding to the catalytic subunit of PI 3-kinases (93). The 4 triple negative cell lines treated with this inhibitor showed some sensitivity to LY 294002 with IC_{50} values ranging from 8-11 μM (Table 3.2).

Inhibition of MEK1/2 is an attractive strategy for therapeutic intervention in cancer because it has the potential to block inappropriate signal transduction, regardless of the upstream position of any oncogenic aberration and additionally ERK 1/2 are the only known substrates for MEK1/2 phosphorylation and signaling. The MEK Inhibitor, U0126, is a potent, cell-permeable inhibitor of MAPK (ERK 1/2) activation by inhibiting the kinase activity of MAP Kinase Kinase (MAPKK or MEK 1/2). U0126 inhibits MEK1 with an IC_{50} of 72 nM and MEK 2 with an IC_{50} of 58 nM (86) (94). The IC_{50} values obtained in the triple negative cell lines tested were within the μM range suggesting that these cell lines are resistant to MEK 1/2 inhibition *in vitro* (Table 3.2).

3.4 Summary

Among the targeted therapies tested, sunitinib malate was moderately active in the Her-2 amplified cell lines and the triple negative cell lines, and dasatinib showed marked activity in most of the triple negative cell lines (Table 3.1).

The panel of triple negative cell lines were tested with single-agent PI3K inhibitor, mTOR inhibitor and MEK1/2 inhibitor. Marked *in vitro* sensitivity was observed with inhibition of mTOR but not the MAPK pathway. Based on our *in vitro* evaluation of a range of inhibitors, dasatinib and rapamycin warrant further investigation in triple negative breast cancer, and sunitinib may have some activity in HER2 positive and triple negative breast cancer. Based on the non-overlapping targets of the multi-target kinase inhibitors tested the dasatinib sensitivity in the triple negative cell lines may be due to targeting SRC and EphA receptors.

Chapter 4. Preclinical evaluation of dasatinib in triple negative breast cancer cell lines

Results in part published in:*Tryfonopoulos D, Walsh S, Collins DM, Flanagan L, Quinn C, Corkery B, McDermott EW, Evoy D, Pierce A, O'Donovan N, Crown J, Duffy MJ. Src: a potential target for the treatment of triple-negative breast cancer.* ***Ann Oncol. 2011*** *Oct;22(10):2234-40.*

4.1 Introduction

Dasatinib showed promising activity in the triple negative breast cancer cell lines. Two previous studies have also shown that dasatinib is selectively active in triple negative/basal like breast cancer cells (33) (95). Based on the anti-proliferative effects of dasatinib in the triple negative cell lines, we chose to further evaluate the effects of dasatinib in combination with chemotherapy drugs in triple negative breast cancer cells *in vitro* and *in vivo*. We also investigated potential predictive markers of response to dasatinib.

4.2 *In vitro* evaluation of dasatinib in combination with chemotherapy

4.2.1 Dasatinib + cisplatin

The three dasatinib-sensitive triple negative breast cancer cell lines (MDA-MB-231, HCC-1143, HCC1937) and one dasatinib resistant cell line (MDA-MB-468) were treated with fixed-ratio combinations of dasatinib with cisplatin (Figure 4.1). Combined treatment with dasatinib and cisplatin was synergistic in the three dasatinib sensitive cell lines (CI<0.9) (Table 4.1). In the dasatinib resistant cell line MDA-MB-468, the combination of dasatinib with cisplatin had no additive effect. CI values were not calculated for MDA-MB-468, as dasatinib did not achieve greater than 50% inhibition at the concentrations tested.

