2004

Binding and fate of plasminogen activator inhibitor type-2 in breast carcinoma cells: rationale for development as a delivery vehicle of cytotoxins for anti-urokinase therapeutic strategies

Fares Al-Ejeh

University of Wollongong

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Binding and Fate of Plasminogen Activator Inhibitor Type-2 in Breast Carcinoma Cells:
Rationale for Development as a Delivery Vehicle of Cytotoxins for Anti-Urokinase Therapeutic Strategies

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

Doctor of Philosophy

From

School of Biological Sciences 2004
University of Wollongong

By

Fares Al-Ejeh
Bachelor (Honours) Biotechnology
STATEMENT OF ORIGINALITY

I, Fares Al-Ejeh, declare that the material of this thesis has not been submitted nor accepted for the award of any degree or diploma in any University. To the best of my knowledge, this thesis does not contain material which has been previously published or written by another person except where due reference is made in the text of this thesis.

Fares Al-Ejeh
ACKNOWLEDGMENT

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Fares Al-Ejeh
ABSTRACT

Plasminogen activator inhibitor type 2 (PAI-2) is one of the inhibitors of the plasminogen activation cascade mediated by urokinase (uPA) which is overexpressed on the surface of metastatic cancer cells. The plasminogen activation cascade is the generator of plasmin proteolytic activity on the cell surface which is required under normal physiological conditions and is largely exploited during cancer invasion and metastasis. The overall aim of this thesis was mainly to characterise the inhibition of cell-surface uPA by exogenously added PAI-2 and the elucidation of the fate of uPA:PAI-2 complexes once formed on the cell surface. PAI-2 was directly confirmed as a potent and rapid inhibitor of cell-surface uPA and specifically targets this antigen. PAI-2 internalised rapidly in a uPA-dependent manner after complex formation with cell-surface receptor (uPAR)-bound uPA. Internalised PAI-2 localised in endosomes/lysosomes where it was presumably degraded. While the majority of uPAR/uPA:PAI-2 complexes formed on the cell surface were internalised, cell-surface uPA appeared to be replenished and uPAR was recycled to the cell surface. These findings were significant as they provide new information regarding cell-surface regulation of the plasminogen activation system as well as a biological rationale for the use of PAI-2 as a delivery vehicle of cytotoxins for anti-uPA therapeutic strategies.

To extend the analyses of PAI-2 binding to cells and its subsequent fate, breast cancer cells were genetically modified to provide material for this study. Modulation of uPA-receptor (uPAR) expression was found to induce changes in
components of the plasminogen activation cascade on the cell-surface. Upregulation of cell-surface uPAR on non-invasive, low uPAR/uPA-expressing T-47D breast cancer cells caused a concomitant increase in cell-surface uPA. This caused an increase in PAI-2 binding capacity to cells, thus directly confirming uPA-specificity of PAI-2. In addition, uPAR upregulation induced an increase in the lysine-dependent binding of plasminogen to the surface of these cells. Although cell-surface uPA was not confidently excluded as receptor for plasminogen binding on uPAR-modulated cells, the increase in plasminogen binding on these cells is ultimately due to increase in cell-surface plasminogen receptors. Finally, upregulation of uPAR also affected cell morphology and all together the data confirmed that uPAR/uPA-upregulation is associated with adoption of a metastatic genotype/phenotype. These results support other interests in our laboratory directed towards concluding whether uPA may act as a plasminogen receptor via an active-site independent epitope and elucidating plasminogen receptors which may be modulated due to modulation of uPAR/uPA expression.

Finally, this thesis aimed at optimising the preparation of PAI-2 as a uPA-targeting vehicle for cancer radiotherapy and radioimaging applications. PAI-2 was modified by the addition of the metal chelator cDTPA; the product was characterised and radiolabelling with a radioisotope suitable for radiotherapy was optimised. These results allowed the development of standard operating procedures for use in pre-clinical and potentially clinical studies.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I</td>
<td>Iodine-125 radioisotope</td>
</tr>
<tr>
<td>$^{225}$Ac</td>
<td>Actinium-225 radioisotope</td>
</tr>
<tr>
<td>#3921</td>
<td>Monoclonal antibody against human A-chain uPA</td>
</tr>
<tr>
<td>#3934</td>
<td>Monoclonal antibody against human uPAR</td>
</tr>
<tr>
<td>#394</td>
<td>Monoclonal antibody against human B-chain uPA</td>
</tr>
<tr>
<td>$\alpha$1AT</td>
<td>$\alpha_1$-antitrypsin</td>
</tr>
<tr>
<td>$\alpha$2AP</td>
<td>$\alpha_2$-anti-plasmin</td>
</tr>
<tr>
<td>$\alpha$2MR/LRP</td>
<td>$\alpha_2$-macroglobulin receptor/low density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin antibiotic</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATF</td>
<td>Amino terminal fragment</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cDTPA</td>
<td>Cyclic anhydride of diethylenetriaminepentacetic acid</td>
</tr>
<tr>
<td>Con.S</td>
<td>Collagen-binding region connecting segment</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DT</td>
<td><em>Diphtheria</em> toxin</td>
</tr>
<tr>
<td>EACA</td>
<td>$\varepsilon$-amino caproic acid</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethyldiaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionisation mass spectroscopy</td>
</tr>
<tr>
<td>GFD</td>
<td>Growth factor-like domain</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK/MAPK</td>
<td>Extracellular signal-regulated kinase/mitogen-activated protein kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forwards scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>gp330</td>
<td>Glycoprotein 330 (megalin)</td>
</tr>
<tr>
<td>GPI anchor</td>
<td>Glycosyl phosphatidyl inositol anchor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Hepatocyte growth factor/scatter factor</td>
</tr>
<tr>
<td>HI</td>
<td>Hydroiodic acid</td>
</tr>
<tr>
<td>HMW-uPA</td>
<td>High molecular weight uPA</td>
</tr>
<tr>
<td>HNO$_3$</td>
<td>Nitric acid</td>
</tr>
<tr>
<td>HPI and HPII</td>
<td>Hemopexin-like domains I and II</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-1 and IL-2</td>
<td>Interleukin-1 and -2</td>
</tr>
<tr>
<td>ITLC-SG</td>
<td>Instant thin layer chromatography strips</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducers and activators of transcription</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low-density lipoprotein receptor family</td>
</tr>
<tr>
<td>LMW-uPA</td>
<td>Low molecular weight uPA</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MMPs</td>
<td>Metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MT-MMPs</td>
<td>Membrane-type metalloproteinases</td>
</tr>
<tr>
<td>MTS/PMS</td>
<td>3-(4,5–dimethylthiazol–2-yl)-5-(3-carboxymethoxyphenyl)–2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitors Type-1</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen activator inhibitor type-2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCI</td>
<td>Protein C inhibitor</td>
</tr>
<tr>
<td>PE</td>
<td>Pseudomonas exotoxin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Plg</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>Pln</td>
<td>Plasmin</td>
</tr>
<tr>
<td>PN-1</td>
<td>Proteinase nexin-1</td>
</tr>
<tr>
<td>pro-uPA</td>
<td>Proenzyme form of uPA</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor associated protein</td>
</tr>
<tr>
<td>RCL</td>
<td>Reactive centre loop</td>
</tr>
<tr>
<td>RCPB</td>
<td>Reactive centre peptide bond</td>
</tr>
<tr>
<td>RGD peptide</td>
<td>Arg-Gly-Asp peptide</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Src kinase</td>
<td>Sarcoma kinase</td>
</tr>
<tr>
<td>sc-tPA</td>
<td>Single chain tPA</td>
</tr>
<tr>
<td>sc-uPA</td>
<td>Single-chain uPA</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate (detergent)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Serpin</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>SPD</td>
<td>Serine proteinase domain</td>
</tr>
<tr>
<td>suPAR</td>
<td>Soluble form of uPAR</td>
</tr>
<tr>
<td>TA</td>
<td>Tranexamic acid</td>
</tr>
<tr>
<td>TAT</td>
<td>Targeted alpha (radiation) therapy</td>
</tr>
<tr>
<td>tc-tPA</td>
<td>Two-chain tPA</td>
</tr>
<tr>
<td>tc-uPA</td>
<td>Two (Twin) chain uPA</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
</tbody>
</table>
tPA  Tissue-type plasminogen activator
uPA  Urokinase-type plasminogen activator
uPAR  Urokinase (uPA) receptor
VLDLR  Very low-density lipoprotein receptor

**Units Used**

- **mole**  mole (6.022 x 10^{23} particles): amount
- **fmole**  femto (10^{-15}) mole
- **MW**  Molecular weight: mass of 1 mole (g/mol)
- **kDa**  kilo Dalton: unit of molecular weight = 1000 g/mol
- **g**  gram
- **kg**  kilo (10^3) gram
- **mg**  milli (10^{-3}) gram
- **µg**  micro (10^{-6}) gram
- **ng**  nano (10^{-9}) gram
- **l**  litre: volume
- **ml**  milli (10^{-3}) litre
- **µl**  micro (10^{-6}) litre
- **M**  Molar: concentration mole/litre
- **mM**  milli (10^{-3}) Molar
- **µM**  micro (10^{-6}) Molar
- **nM**  nano (10^{-9}) Molar
- **pM**  pico (10^{-12}) Molar: unit of concentration
- **% v/v**  concentration expressed as percentage volume ratio
- **% w/v**  concentration expressed as percentage weight/volume ratio
- **m**  metre: length
- **cm**  centimetre, (10^{-2}) metre
- **mm**  milli (10^{-3}) metre
- **µm**  micro (10^{-6}) meter
- **nm**  nano (10^{-9}) meter
- **h**  hour
- **min**  minutes
- **sec**  second
- **kb**  kilo (10^3) base pairs: DNA length sequence length
- **Kd**  Binding affinity or dissociation constant
- **°C**  degrees Celsius: units of temperature
- **MeV and keV**  Mega (10^6) and kilo (10^3) electron volts: Energy
- **rpm**  rounds per minutes: rotation
- **x g**  gravity force of rotation
- **Ci**  Curies: unit of radioactivity
- **mCi**  milli (10^{-3}) Curies
- **µCi**  micro (10^{-6}) Curies
- **cpm**  counts per minute: radioactivity
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Chapter 1: UROKINASE PLASMINOGEN ACTIVATION PATHWAY: CLINICAL RELEVANCE AND THERAPEUTIC TARGETS FOR CANCER METASTASIS AND INVASION

1.1. INTRODUCTION

The term “tumour” describes the abnormal uncontrolled proliferation of cells leading to a mass of cells that have no physiological function. Despite its common use, the term cancer only refers to an advanced stage of tumour progression where tumour cells have gained the ability to invade nearby tissues or spread throughout the body. This process, called metastasis, occurs when cells detach from the original (primary) tumour site and travel through the circulatory systems (lymphatic or bloodstream) to other parts of the body, causing new secondary tumours called metastatic foci. Cancer claims the lives of millions worldwide. In Australia, cancer accounted for 30% of male deaths and 25% of female deaths in year 2000 (Cancer in Australia 2000, 2003) whereas it accounted for 24% of male and 22% of female deaths in the US in year 2001 (Arias et al., 2003). The leading cause of cancer-related mortality, even after extensive treatment regimes, is the inability to control the spread of malignant tumours (Woodhouse et al., 1997). Ongoing research is required to further understand tumour growth, progression and metastasis to allow the development of preventative measures and therapies in order to limit the spread of this disease in humans.
The plasminogen activation system generates plasmin proteolytic activity which is a key player in tumour progression and cancer invasion and metastasis. Proteolysis of the circulatory zymogen plasminogen by these specific plasminogen activators produces the serine protease plasmin. Plasmin has a broad specificity and is capable of degrading fibrin as well as other extracellular matrix (ECM) components, in addition to the activation of other proteinases and growth factors (for general reviews see Danø et al., 1985; Mignatti and Rifkin, 1993; Andreasen et al., 1997). There are two types of mammalian plasminogen activators: the urokinase-type plasminogen activator (uPA) and the tissue-type plasminogen activator (tPA). While tPA has high affinity for binding to fibrin, uPA binds to the urokinase-type plasminogen activator receptor (uPAR), a cell membrane-anchored protein which focuses plasminogen activation activity at the cell surface. The two main inhibitors of these activators are plasminogen activator inhibitors type-1 and 2 (PAI-1 and PAI-2), whereas plasmin is inhibited by $\alpha_2$-anti-plasmin ($\alpha_2$AP). This chapter reviews the components of the plasminogen activation system that leads to proteolysis (Figure 1), their interactions with other intracellular/extracellular components and roles in cancer metastasis. The main focus of this review is the urokinase plasminogen activation pathway of the plasminogen activation system as targeting cell-surface uPA by its inhibitor PAI-2 holds a potential for development of uPA-based cancer therapy strategies.
**1.2. Causes of Cancer**

Multiple factors lead to the transformation of normal cells into tumour cells, including inherited predisposing genes and environmental factors such as ultraviolet light, ionising radiation and tumour-inducing chemicals (chemical carcinogens, e.g. asbestos and talc) (Alberts et al., 2002). Lifestyle and dietary habits such as tobacco smoking, consumption of alcohol, obesity, stress and infection by certain viruses have also been related to generation of tumour cells (Alberts et al., 2002). It is not the place here to review the factors leading to or associated with tumour onset. However, it is important to state that despite the
variety of causes, the generation of tumour cells requires cumulative genetic mutations (mutagenesis) to alter the biology of normal cells. Indeed, a correlation between tumour-cell generation and mutagenesis is clear for three classes of tumour-causing agents; chemical carcinogens, ionising radiation and viruses (Alberts et al., 2002). Furthermore, these genetic mutations must be passed onto progeny in order to maintain the acquired tumour phenotype. In agreement, molecular evidence suggests that tumour cells from one tumour type are usually clones descendants from a single abnormal cell (Alberts et al., 2002). The progeny of the first abnormal tumour cell must undergo further mutations to obtain characteristics that are required for tumour progression such as high rate of proliferation (Alberts et al., 2002).

Cell proliferation in general is controlled by mechanisms that determine whether the cell passes to another round of cell-division cycle, be directed to differentiate and cease proliferation or undergo programmed cell death (apoptosis). Thus, two routes are available in order to acquire the uncontrolled proliferative characteristic of tumour cells (see review by McCormick, 1999; Alberts et al., 2002). The first route is by hyper-activating proliferation stimulatory genes (proto-oncogenes) to produce mutant genes (oncogenes) (reviewed by Bertram, 2001). The second route involves the inactivation of proliferation inhibitory genes (tumour suppressor genes) (reviewed by Bertram, 2001).

Oncogenesis (activation of proto-oncogenes or deactivation of tumour suppressor genes) is a multistep mutational process where each new mutational step leads to a clonal expansion of cells bearing the new mutation. This clonal expansion is required in order to develop the cancerous (malignant) phenotype of transformed tumour cells (McCormick, 1999). Complications in understanding cancer biology arise from the randomness of the genetic
mutations induced by the variable factors and the subsequent clonal expansion required for the establishment of cancerous cells. Nevertheless, all cancers can be expected to involve disruption of the normal restraint on cell proliferation and cell death (Alberts et al., 2002). Furthermore, despite the randomness of the mutation process and the variability of causes leading to cancer, a relatively predictable set of genes may be expected to mutate for the acquisition of the malignant phenotype (Evans, 1991; McCormick, 1999; Bertram, 2001). Cancer metastasis appears to be accounted for by deregulation of expression of so-called “metastasis genes” (Weber and Ashkar, 2000) which aid the immobilisation of tumour cells from the primary tumour cell mass to invade throughout the body. These genes include those that encode proteolysis factors (e.g. serine proteases, metallo-proteases and cathepsins), angiogenic factors (e.g. vascular endothelial growth factor) and factors related to cell adhesion and migration (Mignatti and Rifkin, 1993; Andreasen et al., 1997; Murphy et al., 2000; Weber and Ashkar, 2000; Chapman and Wei, 2001).

1.3. GENERAL MECHANISMS OF INVASION

The general steps for metastasis are similar for cells derived from all solid tumours (Woodhouse et al., 1997). This involves the detachment of invasive tumour cells from the primary tumour site, invasion through surrounding tissue and invasion into blood/lymph vessels (Figure 2). These cells migrate through the circulatory system to a distance locus. Adhesion of the migrating tumour cells to a tissue is the first step for the establishment of a new tumour site where invasion of the new hosting tissue via proteolysis and proliferation is induced to form a new tumour cell mass (Andreasen et al., 1997; Woodhouse et al., 1997; Wang, 2001).
At the cellular level, tumour cell invasion requires local proteolysis at the leading edge of the cells to clear the ECM in front of its path (Figure 3 – A and B). For the cell to move into the area of lysis, the leading edge must attach (adhere) to the ECM, thus proteolysis at the leading edge is stopped (Figure 3 – B and C). Proteolysis then occurs at the rear end to detach the cell (Figure 3 – C and D) and forward-movement results as intracellular contractions occur in the direction of the leading edge (Figure 3 – D and E) (Woodhouse et al., 1997). Taken together, invasion is best defined as a process of networked and
regulated events of proteolysis/adhesion (detachment/attachment) coupled with contractions of the intracellular filaments (cytoskeleton) mimicking pseudopodial movement (Liotta et al., 1991; Condeelis, 1993; Stossel, 1993).

![Figure 3: Tumour cell invasion through the extracellular matrix](image)

**Figure 3: Tumour cell invasion through the extracellular matrix**

Migrating tumour cells adhere to the ECM of a selected tissue (A) and proteolysis at the leading front of the cell breaks the ‘front’ attachments and clears the ECM in front of the cell (B). Proteolysis at this side of the cell is stopped and the leading front adheres deeply into the cleared ECM (C). Proteolysis at the latter end of the cell breaks the ‘back’ attachments (D) and forward (into the ECM) movement occurs as a result of intracellular cytoskeletal rearrangements (E).

Proteolytic processes, coupled with invasion and adhesion, are also necessary for normal physiological functions in the body, including normal blood vessel maintenance, angiogenesis, clot formation and dissolution, tissue growth, tissue remodelling (morphogenesis), embryogenesis and ovulation (Woodhouse et al., 1997; Wang, 2001). It is important to note here that there
are no qualitative differences between tumour cells and normal cells with respect to the basic processes of cell detachment, migration, invasion and the formation of colonies at distant sites. However, the moment and the place of the expression of these characters by tumour cells are incompatible with normal cellular activities (see reviews by Werb et al., 1990; Liotta et al., 1991; van Roy and Mareel, 1992; Andreasen et al., 1997).

