Development of targeted anticancer agents using novel N-alkylisatin derivatives

Vineesh Indira Chandran

University of Wollongong

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Development of Targeted Anticancer Agents using Novel $N$-Alkylisatin Derivatives

A thesis submitted in partial fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

Illawarra Health and Medical Research Institute (IHMRI)
School of Biological Sciences
UNIVERSITY OF WOLLONGONG

By

Vineesh Indira Chandran
B.Sc. Biochemistry, 2000
CERTIFICATION

The work described in this thesis does not contain any material that has been submitted for the award of any higher degree in this or any other University and to the best of my knowledge contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

Vineesh Indira Chandran

17/09/2012

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<tbody>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial Mesenchymal Transition</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>CdK</td>
<td>Cyclin dependent Kinases</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>HER</td>
<td>Human Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>RTKs</td>
<td>Receptor Tyrosine Kinases</td>
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<tr>
<td>HB-EGF</td>
<td>Heparin-Binding EGF-like Growth Factor</td>
</tr>
<tr>
<td>NRG 1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>NRG2</td>
<td>Neuregulin 2</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>PAS</td>
<td>Plasminogen Activation System</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue Plasminogen Activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase Plasminogen Activator</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteases</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>ATF</td>
<td>Amino Terminal Fragment</td>
</tr>
<tr>
<td>GFD</td>
<td>Growth Factor Domain</td>
</tr>
<tr>
<td>RCL</td>
<td>Reactive Centre Loop</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low Density Lipoprotein Receptor</td>
</tr>
<tr>
<td>MBC</td>
<td>Metastatic Breast Cancer</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal Antibodies</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>TKIs</td>
<td>Tyrosine Kinase Inhibitors</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myeloid Leukemia</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>LDCs</td>
<td>Ligand-Drug Conjugates</td>
</tr>
<tr>
<td>RME</td>
<td>Receptor-Mediated Endocytosis</td>
</tr>
<tr>
<td>SCC</td>
<td>Small Cell Carcinomas</td>
</tr>
<tr>
<td>T-DM1</td>
<td>Trastuzumab-Maytansinoid</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-Dependent Cytotoxicity</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum Tolerated Dose</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>SDP</td>
<td>Site-Directed Pegylation</td>
</tr>
<tr>
<td>DAR</td>
<td>Drug per Ligand Ratio</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour Associated Antigen</td>
</tr>
<tr>
<td>NAI-1</td>
<td>5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin</td>
</tr>
<tr>
<td>NAI-2</td>
<td>5,7-dibromo-N-(p-methoxylbenzyl)isatin</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma Protein</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>TfRs</td>
<td>Transferrin Receptors</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray Ionisation-Mass Spectrometry</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>SCCL</td>
<td>Small Cell Carcinoma of the Lung</td>
</tr>
</tbody>
</table>
NAI  \( N \)-Alkylisatin
DMSO  Dimethyl Sulfoxide
PBS  Phosphate Buffered Saline
pI  Isoelectric Point
RT  Room Temperature
MFI  Mean Fluorescence Intensity
DMF  Dimethylformamide
NHS  \( N \)-hydroxysuccinimide
DCC  Dicyclohexylcarbodiimide
BSA  Bovine Serum Albumin
SA  Sinapinic Acid
ACN  Acetonitrile
HMW  High Molecular Weight
TB  Terrific Broth
SEC  Size Exclusion Chromatography
FA  Formic Acid
TRZ  Trastuzumab
Ab  Antibody
EMEA  European Medicines Agency
PEG-PEI  Polyethylenimine-Polyethylene Glycol
SPDP  \( N \)-succinimidyl-3-(2-pyridyldithio) propionate
Q-TOF-MS  Quadrupole-Time of Flight-Mass Spectrometry
MALDI-TOF MS  Matrix-Assisted Laser Desorption/Ionisation-Time-of-Flight Mass Spectrometry
HER-2/ErbB2  Human Epidermal Growth Factor Receptor Type 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>HER-3</td>
<td>Human Epidermal Growth Factor Receptor Type 3</td>
</tr>
<tr>
<td>HER-4</td>
<td>Human Epidermal Growth Factor Receptor Type 4</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase Plasminogen Activator Receptor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor Type 1</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen Activator Inhibitor Type 2</td>
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<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>NAI-1-succ</td>
<td>6-carboxypropanoyl, 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin</td>
</tr>
<tr>
<td>NAI-2-imine</td>
<td>3-(4-((5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene)amino)phenyl)propanoic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>HC</td>
<td>Heavy Chain</td>
</tr>
<tr>
<td>LC</td>
<td>Light Chain</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>mPEG</td>
<td>Maleimide Functional PEG</td>
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</table>
I take this opportunity to express my deepest gratitude to A/Prof. Marie Ranson. Marie, the kind of freedom and support that I got from you during the course of my PhD has really helped me build confidence and self belief from someone who was completely confused, disintegrated and out of place (as I was out of my comfort zone, away from family, away from friends and away from a friendly atmosphere) when I joined your lab. I know, not many students who come through a Masters route manages to get an opportunity to work with you, but I was lucky to get a chance and I really appreciate your willingness to offer me a project. Also, a big thank you for the Matching Postgraduate Research Scholarship and the International Postgraduate Tuition Award.

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Targeted therapy involves selective targeting of tumour and/or tumour associated cells using agents that recognise specific biomarkers which are characteristic of the tumour cell population. The use of ligand-drug conjugates (LDCs) for targeted therapy includes agents that combine the selectivity and specificity of biomarker ligands with the high potency of chemotherapeutic drugs. This not only causes effective reduction of tumour growth, but also minimises off target cell killing and delays the development of resistance which otherwise arises from prolonged exposure to non-specific drugs.

This study focussed on the development of LDCs incorporating novel \( N \)-alkylisatin derivatives. We have previously reported the cytotoxic activity of a series of \( N \)-alkylisatin derivatives that destabilise microtubules and induce apoptotic cell death in a range of cancer cell lines. In this study, two of the most potent \( N \)-alkylisatin derivatives, 5,7-dibromo-\( N-\)(\( p \)-hydroxymethylbenzyl)isatin (NAI-1) and 5,7-dibromo-\( N-\)(\( p \)-methoxybenzyl)isatin (NAI-2), functionalised with an esterase-labile succinate (NAI-1-succ) and an imine (NAI-2-imine) functional linker respectively, were conjugated to well described tumour ligands, namely, transferrin (Tf), plasminogen activator inhibitor type 2 (PAI-2), and trastuzumab (TRZ). These were tested against various human breast carcinoma cell lines that differed in their cell surface expression of relevant targets for these ligands. Both NAI-1-succ-Tf and NAI-2-imine-Tf conjugates were selectively toxic against the cell lines tested. NAI-1-succ-Tf conjugate (IC\(_{50} \) 0.75 \( \mu \)M) was greater than 10 \( \times \) more active compared to the parent compound (IC\(_{50} \) 7.67 \( \mu \)M) against the high Tf receptor (TfR) expressing SK-BR-3 cells after 48 h incubation. The NAI-2-imine-Tf conjugate was equipotent to the parent NAI-2 compound against cells...
expressing moderate to high TfR levels. As the conjugate incorporating the imine-based linker system was less selective for their target, evident by their equipotency to the parent NAI-2 and NAI-2-imine compounds, the imine-based linker system was not used in the development of other LDCs.

Previous studies performed in our laboratory found the NAI-1-succ-PAI-2 conjugate to be equivalent in efficacy to the free NAI-1 compound at 1/20th of the dose, in a metastatic, orthotopic human breast tumour xenograft mouse model. However, our laboratory has also shown that PAI-2 is rapidly excreted from the body. Therefore, to improve the pharmacokinetic and potential tumour uptake properties, a modified form of PAI-2 containing only three potential sulfhydryl sites, C161S-PAI-2, was covalently modified using polyethylene glycol molecules (PEGs) functionalised with a maleimide functional linker (mPEG) to form mPEG-C161S-PAI-2. Purification of mPEG-C161S-PAI-2 by ion exchange was achieved after a series of optimisations and NAI-1-succ was then conjugated to the purified mPEG-C161S-PAI-2 to form mPEG-C161S-PAI-2-NAI-1-succ conjugate. In vitro studies showed mPEG-C161S-PAI-2-NAI-1-succ conjugate to be ~3.5 × more potent than the free NAI-1 and showed selectivity against high uPAR expressing breast cancer cells.

Recent studies have found over amplification of HER-2 and uPA receptors in 70% of HER-2 amplified advanced invasive breast carcinoma cells. Further studies targeting HER-2 and uPA showed that depleting both HER-2 and uPA receptors suppressed cell growth and induced cell apoptosis in SK-BR-3 and ZR-75-1 mammary epithelial cells that over-express HER-2 and uPA receptors. In this direction, NAI-1-succ-TRZ conjugate was prepared by reacting the commercially available recombinant humanised
monoclonal antibody (mAb) TRZ directed against HER-2 receptors, with a 40-fold molar excess of NAI-1-succ in sodium phosphate buffer (pH 6.0). The resultant NAI-1-succ-TRZ conjugate (IC$_{50}$ 0.61 $\mu$M) was ~8 $\times$ more toxic than free NAI-1 (IC$_{50}$ 5.19 $\mu$M) and was highly selective against high HER-2 expressing SK-BR-3 cells. This highly potent NAI-1-succ-TRZ along with NAI-1-succ-C161S-PAI-2 was utilised in a novel combination targeting approach (also called “double targeting”) to specifically target metastatic breast cancer cells that over-express uPA/uPAR and HER-2 receptors. 

*In vitro* double targeting assays using these agents against high uPAR and HER-2 expressing ZR-75-1 breast cancer cells showed increased cell death. This warrants further *in vivo* study of NAI-1-succ-TRZ and NAI-1-succ-C161S-PAI-2 in combination in appropriate mouse models to ascertain their potential synergistic effect in reducing tumour growth compared to treatment with each drug alone. This approach is expected to result in superior efficacy over single agent therapy and due to minimal drug exposure, delay the development of drug resistance.

On the whole, this study successfully developed and proved the selective potency of 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin functionalised with an ester linker in ligand-drug conjugates, namely mPEG-C161S-PAI-2-NAI-1-succ and NAI-1-succ-TRZ *in vitro*. The utility of these conjugates in a novel double targeting treatment strategy to simultaneously target highly characteristic tumour-specific biomarkers in breast cancer warrants further development using appropriate animal tumour models.
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CHAPTER ONE

INTRODUCTION
INTRODUCTION

1.1. General introduction

Disruption of normal cell division and apoptosis is integral to the pathophysiology of the state of malignancy popularly known as “cancer”, and is usually manifested in a state of diverse morphological and physiological changes [1-3]. Current statistics indicate that cancer causes one in eight deaths worldwide causing more deaths than AIDS, tuberculosis, and malaria combined. It is estimated that by 2050, the global burden of cancer is expected to grow to 27 million new cases per annum and there will be as many as 17.5 million cancer deaths (International Agency for Research on Cancer, 2008)\(^1\). In Australia alone, an estimated 114,000 new cases of cancer were diagnosed in 2010 with approximately 43,000 cancer deaths (Australian Institute of Health and Welfare and Australasian Association of Cancer Registries, 2011)\(^2\).

Over the years, breast cancer has been tackled with a series of treatment modalities ranging from local surgical removal to systemic chemotherapeutic agents to modified recombinant monoclonal antibodies and small molecule inhibitors. Depending on the type and intensity of the malignant tumour manifestation, these treatment modalities have been applied in both single and combination mode. However, more often than not, development of resistance in the form of disease relapse puts patients at increased risk for not responding to subsequent treatment optimisations. In a bid to confront this issue, there have been intensive efforts to decipher alternative treatment strategies that can

\(^1\) http://globocan.iarc.fr/factsheets/cancers/all.asp
increase the therapeutic index of chemotherapeutics and also reduce the toxicity caused to normal cells. In this direction, studies are underway to develop antibody-drug conjugates or protein-drug conjugates that combine the highly potent nature of chemotherapeutic agents and the target specificity of monoclonal antibodies (mAbs)/proteins with inhibitory action [4; 5]. It can be expected that such targeted delivery of potent chemotherapeutics would not only increase the therapeutic index of this class of drugs, but also would significantly delay the development of resistance against these anticancer agents. To this end the use of novel drugs such as N-alkylisatin derivatives [6] holds immense significance.

This chapter provides an account of established chemotherapeutics, their mechanism of action and efficacy in both single and combination modes, and how novel chemotherapeutic drugs may be incorporated into such strategies. The use of such drugs in emerging targeted therapies will also be reviewed with a description of validated drug targets and development of ligand-drug conjugates against these targets, including double targeting strategies and their advantages.

1.2. Tumourigenesis and metastasis – overview

Metastasis is responsible for 90% of all cancer-related mortalities [7]. Metastasis is a multi-stage process whereby tumour cells escape from their primary site or site of origin and invade or colonise other immediate or distant locations in the body [8-13] (Figure 1.1).
Figure 1.1. – Diagrammatic representation of tumour growth at a primary site and its local and distant metastatic invasion. Dysregulated epithelial cell proliferation leads to the formation of a primary tumour at an epithelium site. The tumour cells migrate by locally invading the surrounding extracellular matrix (ECM), enters the blood or lymph systems (intravasation), survives and translocates through the bloodstream to microvessels of distant tissues and exits from the bloodstream (extravasation), and finally adapts to the distant microenvironment of these tissues facilitating cell proliferation and the formation of a secondary tumour (colonisation). Modified from Whale et al. [14].

The transformation of a normal cell to a tumour cell with metastatic properties can be broadly divided into two critical phases. In the first phase, acquisition of genetic mutations leading to a series of biological changes including cell de-differentiation, inability to sense cell death (apoptosis) signals, cell cycle dysregulation, and genomic instability [15], contributes to the transformation of a normal cell to a tumour cell and formation of a tumour mass. The second phase involves acquisition of metastatic properties of certain tumour cells within the primary mass that allow for enhanced
motility and invasiveness. This includes epithelial-mesenchymal transition (EMT), enhanced proteolytic activity and the activation of angiogenic programs that gives metastatic properties to the tumour cell [16]. These malignant traits may arise from a subpopulation of cells within the heterogeneous tumour population that exhibit cancer stem cell (CSC) like properties [17-19] with capacity for self-renewal [20-22].

Many chemotherapeutic agents that already exist or are in various stages of development target these dysregulated pathways which otherwise regulate normal cellular behaviour. Of particular relevance to this project are drugs that target components of the cell cycle, especially anti-mitotics and certain cell surface receptors and proteases, the latter of which may more effectively limit metastasis. Prior to discussing examples of these drugs, the cell cycle and the role of growth factors and proteases in cancer will be overviewed in the subsequent subsections.

1.2.1. Cell cycle and cancer

The cell cycle is a series of coordinated processes that a cell undergoes for replicating its genetic material (DNA) and to produce daughter cells. This coordinated set of events can be divided into four sequential phases namely, S phase or synthesis phase where the DNA replication occurs, G2 or gap 2 phase follows S phase where the cell prepares for entry into the M phase (mitosis and cytokinesis) where the duplicated chromosomes are separated into the daughter cells, and G1 or gap 1 phase that follows on from mitosis where the cell is ready to respond to external growth signals. In the absence of appropriate external growth-promoting signals in the G1 phase, the cells go into and remain in a state of quiescence or G0 phase, until it receives any signals to re-enter into the cell cycle [23; 24] (Figure 1.2).
Figure 1.2. – Mammalian cell cycle machinery showing various stages of cellular reproduction (see text for details). Modified from Vermeulen et al. [25].

Under normal conditions, the cell cycle operates in an environment that is tightly regulated by various factors, which helps to maintain the steady state equilibrium between cell death and proliferation. There are mainly two types of regulatory mechanisms that control the cell cycle events; first by highly regulated kinase family members (cyclin-dependent kinases – Cdk) [26] which undergoes phosphorylation and dephosphorylations to enable the cell to relay from one stage to the next [27]. The second type of cell cycle control mechanisms are carried out by the cell cycle
checkpoints that supervise the completion of critical events during the cell cycle and relay signals associated with DNA replication and chromosome segregation to the cells [28]. Cells respond to these signals by delaying the cell cycle progression until the potential dysregulation is repaired or averted [23].

Several cell cycle regulatory molecules are altered in a tumour condition including those that regulate the G1-S transition phase [29]. The G1-S phase regulatory molecules are found downstream of the pRb pathway. The Rb protein, whose gene is located on human chromosome 13, has been found to be mutated in several human cancers [30-32]. Mutations in other molecules which regulate the activity of pRb pathway can also lead to cancer development. One such example is the loss-of-function mutations in the INK4a family members, especially p16\textsuperscript{INK4a}, which is mutated in almost 50% of all familial melanomas [29; 31; 33]. Over-expression of cyclins, particularly the cyclin D1 has been implicated in several cancers. These gain-of-mutations disrupting the Rb pathway are highly common in colon cancers [34]. Cyclin D1 over-expression has also been reported in ductal carcinoma in situ [35] and in more than 50% of mammary carcinomas [36].

1.2.2. Mitotic spindle assembly checkpoints and initiation of tumourigenesis

The mitotic spindle assembly checkpoint is the major cell cycle control mechanism in mitosis. The mitotic spindle functions to accurately segregate the replicated chromosomes into two daughter cells. This is mainly orchestrated by the dynamic behaviour of the microtubules, which are components of the mitotic spindle assembly [37; 38]. Microtubules are cellular polymers that are composed of heterodimers of $\alpha$ and
β-tubulin, which are highly homologous proteins with a molecular weight of approximately 55 kDa [39]. The biological functions of microtubules are mainly determined by their polymerisation dynamics [40-43], which involves non-equilibrium dynamic instability [44] and treadmilling [45-50].

Dysregulated events during mitosis, especially associated with the functioning of the mitotic spindle assembly, are also highly critical in tumourigenesis. It has been found that a defective mitotic spindle checkpoint leads to chromosomal missegregation [51; 52], eventually resulting in aneuploidy and chromosomal instability. For example, heterozygous point mutations in the Bub1 and BubR1 gene, which encode for the multidomain Bub1 and BubR1 protein kinases that are central components of the mitotic spindle assembly checkpoints [53], have been noted in many human cancers [37; 54].

Thus, owing to their crucial role in the orchestration of mitotic events, microtubules serve as pharmaceutically validated targets for anticancer chemotherapy [55; 56]. A plethora of microtubule stabilising and destabilising agents have been developed and some of them have also been approved by US FDA for clinical use including paclitaxel and the vinca alkaloids. These agents are discussed in detail in Section 1.3.

In addition to the defective cell cycle checkpoints and dysregulated mitotic spindle, over-expression of certain cell surface growth factor receptors of the EGFR family, especially HER-2 contribute to tumourigenesis and tumour progression. The biological function and prognostic significance of HER-2 will be discussed in detail in the next few subsections.
1.2.3. Human epidermal growth factor receptors

Human epidermal growth factor receptor type 2 (HER-2/ErbB2) is a transmembrane protein that belongs to the family of type 1 receptor tyrosine kinases (RTKs), which also includes other members such as EGFR/ErbB1, HER-3/ErbB3, and HER-4/ErbB4 (Figure 1.3).

These HER family member receptors are composed of an extracellular ligand binding region consisting of domain I to IV, a transmembrane helix, a highly conserved intracellular domain with kinase activity, and a less conserved cytoplasmic tail. HER-2, unlike other HER family members, is a ligand-less receptor; whereas HER-3 lacks an intracellular kinase domain [58-62] (Figure 1.3).
1.2.3.1. Activation of HER-2 – Ligand binding, dimerisation, and transautophosphorylation

Upon ligand binding, the four different epidermal growth factor receptors dimerise to form ten different homo and heterodimer combinations. The HER-1 receptor, also referred to as EGFR or ErbB1, is activated by six ligands including epidermal growth factor (EGF), TGF-α, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and the EGF-related ligand epiregulin. The HER-3 and HER-4 receptors bind a family of structurally distinct peptide forms of neuregulins, namely neuregulin 1 (NRG 1) and neuregulin 2 (NRG 2) respectively [63-67] (Table 1.1).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
<th>Potential receptor dimer combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-1/ErbB1/EGFR</td>
<td>Epidermal growth factor (EGF), transforming growth factor (TGF)-α, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and the EGF-related ligand epiregulin</td>
<td>HER-1/HER-2, HER-1/HER-3, HER-1/HER-4, HER-1/HER-1</td>
</tr>
<tr>
<td>HER-2/ErbB2</td>
<td>No ligands reported yet</td>
<td>HER-2/HER-1, HER-2/HER-3, HER-2/HER-4</td>
</tr>
<tr>
<td>HER-3/ErbB3</td>
<td>Neuregulin 1 (NRG 1)</td>
<td>HER-3/HER-1, HER-3/HER-2, HER-3/HER-4, HER-3/HER-3</td>
</tr>
<tr>
<td>HER-4/ErbB4</td>
<td>Neuregulin 2 (NRG 2)</td>
<td>HER-4/HER-1, HER-4/HER-2, HER-4/HER-3, HER-4/HER-4</td>
</tr>
</tbody>
</table>
These ligands are composed of a high-affinity binding site localised at the N-terminal and a low-affinity binding region at the C-terminal. Under normal circumstances, these ligands specifically bind to a primary receptor through the high-affinity binding region and activate them. This is followed by binding of this complex to a secondary receptor through the non-specific low-affinity binding region of the ligand [68].

Furthermore, these sequence of events lead to the phosphorylation of specific tyrosine residues in the intracellular kinase domain of HER family member receptors. Finally, such a series of interdependent processes trigger the binding of specific signalling molecules to the cytoplasmic domain, initiating a series of signalling transduction pathways downstream [69; 70]. The vast repertoire of signalling molecules that bind to the intracellular kinase domain of the EGFR family members include SH2 (Src homology 2) domain containing proteins, docking/adaptor proteins like Gab2, and transcription factors [71-75]. These signalling molecules recognise and attach to the phosphorylated tyrosine residues of the highly conserved cytoplasmic domain of HER family of receptors [76] at specific docking sites [77]. Activation of HER family members by ligand binding is essential for the initiation of downstream signalling transduction pathways, leading to cellular proliferation and cell survival [69; 77-79].

1.2.3.2. Role of HER-2 in tumour aggressiveness

The activation of HER family members and the initiation of downstream signalling transduction pathways are critically regulated by a series of events like rapid internalisation through clathrin-coated vesicles, dephosphorylation of the activated receptor, dissociation of ligand-receptor complexes, and degradation of active receptors in lysosomes [80]. Under normal circumstances, binding of ligands to HER receptors
leads to the formation of only a few HER-2 heterodimers and the resultant HER-2-mediated signalling is weak, resulting in normal cell growth [81]. HER-2 receptors are highly essential for cardiac development [82] and signalling initiated from HER-2 receptors protects the heart from oxidative stress [83]. Also, heterodimeric combinations of receptors other than HER-2 also provide weak, but essential signals for normal cell growth [81].

Activating mutations downstream of the HER family members leads to the amplification of HER-2/neu gene, which eventually results in the over-expression of HER-2 receptors at the cell surface at abnormally high levels. This is accompanied by an increase in their kinase activity, which in turn initiates signal transduction pathways resulting in either increased cellular proliferation and/or differentiation, depending on the ligands [84; 85]. In addition to the above factors, when HER-2 receptor is over-expressed, several distinct mechanisms such as low dissociation rates of HER-2 containing heterodimers [84; 86-88]; slow rate of endocytosis of HER-2 receptor [89]; and enhanced rate of recycling and reduced lysosomal degradation of HER-2 containing heterodimers [90; 91] allow for the prolonged retention of HER-2 receptor at the cell surface, thereby extending the duration of signalling by its heterodimeric partners [80].

1.2.3.3. HER-2 over-expression – Potential therapeutic target

During the last decade, HER-2 over-expression on cell surface, implicated as one of the factors contributing to cellular transformation, attained more recognition as a focal therapeutic target against breast carcinoma and to a lesser extent against other carcinomas. This was primarily attributed to the clinical finding that HER-2 was over-expressed in 20–25% of all breast cancer patients, and was often associated with poor
disease-free survival [92-98]. It was also noticed that HER-2 over-expression together with deregulated activation of the intracellular signalling pathways initiated downstream of it imparted increased aggressiveness to tumours [99-108] and also in some cases, induced resistance to certain chemotherapeutics [109-111]. Furthermore, over-expression of HER-2 receptors was found both at the primary tumours and also at the metastatic sites [112; 113]. Consequently, HER-2 over-expression, not only came to be considered as a highly consistent predictor for advanced metastatic breast carcinoma, but also it led to the development of specific inhibitors targeting them. Trastuzumab is a recombinant monoclonal antibody directed against the extracellular domain of HER-2. Trastuzumab and its mode of action will be discussed in detail in later sections.

Other factors that play a critical role in tumour progression and metastasis are the molecules associated with the plasminogen activation system.

1.2.4. Plasminogen activation system (PAS)

PAS is an extracellular proteolytic regulatory system that performs a variety of functions including matrix degradation and fibrinolysis [114]. In humans, plasminogen is primarily produced in the liver [115] and the inactive pro-zymogen binds to blood coagulation factors like fibrin [116], cell surface receptors such as cytokeratin 8 [117] and structural components of the extracellular matrix (ECM) like laminin [118], fibronectin [119], thrombospondin [120], tetranectin [121]. Following binding to the above-mentioned molecules, the PAS is readily activated by the serine proteases tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) [122], leading to the efficient conversion of co-localised plasminogen to active plasmin (Figure 1.4). The
tPA system is mostly associated with fibrin homeostasis. For a detailed review on tPA and its role in fibrinolysis, see [123; 124].

Figure 1.4. Schematic representation of the uPA system. Binding of uPA to its receptor, uPAR allows for the efficient activation of the latter. Active uPA has been found to be primarily responsible for the generation of active plasmin at the cell surface from the inactive plasminogen. Plasmin performs a host of functions including degradation of the extracellular matrix (ECM), activation of matrix metalloproteases (MMP), and release of latent growth factors. Modified from Croucher et al. [125]. The activation of cell bound plasminogen by tPA is not shown.

Under normal conditions, activation of the uPA system is mostly implicated in pericellular proteolysis and tissue remodelling such as wound healing and angiogenesis [114; 126]. However, dysregulation of the uPA system has been strongly implicated in tumour invasion and metastasis [125; 127-132].
1.2.4.1. Biology of uPA/uPAR and its inhibitors

The uPA receptor (uPAR) (CD87) is a single chain 45-65 kDa [133] glycosylphosphatidylinositol (GPI) anchored protein [134] that is composed of three domains (D1, D2, and D3) linked by short peptide linkages [135; 136]. Apart from binding uPA [133], uPAR also binds to other ligands such as vitronectin, fibronectin, integrin [137]. These various binding interactions enables uPAR to play a critical role in a number of cellular activities including cell adhesion and migration, immune response, wound repair, angiogenesis, inflammation and tumour invasion and metastasis [138-142].

uPA consists of three distinct domains: a region with homology to EGF (referred to as the growth factor domain), a kringle domain (both these domains combined is also referred to as amino terminal fragment (ATF), and a carboxy-terminal serine protease domain. The ATF of uPA contains all the determinants required for binding to uPAR at the cell surface (Figure 1.5).

uPA binds to uPAR with a $K_d$ of 0.28 nM [143] and is activated through cleavage of the Lys$_{158}$-Ile$_{159}$ peptide bond. This activation of uPA is brought about by active plasmin, which has been described earlier in Section 1.2.3. to facilitate generation of active plasmin from plasminogen, thus completing the loop for a feed-back-type activation. More information on the structure and function of uPA in complex with uPAR can be found in Huai et al. [144] and Dass et al. [145].
The uPA system is critically regulated by plasminogen activator inhibitors, namely plasminogen activator inhibitor type 1 (PAI-1) [146] and plasminogen activator inhibitor type 2 (PAI-2) that binds to and inhibits uPA [147-150]. Although both PAI-1 and PAI-2 belong to the family of serine protease (serpin) inhibitors and inhibit uPA [151], they have been found to exhibit contrasting functional roles in tumour invasion and metastasis [125; 129; 152; 153]. PAI-1 bound to vitronectin, inhibits uPA, eventually leading to cell migration partially through facilitation of an interaction between vitronectin and co-localised uPAR-integrins [154], whereas PAI-2 does not bind to vitronectin [155; 156]. PAI-1 alone and especially in complex with uPA binds
with high affinity to endocytosis receptors (LRP1 and VLDLR), leading to enhanced endocytosis and stimulation of signalling pathways resulting in increased cell proliferation [157]. While uPA-PAI-2 complex is also endocytosed, the complex does not stimulate signalling pathways, but serves only to inhibit uPA without further biological consequences [125; 158]. As this chapter mainly focuses on the biology and use of PAI-2 in targeting strategies, the functional role of PAI-1 will not be discussed in detail here. Information on PAI-1 can be found in [159-161].