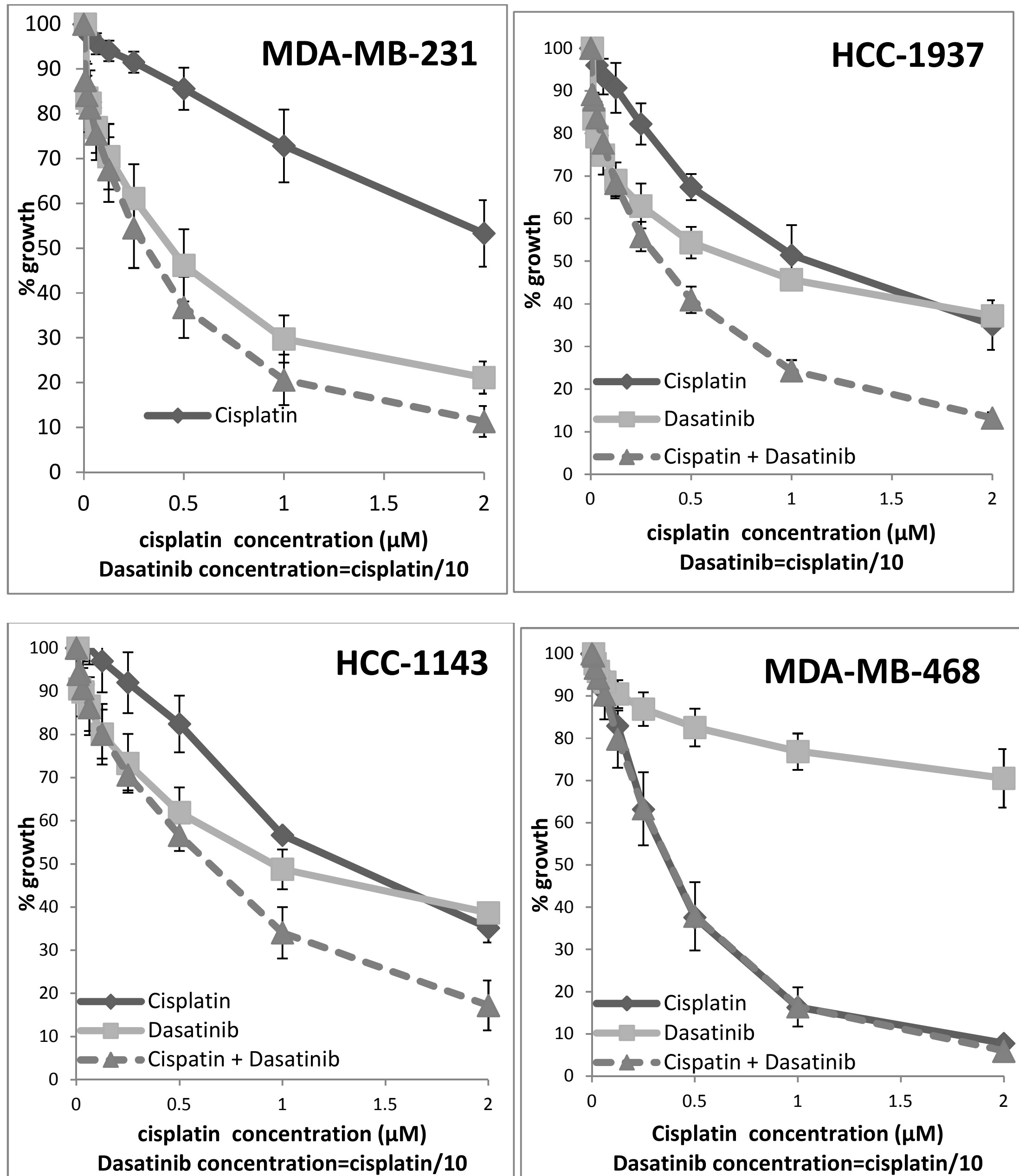

Figure 4.1. *In vitro* testing of dasatinib and cisplatin alone and in combination, in triple negative breast cancer cell lines. The dasatinib concentration (nM) is represented as a ratio of the cisplatin concentration, as indicated. Error bars represent the standard deviation of triplicate experiments.

Table 4.1. Combination index (CI) values determined for *in vitro* combinations of dasatinib with chemotherapy in triple negative breast cancer cell lines.

Cell line	CI at ED_{50} ± SD		
	Docetaxel + Dasatinib	5'-DFUR + Dasatinib	Cisplatin + Dasatinib
MDA-MB-231	0.77 ± 0.41	0.66 ± 0.19	0.79 ± 0.11
HCC-1937	0.83 ± 0.10	0.98 ± 0.40	0.57 ± 0.07
HCC-1143	1.45 ± 0.42	0.47 ± 0.04	0.74 ± 0.13

4.2.2 Dasatinib + 5'-DFUR

In MDA-MB-231 and HCC-1143 cells, combined treatment with dasatinib and 5'-5'-DFUR displayed synergy (CI<0.9), whereas the combination was additive in HCC-1937 cells (CI=0.98) (Figure 4.2, Table 4.1). In the dasatinib resistant cell line MDA-MB-468, the combination of dasatinib with 5'-5'-DFUR displayed a positive interaction. CI values were not calculated for MDA-MB-468, as dasatinib did not achieve greater than 50% inhibition at the concentrations tested.

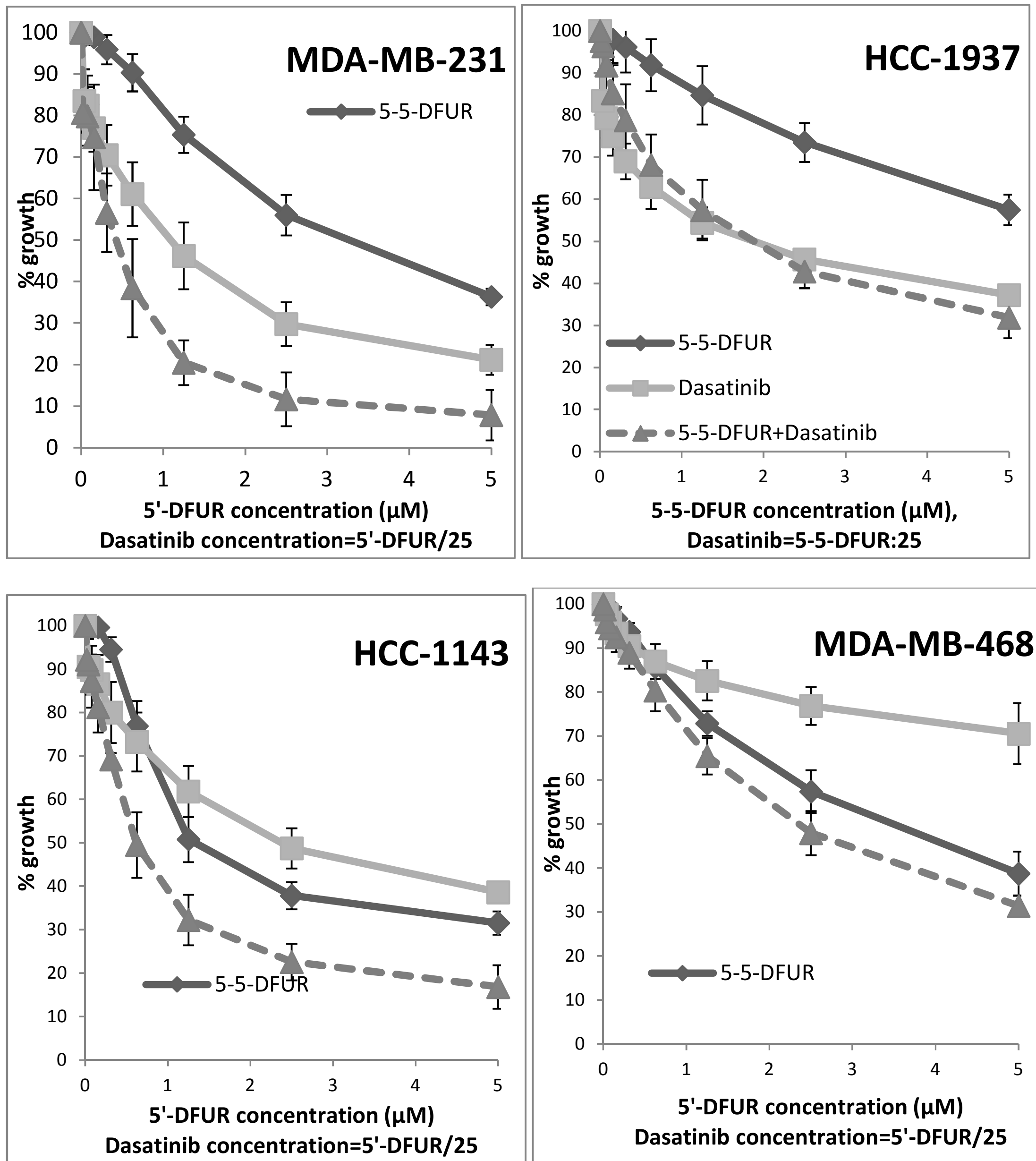

Figure 4.2.*In vitro* testing of dasatinib and 5'-DFUR alone and in combination in triple negative breast cancer cell lines. The dasatinib concentration (nM) is represented as a ratio of the 5'DFUR concentration, as indicated. Error bars represent the standard deviation of triplicate experiments.