Tumour cell invasion into the surrounding ECM is facilitated by a variety of cell surface-associated proteolytic enzymes. The ECM degrading proteinases can be divided into three major classes: metalloproteinases (MMPs: Collagenases, gelatinases and stromelysins), cysteine proteinases (cathepsins B and L, and the aspartyl protease cathepsin D) and serine proteinases (reviewed by Danø et al., 1985; Liotta et al., 1991; Mignatti and Rifkin, 1993; Andreasen et al., 1997; Schmitt et al., 2000; Wang, 2001). New classes of proteinases include the transmembrane serine protease matriptase (Wu, 2003) and membrane-type metalloproteinases (MT-MMPs) (Zucker et al., 2003). The remainder of this thesis focuses on the plasminogen activation system, a cascade of serine proteases. This system, which plays an important role under normal physiological conditions such as tissue remodelling, ovulation and wound healing, has been described as a central pathway exploited by a variety of cancer-related processes (Andreasen et al., 1997; Schmitt et al., 2000).

1.4. Plasminogen activation system: Main components and their interactions

1.4.A. Plasminogen/plasmin

Plasminogen is the single-chain glycoprotein, zymogen form of plasmin with an activity that is at least several hundred-fold lower than that of plasmin. Plasminogen is produced mainly in the liver and its concentration in the blood plasma is approximately 2 µM, but about 40 % of plasminogen is localised
extravascularly (see reviews by Collen, 1980; Danø et al., 1985; Saksela and Rifkin, 1988; Mignatti and Rifkin, 1993). Plasminogen is 709 amino acids long with a molecular weight of 92 kDa (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978). This zymogen can be observed in several isoforms; the native mature circulatory form is called glu-plasminogen for the glutamic acid at the amino terminus (Wallèn and Wiman, 1975). This form can be converted to the lys-plasminogen form by cleavages at Arg$^{67}$-Met$^{68}$, Lys$^{76}$-Lys$^{77}$ and Lys$^{77}$-Val$^{78}$ by plasmin (Wallèn and Wiman, 1972; Collen and DeMaeyer, 1975; Lijnen and Collen, 1982). The conversion from glu-plasminogen to lys-plasminogen induces large conformational changes (Violand et al., 1978) resulting in increased rate of conversion from lys-plasminogen to plasmin compared to conversion of glu-plasminogen to plasmin (Claeys and Vermylen, 1974).

Plasminogen is cleaved by plasminogen activators at the Arg$^{560}$-Val$^{561}$ bond resulting in plasmin (~ 90 kDa), consisting of A and B chains linked by two disulfide bridges (reviews by Andreasen et al., 1997; Ranson and Andronicos, 2003). The C-terminal B-chain contains the serine proteinase domain responsible for the catalytic activity of the active site which comprises the amino acids His$^{602}$, Asp$^{645}$ and Ser$^{740}$ (Sottrup-Jensen et al., 1978). The N-terminal A-chain contains 5 kringle domains which are triple loop structures that can bind to lysine (Sottrup-Jensen et al., 1978) or lysine analogues such as ε-amino caproic acid (EACA: Markus et al., 1978) and tranexamic acid (Markus et al., 1979). The kringle domains also allow the binding of plasminogen/plasmin to lysine residues available on the cell surface or to components of the ECM. Candidate plasminogen/plasmin cellular receptors include tetranectin, α-enolase, actin and cytokeratin 8 (see reviews by Félez, 1998; Ranson and Andronicos, 2003). Binding to receptors significantly enhances the rate of plasminogen activation (Andronicos and Ranson, 2001). Plasmin has
broad substrate specificity and is able to degrade many ECM proteins, such as fibronectin, vitronectin, fibrin and activate zymogen forms of several metalloproteinases (reviews by Andreasen et al., 1997; Ranson and Andronicos, 2003). Plasmin is also responsible for the release of latent growth/angiogenic factors from the ECM (Bass and Ellis, 2002; Rakic et al., 2003).

1.4.B. PLASMINOGEN ACTIVATORS

1.4.B.1. UROKINASE-TYPE PLASMINOGEN ACTIVATOR

Urokinase-type plasminogen activator (uPA) is synthesised as a single-chain, 411-amino acids-long glycoprotein in the proenzyme form (pro-uPA or sc-uPA), with a molecular weight of ~ 50 kDa (Gunzler et al., 1982a; Gunzler et al., 1982b). uPA was initially found in human urine, then in human blood, seminal fluids as well as many cancer tissues (Danø et al., 1985). The human blood plasma concentration of uPA is around 20 pM and mostly complexed with PAI-1 while the remaining fraction is in the zymogen form (see review by Andreasen et al., 1997). uPA is produced by several cell types in vitro including hepatocytes (Busso et al., 1994), monocytes, granulocytes and lymphocytes (Carpén et al., 1986). Pro- or sc-uPA is several hundred-fold less active than uPA (reviews by Andreasen et al., 1997; Schmitt et al., 2000). uPA (also called two chain uPA [tc-uPA] or high molecular weight [HMW] uPA) consists of two polypeptide chains which are linked by a disulfide bridge. Conversion of pro-uPA to uPA occurs by cleavage of the peptide bond Lys\textsuperscript{158}–Ile\textsuperscript{159} (reviewed in Andreasen et al., 1997) (Figure 4). The conversion of pro-uPA to uPA can be catalysed by plasmin (see review by Danø et al., 1985). Other proteinases also have been reported to catalyse this activation, at least in vitro (reviews by Andreasen et al., 1997; Schmitt et al., 2000): e.g. plasma kallikrein and blood coagulation factor XIIa (Ichinose et al., 1986), two trypsin-like proteinases purified from human ovarian tumours (Koivunen et al., 1989), T cell-associated serine proteinase (Brunner et
al., 1990), cathepsin B (Kobayashi et al., 1991), cathepsin L (Goretzki et al., 1992), nerve growth factor-γ (Wolf et al., 1993), human mast cell tryptase (Stack and Johnson, 1994), prostate-specific antigen (Yoshida et al., 1995) and matriptase (Lee et al., 2000). As shown in Figure 4, alternative proteolysis at positions preceding the disulfide bridge gives rise to low molecular weight uPA (LMW-uPA). Low-molecular weight uPA occurring in urine arises by cleavage between Lys^{135} and Lys^{136} by unknown proteases and generates a 33 kDa molecule that can not bind to its receptor but is still active (Steffens et al., 1982). The matrix metalloproteinase Pump-1 cleaves the Glu^{143}–Leu^{144} bond to produce another form of low molecular weight (32 kDa) uPA (Marcotte et al., 1992).

![Figure 4: Structure and activation of pro-uPA](image)

The top panel shows a cartoon representation of pro-uPA as a single-chain polypeptide. The domains from the amino-terminus to the carboxyl-terminus include: (i) growth factor-like domain (GFD; G for short), (ii) kringle domain (K) and (iii) the serine proteinase domain (SPD). The amino-terminal fragment (ATF) in the GFD is 16 amino acids long (residues 14 – 30). The bottom panel describes (1) the proteolysis of pro-uPA which leads to the formation of two-chain, disulfide-linked, high molecular weight active uPA. (2) Proteolysis of pro-uPA at positions NH_3-terminal to the disulfide bridge (S-S) produces low molecular weight uPA.
The two polypeptide chains of active uPA are called the A- and B-chains. The N-terminal A-chain is 158 amino acids long (24 kDa) and contains a kringle domain (Gunzler et al., 1982b) and a growth factor-like domain (GFD) which is homologous to epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) (Gunzler et al., 1982b). The A-chain of uPA contains a 16 amino acid residues-domain (residues 14 – 30) within the GFD, termed the amino terminal fragment (ATF), which is required for binding of uPA to uPAR (Appella and Blasi, 1987; Appella et al., 1987). The C-terminal B-chain is 253 amino acids long (30 kDa) and contains the serine proteinase domain (SPD) with the active-site catalytic triad composed of His204, Asp255 and Ser356 (Strassburger et al., 1983; Verde et al., 1984).

uPA has a restricted substrate specificity with plasminogen being the main substrate (reviews by Danø et al., 1985; Saksela and Rifkin, 1988; Mignatti and Rifkin, 1993). However, uPA is also able to catalyse the activation of hepatocyte growth factor/scatter factor (HGF/SF) (Naldini et al., 1992; Mars et al., 1993) and the latent from of membrane type 1 matrix metalloprotease (Kazes et al., 1998), as well as directly cleave uPAR (Høyer-Hansen et al., 1992) and fibronectin (Quigley et al., 1987; Gold et al., 1989).

1.4.B.2. TISSUE-TYPE PLASMINOGEN ACTIVATOR

Tissue-type plasminogen activator (tPA) is a 70 kDa glycoprotein containing 527 amino acids (Rijken, 1995) and, like uPA, is secreted as a single chain zymogen (sc-tPA) (Pennica et al., 1983; Fisher et al., 1985). tPA was originally identified in tissue extracts and later found in human uterus, plasma and conditioned cell culture fluids (Gerard et al., 1986; Lill, 1987). The concentration of tPA in human plasma is 5 – 10 ng/ml (van Hinsbergh et al.,
1991; Lijnen and Collen, 1995; Rijken, 1995). tPA is also synthesised by other cell types in vitro such as mast cells (Sillaber et al., 1999).

Cleavage of the Arg^{275}-Ile^{276} bond is required for the generation of the active two-chain tPA which consists of A- and B-chains held together by a disulfide bridge. The N-terminal A-chain contains the active site of tPA (Hoylaerts et al., 1982), which also contains a classical serine protease catalytic triad (His^{322}, Asp^{371} and Ser^{478}) (Pennica et al., 1983). The C-terminal B-chain contains a fibrin binding domain (also called terminal finger domain, residues 6-43) (Holvoet et al., 1986), growth factor-like domain (residues 44-91), kringle 2 between residues 91-173 and kringle 1 between residues 180-261 (Rijken, 1995). The ability of tPA to bind to fibrin is primarily attributed to the second kringle and partly to the finger domain (van Zonneveld et al., 1986b, 1986a; Verheijen et al., 1986). Fibrin binding is required for acquiring fully active tPA (Hoylaerts et al., 1982) and the A-chain is necessary for this stimulation (Rijken and Groeneveld, 1986).

1.4.B.3. uPA VERSUS tPA

Although both catalyse the production of plasmin, uPA and tPA are independent gene products with distinctive structural and functional properties as their protein and complement DNA (cDNA) sequences are quite different (Pennica et al., 1983). They only share 40 % homology at the amino acid level (Degen et al., 1986).

In contrast to pro-uPA that has little or no activity, pro-tPA has amidolytic activity, binds to fibrin (Higgins and Vehar, 1987), can activate plasminogen in the presence of fibrin and is inhibited with plasminogen activator inhibitors (Bachmann and Kruithof, 1984; Danø et al., 1985; Hekman and Loskutoff, 1987). In vivo, uPA operates as a fibrin-independent, cellular receptor-bound activator, whereas tPA functions as a fibrin-dependent
circulatory (blood) activation enzyme (review by Chapman, 1997) though it can bind to Annexin-II on endothelial cells (Kim and Hajjar, 2002).

As discussed earlier (Section 1.3), proteolytic activity on the cell-surface (pericellular proteolysis) plays a vital role in cancer metastasis. uPA binds to its cellular receptor whereas plasma uPA is usually complexed and inhibited by PAI-1. Thus, this plasminogen activator primarily functions on the cell surface and is a central molecule for the activation of plasmin-dependent pericellular proteolysis. The rest of this review addresses the urokinase-type plasminogen activation pathway; however, it is appreciated that tPA may have significant functions in certain cancer types such as melanoma (Bizik et al., 1996; de Vries et al., 1996).

1.4.C. UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR

Specific and high-affinity binding of uPA was first described and detected in human blood monocytes (Vassalli et al., 1985) and the lymphoma cell line U937 (Stoppelli et al., 1985). Nielsen et al. (1988) purified and characterised the membrane protein responsible for this binding from U937 cells, designated as uPAR. The binding of uPA to its cell-surface receptor, *in vitro* (Ellis et al., 1991) and *in vivo* (Quax et al., 1991b), significantly increases (at least 20-fold) the rate of its catalytic activity towards plasminogen. This indicates that uPAR plays an important role in localising uPA activity on the cell surface and is a key player in directing uPA-mediated pericellular proteolysis.

1.4.C.1. GENE STRUCTURE

uPAR cDNA (1.4 kilo base pairs [kb]) was cloned from the human lymphoma cell line U937 (Roldan et al., 1990) and the gene mapped to chromosome 19q13.2 (Webb et al., 1994). uPAR cDNA was also cloned from
murine (Kristensen et al., 1991) and bovine (Krätzschmar et al., 1993) sources. The amino acid sequences from these cDNAs are similar to the sequence of the three domains of the human uPAR (see structure in Section 1.4.C.2.). The interspecies conservation at the amino acid level of individual repeats in the domains is more than 60% (Ploug and Ellis, 1994).

1.4.C.2. PROTEIN STRUCTURE

uPAR is a 55 - 60 kDa cysteine-rich glycoprotein attached to the plasma membrane via a covalent linkage of its C-terminus to a glycosyl phosphatidyl inositol (GPI) anchor (Nielsen et al., 1988). The GPI anchor is added during posttranslational processing involving C-terminal truncation of the primary translation product (Ploug et al., 1991a; Ploug et al., 1991b; Møller et al., 1992). The nascent uPAR is translated as a 313 amino acid peptide and preceded by a 21 amino acid signal peptide. The mature uPAR protein is highly glycosylated and decreases to 35 kDa upon deglycosylation; consistent with the fact that its amino acid sequence contains 3 potential glycosylation sites (Behrendt et al., 1990; Roldan et al., 1990).

uPAR consists of three homologous cysteine-rich repeats of about 90 amino acids each (3 domains, Figure 5). The consensus motif of each domain is characterised by a unique pattern of cysteine residues common to other proteins of the Ly6 superfamily (Ploug and Ellis, 1994). Stable receptor fragments can be produced by limited proteolysis (Behrendt et al., 1991; Ploug et al., 1993; Behrendt et al., 1996), which is consistent with the presence of the three domains. The disulfide bridge pattern of the N-terminal domain 1 was determined and found to be similar to that of α-neurotoxins from snake venoms (Ploug et al., 1993; Ohkura et al., 1994). On this basis, Ploug and Ellis (1994) proposed a model for the 3-dimensional structure of uPAR domains (Figure 5).
Figure 5: The structure of urokinase receptor, uPAR

The primary sequence of human uPAR polypeptide is shown as encircled amino acids in the single letter code and disulphide-bonded cysteine residues are joined by a black bar (Ploug and Ellis, 1994). The three domains (D1, D2 and D3) are marked according to Ploug and Ellis (1994) and to date the crystal structure of uPAR has not been resolved. The uPA-binding residues (7-residue-long Ω loop) are shown; NH₂ and COOH represent the amino- and carboxyl- termini, respectively. Inset: schematic representation of the GPI-anchored uPAR where the GPI anchorage is added during posttranslational processing.

1.4.C.3. uPA/uPAR BINDING

The binding affinity (dissociation constant, $K_d$) for the uPA/uPAR interaction varies between 0.1 – 1 nM depending on cell type and assay conditions (e.g. Nykjær et al., 1994). Pro-uPA and uPA bind to uPAR with similar affinity (Cubellis et al., 1986) via their ATF (residues 14 – 30) (Stoppelli et al., 1985; Appella and Blasi, 1987; Appella et al., 1987). The uPA-binding residues of uPAR are located at one side of a 7-residue-long loop (Ω loop, see Figure 5) in domain 1 (Behrendt et al., 1991; Ploug et al., 1995; Magdolen et al., 1996). Two
sub-regions within domain 1 of uPAR are critical for uPA recognition (Pöllänen, 1993) and residue 57 in uPAR is specifically involved in binding (Ploug et al., 1995). The other two domains (2 and 3: residues 88-283) do not have detectable uPA binding activity, however inter-domain interactions contribute critically to the binding as the affinity of uPA to isolated domain 1 is approximately 1500-fold lower than that to the intact receptor (Ploug and Ellis, 1994; Behrendt et al., 1996).

Bound uPA can cleave uPAR after Arg^{83} and Arg^{89} in the linker region between domains 1 and 2 and lead to the release of the uPA binding region (Høyer-Hansen et al., 1992; Høyer-Hansen et al., 1997b). GPI-uPAR has a much higher susceptibility to uPA-mediated cleavage than soluble forms of uPAR that lack the GPI moiety (Høyer-Hansen et al., 2001). Furthermore, uPA-mediated cleavage of GPI-uPAR is accelerated on the cell surface and the specific receptor binding of active uPA is required for the high-efficiency cleavage (Høyer-Hansen et al., 1997b). This form of cleaved uPAR has been identified on several cell lines of neoplastic origin (Høyer-Hansen et al., 1992; Solberg et al., 1994; Høyer-Hansen et al., 1997b; Ragno et al., 1998) and in extracts of experimental tumours (Solberg et al., 1994). Since domain 1 of uPAR contains the binding site for uPA and, as discussed later (Sections 1.5 and 1.6), intact uPAR is required for uPAR/vitronectin-mediated adhesion, the release of domain 1 of uPAR by uPA-mediated cleavage is thus likely to affect both pericellular proteolysis and adhesion (Wei et al., 1994; Kanse et al., 1996; Høyer-Hansen et al., 1997a; Høyer-Hansen et al., 1997b). Høyer-Hansen et al. (1997a) postulated that the cleavage and release of domain 1 of uPAR represents an in vivo regulatory step. Domain 1-deleted uPAR was detected in vivo in uPA/uPAR-overexpressing transgenic mice and was related to keratinocyte differentiation and lowered pericellular proteolytic activity (Zhou et al., 2000).
**1.4.C.4. SOLUBLE uPAR**

A soluble form of uPAR (suPAR) has been identified in the plasma of healthy individuals (Rønne et al., 1995) and the level of suPAR is elevated in patients with some types of cancers (see Section 1.7.). Soluble uPAR (suPAR) may be generated by alternative splicing of the uPAR messenger (m)RNA (Pyke et al., 1993). suPAR functions as a chemoattractant after cleavage between domains 1 and 2 by any of a number of proteinases, including uPA, plasmin or chymotrypsin (Resnati et al., 1996). Currently, the role of suPAR in modulating cancer progression is thought to ensue by either scavenging uPA or alternatively regulating cell signaling (Resnati et al., 1996; Fazioli et al., 1997; Aguirre Ghiso et al., 1999b; Degryse et al., 1999; Nguyen et al., 2000; Jo et al., 2003).