PAI-2 is a 47 kDa single-chain protein [162] belonging to the serpin B clade family that has been found to exist in both cytosolic (47 kDa) and in extracellular glycosylated form (60 kDa) [125; 148; 163]. Extracellular PAI-2 binds to and inhibits uPA by the serpin “suicide” trapping mechanism that involves the reactive centre loop (RCL) of the PAI-2 [125]. It was also found that PAI-2 upon binding to uPA undergoes specific and rapid internalisation facilitated by the LDLR family of receptors and could be localised within endosomes and lysosomes [150; 157; 164]. Therefore, PAI-2 can be exploited to selectively deliver a large dose of drug to tumour cells that over-express the uPA/uPAR. The utility of PAI-2 in targeted therapy will be discussed in detail in Chapter 3.

1.2.4.2. uPA/uPAR and its prognostic significance.

The cell surface over-expression of members of the urokinase plasminogen activation (uPA) system, namely uPA and uPAR, is correlated with poor prognosis in several malignant tumours [132; 149; 165; 166]. Analysis of various tumour tissues by a number of different techniques including ELISA of cytosol extracts, in situ hybridisation and immunohistochemistry have found a varied level of expression of uPA/uPAR, however, a significant increase in the expression of uPAR was observed in
invasive tumour tissues compared to benign tissues [129; 167-173]. On the whole, the cell surface differential expression of uPA system in cancer, together with the fact that it plays a major role in cellular proliferation, adhesion, migration, invasion, and metastasis of tumour cells (Figure 1.6) [114; 139; 141; 174], makes uPA/uPAR an important target for anticancer therapy.

**Figure 1.6. uPA system: components and biological functions.** Binding of uPA to uPAR at the cell surface leads to multiple biological consequences. At the cellular level, activation of uPAR by uPA binding to it leads to initiation of signalling transduction pathways following interaction of uPAR with other cell surface receptors, eventually effecting a host of functions such as cellular adhesion, proliferation, and migration. Binding of uPA to uPAR also leads to extracellular matrix degradation. All of these processes are important for primary tumour growth, angiogenesis, invasion and metastasis. Modified from Mekkawy et al. [175].
1.3. Current treatment modalities

While drugs for potential use against cancer have been reported from the beginning of the 20th century, surgery and radiotherapy were the most preferred forms of treatment modality until the 1960s [176]. The discovery of effective chemotherapeutic agents brought a significant revolution in the treatment of cancer malignancy [177].

Most of the anticancer chemotherapeutics that are used in the clinic today are either natural or derived from natural products such as plants, animals, and microorganisms [178]. The choice of natural products as anticancer drugs has been mainly based on the huge structural and biodiversity of these compounds [179; 180]. Initially, considered only as an alternative source [176], natural products, particularly plant-derived compounds received renewed attention during the last few decades, largely due to the discovery of the many anti-tumour agents from plants. The first plant derived anticancer agents to be developed were vinca alkaloids (vinblastine and vincristine) and the cytotoxic podophyllotyoxins in the 1950s. Since then, periodic screening of plants and other organisms have led to the development of many novel chemotherapeutics with varying cytotoxic activities [181]. Further manipulation of these drugs obtained from either terrestrial or marine sources were carried out by molecular modification, whereby products with superior properties were generated from biosynthetic intermediates. This led to the creation of a class of compounds, which were structural analogues of the parent compound with greater pharmacological properties and reduced side effects [176]. For example, curcumin, which is obtained from the dried rhizomes of *Curcuma longa* and known for its anti-oxidative, anti-proliferative, anti-angiogenic, and anti-tumourigenic properties, was unsuitable to be used as a drug due to its poor
bioavailability. However, structural modification of curcumin following the conversion of the central conjugated beta-diketone to monocarbonyl dienone significantly improved the bioavailability and pharmacokinetic properties and retained the cytotoxic activity of the parent compound [176; 182].

In addition to the above-mentioned structural modifications, introduction of high-throughput screening protocols, combinatorial chemistry, computational chemistry bioinformatics, and other associated complementary tools have enabled researchers to develop natural product derivatives that are far superior than the currently used drugs in clinical practice, particularly in terms of its cytotoxic, pharmacokinetic, and pharmacodynamic properties [176; 178; 180]. However, of the many anticancer agents discovered, only a few have received FDA approval for clinical use to date. The most commonly used chemotherapeutics in the clinic for breast cancer, their mode of action and indications, are given in Table 1.2. All of these drugs are associated with numerous adverse reactions including neutropenia, leukopenia, thrombocytopenia, nausea, vomiting, diarrhea, myalgia, and peripheral neuropathy.
Table 1.2. Examples\(^3\) of commonly used chemotherapy agents in clinic for the treatment of breast cancer.

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Drug name / Generic name</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxanes</td>
<td>Paclitaxel/Taxol</td>
<td>Microtubule inhibitors</td>
</tr>
<tr>
<td></td>
<td>Docetaxel/Taxotere</td>
<td></td>
</tr>
<tr>
<td>Vinca alkaloid</td>
<td>Vinorelbine Tartrate/Navelbine</td>
<td></td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Capecitabine/Xeloda</td>
<td>DNA synthesis inhibitors</td>
</tr>
<tr>
<td></td>
<td>Gemcitabine/Gemzar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
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<tr>
<td></td>
<td>Fluorouracil (Adrucil)</td>
<td></td>
</tr>
<tr>
<td>Anthracyclines</td>
<td>Doxorubicin HCl (Adriamycin PFS and RDF)</td>
<td>DNA-RNA transcription regulators</td>
</tr>
<tr>
<td></td>
<td>Epirubicin HCl</td>
<td></td>
</tr>
<tr>
<td>Alkylating agent</td>
<td>Cyclophosphamide</td>
<td>DNA intercalator/cross-linker</td>
</tr>
</tbody>
</table>

Lynch, Mary Pat, *Oncology Nursing Essentials*, 2002, Professional Publishing Group, Ltd.
http://www.advancedbreastcancercommunity.org/treatment/drugs.htm
http://www.drugs.com/sfx/methotrexate-side-effects.html
http://www.drugs.com/sfx/gemcitabine-side-effects.html
1.4. Drawbacks of chemotherapy

Despite decades of extensive research on anticancer drugs, most of the chemotherapeutics that are in clinical use against early or metastatic breast cancer (MBC) have nonspecific toxicity. By killing rapidly proliferating cells via targeting cell cycle molecules, these anticancer agents do not explicitly discriminate between normal and malignant cells. For the same reason, these non-selective anticancer agents have a narrow therapeutic window and have to be administered in sub-optimal doses, which give them limited efficacy [183-192]. In addition, due to their non-selectivity, in some cases, a high drug concentration is needed to cause a significant reduction in tumour population. This could potentially lead to tumour cells developing resistance against the anticancer agents due to prolonged exposure [193; 194]. On the other hand, combination therapies generally result in higher overall response rates and times to disease progression than with sequential single agents [195-197]. However, this is usually associated with a greater level of toxicity compared to treatment with single agents. Also, the higher overall response rates of combination therapy over single agents may not necessarily translate into superior survival outcomes [198].

On the whole, although single agent and combination chemotherapeutic treatment regimen has provided significant clinical benefit, the development of chemotherapeutic structural analogues and other cytotoxins in the last decade has been largely unrewarding, especially in terms of the overall survival of patients. This also calls for the development of novel agents that can significantly improve the survival of patients with early and advanced MBC. The introduction of recombinant mAbs, small molecule
tyrosine kinase inhibitors (TKIs) and antibody-drug conjugates is a step in this direction.

1.5. Targeted cancer therapy

The introduction of targeted cancer therapy represents a major revolution in the treatment of cancer. Targeted therapies are highly specific for the molecular abnormality that is responsible for the malignant progression in human cancers. Two classes of agents that are commonly used in the clinic are; (1) mAbs those recognise and specifically bind to extracellular and transmembrane antigens at the cell surface; (2) small molecule TKIs that target the intracellular domain of transmembrane molecules or other intracellular molecules. These agents have a greater degree of specificity for the tumour cell and exhibit diverse biological properties [199; 200]. A list of commonly used clinically approved targeted cancer therapeutics is given in Table 1.3.
Table 1.3 Examples\(^4\) of clinically approved and commonly used targeted cancer therapies based on antibodies/small molecule TKIs.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Target Antigen</th>
<th>Indication(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib mesylate (Gleevec(^*))</td>
<td>several tyrosine kinase enzymes</td>
<td>gastrointestinal stromal tumour, certain kinds of leukemia, dermatofibrosarcoma protuberans, myelodysplastic/myeloproliferative disorders, and systemic mastocytosis</td>
</tr>
<tr>
<td>Dasatinib (Sprycel(^*))</td>
<td>several tyrosine kinase enzymes</td>
<td>CML or acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>Nilotinib (Tasigna(^*))</td>
<td>several tyrosine kinase enzymes</td>
<td>CML</td>
</tr>
<tr>
<td>Trastuzumab (Herceptin(^*))</td>
<td>HER-2</td>
<td>breast cancer, gastric or gastroesophageal junction adenocarcinoma</td>
</tr>
<tr>
<td>Lapatinib (Tykerb(^*))</td>
<td>HER-2</td>
<td>advanced or MBC</td>
</tr>
<tr>
<td>Gefitinib (Iressa(^*))</td>
<td>tyrosine kinase enzyme (EGFR)</td>
<td>advanced non-small cell lung cancer</td>
</tr>
<tr>
<td>Erlotinib (Tarceva(^*))</td>
<td>tyrosine kinase enzymes (EGFR)</td>
<td>metastatic non-small cell lung cancer and pancreatic cancer</td>
</tr>
<tr>
<td>Cetuximab (Erbitux(^*))</td>
<td>EGFR</td>
<td>squamous cell carcinoma of the head and neck or colorectal cancer</td>
</tr>
<tr>
<td>Panitumumab (Vectibix(^*))</td>
<td>EGFR</td>
<td>metastatic colon cancer</td>
</tr>
<tr>
<td>Temsirolimus (Torisel(^*))</td>
<td>inhibitor of a serine/threonine kinase called mTOR</td>
<td>advanced renal cell carcinoma</td>
</tr>
<tr>
<td>Everolimus (Afinitor(^*))</td>
<td>immunophilin FK binding protein-12, and inhibits the mTOR kinase</td>
<td>advanced kidney cancer</td>
</tr>
<tr>
<td>Vandetanib (Zactima™)</td>
<td>growth-promoting activity of several tyrosine kinase enzymes, including EGFR, several receptors for vascular endothelial growth factor receptor (VEGF), and RET</td>
<td>metastatic medullary thyroid cancer</td>
</tr>
<tr>
<td>Vorinostat (Zolinza(^*))</td>
<td>inhibits histone deacetylases (HDACs)</td>
<td>cutaneous T-cell lymphoma (CTCL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Target Antigen</th>
<th>Indication (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romidepsin (Istodax®)</td>
<td>inhibits members of one class of HDACs</td>
<td>CTCL</td>
</tr>
<tr>
<td>Bexarotene (Targretin®)</td>
<td>binds to and activates retinoid X receptors</td>
<td>CTCL</td>
</tr>
<tr>
<td>Tretinoin (Vesanoid®)</td>
<td>binds to and activates retinoid acid receptors</td>
<td>acute promyelocytic leukemia</td>
</tr>
<tr>
<td>Bortezomib (Velcade®)</td>
<td></td>
<td>multiple myeloma and mantle cell lymphoma</td>
</tr>
<tr>
<td>Pralatrexate (Folotyn®)</td>
<td></td>
<td>peripheral T-cell lymphoma</td>
</tr>
<tr>
<td>Bevacizumab (Avastin®)</td>
<td>VEGF</td>
<td>glioblastoma, non-small cell lung cancer, MBC, metastatic colorectal cancer,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and metastatic kidney cancer</td>
</tr>
<tr>
<td>Sunitinib (Sutent®)</td>
<td>several tyrosine kinase enzymes</td>
<td>metastatic renal cell carcinoma, gastrointestinal stromal tumour that is not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>responding to imatinib, or pancreatic neuroendocrine tumours</td>
</tr>
<tr>
<td>Pazopanib (Votrient®)</td>
<td>tyrosine kinase enzymes (VEGF)</td>
<td>advanced renal cell carcinoma</td>
</tr>
<tr>
<td>Rituximab (Rituxan®)</td>
<td>CD20</td>
<td>B-cell non-Hodgkin lymphoma and chronic lymphocytic leukemia (CLL)</td>
</tr>
<tr>
<td>Alemtuzumab (Campath®)</td>
<td>CD52</td>
<td>B-cell CLL</td>
</tr>
<tr>
<td>Ofatumumab (Arzerra®)</td>
<td>B-cell CD20</td>
<td>CLL</td>
</tr>
<tr>
<td>Ipilimumab (Yervoy™)</td>
<td>cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)</td>
<td>unresectable or metastatic melanoma</td>
</tr>
<tr>
<td>Tositumomab and 131I-tositumomab</td>
<td>CD20</td>
<td>B-cell non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>(Bexxar®)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ibritumomab tiuxetan (Zevalin®)</td>
<td>CD20</td>
<td>B-cell non-Hodgkin lymphoma</td>
</tr>
</tbody>
</table>
1.5.1. Monoclonal antibodies (mAbs)

This class of anticancer agents recognise and bind to target specific antigens (proteins or hormones) that are either abnormally expressed or are mutant forms of normal antigens present on the cell surface. This has presented researchers with a plethora of opportunities to manipulate them to be used as targeted therapeutics [189]. However, one of the key challenges encountered during the introduction of mAbs as a therapeutic in the initial stages, has been the development of immune response from the human immune system. This incompatibility was rectified to an extent with the help of human antibody phage display libraries and use of transgenic animals that express humanised antibodies [201-207]. Subsequently, the successful introduction of humanised and chimeric immunoglobulin proteins established mAbs as a validated approach for treating malignancies.

Although mAbs were highly specific for their target antigen on breast cancer cells and induced tumour regression characteristics when administered as a single agent [208], the overall limited efficacy of mAbs as a single agent prevented a successful therapeutic outcome [209]. In this scenario, mAbs were administered in combination with chemotherapy regimen to improve the clinical benefit and overall survival of patients with MBC. Advances in this direction followed with a series of investigations revealing the increased efficacy of trastuzumab and bevacizumab in combination with established chemotherapeutics compared to treatment with mAb or chemotherapy alone. For example, when trastuzumab was administered in combination with paclitaxel (taxane-based chemotherapy) as first line of therapy [210] and also after anthracycline treatment regimen [211], gave an improved objective response rate of 36% in the former and 41%
in the latter and an increased median time to progression of the disease compared to patients with HER-2 over-expressing tumours who received trastuzumab alone or paclitaxel alone treatments. For detailed information on clinical trials describing combination treatment modalities involving trastuzumab and chemotherapeutics, refer to [212]; [213]; [214]; [215]; [216]; [217]; [218]; [219]; and [220].

Meanwhile, bevacizumab was also tested in combination with established chemotherapeutics. In an open, randomised phase III study where bevacizumab was administered in combination with paclitaxel for MBC, it was found to increase the progression free survival of patients compared to paclitaxel alone treatment, however, the overall survival scenario remained same for both treatment groups [221]. This finding was confirmed by Chan et al. [222], where bevacizumab was found to be efficacious in combination with taxane (paclitaxel and docetaxel) therapy as a first line treatment for HER-2 negative MBC over the taxane therapy alone and showed improved response rates and progression-free survival for patients who have received taxane therapy in the adjuvant setting.

From the data obtained so far, it can be ascertained that both mAbs and small molecule TKIs were effective as a single agent and considerably increased patient survival. However the development of resistance against both is a major drawback. On the other hand, although combination of mAb/small molecule TKI with chemotherapy improved patient survival over their administration as single agents, it is still not entirely clear on the choice of chemotherapeutics to be administered with mAb/small molecule TKI so as to reduce the risk of increased toxicity [223-227]. In this situation of uncertainty,
alternative forms of therapy should be developed that are highly specific for their targets and have high potency with reduced toxicity-related side effects.

1.5.2. Emergence of ligand-drug conjugates (LDCs)

The development of LDCs may be attributed to the fact that most of the tumour targeting agents such as naked mAbs or small molecule TKIs in cancer therapy have not been effective on their own. At the same time, anticancer chemotherapeutics are cytotoxic, but not tumour specific. However, combining a specific protein/antibody with a highly potent cytotoxin will not only ensure the selective delivery of the complex to malignant cells, but also the presence of a highly potent drug brings about significant reduction in tumour population. In the LDC system, a highly potent drug is attached to a specific ligand through a selectively labile linker. In other words, LDCs can be viewed as sophisticated delivery systems for highly potent antitumour cytotoxic drug. (Figure 1.7).
Figure 1.7. Diagrammatic representation of an LDC showing its major components.

The ligand guides the cytotoxin precursor to the target cancer cell, where the LDC is internalised via receptor-mediated endocytosis and can be chemically and/or enzymatically cleaved to release the parent drug and unleash its cytotoxic activity. A plethora of LDCs, depending on the different types of ligand or drug or linker used, have been studied and optimised for methodical use of targeting different tumour specific biomarkers to date [190; 228; 229]. Examples of LDCs at various stages of clinical trials are given in Table 1.4.
Table 1.4. Examples of LDCs that are currently at various stages of clinical development.

<table>
<thead>
<tr>
<th>Conjugate Name</th>
<th>Target Antigen</th>
<th>Drug Payload</th>
<th>Stage</th>
<th>Indication(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGN-35</td>
<td>CD30</td>
<td>Auristatin</td>
<td>Phase I</td>
<td>CD30-positive hematologic malignancies</td>
<td>[230]</td>
</tr>
<tr>
<td>SGN-75</td>
<td>CD70</td>
<td>Auristatin</td>
<td>Phase I</td>
<td>Renal cell carcinoma</td>
<td><a href="http://clinicaltrials.gov/ct2/show/NCT01015911?cond=">http://clinicaltrials.gov/ct2/show/NCT01015911?cond=</a></td>
</tr>
<tr>
<td>CR011-vcMMAE</td>
<td>Glycoprotein NMB</td>
<td>Auristatin</td>
<td>Phase I/II</td>
<td>Unresectable stage III or stage IV melanoma</td>
<td>[231]</td>
</tr>
<tr>
<td>Trastuzumab-DM1</td>
<td>HER-2</td>
<td>Maytansine</td>
<td>Phase III</td>
<td>HER-2 positive early and advanced metastatic breast carcinoma</td>
<td>[232]</td>
</tr>
<tr>
<td>HuC242-DM4</td>
<td>CanAg</td>
<td>Maytansine</td>
<td>Phase II</td>
<td>CanAg-expressing metastatic or locally-advanced gastric or GE junction cancer</td>
<td>[233]</td>
</tr>
<tr>
<td>AVE9633</td>
<td>CD33</td>
<td>Maytansine</td>
<td>Phase I/II</td>
<td>Acute myeloid leukemia</td>
<td>[234]</td>
</tr>
<tr>
<td>HuN901-DM1</td>
<td>CD56</td>
<td>Maytansine</td>
<td>Phase II</td>
<td>Small cell carcinomas (SCC), neuroendocrine carcinomas, and multiple myeloma</td>
<td>[235]</td>
</tr>
<tr>
<td><strong>Conjugate Name</strong></td>
<td><strong>Target Antigen</strong></td>
<td><strong>Drug Payload</strong></td>
<td><strong>Stage</strong></td>
<td><strong>Indication (s)</strong></td>
<td><strong>References</strong></td>
</tr>
<tr>
<td>-------------------</td>
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<td>----------------</td>
</tr>
<tr>
<td>Inotuzumab ozogamicin</td>
<td>CD22</td>
<td>Calicheamicin</td>
<td>Phase III</td>
<td>Non-Hodgkin's Lymphoma</td>
<td>[236]; [237]; <a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a></td>
</tr>
<tr>
<td>Lorvotuzumab mertansine</td>
<td>CD56</td>
<td>Maytansine</td>
<td>Phase II</td>
<td>Myeloma</td>
<td></td>
</tr>
<tr>
<td>SAR3419</td>
<td>CD19</td>
<td>DM4</td>
<td>Phase I</td>
<td>Non-Hodgkin's Lymphoma</td>
<td></td>
</tr>
<tr>
<td>IMGN-388</td>
<td>alphaV integrin</td>
<td>DM4</td>
<td>Phase I</td>
<td>Solid tumours</td>
<td></td>
</tr>
<tr>
<td>BIIB015</td>
<td>Cripto</td>
<td>DM4</td>
<td>Phase I</td>
<td>Breast cancer</td>
<td></td>
</tr>
<tr>
<td>BT-062</td>
<td>CD138</td>
<td>DM4</td>
<td>Phase I</td>
<td>Myeloma</td>
<td></td>
</tr>
<tr>
<td>Brentuximab vedotin</td>
<td>CD30</td>
<td>vcMMAE</td>
<td>FDA approved</td>
<td>Hodgkin's Lymphoma</td>
<td></td>
</tr>
<tr>
<td>Glembatumumab vedotin</td>
<td>GPNMB</td>
<td>vcMMAE</td>
<td>Phase II</td>
<td>Breast cancer, Melanoma</td>
<td></td>
</tr>
<tr>
<td>PSMA ADC</td>
<td>PSMA</td>
<td>vcMMAE</td>
<td>Phase I</td>
<td>Prostate cancer</td>
<td></td>
</tr>
<tr>
<td>MEDI-547</td>
<td>EphA2</td>
<td>mcMMAF</td>
<td>Phase I</td>
<td>Solid tumours</td>
<td></td>
</tr>
<tr>
<td>ASG-SME</td>
<td>SLC44A4</td>
<td>vcMMAE</td>
<td>Phase I</td>
<td>Pancreatic cancer</td>
<td></td>
</tr>
<tr>
<td>MN</td>
<td>MN</td>
<td>Auristatin</td>
<td>Phase I</td>
<td>Cancer</td>
<td></td>
</tr>
<tr>
<td>MDX-1203</td>
<td>CD70</td>
<td>Duocarmycin</td>
<td>Phase I</td>
<td>Non-Hodgkin's Lymphoma, Renal cell carcinoma</td>
<td></td>
</tr>
</tbody>
</table>
1.5.3. Design considerations for LDCs

The efficacy of LDCs depends on a variety of factors including; (1) tumour antigen selection – it should be highly characteristic of the cell, i.e., highly over-expressed on the cell surface, which will enable the ligand to differentiate between normal and malignant cells; (2) ligand selectivity and affinity for target antigen – high selectivity of the ligand for the antigen ensures non-specific targeting of surrounding normal cells and also affinity for the target antigen must be finely balanced such that it is high enough to mediate prolonged tumour retention and does not undergo non-specific cleavage; (3) size of ligand/conjugate internalisation by tumour cells – optimal tumour penetration requires that the ligand be large enough to be maintained in circulation so that it can accumulate in the tumour, but small enough to successfully diffuse throughout the tumour; (4) efficient internalisation of LDCs; (5) stability of the linker between drug and ligand – highly selective labile nature of the linker system would markedly improve the pharmacokinetic properties as well as reduce general toxicity and other side effects; and (6) potency of drug – optimal potency of the drug would bring about maximum damage to the malignant growth, and at the same time, it would not effect the activity of the ligand component [189].

In addition to the above mentioned properties, the anti-tumour activity of the LDC is enhanced if the tumour associated antigen has a biological role either at the level of tumour growth or in survival pathways. For example, in the case of trastuzumab-maytansinoid (T-DM1) drug, there is an additive effect to the anti-tumour activity of the cytotoxin, as trastuzumab binds to HER-2 receptors, which are over-expressed on
tumour cells and downregulates the signalling pathways, responsible for cellular proliferation or cell survival, initiated from the HER-2 receptors [238-240].

Other important considerations include conjugation methods, drug-to-ligand ratio, effects of drug conjugation on ligand properties [241], valency, and charge [242]. A few of these LDC design considerations like tumour specific antigens, ligands, and linkers will be discussed in detail in the following sections.

### 1.5.3.1. Tumour antigen/biomarker

Selection of an appropriate tumour associated antigen/biomarker is highly critical to the efficacy of an LDC. A plethora of tumour associated antigens have been identified as targets for LDCs and these include various cell surface proteins and glycoproteins, gangliosides, and extracellular proteins [183; 243]. The main properties of a tumour associated antigen considered favourable for targeted selectivity of an LDC are; (1) level of expression of the antigen on the surface of tumour cells relative to normal cells; (2) the rate of internalisation & intracellular trafficking of the antigen-LDC complex in a tumour cell; (3) rate of intracellular processing of the antigen-LDC complex in the intracellular compartments of a tumour cell; (4) post-translational modification of the tumour associated antigens [184; 190; 244-249]; and (5) homogenous expression on tumour cells [250; 251].

To this end, uPA/uPAR and HER-2 discussed in Section 1.2.3. and 1.2.4. represent tumour specific biomarkers suitable for targeting utilising the LDC approach. The development of agents targeting these tumour specific biomarkers will be discussed in detail in Chapter 3 & 4 respectively.
1.5.3.2. Tumour targeting ligands

The critical characteristics of the ligand component that contributes to the effectiveness of an LDC are; (1) high specificity for the antigen; (2) high binding affinity to the antigen; (3) effective induction of immune effector functions like antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC); (4) available sites for modification; (5) inhibitory influence on the biological function of the antigen. Another characteristic of the ligand that is highly critical for the functional success of an LDC is its ability to retain the full biological properties after being manipulated by chemical modification. For example, trastuzumab retained the affinity for HER-2 binding, ability to inhibit HER-2 signalling and Fc-mediated immune-effector activities, such as ADCC after modification by attachment of maytansinoids [252]. Secondly, to achieve the additive effect of the ligand component, along with the cytotoxin payload, it is imperative that the LDC is administered at high doses, since studies have found optimal anti-tumour activity of the ligand in patients at relatively high doses. For example, in clinical studies, the maximum tolerated dose (MTD) for T-DM1 is 3.6 mg/kg when given every 3 weeks, and 2.4 mg/kg when administered weekly [240; 253], and this is similar to the trastuzumab dose (2 mg/kg weekly, after the first dose of 4 mg/kg) administered to patients with MBC [185; 191].

The choice of mAb over small molecule TKIs as the ligand in LDC is primarily based on the comparatively superior adaptive biological properties exhibited by mAbs. It was found that there is little variation in the plasma concentration of mAbs at a given dose between different patients in complete contrast to what has been observed with the small molecule TKIs [254]. Also, it was noticed that unlike the small molecule TKIs,
mAbs exert not only direct inhibitory effects on tumour growth by intervening with the signalling pathways, but also have the ability to induce indirect immune responses from the cell such as ADCC and CDC [255]. Moreover, mAbs were also found to be more specific than the small molecule TKIs for their targets [256].

Most of the mAbs that are utilised in LDCs are humanised IgGs [202; 257; 258]. The primary purpose of the humanisation of antibodies was to reduce the immunogenicity induced upon administration in human patients [202; 259-262]. Other clinical benefits achieved following this process was the higher retention times for these human IgGs in circulation [263] and their superior ability to induce ADCC with human effector cells [264]. A complete list of LDCs utilising mAbs as tumour targeting ligands is provided in the Table 1.4.