4.2.3 Dasatinib + docetaxel

Dasatinib in combination with docetaxel displayed moderate synergy in MDA-MB-231 and HCC-1937 cells (CI<0.9), but was antagonistic in HCC-1143 cells (CI>1.1) (Figure 4.3, Table 4.1). In the dasatinib resistant cell line MDA-MB-468, the combination of dasatinib with docetaxel displayed a positive interaction. CI values were not calculated for MDA-MB-468, as dasatinib did not achieve greater than 50% inhibition at the concentrations tested.

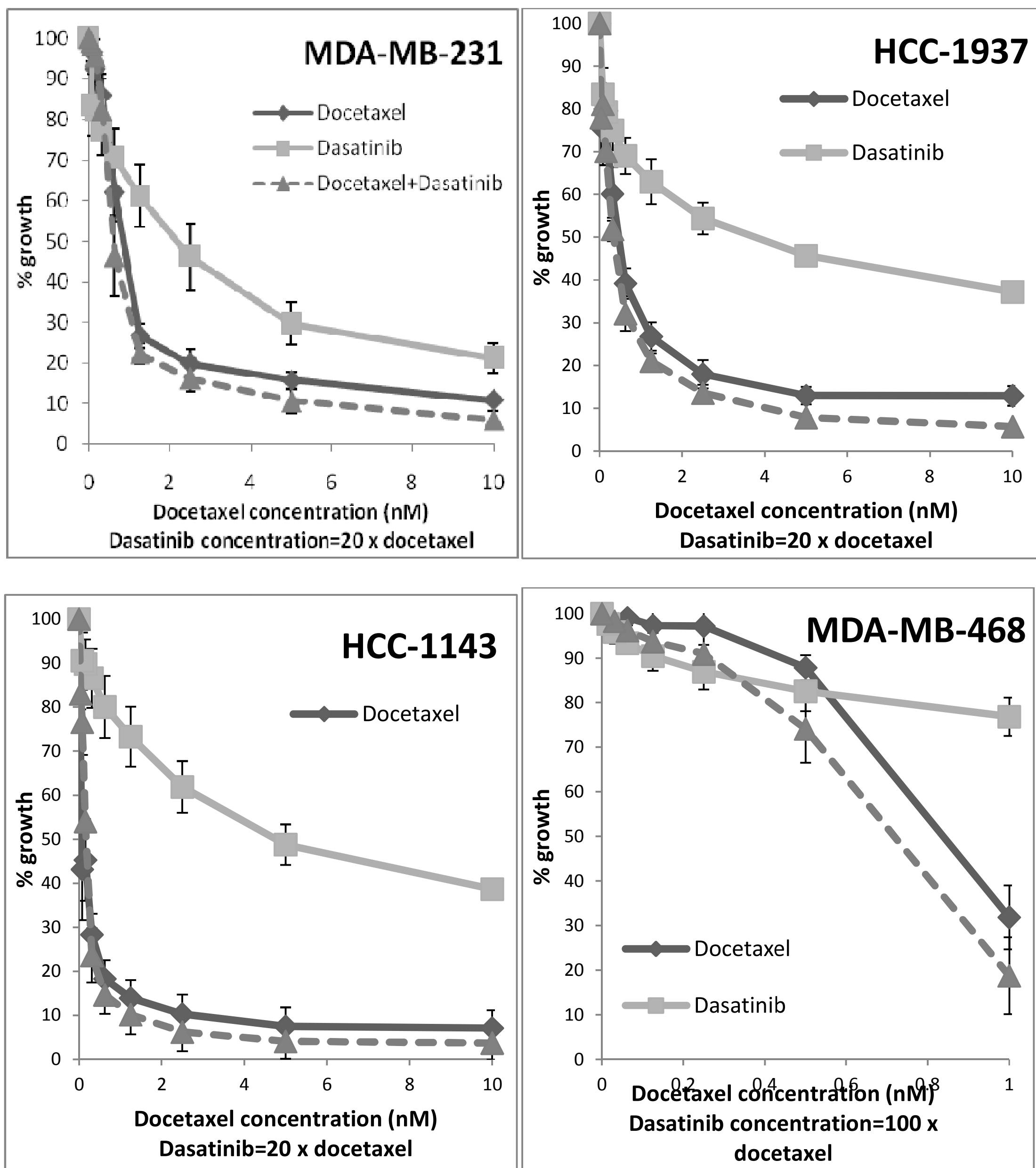

Figure 4.3. *In vitro* testing of dasatinib and docetaxel alone and in combination in triple negative breast cancer cell lines. The dasatinib concentration (nM) is represented as a ratio of the docetaxel concentration, as indicated. Error bars represent the standard deviation of triplicate experiments.

4.3 Biomarkers of dasatinib response

The levels of Src, phosphorylated Src and EphA2, which are targets of dasatinib, were analysed in a panel of triple negative and *HER2* amplified breast cancer cell lines (Figure 4.4A). No difference was observed in the level of Src or phosphorylated Src in dasatinib sensitive and resistant cells. However, EphA2 levels were significantly higher in the dasatinib-sensitive cell lines than in the resistant cells ($p = 0.0385$).

As other investigators (33) (95) found that expression of caveolin 1 and caveolin 2 were associated with response to dasatinib, we examined caveolin 1 and 2 protein levels in our panel of cell lines. Expression of both caveolin 1 and 2 proteins were also significantly higher in dasatinib-sensitive than dasatinib-resistant breast cancer cell lines ($p < 0.00002$ and < 0.0125, respectively) (Figure 4.4A-B).

Following 6-hours of treatment with dasatinib, levels of phosphorylated Src were significantly reduced in all cell lines, irrespective of sensitivity to dasatinib (Figure 4.5).