**1.4.D. SERINE PROTEASE INHIBITORS**

In general, proteinase inhibitors represent more than 10 % of total protein in human plasma of which the majority regulate serine proteases (reviewed by Travis et al., 1990). The serine protease inhibitor (serpin) superfamily includes non-inhibitory serpins and inhibitors of various serine proteases. They are single chain proteins containing conserved domain structure with 80 % of their amino acids organised in secondary elements including 7 – 9 α-helices (lettered A - I) and β-sheets A, B and C (see reviews by Travis et al., 1990; Gettins et al., 1992; Potempa et al., 1994; Stein and Carrell, 1995; Whisstock et al., 1998; Silverman et al., 2001). The most important feature of inhibitory serpins is the presence of a reactive centre loop (RCL) exposed on the surface of the protein which is essential for the inhibitory mechanism of these inhibitors.
1.4.D.1. INHIBITORY MECHANISM OF SERPINS

Serpins are mechanism-based enzyme inhibitors (also called suicide inhibitors) since they irreversibly inhibit their target proteinase by forming a covalent bond with the proteinase. According to Silverman (1996), this class of inhibitors is characterised by their ability to bind to the enzyme where the enzyme acts on the inhibitor as a substrate. Both the enzyme and the inhibitor are then trapped in an inactivated state due to the formation of a covalent bond between the inhibitor and the enzyme. Sufficient evidence indicates that the mechanism of action of serpins on their target proteinases involves such events (see reviews by Travis et al., 1990; Gettins et al., 1992; Potempa et al., 1994; Stein and Carrell, 1995; Whisstock et al., 1998; Silverman et al., 2001). The events of this mechanism is described below and illustrated in Figures 6 and 7.

The amino acid sequence of the RCL is the most variable region among serpins and minor changes within this loop greatly affects the inhibitor specificity (Travis et al., 1990). Serpins have a single reactive site in the RCL loop and the amino acids in this region are numbered as ...P3-P2-P1-P1′-P2′-P3′-...-COOH, where the reactive site is the bond between P1 and P1′ (review by Gettins et al., 1992). This specific bond in the RCL of the serpin (called the reactive centre peptide bond, RCPB) provides a pseudo-substrate for the target proteinase and, as described next, the enzyme is trapped in an inactive form (Andreasen et al., 1997). The serpin initially forms a non-covalent Michaelis-like (reversible) complex with the target proteinase by the interaction of the RCL of the serpin with the active site of the enzyme (Figure 6 – A and B). The serine residue in the active-site of the proteinase attacks the P1-P1′ bond (RCPB) of the serpin and this bond is cleaved then a covalent linkage is formed between the serine residue and the backbone carbonyl of the P1 residue of the serpin. The RCL starts to insert into β-sheets A of the serpin and upon complete loop
insertion the proteinase/serpin undergo conformational changes, Figure 6 – C, where the active site of the enzyme is distorted and the serpin is in high conformational-stability state (reviewed by Silverman et al., 2001). The net result of the conformational rearrangements is the kinetic trapping of the acyl intermediate complex between the serpin and its target (Lawrence et al., 1995; Wilczynska et al., 1995). This rearrangement, also called the stressed (S) to relaxed (R) transition (Whisstock et al., 1998), is essential for efficient inhibition and formation of the stable final complex. The N-terminal portion of the RCL is critical for the inhibition mechanism (Chaillan-Huntington et al., 1997) and variation in the length of this portion of the RCL markedly reduces the efficiency of inhibition and the stability of the final complex (Zhou et al., 2001).
**Figure 6: Inhibitory mechanism of serpins and associated conformational changes**

The active serpin (A) in the S state (native) interacts with the active site of the proteinase via the serpin's RCL (shown in red) to form the initial Michaelis-like complex (B). The proteinase cleaves the P<sub>1</sub>-P<sub>1</sub> bond in the RCL of the serpin and a covalent bond is formed between the carbonyl group of the P<sub>1</sub> residue and the serine residue of the enzyme. This acyl-enzyme intermediate is trapped in a kinetically stable state (R state, C) by conformational changes in the serpin structure where the RCL inserts as a β-sheet (shown in red) between the β-sheets A of the serpin (shown in yellow). The coordinates of the structures shown are available from Protein Data Bank (PDB: http://www.rcsb.org/pdb) and represent: (A) native α<sub>1</sub>-antitrypsin (α<sub>1</sub>AT, PDB entry 1QLP), (B) Michaelis-like complex between Serpin 1 and trypsin (PDB entry 1I99) and (C) covalent complex between α<sub>1</sub>AT and trypsin (PDB entry 1EZX). The structures were modelled using Swiss-PdbViewer (Deep View v 3.6: Guex and Peitsch, 1997) available from (http://www.expasy.org/spdbv).
The pathway discussed above describes the complete inhibition of the target proteinase and the formation of covalently-bound, sodium dodecyl sulphate (SDS)-stable 1:1 stoichiometric serpin:proteinase complex. Nevertheless, some evidence suggests that in some cases, due to unusual stabilisation of the initial Michaelis-like complex, progression of the reaction is blocked and the inhibition of the enzyme by the serpin is reversible (Travis et al., 1990; Potempa et al., 1994; Silverman et al., 2001). Furthermore, experimental data also show that, in some cases, the serpin acts in a non-inhibitory pathway, where the active proteinase cleaves the serpin and the inactive serpin is released before loop insertion occurs (Silverman et al., 2001). This results in release of active proteinase and inactivated serpin where the proteinase reacted on the serpin as a substrate. In summary, as shown in Figure 7, the current model for serpin mechanism of action describes an initial reversible interaction between the serpin (I) and the proteinase (P) to form a short-lived Michaelis-like complex (IP). The carbonyl group of P₁ residue of the serpin is attacked by the active-site serine residue of the protease. The P₁-P₁⁻ bond is cleaved and through a tetrahedral transition state the reaction proceeds to produce the acyl-enzyme intermediate complex (IP*). The serpin then undergoes the dramatic conformational change and loop insertion to produce the stable inhibited serpin/proteinase complex (IP_{cpx}). If loop insertion is not rapid enough to compete with de-acylation, then the reaction proceeds to produced cleaved inactivated serpin (I*) and active enzyme (E) mimicking the normal pathway for the serine protease acting on its substrate.
Inhibitory serpins (I) interact with the target proteinase (P) and form the initial reversible, short lived Michaelis-like complex (IP). The association/dissociation rates of this complex are described by $k_{on}$ and $k_{off}$, respectively. The acyl-enzyme intermediate complex (IP*) is formed at a rate of $k_2$ through a tetrahedral transition state after the cleavage of the P$_1$-P$_1$' of the serpin by the active-site serine residue of the proteinase. The serpin then undergoes dramatic conformational change and loop insertion to produce the stable inhibited serpin/proteinase complex (IP$_{cpx}$) at a rate of $k_4$. Alternatively, the reaction proceeds to produce cleaved inactivated serpin (I*) (i.e. de-acylation) and active enzyme (E) at a rate of $k_3$ mimicking the normal pathway for the serine protease acting on its substrate. The final fate of the acyl-enzyme intermediate complex (IP*) depends on whether the rate of loop insertion ($k_4$) can compete with the rate of de-acylation ($k_3$). The Figure was modified from Zhou et al. (2001).

Loop insertion is not only essential for the inhibitory mechanism of serpins, but also plays a role in serpin biology. For example, some serpins may adopt an alternative conformation compared to that of the native form (active ‘S’ state, Figure 6 - A) where the RCL is inserted into β-sheets A as in the cleaved form. This latent form of serpins, Figure 8 – A, is inactive and requires specific interaction to produce the active form which can interact with target proteinase and produce the cleaved (‘R’ state, Figure 8 – B) (reviewed by Whisstock et al., 1998; Silverman et al., 2001). Another consequence of serpins’ conformational elasticity is the formation of dimers, which are commonly observed under certain conditions and explained by loop insertion from one serpin molecule into the β-sheets A of another (Silverman et al., 2001).
Serpins adopt three conformational states. The native ‘S’ state where the serpin is active and the RCL is exposed for reaction with target proteinase (see Figure 6 – A), (A) the latent conformation where the RCL (shown in red) inserts in the β-sheets A (shown in yellow) and (B) the ‘R’ state where the P1-P1’ bond is cleaved (by proteinase) and the RCL is inserted into the β-sheets A. The latent conformation resembles the ‘R’ cleaved state; however it can be converted to the active native state under certain conditions since bond cleavage has not occurred. The structures were modelled using Swiss-PdbViewer from the coordinate files of **A**: latent anti-thrombin III (PDB entry 2ANT) and **B**: cleaved α1-antitrypsin (PDB entry 7API).
The serpin superfamily includes various members from different organisms. Only the function and biology of serpins related to the urokinase-plasminogen activation pathway will be reviewed here. For a list of other serpins and their targets see reviews by Potempa et al. (1994), Whisstock et al. (1998) and Silverman et al. (2001). PAI-1, PAI-2 and α2AP belong to the serpin superfamily and while PAI-1 and PAI-2 target both uPA and tPA, α2AP is the primary inhibitor of plasmin. Two other serpins, proteinase nexin-1 (PN-1) and protein C inhibitor (PCI), also inhibit uPA and tPA at physiologically relevant rates, though they are not specific and react more slowly with these proteinases than PAI-1 and PAI-2 (Sprengers and Kluft, 1987; España et al., 1993a; España et al., 1993b). The crystal structures of both PAI-1 (Nar et al., 2000) and PAI-2 (Jankova et al., 2001; Saunders et al., 2001) confirm that these act as classic serpins and form SDS-stable 1:1 stoichiometric complexes with the catalytic site on their target protease. Since one of the aims of this thesis relates to targeting the urokinase-type plasminogen activation pathway as a potential anti-cancer strategy, the following sections only address the two main inhibitors of uPA/tPA activities, PAI-1 and PAI-2.

1.4.D.2. PAI-1

PAI-1 was first described in conditioned media of human endothelial cells (Loskutoff and Edgington, 1977) and later detected in a wide variety of cell types (see review by Kruithof, 1988). PAI-1 level in plasma is variable but is approximately 20 ng/ml (Kruithof et al., 1987). It is a single chain glycoprotein with 379 amino acids (~ 52 kDa) and an isoelectric point (pI) of 4.5 – 5.0 (Erickson et al., 1984; Andreasen et al., 1986a). The active site is located at Arg346-Met347 (Andreasen et al., 1986b; Ny et al., 1986; Sanzo et al., 1987) and the sequence contains an amino terminal signal peptide (21 – 23 amino acids) that is cleaved in the mature protein (Andreasen et al., 1986b; Ginsburg et al.,
1986; Ny et al., 1986; Pannekoek et al., 1986; Wun and Kretzmer, 1987). PAI-1 is highly sensitive to oxidation (Baker et al., 1990b), relatively unstable and rapidly converts into the latent conformation at 37°C (Levin and Santell, 1987; Mimuro et al., 1987; Reilly et al., 1990). This latent form can be re-activated by denaturation (Hekman and Loskutoff, 1985) and heat (Katagiri et al., 1988). Interaction of active PAI-1 with the matrix protein vitronectin stabilises PAI-1 in this active form and reduces the formation of latent PAI-1 (see Section 1.5.A.2. for more details).

PAI-1 efficiently inhibits tPA and uPA (Erickson et al., 1984; Colucci et al., 1986) with similar second order rate constants for both targets; approximately $2 \times 10^7 \text{M}^{-1}\text{s}^{-1}$ (Declerck et al., 1988; Thorsen et al., 1988). In the presence of vitronectin, the second order rate constant of PAI-1 towards uPA is lowered to $7.9 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ (Ellis et al., 1990) and by 35% towards tPA (Declerck et al., 1988). PAI-1 is a less efficient inhibitor of pro-tPA ($5.5 \times 10^6 \text{M}^{-1}\text{s}^{-1}$) compared to tPA (Thorsen et al., 1988) and inhibition of tPA is lowered when tPA is bound to fibrin (Kruithof et al., 1984). Similarly, the second order constant towards uPA is lowered when uPA is bound to cell-surface uPAR ($4.5 \times 10^6 \text{M}^{-1}\text{s}^{-1}$) (Ellis et al., 1990).

1.4.D.3. PAI-2

PAI-2 inhibitory activity was first described in partially purified extracts from human placenta (Kawano et al., 1970). Pure PAI-2 was later prepared from placenta and human monocyte/macrophage lineages (see reviews by Dear and Medcalf, 1995; Kruithof et al., 1995). PAI-2 exists in two forms; a non-glycosylated form with a molecular weight of 47 kDa and pI of 5.0 (Kruithof et al., 1986) and a 60 kDa glycosylated form with pI of 4.4 (Kruithof et al., 1986; Genton et al., 1987; Wohlwend et al., 1987b). These two forms also have distinct
cellular localisation; the 47 kDa PAI-2 being intracellular and the 60 kDa PAI-2 being secreted (Genton et al., 1987; Wohlwend et al., 1987a, 1987b).

PAI-2 is an efficient inhibitor of uPA (second order rate constant of $10^6$ M$^{-1}$s$^{-1}$) and tPA ($2 \times 10^5$ M$^{-1}$s$^{-1}$) (Kruithof et al., 1986; Thorsen et al., 1988; Mikus et al., 1993). The second order rate constant for PAI-2 inhibition of pro-tPA is much lower than that of tPA ($10^3$ M$^{-1}$s$^{-1}$) (Åstedt et al., 1985; Kruithof et al., 1986) and fibrin bound tPA appears to be protected from inhibition by PAI-2 (Leung et al., 1987). As in the case of PAI-1, the second order rate constant for the inhibition of cell-surface uPAR-bound uPA by PAI-2 is lower than that of soluble uPA (Ellis et al., 1990). All together, PAI-2 may be accepted to function primarily as an inhibitor of uPA and only has a minor role in controlling tPA-mediated fibrinolysis. This serpin would preferentially target the urokinase plasminogen activation pathway on the cell surface, thus an expanded review of the structure/function of PAI-2 is described below.

1.4.D.3.I. PAI-2 GENE STRUCTURE AND EXPRESSION

PAI-2 gene is 16.5 kb long and consists of 8 exons and 7 introns located on chromosome 18q21-23 (Webb et al., 1987; Webb et al., 1994). Three other ov-serpins (see Section 1.4.D.3.II.); squamous cell carcinoma antigen (SCCA-1 and SCCA-2) genes and the maspin gene, are located 300 kb centromeric from the PAI-2 gene (Schneider et al., 1995). In addition, the bcl-2 proto-oncogene, regulator of apoptosis (Vaux et al., 1994), lays 600 kb centromeric to the PAI-2 gene suggesting a role for PAI-2 in apoptosis (Silverman et al., 1991; Schneider et al., 1995).

PAI-2, in both forms, is 415 amino acids long encoded by a single mRNA of approximately 2.0 kb length (see reviews by Dear and Medcalf, 1995; Kruithof et al., 1995). The transcription initiation site for PAI-2 gene resides between 22 to 25 base pairs (bp) downstream of a consensuses TATAAAA sequence
(Kruithof and Cousin, 1988; Ye et al., 1989; Samia et al., 1990) and the promoter region contains two alu repeat sequences, 1390 - 1100 and 870 - 580 (Kruithof and Cousin, 1988). Two closely spaced activator protein (AP)-1 like elements (at -116 to -97) and a cyclic adenosine monophosphate (cAMP) response element (CRE)-like element (-189 to -182) in the PAI-2 promoter are essential for basal gene transcription and for phorbol ester-mediated induction of PAI-2 expression (Cousin et al., 1991). Retinoic acid receptor/glucocorticoid element (at -1659 to -1620) mediates the retinoic acid response of the PAI-2 gene (Schuster et al., 1994). The region around -1150, homologous to dioxin response element, may mediate the response of PAI-2 gene to the toxin dioxin (Sutter et al., 1991). The sequences at -3.4 and at -4.1 kb in PAI-2 promoter bind a protein with a similar size to that of an interferon-gamma activated DNA binding element (Antalis et al., 1993). As suggested by the structure and characteristics of the promoter of PAI-2, various agents affect the expression of PAI-2 gene. These agents are summarised in Figure 9. For more details refer to reviews by Bachmann (1995), Dear and Medcalf (1995) and Kruithof et al. (1995).

1.4.D.3.II. PAI-2 BIOCHEMISTRY

The polypeptide chain of PAI-2 has three potential N-glycosylation sites (Asn75, Asn115 and Asn339), which all appear occupied in the glycosylated form of PAI-2 (Ye et al., 1988), and the reactive centre (P1-P1') maps to Arg380-Thr381 bond (Kiso et al., 1988). There are two common variants of PAI-2 which differ at three amino acid positions; Asn120, Asn404 and Ser413 for Type A compared to Asp120, Lys404 and Cys413 for Type B (Ye et al., 1987; Ye et al., 1989). The extra cysteine in Type B PAI-2 may mediate dimerisation (Mikus et al., 1993) and the formation of disulfide bridges with other proteins as that described with vitronectin in human placenta (Wun and Reich, 1987; Radtke et al., 1990).
Figure 9: Regulation of PAI-2 gene expression

Agents that affect PAI-2 gene expression are classified in the groups shown in balloons. Shaded balloons indicate suppressors of PAI-2 gene expression while the remaining (clear) are activators of PAI-2 gene expression (modified from Dear and Medcalf, 1995). These agents have been shown experimentally to modulate PAI-2 expression in several different cell lines. **Abbreviations:** EGF: epidermal growth factor, M-CSF: macrophage colony stimulating factor, GM-CSF: granulocyte-macrophage colony stimulating factor, TNF: tumour necrosis factor, IL-1 and IL-2: interleukin-1 and -2.