In comparison to mAbs, there are fewer examples of LDCs utilising proteins and/or peptides. Naturally occurring inhibitors with selective and specific affinity for well described tumour specific biomarkers are useful in this context. PAI-2 is an example of a naturally occurring inhibitor used in LDC therapy system. The biological role of PAI-2 was discussed in Section 1.2.4.1. and the information on the use of PAI-2 as a targeting moiety with examples will be discussed in detail in Chapter 3. Naturally occurring macromolecules as targeting ligands are often modified to impart superior biodistribution, tumour retention, pharmacokinetic and pharmacodynamic properties to improve their efficacy in a LDC system. Attachment of polyethylene glycol (PEG) to macromolecules is one of those modification techniques explored extensively.
1.5.3.2.1. Covalent modification of macromolecules by PEGylation

Until recently, most biopharmaceutical preparations as well as natural protein inhibitors were less preferred for use in LDC than mAbs due to their inferior pharmacokinetic and pharmacodynamic properties such as short half-life, immunogenicity, proteolytic decay, and low solubility [265; 266]. Although various strategies were developed to counter these drawbacks, it was not until the introduction of polyethylene glycol (PEG) in 1970, that a comprehensive elimination of the drawbacks was achieved following PEGylation appropriate for peptide and protein modifications [267; 268].

PEG is a chemical compound that is composed of repeating ethylene glycol units. PEG molecules mask the covalently attached protein or peptide preventing it from causing an immunogenic response. PEGylation can also shield the protein or peptide, which significantly reduces the likelihood of the degradation of the protein or peptide from proteolytic digestion. This consequently increases the half-life of the PEGylated protein or peptide. Moreover, due to the physicochemical properties of PEG, PEGylated proteins or peptides are highly soluble in solution, and their biodistribution profile is highly enhanced [269].

PEGylation of proteins is usually achieved by the chemical reaction between the various functional groups on the amino acid side chains in the protein and activated appropriate PEGylation molecules. Synthesis of PEGylated protein or peptides depends on a variety of factors such as protein concentration, PEG–to–protein ratio, reaction pH, temperature, reaction time, and protein characteristics (molecular weight, surface area, polarity, local amino acid conditions at the PEGylation site, such as lysine pKa, and site
accessibility). A range of proteins and peptides have been modified using PEG molecule for various manipulative and therapeutic purposes [270].

There are three important factors that affect the efficiency of PEGylation. These are: (1) The number of PEG chains attached to a polypeptide, (2) Molecular weight and structure of PEG chains, and (3) The attachment site of PEG [269], which can be either random or site-directed. Random PEGylation of macromolecules involves targeting of ε-amino groups on the side-chains of lysine residues of proteins. Although these PEGylation reactions were quick and efficient due to the relative abundance of lysine residues, it often results in highly complex heterogeneous species, which presents a significant challenge for their purification [270]. In this context, to obtain highly defined PEGylation products, site-directed PEGylation is now preferred.

### 1.5.3.2.2. Site-directed PEGylation

Site-directed PEGylation (SDP) reactions are one of the most popular methods used for the preparation of a highly defined or homogenous PEGylated product. It is mainly carried out by targeting either the N-terminal or the cysteine residues present on the protein. An example of N-terminal PEGylation of a protein ligand is the development of Neulasta, which is an N-terminally mono-PEGylated G-CSF containing a 20 kDa PEG [270].

PEGylation of thiol groups on cysteine residues is gaining immense popularity. The thiol-specific reagents used in the site-specific PEGylation reaction includes maleimide, pyridyl disulfide, vinyl sulfone, and other agents, with the maleimide-PEG group being the most preferred due to the high stability of the linker containing the particular group.
Two well known approaches to achieving site-directed PEGylation are PEGylation of thiol groups of either native or genetically introduced cysteine residues. This not only reduces the heterogeneity of the PEGylated product, but also it gives an opportunity to produce site-specific PEGylated species. An example of this is human interferon α2a, in which several cysteine analogues were used for site-specific PEGylations [271; 272].

Taken altogether, LDCs offer a highly promising treatment approach which needs intensive and immediate attention as an alternative system over the conventional single or combination agent chemotherapeutics. Moreover, LDCs have also been explored recently as an effective strategy to target cancer stem cell (CSC) specific antigens [273; 274] as several CSC-associated antigens are potential targets of LDC therapeutics [191; 247; 275; 276].

1.5.3.3. Receptor-mediated endocytosis – Internalisation & intracellular trafficking of LDCs

Receptor-mediated endocytosis (RME) is a biological process which aid in the entry of extracellular macromolecules (biological and other molecules) to the intracellular space [277-279]. RME involves the binding of an extracellular ligand to a cell surface receptor and the ligand-receptor complex is transported to the intracellular environment in coated vesicles [280-283]. LDCs also utilise this channelised endocytic pathway to target malignant cells. In this antigen-mediated uptake, the internalised antigen-LDC complexes traffic along the endosomal-lysosomal pathway before the active drug is released through the action of proteases, esterases or the low pH environment on the linker systems within the LDC (Figure 1.8).
Figure 1.8. Receptor-Mediated Endocytosis (RME) – A schematic representation showing internalisation of an LDC via clathrin coated pits followed by intracellular trafficking of the receptor-LDC complex bound to the cell surface receptors/antigens in vesicles, also called early endosomes. Subsequently the vesicle loses the clathrin coat and fuses to the late endosomes, where the LDC dissociates from receptors/antigens and the receptors/antigens are recycled back to the cell surface (not shown). Thereafter, the LDC is transferred to the lysosomes, where it gets cleaved into its individual components through lysosomal degrading enzymes or the acidic environment. Modified from Schrama et al. [186].

1.5.3.4. The linker component of LDCs.

The stability of an LDC is ultimately decided by the linker system between the ligand and the drug components. The presence of a highly selectively labile linker that undergoes cleavage only at the specified location would finally lead to maximised efficacy of the LDC. Therefore, such a LDC design would be expected to exhibit improved physiochemical properties, which would be evident by its improved pharmacokinetic properties, reduced general toxicity and side effects, and increased efficacy due to higher selectivity [189; 241].
The critical factors that influence the suitability of a linker in the LDC are; (1) the site of attachment of the linker to the ligand; (2) the average number of attachment sites per ligand molecule; (3) and the selective lability/cleavability of the linker system. Most of the LDCs that are at various stages of clinical trials have drug attachment to the ligand either through the amino group of lysine residues or the thiol group of the cysteine residues [190; 191; 284-286].

The number of molecules of drug attached to the ligand is primarily a function of the number of attachment sites on the ligand. However, the suitability of the linker system, for example, the length of the linker and also its reactivity plays a crucial role towards the drug per ligand ratio (DAR) [187; 189; 190; 284; 287; 288]. DAR is determined empirically on the basis of; (1) impact on ligand, for example antigen-binding affinity; (2) and stability and solubility of the resulting LDC. Most of the LDCs that are at various stages of development have, on an average, 3-4 molecules of drug attached to the ligand component [191]. A few examples include T-DM1 (DAR 3-4), Gemtuzumab ozogamicin (DAR 4-6) [289; 290], and inotuzumab ozogamicin (DAR 5-7) [291]. An optimal DAR is highly critical for the efficacy of the LDC, as a low DAR can result in low potency of the LDC, whereas a high DAR can adversely affect the structural and functional stability of the ligand [191]. The effect of a high DAR on the pharmacokinetic properties of the LDC was demonstrated by [292], where they reacted different molar ratios of auristatin to antibody to produce anti CD30 antibody-MMAE conjugates with 2, 4, and 8 molecules of auristatin attached to the antibody. Following further investigations, they found that antibody-auristatin conjugate with higher MMAE load had faster clearance rates and produced increased toxicity in mice [292].
Finally, the most important functional characteristic of the linker system is its selective cleavability. As LDCs score over the chemotherapeutics and other small molecule inhibitors for their longer retention in circulation, it is imperative that the linker system is stable in circulation and undergo cleavage only at the target site within cells. This not only increases the efficacy of the LDC, but also reduces the general toxicity due to cleavage at the extracellular space.

Over the last two decades, a range of linker systems have been developed and they have been found to significantly alter the physiochemical properties of the LDC to a greater extent. These include the acid-labile linkers, enzyme-cleavable linkers, and the thioether linkers [293]. A few selected linker systems have been discussed in more detail in the following subsections 1.5.4.4.1.

1.5.3.4.1. Acid labile linkers

Examples of acid-labile linker system include imines, hydrazones, and cis-aconityl. These have received a lot of attention due to their highly selectively labile nature only undergoing cleavage in the low pH environment of the late endosomes/lysosomes [186; 294; 295]. In recent years, the imine linker system has been developed for use in LDCs [296] (Figure 1.9A). Doxorubicin conjugated to the anti-melanoma monoclonal antibody (mAb) via the cis-Aconityl linker (Figure 1.9B) [297], and a hydrazone linker holding doxorubicin and anti-lymphoma mAb in a conjugate [298] are examples of this linker system. Similarly, acid-labile hydrazone linker system has also been utilised in a conjugate set up, where it was used to link doxorubicin to BR64, an anti-carcinoma
mAb that identifies Le\(^{y}\) related tumour associated antigens (TAA) which are over-expressed on the surface of cells of lung, colon and breast [299].

Figure 1.9. (A) Example of an imine linker and (B) Example of a cis-Aconityl linker. R indicates side chains attached to the imine linker. Modified from Singh et al. [300].

1.5.3.4.2. Enzyme-cleavable linkers

Ester or peptide-based linkers are highly established enzyme-cleavable linkers that release the active drug under the influence of non-specific esterases or proteases enzymes that are present in the lysosomal compartment [186; 241; 295; 301]. An esterase labile succinate linker has been used successfully to conjugate novel anti-mitotics based on the indole isatin to validated targeting ligands such as transferrin and PAI-2 [286]. An example of ester liker is given in Figure 1.10.
1.5.3.4.3. Non-reducible sulfide (thiol) linkers

An example of this class of linker system includes the maleimide-thiol linker. These linkers release the free active drug upon catabolic degradation of internalised LDCs in the lysosomal compartment of the cell [186; 241; 302; 303] (Figure 1.11). Conjugation through the maleimide functional linker has been gaining popularity as it has been found to preserve the activity and physical properties of the protein carrier since its pI remains unchanged [304]. The best studied examples involving such a maleimide functional linker system are the T-DM1 drug [305] and anthracycline (doxorubicin & daunorubicin) conjugates, DOX-3-′-aminoalkylmaleimide derivatives employing the maleimide-thioether linkage [306] (Figure 1.11).

Figure 1.10. Example of an ester linker.
1.5.3.4.4. Reducible (cleavable) di-sulfide linkers

Another class of linkers which are less explored in terms of preclinical studies are the reducible di-sulfide linkers (Figure 1.12). These linkers are selectively cleaved in the cytosol due to the reductive intracellular environment compared to the extracellular surrounding [186; 294; 302].

Figure 1.12. Example of an SPDP linker incorporating the drug DM and the antibody trastuzumab. Taken from Phillips et al. [305].
1.5.3.5. Drug component of LDCs – Use of isatin derivatives

The potency of the drug component is a critical factor for the efficacy of an LDC. As shown in Table 1.4, numerous drugs have been incorporated into LDCs including cytotoxins. An example of such a novel class of cytotoxins are based on isatin (1H-indole-2,3-dione) [307]. The synthetic versatility of isatins allows for the generation of a large number of structurally diverse derivatives including analogues derived from the substitution of the aryl ring, and/or derivatisation of the isatin nitrogen and C2/C3 carbonyl moities [307]. These highly potent cytotoxins are derivatives of \( N \)-substituted alkylisatins (Figure 1.13).

![Image of N-substituted alkylisatin](image)

**Figure 1.13. \( N \)-substituted alkylisatin.** Based on the indole isatin and synthetically derived by substitutions in the aromatic ring at the \( N1 \) position [6].

*In vitro* studies with a panel of selected \( N \)-alkylisatin derivatives against different cell lines including PC-3 (prostate), HCT-116 (colorectal), MCF-7 (mammary epithelial), revealed that di-brominated \( N \)-substituted isatin molecules possess high anti-cancer activity. These derivatives were found to destabilise microtubules and thereby inhibit tubulin polymerisation, alter lymphocyte morphology, induce G2/M cell cycle arrest.
and activate effector caspase-3 & caspase-7, thereby bringing about cell apoptosis [308]. The most potent derivatives gave IC$_{50}$ values in the nanomolar range in vitro. $N$-alkylated isatin derivative, 3/131 (5,7-dibromo-$N$-(p-hydroxymethylbenzyl)isatin) is of particular value as it can be easily derivatised for conjugation via linkers to targeting ligands through its hydroxymethyl group (See figure 2.3A, Chapter 2). This derivative is still highly active with IC$_{50}$ values in the low micromolar range against a range of cancer cell lines including breast carcinoma cells [286], and was 10 × more potent than the currently used anti-mitotics such as taxol and vinblastine following a 24 h treatment [308]. A complete list of all $N$-alkylisatin derivatives and their IC$_{50}$ values against different cell lines can be found in [308]. However, a problem common to all small molecule anti-mitotics are that they will accumulate in tissues with high proliferative potential such as the gut causing adverse side effects. These drugs are also often insoluble in aqueous solutions, thus various methods are routinely employed to increase and/or maintain the solubility of pharmaceuticals including the use of co-solvents, surfactants and complexing agents, as well as salt formation and pH adjustment [309].

1.6. Future drug treatment programs

1.6.1. Double targeting breast cancer using two LDCs

Although LDCs can be expected to be highly efficacious compared to other treatment modalities such as chemotherapy or antibodies alone or in combination, development of resistance against the LDC at some stage of its administration is a potential risk. Therefore, it is imperative to utilise LDC so as to cause maximum damage to the tumour cell population within this narrow window. In this context, it can be anticipated that simultaneously targeting advanced MBCs with two different LDCs will not only need a low-dose administration as it could cause more cell killing compared to
individual drugs, but also because the extend of damage it causes might significantly prolong the time of relapse of the tumour condition.

In this strategy, identification of appropriate co-existing interdependent tumour specific biomarkers remains the key to its effectiveness. Urban et al. [310] found uPA receptor to be the single most highly consistent predictor of metastasis in HER-2 over-expressing breast cancer patients. This finding was further supported by Meng et al. [311] who found that the higher the HER-2 ratio in tumour cells, the more likely there was a co-amplification of uPAR gene. Therefore, targeting metastatic breast tumours that over-express HER-2 and uPA receptors with a combination LDC approach can be expected to be improve outcomes for cancer patients.

### 1.7. Project rationale and aims

Although treatment with chemotherapy in single and combination mode has been highly beneficial, tumour relapse owing to development of resistance is a major drawback for further treatment optimisations. The \( N \)-alkylisatin class of drugs are highly toxic against malignant cells, but lack of specificity for the tumour population, as is the case with other chemotherapeutics, is a major limiting factor. Conjugating highly potent chemotherapeutics/cytotoxins with mAbs/natural inhibitors that recognises specific tumour associated antigens to form LDCs not only rectifies the non-selectivity and low potency of chemotherapeutics and mAbs respectively, but also it acts as a delivery platform to counter other inferior properties of the drugs such as decreased solubility issues.
Thus the **overall aim** of this project was to develop and test the potency and selectivity of LDCs incorporating $N$-alkylisatin and the validated target ligands transferrin, PAI-2, and trastuzumab against a panel of breast cancer cell lines that differ in their expression of the corresponding receptors/antigens for these ligands.

**The specific aims of the project were to;**

1. Compare the utility of a succinate versus imine linker system for the conjugation of $N$-alkylisatin derivatives to transferrin. This would involve characterisation of the selectivity and cytotoxicity of the transferrin-conjugates thus formed and allows the determination of optimised conditions for use in the development of other LDCs (**Chapter 2**).

2. Develop and characterise the *in vitro* selectivity and potency of PEGylated PAI-2 conjugated to $N$-alkylisatin derivatised with a succinate linker system. This would require optimisation of conditions for the PEGylation of a mutant form of PAI-2 (C161S-PAI-2) using a maleimide functionalised PEG (mPEG) and to develop ion-exchange chromatography methods to separate the different PEGylated PAI-2 species potentially generated. Method development for characterisation of the extent of PEGylation would also be required (**Chapter 3**).

3. Develop and characterise $N$-alkylisatin conjugated to trastuzumab via the succinate linker system as a novel LDC. An additional aim was to utilise the PAI-2 and trastuzumab-$N$-alkylisatin containing LDCs in preliminary *in vitro* double targeting experiments against breast cancer cell lines that differ in HER-2 and uPA/uPAR expression (**Chapter 4**).
CHAPTER TWO

PREPARATION AND CHARACTERISATION OF TRANSFERRIN-N-ALKYLISATIN CONJUGATES AS TARGETED ANTI-CANCER AGENTS
PREPARATION AND CHARACTERISATION OF TRANSFERRIN-ISATIN CONJUGATES AS TARGETED ANTI-CANCER AGENTS

2.1. INTRODUCTION

2.1.1. Transferrin

Transferrin (Tf) is a single-chain glycoprotein [312], which is found in the serum of almost all body fluids [313-316]. It is synthesised mainly in the hepatocytes [317] and functions primarily as an iron (Fe$^{3+}$) transporter due to its iron-binding properties [318; 319]. In humans and other higher animals, transport of iron has been, predominantly, observed to be receptor mediated, mainly in the form of iron-bound Tf (Fe$^{3+}$-Tf) [320-326]. This process has been found to be triggered by the binding of iron-bound Tf to specific transferrin receptors (TfRs) on the cell surface [327-331].

2.1.2. The transferrin receptor (TfR) – Transferrin (Tf) complex

Tf consists of two homologous lobes (N- and C-lobe) and each lobe contains two domains (N1, N2 and C1, C2), connected by a flexible hinge (Figure 2.1). In the iron-free conformation, the two domains of each lobe are well separated, while the two domains are closed to coordinate the Fe$^{3+}$ in the iron-bearing form [312]. Tf delivers iron to cells after forming a complex with TfR at the surface of cells. The structure of the dimeric TfR ectodomain consists of three domains (a protease-like, apical, and helical domain). At slightly alkaline extracellular pH of 7.4, Tf can bind one or two
ferric ions, and two iron-bearing Tf molecules can bind the dimeric TfR; however, iron-free Tf is not recognised by TfR at this physiological pH [332].

Once the iron-bound Tf binds to TfRs, the entire Tf-TfR complex is internalised in clathrin-coated pits. These clathrin-coated pits invaginate to form vesicles, which in turn fuses with the endosomes [333; 334]. Acidification of the contents of the vesicle (endosomes) releases iron from Tf, thereby facilitating the delivery of iron to the cytoplasm and also enables the emptied Tf (apo Tf) molecules to remain tightly bound to TfR [332]. The vesicle is then recycled back to the surface of the cell [335; 336], where the extracellular pH causes the dissociation of the apoTf molecules from the TfR allowing the internalisation cycle to continue [332].
2.1.3. Role of TfR in cancer

Interestingly, expression of TfR on both normal and malignant cells has been observed to differ at different stages of growth and differentiation [337-340]. TfRs have been found to be highly expressed on tumour cells [341-348] and in breast cancer, TfR expression has been shown to be up to five times higher in malignant versus benign tissues [349; 350]. Furthermore, in a recent clinical study, TfR was identified as a marker of poor prognosis in breast cancer and to predict patient response to tamoxifen [351]. This phenomenon has been largely attributed to the increased demand for iron by cells for DNA synthesis [352-354]. Thus, TfRs serve as potential targets for ligand-directed anti-cancer therapy.

2.1.4. Transferrin-drug conjugates

Tf has been extensively utilised as a carrier protein to release a range of chemotherapeutics via RME to the intracellular space of tumour cell lines. A general scheme of drug-Tf-TfR complex formation and its intracellular trafficking is essentially given in Chapter 1, Figure 1.8.

Conjugation of therapeutics like adriamycin [355]; doxorubicin [356]; cisplatin; chlorambucil [357]; daunorubicin insulin and toxin CRM107, a genetic mutant of diphtheria toxin [358-362] to Tf through selectively cleavable linkers are a few examples along this line [312]. These Tf conjugates have been found to be highly effective compared to treatment with the drug component alone. For example, Tf-doxorubicin conjugates linked via acid-labile phenylacetyl hydrazone linkers exhibited enhanced cytotoxic activity and reduced general toxicity compared to treatment with
free drug alone in different cancer cell lines [363-365]. Similarly, daunorubicin insulin conjugated to Tf through glutaraldehyde disulfide linkers were found to be almost $10 \times$ more effective than treatment with daunorubicin insulin alone in a small cell carcinoma of the lung (SCCL) cell line [358]. Tf conjugated to chlorambucil by an acetaldehyde carboxylic hydrazone bond was found to be $3-18 \times$ more active than chlorambucil with preliminary in vivo investigations indicating the improved tolerability of the Tf-chlorambucil conjugate compared to free chlorambucil compound [357].

More recently, artemisinin, a natural product containing an endoperoxide group which can be activated by intracellular iron to release toxic radical species was conjugated to Tf via the $N$-glycoside chains. Tf conjugates of artemisinin were found to be highly cytotoxic and induced apoptosis in DU 145 prostate cancer cells [366]. On further investigations in a rat model, Tf-artemisinin conjugates were shown to significantly retard the growth rate of the breast tumour with reduced side effects [367]. Despite these promising results, to date, no drug-Tf conjugate has been approved for clinical use, which could be attributed to the expression of TfR on normal cells. Meanwhile, the ability of Tf to undergo modification and the channelised pathway it follows after forming complex with TfR provides sufficient reason for utilising Tf as a model system to optimise conditions for the development and exploration of other novel LDCs, which will be discussed in Chapter 3 & 4.

In this study, two highly potent cytotoxins, 5,7-dibromo-$N$-(p-hydroxymethylbenzyl)isatin (NAI-1) (Figure 2.2A) and 5,7-dibromo-1-(4-methoxybenzyl)isatin (NAI-2) (Figure 2.2B), were used. NAI-1 and NAI-2 were functionalised with an enzyme cleavable ester linker and an acid sensitive imine linker
respectively to form 6-carboxypropanoyl, 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin (NAI-1-succ) (Figure 2.2C) and 3-((5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene)amino)phenyl)propanoic acid (NAI-2-imine) (Figure 2.2D). Acid-sensitive linking agents, such as imines, hydrazones, acetals and cis-aconityls have received considerable attention due to their highly versatile nature [300; 368]. Hydrolytic studies conducted by [296] on a series of imino acid derivatives found the para-phenylpropionic acid derivative, which is utilised in NAI-2-imine to be highly stable at pH 7.4 (half life > 4 h), but labile with a half-life of 17 min. Also, hydrolytic studies conducted by [286] to determine the lability of succinate linker in NAI-1-succ (pro-drug) found the pro-drug to drug conversion to be highly enzyme-dependent undergoing cleavage only in an esterase solution at pH 7.4 and 37º C (half life 30.5 min).

The specific aims of this chapter were:

1. To prepare the active form of the linker-attached isatin derivatives NAI-1-succ & NAI-2-imine (Figure 2.3 B & 2.4 B) and conjugated to Tf.
2. To determine the in vitro cytotoxicity of Tf–6-carboxypropanoyl, 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin) conjugate (NAI-1-succ-Tf) (Figure 2.3C) and transferrin-3-((5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene)amino)phenyl)propanoic acid (NAI-2-imine-Tf) (Figure 2.3C) against selected mammary epithelial and human monocytic cell lines that varied in their expression levels of TfR. In addition, to compare the activities of the free isatin (NAI-1 & NAI-2) and their functionalised derivatives (NAI-1-succ & NAI-2-imine) against these cell lines.
Figure 2.2. Scheme showing structures of the N-alkylisatin derivatives with and without linkers used in this study. (A) 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin (NAI-1); (B) 5,7-dibromo-1-(4-methoxybenzyl)isatin (NAI-2); (C) 6-carboxypropanoyl, 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin (NAI-1-succ); (D) (Z)-3-(4-((5,7-dibromo-1-(4-methoxybenzyl)-2-oxoindolin-3-ylidene) amino) phenyl) propanoic acid (NAI-2-imine).
Figure 2.3. Potential schematic representation of the synthesis of NAI-1-succ-Tf conjugate and its selective release in a tumour cell. Firstly, NAI-1-succ (A) is converted to its active form (B) in the presence of NHS & DCC. Following this, the active form of NAI-1-succ is reacted with Tf via its free amino groups to form NAI-1-succ-Tf conjugate (C). Upon RME of the conjugate by the targeted cell, the NAI-1 native cytotoxin (D$_1$) is expected to be selectively released inside the tumour cell by the action of lysosomal esterases.
Figure 2.4. Potential schematic representation of the synthesis of NAI-1-succ-Tf conjugate and its selective release in a tumour cell. Firstly, NAI-1-succ (A) is converted to its active form (B) in the presence of NHS & DCC. Following this, the active form of NAI-1-succ is reacted with Tf via its free amino groups to form NAI-2-imine-Tf conjugate (C). Upon RME of the conjugate by the targeted cell, the NAI-1 native cytotoxin (D₁) is expected to be selectively released inside the tumour cell due to the acidic conditions of late endosomes/lysosomes.
2.2. MATERIALS & METHODS

2.2.1. Chemicals, reagents, proteins and antibodies. Human apo-transferrin (Tf), hanks balanced salt modified and non-modified, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). Alexa Fluor™ 488 Protein Labelling Kit, anti-Alexa Fluor® 488, and rabbit IgG quenching antibody were purchased from Molecular Probes™ (Eugene, OR, USA). Mouse monoclonal antibody, IgG1 was obtained from Millipore Australia Pty Ltd (Victoria, Australia). Anhydrous dimethylformamide (DMF), N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich (MO, USA). RPMI-1640 powder and foetal calf serum were from Trace Bioscientific (NSW, Australia). Trypsin/EDTA (0.05% trypsin, 4Na EDTA) was obtained from Invitrogen. Desalting PD-10 column was purchased from GE Healthcare. NAI-1, NAI-2, NAI-1-succ, and NAI-2-imine were synthesised in-house by Dr. Julie Locke, Dr. Lidia Matesic and Ana Zivanovic, School of Chemistry, University of Wollongong (NSW, Australia). BIO-RAD Quick Start™ Bovine Serum Albumin (BSA) Standard and Bio-Rad DC™ Protein Assay Kit (Reagent A & B) were obtained from BIO-RAD, Hercules, CA. CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) reagent was purchased from Promega Corporation (Sydney, Australia).

2.2.2. Cell lines. The human mammary epithelial carcinoma cell lines; MDA-MB-231, MCF-7, and SK-BR-3 and the human monocytic U937 and THP1 cell lines were purchased from American Type Culture Collection (ATCC, VA, USA). All cell lines
were routinely cultured in RPMI-1640 (10.4 g/L) and supplemented with 2 mM L-glutamine and 5% (v/v) fetal calf serum (FCS) at 37° C in 95% humidified atmosphere, containing 5% CO₂. SK-BR-3 cells were additionally supplemented with 10% (v/v) foetal calf serum (FCS) and 5 µg/L Insulin and the U937 cells were supplemented with 10% (v/v) foetal calf serum (FCS). All cells lines were also tested routinely for mycoplasma contamination.

2.2.3. General conjugation procedure. The active form of NAI-1-succ (Figure 2.4B) and NAI-2-imine (Figure 2.5B) was prepared based on the method described by Vine et al. [286] and Indira Chandran et al. [369] respectively. Briefly, NAI-1-succ or NAI-2-imine (2.7 mg, 5.0 µmol) was dissolved in anhydrous DMF (70 µL). N-hydroxysuccinimide (NHS, 0.59 mg, 5.0 µmol) in DMF (17 µL) and dicyclohexylcarbodiimide (DCC, 6.18 mg, 30 µmol) in DMF (50 µL) were then added and the reaction mixture shaken and kept at RT for 15-20 min. A 20-fold M excess of crude orange product was then conjugated to free lysine residues of Tf (>1 mg/mL) in PBS (pH 8.4) at RT, with shaking. After 3 h, the reaction mixture was centrifuged (maximum speed for 2-5 min) to remove any precipitate. The conjugate was then purified by gel filtration (PD-10 column), with PBS (pH 7.4) as eluant. The extent of conjugation was then qualitatively characterised by UV/Vis spectrophotometry by measuring for Tf at 280 nm and isatin at 432 nm. Subsequently, the fractions corresponding to the protein peak (as determined by UV/Vis spectrophotometry at 280 nm) were pooled, and stored at 4° C for future cytotoxicity assays. Protein concentration was determined using the standard Lowry protein determination assay and the amount of isatin bound to protein determined by electrospray ionisation mass spectrometry (ESI-MS) analysis.
2.2.4. Alexa488 fluorescent dye labelling of Tf and NAI-1-succ-Tf. Tf or NAI-1-succ-Tf were labelled using Alexa Fluor™ 488 according to manufacturer’s instructions and will be referred to as Tf-Alexa488 or NAI-1-succ-Tf-Alexa488, respectively. Briefly, around 50 μL of 1 M bicarbonate solution (prepared by dissolving component B (sodium bicarbonate) in dH₂O) was added to approximately 0.5 mL of 2 mg/mL Tf solution to raise the pH of the reaction mixture to pH ~8.0. Following this, the reaction mixture was transferred to a vial of reactive dye (component A) and then stirred at RT for 1 h. Subsequently, the Alexa488 labelled Tf was purified by gel filtration using disposable PD-10 desalting columns. On determination of the degree of labelling, an average of 5.19 fluorophore molecules of Alexa488 dye was found attached per molecule of Tf, calculated as per the equation.