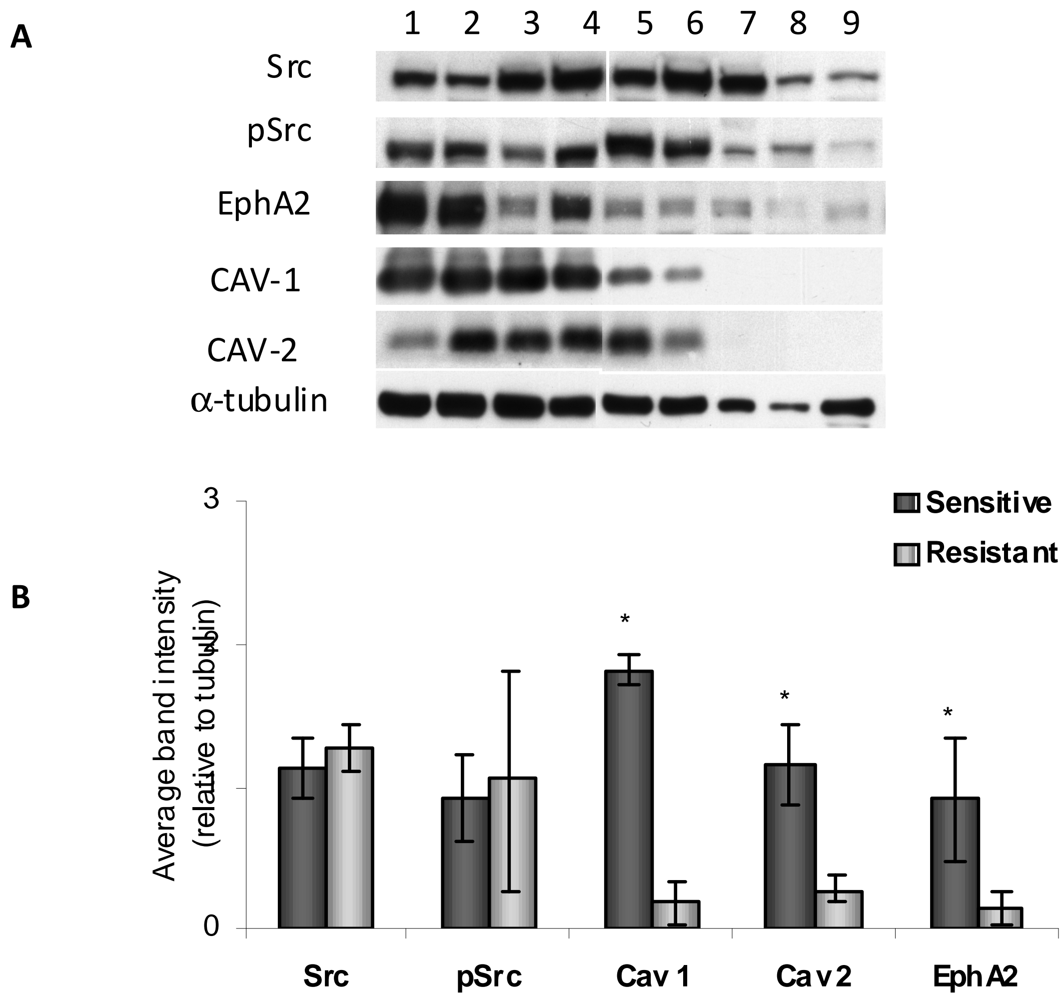

Figure 4.4.(A)Representative immunoblotting for Src, phospho-Src (Y419), EphA2, caveolin 1 (CAV-1) and caveolin 2 (CAV-2) in the breast cancer cell lines (*dasatinib sensitive*: 1. MDA-MB-231, 2. HCC1937, 3. HCC1143, 4. JIMT-1; *dasatinib resistant*: 5. MDA-MB-468, 6. BT20, 7. SKBR3, 8. BT474, 9. HCC1419). **(B)** Densitometry analysis of triplicate blots for Src, phospho-Src, CAV-1, CAV-2 and EphA2 (relative to tubulin) in dasatinib sensitive and resistant cell lines. * indicates $p<0.05$ using the Students T-test.

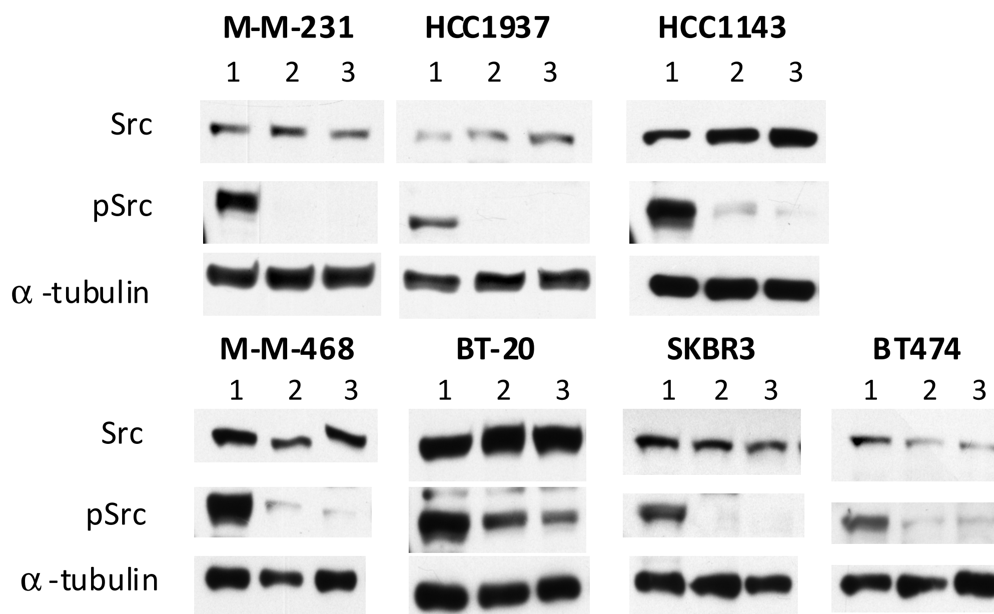

Figure 4.5 Immunoblotting for Src and phospho-Src (Y419) in (1) control cells and cells treated with (2) 100 nM or (3) 200 nM dasatinib for 6 hours (single determination).

4.4 *In vivo* testing of dasatinib plus chemotherapy in triple negative breast cancer

Based on previous results, the combination of dasatinib and cisplatin was chosen for *in vivo* study due to stronger synergy. In addition, since anthracycline and taxane treatment is commonly used as adjuvant chemotherapy in triple negative breast cancer other non-overlapping chemotherapeutics become of interest for use in the 1st or subsequent lines of treatment.

To determine the safe dose of cisplatin to be administered in combination with dasatinib, 8 groups of Balb-C mice (3 mice/group) were treated with fixed dose of dasatinib (15 mg/kg, po, 5 days/week) combined with escalating doses of cisplatin (1 mg/kg, 2 mg/kg, 3 mg/kg and 4 mg/kg, ip, once a week). No significant toxicities were observed with the 4 mg/kg dose, thus this dose was selected for the efficacy study.