PAI-2 does not have a cleavable signal peptide (Ye et al., 1988; von Heijne et al., 1991) as the case in ovalbumin (a non-inhibitory serpin). In fact, evidence suggests that PAI-2 belongs to a subfamily of serpins called ov-serpins, which includes ovalbumin, chicken gene Y and other serpins (reviewed by Bachmann, 1995; Silverman et al., 2001). Members of this subfamily show additional amino acid sequence homology compared to other serpins, lack N-terminal and C-terminal extensions, often contain a non-conserved loop between their C and D
helices (C-D interhelical region) and lack a cleavable signal peptide (Bachmann, 1995). As many other ov-serpins, PAI-2 is mainly found intracellularly in almost all cell types studied (Genton et al., 1987; Wohlwend et al., 1987b; Medcalf et al., 1988a; Medcalf et al., 1988b; Belin et al., 1989). However, extracellular traces of PAI-2 under normal conditions were reported and under certain conditions secreted PAI-2 may represent a significant part of total PAI-2, indicating that cellular targeting of synthesised PAI-2 may be modulated (Ye et al., 1988; Quax et al., 1990). The differential translocation of the two forms of PAI-2, which are encoded by a single mRNA species, may be explained by the presence of two internal hydrophobic signal regions. Increasing the hydrophobicity of these regions, which are homologous to an internal hydrophobic signal region in ovalbumin, enhances the extracellular translocation of PAI-2 (von Heijne et al., 1991; Belin, 1993). These regions may explain the secretion of PAI-2 to the endoplasmic reticulum (ER)-Golgi apparatus pathway, however other evidence suggests that secretion of PAI-2 by monocytes occurs via ER-Golgi-independent and vesicle-independent pathway (Ritchie and Booth, 1998). To date, the exact mechanism of PAI-2 secretion is unknown.

1.4.D.3.III. FUNCTIONS OF PAI-2

The principal known target proteinases of PAI-2 are uPA and tPA. This is intriguing considering that PAI-2 is mainly localised intracellularly and its secretory pathway is poorly understood. Thus, the physiological role of PAI-2 has been a subject for research. PAI-2 levels in plasma are normally below detection limits, however, intracellular (47 kDa form) and extracellular PAI-2 (60 kDa form) seem to play physiological roles under certain conditions such as pregnancy, cancer, inflammation, diseases and in apoptosis (Dear and Medcalf, 1995; Kruithof et al., 1995).
During pregnancy, plasma concentrations of PAI-2 increase from below detection limits to a maximum of 250 ng/ml at term (Kruithof et al., 1987; Lecander and Åstedt, 1987; Wright et al., 1988; Koh et al., 1992; Stegnar et al., 1993; Halligan et al., 1994). Changes in PAI-2 concentration have been shown to occur in cases of pre-eclampsia and intrauterine growth retardation, indicating a role of PAI-2 in securing hemostasis during pregnancy (reviewed by Dear and Medcalf, 1995; Kruithof et al., 1995; Åstedt et al., 1998). This role may be related to regulation of uPA activity since PAI-2 cleaved at the reactive centre is observed in placental extracts (Kiso et al., 1991). PAI-2 levels are modulated during cancer and as discussed in later sections (see Section 1.7); elevated PAI-2 levels appear to be a marker of low metastatic activity and good prognosis in a few cancer models. Induction of PAI-2 expression by inflammatory mediators (e.g. phorbol esters) and down-regulation by anti-inflammatory agents (e.g. dexamethasone) suggest a role for PAI-2 in inflammation (Dear and Medcalf, 1995; Kruithof et al., 1995). The precise role of the predominant intracellular form of PAI-2 is still not clearly elucidated. PAI-2 seems to play a role in keratinocytes differentiation (Lavker et al., 1998; Risse et al., 1998; Williams et al., 1999; Risse et al., 2000), prevention of apoptosis (Kumar and Baglioni, 1991; Jensen et al., 1994; Dickinson et al., 1998; Jensen et al., 1999) and protection against viral or bacterial infections (Gan et al., 1995; Antalis et al., 1998; Shafren et al., 1999). Recently, a novel intracellular role of PAI-2 as a retinoblastoma protein (Rb)-binding protein was reported (Darnell et al., 2003). Rb is a ubiquitous regulator of transcription involved in activities such as cell cycle control, apoptosis, differentiation and tumour suppression (Harbour and Dean, 2000b, 2000a). Binding of PAI-2, via its unique C-D interhelical region, to Rb in the nucleus inhibits the turnover of Rb which leads to increase of Rb protein levels and activity (Darnell et al., 2003). These recent results provide
further insights to the mechanism by which intracellular PAI-2 may mediated its effects, however, further work is still required to elucidate the complete roles of intracellular PAI-2.

1.5. THE UROKINASE PLASMINOGEN ACTIVATION PATHWAY: NON-PROTEOLYTIC FUNCTIONS

Members of the urokinase-plasminogen activation pathway of the plasminogen activation system are associated with other molecules present on the cell surface and/or the ECM, which appear to expand the functions of this pathway further than just generating proteolytic activity on the cell surface. This section addresses these other molecules which associate with this pathway.

1.5.A. VITRONECTIN

Vitronectin is a 78 kDa glycoprotein that contains a somatomedin B domain, an integrin-binding RGD (single-letter amino acid code) sequence, a collagen-binding region and two hemopexin-like domains (reviewed by Felding-Habermann and Cheresh, 1993; Hess et al., 1995). This glycoprotein is primarily a circulatory plasma protein (plasma concentration of 4 µM) and it is deposited extravascularly in the ECM during injury and repair (Dvorak et al., 1995; Hess et al., 1995). Plasma vitronectin is in the closed form (native, Figure 10), while the extended form is believed to be the one deposited in the ECM (Hess et al., 1995). Cell-surface receptors of the integrin family (see Section 1.5.B.), especially those containing αv chain (e.g. αvβ1, αvβ3, αvβ5), bind to the RGD sequence of vitronectin (Felding-Habermann and Cheresh, 1993; Schnapp et al., 1995). Interestingly, evidence suggests important functional binding between vitronectin and components of the urokinase plasminogen activation system.
1.5.A.1. uPAR/VITRONECTIN INTERACTION

uPAR is a high affinity receptor for the matrix (extended) form of vitronectin (Wei et al., 1994; Kanse et al., 1996). The vitronectin-binding site on uPAR is within domains 2 and 3 (Wei et al., 1994) while the uPAR-binding residues on vitronectin are located at the amino-terminal somatomedin B domain (Deng et al., 1996; Waltz et al., 1997). Vitronectin-uPAR binding is not blocked by RGD peptides, distinguishing this interaction from the known integrin–vitronectin interactions (Preissner et al., 1988). Intact uPAR is required for efficient vitronectin binding (Høyer-Hansen et al., 1997a) and this binding is enhanced by the presence of uPA on uPAR (Wei et al., 1994). This enhancement of the adhesion of uPAR to vitronectin is independent of the proteolytic activity of uPA (Chang et al., 1998; Sidenius et al., 2002).

1.5.A.2. PAI-1/VITRONECTIN INTERACTION

PAI-1 in its native form binds to vitronectin and this binding stabilises PAI-1 in active conformation (Reilly et al., 1992), thus protecting PAI-1 from converting into its latent form. The amino terminus of vitronectin (Somatomedin B domain) is involved in the PAI-1/vitronectin interaction and the binding site...
for PAI-1 overlaps with those of uPAR and integrins (Seiffert and Loskutoff, 1991; Royle et al., 2001). This is in agreement with data indicating that active PAI-1 blocks uPAR/vitronectin interaction (Deng et al., 1996; Kanse et al., 1996; Waltz et al., 1997; Loskutoff et al., 1999) and competition between PAI-1 and integrins for binding to vitronectin (Stefansson and Lawrence, 1996; Kjøller et al., 1997).

The quantity of ECM-deposited PAI-1 correlates strongly with the availability of ECM-associated vitronectin (Mimuro et al., 1987; Declerck et al., 1988; Mimuro and Loskutoff, 1989a, 1989b). Furthermore, the distribution of PAI-1 in the extracellular space is almost the same as that of vitronectin (Barnes et al., 1983; Hayman et al., 1983; Neyfakh et al., 1983). Thus, vitronectin appears to function as a ‘carpet’ for presenting active PAI-1 in the ECM. The vitronectin-PAI-1 complex dissociates simultaneously with the appearance of the inactive enzyme–PAI-1 product upon the reaction of vitronectin-associated PAI-1 with uPA or tPA (Declerck et al., 1988; Salonen et al., 1989; Hess et al., 1995; Deng et al., 1996). The significance and consequences of these interactions are discussed later (Section 1.6.).

1.5.A.3. uPA/VITRONECTIN INTERACTION

Moser et al. (1995) showed a concentration-dependent reversible binding of uPA to vitronectin in vitro (K_d approximately 97 nM). This binding involves the hemopexin domain fragment of vitronectin and the N-terminal fragment of uPA. PAI-1 does not compete with uPA for binding to vitronectin, suggesting that both molecules may co-localise on vitronectin (Moser et al., 1995). To date, functional consequences of uPA binding to vitronectin or co-localisation of uPA and PAI-1 on vitronectin have not been demonstrated in vivo.
1.5.B. INTEGRINS

Integrins are heterodimeric transmembrane glycoprotein receptors composed of distinct $\alpha$ and $\beta$ subunits, which bind to ECM proteins such as vitronectin, collagens, fibrin, laminin and fibronectin (Hynes, 1992; Chapman, 1997). These receptors mediate adhesion of cells to the ECM via RGD moiety as well as non-RGD dependent interactions with intracellular signalling proteins and cytoskeletal elements (Hemler, 1998). Integrins undergo reversible activation upon binding to their ligands. This activation is characterised by conformational changes in the extracellular domains, reorganisation of intracytoplasmic connections (actin-cytoskeleton) and redistribution of integrins on the cell surface (Diamond and Springer, 1994; Yednock et al., 1995).

A comprehensive study on the association of integrins with uPAR found that $\beta_1$ integrins and uPAR co-localise with close proximity (7 nm) at focal contacts between carcinoma cells and fibronectin, laminin or vitronectin matrices (Xue et al., 1997). On the other hand, uPAR and $\beta_3$ integrins co-localise only when cells attach to vitronectin. Furthermore, uPAR co-localises with $\alpha_5$ integrins on cells attached to fibronectin, with $\alpha_5$ and $\alpha_v$ integrins on cells attached to vitronectin and with $\alpha_3$ and $\alpha_6$ integrins on cells attached to laminin (Xue et al., 1997). The formation of complexes between uPAR and $\beta_1$ integrins alters the phenotype (not the expression) of these integrins, thus affecting integrin functions (Wei et al., 1996; Wei et al., 2001). uPAR also interacts with the $\beta_2$ integrin CD11b/CD18 (termed Mac-1) (Sitrin et al., 1996) and the interaction of $\beta_2$ integrins with uPAR is essentially identical to that of $\beta_1$ integrins and uPAR (Wei et al., 1996). uPAR-dependent adhesion to immobilised
vitronectin is enhanced by uPAR-integrin-calveolin* multimeric complexes on the cell membranes (Wei et al., 1996; Chapman, 1997) and uPA/uPAR complexes are concentrated at these multimeric complexes (Stahl and Mueller, 1995).

The association of uPAR with various members of the integrin family and its ability to induce their activation indicate additional functions for the plasminogen activation pathway. As discussed in Section 1.6., uPAR may modulate integrin functions to focus proteolysis at specified sites and utilise the intracellular domains of integrins for signal transduction.

1.5.C. LOW-DENSITY LIPOPROTEIN RECEPTOR (LDLR) FAMILY

Currently, there are more than six known mammalian members of the low-density lipoprotein receptor family of endocytosis (LDL receptors), which mediate the endocytosis of various types of ligands (Nykjær and Willnow, 2002; Strickland et al., 2002). Specifically, the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2MR/LRP), the epithelial glycoprotein 330 (megalin, gp330) and the very low-density lipoprotein receptor (VLDLR) of the LDLR family are known to mediate the endocytosis of components of the plasminogen activation system (reviewed by Andreasen et al., 1994; Andreasen et al., 1997; Strickland et al., 2002).

Cell-surface uPAR-bound uPA/PAI-1 complex is rapidly internalised and degraded in lysosomes in a process mediated by α2MR/LRP (Cubellis et al., 1990; Jensen et al., 1990; Nykjær and Willnow, 2002), gp330 (Moestrup et al., 1993) and VLDLR (Argraves et al., 1995; Heegaard et al., 1995). Similarly, PAI-1 mediates binding of tPA to α2MR/LRP (Owensby et al., 1989; Owensby et al.,

* Caveolae are plasma membrane structures that contain calveolin; a protein associated with intracellular signalling pathways and cytoskeletal elements.
1991; Bu et al., 1993; Grobmyer et al., 1993) and gp330 (Willnow et al., 1992) and these complexes are internalised and degraded. The uPA/PN-1 complex and the uPA/PCI complex are also endocytosed by α2MR/LRP and VLDLR (Conese et al., 1994). The endocytosis of uPA:PAI-2 complexes was only recently illustrated by definitive evidence (see Chapter 4, Al-Ejeh et al., 2004). The internalisation of uPA:PAI-2 complexes is mediated in part by members of the LDLR family; however, other mediators seem to play a role in this internalisation (see Chapter 4 for more details).

1.5.D. INTERACTION WITH SIGNALLING MOLECULES

Studies indicate that uPAR transmits intracellular signals despite the lack of transmembrane structure in this extracellularly GPI-anchored receptor. The expression of uPAR and its ability to bind uPA are required for such signalling (Resnati et al., 1996) and uPA-mediated signalling is independent of its proteolytic activity (Sidenius et al., 2002; Liu et al., 2003). To date, molecular adaptors that may mediate uPAR signalling include integrins, calveolin and G-protein-coupled receptor (GPCR) (reviewed by Ossowski and Aguirre-Ghiso, 2000; Blasi and Carmeliet, 2002). Other less defined signal mediators have been proposed to mediate uPAR signalling such as α2MR/LRP and mannose-6-phosphate/insulin-like growth factor II receptor (Blasi and Carmeliet, 2002).

The uPA-uPAR/mediator association have been shown to modulate several signalling pathways. It is beyond the scope of this thesis to discuss the growing evidence of the signalling role of uPA/uPAR complexes (For more details see reviews by Conese and Blasi, 1995; Andreasen et al., 1997; Chapman, 1997; Dear and Medcalf, 1998; Konakova et al., 1998; Preissner et al., 2000; Blasi and Carmeliet, 2002). In brief, however, signalling pathways which are linked to uPA/uPAR include cytosolic kinase pathways (tyrosine kinases,
serine/threonine kinases and protein kinase C), focal adhesion kinase (FAK) pathway, extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, intracellular calcium mobilisation, Src (sarcoma) family kinases, Janus kinase/signal transducers and activators of transcription (JAK/STAT) in addition to cytoskeletal elements such as vinculin and actin. These signalling pathways regulate the proteolytic as well as the non-proteolytic functions of the urokinase plasminogen pathway such as cellular adhesion, chemotaxis, proliferation, differentiation and LDLR-mediated endocytosis (see Section 1.6.). For example, FAK and ERK/MAPK signalling pathways are activated while the p38 MAPK pathway is down-regulated due to interaction between integrin and uPAR (Liu et al., 2002), thus affecting epidermal growth factor-receptor-dependent proliferation. Gradients of uPA, pro-uPA or the ATF of uPA display a chemotactic effect on cells which express uPAR (Resnati et al., 1996) and a GPCR member is implied in transduction of signals for cytoskeletal adjustment required for this chemotactic activity (Fazioli et al., 1997; Degryse et al., 1999; Nguyen et al., 2000; Resnati et al., 2002).

1.6. UROKINASE PLASMINOGEN ACTIVATION PATHWAY: THE OVERALL PICTURE

The components of the urokinase-plasminogen activation pathway and their interactions which result in the generation and control of plasmin on the cell surface were introduced. However, studies indicate that the urokinase plasminogen activation pathway is more than just a provider of proteolytic activity required for ECM degradation. These studies improve the understanding of the role of this pathway in cell migration which emerges from characterisation of cross-talk between components of this pathway and other cellular components (e.g. integrins and signalling mediators) as well as components in the microenvironment (e.g. ECM components such as
vitronectin). Given these biochemical and physical interactions, the overall organisation and functions of the urokinase plasminogen activation pathway is discussed in this section.

1.6.A. LOCALISED PROTEOLYSIS

Localisation studies describe the accumulation of uPAR and pro-uPA/uPA [pro/uPA for abbreviation] at focal adhesion sites (cell-to-substratum) and cell-to-cell contact sites (Pöllänen et al., 1987; Glass et al., 1988; Hébert and Baker, 1988; Takahashi et al., 1990; Del Rosso et al., 1992; Jensen and Wheelock, 1992; Bastholm et al., 1994a; Bastholm et al., 1994b; Reinartz et al., 1995; Sitrin et al., 1996). Integrins also accumulate at these sites and their cytoplasmic domains interact with intracellular filaments (see reviews by Clark and Brugge, 1995). The accumulation of pro/uPA:uPAR at focal-contacts occurs on specific ECM substrates, e.g. vitronectin (Ciambrone and McKeown-Longo, 1990; Nusrat and Chapman, 1991; Waltz and Chapman, 1994; Wei et al., 1994; Wei et al., 1996). In vivo, only uPAR occupied with pro/uPA, but not unbound uPAR, accumulates at these specific sites on the cell surface (Myöhänen et al., 1993). The accumulation of pro/uPA:uPAR complexes at focal adhesion sites (see Figure 11) may be explained by uPAR/vitronectin and/or uPAR/integrin interactions described earlier (Section 1.5.).