Firstly, absorbance value of samples (Tf-Alexa488 or NAI-1-succ-Tf-Alexa488) was measured at 280 nm for protein and 490 nm for Alexa488 dye using SpectraMax® Plus 384 Microplate Spectrophotometer.

Concentration of Tf in the eluate fractions obtained after purification:

\[
\text{Tf concentration (M)} = \frac{(A_{280} - (A_{490} \times 0.11)) \times \text{dilution factor}}{72,500}
\]

72,500 cm⁻¹M⁻¹ is the molar extinction coefficient of apo-Tf at 280 nm [370] and 0.11 is a correction factor to account for absorption of the dye at 280 nm.

Degree of labelling:

\[
\text{moles dye per mole Tf} = \frac{A_{490} \times \text{dilution factor}}{71,000 \times \text{Tf concentration (M)}}
\]
71,000 cm\(^{-1}\)M\(^{-1}\) is the approximate molar extinction coefficient of the Alexa Fluor® 488 dye at 494 nm.

**2.2.5. Cell surface TfR detection assay.** Cell surface detection of TfRs was done as previously described by Lee et al. [163] with minor modifications. Briefly, adherent cells were detached using Trypsin/EDTA (0.05% trypsin, 4Na EDTA) and resuspended in Hanks’ binding buffer (Phenol red free Hanks’ balanced salts 9.7 g/L, HEPES 4.8 g/L, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.1% BSA, pH 7.4) (Appendix 1) at a density of 1 \(\times\) 10\(^6\) cells/mL and incubated in binding buffer at room temperature for 1 h to allow receptor recycling. After a washing step, the cells were incubated with 10 µg/mL Tf-Alexa488 or NAI-1-succ-Tf-Alexa488 in binding buffer for 30 min on ice. Following further washing, cell surface associated Alexa488 fluorescence was then analysed by dual colour flow cytometry with 5 µg/mL propidium iodide (PI) to distinguish between viable and non-viable cell population as previously described [371] using a Becton and Dickinson FACScan Flow Cytometer. This technique allows non-viable (PI positive) cells to be gated out so that potential intracellular Tf fluorescence is excluded from the analysis.

**2.2.6. Internalisation assay of Tf and NAI-1-succ-Tf.** Internalisation assays were performed as previously described by Croucher et al. [164] with some modifications. Briefly, cells were harvested using Trypsin/EDTA (0.05% trypsin, 4Na EDTA) and resuspended at 1 \(\times\) 10\(^6\) cells/mL in Hanks’ binding buffer prior to incubating cells with 25 µg/mL Tf-Alexa488 or NAI-1-succ-Tf-Alexa488 for 45 min on ice to allow cell surface receptor binding, but with minimal endocytosis. After washing to remove any unbound Tf-Alexa488 or NAI-1-succ-Tf-Alexa488, the 0 min sample
was removed and washed once in ice-cold binding buffer before incubating immediately with 4 μg/mL anti-Alexa Fluor® 488 IgG quenching antibody in ice-cold binding buffer for 30 min to prevent further endocytosis of Tf-Alexa488 or NAI-1-succ-Tf-Alexa488. Meanwhile, the remaining cells were re-suspended in binding buffer pre-equilibrated to 37°C and cell sample removed after 45 min and plunged on ice. These samples were also then incubated with 4 μg/mL anti-Alexa Fluor® 488 IgG quenching antibody in ice-cold binding buffer for 30 min after washing them once in ice-cold binding buffer. Subsequently, all the samples were washed and analysed by flow cytometry as described in the previous section.

2.2.7. Electrospray ionisation mass spectrometry (ESI-MS). Positive ion mass spectra of Tf and NAI-1-succ-Tf conjugates were acquired on a quadrupole time of flight mass spectrometer (Q-TOF-MS) using Waters Synapt(tm) HDMS mass spectrometer (Wythenshawe, UK), fitted with a Z-spray ESI source as previously described by Vine et al. [286] with minor modifications. Tf and NAI-1-succ-Tf conjugate samples were prepared for ESI-MS by buffer (Appendix 1) exchanging into a 10 mM ammonium acetate buffer (pH 6.8) containing 0.1% formic acid at a final concentration of Tf (25 μM) and NAI-1-succ-Tf (11.2 μM) by standard dialysis using PD-10 desalting columns. The samples were injected into the spectrometer and the mass spectrum acquired with a capillary of 2.3, cone of 150, reflectron of 35.6, collision energy of 2.0, source temperature of 40°C, desolvation temperature of 100°C and a resolution of 4000 (LM Resolution - 5.0 and HM Resolution - 5.0). Cesium iodide in 70% isopropanol was used for external calibration. The data is presented as raw data, on a mass scale. Masses were calculated using MassLynx MS software (Waters). All
analyses were performed by Dr Thitima Urathamakul (Mass Spectrometry Facility Manager, School of Chemistry, University of Wollongong)

2.2.8. In vitro cytotoxicity assay. In vitro cytotoxicity assays were performed as described previously by Indira Chandran *et al.* [369]. Briefly, cells (5,000 cells/well) were seeded in 96-well plates and incubated overnight at 37° C in 95% humidified atmosphere, containing 5% CO₂. On the following day, Tf, NAI-1, NAI-2, NAI-1-succ, NAI-2-imine, NAI-1-succ-Tf or NAI-2-imine-Tf were added to triplicate of wells at decreasing concentrations before incubating them for 24 or 48 h at the above-mentioned conditions. Subsequently, MTS reagent was added to appropriate wells and after a 3 h incubation period, the absorbance was measured at 490 nm using SpectraMax® Plus 384 Microplate Spectrophotometer with SoftMax® Pro Software. The cytotoxicity of the compounds was expressed in terms of IC₅₀ values (concentration of test compounds, in terms of *N*-Alkyisatin (NAI), required to inhibit the metabolic activity of 50% of the total cell population) after normalising to DMSO, Tf only or and PBS-treated control cells. These values were calculated from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism™ software v5.02 (GraphPad Software Inc.). In all IC₅₀ calculations using GraphPad prism software, either cells + PBS or cells + DMSO absorbance values subtracted from baseline absorbance values (media + PBS or media + DMSO) were taken as 100% cell viability (100% value) control and cells + highest concentration of test compound treatment was taken as 0% cell viability (0% value) control.

2.2.9. Statistical Analyses. Statistical significance of treatment groups as compared to control groups was determined using unpaired students T-test (GraphPad
Prism V 5.1; San Diego, CA, USA). *P* values < 0.05 were considered statistically significant.

### 2.3. RESULTS

#### 2.3.1. Detection of TfRs

The expression of TfRs on the surface of U937 and THP1 human monocytic cell lines and breast carcinoma cells such as MDA-MB-231, MCF-7, and SK-BR-3 cells was determined using Tf-Alexa488 and flow cytometry. As the relative shift in fluorescence produced by the binding of Tf-Alexa488 to TfRs on cells indicate ([Figure 2.5](#)), all the cell lines tested expressed significant level of TfRs, with the carcinomic cells expressing higher levels of TfRs on their surface in comparison to the monocytic cell lines tested. Previous studies performed with a non-specific Alexa488 labelled protein (BSA) in our laboratory showed no significant shift of fluorescence compared to the autofluorescence indicating that the Tf-Alexa488 binding was specific [163].
Figure 2.5. Representative fluorescence histograms showing the relative cell surface expression of TfRs on (A) U937, (B) THP-1, (C) MDA-MB-231, (D) MCF-7, and (E) SK-BR-3 Cells. The shift to the right of the No Fill Peak from the Dark Filled Peak represents fluorescence of cells produced by binding of Tf-Alexa488 to TfR on the cell surface.
The MCF-7 cells were found to express very high amount of TfRs (GM – 252.3 ± 2.63), while U937 cells were found to express the least (GM – 33.03 ± 7.82). The amount of TfRs on the surface of cell lines that were tested can be summarised in the following order MCF-7>>SK-BR-3>MDA-MB-231>THP-1>U937. For the ease of comparison, the relative expression of TfR on above cell lines will be categorised as high (MCF-7), medium (SK-BR-3), and low (MDA-MB-231, THP-1 & U937) (Figure 2.6).

Figure 2.6. Bar graph representing the relative levels of expression of TfRs on various cell lines tested with each bar being the mean of triplicate of values ± SDEV. *P <0.05 MCF-7 cells versus all other cell lines.

2.3.2. Conjugation of NAI-1-succ and NAI-2-imine to Tf. The active form of NAI-1-succ and NAI-2-imine was prepared according to the method described by [286] and [369] respectively. Conjugation of the active form of NAI-1-succ or NAI-2-imine to Tf was achieved by incubating both reactants in PBS (pH 8.5) for 3 h under
shaking conditions. The pH of 8.5 was chosen for this reaction, as the isoelectric point (pI) of apo-Tf was noted to be in the range of 5.5–6.2 [372]. The conjugated products were purified by size-exclusion chromatography and subsequently visualised at 280 nm for Tf and 432 nm for isatin (Figure 2.7 & 2.8). Upon purification by PD-10 desalting columns, some residual orangish-yellow coloured product remained on the column which could be unconjugated or free isatin from the conjugation reaction.

The NAI-2-imine-Tf conjugate (4) was characterised by UV/Vis spectrophotometry at two wavelengths (280 nm for protein and 432 nm for isatin) (Figure 2.8). To rule out any non-specific interactions between NAI-2-imine and Tf, the NAI-2-imine was reacted with Tf in the absence of the coupling reagents (i.e. DCC and NHS) and the protein fraction collected after size exclusion chromatography. The low absorbance value at 432 nm (OD = 0.154) in the eluted fraction, with a corresponding high absorbance value at 280 nm (OD = 1.569) for Tf, indicates negligible non-specific attachment of NAI moieties onto Tf molecules in the absence of coupling agents (Figure 2.8A). In the presence of coupling reagents, an increase in absorbance at 432 nm (OD = 0.741), corresponding to incorporated NAI, was noted to co-elute with the protein peak at 280 nm (OD = 1.595), indicating attachment of NAI-2-imine (3) to Tf (Figure 2.8B).
Figure 2.7. Size exclusion elution profiles of NAI-1-succ-Tf conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of Tf (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1-succ.
Figure 2.8. Size exclusion chromatography elution profiles of (A) Tf and NAI-imine after conjugation without the coupling reagents NHS and DCC and (B) Tf and NAI-imine after conjugation with the coupling reagents NHS and DCC. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of Tf (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-2-imine.
A representative Q-TOF ESI mass spectrometric analysis (Figure 2.9) of the NAI-1-succ-Tf conjugate with 20-fold molar excess of active isatin ester revealed incorporation of an average of 2-3 molecules of NAI-1 per Tf molecule. These NAI-1-succ-Tf conjugates were used in cytotoxicity experiments. In the case of NAI-2-imine-Tf conjugate, because of the acidic conditions required for MS and the acid-sensitive nature of the imine linker used in this study, the conjugate was not amenable to further characterisation using this method.

Figure 2.9. A representative positive ESI–MS analysis of NAI-1-succ-Tf conjugate in 10mM NH₄OAc containing 0.1% formic acid using Q-TOF MS analyser. To ascertain the number of molecules of NAI-1 attached to per molecule of Tf, the molecular weight of apo-Tf was taken as 79574.75 Da (B, lower panel) and isatin derivative as 525.14 g/mol. The average increase in molecular weight of Tf upon attachment of isatin molecules calculated from the spectrum (A, upper panel) indicated a maximum of 5 molecules and an average of 2-3 molecules of isatin attached per molecule of Tf.
2.3.3. Confirmation of NAI-1-succ-Tf-Alexa488 cell uptake. The ability of NAI-1-succ-Tf-Alexa488 to bind to Tf receptors and undergo endocytosis was ascertained on MCF-7 human mammary epithelial cells using internalisation assays. Under these conditions, any fluorescence emitted by internalised NAI-1-succ-Tf-Alexa488 would be protected from quenching by the anti-Alexa Fluor® 488 IgG quenching antibody.

At the zero time point, after cell surface loading of either Tf-Alexa488 or NAI-1-succ-Tf-Alexa488 (Figure 2.10) and prior to initiation of endocytosis by incubation at 37° C, considerable shift in the fluorescence intensity compared to the autofluorescence was noted even in samples removed at 0 min indicating that some internalisation must have occurred, which cannot be quenched over the preloading step even though the cells were kept on ice. The relative increase in fluorescence intensity of cells removed after 45 min of incubation to 0 min at 37° C indicates that both Tf-Alexa488 and NAI-1-succ-Tf-Alexa488 was indeed internalised.
Figure 2.10. Tf and NAI-1-succ-Tf internalisation. Graph (A & B) and bar graph (C) showing the relative internalisation of Alexa488 fluorescent dye labelled Tf and NAI-1-succ-Tf conjugate in samples removed at 40 min compared to the 0 min sample in human monocytic U937 cells. (A) Tf alone, solid peak represents total autofluorescence, dark peak represents fluorescence of cells produced by binding of Alexa Fluor™ 488 Protein labelled Tf to TfR on the cell surface assuming there is no internalisation at zero time point, dotted peak represents the fluorescence of cells produced by internalised Tf-Tf-Alexa488 complexes after 40 min of incubation in Hanks’ binding buffer at RT; (B) NAI-1-succ-Tf conjugate, solid peak represents total autofluorescence, dark peak represents fluorescence of cells produced by binding of Tf-Alexa488 to TfR on the cell surface assuming there is no internalisation at zero time point, dotted peak represents the fluorescence of cells produced by internalised TfR-Tf-Alexa488 complexes after 45 min of incubation in Hanks’ binding buffer at RT. (C) Relative internalisation of Tf-Alexa488 alone and NAI-1-succ-Tf-Alexa488 in terms of the mean fluorescence intensity (MFI). Blue bars represent 0 min samples and Red bars represent 45 min samples with each bar being the mean of triplicate of values ± SDEV. ***P <0.005 extremely significant, *P <0.05 significant.
2.3.4. Cytotoxic activity of NAI-1 and NAI-1-succ-Tf conjugate. The potency of the free isatin derivative, NAI-1 and the conjugate, NAI-1-succ-Tf conjugate was ascertained against a range of cell lines that differed in their sensitivity to free isatin as well as in their levels of cell surface TfR. Since it was also observed upon routine culturing that the human monocytic and mammary epithelial cell lines used in this experiment differed in their growth properties/population doubling time (U937 – 24 h > THP-1 – 26 h > MDA-MB-231 & MCF-7 – 29 h > SK-BR-3 – 48 ± 7.3 h) [373-375], with a view to negate any potential effects of proliferative disparity among the different cell lines used, cytotoxicity assays were conducted over 24, 48 and 72 h.

After 24 h of incubation, NAI-1 was found to be most effective against MDA-MB-231 cells compared to other cells. The significantly high IC$_{50}$ value of 13.65 μM against SK-BR-3 compared to other cell lines, even at the highest concentration of NAI-1 (6.25 μg/mL) tested, reflects its slower doubling time (Table 2.1 & Appendix 2.3). Increasing the incubation time to 48 h significantly enhanced the cytotoxic effect of NAI-1 against all cell lines tested (Table 2.2, Figure 2.11 & Figure 2.12). No enhanced potency was observed for the different cell lines upon extending the incubation time to 72 h (Table 2.3 & Appendix 2.4). This suggested that cells with high proliferation rates like MDA-MB-231 and U937 cells are more sensitive to NAI-1, which is consistent with the cytotoxic effect of most of the chemotherapeutic drugs [376-379]. Meanwhile, as expected, NAI-1-succ was highly ineffective and did not reach IC$_{50}$ values against the different cell lines tested after 24 h and 48 h incubation (Appendix 2.1 & 2.2).
Delivery of the NAI-1 to these cell lines via the NAI-1-succ-Tf conjugate form abolished the high variation, based on proliferation rates, in the cytotoxic activity of the NAI-1 between different cell lines, especially after 48 h incubation (Tables 2.1 & 2.2), suggesting their highly targeted receptor mediated delivery into the cells. Moreover, the conjugate exhibited significantly enhanced potency compared to the NAI-1 at 24 h, and this effect was even more evident at 48 h, especially against SK-BR-3 cells, with IC\textsubscript{50} values ~10 × greater to that obtained for the NAI-1 (Table 2.2). Meanwhile, extending the incubation of the conjugates with all cell lines to 72 h did not enhance the cytotoxic activity of the conjugates, suggesting that 48 h incubation was sufficient for maximum cell death (Table 2.3).
Table 2.1. Cytotoxicity of the NAI-1-succ-Tf conjugate, NAI-1, and NAI-1-succ after 24 h presented as IC₅₀ values.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TfR level</th>
<th>(NAI-1-succ)</th>
<th>(NAI-1)</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>*****</td>
<td>NR b</td>
<td>8.81 (+1.08)</td>
<td>1.41 (+0.12)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>**</td>
<td>NR</td>
<td>4.02 (+0.63)</td>
<td>0.75 (+0.19)</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>***</td>
<td>NR</td>
<td>13.65 (+3.14)</td>
<td>1.36 (+0.34)</td>
</tr>
<tr>
<td>U937</td>
<td>**</td>
<td>NR</td>
<td>5.39 (+0.09)</td>
<td>1.36 (+0.27)</td>
</tr>
<tr>
<td>THP-1</td>
<td>**</td>
<td>NR</td>
<td>0.25 (+0.01)</td>
<td>NT c</td>
</tr>
</tbody>
</table>

*IC₅₀ values were calculated based on moles of cytotoxin from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism V. 5 (GraphPad Software Inc.). Values are the mean of at least two separate experiments ± SDEV.

b NR: Not reached, even at the highest concentration tested (i.e. 11.9 μM).
c NT: Not tested.

***** high, *** medium, ** low
Table 2.2. Cytotoxicity of the NAI-1-succ-Tf conjugate, NAI-1, and NAI-1-succ after 48 h presented as IC₅₀ values.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TfR level</th>
<th>(NAI-1-succ)</th>
<th>(NAI-1)</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>*****</td>
<td>NR</td>
<td>4.57 (+0.75)</td>
<td>0.67 (+0.22)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>**</td>
<td>NR</td>
<td>1.43 (+0.4)</td>
<td>0.52 (+0.15)</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>***</td>
<td>NR</td>
<td>7.67 (+3.02)</td>
<td>0.75 (+0.05)</td>
</tr>
<tr>
<td>U937</td>
<td>**</td>
<td>NR</td>
<td>2.85 (+0.49)</td>
<td>0.93 (+0.03)</td>
</tr>
<tr>
<td>THP-1</td>
<td>**</td>
<td>NR</td>
<td>0.21 (+0.14)</td>
<td>1.67 (+0.07)</td>
</tr>
</tbody>
</table>

- IC₅₀ values were calculated based on moles of cytotoxin from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism V. 5 (GraphPad Software Inc.). Values are the mean of at least two separate experiments ± SDEV.
- NR: Not reached, even at the highest concentration tested (i.e. 11.9 µM).
- ***** high, *** medium, ** low
Table 2.3. Cytotoxicity of the NAI-1-succ-Tf conjugate, NAI-1, and NAI-1-succ after 72 h presented as IC$_{50}$ values.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TfR level</th>
<th>(NAI-1-succ)</th>
<th>(NAI-1)$^b$</th>
<th>Conjugate$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>*****</td>
<td>NT$^c$</td>
<td>4.05 (±1.12)</td>
<td>0.79</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>**</td>
<td>NT</td>
<td>1.38 (±0.34)</td>
<td>0.42</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>***</td>
<td>NT</td>
<td>5.19 (±1.69)</td>
<td>1.15</td>
</tr>
<tr>
<td>U937</td>
<td>**</td>
<td>NT</td>
<td>0.68 (±0.03)</td>
<td>NT</td>
</tr>
<tr>
<td>THP-1</td>
<td>**</td>
<td>NT</td>
<td>0.27 (±0.01)</td>
<td>NT</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ values were calculated based on moles of cytotoxin from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism V. 5 (GraphPad Software Inc.). Values are the mean of at least two separate experiments ± SDEV.

$^b$ Values are the mean of at least two independent experiments ± SDEV.

$^c$ NT: Not tested.

$^d$ Values derived from one experiment.

***** high, *** medium, ** low
Figure 2.11. Representative dose response curves showing cytotoxic activity of NAI-1, and NAI-1-succ-Tf conjugate against human monocytic cell lines (A, B) U937, (C, D) THP-1 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 48 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All value points in the curves are the means of at least triplicate of values ± SDEV.
Figure 2.12. Representative dose response curves showing cytotoxic activity of NAI-1, and NAI-1-succ-Tf conjugate against human breast carcinoma (A, B) MCF-7, (C, D) MDA-MB-231, (E, F) SK-BR-3 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 48 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All value points in the curves are the means of at least triplicate of values ± SDEV.

2.3.5. Cytotoxicity of NAI-2, NAI-2-imine and NAI-2-imine-Tf in vitro.

The cytotoxic activity of NAI-2, NAI-2-imine and the NAI-2-imine-Tf conjugate was
determined in two mammary carcinoma cell lines (MDA-MB-231 and MCF-7), and a human monocytesic U937 cell line, which express differing levels of cell surface TfR (Figure 2.6 & 2.7). The IC50 values after 48 h and 72 h treatment were determined from dose response curves (Figure 2.13) and are summarised in Table 2.4 & 2.5.

NAI-2 was cytotoxic towards all three cell lines tested but was significantly more potent against MDA-MB-231 cells (IC50 = 0.58 µM) compared to U937 cells (IC50 = 1.27 µM) and MCF-7 cells (IC50 = 1.67 µM) after 48 h (Figure 2.13, Table 2.4). Extending the incubation time to 72 h, however, did not produce any marked difference in the cytotoxic activity of NAI-2 against the different cell lines tested (Table 2.5). NAI-2-imine also showed a similar pattern of cytotoxic activity against the three cell lines tested, with the compound being most active against MDA-MB-231 cells after 48 h (IC50 = 0.94 µM) and 72 h (IC50 = 0.49 µM) treatment (Appendix 2.5 & 2.6 for dose response curves of NAI-2 and NAI-2-imine against different cell lines after 72 h treatment).

Here, unlike NAI-1 and NAI-1-succ, the IC50 values obtained with NAI-2-imine against different cell lines tested were not significantly different from the respective IC50 values obtained with NAI-2 (Table 2.4 & 2.5). This was more evident in the case of MCF-7 and MDA-MB-231 cells after 72 h incubation time.

Comparison of the free drug NAI-2 to the NAI-2-imine-Tf conjugate across cell lines demonstrates that the cytotoxic effect of the conjugate is TfR dependent. Here, IC50 values reveal that NAI-2-imine-Tf is equipotent to the free drug against the higher TfR expressing MCF-7 cell line, while the conjugate is 5 × less active than the free drug.
against the lower TfR expressing MDA-MB-231 cell line (Figure 2.13, Table 2.4). The trend observed for the NAI-2-imine-Tf conjugate against the mammary epithelial cell lines is highly consistent with TfR expression levels, that is, the NAI-2-imine-Tf conjugate is $1.25 \times$ more potent against MCF-7 cells compared to MDA-MB-231 cells and the amount of TfR expressed on MCF-7 cells is $1.49 \times$ higher than on MDA-MB-231 cells (Table 2.4). However, the potency of NAI-2-imine-Tf against U937 cells which expresses very low level of TfRs did not follow the general trend based on the relative TfR levels. NAI-2-imine-Tf was found to be most potent against U937 cells after 48 h ($IC_{50} = 1.17 \, \mu M$) and 72 h ($IC_{50} = 0.83 \, \mu M$) treatment (Figure 2.13, Table 2.4 & 2.5).
Table 2.4. Cytotoxicity of the NAI-2-imine-Tf conjugate, NAI-2, and NAI-2-imine after 48 h presented as IC_{50} values.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tfr</th>
<th>(NAI-2-imine)</th>
<th>(NAI-2)</th>
<th>NAI-2-imine-Tf Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>*****</td>
<td>2.19 (± 0.46)</td>
<td>1.67 (± 0.05)</td>
<td>2.00 (± 0.42)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>**</td>
<td>0.94 (± 0.06)</td>
<td>0.58 (± 0.02)</td>
<td>2.51 (± 0.11)</td>
</tr>
<tr>
<td>U937</td>
<td>**</td>
<td>1.75 (± 0.34)</td>
<td>1.27 (± 0.52)</td>
<td>1.17 (± 0.38)</td>
</tr>
</tbody>
</table>

IC_{50} values were calculated based on moles of cytotoxin from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism V. 5 (GraphPad Software Inc.). Values are the mean of at least two separate experiments ± SDEV.

***** high, *** medium, ** low

Table 2.5. Cytotoxicity of the NAI-2-imine-Tf conjugate, NAI-2, and NAI-2-imine after 72 h presented as IC_{50} values.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tfr</th>
<th>(NAI-2-imine)</th>
<th>(NAI-2)</th>
<th>NAI-2-imine-Tf Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>*****</td>
<td>2.03 (± 0.29)</td>
<td>1.96 (± 0.08)</td>
<td>2.18 (± 0.11)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>**</td>
<td>0.49 (± 0.01)</td>
<td>0.49 (± 0.07)</td>
<td>2.41 (± 0.003)</td>
</tr>
<tr>
<td>U937</td>
<td>**</td>
<td>2.43 (± 0.15)</td>
<td>1.56 (± 0.20)</td>
<td>0.83 (± 0.07)</td>
</tr>
</tbody>
</table>

IC_{50} values were calculated based on moles of cytotoxin from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism V. 5 (GraphPad Software Inc.). Values are the mean of at least two separate experiments ± SDEV.

***** high, *** medium, ** low
Figure 2.13. Representative dose response curves showing cytotoxic activity of NAI-2, NAI-2-imine, and NAI-2-imine-Tf conjugate against (A) U937, (B) MDA-MB-231, (C) MCF-7 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 48 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All value points in the curves are the means of at least triplicate of values ± SDEV.

An interesting observation on comparing the cytotoxic activity of NAI-1 and NAI-2 compounds was that the free isatin derivative NAI-2 was more potent against the mammary epithelial MDA-MB-231 and MCF-7 cells and the human monocytic U937
cells (Figure 2.14). However, when delivered to the cells in the conjugate form, the NAI-2 derivative was ~3 × less active than its analog NAI-1.

Figure 2.14. Comparison of the cytotoxic activity of (A) NAI-1 and NAI-2, (B) NAI-1-succ-Tf and NAI-2-imine-Tf against MDA-MB-231, MCF-7, and U937 cells after 48 h treatment. All data points are means of triplicates of at least three independent experiments +/- STDEV. ***P <0.005 extremely significant, *P <0.05 significant, #P >0.05 statistically insignificant.
2.3.6. Morphological assessment of the cytotoxic activity of Tf, NAI-1, NAI-1-succ-Tf, and NAI-2-imine-Tf conjugate. Morphological assessment of individual cell lines treated with NAI-1, NAI-1-succ-Tf, and NAI-2-imine-Tf conjugate found the drug to be cytotoxic as cells showed characteristic features of apoptotic cell death, such as membrane blebbing and condensed chromatin (Figure 2.15 & Figure 2.16). At low concentrations, the NAI-2-imine-Tf conjugate was anti-proliferative as cells appeared viable and were of a lower density than the corresponding Tf only controls (Figure 2.16).