For the efficacy study, MDA-MB-231 xenografts were treated with: (i) vehicle (50% propylene glycol), (ii) dasatinib (15 mg/kg), (iii) cisplatin (4 mg/kg), (iv) dasatinib and cisplatin and (v) no treatment. Treatment with cisplatin alone reduced tumour growth (Figure 4.6). Treatment with dasatinib alone also reduced tumour growth and a greater effect was observed for the combination of dasatinib with cisplatin. However, the vehicle alone also had a significant effect on tumour growth. Thus it was not possible to determine whether the enhanced effect of dasatinib plus cisplatin was due to dasatinib or caused by the vehicle.

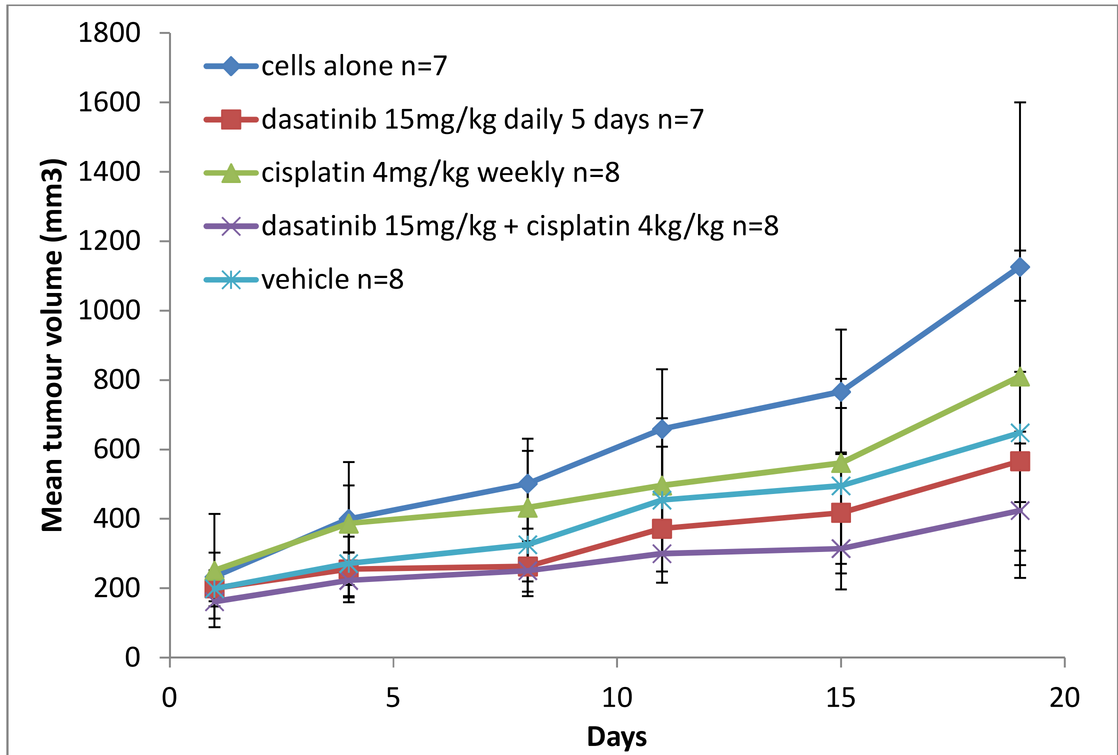

Figure 4.6 Growth of MDA-MB-231-Luc xenograft tumours in Balb-C nude mice following treatment with (i) vehicle (50% propylene glycol)(n=7), (ii) dasatinib (15 mg/kg)(n=7), (iii) cisplatin (4 mg/kg)(n=8), (iv) dasatinib and cisplatin (n=8) and (v) no treatment (n=7) for 19 days.

4.5 Summary

Our results presented in chapter 3 suggest that dasatinib sensitivity in triple negative cell lines may be due to targeting SRC or EphA receptors. Western blot analysis showed no differential expression of SRC or phosphorylated SRC between dasatinib sensitive and dasatinib resistant cell lines. On the contrary, expression of EphA 2 was higher in the dasatinib-sensitive cell lines, suggesting that EphA2 inhibition may contribute to dasatinib sensitivity in the triple negative cells.Caveolin-1 and -2 expression were also higher in the dasatinib-sensitive cell lines. Finally, levels of phosphorylated SRC were lower following dasatinib treatment in all cell lines (dasatinib-sensitive and –resistant), indicating that although SRC is a target of dasatinib it is not a valid pharmacodynamic biomarker of response in triple negative cells.

Combined treatment with dasatinib and cisplatin was synergistic in 3 out of 4 triple negative cell lines. The combination of dasatinib with 5'-DFUR and docetaxel was also additive in 3 out of 4 triple negative cell lines. Therefore the best chemotherapy partner for dasatinib in triple negative cell lines proved to be cisplatin, which was chosen for the *in vivo* studies. Unfortunately the results of the *in vivo* study were inconclusive due to an unanticipated effect of the vehicle alone on tumour growth, even though the vehicle used has been used in previously published studies with dasatinib. Thus, the *in vivo* testing would need to be repeated with an alternative vehicle for the dasatinib.

Chapter 5. Evaluation of combinations of sunitinib with current therapies for HER2 positive and triple negative breast cancer

5.1 Introduction

Based on previous results (See Chapter 3) and on the strong interaction between the Her-2 and VEGF pathways, sunitinib malate was the most appropriate agent to use in combination with trastuzumab for the Her-2 amplified cell lines. For the triple negative cells, was tested in combination with cisplatin.

5.2 Sunitinib plus trastuzumab in HER-2 positive breast cancer cell lines

In vitro, some of the Her-2 positive cell lines (SKBR-3, BT-474) showed moderate sensitivity to sunitinib malate (Table 3.1). Sunitinib was tested in fixed ratio combinations with trastuzumab in two trastuzumab sensitive cell lines (SKBR3 and BT474) and two trastuzumab-resistant cell lines (JIMT-1 and HCC-1419). Combined treatment with sunitinib and trastuzumab showed a slightly improved response compared to either drug alone, in the 2 trastuzumab sensitive cell lines (SKBR3 and BT474) (Figures 5.1 and Table 4). No significant enhancement was observed in the trastuzumab resistant cell lines (JIMT-1 and HCC-1419).