During cell migration, uPAR polarises to the leading edge of the cell in vitro (Estreicher et al., 1990; Okada et al., 1995) and in vivo (Del Vecchio et al., 1993). Similarly, uPA also accumulates at the leading edge of cells (Pöllänen et al., 1987). The localisation of uPA/uPAR at focal adhesion sites and leading edges of migrating cells indicate the role of uPAR-bound uPA during phases of cell migration, invasion and tissue remodelling (Danø et al., 1985; Saksela and Rifkin, 1988; Gold et al., 1989). This role is convincingly related to the generation of plasmin on the cell surface since uPA-mediated plasminogen
activation occurs much faster in the presence of cells than their absence, both
\textit{in vitro} (Ellis \textit{et al.}, 1991) and \textit{in vivo} (Quax \textit{et al.}, 1991b). The co-localisation of
plasminogen with uPA at cell surfaces provides a mechanism for accelerated
plasminogen activation by cells (Andronicos and Ranson, 2001). This is because
binding leads to an activation susceptible conformation of glu-plasminogen; an
important requirement for efficient generation of cell-surface plasmin (reviewed
by Ranson and Andronicos, 2003). Plasmin generation on the cell surface is also
dependant on binding of uPA to uPAR, since blocking uPAR/uPA binding
inhibits it (Stephens \textit{et al.}, 1989; Ellis \textit{et al.}, 1991; Rønne \textit{et al.}, 1991) and
accelerated cell-surface plasmin generation is not observed in uPAR-deficient
mice (Bugge \textit{et al.}, 1995). Thus, cell-surface-associated plasmin generated by
uPAR-bound uPA confers plasmin-dependent degradation of ECM (Bergman \textit{et al.}, 1986; Cajot \textit{et al.}, 1989; Schlechte \textit{et al.}, 1989; Cohen \textit{et al.}, 1991; Quax \textit{et al.}, 1991a; Quax \textit{et al.}, 1991b; Reiter \textit{et al.}, 1993). Conclusively, the correlation
between plasmin-dependent proteolysis and uPA/uPAR binding in addition to
the directed distribution of uPAR-bound uPA at focal adhesion sites on the cell
surface indicate the conception of localised pericellular proteolysis at the
invading fronts of migrating cells (see Figure 11).

Plasmin bound to the cell surface is protected from inhibition by its
efficient inhibitory serpin, $\alpha_2$AP (Stephens \textit{et al.}, 1989; Plow and Miles, 1990;
Ellis \textit{et al.}, 1991; Duval-Jobe and Parmely, 1994; Falcone \textit{et al.}, 1994). Thus,
control of plasmin activity on the cell-surface appears to be controlled by the
level of plasmin generation rather than the activity of the generated plasmin.
PAI-1 and PAI-2 can react with uPAR-bound uPA on the cell surface (Cubellis \textit{et al.}, 1989; Pöllänen \textit{et al.}, 1990). Although the reaction on the cell-surface may
be slower than in fluid phase (Ellis \textit{et al.}, 1990), PAI-1 (Cajot \textit{et al.}, 1990;
Shirasuna \textit{et al.}, 1993), PAI-2 (Laug \textit{et al.}, 1993) and PN-1 (Bergman \textit{et al.},
Inhibit plasmin-dependent ECM degradation initiated by uPAR-bound uPA. Figure 11: The urokinase plasminogen activation pathway on the cell surface

Accelerated plasmin generation on the cell-surface requires the co-localisation of receptor-bound plasminogen (plg) and binding of pro/uPA to uPAR. Interactions between uPAR with vitronectin and integrins and the interaction between vitronectin and integrins account for the accumulation of uPA/uPAR complexes at sites of focal adhesion. The accumulation of uPAR-bound pro/uPA at focal adhesion sites also leads to localised plasmin-dependent pericellular proteolysis at these sites. The generation of plasmin on the cell-surface allows the plasmin-mediated functions indicated in the Figure and discussed elsewhere in this review. Plasminogen activator inhibitors (PAIs) inhibit the uPA catalytic activity and thus control cell-surface plasminogen generation. uPAR/uPA:PAIs complexes are internalised by LDLRs. Intracellular signals which are mediated by various signal transduction adaptors may be initiated due to uPA/uPAR binding or during the internalisation of uPAR/uPA:PAIs. Note: Plasmin generated on the cell-surface remains bound to the cell-surface; this is not shown in the Figure for clearer presentation.

As discussed earlier (Section 1.5.C.), serpin/uPA complexes are endocytosed readily after complex formation, thus clearing the cell surface of...
inhibited uPA. Endocytosis of uPAR-bound uPA/serpin complexes is much faster than that of fluid-phase complexes (Olson et al., 1992; Conese et al., 1994). Furthermore, the affinity of $\alpha_2$MR/LRP to uPA:PAI-1 complex is approximately 100-fold higher than the affinities for un-complexed components (Nykkjaer et al., 1994). This indicates preferential clearance of complexes when bound to cell-surface uPAR and suggests another control-point of the urokinase plasminogen activation pathway on the cell surface. However, in the case of PAI-1, both uPAR and the $\alpha_2$MR/LRP are recycled to the cell surface (Nykaer et al., 1997). This recycling in addition to the binding properties of PAI-1 and uPAR to vitronectin, which have not been demonstrated for PAI-2, suggests that PAI-1 may play as a switch molecule in the process of cell migration.

1.6.B. FUNCTIONAL DUALITY

Cell invasion through the ECM (see Section 1.3.) involves interplay of proteolytic and adhesive functions (detachment/attachment) at the leading and posterior ends of the cell. uPAR-bound uPA, which generates plasmin, is localised at focal adhesion sites, polarises to the leading edge of the cell and leads to degradation of the ECM in front of the cell. This proteolytic activity is inhibited when uPAR-bound uPA encounters active PAI-1 embedded on vitronectin. uPA:PAI-1 complexes are endocytosed and uPAR along with the endocytosis receptor are recycled back to the cell surface where uPAR may be able to bind to integrins and/or PAI-1-free vitronectin. Alternatively, the interaction of PAI-1 with vitronectin may also suppress integrin/vitronectin-mediated adhesion independent of its serpin activity (Kjoller et al., 1997), thus modulating cellular adhesion. In the case of internalisation of uPA:PAI-1 complex, binding of uPA to recycled uPAR may initiate another round of proteolysis and result in further ECM degradation and decrease in attachment.
This scenario implicates proteolytic/adhesive interplay through multiple interactions between uPA, uPAR, vitronectin, integrin and PAI-1. Similar proteolytic activity followed by detachment/attachment events at the posterior end of the cell may collectively lead to the migration and invasion of the cell through the ECM.

Recent evidence indicates that PAI-1 can activate signalling systems and stimulate cell migration during chemotaxis, chemokinesis and wound healing assays (Degryse et al., 2004). These effects of PAI-1 are blocked when α2MR/LRP is blocked by antibodies or antagonist, suggesting that α2MR/LRP mediates the signalling activation and affects of PAI-1 in these assays (Degryse et al., 2004). These findings along with other growing evidence (Deng et al., 1996; Chapman, 1997; Brooks et al., 2000), suggest that uPA and PAI-1 co-operate in the migratory process by facilitating attachment to and subsequent detachment from vitronectin in the extracellular matrix. Association of uPAR with integrins/vitronectin also links uPAR with intracellular signalling/cytoskeletal elements (Porter and Hogg, 1998; Chen et al., 2001; Kjøller and Hall, 2001) and facilitates cell migration and invasion (Yebra et al., 1996; Carriero et al., 1999; Yebra et al., 1999; Khatib et al., 2001; Prifti et al., 2002). Furthermore, these interactions even modulate the expression of uPAR/uPA/PAI-1 (Hapke et al., 2001a; Hapke et al., 2001b) and plasminogen receptors (Kim et al., 1996). This dynamic organisation and interplay/cross-talk of plasminogen activation pathway components and other microenvironmental factors need intracellular signalling to orchestrate the organisation of the events (see Section 1.5.D.). As discussed in the previous section (Section 1.5.), signalling and other non-proteolytic interactions of the urokinase plasminogen activation pathway provide further evidence for the critical involvement of this pathway in tumour cell invasion and metastasis.
1.7. **UROKINASE PLASMINOGEN ACTIVATION PATHWAY: REGULATION AND CANCER**

Plasmin generation on the cell surface requires the presence of uPAR, uPA, plasminogen and plasminogen receptors. PAI-1 and PAI-2 are required for controlling uPA-mediated plasminogen activation. Thus, the first level of control of this pathway is the synthesis of these components, which can be regulated by a wide variety of factors including hormones, phorbol esters, growth factors, retinoids and cytokines (reviewed by Danø *et al.*, 1985; Saksela and Rifkin, 1988; Andreasen *et al.*, 1990; Blasi *et al.*, 1990; Besser *et al.*, 1996). The control of expression of these components occurs at both transcriptional and posttranscriptional levels (Vassalli *et al.*, 1991). In brief, under normal physiological conditions, complex regulation of plasminogen activators and PAIs synthesis allows this system to be precisely controlled in time and space and in a manner that depends upon hormones and growth factor balance and other microenvironmental conditions.

In cancer, the precise regulation of the plasminogen activation system appears to be lost or modified. Early studies provided evidence for the particular involvement of uPA in the invasive and metastatic properties of malignant tumours. These include the close correlation between oncogene transformation and high increase in uPA synthesis (Unkeless *et al.*, 1973; Unkeless *et al.*, 1974), the immunohistochemical localisation of uPA in invading areas of tumours (Skriver *et al.*, 1984; Grøndahl-Hansen *et al.*, 1991) and the inhibition of cellular invasion and metastasis by anti-catalytic antibodies to uPA in model systems (Ossowski and Reich, 1983; Mignatti *et al.*, 1986; Hearing *et al.*, 1988; Ossowski, 1988; Reich *et al.*, 1988). During the last two decades, an overwhelming number of reports implicate the important role of the plasminogen activation pathway in cancer invasion and metastasis *in vitro* and
in vivo. In the space of this thesis, it is impossible to review all the available data to date which describe the effect of the different components of this pathway on cancer invasion and metastasis (for detailed reviews refer to Duffy et al., 1999; Murphy and Gavrilovic, 1999; Andreasen et al., 2000; Dunbar et al., 2000; Choong and Nadesapillai, 2003; Ranson and Andronicos, 2003; Reuning et al., 2003a; Reuning et al., 2003b; Rosenberg, 2003; Sidenius and Blasi, 2003). In summary, in vitro evidence for the role of urokinase plasminogen activation pathway in cancer metastasis and invasion include: (1) correlation between metastatic and invasive potential of cancer cells and the expression of uPA, uPAR and plasminogen binding; (2) retardation of cancer cell migration and invasion by inhibiting the enzymatic activity of uPA or binding of uPA to uPAR; (3) increased migration and invasion when cells are transfected with uPAR or uPA cDNAs whereas migration decreased when uPAR- or uPA-overexpressing cancer cells are transfected with anti-sense cDNAs and (4) inhibition of invasion and metastasis when invasive cancer cells are treated with PAI-2 or transfected with PAI-2 cDNA. In vivo evidence for the role of this pathway in cancer invasion and metastasis include: (1) correlation between increased uPAR, uPA and PAI-1 expression and plasmin generation with cancer metastasis in animal models; (2) inhibition of metastatic cancers in animal models when treated with anti-uPA antibodies or when pre-transfected with uPA anti-sense oligonucleotides; (3) decreased metastasis in animal models treated with uPA/uPAR binding antagonists and (4) suppression of tumour growth and invasion in animal models inoculated with PAI-2 or with invasive cancer cells transfected with PAI-2 cDNA.

The established role of the urokinase plasminogen activation pathway in cancer metastasis and invasion initiated the interest in evaluating its clinical significance in cancer patients. Early studies established that the level of uPA in
malignant tumours is significantly higher than that in the corresponding normal or benign tumours of the same tissue (for reviews see Duffy, 1993a; Duffy, 1996). A considerable number of studies have since evaluated the prognostic significance of uPA, uPAR, PAI-1 and PAI-2 in a wide variety of human cancers. Table I summarises the outcome of such studies undertaken between 1999 to date. For extensive reviews of studies prior to 1999 concerning this topic refer to Andreasen et al. (1997), Schmitt et al. (1997), Brunner et al. (1999), Duffy et al. (1999), Look and Foekens (1999) and Mazar et al. (1999).

uPA and/or PAI-1, as evident in earlier reviews and Table I, appear to be the most common and best clinical and prognostic factors of the urokinase plasminogen activation pathways in many cancers including breast, prostate and colorectal cancers. In fact, the prognostic value of uPA/PAI-1 in axillary node-negative breast cancer patients was recently validated through randomised controlled trials (RCTs). RCTs ensure low risk of incorporating false-positive or false-negative results, thus uPA/PAI-1 were justified as strong prognostic markers in breast cancer patients by Level 1 of evidence (LOE-1 which is highest level of evidence) (Duffy, 2004). Assay of uPA and PAI-1 may therefore help identify high risk node-negative breast cancer patients who would benefit from adjuvant chemotherapy (Duffy, 2004).
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<tr>
<th>Antigen</th>
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<td></td>
<td>Multiple myeloma</td>
<td>Marker of deeper myometrial invasion (Gerstein et al., 2003) (EI)</td>
<td></td>
<td>Soluble uPAR in plasma predicts extra-bone marrow involvement and poor prognosis (Rigolin et al., 2003) (IF)</td>
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<td>Bladder cancer</td>
<td>Urinary uPA has prognostic ability (Shariat et al., 2003a) (ELISA and EI)</td>
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<td>uPAR has prognostic ability (Shariat et al., 2003a) (ELISA and EI)</td>
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<td>Pancreatic cancer</td>
<td>Potential prognostic indicator (Harvey et al., 2003) (IHC and ISH)</td>
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<td>uPAR overexpression correlates with poorer survival (Harvey et al., 2003) (IHC and ISH)</td>
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<td>Prostate cancer</td>
<td>Serum levels alone or with uPAR could be predictor of progression and prognosis (Miyake et al., 1999a; Miyake et al., 1999b) (ELISA)</td>
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<td>Serum levels alone or with uPA could be predictor of progression and prognosis (Miyake et al., 1999a; Miyake et al., 1999b) (ELISA)</td>
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<td>Serum levels of soluble uPAR is a possible diagnostic marker for early detection (McCabe et al., 2000) (ELISA)</td>
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EI: enzyme immunoassays; IFA: Immunofluorescent assays (e.g. flow cytometry); ELISA: enzyme-linked immunosorbent assay; IHC: immunohistochemistry; ISH: in situ hybridization; EA: enzymatic assay; NB: northern blots; RT-PCR: reverse transcription-PCR; IRA: Immuonoradioactivity assay; VA: Various Assays (generally a review article)
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<tr>
<td>Ovarian cancer</td>
<td>● Strong prognostic relevance (Konecny et al., 2001) (ELISA)</td>
<td>● Plasma levels correlate with presence of malignancy and higher stage of disease (Ho et al., 1999) (ELISA)</td>
<td>● Statistically independent strong prognostic factor (Kuhn et al., 1999) (ELISA)</td>
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<td>● Predictor for overall survival (Tecimer et al., 2000) (ELISA)</td>
<td>● Strong prognostic relevance (Konecny et al., 2001) (ELISA)</td>
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<td>Melanoma</td>
<td>● Provides significant additional prognostic information (Stabuc et al., 2003) (ELISA)</td>
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<td>Gastric cancer</td>
<td>● uPA enzymatic activity is a prognostic factor (Okusa et al., 1999) (EA and IHC)</td>
<td>● Correlated with tumour size, depth, lymph node involvement, differentiation and vascular invasion (Kaneko et al., 2003) (IHC)</td>
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<td></td>
<td>● Associated with a poor outcome (Kaneko et al., 2003) (IHC)</td>
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<td>Colon cancer</td>
<td>● Increase in tumour and serum levels is associated with a worse prognosis (Berger, 2002) (VA)</td>
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</table>
| Colorectal cancer           | • Combined analysis of uPA and PAI-1 is a useful prognostic factor (Fuji et al., 1999) *(IHC and RT-PCR)*  
• Major prognostic impact (Herszenyi et al., 1999) *(ELISA)*  
• Independent prognostic factors (Yang et al., 2000) *(IHC)*  
• Marker of late stages of this cancer (Papadopoulou et al., 2002) *(ELISA and IHC)* | • PAI-1 level over uPAR or uPAR-bound uPA associate with tumour progression (Abe et al., 1999) *(ELISA)*  
• Combined analysis of uPA and PAI-1 is a useful prognostic factor (Fuji et al., 1999) *(IHC and RT-PCR)*  
• Major prognostic impact (Herszenyi et al., 1999) *(ELISA)*  
• Marker of later stages (Papadopoulou et al., 2002) *(ELISA and IHC)*  
• PAI-1 genotype may be a useful prognostic marker (Loktionov et al., 2003) *(Genotyping)* | • Higher expression related to poor prognosis (Abe et al., 1999) *(ELISA)*  
• Plasma level of soluble uPAR independently predict survival (Stephens et al., 1999) *(ELISA)*  
• Independent prognostic factors (Yang et al., 2000) *(IHC)*  
• Soluble uPAR has an independent prognostic information in rectal cancer patients and high values associate with shorter survival (Fernebro et al., 2001) *(ELISA)* | | | |
| Endometrial cancer          | • Elevated levels correlate with unfavourable prognosis (Taponeco et al., 2001; Tecimer et al., 2001) *(ELISA and IHC)*  
• High level correlates with unfavourable prognosis (Gerstein et al., 2003) *(EI)* | • Elevated levels appear correlated with unfavourable prognosis (Taponeco et al., 2001; Tecimer et al., 2001) *(ELISA and IHC)* | • Useful prognostic marker for biologically aggressive forms (Memarzadeh et al., 2002) *(IHC)* | | • High tumour tissue concentration independent marker for shorter progression-free survival (Nordengren et al., 2002) *(ELISA)* |
| Squamous cell carcinoma     | • Predictive of poor survival (Shiomi et al., 2000) *(IHC and ISH)*  
• Tumour and serum levels potentially predict survival probability (Strojan et al., 2000) *(ELISA and IRA)* | • Parameter for prediction of prognosis (Sakakibara et al., 2004) *(RT-PCR)* | • Elevated levels of soluble uPAR in plasma, but not significantly correlated to metastasis or recurrence (Schmidt and Hoppe, 1999) *(ELISA)* | | • Expression of PAI-2 in fibroblasts surrounding these carcinomas is protective (Shiomi et al., 2000) *(IHC and ISH)* |