Figure 2.15. Morphological effects of (A) vehicle control, (B) 1 μM NAI-1, (C) 0.2 μM Tf and (D) 0.2 μM NAI-1-succ-Tf equiv. protein conc. on U937 cells after 24 h. Images were viewed at 1000 × magnification using light microscopy on a Leica 12-megapixel high-performance FireWire camera system. Arrows indicate elongated morphology in conjugate treated cells.
Figure 2.16. Morphological effects of Tf and NAI-imine-Tf on MCF-7 and MDA-MB-231 cells after 48 h. Cells were treated with a high concentration (2.75 µM) of Tf (A MCF-7 and E MDA-MB-231), a low concentration (0.34 µM) of Tf (B MCF-7 and F MDA-MB-231), a high concentration (2.75 µM) of NAI-imine-Tf (C MCF-7 and G MDA-MB-231), and a low concentration (0.34 µM) of NAI-imine-Tf (D MCF-7 and H MDA-MB-231). Images were viewed at 400 × magnification using light microscopy on a Leica 12-megapixel high-performance FireWire camera system.
2.4. DISCUSSION

Targeted drug therapy involves selective targeting of tumour cells using agents that recognise specific biomarkers which are characteristic of the tumour cell population. This not only brings about effective reduction of tumour growth, but also minimises cell killing due to bystander effect. Exploitation of TfR remains a current focus of anticancer drug development. TfR is used as a biomarker for the targeting of toxins [380-385], siRNA [386], radionuclide’s [387; 388], nanoparticles [389], and viruses [390-392] to TfR positive cancer cells both \textit{in vitro} and \textit{in vivo} [393]. For example, tumour regression has been reported for a human Tf-diphtheria toxin conjugate in patients with malignant brain tumours [362]. This demonstrates the utility of Tf with well-established evidence of its cellular uptake via RME provides an ideal system for the evaluation and validation of novel drug conjugates. In this \textit{in vitro} study, potent isatin-based microtubule destabilisers (NAI-1 & NAI-2) were conjugated to Tf via an ester and imine bond respectively and were shown to be preferentially delivered to tumour cells that over-express TfR. Here, cytotoxicity assays against mammary epithelial breast carcinoma cells and human monocytic leukemic cells showed that isatin-based targeted drugs are more/as potent as the free drug when delivered to tumour cells in a ligand-dependent manner over the ligand-independent form, confirming the rationale for using NAI-Tf conjugates as targeted therapeutics.

The high efficacy of NAI-1-succ-Tf conjugates compared to the free isatin derivative, NAI-1 is likely due to their selective delivery to the cells by binding to the TfR on the surface of the cell lines tested [327-331]. This receptor-mediated cytotoxicity was more evident following extending the incubation time of the NAI-1-succ-Tf conjugates with
different cells to 48 h (Table 2.2). The cytotoxic activity of the NAI-1-succ-Tf conjugate was consistent with the relative TfR expression levels, on different cell lines tested, in the following order (high TfR > medium TfR > low TfR) (Figure 2.6 & 2.7 and Table 2.1, 2.2, & 2.3). However, variations in the IC_{50} values, contradictory to their relative TfR expression levels, were also observed in the case of U937 and MDA-MB-231 cells (Table 2.1, 2.2, & 2.3, Figure 2.6 & 2.7). This pattern remained unchanged upon extending the incubation time to 48 and 72 h. Normally, NAI-1-succ-Tf would be expected to be highly potent against cells that express high TfR levels. This inconsistency in the receptor-mediated cytotoxicity of U937 and MDA-MB-231 could probably be due to the high proliferation rate of MDA-MB-231 and U937 cells, as observed during routine culturing of these cell lines. Further investigations, however, should be carried out to establish the contribution of cellular proliferation on the receptor mediated cytotoxic activity of NAI-1-succ-Tf conjugates.

The cytotoxic activity of NAI-2-imine-Tf conjugate against MCF-7 and MDA-MB-231 breast cancer cells followed the pattern expected due to the relative difference in cell surface TfR expression levels and suggests a highly selective nature for tumour cells that over-express TfRs. Furthermore, the potency of the NAI-2-imine-Tf conjugate compared to the NAI-2/NAI-2-imine derivatives compares very favourably to other Tf-drug conjugates reported in the literature, which were found to be an order of magnitude less potent than the free drug against various cancer cell lines [356; 366; 394]. However, the fact that NAI-2-imine-Tf conjugate was found to be most potent against U937, which has a low level expression of cell surface TfR compared to MCF-7 and MDA-MB-231 cells (Table 2.4 & 2.5), is in disagreement to the pattern of cytotoxicity
expected against different cell lines with differential TfR expression levels. As reasoned before, this could be due to the high proliferation rate of U937 cells.

Meanwhile, the comparable cytotoxic activity of NAI-2-imine to NAI-2 against the different cell lines tested, especially MCF-7 and MDA-MB-231 cells (Table 2.4 & 2.5), indicates that the linker is undergoing nonspecific cleavage either at the cell surface or in the intracellular space, which could be contributing to the inconsistent cytotoxic activity of NAI-2-imine-Tf. This inference is in complete contradiction to the hydrolytic studies performed by Matesic et al. [296], who found that the NAI-2-imine derivative used in this study is stable at physiological pH and undergoes acid-catalysed hydrolysis only at a pH 4.5 with a half life of 17 min. However more investigations need to be done to corroborate this observation.

The high potency of NAI-2 compared to its analog form, NAI-1, against the different cell lines tested indicates a difference in the mode of action of these compounds, which needs further investigation. Interestingly, when the NAI-2 analog was delivered to cells in the conjugate form, it was less potent than the NAI-1-suce-Tf conjugate (Figure 2.14). This contradiction could likely be explained by the relative efficiency of the linker system utilised in these conjugates. Although the acid sensitive linker system was employed owing to its highly selective and labile nature, the low potency of NAI-2-imine-Tf compared to its analog conjugate indicates the potential unsuitability of this linker system. More investigations, however, should be done to confirm this observation.
Meanwhile, the receptor-dependent cytotoxicity of the NAI-1-succ-Tf and NAI-2-imine-Tf conjugate itself indicates that the structural integrity of Tf is maintained after conjugation and suggests that chemical modification of Tf with NAI-1-succ or NAI-2-imine did not alter the activity of the protein. This fact was further corroborated by internalisation assays that showed receptor mediated delivery of the conjugate to the intracellular space of cells confirming the activity of the conjugate [286].

On the whole, NAI-1-succ-Tf and NAI-2-imine-Tf conjugates can be expected to be highly efficacious against leukemic and malignant solid tumours that are composed of a large population of highly proliferative cells. Preliminary in vivo studies in a metastatic, orthotopic human breast tumour xenograft mouse model showed the NAI-1-succ-Tf conjugate to be effective with less toxicity. However, given the fact that TfRs are also expressed on otherwise normal proliferative cells, further detailed toxicity assessment still needs to be conducted to ascertain its efficacy under in vivo conditions. Prior to that, more investigations need to be done to ascertain its linker stability under in vitro conditions, which can lead to further optimisations of the prodrug and its selective delivery to various tumours.

It can be anticipated that targeting characteristic tumour biomarkers which are highly over-expressed on metastatic malignant tumours using agents that are highly specific for them can cause significant tumour reduction. In this direction, LDCs targeting uPA and HER-2 receptor systems (discussed in detail in Chapter 1), will be developed and discussed in the next few Chapters.
CHAPTER THREE

DEVELOPMENT AND CHARACTERISATION OF PEGYLATED PAI-2 AND ITS USE AS PEG-PAI-2-N-ALKYLISATIN CONJUGATES IN TARGETED THERAPY
DEVELOPMENT AND CHARACTERISATION OF PEGYLATED PAI-2 AND ITS USE AS PEG-PAI-2-N-ALKYLISATIN CONJUGATES IN TARGETED THERAPY.

3.1. INTRODUCTION

Targeted anti-cancer agents utilising naturally occurring inhibitor proteins, although a relatively less populated field, have received major attention as ligand-drug therapeutics owing mainly to their non-toxic and non-immunogenic properties [356; 357]. However, advances in this field in the last decade have been somewhat slow partly due to the limited availability of natural proteins/inhibitors against specific tumour biomarkers and also due to the rapid developments occurring in the field of antibody-based therapeutics [395]. One of the best examples of an LDC utilising a natural protein is the Tf-drug conjugate, which has been widely investigated for targeting many chemotherapeutic agents and other cytotoxins to malignant tumours (refer to Chapter 2).

3.1.1. PAI-2 as a targeting agent for ligand-drug therapeutics

The urokinase plasminogen system along with its specific inhibitors present an ideal scenario to be exploited for targeting malignant tumours (discussed in Chapter 1, Section 1.2.4.1 & 1.2.4.2.). The fact that PAI-2 binds to uPA/uPAR and undergoes rapid and efficient RME [164; 396-398], has been previously exploited for the delivery of radioisotopes and cytotoxins as ligand-directed anticancer agents targeting the uPA system. For example, PAI-2 has been conjugated to the $\alpha$–emitting radioisotope $^{213}$Bismuth ($^{213}$Bi) via the metal chelator cDTPA and has registered impressive activity
against MBC cells under in vitro [399] and in vivo conditions [400; 401]. PAI-2 has also been conjugated to 5-FUdrsucc, which was found to be highly selective against uPA expressing breast cancer cells [402]. However, although the PAI-2-drug conjugates showed efficient receptor-mediated cytotoxicity in vitro, the short in vivo half-life of PAI-2 [403; 404] is a major drawback that may significantly limit the tumour uptake properties of PAI-2 conjugated drugs to reach maximal therapeutic levels. Indeed <2% of total injected dose of radioiodinated PAI-2 appears to be taken up by uPA expressing tumours [403].

3.1.2. Site-directed PEGylation of PAI-2

Covalent modification of proteins utilising polyethylene glycol (PEG) molecules is a common practice used to improve the pharmacokinetic and pharmacodynamic properties of macromolecules. For a complete review of FDA approved PEGylated products and those undergoing clinical trials, refer to [269] and [270]. Site-directed PEGylation through specific amino acid residues present on the genetically modified protein that are low in number or are distinct creates PEG-protein conjugates that are highly defined in terms of its heterogeneity [270]. A description of the effects of PEG on PEGylated proteins and different types of PEGylation strategies were discussed in Chapter 1, Section 1.5.3.2.1 & 1.5.3.2.2.

Site-directed mutagenesis was performed with PAI-2 cDNA to limit the number of cysteine residues available for modification (performed by Dr. Sergei Lobov, UoW). As a result, the mutant form of PAI-2, i.e., C161S-PAI-2 has a total of three cysteine residues; one of them (C145) is surface exposed and are easily accessible for modification and the other two cysteine residues potentially linked by disulfide bond
(C405-S-S-C5), which is deeply embedded inside the molecule, are barely accessible
[405] (Figure 3.1).

Figure 3.1. Ribbon structure of PAI-2 delta CD loop mutant showing the cysteine amino acid residues at C161, C145, C5 and C405. In the delta CD loop mutant form of PAI-2, amino acid residues 66-98 are deleted. In C161S-PAI-2, the cysteine at position 161 was replaced by a serine residue (Sourced from Dr. Sergei Lobov).

Subsequently, site-directed PEGylation of PAI-2 through the cysteine residues was conducted to regulate the number of molecules of PEG that attach to PAI-2 to obtain a PEG-protein conjugate with defined ratio. This thus limits the heterogeneity as well as to enhance the therapeutic benefit of C161S-PAI-2. PEGylation of PAI-2 through the
cysteine residues can also be expected to not block NAI-1-succ conjugation to PAI-2 via the lysine residues. The full scheme of PEGylation and attachment of NAI-1 to C161S-PAI-2 and subsequent RME is shown in **Figure 3.2 & 3.3**.

![Figure 3.2](image)

**Figure 3.2. Schematic representation of the PEGylation of C161S-PAI-2, conjugation of NAI-1 to PEGylated C161S-PAI-2.** The PEGylation of C161S-PAI-2 occurs through the covalent linkage between the terminal maleimide functional group of the PEG and the free thiol group of the cysteine residues present on the C161S-PAI-2 protein. PEGylated C161S-PAI-2 protein is reacted with activated NAI-1-succinate ester, which binds to available surface lysine residues of mPEG-C161S-PAI-2 to form mPEG-C161S-PAI-2-NAI-1-succ conjugate.
Figure 3.3. Schematic representation of the release of NAI-1 free drug following cleavage by lysosomal esterases inside the tumour cell. The mPEG-C161S-PAI-2-NAI-1-succ conjugate specifically binds to uPA/uPAR complex on the cell surface and undergoes RME with the help of LDLR family of receptors. The conjugate undergoes cleavage by esterases in the lysosomal compartment to release the free drug (NAI-1), which in turn kills the cell.

The specific aims of this chapter were:

1. To optimise the PEGylation and purification of a mutant form of PAI-2 (i.e., C161S-PAI-2 ΔCD loop) by attaching a 12 kDa or 20 kDa maleimide functional PEG (mPEG) group to yield mPEG-C161S-PAI-2 molecules, which could be used for further studies.

2. To optimise the conjugation of a highly potent cytotoxin, 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin (NAI-1) to mPEG-C161S-PAI-2 obtained from aim 1 via an enzyme labile ester linker.
3. To determine the cytotoxic activity of the mPEG-C161S-PAI-2-NAI-1-succ conjugate by in vitro cytotoxicity assays against MDA-MB-231 mammary epithelial carcinoma cells that highly express uPA/uPAR.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals, reagents, proteins and antibodies. M15 [pREP4] E. coli was obtained from QIAgen (Victoria, Australia). Anhydrous dimethylformamide (DMF), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), hanks balanced salt modified and non-modified, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. RPMI-1640 powder and foetal calf serum were from Trace Bioscientific (NSW, Australia). Trypsin/EDTA (0.05% trypsin, 4Na EDTA) was obtained from Invitrogen. Polyethylene glycol functionalised with a maleimide group (mPEG-maleimide) MW 12,000 Da and MW 20,000 Da was obtained from Yushi-Seihin Co., Ltd., (Keypoint, # 15-08, Singapore 199597). IPTG was purchased from Applichem. Ampicillin and kanamycin were purchased from Amresco. TALON metal affinity resin was purchased from Clontech. Desalting PD-10 columns were purchased from GE Healthcare. Hi Screen Capto Q ImpRes strong anion exchange column was obtained from Quantum Scientific. NAI-1 and NAI-1-succ were synthesised in-house by Dr. Julie Locke and Dr. Lidia Matesic, Intelligent Polymer Research Institute and School of Chemistry, University of Wollongong (NSW, Australia). BIO-RAD Quick Start™ Bovine Serum Albumin (BSA) Standard and Bio-Rad DCTM Protein Assay Kit (Reagent A & B) were obtained from BIO-RAD, Hercules, CA. CellTiter 96 AQueous
One Solution Cell Proliferation Assay (MTS) reagent was purchased from Promega Corporation (Sydney, Australia). High molecular weight (HMW)-uPA and Mouse anti-human PAI-2 (#3750) were purchased from American Diagnostica, Inc. (Greenwich, CT). Mouse monoclonal antibody, IgG1 and Goat anti-mouse HRP (#AP124P) were purchased from Millipore Australia Pty Ltd (Victoria, Australia). Sinapinic acid (SA) was obtained from LaserBio Labs (LBL) (Sophia-Antipolis Cedex, France).

3.2.2. Cell lines. The human mammary epithelial carcinoma cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC, VA, USA). Refer to Section 2.2.2 for routine culturing conditions.

3.2.3. Expression and purification of C161S-PAI-2 ΔCD loop mutant. C161S-PAI-2 ΔCD loop hexa his-tagged mutant was expressed and purified as described by Cochran et al. [406] with minor modifications and will be referred to as C161S-PAI-2. Briefly, purified pQE9/C161S-PAI-2 ΔCD-loop vector was transformed into electro competent M15 [pREP4] cells. Cells were cultured overnight at 37° C with shaking in LB containing 100 μg/mL ampicillin and 25 μg/mL kanamycin. A 15 mL aliquot of this starter culture was added to 1 L T-broth (Appendix 1) and grown to an OD (600) value of ~1.0. Subsequently, C161S-PAI-2 expression was induced by the addition of 0.5 mM IPTG and the culture incubated for a further 4-5 h. Cells were collected by centrifugation at 10,000 g for 10 min. Pelleted cells were resuspended in 25 mL of ice-cold loading buffer (Appendix 1) and lysed using a EmulsiFlex-C5 Homogenizer (Avestin, Canada). The cell lysate was then spun at 17,000 g for 30 min to remove the cell debris. The supernatant thus obtained was incubated with the pre-equilibrated TALON metal affinity resin at 4° C for 1 h under gentle rotation. The
unbound proteins were removed using 10 column volumes of loading buffer followed by 10 column volumes of wash buffer (Appendix 1). Bound protein was eluted from the column using elution buffer (Appendix 1). Purification was analysed using SDS-PAGE under reducing conditions and protein concentration was ascertained using Bio-Rad Protein Assay Kit and BSA Standards. Samples were then buffer exchanged into phosphate buffer (pH 7.4) to remove imidazole and stored in 0.02% sodium azide at 4°C for future use.

3.2.4. Characterisation of C161S-PAI-2 by SDS-PAGE. SDS-PAGE was performed to analyse the purified and modified PAI-2 forms. Briefly, protein samples were prepared in PBS (pH 7.5) containing 5 μL of 5 × reducing sample buffer or 5 × non-reducing sample buffer (Appendix 1) and denatured by boiling at 95°C for 5 min. Meanwhile, 4% stacking (Appendix 1) and 10% resolving gel (Appendix 1) was prepared using a Mini PROTEAN3 gel system (BioRad, Hercules, CA, USA). The samples were then electrophoresed at 120 V for 90 min at RT until the dye front had run to the bottom of the gel. Gels were stained in Coomassie staining solution (Appendix 1) by rocking at RT O/N. Gels were then destained using rapid destain solution (Appendix 1) by rocking at RT O/N. Subsequently standard curves were generated using dual colour precision plus protein prestained standards to determine the molecular weight of the samples.

3.2.5. Western blot analysis. Western blot analysis was performed to confirm the status of the purified PAI-2 forms using a Mini Trans-Blot Cell system (Bio-Rad, Hercules, CA, USA). Firstly, the PVDF membrane was activated by soaking in 100% methanol and equilibrated in transfer buffer (Appendix 1). The blot system was
assembled as per manufacturers’ instructions after soaking all components in transfer buffer. Thereafter, the proteins were transferred to the PVDF membrane at 100 V for 90 min at RT. The PVDF membrane was blocked with 10% (w/v) powdered milk in TBST (Appendix 1) for 1 h at RT. The membrane was then incubated with mouse anti-human PAI-2 primary antibody (1:500 dilution) in 2% (w/v) powdered milk in TBST O/N at 4°C. Subsequently, the membrane was re-blocked with 6% (w/v) powdered milk in TBST and then incubated with goat anti-mouse HRP secondary antibody (1:5000 dilution) 2% (w/v) powdered milk in TBST for 1 h at RT. The PVDF membrane was washed at each step and finally washed in TBS (Appendix 1) before analysis using SuperSignal West Pico Chemiluminescence Substrate and Hyperfilm ECL.

3.2.6. Conjugation of mPEG to C161S-PAI-2. Freshly prepared C161S-PAI-2 was buffer exchanged into phosphate buffer (pH 7.5). A 20–30 molar excess of 12 or 20 kDa mPEG solution (10 mM stock solution freshly prepared by dissolving 12 or 20 kDa mPEG in distilled water) was added to C161S-PAI-2 in phosphate buffer (pH 7.5) and incubated at RT for 0–8 h with gentle shaking. Where indicated, the reaction was stopped by the addition of 6x SDS buffer followed by heating the mixture at 95°C for 5 min. The mPEG-C161S-PAI-2 conjugate mixture was then fractionated by a 10% non-reducing SDS-PAGE to visualise the extent of PEGylation of C161S-PAI-2 at various time points or the reaction mixture was stored at 4°C without purification for future use.

3.2.7. Purification of mPEG-C161S-PAI-2 by ion exchange chromatography. The purification of PEGylated C161S-PAI-2 was performed on
the AKTA Purifier (UNICORN 5.01) using a Hi Screen Capto Q ImpRes anion exchange column.

The pH of mPEG-C161S-PAI-2 was adjusted to the composition of the start buffer (20 mM Tris-HCl, pH 8.0) by exchanging into the desired buffer to ensure the sample was sufficiently negatively charged. The concentration of the sample was then determined by Lowry assay.

The column was washed with 5 column volumes of start buffer until the baseline, eluent pH, and conductivity were stable. This was followed by washing with 5 column volumes of elution buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0), and thereafter the excess NaCl in the column was washed away with 5 column volumes of start buffer. The sample was applied to the column and subsequently the column was washed with 5 column volumes of start buffer and flow through was collected. Thereafter, samples were eluted using a gradient of increasing ionic strength up to 500 mM NaCl (100% B) with a flow rate of 0.5 mL/min for 60 min. The column was then washed with 5 column volumes of 1 M NaCl (100% B) to elute any remaining ionically-bound material followed by re-equilibration of the column with 5–10 column volumes of start buffer until eluent pH and conductivity reach the required values. The concentration of the eluate fractions was determined by Lowry assay.


Sample preparation. The MALDI TOF analyses of C161S-PAI-2 and modified mPEG-C161S-PAI-2 was performed according to the method described by Seyfried et al. [407] with slight modifications. Firstly, for matrix preparation, a mixture of acetonitrile (ACN), distilled water (dH2O) and trifluoroacetic acid (TFA) in 40:60:0.1
ratio was added to a matrix aliquot of sinapinic acid (SA) to achieve a final concentration of 10 mg/mL. The mixture was mixed thoroughly by vortexing for 30 s and then spun in a microcentrifuge at 14,000 rpm to separate undissolved matrix and the supernatant was used for further analysis. For sample preparation in the case of C161S-PAI-2, the dried droplet technique [408] was utilised. After the deposition of 0.5 μL of matrix solution onto the target plate, approximately 0.5 μL of C161S-PAI-2 in 10 mM Tris buffer (pH 8.0) was injected into the matrix droplet and dried at RT. For mPEG-C161S-PAI-2 and mPEG-C161S-PAI-2-NAI-1-succ, the volume technique was applied. Approximately 1 μL of matrix and 1 μL of mPEG-C161S-PAI-2/ mPEG-C161S-PAI-2-NAI-1-succ in 10 mM Tris buffer (pH 8.0) were mixed thoroughly in an eppendorf tube and 1 μL of the homogeneous solution was deposited on the target plate and dried at RT.

**Analyses.** All measurements were performed in the linear positive ion mode with an AXIMA Confidence instrument (Shimadzu Biotech, Manchester, UK) equipped with a nitrogen laser and applying a source voltage of 20,000 V, einzel lens voltage of 6,500 V, pulsed extraction voltage of 2,625 V, reflectron voltage of 24,400 V, linear detector voltage of 2,700 V, and reflection detector voltage of 1,850 V. All analyses were performed by Dr. David Harman (NMR and MS Manager, ARC Centre of Excellence for Electromaterials Science, Intelligent Polymer Research Institute, University of Wollongong) using the Shimadzu Biotech Axima Confidence software (version 2.8.3).

**3.2.9. Conjugation of NAI-1-succ to mPEG-C161S-PAI-2.** The active ester of NAI-1-succ was prepared based on the method described by Vine *et al.* [286]. Briefly, NAI-1-succ (2.7 mg, 5.0 μmol) was dissolved in anhydrous DMF (70 μL). N-hydroxysuccinimide (NHS, 0.59 mg, 5.0 μmol) in DMF (17 μL) and
dicyclohexylcarbodiimide (DCC, 6.18 mg, 30 μmol) in DMF (50 μL) were then added and the reaction mixture shaken and kept at RT for 15-20 min. A 30-fold molar excess of crude orange product was then conjugated to free lysine residues of mPEG-C161S-PAI-2 (~1 mg/mL) in PBS (pH 8.5) at RT, with gentle shaking. After 4 h incubation, the reaction mixture was centrifuged (maximum speed for 2-5 min) to remove any precipitate. The conjugate was then purified by gel filtration using PD-10 desalting column, with PBS (pH 7.5) as eluant. Fractions corresponding to the conjugated protein peak (as determined by UV/Vis spectrophotometry for C161S-PAI-2 at 280 nm and for NAI-1 at 432 nm) were pooled, and stored at 4° C for future cytotoxicity and characterisation studies. Protein concentration was determined using the Lowry assay. The number of NAI-1 molecules bound to mPEG-C161S-PAI-2 was determined by MALDI-TOF MS analysis.

3.2.10. Activity of mPEG-C161S-PAI-2 Forms. The inhibitory activity of mPEG-C161S-PAI-2 and mPEG-C161S-PAI-2-NAI-1 conjugate was ascertained by the ability of the PAI-2 to form stable complexes with uPA as previously described [399]. Briefly, mPEG-C161S-PAI-2 or mPEG-C161S-PAI-2-NAI-1 conjugate was incubated with equimolar amounts of uPA in PBS (pH 7.4) for 20 min at RT and complex formation was visualised by Coomassie blue staining of samples fractionated by SDS-PAGE under non-reducing conditions.

3.2.11. In vitro cytotoxicity assay. In vitro cytotoxicity assays were performed as described previously by Vine et al. [286]. Briefly, cells (5,000 cells/well) were seeded in 96-well plates and incubated overnight at 37° C in 95% humidified atmosphere, containing 5% CO2. On the following day, mPEG-C161S-PAI-2 and mPEG-C161S-
PAI-2-NAI-1-succ conjugate was added to triplicate wells at decreasing concentrations followed by incubation for 24 h at the above-mentioned conditions. Subsequently, MTS reagent was added to all wells and after a 3 h incubation period, the absorbance was measured at 490 nm using SpectraMax® Plus 384 Microplate Spectrophotometer with SoftMax® Pro Software. The cytotoxicity of the compounds was expressed in terms of IC₅₀ values (concentration of conjugate required to inhibit the metabolic activity of 50% of the total cell population) after taking into account the PBS-treated control cells. These values were calculated from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism™ software v5.02 (GraphPad Software Inc.). In all IC₅₀ calculations using GraphPad prism software, cells + PBS absorbance values subtracted from baseline absorbance values (media + PBS) were taken as 100% cell viability (100% value) control and cells + highest concentration of test compound treatment was taken as 0% cell viability (0% value) control.

3.2.12. Statistical analyses. Statistical significance of treatment groups as compared to control groups was determined using unpaired students T-test (GraphPad Prism V 5.1; San Diego, CA, USA). P values < 0.05 were considered statistically significant.

3.3. RESULTS

3.3.1. Expression and purification of C161S-PAI-2 ACD loop mutant.
The expression and purification of C161S-PAI-2 mutant using the pQE9 system regularly yielded ~15 mg of relatively pure protein from a 1L terrific broth (TB) culture
(Appendix 1) with some impurities. Analysis of the purified C161S-PAI-2 mutant by a 10% non-reducing SDS-PAGE revealed a dense band migrating at ~45 kDa consistent with the size of C161S-PAI-2 ΔCD loop mutant (Figure 3.4A, Lane 2) and this was confirmed as PAI-2 by western blotting (Figure 3.4B). Another band migrating at ~95 kDa was also observed, which are C161S-PAI-2 polymers. This is supported by studies performed by [155] who found that PAI-2 forms polymers spontaneously under non-reducing conditions.

Figure 3.4. (A) SDS-PAGE analysis of purified C161S-PAI-2 eluate fractions fractionated on a 10% acrylamide gel under non-reducing conditions. Lane 1 – Marker, Lane 2 – C161S-PAI-2 sample. (B) Western blot – Fractionated samples were transferred to PVDF membrane and the PAI-2 protein detected using an anti-PAI-2 mAb.
3.3.2. PEGylation and characterisation of C161S-PAI-2. Conjugation of mPEG to C161S-PAI-2 and further optimisations were carried out under various conditions by altering the reaction time, temperature and molar ratio of the amount of mPEG reacted with C161S-PAI-2, in order to optimise the production of a homogenous species of mPEG-C161S-PAI-2 conjugate. Currently, there are several methods in use to determine the extent of PEGylation of a mPEG-protein conjugate such as size exclusion chromatography (SEC), electrophoretic methods like SDS-PAGE, light scattering and mass spectrometry [270; 409; 410]. Here, the molecular weight of the PEGylated protein was determined by SDS-PAGE analysis under non-reducing conditions.