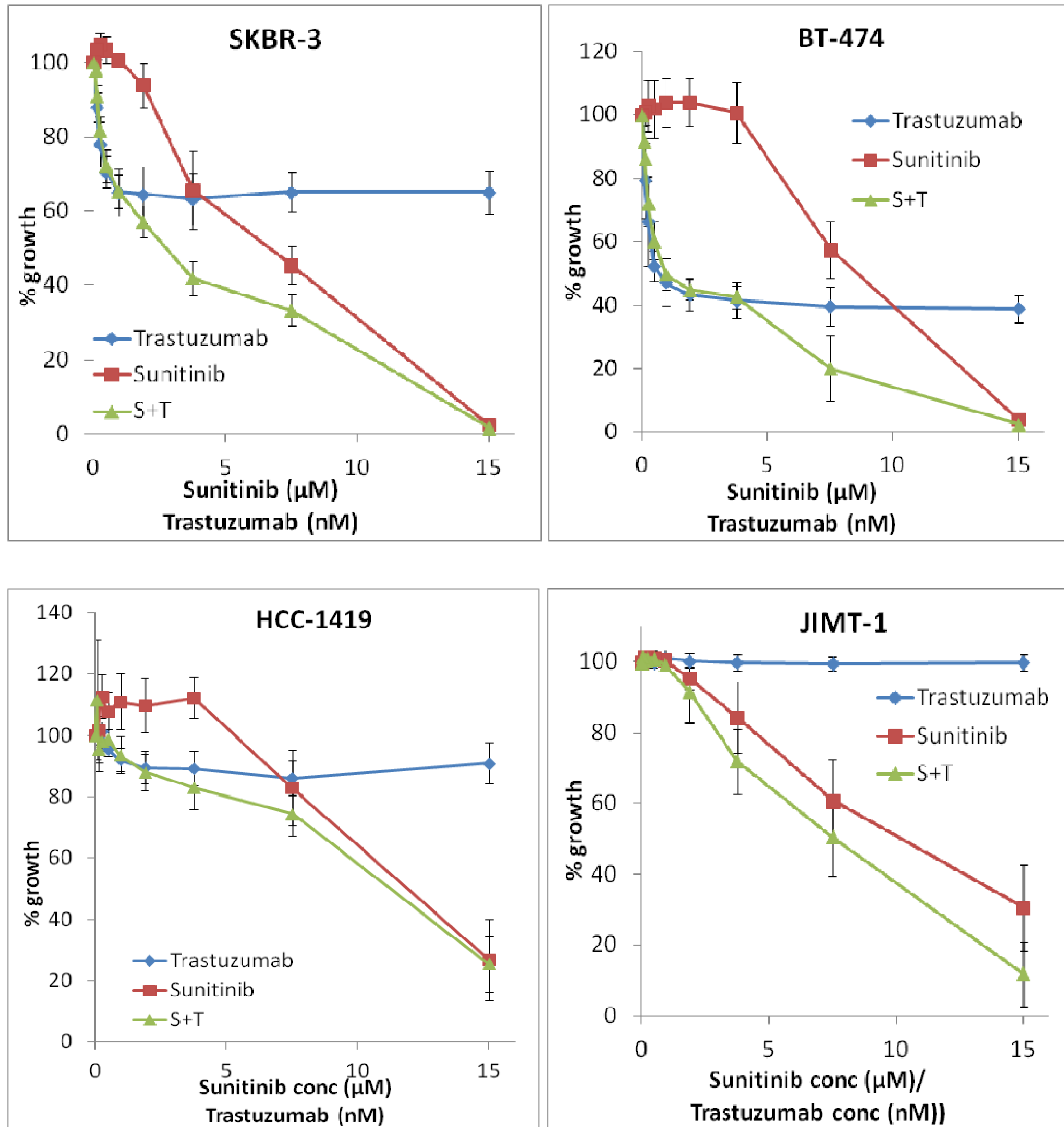

Figure 5.1. Fixed ratio combination assays with sunitinib and trastuzumab in four Her-2 positive breast cancer cell lines. Error bars represent the standard deviation of triplicate experiments.

Table 5.1. Combination treatment with sunitinib and trastuzumab in Her-2 positive cell lines. Triplicate experiment results.

Cell Line	IC_{50} (±stddev)	% Growth (± stddev)		
	Sunitinib (μM)	Sunitinib (7.5 μM)	Trastuzumab (7.5 nM)	Sunitinib (7.5 μM) + trastuzumab (7.5 nM)
SKBR-3	5.4 ± 0.9	45.4 ± 5.1	65.2 ± 5.2	33.3 ± 4.3*
JIMT-1	7.3 ± 1.9	60.9 ± 11.3	99.5 ± 1.9	50.4 ± 10.9
BT-474	5.0 ± 1.3	57.6 ± 6.1	39.6 ± 6.2	20.1 ± 10.1*
HCC-1419	8.4 ± 2.9	90.9 ± 16.3	86.1 ± 5.6	74.5 ± 7.3

5.3 Sunitinib and cisplatin in triple negative breast cancer cell lines

Sunitinib malate showed modest *in vitro* activity in the triple negative cell lines. Since angiogenesis is a possible target in triple negative breast cancer we combined sunitinib with cisplatin, an active chemotherapy drug in the triple negative cell line which was more sensitive to sunitinib (MDA-MB-231) and in the BRCA-1 deficient cell line (HCC-1937) which is more sensitive to platinum agents. The combination of these agents was antagonistic in both cell lines with combination indexes (CI) over 1.1(Table 5.2 and Figure 5.2).

Table 5.2. Combinations of cisplatin with sunitinib in triple negative cell lines.

Cell line	**Sunitinib + Cisplatin CI at ED_{50} ± SD**
MDA-MD-231	2.01 ± 0.62
HCC-1937	1.37 ± 0.9

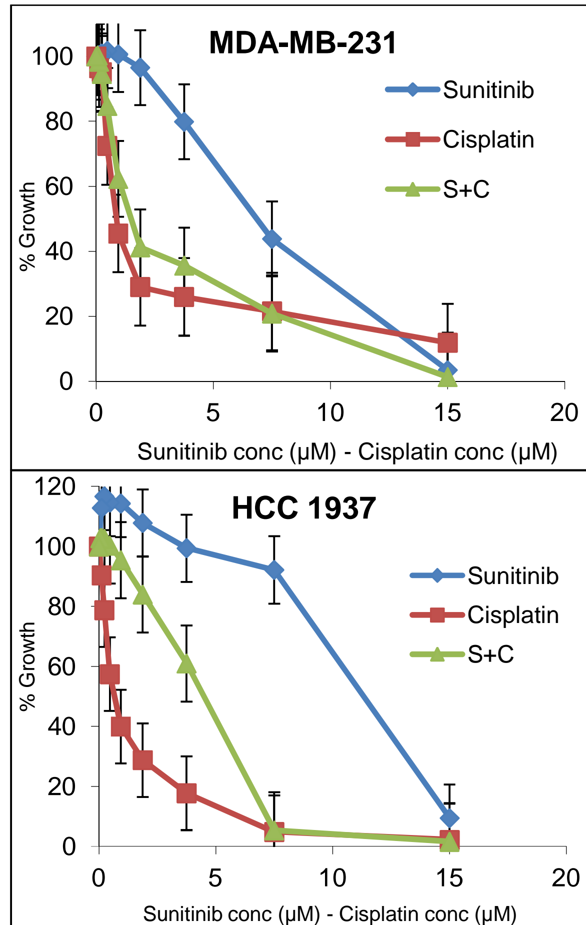

Figure 5.2. Fixed ratio combination assays with cisplatin and sunitinib in triple negative cell lines. Error bars represent the standard deviation of triplicate experiments.