EI: enzyme immunoassays; IFA: Immunofluorescent assays (e.g. flow cytometry); ELISA: enzyme-linked immunosorbent assay; IHC: immunohistochemistry; ISH: in situ hybridization; EA: enzymatic assay; NB: northern blots; RT-PCR: reverse transcription-PCR; IRA: Immunoradioactivity assay; VA: Various Assays (generally a review article)
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<tr>
<td>Breast cancer</td>
<td>● Prognostic role (de Witte et al., 1999a; de Witte et al., 1999b) <em>(ELISA)</em>&lt;br&gt;● Independent predictive factors of poor overall survival and relapse-free survival (Foekens et al., 2000) <em>(ELISA)</em>&lt;br&gt;● Clinical relevance and prognosis is best when combined with PAI-1 (Harbeck et al., 2002a; Harbeck et al., 2002b) <em>(ELISA)</em>&lt;br&gt;● High cytosolic levels correlate significantly with shorter disease-free survival (Levicar et al., 2002) <em>(ELISA)</em>&lt;br&gt;● Independent prognostic value in primary breast cancer (Look et al., 2002; Luqmani et al., 2002) <em>(VA)</em>&lt;br&gt;● Cytosolic levels at diagnosis may be predictive of early relapse in primary cancer (Dazzi et al., 2003) <em>(ELISA)</em>&lt;br&gt;● Prognostic marker validated by level-1-evidence for node-negative patients (Qin et al., 2003a; Thomssen et al., 2003; Duffy, 2004) <em>(VA and ELISA for Qin et al.)</em></td>
<td>● Prognostic role (de Witte et al., 1999a; de Witte et al., 1999b) <em>(ELISA)</em>&lt;br&gt;● Tumour-associated levels as independent prognostic information in a follow-up period of ~10 years (Harbeck et al., 1999a; Harbeck et al., 1999b; Harbeck et al., 2000) <em>(ELISA)</em>&lt;br&gt;● Independent predictive factors of a poor overall survival and relapse-free survival (Foekens et al., 2000) <em>(ELISA)</em>&lt;br&gt;● Patients with low levels of PAI-1 have better disease-free survival (Borstnar et al., 2002) <em>(ELISA)</em>&lt;br&gt;● Clinical relevance and prognosis is best when combined with uPA (Harbeck et al., 2002a; Harbeck et al., 2002b) <em>(ELISA)</em>&lt;br&gt;● High cytosolic levels correlate significantly with shorter disease-free survival (Levicar et al., 2002) <em>(ELISA)</em>&lt;br&gt;● Independent prognostic value in primary cancer (Cufer et al., 2002; Look et al., 2002; Luqmani et al., 2002) <em>(ELISA)</em>&lt;br&gt;● Independent prognostic marker for recurrence-free survival in primary cancer (Hansen et al., 2003) <em>(IHC and ELISA)</em>&lt;br&gt;● Prognostic marker validated by level-1-evidence for axillary node-negative patients (Qin et al., 2003a; Qin et al., 2003b; Thomssen et al., 2003; Duffy, 2004) <em>(VA and ELISA for Qin et al.)</em></td>
<td>● Elevated levels of cytosolic uPAR is an independent predictors of poor overall survival and relapse-free survival (de Witte et al., 2001) <em>(ELISA)</em>&lt;br&gt;● Increased uPAR mRNA in primary cancer may be a predictor of poor overall survival (Pacheco et al., 2001) <em>(NB)</em>&lt;br&gt;● High level of age-dependent soluble uPAR in serum independently associate with poor outcome (Riisbro et al., 2002) <em>(ELISA)</em>&lt;br&gt;● uPAR provides useful information for cancer diagnosis and prognosis (Qin et al., 2003a) <em>(ELISA)</em></td>
<td>● Intermediate and low levels show poor overall survival and relapse-free survival (Foekens et al., 2000) <em>(ELISA)</em>&lt;br&gt;● Patients with high levels of PAI-2 have better disease-free survival (Borstnar et al., 2002) <em>(ELISA)</em></td>
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EI: enzyme immunoassays; IFA: Immunofluorescent assays (e.g. flow cytometry); ELISA: enzyme-linked immunosorbent assay; IHC: immunohistochemistry; ISH: in situ hybridization; EA: enzymatic assay; NB: northern blots; RT-PCR: reverse transcription-PCR; IRA: Immunoautoradioactivity assay; VA: Various Assays (generally a review article)
1.8. UROKINASE PLASMINOGEN ACTIVATION PATHWAY AND CANCER THERAPY

The identification of strong tumour cell markers, especially to identify metastatic cells, is one of the main directives of cancer research. Since the leading cause of cancer-related mortality is the inability to control the spread of malignant tumours (Woodhouse et al., 1997), characterisation of strong tumour/malignancy markers is vital for therapeutic targeting purposes. It is beyond the scope of this thesis to review the efforts and the success of studies endeavouring to identify such markers (see review by Dubowchik and Walker, 1999). As discussed in the previous section (Section 1.7.), uPA appears be a strong marker of cancer progression and metastasis and is currently validated by LEO-1 as a clinical and prognostic marker in breast cancer (Duffy, 2004) and may be validated for other human cancers in the near future. Modulation of uPA expression and the use of antagonist of the uPA/uPAR binding have shown success in regulating cancer metastasis and invasion (Crowley et al., 1993; Achbarou et al., 1994; Wilhelm et al., 1995; Min et al., 1996; Shapiro et al., 1996). The therapeutic potential of modulating gene expression in humans is limited due to the requirement of cancer cell-specific gene delivery methods. Thus, antagonists of the uPA/uPAR binding or uPA inhibitors, natural (e.g. PAI-2) or engineered (see review by Rockway et al., 2002), seem more feasible for clinical applications. Although such antagonists/inhibitors may be administered to inhibit tumour growth, migration or differentiation, tactics such as cytotoxins or toxic radiation for the killing and clearance of tumour cells present more valuable curative advantages.

Several biological and chemical agents with different cytotoxic mechanisms are readily available for the targeted or non-targeted killing of cells. A particular targeted uPA-based anti-cancer strategy involves the production of
uPA-toxin conjugates. Examples of such successful strategies include uPA-
*Pseudomonas* exotoxin (PE) (Rajagopal and Kreitman, 2000), ATF-saporin (plant
ribosome-inactivating protein) (Cavallaro *et al.*, 1993; Fabbrini *et al.*, 1997;
Fabbrini *et al.*, 2000), ATF-*Diphtheria* toxin (DT) (Vallera *et al.*, 2002;
Rustamzadeh *et al.*, 2003) and PAI-1-(A-chain)*Cholera* toxin (Jankun, 1992)
conjugates. Another example of uPA-based therapy is a recombinant fusion
protein consisting of anthrax toxin lethal factor and *Pseudomonas* exotoxin
which is engineered to be only cleaved and internalised by uPA-overexpressing

Alternatively, new hopes for cancer therapy arise from recent
characterisation of radionuclides with favourable radiophysical states which are
efficient for cytotoxic targeting. γ-emitting radionuclides are widely used for
radioimaging; however, γ rays do not contribute significantly to therapeutic
efficacy (Chatal and Hoefnagel, 1999). Isotopes emitting β particles are more
often used for therapy (e.g. 131Iodine, 90Yttrium, 186Rhenium, and 67Copper) and
are characterised by low linear energy transfer released over a relatively long
distance, resulting in inefficient local killing of target cells coupled with toxicity
to distant normal tissues (Waldmann, 1991; Bender *et al.*, 1992; O’Donoghue *et
al.*, 1995; Griffiths *et al.*, 1999). On the other hand, α-emitting radionuclides
release high-energy emissions (6 - 9 Mega electron volts [MeV]; ten times as
great as β- or γ-emitters) over a short distance (40 to 80 µm) (Waldmann, 1991;
Behr *et al.*, 1998; Behr *et al.*, 1999; Chatal and Hoefnagel, 1999; Griffiths *et al*.,
1999). Thus, using α-emitters, much greater fraction of the total energy is
deposited in the targeted cells and very few nuclear hits are required to kill
these cells (Lloyd *et al.*, 1979; Kassis *et al.*, 1986). Monoclonal antibodies
conjugated to metal chelators, such as cDTPA or its derivates, and labelled with
α–emitting radionuclides (e.g. 213Bismuth and 211Astatine) are starting to show
promise in radio-immunotherapy. In preclinical studies, McDevitt et al. (2000) and Nikula et al. (1999) have proven the efficacy of $^{213}$Bismuth-labelled monoclonal antibodies for radio-immunotherapy of prostate cancer and leukaemia and are conducting a phase I trial for end stage leukaemia (Jurcic et al., 2002). $^{213}$Bismuth-labelled antibody has been validated for radio-immunotherapy of multiple myeloma (bone marrow cancer) (Couturier et al., 1999). $^{211}$Astatine-labelled antibodies have been used clinically in a phase I trial to treat malignant gliomas in humans (Zalutsky and Vaidyanathan, 2000).

There are many other examples of the use of DTPA or its derivatives for chelating metals to agents used for imaging or targeting α-radiation to tumours (targeted alpha therapy, TAT) (Merlo et al., 1997; Boerman et al., 1999; Gruaz-Guyon et al., 2001; Gulec et al., 2001; McDevitt et al., 2001; Wen et al., 2001).

1.9. RATIONALE AND OBJECTIVES

Given the functional duality (proteolytic and non-proteolytic functions) of the urokinase plasminogen activation pathway, it is not surprising that this pathway is critical in processes of cancer invasion and metastasis. Growing evidence suggest that uPA and PAI-1 co-operate in the migratory process by facilitating attachment to and subsequent detachment from vitronectin in the extracellular matrix (Deng et al., 1996; Chapman, 1997; Brooks et al., 2000; Degryse et al., 2004). In agreement, high levels of uPA and/or PAI-1 have shown a prognostic significance in many types of cancers (see Section 1.7.). This invites the clinical use of uPA as a target for therapeutic strategies against cancer. The low level of PAI-2 in metastatic cancers together with the good prognosis in cancer patients with high PAI-2 expression indicate the possible use of PAI-2 for the development of uPA-based targeted therapies. Exogenous PAI-2 (either as a free molecule or coupled to radionuclides) localises within
uPA-expressing colon carcinoma xenografts (Hang et al., 1998) and significantly reduces tumour growth and metastases with no evidence of toxicity (Shinkfield et al., 1992; Åstedt et al., 1995; Evans and Lin, 1995; Li et al., 2002; Allen et al., 2003). Thus, despite the presence of PAI-1 in malignant tumours, it is apparent that uPA is accessible to exogenously administered PAI-2 within several tumour types in vivo. PAI-2 can specifically access and inhibit uPA while sparing thrombosis as suggested by the low second order inhibition rate constant towards tPA and that fibrin-bound tPA is protected from PAI-2 inhibition (Kruithof et al., 1995). Furthermore, unlike PAI-1 which is subject to oxidation (Baker et al., 1990b), stable cytotoxin-PAI-2 conjugates may be easily achieved in vitro (Ranson et al., 2002).

The primary aim of this thesis was to provide further biological rationale for the preliminary success of the anti-uPA targeted therapy using PAI-2 linked to $^{213}$Bismuth reported by Li et al. (2002), Ranson et al. (2002) and Allen et al. (2003). This involved the direct in vitro determination of the inhibition kinetics of cell-surface uPA by PAI-2 on invasive and non-invasive breast cancer cells. The controversial fate of uPAR-bound uPA:PAI-2 complexes was also addressed as a part of these studies. An additional aim was to modulate uPAR-expression in breast cancer cells to assess the role of uPAR/uPA in the acquisition of metastatic phenotype and extend the analyses of PAI-2 binding to cells. Lastly, this thesis also aimed to optimising the protocol used for the preparation of PAI-2-$^{213}$Bismuth ($\alpha$-PAI-2). This involved the development of standard operating procedures for the conjugation of PAI-2 to cDTPA and radiolabelling of the conjugate with $^{213}$Bismuth for radiotherapy.
Chapter 2: MATERIALS AND GENERAL CELL AND PROTEIN ANALYSIS METHODS

2.1. MATERIALS

Lipofectamine™ 2000, pcDNA3 plasmid, RPMI-1640 powder and T7-promoter primer were purchased from Invitrogen® Life Technologies, NSW, Australia. Foetal calf serum (FCS) was obtained from ThermoTrace®, VIC, Australia. Acetonitrile (100 %), ammonium persulfate, ammonium bicarbonate (NH₄HCO₃), ampicillin, bovine serum albumin (BSA fraction V), β-mercaptoethanol, Brilliant Blue R250, bromophenol blue, chloroform, dimethyl sulfoxide (DMSO), ethidium bromide, ethyldiaminetriacetic acid (EDTA), Hank’s and phenol red-free Hank’s balanced salt powders, HEPES, hydroiodic acid (HI), insulin, lysozyme, nitric acid (HNO₃), paraformaldehyde, pH strips (pH range 0 - 14), phenol: chloroform, potassium phosphate (KH₂PO₄), propidium iodide, rabbit anti mouse IgG monoclonal antibody (IgG) conjugated to Cy3 or FITC, sodium monophosphate (Na₂HPO₄), TEMED, tranexamic acid (TA), Tris-HCl, trypsin-EDTA, urea and xylene cyanol were purchased from Sigma-Aldrich®, MO, USA. The colorimetric substrate for uPA Spectrozyme®-UK and mouse monoclonal antibodies raised against human A-chain uPA (#3921), B-chain uPA (#394), PAI-2 and uPAR (#3934) were all obtained from American Diagnostica Inc. (ADI), CT, USA. Another mouse monoclonal antibody raised against human uPAR (MAB807) was purchased from R&D Systems Inc., MN, USA. Rabbit anti-human uPAR polyclonal antibody was provided by David Croucher (School of Biological Sciences, University of Wollongong, Australia). Mouse
isotype control subclass IgG1 antibody was from Silenus, Sydney, NSW, Australia. Boric acid, calcium chloride (CaCl₂), gelatine powder, glucose, glycine, magnesium chloride (MgCl₂), magnesium sulphate (MgSO₄), potassium acetate, potassium chloride (KCl), sodium acetate, sodium chloride (NaCl) and sodium dodecyl sulphate (SDS) were obtained from BDH Chemicals Australia Pty. Ltd., VIC, Australia. Absolute alcohol, citric acid, isopropanol, glacial acetic acid, hydrochloric acid (HCl), methanol and sodium hydroxide tablets (NaOH) were purchased from Ajax Chemicals, NSW, Australia. Agar, bactotryptone, trypton and yeast extract were obtained from Oxoid Australia Pty Ltd, VIC, Australia. Hind III, Not I, Pst I and Xho I restriction enzymes and their compatible 10 x buffers were purchased from Roche Applied Science, IN, USA. The Cy5 mono-reactive dye pack, PD-10 columns (Sephadex G-25 columns), T4 DNA ligase and 10 x ligation buffer were from Amersham Biosciences, Uppsala, Sweden. Qiagen® plasmid midi-prep kit and Superfect® transfection reagent were purchased from Qiagen Pty Ltd, VIC, Australia. BigDye® terminator mix v2.0 was obtained from Applied Biosystems, CA, USA and PAGE-PLUS™ purchased from Amresco Inc., OH, USA. AG® 501-X8 resin, BioRad® DC protein concentration assay kit, broad range unstained and prestained markers and precision plus unstained marker were from BioRad Laboratories Inc., CA, USA. Prestained protein ladder marker (10 – 160 kDa) was obtained from Fermentas Inc., MD, USA. Agarose, glycerol and polyacrylamide were purchased from Progen Industries Limited, QLD, Australia. Human recombinant PAI-2 (molecular weight 47 kDa) and Actisolv uPA were provided by PAI-2 Pty Ltd, Sydney, Australia. Iodo-beads were purchased from Pierce Chem. Co., IL, USA. Iodine-125 (¹²⁵I) was obtained from PerkinElmer™, MA, USA. Actinium-225 (²²⁵Ac) and prepacked columns containing resin (AG® 50W-X4) were purchased from the Oak Ridge National Laboratory, United States Department of Energy,
USA. Whatman #1 filter paper was purchased from Whatman International Ltd., Kent, UK. Cyclic anhydride diethylenetriaminepentacetic acid (cDTPA) was purchased from MP Biomedicals Inc., formerly ICN Biomedicals, CA, USA. Glu-Plasminogen was purified from human plasma and conjugated to FITC and Cy5 as described earlier (Andronicos et al., 1997). uPA and the goat anti-human uPA polyclonal antibody were obtained from Chemicon® International, CA, USA. LysoTracker® Yellow DND-68 and transferrin-Alexa488 were purchased from Molecular Probes, Inc., OR, USA. Lambda (λ) DNA and the CellTitre 96® AQueous Non-Radioactive Cell Proliferation Assay were obtained from Promega® Corporation, WI, USA. Instant thin layer chromatography (ITLC-SG) strips were purchased from Gelman Science, MI, USA. The 0.22 µm sterile filter units and the 30 kDa cut-off microconcentrators were obtained from Millipore, MA, USA. Gamma (γ) counter tubes were purchased from Sarstedt®, NSW, Australia. AtomLab 200 dose calibrator was purchased from Pinestar Technology Inc., PA, USA.

2.2. GENERAL METHODS†

2.2.A. TISSUE CULTURE AND ANALYSIS

2.2.A.1. CELL LINES

The human breast cancer cell lines - MDA-MB-231, T-47D and MCF-7 (American Type Culture Collection, ATCC®, VA, USA) - were routinely cultured at 37°C in a Heracell incubator (Kendro® Laboratory Products, Langenselbold, Germany) in atmospheric air containing humidified 5 % CO₂. Cells were grown in culture media [10.4 g/l of RPMI-1640 and 2 g/l sodium bicarbonate; solution was adjusted to pH 7.2 and filter-sterilised using 0.22 µm sterile filters units].

† All buffers and solutions in this thesis are described in alphabetical order in Appendix 4 (Section 7.4.)
Culture media was supplemented with 5 % (v/v) foetal calf serum (FCS) and T-47D cells were also supplemented with 0.26 units/ml insulin. Cells were routinely passaged at confluency using Trypsin-EDTA. The MDA-MB-231 cell line has been shown previously to display a metastatic phenotype (high uPA, uPAR and plasminogen binding and activation capacity) and metastasise in nude mouse models (Andronicos et al., 1997; Ranson et al., 1998; Li et al., 2002). The T-47D and MCF-7 cells display a non-metastatic phenotype (low uPA, uPAR, plasminogen binding and activation capacity) (Ranson et al., 1998).