Initially, a 20 molar excess of the 20 kDa mPEG was reacted with C161S-PAI-2 at RT for a total of 3 h. Samples were removed at the end of 0, 1, 1.2, 2, and 3 h and immediately added to SDS sample buffer and heated at 95° C to stop any further reaction and were analysed by SDS-PAGE to ascertain the degree of PEGylation. Characterisation of the mPEG-C161S-PAI-2 conjugate mixture revealed a band migrating at ~85 kDa, which grew dense when the incubation time was increased from 0-3 h. This corresponds to two molecules of mPEG (total MW = 40 kDa) attached to the C161S-PAI-2 (di-PEGylated C161S-PAI-2). Another dense band at ~45 kDa was also observed consistent with the size of unPEGylated C161S-PAI-2. As expected, there was a significant amount of unreacted mPEG molecules in the mixture as can be seen by the uneven migration of bands (Figure 3.5, Lane 3-7).
Figure 3.5. SDS-PAGE analysis of C161S-PAI-2 PEGylation over 0-3 h at RT using 20 molar excess of 20 kDa mPEG. Samples were taken at each time point and fractionated on a 10% acrylamide gel and visualised by Coomassie staining. Lane 1 – Marker, Lane 2 – C161S-PAI-2, Lane 3 – mPEG-C161S-PAI-2 (0 h), Lane 4 – mPEG-C161S-PAI-2 (1 h), Lane 5 – mPEG-C161S-PAI-2 (1.2 h), Lane 6 – mPEG-C161S-PAI-2 (2 h), Lane 7 – mPEG-C161S-PAI-2 (3 h).

To increase the extent of PEGylation of C161S-PAI-2, the incubation time of the reactants (C161S-PAI-2 and 20 kDa mPEG), was increased to a total of 6 h at RT. Samples were removed at the end of 0, 2, 4, and 6 h and immediately added to SDS sample buffer and heated at 95° C to stop any further reaction and analysed by SDS-PAGE to ascertain the degree of PEGylation. Analysis of the mPEG-C161S-PAI-2 mixture at the end of 6 h reaction time revealed a dense band migrating at ~85 kDa consistent with the size of a di-PEGylated C161S-PAI-2. Similar to the previous PEGylation, here, there was no significant increase in the amount of PEGylated C161S-PAI-2 product upon extended incubation time, especially after 2-4 h, as evidenced by
the SDS-PAGE (Figure 3.6, Lane 4 & 5). Meanwhile, the reaction mixture also contained a large amount of unreacted C161S-PAI-2 and mPEG molecules.

![Figure 3.6. SDS-PAGE analysis of C161S-PAI-2 PEGylation over 0-6 h at RT using 20 molar excess of 20 kDa mPEG. Samples were taken at each time point and fractionated on a 10% acrylamide gel and visualised by Coomassie staining. Lane 1 – Marker, Lane 2 – C161S-PAI-2, Lane 3 – mPEG-C161S-PAI-2 (0 h), Lane 4 – mPEG-C161S-PAI-2 (2 h), Lane 5 – mPEG-C161S-PAI-2 (4 h), Lane 6 – mPEG-C161S-PAI-2 (6 h).](image)

In order to further increase the yield of the 85 kDa mPEG-C161S-PAI-2 conjugate with less residual unreacted C161S-PAI-2 and mPEG species, a 30 molar excess of 20 kDa mPEG was reacted with C161S-PAI-2 at RT. Here the conjugation was carried out only
for 4 h as it was noticed in the previous conjugation that extending the incubation to more than 4 h does not significantly increase the extent of PEGylation. Analysis of the samples showed two bands which were in the size range of 75-110 kDa, which could be di-PEGylated C161S-PAI-2 (~85 kDa) and tri-PEGylated C161S-PAI-2 (~105 kDa) (Figure 3.7, Lane 3-5).

As part of further optimisation experiments, the effect of temperature on PEGylation rate was ascertained. The PEGylation of C161S-PAI-2 was performed at 37°C with all other reaction conditions remaining the same as in the previous conjugation (described in Figure 3.7). Analysis of the PEGylated C161S-PAI-2 products obtained by SDS-
PAGE revealed that the conjugation reaction was not substantially improved by incubation at 37° C and the degree of attachment of mPEG to C161S-PAI-2 remained unchanged after 2 h of incubation time (Figure 3.8). A mixture of what appears to be di- and tri-PEGylated C161S-PAI-2, ~85 kDa and ~105 kDa respectively, was obtained at the end of this reaction with a large amount of unreacted C161S-PAI-2 and mPEG molecules.

Figure 3.8. SDS-PAGE analysis of C161S-PAI-2 PEGylation over 0-4 h at RT using 30 molar excess of 20 kDa mPEG. Samples were taken at each time point and fractionated on a 10% acrylamide gel and visualised by Coomassie staining. Lane 1 – Marker, Lane 2 – C161S-PAI-2, Lane 3 – mPEG-C161S-PAI-2 (0 h), Lane 4 – mPEG-C161S-PAI-2 (2 h), Lane 5 – mPEG-C161S-PAI-2 (4 h).
Thereafter, PEGylation using a smaller 12 kDa mPEG was examined. A 30 molar excess of 12 kDa mPEG was reacted with C161S-PAI-2 at RT for 4 h. Analysis of the mPEG-C161S-PAI-2 product by SDS-PAGE found a dense band migrating at ~70 kDa indicating attachment of two molecules of 12 kDa mPEG per molecule of C161S-PAI-2 (di-PEGylated C161S-PAI-2) as the major species and some higher species migrating at ~80 kDa, which could be tri-PEGylated C161S-PAI-2. In this PEGylation reaction, more than ~30% of the total C161S-PAI-2 was PEGylated, as evidenced by densitometry analysis of SDS-PAGE (Figure 3.9A). However, there was still a significant amount of unconjugated residual C161S-PAI-2 and mPEG present in the reaction mixture (Figure 3.9A, Lane 3).

In order to improve the efficiency of the PEGylation reaction, freshly prepared C161S-PAI-2 was used along with reaction conditions described in Figure 3.9A. Analysis of this reaction mixture by SDS-PAGE revealed a significant improvement in the degree of PEGylation with greater than ~60% of the total C161S-PAI-2 PEGylated (Figure 3.9B). The mPEG-C161S-PAI-2 species, as evidenced by the SDS-PAGE contained a mixture of apparently di-PEGylated (~70 kDa) and tri-PEGylated C161S-PAI-2 (~80 kDa), with the di-PEGylated C161S-PAI-2 being the major species. A minor species migrating at ~100 kDa and ~150 kDa was also observed (Figure 3.9A & B, Lane 2 & 3).
Figure 3.9. SDS-PAGE analysis of C161S-PAI-2 PEGylation over 4 h at RT using 30 molar excess of 12 kDa mPEG and fractionated on a 10% acrylamide gel and visualised by Coomassie staining. (A). Reactions performed using stored C161S-PAI-2. Lane 1 – Marker, Lane 2 – C161S-PAI-2, Lane 3 – mPEG-C161S-PAI-2 (PEGylated and fractionated after 4 h incubation). (B). Reactions performed using freshly prepared C161S-PAI-2. Lane 1 – Marker, Lane 2 – C161S-PAI-2, Lane 3 – mPEG-C161S-PAI-2 (freshly prepared C161S-PAI-2, PEGylated and fractionated after 4 h incubation).

3.3.3. Purification and characterisation of mPEG-C161S-PAI-2.

PEGylated C161S-PAI-2 conjugates were separated from free C161S-PAI-2 and mPEG by FPLC using an anion exchange column attached to the AKTA Explorer.

Initially, the separation of mPEG-C161S-PAI-2 conjugates containing the 20 kDa mPEG was carried out using a Mono Q anion exchange column [411]. However, the removal of excess free C161S-PAI-2 and mPEG proved inefficient and all the PEGylated C161S-PAI-2 and other constituents eluted in the flow through and in the
subsequent fractions (A1-A5) indicating inefficient binding of the protein to the column (Figure 3.10, Lane 3-8).

Figure 3.10. Samples fractionated by SDS PAGE showing the compounds of the different elution fractions resolved in an anion exchange Mono Q column. Samples were then fractionated under non-reducing conditions using a 10% acrylamide SDS PAGE and visualised by staining with Coomassie blue. Lane 1 – Marker, Lane 2 – C161S-PAI-2 starting material sample, Lane 3 – Flow through, Lane 4 – Fraction A1, Lane 5 – Fraction A2, Lane 6 – Fraction A3, Lane 7 – Fraction A4, Lane 8 – Fraction A5.

PEGylated C161S-PAI-2 containing 12 kDa mPEG was purified using a Hi Screen Capto Q ImpRes anion exchange column, which is highly specific for the separation of 12 kDa mPEG-protein conjugates from free mPEG and C161S-PAI-2 [411]. Analysis of the ion-exchange output profile of a representative purification showed elution of some charged species (Figure 3.11, Peak 1, 2 & 3) at around 20-25% gradient of 100% 500 mM NaCl.
Figure 3.11. Ion exchange purification of mPEG-C161S-PAI-2. mPEG-C161S-PAI-2 was injected onto a Hi Screen Capto Q ImpRes anion exchange column and eluted using a linear NaCl gradient (0 – 500 mM) (100% B) at a flow rate of 0.5 mL/min. The blue line indicates the elution of protein. The light green line indicates NaCl concentration in the column. The light blue represents % conductivity. The dark green line denotes the relative pressure in the column.

Samples of the starting material C161S-PAI-2 and unpurified mPEG-C161S-PAI-2 as well as samples from the eluate fractions were fractionated by SDS-PAGE under non-reducing conditions (Figure 3.12).

Analysis of the SDS-PAGE showed what appears to be approximately 20% of tri-PEGylated and ~60% of di-PEGylated C161S-PAI-2 species (Figure 3.12, Lane 5, 6 & 7) and some residual C161S-PAI-2 species (Figure 3.12, Lane 9). Further analysis indicated that the free uncharged 12 kDa mPEG molecules yielded from the PEGylation reaction did not bind to the column as expected and was found in the flow through fraction (Figure 3.12, Lane 4). The tri-PEGylated C161S-PAI-2, di-PEGylated C161S-
PAI-2, and the free C161S-PAI-2 eluted in the later fractions, in that order which was dependent on the relative negative charge density on these species (tri-PEG-C161S-PAI-2 < di-PEG-C161S-PAI-2 < C161S-PAI-2) (Figure 3.12, Lanes 5 – 10). Some cross-contamination of different PEGylated species and C161S-PAI-2 was also observed in these eluate fractions obtained from the ion exchange, which needs further optimisation (Figure 3.12, Lane 5, 6, 7 & 8). However, fractions in Lane 5 & 6 were relatively free of unconjugated C161S-PAI-2 and contained >90% of either tri-PEG (Figure 3.12, Lane 5) or di-PEG (Figure 3.12, Lane 6 & 7) and they were thus used for further conjugation experiments.

Figure 3.12. Samples fractionated by SDS PAGE showing the compounds of the different elution fractions resolved using a strong anion exchange Hi Screen Capto Q ImpRes column. Samples were then fractionated under non-reducing conditions using a 10% acrylamide SDS PAGE and visualised by staining with Coomassie blue. Lane 1 – Marker, Lane 2 – C161S-PAI-2 starting material sample, Lane 3 – mPEG-C161S-PAI-2 control, Lane 4 – Flow through, Lane 5 – Fraction A8, Lane 6 – Fraction A9, Lane 7 – Fraction A10, Lane 8 – Fraction A11, Lane 9 – Fraction A12, Lane 10 – Fraction A13.
3.3.3.1. Characterisation of mPEG-C161S-PAI-2 by MALDI-TOF MS.

A MALDI-TOF MS was performed to confirm the number of molecules of mPEG attached to C161S-PAI-2. A representative MALDI-TOF MS analysis (Figure 3.13) of purified mPEG-C161S-PAI-2 fractions (fractions 5 & 6 from Figure 3.11) obtained from Hi Screen Capto Q ImpRes anion exchange column showed PEGylated C161S-PAI-2 species with attachment of one (Figure 3.13B) and two (Figure 3.13C) molecules of mPEG per C161S-PAI-2 molecule. mPEG-C161S-PAI-2 was then stored at 4° C for future experiments.
Figure 3.13. A representative linear positive ion mode MALDI-TOF MS analysis of ion exchange purified mPEG-C161S-PAI-2 with sinapinic acid as the matrix using AXIMA Confidence instrument and Shimadzu Biotech Axima Confidence software (version 2.8.3). The average molecular weight of unmodified C161S-PAI-2 was found to be 44890.8 Da (panel A) and the molecular weight of mPEG was taken as 12000 Da. An increase in molecular weight of C161S-PAI-2 to 57069.1 (panel B) and 69718.2 (panel C) indicates attachment of 1 and 2 molecules of mPEG to C161S-PAI-2 molecule.
3.3.4. Preparation, visualisation, and characterisation of mPEG-C161S-PAI-2-NAI-1-succ conjugate. Previous conjugation experiments involving PAI-2 conjugated to NAI-1 utilised a 20-fold molar excess of NAI-1-succ, which resulted in optimum drug incorporation without affecting the activity of PAI-2 protein [286]. Here, a 30-fold molar excess of the active ester of NAI-1-succ was reacted with mPEG-C161S-PAI-2 to counter the likely effect of steric hindrance due to the presence of PEG molecules.

The conjugated product was purified by size-exclusion chromatography and subsequently visualised at 280 nm for C161S-PAI-2 and 432 nm for isatin (Figure 3.14). Upon purification by PD-10 desalting columns, some residual orangish-yellow coloured product remained on the column which could be unconjugated or free isatin from the conjugation reaction. Determination of the number of molecules of NAI-1 attached per molecule of mPEG-C161S-PAI-2 was first carried out using the absorbance values of mPEG-C161S-PAI-2-NAI-1-succ conjugate measured at A280 & A432 and the extinction coefficients of C161S-PAI-2 at A280 and the NAI-1 drug at A432 mathematically (Appendix 3). The NAI-1 compound also absorbs at A260 and A310 (Vine et al., unpublished data). After negating the contribution of NAI-1 to the measured absorbance at A280, a total of approximately 15 molecules of NAI-1 was calculated incorporated onto per molecule of mPEG-C161S-PAI-2. This is highly inconsistent with the number of lysine residues (~7) [400] and [403] present on PAI-2 that is available for chemical modification.
Figure 3.14. Size exclusion chromatography elution profiles of a representative mPEG-C161S-PAI-2-NAI-1-succ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of mPEG-C161S-PAI-2 (and in later fractions, the excess coupling reagents, NHS and DCC). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1-succ.

The quantification of NAI-1 attachment mPEG-C161S-PAI-2 was attempted by MALDI-TOF MS. A representative MALDI-TOF MS analysis (Figure 3.15) of the mPEG-C161S-PAI-2-NAI-1-succ conjugate revealed incorporation of an average of ~2 molecules of NAI-1 per mPEG-C161S-PAI-2 molecule. This mPEG-C161S-PAI-2-NAI-1-succ conjugate was then stored at 4° C and used in cytotoxicity experiments.
Figure 3.15. A representative linear positive ion mode MALDI-TOF MS analysis of mPEG-C161S-PAI-2-NAI-1-succ conjugate with sinapinic acid as the matrix using AXIMA Confidence instrument and Shimadzu Biotech Axima Confidence software (version 2.8.3). The average molecular weight of mPEG-C161S-PAI-2 was taken as 57069.1 Da from (A) and the molecular weight of NAI-1-succ was taken as 525.14 g/mol. The average increase in molecular weight of mPEG-C161S-PAI-2 upon attachment of isatin molecules calculated from the figure indicated a total of ~2 molecules of isatin attached per molecule of mPEG-C161S-PAI-2.

3.3.5. mPEG-C161S-PAI-2-NAI-1-succ conjugate is active after modification. The uPA inhibitory activity of the modified C161S-PAI-2 was ascertained by its ability to form stable covalent complexes with uPA. Analysis of the fractionated samples by SDS-PAGE showed formation of ~100000 Da stable complexes of modified C161S-PAI-2 with high molecular weight (~55000 Da) uPA (Figure 3.16, Lane 6). This is consistent with the results obtained for unmodified C161S-PAI-2 (Figure 3.16, Lane 5).
Figure 3.16. Samples fractionated by 10% acrylamide SDS PAGE under non-reducing conditions showing the ability of a) unmodified and b) modified C161S-PAI-2 to form stable complexes with uPA. Samples were then visualised by staining with Coomassie blue. Lane 1 – Marker, Lane 2 – C161S-PAI-2, Lane 3 – mPEG-C161S-PAI-2-NAI-1-succ, Lane 4 – uPA, Lane 5 – C161S-PAI-2:uPA complex, Lane 6 - mPEG-C161S-PAI-2-NAI-1-succ:uPA complex.
3.3.6. *In vitro* potency of mPEG-C161S-PAI-2-NAI-1-succ conjugate.

The cytotoxic activity of mPEG-C161S-PAI-2-NAI-1-succ conjugate was ascertained against the MDA-MB-231 breast cancer cell line, previously determined in our laboratory to be high uPA/uPAR expressing [286]. Here, the mPEG-C161S-PAI-2-NAI-1-succ conjugate was incubated for 24 h, as it was found that PAI-2-N-AIE with similar conjugation efficiency, in terms of the number of molecules of NAI-1 attached to C161S-PAI-2, was most active against the MDA-MB-231 cells after 24 and 48 h [286].

To enable efficient binding and internalisation of more conjugates into the tumour cells, exogenous uPA was added to the cells, to saturate all potentially unoccupied uPAR (as described by Al-Ejeh *et al.* [150]), after 24 h incubation and before the addition of mPEG-C161S-PAI-2 and mPEG-C161S-PAI-2-NAI-1-succ conjugate.

On analysis of the dose response curves, mPEG-C161S-PAI-2 was found to be highly proliferative at a concentration of 1 µM and above (*Figure 3.17 A*). After normalising to the mPEG-C161S-PAI-2 and PBS controls, mPEG-C161S-PAI-2-NAI-1-succ conjugate was found to be highly cytotoxic against MDA-MB-231 cells with an IC$_{50}$ value of 0.26 µM (*Figure 3.17 B*). The mPEG-C161S-PAI-2-NAI-1-succ conjugate was greater than 16 × more active than the NAI-1 parent compound (IC$_{50}$ 4.4 µM) (*Figure 3.17 C*). Interestingly, mPEG-C161S-PAI-2-NAI-1-succ conjugate was nearly equipotent to the C161S-PAI-2-NAI-1-succ parent conjugate.
Figure 3.17. Representative dose response curves showing cytotoxic activity of mPEG-C161S-PAI-2-NAI-1-succ, C161S-PAI-2-NAI-1-succ conjugate and NAI-1 parent compound against MDA-MB-231 cells; (A) mPEG-C161S-PAI-2-NAI-1-succ (normalised to PBS control only). Dark line represents mPEG-C161S-PAI-2 and dashed line represents mPEG-C161S-PAI-2-NAI-1-succ conjugate; (B) mPEG-C161S-PAI-2-NAI-1-succ conjugate (curve normalised to mPEG-C161S-PAI-2 control); (C) NAI-1 parent compound; and (D) C161S-PAI-2-NAI-1-succ conjugate (curve normalised to C161S-PAI-2 control). Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 24 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All value points in the curves are the means of at least triplicate of values ± SDEV.
3.4. DISCUSSION

PEGylation of proteins involving the covalent attachment of PEG molecules to the biologically active protein should be carried out in a way that favourably alters the physicochemical properties of the protein, and at the same time, does not lead to a significant loss in activity [412; 413]. Here, 12 and 20 kDa mPEG was attached to C161S-PAI-2 protein via the cysteine residues to form mPEG-C161S-PAI-2. The mutant C161S-PAI-2 was generated as described in Section 3.1.2. in order to minimise multisite or nonspecific PEGylation. Modification of C161S-PAI-2 mutant by attachment of mPEG through the cysteine residues gives site-directed mPEG-C161S-PAI-2 species with a defined PEG-to-protein ratio. The mPEG-C161S-PAI-2 was further modified through the attachment of a highly potent cytotoxin, NAI-1 to form mPEG-C161S-PAI-2-NAI-1-succ conjugate to be used in targeted therapy approach. Subsequently, uPA inhibitory and in vitro cytotoxicity assays showed that the C161S-PAI-2 protein was active after modification and retained the cytotoxic activity in comparison to the parent C161S-PAI-2-NAI-1 succ conjugate.

A range of methods have previously been used to drive the PEGylation reaction to produce site-specific PEG-protein conjugates with a suitable degree of PEGylation. This includes use of protective agents, various types (branched or linear) and sizes of PEG, mutations of reactive amino acid residues, and also by taking advantage of specific amino acid reactivity under various solution pH conditions [267; 268; 413-419]. PEGylation of C161S-PAI-2 using the 20 kDa mPEG yielded mostly di-PEGylated and a small proportion of tri-PEGylated C161S-PAI-2 species with a large amount of unreacted C161S-PAI-2 protein and mPEG molecules. However, further quantitative analysis of the PEGylated C161S-PAI-2 conjugate by MALDI-TOF MS should be
performed to confirm this result. Optimisation reactions including increase in incubation
time and large molar excess of mPEG did not substantially reduce the heterogeneity of
PEGylated species or the amount of unreacted C161S-PAI-2 as evidenced by the SDS-
PAGE analysis (Figures 3.4, 3.5, 3.6, and 3.7).

The high molecular weight 20 kDa di- or tri-PEGylated C161S-PAI-2 species correlated
with the number of cysteine residues available for modification. The large amount of
unreacted C161S-PAI-2 and mPEG molecules at the end of the reaction can be
attributed to the high hydrodynamic radius/gyration radius of the mPEG-C161S-PAI-2
conjugate, which could have played a significant role in preventing the formation of
more di- or tri-PEGylated C161S-PAI-2 conjugates. This result is highly consistent with
studies done by Pabst et al. [411], who found that high hydrodynamic radius of a PEG-
protein conjugate can limit the steric availability of other available PEGylation sites.

Another potential drawback of a high molecular weight PEG-protein conjugate with a
high hydrodynamic radius is the adverse impact on the efficiency of its separation from
other components [411]. In this regard, purification of the 20 kDa mPEG-C161S-PAI-2
by Mono Q anion exchange column to obtain homogenous mPEG-C161S-PAI-2 species
resulted in inefficient binding of mPEG-C161S-PAI-2 to the column (Figure 3.10). It
can be assumed that the increased hydrodynamic radius of the high molecular weight
PEG-protein conjugate had a direct impact on the transport and diffusion properties of
the mPEG-C161S-PAI-2 conjugate in the ion exchange column, leading to the
inefficient separation of the PEG-protein conjugate. Also, the fact that PEG masks the
surface of the protein [269] significantly decreasing the affinity of the negatively
charged mPEG-C161S-PAI-2 conjugate to the column cannot be ruled out as a
possibility leading to the low binding and consequently inefficient separation. Moreover, the inefficient separation could also be attributed to the inappropriate selection of the column with the required properties as studies have found that dynamic binding capacity of the column towards the substrates is a highly critical factor for their efficient binding and separation [411].

Analysis of the purified 12 kDa mPEG bound to C161S-PAI-2 by MALDI-TOF MS revealed mPEG-C161S-PAI-2 species with attachment of 1-2 molecules of mPEG to C161S-PAI-2 (Figure 3.13). This is contrary to the tri- and di-PEGylated C161S-PAI-2 species observed earlier in SDS-PAGE analysis. This discrepancy in the SDS-PAGE analysis can be attributed to the hindered diffusion properties of mPEG-C161S-PAI-2 in the acrylamide gel due to the presence of PEG molecules, which make the modified protein forms, migrate higher than their normal size.

The analysis of mPEG-C161S-PAI-2-NAI-1-succ to determine the number of molecules of NAI-1 attached to mPEG-C161S-PAI-2 was initially performed by UV/Vis spectroscopy and confirmed by MALDI-TOF MS. This UV/Vis spectroscopic analysis technique has been used successfully for many conjugate drugs like maytansinoid DM1 [420], methotrexate [421], CC-1065 analogues [422], adriamycin [423], doxorubicin (DOX) [424], calicheamicin analogues [425; 426] and dipeptide-linked auristatins such as maleimidocaproyl-valine-citrulline-p-aminobenzylxycarbonyl-MMAE (“vc-MMAE”) [292]. The data obtained from UV/Vis spectroscopy, which suggested that ~15 molecules of NAI-1 was attached per molecule of mPEG-C161S-PAI-2, was highly inconsistent with the number of surface lysine residues available for modification. This inconsistency was confirmed by MALDI-TOF MS (Figure 3.15), which showed that
there was only an average of ~2 molecules of NAI-1 attached per molecule of mPEG-C161S-PAI-2 and this correlates well with studies performed by Stutchbury et al. [400] and Ranson et al. [403], who showed that there are only ~7 lysine residues available in the PAI-2 for modification. This also indicates that UV/Vis spectroscopy is a highly unsuitable technique for the analysis of mPEG-C161S-PAI-2-NAI-1-succ conjugate and concurs with previous findings which indicate that UV/Vis spectroscopic analysis is only reliable for conjugates that have a large difference in their $A_{\text{max}}$ values [427].

Although, PEGylation has been found to affect the physical properties of the protein in terms of steric hindrance and altered binding properties of the protein, most of the studies reported till date indicated to an unchanged secondary structure of the protein, resulting in very little effect on the biological activity of the protein [428; 429]. This was proved by the ability of mPEG-C161S-PAI-2-NAI-1-succ conjugate, with an incorporation of 1-2 molecules of 12 kDa mPEG and around 2-3 molecules of NAI-1 cytotoxin, to form stable covalent complexes with uPA (Figure 3.16). The uPA binding activity of the modified C161S-PAI-2 protein proves that there was very little effect of the modification on its biological activity.

The uPA inhibitory activity of the mPEG-C161S-PAI-2-NAI-1-succ conjugate was again confirmed by the fact that it was found to be equipotent to C161S-PAI-2-NAI-1-succ conjugate against MDA-MB-231 breast cancer cells. The development of mPEG-C161S-PAI-2-NAI-1-succ conjugate was a result of the poor pharmacokinetic properties of the PAI-2, as evidenced by its rapid renal excretion rate [403; 404]. Previous studies have shown that PEGylation of proteins significantly alters the physicochemical properties such as absorption rate, bioavailability, biodistribution,
pharmacokinetic and pharmacodynamic profile of the PEG-protein conjugate [270]. In this context, mPEG-C161S-PAI-2-NAI-1-succ conjugates can be expected to exhibit improved pharmacokinetic and pharmacodynamic profile under *in vivo* conditions. These findings warrant further investigations to ascertain the efficacy of mPEG-C161S-PAI-2-NAI-1-succ conjugate *in vivo*. 
CHAPTER FOUR

DEVELOPMENT OF $N$-ALKYLISATIN-TRASTUZUMAB CONJUGATES: POTENTIAL FOR USE IN DOUBLE TARGETING ASSAYS

IN VITRO
DEVELOPMENT OF $N$-ALKYLISATIN-TRASTUZUMAB CONJUGATES: POTENTIAL FOR USE IN DOUBLE TARGETING ASSAYS IN VITRO.

4.1. INTRODUCTION

4.1.1. Herceptin/Trastuzumab

Trastuzumab (TRZ) (Herceptin®, Genentech Inc., South San Francisco, CA) is a humanised recombinant mAb directed against the ectodomain of HER-2 receptors that are over-expressed on malignant cells [430-432]. The antibody (Ab) was humanised by complementarity-determining region grafting of the murine 4D5 Ab together with the human IgG1 constant regions [430]. In vitro and in vivo studies conducted with TRZ demonstrated similar results as its murine counterpart [433], following which clinical trials done showed high efficacy rates for TRZ treatment regimen against HER-2/neu over-expressing advanced MBC. This led to its approval in 1998 by the United States Food and Drug Administration (FDA) [430] & European Medicines Agency (EMEA). Following further successful experimentations, TRZ was also approved for treatment of HER-2 over-expressing early and advanced invasive stage MBC by FDA & EMEA in 2006.

Based on the clinical data analysis, it has been proposed that TRZ induces transgression of HER-2 over-expressing tumours by HER-2 downregulation, partly assisted by TRZ
mediated internalisation of HER-2 [434; 435]; ADCC [430; 436-439]; and affects microvessel formation by inhibiting angiogenesis [440].

4.1.2. Combination therapy – TRZ and chemotherapeutics

Although TRZ was found to be active against HER-2 over-expressing MBC, it was noticed that targeting over-expressed HER-2 receptors in an advanced metastatic condition with TRZ alone have yielded relatively low objective response rates compared to their efficacy when administered in combination with chemotherapy [227]. This observation also supports the finding that mAbs, in general, have moderate antitumour activity [189].