5.4 Summary

In trastuzumab sensitive Her-2 amplified cell lines, the combination of sunitinib malate and trastuzumab was more active than either drug alone. Sunitinib would also be expected to have anti-angiogenic effects in vivo, in addition to its direct anti-tumour effects. Indeed a preliminary exploratory study of trastuzumab with docetaxel and sunitinib showed encouraging anti-tumour activity in the first line treatment of advanced HER2 positive breast cancer (96).

In the two triple negative cell lines, the combination of sunitinib and cisplatin was antagonistic. Sunitinib is currently being tested in a number of clinical trials in combination with cisplatin-based regimens in other cancer types but our *in vitro* results suggest this would not be a beneficial combination in triple negative breast cancer.

Chapter 6. Discussion

6.1 Discussion

Translational research has provided us with an abundance of molecularly targeted agents. Some of these agents have led to clinical benefit, most of them though have failed to provide benefit, possibly due to premature clinical use and lack of patient selection. This project aimed to systemically assess targeted agents and appropriate combinations in Her-2 amplified and triple negative cell lines *in vitro*. Using the most active targeted agent we attempted to identify reliable biomarkers for patient selection and response assessment. Finally we tested the most active combination *in vivo* using xenograft nude mice models.

6.2 Her-2 amplified cell lines

Trastuzumab monotherapy provides long-term responses in a minority of breast cancer patients (55). Several studies have reported data on mechanisms of trastuzumab resistance and alternative escape pathways (97) (46) (98) (99) (100). In this study we tested a number of multi-target kinase inhibitors to determine if combination therapy could improve response to trastuzumab. In general, the Her-2 positive cell lines displayed resistance to the inhibitors tested, with the exception of the Her-2 amplified cell line JIMT-1 which was sensitive to dasatinib. Interestingly, this cell line has been classified as borderline between basal and Her-2 amplified as it expresses basal and luminal cytokeratins (101). Sunitinib had modest activity, at concentrations higher than biologically achievable, as the mean maximum plasma concentration (Cmax) of the standard oral once-daily dose of 50 mg/day sunitinib is reported to be 0.18 μM (102). However, as trastuzumab resistance has been associated with higher levels of VEGFR and sunitinib targets VEGFR-1,-2,-3, trastuzumab was tested in combination with sunitinib. The combination treatment increased the effectiveness compared to either drug alone. Even the trastuzumab-resistant Her-2+ cell lines (JIMT-1, HCC-1419) displayed greater growth inhibition, suggesting that the VEGF pathway may play a role in resistance to trastuzumab. Other investigators have found similar effects with dual inhibition of VEGF and Her-2 *in vitro* and *in vivo* (103). Furthermore, this combination may have greater

benefit *in vivo* due to the anti-angiogenic effects of sunitinib, which are not assessed using in vitro proliferation assays. However, the emergence of novel, potentially more effective targeting agents (T-DM1, pertuzumab, etc) and the premature discontinuation of the sunitinib clinical trials in breast cancer reduced our interest in further evaluating this combination.

6.3 Triple negative cell lines

Previous studies have shown that triple negative breast cancer cell lines are more sensitive to dasatinib than luminal or Her-2 positive breast cancer cell lines (33). Our results validated this observation, as triple negative cell lines were more sensitive compared to Her-2 positive cell lines. Among the triple negative cell lines, the MDA-MB-468 and the BT-20 cells were resistant to dasatinib treatment. Interestingly both of these cell lines have amplified EGFR. There is significant evidence for cross-talk between EGFR and Src signalling, and Src has been implicated in resistance to EGFR targeted therapies. However, it is not yet known whether increased EGFR signalling can contribute to resistance to EGFR inhibition. The mesenchymal MDA-MB-231 cells were the most sensitive, followed by the basal BRCA-1 mutated HCC-1937 and the basal HCC-1143. These results are consistent with the results obtained by Finn et al (33), who also showed that cell lines, including the MDA-MB-231 cells, which have undergone an epithelial-to-mesenchymal transition displayed the greatest sensitivity to dasatinib. Our laboratory has also shown that dasatinib inhibited cellular invasion and migration in the MDA-MB-231 cell line (104).

We compared sensitivity to dasatinib with sensitivity to other multi-target kinase inhibitors and investigated the specific targets that may be responsible for sensitivity to dasatinib. The molecular targets of dasatinib are Abl, PDGFR, c-Kit, Src and EphA receptors (Table 3.1). Abl is a target of imatinib mesylate, PDGFR and c-Kit are targets of imatinib, sunitinib and pazopanib, therefore Src and EphA receptors are non-overlapping targets which may contribute to the observed sensitivity of triple negative cell lines to dasatinib and resistance to the other kinase inhibitors. Other investigators, using gene expression profiling have

also identified among others EphA2 and Src as targets of dasatinib in triple negative breast cancer (95) (33) (30) (105). However, all triple negative cell lines tested were resistant to PP2, a laboratory grade selective Src inhibitor. These results suggest that targeting EphA receptors contributes to sensitivity to dasatinib in triple negative breast cancer cells. Consistent with this hypothesis, protein expression analysis in the panel of cell lines showed that dasatinib-sensitive cell lines had higher levels of expression of EphA2 compared to dasatinib-resistant cell lines. EphA2 receptor overexpression has been detected in a variety of cancers, including the majority of breast cancers (106) (107). In addition, higher levels of EphA2 protein have been detected in triple negative breast cancer cell lines as opposed to nontransformed epithelial cells. Upregulation of Eph receptor signalling has been implicated in tumour growth, invasion, resistance to anoikis and neovascularisation (108). In addition,we found elevated caveolin-1 and -2 levels in the dasatinib-sensitive cells. Caveolins interact closely with the Src family kinase and have been identified as markers of basal breast cancer (109) (110).