All cell experiments were performed using cells cultured for 48 – 72 h without a change of media. Cells were detached using sterile 1 x phosphate buffered saline (PBS) containing 5 mM EDTA [PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na₂HPO₄, 0.24 g/l KH₂PO₄; pH adjusted to 7.4; then EDTA was added at 5 mM final concentration]. Detached cells were centrifuged at 652 x g for 3 min at 4°C and cell pellets were washed and resuspended at 1 x 10⁶ cells/ml in ice-cold binding buffer [Hank’s buffered saline solution: 9.5 g/l Hank’s powder, 1 mM HEPES and 0.1 % w/v bovine serum albumin (BSA); 1 mM CaCl₂ and 1 mM MgCl₂; pH 7.4]. Cell and viability were assessed by trypan blue exclusion and visualisation in hemocytometer. Unless specified otherwise, all surface pro-uPA was activated by incubation of cells at 1 x 10⁶ cells/ml for 5 min at room temperature (RT) with 5 µg/ml plasminogen solution in RPMI-1640, washed then resuspended at 1 x 10⁶ cells/ml in binding buffer for further experimentation.

**2.2.A.2. FLOW CYTOMETRY**

Cell-surface antigens were detected by indirect immunofluorescence staining as previously described (Ranson et al., 1998). The concentration and the specificity of the primary and secondary antibodies used are specified in the Figure legends. Plasminogen binding was detected directly using 50 µg/ml
plasminogen-FITC conjugate in the absence or presence of 1 mM tranexamic acid (TA, a lysine analogue) as described by Ranson et al. (1998). Plasminogen was purified from human plasma and conjugated with FITC as described by Andronicos et al. (1997). After the direct and indirect staining, cells were washed and resuspended in PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ and the vital fluorescent stain propidium iodide (PI) at 5 µg/ml. Flow data was acquired using the Becton-Dickinson FACSCalibur™ flow cytometry system (BD Biosciences, CA, USA) and the instrument calibrated with reference settings for each cell line. Cell-associated fluorescence was measured by dual-colour flow cytometry which allows a differentiation between cell-surface and total fluorescence (Ranson et al., 1998). Briefly, PI positive cells (non-viable) were excluded from the remaining cells which were intact (PI negative) and hence indicate cell-surface detection. Flow cytometry data was analysed using WinMDI v2.8 (Scripps Research Institute, CA, USA) and only viable cell-associated fluorescence are reported.

2.2.A.3. CONFOCAL MICROSCOPY

Confocal microscopy was used to visualise the distribution and localisation of cell-surface and internalised antigens. For assays on attached cells, these cells were cultured for 48 – 72 h on sterile glass-slide cover slips, washed and blocked for 1 h at room temperature with phenol red-free binding buffer [prepared as for normal binding buffer however using phenol red-free Hank’s powder]. Cells were gently washed using ice cold PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ before staining. For assays with suspension cells, 48 h-cultures were detached with EDTA and washed with ice cold PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ before staining. Staining assays were performed using antibodies or fluorochrome-conjugated antigens which are specified for each assay. Generally, in staining assays involving plasminogen binding, cells
were first incubated with 50 µg/ml plasminogen-Cy5 [prepared as described by Andronicos and Ranson (2001)] for 1 h on ice in the absence or presence of 5 mM TA. Cells were then washed 3 x 5 min incubation in ice-cold phenol red-free binding buffer. For double staining assays, cells were then incubated for 30 min on ice in 20 µg/ml of primary antibody, washed three times as described earlier and then incubated for 30 min on ice in 1:50 dilution of secondary antibody (anti-immunoglobulin antibodies conjugated to FITC or Cy3) in phenol red-free binding buffer. Cells were washed and cover slips were air-dried and mounted on glass slide using mounting buffer [1 x PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ and 10 % glycerol]. Cells were not fixed prior staining as earlier assays found cell-fixing (using paraformaldehyde) resulted in disruption of cell-surface and reduced cell viability (data not shown), thus slides were analysed immediately.

All confocal laser scanning microscopy images were acquired using a Leica TCS SP system and the UV 63x1.32 NA oil PLAPO immersion objective lens (Leica, Heidelberg, Germany). FITC and Cy3 fluorochromes were excited using the 488 nm and the 583 nm spectral lines of the Argon ion laser, respectively. Cy5 fluorochrome was excited with the 633 nm spectral line of the Helium-Neon laser. The emissions of Cy3, FITC and Cy5 were collected at 525-540 nm, 545-560 nm, and greater than 650 nm, respectively. Furthermore, transferrin-Alexa488 and LysoTracker® Yellow DND-68 were excited using the 488 nm spectral line of the Argon ion laser and their emissions were collected at 510-530 and 545-560 nm, respectively. The signal from each of the fluorochromes used did not leak into the channels used for detection of the other fluorochromes. All confocal images were analysed and manipulated using the softwares Volocity (version 2.0.1 Build 85, Improvision Ltd., MA, USA) and
2.2.A.4. CELL PROLIFERATION/CYTOTOXICITY ASSAYS

Cell proliferation was determined using the colorimetric CellTitre 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega® Corporation, WI, USA). This assay is based on the conversion of the substrate 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS) to a coloured product by metabolically active cells. The effect of various agents on cell proliferation was also determined using this assay since cytotoxic or proliferative agents result in modulation of metabolism.

In order to establish the relationship between cell number and absorbance of the product produced from the substrate of this assay, increasing number of cells was transferred in triplicate to 96-wells plate in a final 100 µl of phenol red-free binding buffer containing 5 % (v/v) FCS. The assay substrate, MTS/PMS (20 µl), was added to cell solutions in addition to control triplicate wells containing 100 µl of phenol red-free binding buffer only. The plate was incubated at 37°C in the humidified 5 % CO₂ incubator for 3 h and then the absorbance at 490 nm was measured using the Spectramax® 250 UV plate reader (Molecular Devices Corporation, CA, USA). A standard curve for the relationship between cell number and absorbance was constructed after blanking all values to the absorbance reading of control wells. Further cell proliferation assays were performed using the number of cells which falls in the linear range of the correlation between cell number and the absorbance of the product from the breakdown of the MTS/PMS substrate.
2.2.B. PROTEIN ANALYSIS METHODS

2.2.B.1. PROTEIN CONCENTRATION ASSAY

The BioRad® DC protein concentration assay kit was used to determine the concentration of protein samples against standard BSA solutions. Aliquots (5 µl) of standard BSA solutions (0 - 1 mg/ml in PBS) and protein samples were added in duplicates to 96-wells plate. For each well, 25 µl of Reagent A from the kit was added and then two 100 µl volumes of Reagent B were mixed. The plate was stored on ice for 5 min for maximal colour development and the absorbance of all wells was measures at 750 nm using the Spectramax® 250 plate reader. The concentrations of protein samples were interpolated using the curve constructed from the standard BSA solutions and their absorbencies.

2.2.B.2. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

PAGE, unless otherwise specified, was performed using gels composed of 4 % stacking gel and 12 % resolving gels as per Laemmli (1970). Briefly, the resolving gel [12 % v/v of liquid 40 % acrylamide, 0.1 % w/v sodium dodecyl sulphate (SDS), 375 mM Tris-HCl pH 8.8, 0.1 % v/v TEMED and 0.05 % w/v ammonium persulphate (APS)] was poured and set. Stacking gel [4 % v/v of liquid 40 % acrylamide; 126 mM Tris-HCl pH 6.8; 0.1 % w/v SDS; 0.1 % v/v TEMED and 0.05 % w/v APS] was poured on top of stacking gel and a comb was inserted to form wells. Samples for PAGE were prepared in a 20 µl final volume and 5 µl of 5 x non-reducing sample buffer was added [60 mM Tris-HCl; 2 % w/v SDS; 10 % v/v glycerol; 0.01 % w/v bromophenol blue]. For reducing conditions 5 µl of 5 x reducing sample buffer [non-reducing sample buffer but including 5 % w/v 2-β-mercaptoethanol] was added and samples were boiled for 5 min. Electrophoresis was carried out for 1 h at 150 volts against 15 µl of SDS-
PAGE markers (specified for each gel) using 1 x running buffer [3.03 g/l Tris, 14.4 g/l glycine and 1 g/l SDS]. Gels were stained overnight in Coomassie blue stain [0.1 % w/v Brilliant Blue R250, 40 % v/v methanol and 10 % v/v glacial acetic acid]. Gels were destained in destain buffer [40 % methanol and 10% glacial acetic acid] and documented using the Novex® Gel Document System (Novel Experimental Technology, CA, USA). Gels were analysed by the software LabImage® (version 2.62, Labsoft Diagnostics AG, Germany). Standard curves were constructed for the relationship between the size of bands in the molecular marker lane and the distance travelled by these bands. The molecular weights of bands observed on the gel were interpolated from these standard curves.

2.2.B.3. RADIO- AND FLUORO-IMAGING OF SDS-PAGE GELS

The Storm® scanner (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden) was used to acquire images of SDS-PAGE gels used to analyse radiolabelled or fluorochrome-conjugated proteins. In the case of radiolabelled proteins, the SDS-PAGE gel was exposed to a phosphor screen (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden) for 3 h prior to Coomassie blue staining. The phosphor screen was scanned at 100 micron resolution using the phosphor imager scanner-head. In the case of fluorochrome conjugated-proteins (Cy5), the gel was scanned using the red fluorescence/chemiluminescence scanner-head at 100 micron resolution and 800 volts photomultiplier. Images obtained after scanning were processed with the associated software (ImageQuant™, Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden). SDS-PAGE gels were stained in Coomassie blue stain solution and destained as described above (Section 2.2.B.2.) then superimposed on the radio- or fluoro-image of the gels.
2.2.B.4. CONFIRMATION OF MODIFIED PAI-2 ACTIVITY

The effect of PAI-2 modification on its biological function was determined by two methods; complex formation with uPA and inhibition of the activity of uPA. The ability of modified PAI-2 to form SDS-stable complexes with uPA was compared to unmodified PAI-2 as previously described (Hang et al., 1998; Ranson et al., 2002). Briefly, modified and unmodified PAI-2 were incubated for 90 min at 37°C with equimolar amount of uPA in a final 20 µl of 1 x PBS, then fractionated by SDS-PAGE under reducing conditions (see Section 2.2.B.2.).

The activity of modified PAI-2 was investigated by its ability to inhibit active uPA from acting on the synthetic colorimetric uPA substrate, Spectrozyme®-UK. Assays were performed in triplicate in 96-wells plate with 150 µl final volume in each well and all solutions were prepared in 0.1 % gelatine buffer [0.1 g gelatine dissolved in 100 ml of dH2O containing 150 mM NaCl, 20 mM Tris-HCl and adjusted to pH 8.4]. Unmodified and modified PAI-2 or BSA (negative control) at 2, 1 or 0.1 µg/ml were mixed with 20 IU uPA (100 µl of 200 IU/ml actisolv uPA stock) in a final 150 µl of buffer. Standard curve was prepared by 1:2 serial dilutions of 20 IU uPA in a final 150 µl of buffer (see Appendix 3 for example, Section 7.3.). Reactions were incubated at 37°C for 30 min. The Spectrozyme®-UK substrate (50 mM stock in DMSO) was prepared at 2 mM in 0.1 % gelatine buffer and 50 µl aliquots were added to all wells including a triplicate blank wells (150 µl buffer only). The appearance of coloured product was assayed kinetically at 37°C for 10 min measuring absorbance at 405 nm every 15 sec using the Spectramax® plate reader.

2.2.B.5. INSTANT THIN LAYER CHROMATOGRAPHY (ITLC)

Instant thin layer chromatography was generally used for assessing percentage incorporation of radionuclide by protein or the purity of radiolabelled
protein from free contaminating radioisotope. Marks were placed 1 cm from both the bottom and top edges and at the middle of the 1 x 9 cm ITLC-SG strips. Sample (1 - 2 µl) was applied at the bottom 1 cm-mark and the strip was placed vertically in a minimal volume of mobile phase (0.1 M citrate pH 5.5, unless specified otherwise). Chromatography was performed until the solvent reached the top 1 cm-mark then the strip was cut into two pieces at the middle mark. The two pieces of each strip were placed in separate gamma counter tubes and radioactivity was measured. Since protein movement is retarded on these strips, radionuclide incorporation on the protein or purity was calculated as the percentage of activity at the bottom section (origin, Ori) over the sum of the activity at the bottom and top (solvent front, SF) sections.
Chapter 3: MODULATION OF THE EXPRESSION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (uPAR): ASSOCIATED PHENOTYPIC AND FUNCTIONAL CHARACTERS

3.1. INTRODUCTION

Expression levels of urokinase plasminogen activator (uPA) and its receptor (uPAR) strongly correlate with a malignant tumour cell phenotype (Andreasen et al., 1997; Wang, 2001). The role of uPAR as an integral membrane protein that specifically binds uPA has been long established. More recently, data suggests that uPA/uPAR may have two complementary functions; the established role as an activator of the proteolytic cascade on the surface of cancer cells (reviews by Mignatti and Rifkin, 1993; Andreasen et al., 1997) and another non-proteolytic role as a component of a signal transducing assembly and cell adhesion factor (Busso et al., 1994; Chapman, 1997; Fazioli et al., 1997).

Expression of the various components of the plasminogen activation system is under regulation by hormones, cytokines and growth factors both under normal physiological conditions and during cancer. For example, the cytokines interleukin-2 (IL-2) and interleukin-1β (IL-1β) up-regulate both uPA and uPAR (Ogura et al., 2001; Al-Atrash et al., 2002). Basic fibroblast growth factor (bFGF) or transforming growth factor (TGF)-alpha increase invasion activity and associate with increases in cellular mRNA levels of uPA and uPAR (Mori et al., 2000). The upregulation of uPA and uPAR by these, and other factors, is mediated largely by classical mitogenic signalling pathways (Aguirre
Ghiso et al., 1999a). Evidence suggests that upon experimental oncogenic transformation or in spontaneous human cancers, mitogenesis and expression of uPA and uPAR are activated through common signalling complexes and pathways (Aguirre Ghiso et al., 1999a). Interestingly, several examples of these mitogenic signalling pathways have been shown to be activated by uPA binding to uPAR and/or the interaction of uPAR with integrins and/or vitronectin and mediate the role of uPA/uPAR in cancer invasion and metastasis. The activation of these pathways have been described by the interaction of several adaptors such as integrins, calveolin and G-protein-coupled receptor (GPCR) with uPAR (reviewed by Ossowski and Aguirre-Ghiso, 2000; Blasi and Carmeliet, 2002).

The ability to modulate the expression of physiologically important proteins in cultured mammalian cells after delivering the encoding cDNA or mRNAs (transfection) have important applications for analysing their in vivo functions. Utilising these techniques, early in vitro and in vivo studies investigated the role of uPA/uPAR in cancer invasion and metastasis. For example, uPAR downregulation via anti-sense transfection techniques - delivery of anti-sense cDNA or mRNA - renders many human cancers dormant (Kook et al., 1994; Mohanam et al., 1997; Yu et al., 1997; Mohan et al., 1999; Lakka et al., 2001; Liao et al., 2001; Wang et al., 2001). Such studies showed that, as an activator of the proteolytic cascade, uPA/uPAR enhances local invasion, intravasation and metastasis formation (for more details and examples see Andreasen et al., 1997). While these early studies addressed the end-point result on cancer invasion and metastasis, a growing number of investigations are interested in understanding the mechanisms underlying the effects of modulating the expression of uPA/uPAR. For example, evidence suggests that uPAR downregulation leads to abolishment of
uPA/uPAR/integrin(α5β1)–dependent signal transduction; reducing the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway activation (Aguirre Ghiso et al., 1999b) or focal adhesion kinase (FAK) mitogenic signalling (Aguirre Ghiso, 2002) and resulting in cancer cell dormancy. In contrast, overexpression of uPAR through its interaction with integrins is responsible for generating a feedback loop where ERK activity increases and this feeds back by increasing the expression of uPAR (Aguirre-Ghiso et al., 2001). A similar positive feedback loop is also activated by the binding of uPA to uPAR that activates ERK signalling which in turn increases uPAR on the cell surface (Ma et al., 2001). In addition to activation of ERK proteins, binding of uPA to uPAR activates myosin light chain kinase (MLCK) thus affecting cell motility (Nguyen et al., 1999). The level of uPAR occupancy affects cysteine- and metallo-protease expression during macrophage differentiation (Rao et al., 1995) which is mediated by activation of FAK and MAPK following the occupancy of uPAR by uPA (Tang et al., 1998).

Given the crosstalk between uPA/uPAR and other cell-surface adaptors (e.g. integrins), extracellular proteins (e.g. vitronectin) and intracellular signalling pathways, modulation of uPAR expression is expected to affect various cell-surface markers and cell behaviour including the ability to bind uPA-inhibitors e.g. PAI-2.

3.1.A. RATIONALE AND AIMS

The aim of this chapter was to modulate the expression of uPAR on invasive and non-invasive breast cancer cell lines using transfection methodology. This involved the cloning of uPAR cDNA in sense and antisense orientations into an expression vector and then extensive optimisation of transfection protocols for the different cell lines used. The transfected cells were used for PAI-2 binding studies (see Chapter 4) and also provided materials for
studies concerning the relationship between uPA/uPAR overexpression and plasminogen binding capacity on cancer cells (Stillfried, G., Al-Ejeh, F. and Ranson, M., manuscript in preparation).

3.2. METHODS

3.2.A. BIOINFORMATICS TOOLS

The sequence of human uPAR cDNA (ID: X51675 Roldan et al., 1990), and pBSM13+ were obtained from the National Centre for Biotechnology Information (NCBI). Sequence alignments were performed using ClustalW® (version 1.7, Thompson et al., 1994). Restriction enzyme sites on DNA stretches were detected using the software pDRAW32 (the DNA analysis software version 1.1.61 by AcaClone® software). The software pDRAW32 was also used for drawing maps of plasmids and genes based on their sequences.