In a clinical study where TRZ was administered in combination with paclitaxel (taxane-based chemotherapy) as first line of therapy [210] and also as first line of therapy after anthracycline [441], they observed an ORR of 36% in the former and an ORR of 41% in the latter and an increased median TTP of the disease compared to patients with HER-2 over-expressing tumours who received TRZ alone and paclitaxel alone treatments. In another study conducted by Breast Cancer International Research Group (BCIRG) 006, comparing TRZ added to anthracyline or docetaxel (taxane-based chemotherapy) and TRZ alone and the same chemotherapy drugs alone, it was found that the former resulted in longer time to disease progression, a higher rate of objective response, and a significant reduction in the risk of death in patients with HER-2 positive MBC [219]. Meanwhile, when TRZ was administered in combination with vinorelbine, a vinca alkaloid having high anti-tumour activity [213], as a first line of therapy, an ORR in the range of 68-84% was noticed in patients with HER-2 over-expressing tumours [214; 217; 442]. In an another BCIRG study 101 aimed at ascertaining the efficacy of TRZ in
combination with non-anthracyline regimen such as platinum salts (cisplatin) and
docetaxel, an ORR of 79% and a median TTP of 9.9 months was observed in patients
with HER-2 over-expressing tumours [212].

However, it still remains to be ascertained with accuracy, firstly, if a strategy of
administering TRZ sequentially or concurrently would produce beneficial results;
secondly, the optimal duration of TRZ administration; finally, the choice of
chemotherapy drugs to be administered with TRZ in the wake of cardiac risk factors
[227]. In this atmosphere of uncertainty surrounding TRZ, conjugation of previously
established or potent novel chemotherapeutic agents to TRZ in the form of ADCs would
offer an attractive alternative mode of treatment, referred to as ‘targeted therapy’.
Moreover, delivery of chemotherapeutics as conjugates with TRZ would not only
reduce their general toxicity, but also will increase the solubility and circulation time of
chemotherapeutics, thereby increasing their efficacy [443]. On the whole, it can be
assumed that such an action would impart the end product with high tumour specificity
and increased potency.

4.1.3. TRZ-drug conjugates

To date, several drug molecules have been conjugated to TRZ for targeted delivery to
malignant cells. Some of the well investigated conjugates involving TRZ are the TRZ-
polyethylenimine-polyethylene glycol (PEI-PEG) conjugates, where the PEG-PEI was
conjugated to TRZ via an N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) linker
[444].
Another TRZ-drug conjugate, where TRZ was coupled to geldanamycin (GA), a highly cytotoxic ansamycin benzoquinone antibiotic, exerts its cytotoxicity by binding to the protein chaperone, heat shock protein 90 (hsp90) [445]. This immunoconjugate was found to be highly cytotoxic against HER-2 over-expressing human breast carcinoma cells than TRZ or geldanamycin by itself. It was noticed that at very low concentrations of TRZ-GA immunoconjugate (15 µg/mL), it induced 86% reduction in HER-2 expression levels compared to the 20% by TRZ or geldanamycin alone [446].

The most recent in this list is the TRZ-maytansinoid conjugates where the latter was linked to TRZ via a disulfide and nonreducible thioether linker [305]. In this study, TRZ-maytansinod conjugates exhibited high selectivity for HER-2 over-expressing tumours in experiments performed both in vitro and in vivo [305]. They reported that presence of a non-reducible thioether linker imparted increased activity to the TRZ-maytansinoid conjugates compared to conjugates involving reducible disulfide linkers.

4.1.4. Double targeting

Over amplification of HER-2 and uPA receptors were found in 70% of HER-2 amplified advanced invasive breast carcinoma cells [310]. Further studies targeting HER-2 and uPA showed that depleting both HER-2 and uPA receptors suppressed cell growth and induced cell apoptosis in SK-BR-3 and ZR-75-1 mammary epithelial cells that over-express HER-2 and uPA receptors [447]. Hence, over-expression of different receptor forms on malignant cells presents an opportunity to target them simultaneously bringing about increased reduction in tumour growth as well as minimising the exposure time of drugs to tumour cells.
In this direction, the specific aims of this study were;

1. To develop NAI-1-succ-TRZ conjugate by linking NAI-1 to TRZ through an ester-labile succinate linker (scheme of the synthesis of NAI-1-succ-TRZ conjugate and its targeting to breast cancer cells and also the double targeting is given in Figure 4.2) and characterise NAI-1-succ-TRZ conjugate by ESI-MS/MALDI-TOF MS.

2. To determine the cytotoxic activity of the NAI-1-succ-TRZ conjugate by in vitro assays against selected high (SK-BR-3 and ZR-75-1) and low (MDA-MB-231) HER-2 over-expressing breast cancer cells.

3. To target both HER-2 and uPA receptors simultaneously using NAI-1-succ-TRZ conjugate and NAI-1-succ-C161S-PAI-2 conjugate (scheme of synthesis of NAI-1-succ-C161S-PAI-2 conjugate is given in Chapter 3) by combination cytotoxicity assays to establish the rationale of double targeting HER-2 and uPA receptors leading to increased tumour reduction.
Figure 4.1. Schematic representation of the synthesis, internalisation, and intracellular trafficking of NAI-1-succ-TRZ conjugate. Firstly, NAI-1-succ (A) is converted to its active form (B) in the presence of NHS & DCC. Following this, the active form of NAI-1-succ is reacted with TRZ via its free amino groups to form NAI-1-succ-TRZ conjugate (C). Thereafter, NAI-1-succ-TRZ conjugate is expected to bind to HER-2 receptors on the surface of MBC cells (D) and undergo RME. The NAI-1-succ-TRZ conjugate should then be targeted to lysosomes, where the NAI-1 native cytotoxin (E₁) will be selectively released inside the tumour cell following cleavage by lysosomal esterases. The green and yellow shaded regions in TRZ are the antigen-binding complementarity determining region (CDR) loops.
Figure 4.2. Schematic representation of double targeting malignant tumour cells that highly over-express HER-2 and uPA receptors using NAI-1-succ-TRZ and NAI-1-succ-C161S-PAI-2 conjugates.
4.2. MATERIALS & METHODS

4.2.1. Chemicals, reagents, proteins and antibodies.

Trastuzumab/Herceptin® (in lyophilised powder form) was a kind gift from Genentech Inc., South San Francisco, CA, USA. Mouse monoclonal antibody, IgG1 was obtained from Millipore Australia Pty Ltd (Victoria, Australia). Anhydrous dimethylformamide (DMF), N-hydroxysuccinimide (NHS) dicyclohexylcarbodiimide (DCC), hanks balanced salt modified and non-modified, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. RPMI-1640 powder and foetal calf serum were from Trace Bioscientific (NSW, Australia). Trypsin/EDTA (0.05% trypsin, 4Na EDTA) was obtained from Invitrogen. Desalting PD-10 column was purchased from GE Healthcare. NAI-1 and NAI-1-succ were synthesised in-house by Dr. Julie Locke, Dr. Lidia Matesic and Ana Zivanovic, School of Chemistry, University of Wollongong (NSW, Australia). BIO-RAD Quick Start™ Bovine Serum Albumin (BSA) Standard and Bio-Rad DC™ Protein Assay Kit (Reagent A & B) were obtained from BIO-RAD, Hercules, CA. CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) reagent was purchased from Promega Corporation (Sydney, Australia). Goat anti-mouse IgG (Fab specific) FITC conjugate was purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). (Methyl-PEO12)3-PEO4-NHS Ester (~2500 Da, #22421) was purchased from PIERCE (Rockford, IL 61105). Monoclonal antibody against uPAR (#3936) was purchased from American Diagnostica, Inc. (Greenwich, CT). Monoclonal anti-c-erbB-2 antibody (#E2777) produced in mouse was purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). Sinapinic acid (SA) was obtained from LaserBio Labs (LBL) (Sophia-Antipolis Cedex, France).
4.2.2. Cell lines. The human epithelial (mammary gland; breast) cell lines; MDA-MB-231, SK-BR-3 and ZR-75-1 cell lines were purchased from American Type Culture Collection (ATCC, VA, USA). All cells were routinely cultured in RPMI-1640 (10.4 g/L) with 2 mM L-glutamine and supplemented with 5% (v/v) fetal calf serum (FCS) at 37°C in 95% humidified atmosphere, containing 5% CO₂.

4.2.3. Cell surface receptor detection. Membrane bound endogenous proteins were detected using immunofluorescence assays, essentially as previously described by Ranson et al. [371] with minor modifications. Briefly, cells were washed once and resuspended at a final concentration of 1-2×10⁶ cells/mL in ice-cold binding buffer (phenol red-free Hanks buffered salt solution, pH 7.4, containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.1% BSA (Appendix 1)) containing 10 μg/mL of primary antibody and incubated on ice for 30 – 45 min. Following incubation, cells were washed twice, resuspended in ice-cold binding buffer and then incubated with the appropriate 1:100 FITC-conjugated secondary antibody for detection of bound primary antibodies by flow cytometry. In all flow cytometry analyses viable cell populations were selected through propidium iodide (PI) exclusion. Isotype matched control antibodies were used to assess non-specific binding due to the primary antibodies. All data obtained was analysed using FlowJo software version 7.6.1 (Treestar Inc) and was restricted to PI negative cells. All geometric mean fluorescence values reported represent mean fluorescence intensity for specific binding ± SDEV. All assays were conducted in triplicate.

4.2.4. Conjugation of NHS functional PEG to TRZ. A 20 molar excess of ~2500 Da NHS functional PEG solution (125 mM) was added to TRZ (~3 mg/mL) in PBS (pH 7.5) and incubated at RT for 3 h with gentle shaking. Where indicated, the
reaction was stopped by the addition of $5 \times$ SDS buffer followed by heating the mixture at 95° C for 5 min. The PEG-TRZ conjugate mixture was then fractionated by a 10% non-reducing SDS-PAGE to visualise the extent of PEGylation of TRZ or the reaction mixture was subjected to MALDI-TOF MS analysis.

**4.2.5. Optimised conjugation of NAI-1-succ to TRZ.** The active ester of NAI-1-succ was prepared based on the method described by Vine *et al.* [286]. Briefly, NAI-1-succ (2.7 mg, 5.0 μmol) was dissolved in anhydrous DMF (70 μL). N-hydroxysuccinimide (NHS, 0.59 mg, 5.0 μmol) in DMF (17 μL) and dicyclohexylcarbodiimide (DCC, 6.18 mg, 30 μmol) in DMF (50 μL) were then added and the reaction mixture shaken and kept at RT for 15-20 min. Various buffers, molar excess of NAI-1-succ, TRZ concentration and reactant incubation times were tested as described in **Table 4.1.** TRZ was buffer exchanged into the desired buffer using PD-10 columns to remove the formulation components (histidine hydrochloride, histidine, trehalose dehydrate, and polysorbate 20). For a stable conjugation, a 40-fold molar excess of crude orange product was conjugated to free lysine residues of TRZ (~5 mg/mL) in 20 mM sodium phosphate buffer (pH 6.0) at RT, with gentle stirring. After 4 h incubation, the conjugate was then purified by gel filtration using PD-10 desalting column, with 10 mM histidine buffer (pH 6.0) (**Appendix 1**) as eluant. Fractions corresponding to the protein peak (as determined by UV/Vis spectrophotometry, 280 nm) were pooled, and stored at 4° C for characterisation and future cytotoxicity studies. Protein concentration was determined using the Lowry assay. The number of NAI-1 molecules bound to TRZ was determined by ESI-MS and MALDI-TOF MS analysis.
4.2.6. Electrospray ionisation mass spectrometry (ESI-MS). Positive ion mass spectra of TRZ and NAI-1-succ-TRZ conjugate were acquired on a quadrupole time of flight mass spectrometer (Q-TOF-MS) (Micromass Q-TOF Ultima, Waters, Wyntheshawe, UK) fitted with a Z-spray ionisation source as described previously by Vine et al. [286]. TRZ and NAI-1-succ-TRZ conjugate samples were prepared for ESI-MS by buffer exchanging into 10 mM ammonium acetate buffer (pH 6.8) containing 0.1% formic acid at a final concentration of TRZ (5 μM) and NAI-1-succ-TRZ (5 μM) by standard dialysis using PD-10 desalting columns. The samples were then injected into the spectrometer and the mass spectrum acquired with a capillary voltage of 1.5 kV, sampling cone of 100.0, extraction cone 4.0, reflectron voltage of 2.16 kV, source temperature of 30°C, desolvation temperature of 150°C, resolution of 9000 (LM Resolution - 4.7 and HM Resolution - 15.0) and a trap and transfer collision energy 6.0 and 4.0 respectively. Cesium iodide in 70% isopropanol was used for external calibration. The data is presented as raw data, on a mass and m/z scale. Masses were calculated using MassLynx MS software (Waters). All analyses were performed by Dr Thitima Urathamakul (Mass Spectrometry Facility Manager, School of Chemistry, University of Wollongong) and Dr. Sydney Liu Lau (Research Officer, Bioanalytical Mass Spectrometry Facility, Mark Wainwright Analytical Centre, The University of New South Wales).

4.2.7. Matrix-assisted laser desorption/ionisation – time-of-flight mass spectrometry (MALDI-TOF MS).

Sample preparation. The MALDI TOF analysis of 2.5 kDa NHS functional PEGylated form of reduced TRZ and NAI-1-succ-TRZ conjugate was performed according to the method described by Seyfried et al. [407] with slight modifications. Firstly, for matrix
preparation, a mixture of acetonitrile (ACN), distilled water (dH₂O) and trifluoroacetic acid (TFA) in 40:60:0.1 ratio was added to a matrix aliquot of sinapinic acid (SA) to achieve a final concentration of 10 mg/mL. The mixture was mixed thoroughly by vortexing for 30 s and then spun in a microcentrifuge at 14,000 rpm to separate undissolved matrix and the supernatant was used for further analysis. For the preparation of both TRZ and NAI-1-succ-TRZ samples, the volume technique was applied. Approximately 1 μL of matrix and 1 μL of TRZ/NAI-1-succ-TRZ in 10 mM Tris buffer (pH 8.0) were mixed thoroughly in an eppendorf tube and 1 μL of the homogeneous solution was deposited on the target plate and dried at RT.

**Analyses.** All measurements were performed in the linear positive ion mode with an AXIMA Confidence instrument (Shimadzu Biotech, Manchester, UK) equipped with a nitrogen laser and applying a source voltage of 20,000 V, einzel lens voltage of 6,500 V, pulsed extraction voltage of 2,625 V, reflectron voltage of 24,400 V, linear detector voltage of 2,700 V, and reflection detector voltage of 1,850 V. All analyses were performed by Dr. David Harman (NMR and MS Manager, ARC Centre of Excellence for Electromaterials Science, Intelligent Polymer Research Institute, University of Wollongong) using the Shimadzu Biotech Axima Confidence software (version 2.8.3).

**4.2.8. In vitro cytotoxicity assay.** In vitro cytotoxicity assays were performed as described previously by Vine et al. [308]. Briefly, cells (5,000 cells/well) were seeded in 96-well plates and incubated overnight at 37° C in 95% humidified atmosphere, containing 5% CO₂. On the following day, TRZ and NAI-1-succ-TRZ conjugate were added to triplicate wells at decreasing concentrations which were then incubated for 72 h at the above-mentioned conditions. Subsequently, MTS reagent was added to appropriate wells and after a 3 h incubation period, the absorbance was measured at 490
nm using SpectraMax® Plus 384 Microplate Spectrophotometer with SoftMax® Pro Software. The cytotoxicity of the compounds was expressed in terms of IC₅₀ values (concentration of test compounds required to inhibit the metabolic activity of 50% of the total cell population) after normalising to DMSO, TRZ only and/or PBS-treated control cells. These values were calculated from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism™ software v5.02 (GraphPad Software Inc.). In all IC₅₀ calculations using GraphPad prism software, cells + PBS absorbance values subtracted from baseline absorbance values (media + PBS) were taken as 100% cell viability (100% value) control and cells + highest concentration of test compound treatment was taken as 0% cell viability (0% value) control.

4.2.9. Double targeting assays. Double targeting assays were performed to determine the synergy/additivity between different drug conjugate combinations. Briefly, cells (5,000 cells/well) were seeded in 96-well plates and incubated overnight at 37°C in 95% humidified atmosphere, containing 5% CO₂. On the following day, NAI-1-succ-C161S-PAI-2 and NAI-1-succ-TRZ conjugate alone and in combination were added to triplicate wells at decreasing concentrations which were then incubated for 72 h at the above-mentioned conditions. Subsequently, MTS reagent was added to appropriate wells and after a 3 h incubation period, the absorbance was measured at 490 nm using SpectraMax® Plus 384 Microplate Spectrophotometer with SoftMax® Pro Software. The cytotoxicity of the compounds was expressed in terms of IC₅₀ values (concentration of test compounds required to inhibit the metabolic activity of 50% of the total cell population) after normalising to NAI-1-succ-C161S-PAI-2, NAI-1-succ-TRZ conjugate only or and PBS-treated control cells. These values were calculated
from Log[inhibitor] versus normalised response curves (variable slope) equation, generated using GraphPad Prism™ software v5.02 (GraphPad Software Inc.).

4.2.10. Statistical analyses. Statistical significance of treatment groups as compared to control groups was determined using unpaired students T-test (GraphPad Prism V 5.1; San Diego, CA, USA). $P$ values $< 0.05$ were considered statistically significant.

4.3. RESULTS

4.3.1. Detection of cell surface uPAR and HER-2 receptors. The expression of uPAR and HER-2 receptors on MDA-MB-231, SK-BR-3 and ZR-75-1 mammary epithelial cells was determined using flow cytometry. An IgG1 mAb was used as an isotype matched control for this experiment. The relative shift in fluorescence of the uPAR and HER-2 peaks compared to the istotype control peak indicate that the MDA-MB-231 cells express high levels of uPAR, but negligible levels of HER-2 at the cell surface (Figure 4.3 A). Relative to MDA-MB-231 cells, SK-BR-3 cells express very high levels of HER-2, and comparable levels of uPAR at the cell surface (Figure 4.3 B). This is consistent with the reported characteristics of this cell line [447]. In the case of ZR-75-1 cells, there were comparable levels of uPAR to the MDA-MB-231, and high expression of HER-2 (Figure 4.3 C) [447].
Figure 4.3. Representative fluorescence histograms showing the relative cell surface expression of HER-2 and uPA receptors on (A) MDA-MB-231, (B) SK-BR-3, and (C) ZR-75-1 Cells. The blue peak represents autofluorescence, black peak represents isotype matched control, green peak represents HER-2 level, and the red peak represents uPAR level. The relative shift to the right of the green and red peaks from the black peak represents cell surface levels of HER-2 and uPAR respectively. For all experiments, the anti-uPAR mAb (ADI #3936) and the anti-c-erbB-2 mAb (Sigma Aldrich #E2777) was used as the primary antibody.
4.3.2. Conjugation of NAI-1-succ to TRZ. The active form of NAI-1-succ was prepared according to the method described by Vine et al. [286]. The optimisation of the conjugation of the active form of NAI-1-succ to TRZ was carried out under the conditions summarised in Table 4.1.

Initially, the conjugation reaction was carried out in phosphate, HEPES, and carbonate buffers (Appendix 1) at different conditions of pH, molar excess of NAI-1, and concentration of TRZ (Table 4.1). Under the above-mentioned altered reaction conditions, the level of attachment of NAI-1 to TRZ was found to be very low upon visualisation by UV/Vis spectrophotometry (TRZ measured at 280 nm and NAI-1 measured at 432 nm) (Appendix 4.1-4.5).
Table 4.1. NAI-1-succ-TRZ conjugation optimisations.

<table>
<thead>
<tr>
<th>Pre-treatment of TRZ</th>
<th>Buffer used</th>
<th>Buffer pH</th>
<th>Conc of TRZ after buffer exchanging (*mg/mL)</th>
<th>Molar excess of activated NAI-1-succ added</th>
<th>Incubation time of reactants</th>
<th>Stirring / Shaking</th>
<th>NAI-1 attachment to TRZ</th>
<th>PEG attachment to TRZ</th>
<th>A280/A432 ratio$^\nu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>8.0</td>
<td>1.8</td>
<td>20</td>
<td>3h</td>
<td>Shaking</td>
<td>Very low</td>
<td></td>
<td>&gt;10$^{A4.1}$</td>
</tr>
<tr>
<td>HEPES (150 mM NaCl and 20 mM HEPES)</td>
<td>7.5</td>
<td>2.3</td>
<td>20</td>
<td>3h</td>
<td>Shaking</td>
<td>Very low</td>
<td></td>
<td>&gt;10$^{A4.2}$</td>
<td></td>
</tr>
<tr>
<td>0.1 M Carbonate</td>
<td>8.5</td>
<td>2.0</td>
<td>20</td>
<td>O/N</td>
<td>Mild shaking</td>
<td>Very low</td>
<td></td>
<td>&gt;10$^{A4.3}$</td>
<td></td>
</tr>
<tr>
<td>0.1 M Carbonate</td>
<td>9.5</td>
<td>2.2</td>
<td>20</td>
<td>O/N</td>
<td>Mild shaking</td>
<td>Very low</td>
<td></td>
<td>&gt;10$^{A4.4}$</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>7.0</td>
<td>4.0</td>
<td>30</td>
<td>O/N</td>
<td>No shaking</td>
<td>Very low</td>
<td></td>
<td>&gt;10$^{A4.5}$</td>
</tr>
<tr>
<td>Partially reduced* TRZ in PBS buffer and reacted with 2.5 kDa NHS functional PEG</td>
<td>7.5</td>
<td>4.0</td>
<td>5h</td>
<td>Mild shaking</td>
<td>High</td>
<td>See Figure 4.3 &amp; 4.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially reduced* TRZ in PBS buffer</td>
<td>PBS</td>
<td>7.5</td>
<td>5.0</td>
<td>30</td>
<td>4h</td>
<td>Mild stirring</td>
<td>Low</td>
<td></td>
<td>~10$^{A4.6}$</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>9.0</td>
<td>5.0</td>
<td>80</td>
<td>1h</td>
<td>Mild stirring</td>
<td>Low</td>
<td></td>
<td>~11.7$^{A4.7}$</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>9.0</td>
<td>6.5</td>
<td>40</td>
<td>2h</td>
<td>Mild stirring</td>
<td>Low</td>
<td></td>
<td>~12.0$^{A4.8}$</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>9.0</td>
<td>6.5</td>
<td>30</td>
<td>4h</td>
<td>Mild stirring</td>
<td>Low</td>
<td></td>
<td>~8.7$^{A4.9}$</td>
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<tr>
<td>PBS</td>
<td>PBS</td>
<td>9.0</td>
<td>7.0</td>
<td>30</td>
<td>4h</td>
<td>Mild stirring</td>
<td>Average</td>
<td></td>
<td>~6.5$^{A4.10}$</td>
</tr>
<tr>
<td>20 mM sodium phosphate</td>
<td>PBS</td>
<td>6.0</td>
<td>5.0</td>
<td>40</td>
<td>4h</td>
<td>Mild stirring</td>
<td>High</td>
<td></td>
<td>~4.2 Fig 4.5</td>
</tr>
</tbody>
</table>

*DTT (in reduction buffer containing 0.025 M sodium borate pH 8.0, 0.025 M NaCl, and 1 mM DTPA) was added to TRZ in phosphate buffer at a final conc of 5 mM and incubated for 2h at 37°C. The reaction was stopped by buffer exchanging into PBS buffer (pH 7.5).

A280/A432 ratio values represent qualitative efficiency of conjugation with low $\rightarrow$ high A280/A432 ratio values indicating high $\rightarrow$ low conjugation efficiency respectively. $^\nu$See Appendix 4 for representative visualisations.
In order to determine the role of steric hindrance preventing a stable and efficient conjugation of NAI-1-suce to TRZ, a 2500 Da NHS ester functional PEG, which reacts with TRZ in a similar way to NAI-1-suce, was conjugated to reduced TRZ at different reaction conditions (as mentioned in Table 4.1).

Characterisation of the unreduced, reduced, and PEG-TRZ conjugate by SDS-PAGE showed a dense band migrating above ~250000 Da, which could be TRZ (~148000 Da) polymer (Figure 4.4, Lane 2). Under reducing conditions, there was a dense band migrating at ~60000 Da and ~27000 Da, which could be the heavy chain (HC) and light chain (LC) of TRZ (Figure 4.4, Lane 3). Characterisation of the 2500 Da NHS functional PEGylated reduced TRZ showed a band migrating at ~65000 Da (Figure 4.4, Lane 4). A few faint bands were also observed upon further analysis in the 25000-37000 Da range indicating attachment of ~3 molecules of 2500 Da NHS ester functional PEG to the LC (~25000-30000 Da) of TRZ (Figure 4.4, Lane 4).

A MALDI-TOF mass spectrometric analysis (Figure 4.5) was done to confirm the PEGylation of TRZ characterised by SDS-PAGE as shown in Figure 4.4. The molecular weight of the LC of TRZ was found to be 28216.61 Da with peaks at 30538.56 Da and 32858.43 Da indicating attachment of 1-2 molecules of 2500 Da NHS functional PEG to the LC (Figure 4.5). The peak at 60441.33 Da was consistent with the size of HC (~55000-60000 Da) with subsequent peaks at 62580.31 Da and 64731.73 Da suggesting attachment of 1-2 molecules of 2500 Da NHS functional PEG to the HC (Figure 4.5). The peak at 90775.21 Da and ~120000 Da indicated the presence of
dimers of HC & LC and dimers of HC respectively. The peak at ~150000 Da was consistent with the size of the TRZ molecule (~148000 Da) (Figure 4.4).

Figure 4.4. SDS-PAGE analysis of 2500 Da NHS ester functional PEG-TRZ conjugate fractionated on a 10% acrylamide gel under non-reducing conditions. Lane 1 – Marker, Lane 2 – unreduced TRZ, Lane 3 – reduced TRZ, Lane 4 – PEGylated reduced TRZ conjugate. Approximately 20 µg of each sample was used for analysis. HC = ~ 55000 Da and LC = ~ 25000 Da.
Figure 4.5. A representative linear positive ion mode MALDI-TOF MS analysis of 2500 Da NHS functional PEGylated form of reduced TRZ with sinapinic acid as the matrix using AXIMA Confidence instrument and Shimadzu Biotech Axima Confidence software (version 2.8.3). In order to determine the extent of PEGylation of reduced TRZ, the molecular weight of the light and heavy chains was taken as 28216.61 Da and 60441.33 Da respectively. The peak at 32858.43 Da and 64731.73 Da indicates attachment of a maximum of 2 molecules of 2500 Da NHS functional PEG to the light and heavy chains of TRZ molecule respectively. The peaks at 90775.21 and 120000 Da represent dimers of heavy and light chains.
On the basis of the successful PEGylation of TRZ, activated NAI-1-succ was conjugated to reduced TRZ in PBS (pH 7.5) (Appendix 1) under different reaction conditions as summarised in Table 4.1. Upon analysis of the conjugate by UV/Vis spectrophotometry, a trace attachment of NAI-1-succ to TRZ was observed. In line with the previous optimisations, it was assumed that a higher buffer pH and a high concentration of TRZ would facilitate attachment of more molecules of NAI-1-succ to TRZ. Therefore, the pH of the phosphate buffer was increased to 9.0 and the concentration of TRZ was increased to ~7.0 mg/mL, with all other conditions not changed from the previous reaction (Table 4.1). However, no improvement was observed after a series of optimisations.

Finally, a stable conjugation was achieved with reaction conditions as recommended by Dr. Fred Jacobson (Principal Scientist, Genentech), whereby a 40-fold excess of active NAI-1-succ was reacted with TRZ in 20 mM sodium phosphate buffer (pH 6.0) (Appendix 1) for 4 h under mild stirring conditions.