There was no difference in expression of Src and phosphorylated Src between dasatinib sensitive and dasatinib resistant cells. Src expression or activation thus does not appear to correlate with sensitivity to dasatinib. Other investigators have also reported a lack of correlation between Src/phosphorylated-Src levels and dasatinib growth inhibitory ability in breast and other cancers (95) (105) (111) (112) (113). With few exceptions though (hormone receptors, Her-2, etc) measurement of target expression does not correlate effectiveness. Even within a panel of Her-2 amplified cell lines, the levels of Her2 protein do not predict sensitivity to trastuzumab (98). Thus, these findings do not necessarily rule out Src as a target of dasatinib in triple negative breast cancer. Src holds part in a complex network of interacting proteins, including EGFR, Her-2, PDGFR, VEGF, etc and activation of this pathway is very common in cancer (114). In order to rule out Src as an important target of dasatinib in triple negative breast cancer it would be necessary to test for sensitivity to dasatinib in an Src negative cell line. All of the cell line tested in this

study expressed Src kinase to varying degrees. Evaluating the effect of Src kinase and/or EphA2 knockdown in triple negative breast cancer cells may also help to elucidate the individual importance of these two targets in sensitivity to dasatinib. Interestingly, in a collaborative study, we compared Src expression in triple-negative and non triple-negative patient tumour samples and showed that although both cytoplasmic and membrane Src were expressed in the majority of breast cancers, the frequency of expression was significantly higher in triple-negative than in non triple-negative samples, suggesting that Src is a potential target for the treatment of patients with this subtype of breast cancer (104).

Other groups have reported predictive gene signatures (EphA2, CAV1, CAV2, ANXA1, PTRF and IGFBP2) (95) and (CAV1, Moesin(MSN) and *yes*-associated protein (YAP1)) (33). All these genes are either targets for dasatinib or substrates for Src kinases and are particularly expressed in triple-negative and basal-type breast cancer cell lines. In our study, in agreement with previous reports showing an association between dasatinib sensitivity and high mRNA levels for *EPHA2, CAV1* and *CAV2*, we found similar relationships at the protein level, supporting those 3 genes as biomarkers of dasatinib response (figure 3 A,B) (33) (95) (113).

The activity of dasatinib is being tested in several clinical trials. Preliminary results of a phase II study using single-agent dasatinib in locally advanced or metastatic triple negative breast cancer patients reported modest results, with a response rate of 4.6% and clinical benefit rate of 9.3% (115). We sought the most active chemotherapeutic agent to combine with dasatinib in triple negative breast cancer cell lines, in order to optimise its efficacy. Docetaxel and 5'-deoxy-5'-fluoruridine (a prodrug of 5'-fluoruracil and a metabolite of capecitabine) were chosen as they are commonly used in 1st-line treatment of metastatic breast cancer, whereas cisplatin was also tested due to its emerging activity in triple negative breast cancers (116) (13). Based on our data (Table 4.1 and figures 4.1-4.3) there was synergistic activity with all 3 combinations in most cases, but the strongest synergism was observed with the combination of dasatinib and

cisplatin in the 3 dasatinib-sensitive cell lines. In solid tumours dasatinib has been reported to synergise with capecitabine and doxorubicin *in vitro* (105) (115). A phase I study of dasatinib in combination with capecitabine in metastatic breast cancer patients showed encouraging activity (117). Other investigators have reported that in colon cancer patients, oxaliplatin activates Src through a ROS-dependent mechanism and combination treatment with dasatinib is synergistic in a cell-line dependent manner, with the level of Src activation correlating with extent of synergy *in vitro* (118). To our knowledge there are no reports of synergistic activity of dasatinib with cisplatin, especially in the highly relevant area of triple negative breast cancer. This was the reason this combination was chosen for *in vivo* study. Unfortunately the results of the *in vivo* study were inconclusive due to an unanticipated effect of the vehicle alone on tumour growth, even though the vehicle used has been used in previously published studies with dasatinib (119) (120). No explanation could be found in the relevant literature explaining this unexpected event. Thus, the *in vivo* testing would need to be repeated with an alternative vehicle for dasatinib.

Finally, testing of a panel of triple negative cell lines with novel cell signalling inhibitors of MEK 1/2, m-TOR and PI3-K as single agents showed that mTOR is a more attractive treatment target compared to PI3-K and the MAPK pathway, at least *in vitro.* This is not surprising, as loss of PTEN is a common finding in triple negative breast cancer thus leading to increased activation of mTOR (121). Targeted treatment against mTOR is currently under extensive clinical study in triple negative breast cancer. The MAPK pathway is equally important in triple negative breast cancer (40) (41). Lack of activity of single agent MEK 1/2 inhibitor is not unexpected as the MAPK pathway is often coactivated along with the PI3K-Akt-mTOR pathway and therefore combination treatment might be needed (42).

6.4 Conclusions

Triple negative breast cancer is still a highly unmet medical need and therefore target selection and preclinical testing are needed to provide rational drug

combinations for clinical testing. Based on our *in vitro* evaluation of a range of inhibitors:

- dasatinib and rapamycin are active and therefore warrant further investigation in triple negative breast cancer.
- protein levels of *EPHA2, CAV1* and *CAV2* appear to be useful biomarkers of dasatinib response.
- the combination of dasatinib and cisplatin exhibited synergy in inhibiting growth of triple negative cell lines.

Future clinical trials combining dasatinib with cisplatin and testing the validity of *EPHA2, CAV1* and *CAV2* as predictive biomarkers may thus be warranted in patients with advanced triple negative breast cancer.

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DISSEMINATION OF RESULTS

1.Possible targets for dasatinib sensitivity in triple negative breast cancer. Poster (Abstract 576) 6th European Breast Cancer Conference, Berlin 2008

2. Preclinical evaluation of sunitinib, alone and in combination with trastuzumab, in HER2 positive breast cancer. Poster publication, ASCO 2008

3. Possible targets for dasatinib sensitivity in triple negative breast cancer. Poster, 1st IMPACT conference, Brussels 2009

4. Activity of dasatinib with chemotherapy in triple negative breast cancer cells, Poster publication(e14605), ASCO 2009

5. Src: a potential target for the treatment of triple-negative breast cancer, 10, 2011, Ann Oncol, Vol. 22, pp. 2234-40.

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