4.3.3. Visualisation and characterisation of NAI-1-succ-TRZ conjugate.

The NAI-1-succ-TRZ conjugate obtained under optimal reaction conditions (Table 4.1) was purified by size exclusion chromatography and characterised by UV/Vis spectrophotometry, ESI-MS, and MALDI-TOF MS. The NAI-1-succ-TRZ conjugate was purified by buffer exchanging into 10 mM histidine buffer. Upon purification by PD-10 desalting columns, some residual orangish-yellow coloured product remained on the column which could be unconjugated or free isatin from the conjugation reaction. The eluate fractions obtained after purification were measured at two wavelengths (280 nm for TRZ and 432 nm for isatin). An increase in absorbance at 432 nm (OD = 0.32) in fraction 3, corresponding to incorporated NAI-1, was noted to co-elute with the TRZ
peak at 280 nm (OD = 1.72), indicating attachment of NAI-1-succ to TRZ (Figure 4.6). Here, to rule out any non-specific interactions between NAI-1-succ and TRZ, the NAI-1-succ was also reacted with TRZ in the absence of the coupling reagents (i.e. DCC and NHS) and the fractions collected after size exclusion chromatography. The low absorbance value at 432 nm (OD = 0.0325) in the eluted fraction, with a corresponding high absorbance value at 280 nm (OD = 1.2397) for TRZ, indicates negligible non-specific attachment of NAI moieties onto TRZ molecules in the absence of coupling agents (Appendix 5, 5.1).

Figure 4.6. A representative size exclusion chromatography elution profile of an optimal NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
A Q-TOF ESI MS of the purified TRZ and NAI-1-succ-TRZ (Figure 4.7) was performed to ascertain the number of molecules of NAI-1 incorporated onto each TRZ molecule. Analysis of the TRZ alone was found to be highly heterogeneous with up to 2 molecules of glycans (160 Da) attached per molecule of TRZ (panel A, Figure 4.7). This is consistent with previous studies which found TRZ to be heavily glycosylated with the attachment of several N-linked 160 Da glycans [448; 449]. The ESI-MS spectrum the NAI-1-succ-TRZ was highly heterogenous and indicated incorporation of an average of 1 molecule of NAI-1 onto glycosylated TRZ molecule (panel B, Figure 4.7).
Figure 4.7. ESI-MS of TRZ (A) and NAI-1-succ-TRZ conjugate (B). A representative positive ESI–MS analysis of TRZ and NAI-1-succ-TRZ conjugate in 10 mM ammonium acetate buffer containing 0.1% formic acid using Q-TOF MS analyser. Panel (A) shows glycosylated TRZ with the attachment of 1-2 molecules of glycans (160 Da). To ascertain the number of molecules of NAI-1 attached per molecule of TRZ, the molecular weight of TRZ was taken as 148398.4 Da (panel A) and isatin derivative as 525.14 g/mol. The average increase in molecular weight of TRZ upon attachment of isatin molecules calculated from the spectrum (148917.6 Da) (panel B) indicated a maximum of 1 molecule of isatin attached per molecule of TRZ.
With the aim of achieving a highly resolved spectrum to determine the number of molecules of NAI-1 attached to TRZ accurately, a MALDI-TOF analysis was performed (Figure 4.8). Analysis of the NAI-1-succ-TRZ conjugate by MALDI-TOF mass spectrometry indicated an attachment of a maximum of 2 molecules of NAI-1 to TRZ (Figure 4.8, Panel B), evident by the peak at 147794.3 Da. These NAI-1-succ-TRZ conjugates were used in cytotoxicity experiments.
Figure 4.8. A representative linear positive ion mode MALDI-TOF MS analysis of NAI-1-succ-TRZ conjugate with sinapinic acid as the matrix using AXIMA Confidence instrument and Shimadzu Biotech Axima Confidence software (version 2.8.3). In order to determine the number of molecules of NAI-1 attached to TRZ, the molecular weight of TRZ was taken as 146558.6 Da. The increase in the molecular weight to 147794.3 Da indicates attachment of at least 2 molecules of NAI-1 to TRZ molecule.
4.3.4. *In vitro* potency of NAI-1-succ-TRZ conjugate. The cytotoxic activity of NAI-1-succ-TRZ conjugate was ascertained against high HER-2 receptor expressing SK-BR-3 and ZR-75-1 cells and compared to the lower HER-2 expressing MDA-MB-231 breast cancer cells. Analysis of the dose response curves showed the effect of TRZ to be essentially cytostatic across all the cell lines tested (*Figure 4.9*). After normalising to the TRZ and PBS controls, NAI-1-succ-TRZ conjugate was highly cytotoxic against all the cell lines tested (i.e., IC$_{50}$ were in the nanomolar range). All cell lines were less sensitive to free NAI-1, with the SK-BR-3 cell line being relatively insensitive to NAI-1 (IC$_{50}$ 5.19 µM), reflecting their lower proliferation rates compared to the other cell lines (*Table 4.2*).

However, in comparison to the NAI-1 parent compound, NAI-1-succ-TRZ conjugate was greater than 8 × more potent against SK-BR-3 cells (*Table 4.2*) suggesting high selectivity and efficient receptor-mediated delivery of the drug. NAI-1-succ-TRZ conjugate was only nearly equipotent compared to the parent NAI-1 compound against the lower HER-2 expressing MDA-MB-231 cells (*Table 4.2*). The NAI-1-succ-TRZ conjugate was greater than 3 × more potent compared to NAI-1 against ZR-75-1 cells, consistent with the high HER-2 levels as measured by flow cytometry (*Figure 4.3*).
Figure 4.9. Representative dose response curves showing cytotoxic activity of TRZ and NAI-1-succ-TRZ conjugate against (A, B) MDA-MB-231, (C, D) SK-BR-3 and (E, F) ZR-75-1 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 72 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All value points in the curves are the means of at least triplicate of values ± SDEV.
Table 4.2. Cytotoxicity of the NAI-1-succ-TRZ conjugate compared to unconjugated NAI-1 after 72 h treatment presented in terms of IC$_{50}$ values.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative HER-2 levels</th>
<th>IC$_{50}$ (μM) ± SDEV$^#$</th>
<th>NAI-1</th>
<th>NAI-1-succ-TRZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231$^+$</td>
<td>*</td>
<td>1.38 (±0.34)$^\Psi$</td>
<td>0.87 (±0.25)</td>
<td></td>
</tr>
<tr>
<td>SK-BR-3$^+$</td>
<td>****</td>
<td>5.19 (±1.69)$^\Psi$</td>
<td>0.61 (±0.09)</td>
<td></td>
</tr>
<tr>
<td>ZR-75-1$^+$</td>
<td>***</td>
<td>1.15 (±0.56)</td>
<td>0.35 (±0.12)</td>
<td></td>
</tr>
</tbody>
</table>

$^\#$IC$_{50}$ values were calculated based on the average number of moles of NAI-1 from Log[inhibitor] vs. normalised response curves (variable slope), generated using GraphPad Prism V.5 software. Values are the mean of at least triplicate experiments performed in triplicate ± SDEV. $^+$Cell density was reduced to 5,000 cells/well, to counter the high proliferative capacity of this cell line. The IC$_{50}$ values of the NAI-1-succ-TRZ conjugate correspond to an average of 1 molecule of NAI-1 per molecule of TRZ. $^{****}$ very high, $^{***}$ high, and $^*$ low. $^\Psi$Tested earlier in Chapter 2. $\infty$Refer to Appendix 2. For NAI-1-succ-TRZ versus NAI-1 treatment against ZR-75-1 and SK-BR-3 cells, $P < 0.05$.

4.3.5. Double targeting cytotoxicity assays. The potential additive effect of NAI-1-succ-TRZ and NAI-1-succ-C161S-PAI-2 was determined by double targeting cytotoxicity assays against MDA-MB-231 and ZR-75-1 cells. Here, MDA-MB-231 cells which has a low expression of HER-2 and high levels of uPA receptors (Figure 4.3) was taken as a negative control, whereas ZR-75-1 cells highly express both HER-2 and uPA receptors (Figure 4.3).

Initial investigations were conducted using TRZ alone, NAI-1-succ-C161S-PAI-2 alone and their combination against MDA-MB-231 cells. As expected, the effect of TRZ was
cytostatic even at the highest concentration (1 µM) tested (Figure 4.10). In terms of
cytotoxic activity, there was no difference between NAI-1-succ-C161S-PAI-2 alone and
the combination treatment, which is along expected lines as the effect of TRZ was
cytostatic after 72 h incubation.

Figure 4.10. Cytotoxic activity of NAI-1-succ-C161S-PAI-2 conjugate and TRZ
alone and in combination against MDA-MB-231 cells. Briefly, cells were incubated
with a range of concentrations of the above-mentioned compounds for 72 h and cell
viability determined using CellTiter 96® AQueous MTS Reagent in reference to
appropriate vehicle treated controls. All value points in the curve are the means of at
least triplicate of values ±SDEV. The blue curve represents TRZ treatment, red curve
represents NAI-1-succ-C161S-PAI-2 conjugate treatment and the green curve
represents combination treatment.

Double targeting of ZR-75-1 cells was performed using NAI-1-succ-TRZ (0.5 µM) and
NAI-1-succ-C161S-PAI-2 (0.5 µM) conjugates in 1:1 ratio for 72 h. Analysis of the
combination treatment in comparison to each drug alone after normalising to the PBS
control resulted in a significant decrease in cell viability (Figure 4.11).
Figure 4.11. Combined effect of NAI-1-succ-TRZ (0.5 µM) and NAI-1-succ-C161S-PAI-2 conjugate (0.5 µM) (1:1) on the viability of ZR-75-1 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 72 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. Values are the mean of triplicates ± SDEV. *P <0.05

4.4. DISCUSSION

Recent studies by Urban et al. [310] and Meng et al. [311] have found co-expression of HER-2 and uPA receptors as a characteristic feature of highly aggressive MBCs. However, the exact correlation and interdependence of these receptor forms in MBCs is yet to be clearly established. In this study, the development of NAI-1-succ-TRZ conjugates was an effort in the direction of utilising them, alongside NAI-1-succ-C161S-PAI-2 conjugates, in combination treatment strategies to double target HER-2 and uPA receptors that are co-expressed on certain breast cancer cells.

Quantitative characterisation to determine the number of molecules of NAI-1 attached to TRZ performed by ESI-MS (Figure 4.7, Panel B) revealed one molecule of NAI-1 attached
per molecule of TRZ. The difficulty in getting a good charge envelope for NAI-1-succ-TRZ conjugate by ESI-MS may be attributed to the heterogeneity of the conjugate due to the N-linked glycans and the relative purity of the conjugate. In this scenario, NAI-1-succ-TRZ conjugate was analysed by MALDI-TOF MS (Figure 4.8, Panel B) with the aim of achieving an enhanced desolvation and hence resolution of the conjugate and to detect the presence of more charged species indicating attachment of more than one NAI-1 moiety per molecule of TRZ. The charge envelope of NAI-1-succ-TRZ at 147794.3 Da detected by MALDI-TOF analysis indicated attachment of at least 2 molecules of NAI-1 to TRZ molecule (146558.6 Da).

The in vitro cytotoxic activity of NAI-1-succ-TRZ conjugate (Table 4.2) compared to NAI-1 against high HER-2 expressing SK-BR-3 cells indicates the receptor-mediated selective delivery of NAI-1-succ-TRZ conjugates into these cells. This also proves that modification of TRZ following attachment of NAI-1 moieties does not interfere with its ability to bind to HER-2 receptors.

Combination treatment is an established therapeutic strategy, which has been widely adopted in the form of chemotherapeutic doublets, combination of mAbs, combination of small molecule TKIs, and combination of mAbs and small molecule TKIs. A plethora of combination studies have been conducted utilising TRZ and other agents including chemotherapeutics like paclitaxel [441; 450], docetaxel [451]; mAbs like pertuzumab [452]; and small molecule TKIs like lapatinib [453]. In most cases, combination treatment has brought about increased response rates, time to progression, and survival. For example, TRZ and pertuzumab synergistically inhibited the survival of BT474 cells by blocking receptor signalling through the Akt molecule. Similarly, combination of TRZ with lapatinib significantly increased the progression free survival and clinical
benefit rate (CBR) in HER-2 positive MBC patients who have progressed on a trastuzumab-based therapy regimen.

Treatment of NAI-1-succ-TRZ and NAI-1-succ-C161S-PAI-2 conjugates alone and in combination against ZR-75-1 cells, revealed the combination treatment to cause more cell death compared to each treatment alone (Figure 4.11). This combination cytotoxic effect can be assumed to be more evident under in vivo conditions since breast cancer is highly heterogeneous in nature. Therefore, further in vivo investigations should be performed to ascertain if simultaneous treatment with NAI-1-succ-TRZ and NAI-1-succ-C161S-PAI-2 conjugates leads to increased tumour reduction. This approach of simultaneously targeting uPAR and HER-2 receptors can be expected to result in superior efficacy over single agent therapy and due to minimal drug exposure, a significant delay in the development of drug resistance. Further detailed studies should also be undertaken to comprehensively define the extent of interaction between uPAR and HER-2 receptors both at the extracellular and intracellular level. Such an effort can be assumed to not only support the rationale of targeting uPAR and HER-2 receptors in a combination treatment approach, but also they might help to identify novel functional interactions that can be targeted to effectively counter the increased aggressiveness of HER-2 and uPAR co-expressing tumours.
CHAPTER FIVE
CONCLUSIONS AND FUTURE DIRECTIONS
CONCLUSIONS AND FUTURE DIRECTIONS

Treatment of breast cancer has undergone significant evolution from being primarily treated with surgery and radiation therapy individually and in combination to treatment with highly potent chemotherapeutics. Although these treatment strategies have significantly improved patient survival rates, development of resistance and the toxicity related side effects are still a major drawback. Recently, the discovery of biological targets that are specific to tumours has revolutionised the area of breast cancer therapy. In this direction, deciphering the role of these biomarkers, which are either highly or selectively expressed on tumour and tumour associated stromal cells, has immensely contributed to designing target specific treatment approaches towards malignant tumour cells [4; 5].

Over-expression of certain receptors or ligands on malignant tumour and/or stromal cells linked closely to carcinogenesis, invasion and metastasis include TfR [351], HER-2 [69; 77-79], and uPA and its receptor [122; 149; 165; 166]. These receptors upon activation both through the binding of a ligand or by forming dimers with other receptors, have been found to trigger a host of intracellular and extracellular dysregulated functions resulting in increased cellular proliferation and have been the focus of intense research in recent years. A multitude of agents, in the form of mAbs and small molecule TKIs, have already been developed specifically targeting these receptors [199; 200]. Although these agents were determined to be highly specific for their targets, preclinically off-target toxicity resulting in reduced efficacy together with the development of drug resistance meant new improved drugs needed to be explored.
This led to the development of LDCs, which combines the specificity of a targeted drug and the potency of a cytotoxin sufficient to kill malignant cells. A series of LDCs targeting various tumour-associated antigens are at various stages of clinical trials (refer to Chapter 1, Table 1.4). In this direction, this thesis dealt with the development of cancer-targeting LDCs incorporating novel NAI derivatives.

Tf has been widely used as a ligand for the synthesis of many LDCs in recent times due to the fact that Tf specifically binds to TfRs on the cell surface and undergoes RME [332-334], which can be exploited to deliver a potent cytotoxin payload of drug to malignant tumour cells. However, to date none of the Tf-drug conjugates has made it into the clinic, as TfR is expressed ubiquitously on both normal, although at relatively low levels, and malignant cells [341-350]. In this study, Tf was used as a model ligand to test and optimise the suitability of various linker systems (ester-based and imine-based linkers) and drugs (NAI-1 and NAI-2) involved in the synthesis of LDCs. Both NAI-1 and NAI-2 conjugated to Tf through the ester and imine functional linker system respectively were found to highly toxic against various breast cancer and human monocytic leukemic cells (refer to Chapter 2, Table 1-5). Although NAI-2-imine-Tf conjugates were highly potent against different cell lines tested, they were not found to be significantly toxic in comparison to the parent NAI-2 and NAI-2-imine compounds. The difference in the cytotoxic activity of NAI-2 and NAI-2 imine form was also found to be minimal. This could be possible if the imine linker attached to NAI-2 is unstable under physiological conditions. Interestingly, this finding is in complete contrast to the hydrolytic studies performed by Matesic et al. [296] who found NAI-2-imine to be highly selectively labile and undergoes cleavage only under high acidic conditions with a half life of 17 min. In this scenario, further optimisation studies should be conducted
to determine the selective lability of the imine linker and also its usefulness in a LDC therapy. Due to these difficulties, the imine linker was not used for the synthesis of other LDCs.

On the basis of successful conjugation of NAI-1 to Tf via the ester functional linker system and its high potency and selectivity, NAI-1 was conjugated to the targeting ligand PAI-2 which is highly specific for uPA/uPAR that are over-expressed on MBCs. However, although the NAI-1-succ-C161S-PAI-2 conjugate showed efficient receptor-mediated cytotoxicity in vitro, the short in vivo half-life of PAI-2 [403; 404] is a major drawback that may significantly limit the tumour uptake properties of conjugated drugs to reach maximal therapeutic levels. Therefore, PAI-2 was covalently modified using PEG molecules to improve pharmacokinetic and pharmacodynamic properties. The modified mPEG-C161S-PAI-2-NAI-1-succ conjugate was found to be active and equipotent to the parent NAI-1-succ-C161S-PAI-2 conjugate (refer to Chapter 3, Section 3.3.5. & 3.3.6.). Future studies should focus on ascertaining the efficacy, pharmacokinetic, and pharmacodynamic properties of mPEG-C161S-PAI-2-NAI-1-succ conjugate in vivo.

Studies by Urban et al. [310] and Meng et al. [311] found compelling evidence that uPA receptors are co-amplified and co-expressed on MBC cells along with HER-2 receptors, which are over-expressed in 25-30% of MBCs [92-98]. Although the exact level of interaction between these receptors both at the extracellular and intracellular level is still not clear, recent studies by Li et al. [447] found that downregulation of uPAR synergises with targeting of HER-2 mainly through the ERK pathway in MBC cells. In this context, it was hypothesised that targeting both the above-mentioned
receptor systems simultaneously would result in an increased inhibition of metastatic
tumour growth in comparison to treatment with drugs targeting each receptor alone. In
this direction, NAI-1 cytotoxin was successfully conjugated to TRZ, after a series of
reaction condition optimisations. The resultant NAI-1-succ-TRZ conjugate was found to
be highly cytotoxic against high HER-2 expressing ZR-75-1 and SK-BR-3 breast cancer
cells and more importantly, greater than 3 × and 8 × respectively more effective than the
NAI-1 parent compound (refer to Chapter 4, Section 4.3.3. and Table 4.2). This also
indicates the receptor-mediated selective delivery of NAI-1-succ-TRZ to HER-2
positive breast cancer cells. Preliminary combination studies using NAI-1-succ-C161S-
PAI-2 and NAI-1-succ-TRZ conjugates against SK-BR-3/ZR-75-1 cells showed
promising results (refer to Chapter 4, Section 4.3.4.). This effect can be expected to
more evident in vivo since HER-2 positive tumours are highly heterogeneous. Future
studies in this area should focus on ascertaining the efficacy of this combination
treatment in appropriate mouse models and also detailed investigations should be
conducted to further elucidate the exact nature of interaction between HER-2 and uPA
receptors both at the extracellular and intracellular level in order to prove the rationale
of targeting these receptors simultaneously.

Previous studies performed by Liu et al. [454] demonstrated that EGFR mediates the
uPAR-integrin-fibronectin interaction induced cellular proliferation pathway in HEp3
human cancer cells that over-express uPA receptors. Another study by Guerrero et al.
[455] found that uPAR stimulation, with the amino-terminal fragment (ATF) of
urokinase devoid of proteolytic activity, transactivates the EGFR in MCF-7 cells
through Src and a metalloproteinase. Monaghan-Benson & McKeown-Longo, [456]
showed that constitutively activated uPAR activates EGFR through the Src molecule.
Activated EGFR dimerise with integrin and this event was subsequently found to activate ERK molecules downstream leading to cellular proliferation. These findings point towards a potential crosstalk between EGFR family members, especially HER-2, and uPA receptors and correlates well with previous clinical findings of co-expression of HER-2 and uPA receptors on MBC cells by Urban et al. [310] and Meng et al. [311]. In view of the developments so far, future work should focus on comprehensively defining the extent of extracellular interaction, if any, between HER-2 and uPA receptors over-expressed on the surface of MBC cells. Also manifestations of those interactions in the form of signals and their mode of transmission via transmembrane and also to the intracellular space would be equally important. Such an attempt to correlate the activity and expression of the HER-2 and uPA receptor systems would provide a new set of co-existing interdependent highly specific tumour biomarkers that would help design more effective therapeutics against tumour that over-express both HER-2 and uPA receptors together or either one of them independently on cell surface of advanced MBC conditions. It will support the theme of aggressiveness, in terms of invasion and distant metastasis, associated with tumours that highly over-express HER-2 and uPA receptors on the cell surface.

This is the first time that two different LDCs are used to target tumour-specific biomarkers present on malignant tumour cells. The highly promising cytotoxic effect found between NAI-1-succ-TRZ and NAI-1-succ-C161S-PAI-2 in combination warrants further detailed preclinical investigations. Successful outcome of this double targeting treatment approach has significant clinical implications, in terms of delay in the development of resistance and improvement in the overall survival rate of patients suffering from highly aggressive MBC.
CHAPTER SIX

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REFERENCES


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APPENDIX 1

Buffers and solutions

Phosphate buffered saline (PBS), pH 7.4
12 mM Na2HPO4
2 mM Na2HPO4
2.7 mM KCl
137 mM NaCl

Phosphate buffered saline (PBS), pH 8.5
12 mM Na2HPO4
2 mM Na2HPO4
2.7 mM KCl
137 mM NaCl

Hanks binding buffer, pH 7.4
0.98% (w/v) Hanks balanced salts
20 mM HEPES
1 mM CaCl2
1 mM MgCl2
0.1% (w/v) BSA

Sodium phosphate buffer
20 mM NaH2PO4
pH 6.0

Histidine buffer
10 mM histidine
pH 6.0

HEPES buffer
150 mM NaCl
20 mM HEPES
pH 7.5

Carbonate buffer (pH 8.5)
0.1 M sodium bicarbonate (NaHCO3)
0.1M sodium carbonate, decahydrate (Na2CO3•10H2O)

Carbonate buffer (pH 9.5)
0.1 M sodium bicarbonate (NaHCO3)
0.1M sodium carbonate, decahydrate (Na2CO3•10H2O)
**DTT reduction buffer**
1 M DTT
0.025 M sodium borate pH 8.0
0.025 M NaCl
1 mM DTPA

**Tris-HCl buffer**
10 mM Tris-HCl
pH 8.0

**Mass spectrometry sample buffer (pH 6.8)**
10 mM NH₄OAc
0.1% formic acid

**Bacterial culture media**

**Lauria-Bertani (LB) broth**
1% (w/v) tryptone
0.5% (w/v) yeast extract
170 mM NaCl

**LB agar**
1% (w/v) tryptone
0.5% (w/v) yeast extract
170 mM NaCl
1.5% (w/v) agar

**YENB medium**
0.8% (w/v) tryptone
0.75% (w/v) yeast extract

**Terrific broth**
1.2% (w/v) tryptone
2.4% (w/v) yeast extract
0.4% (v/v) glycerol

**SDS-PAGE**

**5 x non-reducing sample buffer**
225 mM Tris-Cl
20% (v/v) glycerol
2 mM SDS
0.02% (w/v) bromophenol blue
**5 x reducing sample buffer**
225 mM Tris-HCl
20% (v/v) glycerol
2 mM SDS
0.02% (w/v) bromophenol blue
250 mM DTT

**4% stacking acrylamide gel**
9% (v/v) 37.5:1 Bis:acrylamide
24.3% (v/v) 0.5 M Tris-HCl (pH 6.8)
0.097% (w/v) SDS
0.0485% (w/v) APS
0.485% (v/v) TEMED

**10% resolving acrylamide gel**
25% (v/v) 37.5:1 Bis:acrylamide
24.3% (v/v) 1.5 M Tris-HCl (pH 8.8)
0.097% (w/v) SDS
0.0485% (w/v) APS
0.485% (v/v) TEMED

**SDS-PAGE running buffer (pH 8.3)**
25 mM Tris
120 mM glycine
2 mM SDS

**Coomassie staining solution**
0.2% (w/v) Coomassie blue R-250
40% (v/v) methanol
10% (v/v) glacial acetic acid

**Rapid destain**
40% (v/v) methanol
10% (v/v) glacial acetic acid

**Final destain**
10% (v/v) glacial acetic acid
4% (v/v) glycerol

**Western blotting**

**Transfer buffer**
25 mM Tris base
200 mM glycine
20% (v/v) methanol
**TBS**  
50 mM Tris base  
150 mM NaCl

**TBST**  
50 mM Tris base  
150 mM NaCl  
0.05% (v/v) Tween 20  
pH 8.0

**Ion-exchange chromatography**

**Start buffer**  
20 mM Tris-HCl  
pH 8.0

**Elution buffer**  
20 mM Tris-HCl  
0.5 M NaCl  
pH 8.0

**Washing buffer**  
20 mM Tris-HCl  
1 M NaCl  
pH 8.0
APPENDIX 2

Dose response curves

Appendix 2.1. Representative dose response curves showing cytotoxic activity of NAI-1-succ against (A) MCF-7, (B) MDA-MB-231, (C) SK-BR-3, and (D) U937 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 24 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
Appendix 2.2. Representative dose response curves showing cytotoxic activity of NAI-1-succ against (A) MCF-7, (B) SK-BR-3, (C) U937 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 48 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
Appendix 2.3. Representative dose response curves showing cytotoxic activity of NAI-1 and NAI-1-succ-Tf against (A) MCF-7, (B) MDA-MB-231, (C) SK-BR-3, (D) U937, and (E) THP-1 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 24 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
Appendix 2.4. Representative dose response curves showing cytotoxic activity of NAI-1 and NAI-1-succ-Tf against (A) MCF-7, (B) MDA-MB-231, (C) SK-BR-3, (D) U937, and (E) THP-1 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 72 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
Appendix 2.5. Representative dose response curves showing cytotoxic activity of NAI-2 and NAI-2-imine against (A) MCF-7, (B) MDA-MB-231, (C) U937 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 72 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
Appendix 2.6. Representative dose response curves showing cytotoxic activity of NAI-2-imine-Tf against (A) MCF-7, (B) MDA-MB-231, (C) U937 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 72 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
Appendix 2.7. Representative dose response curve showing cytotoxic activity of NAI-1 against ZR-75-1 cells. Briefly, cells were incubated with a range of concentrations of the above-mentioned compounds for 72 h and cell viability determined using CellTiter 96® AQueous MTS Reagent in reference to appropriate vehicle treated controls. All values in the curve are the means of at least triplicate of values ± STDEV.
APPENDIX 3

A representative calculation of number of molecules of NAI-1 per molecule of mPEG-C161S-PAI-2.

Concentration of mPEG-C161S-PAI-2 = 15.8 µM

Concentration of NAI-1 = Absorbance of NAI-1 measured at 432 nm
Extinction coefficient of NAI-1 X path length

= \frac{0.11}{1400 \times 0.3}

= 260 µM

Number of molecules of NAI-1 incorporated onto per molecule of mPEG-C161S-PAI-2;

= \frac{\text{Concentration of NAI-1}}{\text{Concentration of mPEG-C161S-PAI-2}}

= \frac{260}{15.8}

= 16.5 molecules

Molar extinction coefficient of NAI-1 at 432 nm is 1400 cm⁻¹M⁻¹
Pathlength of a 96-well quartz plate is 0.3 cm
Appendix 4

Visualisation of NAI-1-succc-TRZ conjugates.

Appendix 4.1. A representative size exclusion chromatography elution profile of NAI-1-succc-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.2. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.3. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.4. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.5. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.6. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.7. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.8. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.9. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
Appendix 4.10. A representative size exclusion chromatography elution profile of NAI-1-succ-TRZ conjugate. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
APPENDIX 5

A representative visualisation of NAI-1-succ-TRZ conjugates without coupling reagents (NHS & DCC).

Appendix 5.1. Size exclusion chromatography elution profiles of NAI-1-succ-TRZ after conjugation without the coupling reagents NHS and DCC. A280 represents the absorbance profile of the eluted fractions measured at 280 nm, indicating the absence/presence of TRZ (and in later fractions, the excess coupling reagents). A432 represents the absorbance profile of the eluted fractions measured at 432 nm, indicating the absence/presence of NAI-1.
APPENDIX 6

Thesis Publications