3.2.B. PRODUCTION OF PLASMID CONSTRUCTS AND ANALYSIS

3.2.B.1. AGAROSE GEL ELECTROPHORESIS

Electrophoresis was performed using specified volumes of DNA with DNA sample buffer [5 x concentration: 30 % glycerol; 0.125 % w/v xylene cyanol, 0.125 % w/v bromophenol blue]. The molecular weight marker, λDNA digested with Hind III restriction enzyme (lab preparation at 37 ng/µl), was mixed (5 µl) with 1 µl of 5 x DNA sample buffer. Solutions were loaded onto a set agarose gel [1.2 % w/v agarose dissolved in 1 x Tris-Borate-EDTA (TBE) buffer]. Electrophoresis was performed at 70 volts for 1 h in 1 x TBE [10 x buffer prepared at 108 g/l Tris-HCl; 55 g/l boric acid, 40 mM EDTA pH 8.0 and diluted 1 in 10 for use at 1 x concentration]. Gels were stained in 1 µg/ml ethidium bromide solution for 30 min, destain in distilled water (dH2O) for 30 min or more (as required) then visualised and documented using the Novex® gel document system.
All gels were analysed using Gel-Pro Analyzer™ software (version 3.1, Media Cybernetics, MD, USA). A standard curve was constructed for the relationship between the distance travelled and the molecular weight of the bands in the molecular weight marker lane. The sizes of bands in samples loaded were interpolated from this standard curve. Similarly, a standard calibration curve was constructed for the relationship between the amount of bands in the molecular weight marker and the intensity of their staining. The amount of DNA in loaded samples was calculated based on these calibration curves. Spectrophotometry was used to determine the purity of DNA preparation expressed as the ratio of absorbance at 260 nm (DNA) to the absorbance at 280 nm (protein) as per Maniatis et al. (1989).

3.2.B.2. RESTRICTION ENZYME DIGESTION

Analytical restriction enzyme digestions were performed using 2 µg of DNA, 2 µl of the compatible 10 x restriction enzyme buffer and 1 µl restriction enzyme (approximately 1000 units) diluted with dH2O to final volume of 20 µl and mixed gently. The reaction was spot-centrifuged and incubated at 37°C for 3 h or overnight prior to agarose gel electrophoresis (Section 3.2.B.1.). For preparative restriction enzyme digestion, the volumes and amounts were increased and incubated at 37°C for 24 h.

3.2.B.3. ELECTROELUTION OF DNA FROM AGAROSE

This was performed as described by Maniatis et al. (1989). Briefly, DNA of interest was separated using agarose gel electrophoresis followed by ethidium bromide staining (Section 3.2.B.1.). Stained DNA fragments were visualised using a hand-held ultra-violet lamp and the bands of interest were excised using a razor blade. One end of a washed dialysis tube (prepared by boiling in 1 mM EDTA (pH 8.0) for 10 min and then washed in water) was sealed and the
slice of agarose containing the band of interest was transferred into the tube. Approximately 350 µl of 1 x TBE buffer was added, enough to keep the excised agarose in constant contact with buffer. The other end of the dialysis tube was sealed forming a bag. The bag was immersed in 1 x TBE buffer and electrophoresis was performed. The hand-held ultra-violet light was used to visualise the movement of the DNA fragments out of the gel piece and electrophoresis was stopped. The buffer containing the DNA fragments were collected and phenol/chloroform extractions followed by ethanol precipitation with 0.3 M sodium acetate (pH 5.5) were performed as described by Maniatis et al. (1989).

3.2.B.4. cDNA LIGATION

A mixture was prepared in a final 8.5 µl of dH₂O containing 50:1 mass-ratio mixture of insert (uPAR cDNA): linearised cloning vector (pcDNA3) and was incubated at 45°C for 5 min. The DNA mix was cooled on ice and 1 µl of 10 x ligation buffer and T4 DNA ligase (0.5 µl of 400 U/µl) were added. Reaction was incubated at room temperature overnight and then frozen for further use.

3.2.B.5. DYE TERMINATOR CYCLE SEQUENCING

DNA strands were sequenced using an automated ABI 377 sequencer (Applied Biosystems, CA, USA). Cycle sequencing reactions were carried out as per manufacturer’s instructions (Applied Biosystems, CA, USA) in final 10 µl volumes containing 3.5 µl of BigDye™ terminator mix v2.0, 3.2 µM of primer for the universal promoter T7, 3 % v/v DMSO and 200 - 300 ng of plasmid DNA. Thermocycling conditions were one cycle at 95°C for 3 min, followed by 28 cycles at 95°C for 15 sec and 3.5 min at 60°C. Reactions were precipitated by the addition of 2 µl of DNA precipitation buffer (1.5 M sodium acetate pH 8; 250 mM EDTA) and 10 µl of dH₂O. Ice-cold 100 % ethanol was added at five-volumes
of the reactions and the samples incubated on ice for 5 min. These were centrifuged at 12500 g for 15 min, supernatant discarded and pellets washed once with ice-cold 70 % ethanol before air-drying. Reactions were resuspended in 3.5 µl DNA loading buffer and denatured at 90°C for 3 min before plunging on ice until running sequencing gel. Sequencing gels were prepared in a final 50 ml volume of dH₂O containing 1 x TBE buffer, 6 M Urea and 4.8 % PAGE-PLUS™ both deionized with AG® 501-X8 resin. Solutions were filtered using 0.22 µm filters and the gel was polymerized with 35 µl TEMED and 350 µl of 10 % APS.

3.2.B.6. REPARATION OF uPAR SENSE AND ANTISENSE EXPRESSION VECTORS: RATIONALE OF cDNA CLONING

The pcDNA3 plasmid was used as an expression vector as it contains the high efficiency Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells (Invitrogen® Life Technologies, NSW, Australia). The uPAR cDNA insert (Roldan et al., 1990) was provided as pBluescriptM13+/uPAR+ (pBSM13+/uPAR+) by Professor E. Kruithof (Geneva). The uPAR cDNA was cloned in the sense orientation into the plasmid pcDNA3 (Figure 12) (Ranson M., unpublished data). This plasmid, designated pcDNA3/uPAR+, was confirmed to contain the uPAR cDNA in the sense orientation by restriction enzyme analysis (Figure 12). In order to clone the cDNA in the antisense orientation into pcDNA3 plasmid (designated pcDNA3/uPAR-), uPAR cDNA was released from pBSM13+/uPAR+ plasmid using Not I and Hind III restriction enzyme digestion and the pcDNA3 plasmid was linearised using the same restriction enzyme combination (Figure 13). The uPAR cDNA and linearised pcDNA3 plasmid were purified by electroelution and DNA extraction/precipitation as described earlier (Section 3.2.B.3.). cDNA was ligated into the linearised pcDNA3 as described in Section 3.2.B.4. to produce the pcDNA3/uPAR- plasmid. The presence of the uPAR cDNA in the antisense orientation was confirmed by restriction enzyme analysis (Figure 13).
Figure 12: Confirmation of uPAR cDNA inserted into pcDNA3 in sense direction

The ~1150 kb uPAR cDNA was excised from pBSM13+/uPAR+ by restriction with Not I and Xho I, as confirmed by agarose gel electrophoresis. Excised uPAR cDNA was ligated with Not I/Xho I digested pcDNA3 mammalian expression vector. The produced plasmid (pcDNA3/uPAR+, prepared by Ranson M., unpublished data) was confirmed to contain the uPAR cDNA in the sense orientation by restriction digestion followed by agarose gel electrophoresis.
Figure 13: uPAR cDNA cloning in antisense orientation into pcDNA3 vector

The ~1150 kb uPAR cDNA was excised from pBSM13+/uPAR+ by restriction with Not I and Hind III, as confirmed by agarose gel electrophoresis. The pcDNA3 mammalian expression vector was also digested with Not I and Hind III. Digested pcDNA3 and uPAR cDNA were electroeluted from agarose gel then ligated to produce pcDNA3/uPAR- plasmid which was confirmed to contain the uPAR cDNA in the antisense orientation by restriction digests followed by agarose gel electrophoresis.
3.2.B.7. Transformation of competent *Escherichia coli*

Luria Broth [LB 100 ml: 10 g/l trypton, 5 g/l yeast extract and 10 g/l NaCl] was inoculated with 2 ml of freshly prepared overnight culture of *Escherichia coli* (*E. coli*). The inoculated medium was incubated at 37°C with shaking at 180 rpm until the optical density at 600 nm wavelength reached 0.5 (OD$_{600\text{ nm}}$ = 0.5 approximately after 3 h using LB as a blank). Grown bacteria were centrifuged at 3020 \( \times \) g for 10 min at 4°C. The pellet was incubated on ice for 10 min and then resuspended in 50 ml of ice-cold 0.1 M MgCl$_2$. The solution was centrifuged at 3020 \( \times \) g for 10 min at 4°C and the pellet was resuspended in 5 ml of ice-cold 0.1 M CaCl$_2$. Solution was divided to 1 ml aliquots and the competent *E. coli* was stored 4°C until further use (maximum 7 days storage).

Plasmids constructs (5 \( \mu l \), ~5 \( \mu g \)) were mixed with 100 \( \mu l \) of competent *E. coli* cells and incubated on ice for 30 min. Transformation reaction was heat-shocked at 42°C for 45 seconds and then placed on ice for 2 min. SOC medium [900 \( \mu l \) sterile solution containing: 2 % w/v Bactotryptone, 0.5 % w/v yeast extract; 10 mM NaCl; 2.5 mM KCl; 20 mM MgCl$_2$; 10 mM MgSO$_4$ and 20 mM Glucose] was added and the reaction was incubated at 37°C with 180 rpm shaking for 1 h. Aliquots (10, 100 and 890 \( \mu l \)) of the transformation reaction were spread onto three separate Z-agar plates [sterile 1.5 % w/v agar in LB] containing 100 \( \mu g/ml \) ampicillin (Amp) antibiotic. Plates were incubated at 37°C overnight and colonies were randomly selected and screened for the presence of plasmid containing the uPAR cDNA in the designated orientations. Briefly, single colonies were used to inoculate separate 3 ml aliquots of LB media containing 100 \( \mu g/ml \) Amp for plasmid mini-preparations as described by Maniatis *et al.* (1989). Isolated plasmids were analysed by restriction enzyme
mapping to confirm the presence and orientation of uPAR cDNA (Section 3.2.B.2.).

3.2.B.8. PLASMID MIDI-PREPARATION

Colonies containing the pcDNA3 plasmid with the insert uPAR cDNA in the desired orientations were used to make overnight starting cultures which were then used to inoculate 50 ml of LB media containing 100 µg/ml Amp. After overnight incubation at 37°C with constant 180 rpm shaking, samples of the cells (100 µl) were used to prepare glycerol: cells (7:1 v:v) stocks for storage at –80°C. The remainder of the bacterial cell culture was centrifuged at 6000 x g for 15 min at 4°C. Plasmid midi-preparations were then performed to extract the DNA from bacterial cells as per manufacturer’s instructions using the Qiagen® plasmid midi-prep kit. The DNA pellet produced was resuspended in water, then chloroform/phenol extractions and ethanol precipitation were performed as described by Maniatis et al. (1989). The precipitated plasmids were sterilised by rinsing in 100 % ethanol in a biohazard sterile hood, the final sterile extracted DNA solution was resuspended in sterile TE buffer [10 mM Tris-HCl, pH 7.4; 1 mM EDTA] and frozen at -20°C until further use. The DNA concentration was determined specifically for each plasmid preparation from standard curves constructed from the molecular weight marker for the relationship between the amount of DNA and intensity of ethidium bromide staining. The absorbance at 260 nm (DNA) and 280 nm (protein) measured for plasmid solutions allowed measurement of the purity of DNA.

The concentration of plasmid DNA solutions ranged between 0.8 and 1.2 µg/µl and were in the range of pure DNA preparation (A_{260}/A_{280} = 1.8 - 2) (data not shown, see Section 7.2 - Appendix 2 for example). Dye terminator cycle sequencing (Section 3.2.B.5.) of the plasmid constructs using T7 promoter-
primer confirmed the presence of the uPAR cDNA in the sense (Figure 14) and antisense (Figure 15) orientations.

Figure 14: Alignment of sequence from pcDNA3/uPAR and Human uPAR gene

The sequence from the chromatogram produced from the ABI prism cycle sequencer (black) was exported and aligned with human uPAR gene sequence (red, NCBI accession #: X51675, Roldan et al., 1990) using the ClustalW® program. Matched pairs are marked by ( | ) and the sequence shows almost complete homology to the uPAR cDNA gene when both aligned in the same 5’ → 3’ orientation (sense).
Figure 15: Alignment of sequence from pcDNA3/uPAR and human uPAR gene

The sequence produced from the ABI prism cycle sequencer (black) was aligned with human uPAR gene sequence (red, NCBI accession #: X51675, Roldan et al., 1990) using the ClustalW® program. Matched pairs are marked by ( | ) and the sequence shows almost complete homology to the uPAR cDNA gene when aligned in the opposite orientation (3' → 5') (antisense).
3.2.C. **TRANSIENT TRANSFECTION OF CANCER CELLS WITH uPAR cDNA**

Transient transfection of mammalian cells was optimised using the Lipofectamine™ 2000 and Superfect® transfection reagents. The pcDNA3/uPAR sense (pcDNA3/uPAR+) plasmid was used with MCF-7 and T-47D cell lines and pcDNA3/uPAR antisense (pcDNA3/uPAR-) plasmid with the MDA-MB-231 cell line. Transfection was performed as per manufacturer’s instruction as follows. Cells cultured for 48 h were detached using Trypsin-EDTA and resuspended at $1 \times 10^6$ cells/ml in RPMI-1640 containing 5 % FCS. Cells were seeded into 6-wells plates containing 2 – 2.5 ml RPMI-1640 containing 5 % FCS in order to achieve subconfluent culture after overnight incubation at 37°C. After this incubation complexes between plasmid DNA and reagent for each well were formed by incubating together in 200 µl RPMI-1640 for 20 min at RT after which 300 µl of RPMI-1640 containing 5 % FCS was added to the plasmid DNA: reagent complexes. Control treatments were prepared identically either in the absence of reagent (plasmid DNA only control), absence of plasmid DNA (reagent only control) or in the absence of both plasmid DNA and reagent (negative control). While the manufacturer’s instructions recommend the addition of the final 500 µl transfection solution directly to the 2 – 2.5 ml cell culture media, it was found that removal of culture media and incubation of cells only with the transfection solution overnight then supplementing the cells with 2 ml of culture media significantly increased the transfection efficiency (data not shown).

Transfection optimisation in terms of cell-surface uPAR levels as detected by flow cytometry involved variation in the number of cells, amount of plasmid DNA, ratio of plasmid DNA to reagent and the time of culturing after transfection.
3.3. Results

3.3.A. Optimisation of transient transfection of cancer cells

Initial transfection experiments using the Superfect® reagent in the absence or presence of pcDNA3/uPAR- showed this reagent to be cytotoxic to all cell lines used (Figure 16).

Figure 16: Cytotoxic effect of Superfect® reagent and plasmid DNA on MDA-MB-231 cells

Density plots showing fluorescence of propidium iodide (PI) detected by FL-2 detector on Y-axis plotted against forwards scatter (FSC) on the X-axis. Cell viability of transfected cells was assessed in comparison to a gate established for viable population (excluding PI) of untreated cells (region R1). Cell populations presented were identically treated during the transfection procedure where an overnight culture of $5 \times 10^5$ MDA-MB-231 cells per well in 6-wells plates were seeded overnight then incubated overnight with transfection solution: (A) in the absence of pcDNA3/uPAR- plasmid and Superfect reagent, (B) presence of 7.5 µl reagent alone, (C) 1.5 µg DNA with 3 µl Superfect, (D) 1.5 µg DNA with 4.5 µl Superfect or (E) 1.5 µg DNA with 7.5 µl Superfect. Cells were supplemented with 2 ml of culture media, incubated for further 24 h and viability was assessed (i.e. 48 h after transfection) by the incorporation of propidium iodide (PI) dye.
Treatment of cells with the Superfect® reagent alone showed a slight toxicity in comparison to untreated cells. However, fluorescence due to incorporation of PI dye significantly increased when cells were transfected using the same amount of DNA with increasing volumes of the Superfect® reagent (Figure 16). This observed toxicity due to transfection with the Superfect® reagent was also seen with the other cell lines (data not shown), thus the Superfect® reagent was abandoned.

3.3.A.1. Transient transfection of MDA-MB-231 cells

Initial transfection experiments of MDA-MB-231 cells with Lipofectamine™-2000 transfection reagent showed that 24 h after addition of 1 µg pcDNA3/uPAR plasmid DNA and 2 µl of reagent, uPAR expression significantly decreased by 30% in comparison to that with non-transfected MDA-MB-231 cells (Figure 17 - A, p < 0.05, n = 4). The cell-surface expression of uPAR on cells transfected using the 1:2 ratio was significantly different from all other reagent-treated cells (Figure 17 – A, p < 0.05, n = 4). Treatment of cells with the 1:2 plasmid DNA to reagent ratio displayed the lowest PI incorporation, indicating lower toxicity compared to the other treatments (data not shown). Finally, the expression of uPAR on the surface of cells treated with other plasmid DNA to reagent ratios or reagent alone was not significantly different from that of control MDA-MB-231 cells (Figure 17 - A, p > 0.05, n = 4). For further optimisation, the 1:2 pcDNA3/uPAR plasmid (µg): Lipofectamine™-2000 (µl) ratio was kept constant but the amounts was increased in addition to increasing the transfection time from 24 h to 48 h. Control MDA-MB-231 cells assayed at 48 h past transfection (Figure 17 – B) showed higher uPAR expression compared to cells assayed at 24 h (Figure 17 - A).
MDA-MB-231 cells (5 x 10^5 cells/well in 2.5 ml of RPMI-1640 + 5 % FCS) were added to 6-wells plate, incubated overnight and attached cells were washed and treated as follows. (A) Cells were incubated in transfection solution for 24 h and then assayed by flow cytometry. Transfection solutions were prepared using 1 µg pcDNA3/uPAR- in the absence (DNA only control) or presence of different volumes of Lipofectamine™-2000 (LF) reagent. For the reagent control (LF only control), transfection solution was prepared using 4 µl of Lipofectamine™-2000 in the absence of plasmid DNA. (B) Cells were incubated in transfection solution for 24 h then supplemented with 2 ml of RPMI-1640 + 5 % FCS and incubated for another 24 h then assayed by flow cytometry (i.e. 48 h after transfection). Transfection solutions were prepared using 1 µg pcDNA3/uPAR- in the absence Lipofectamine™-2000 reagent (DNA only control) or using 1, 1.5, 2 and 2.5 µg of pcDNA3/uPAR- complexed with 2, 3, 4 and 5 µl of Lipofectamine™-2000 reagent, respectively. uPAR expression was determined using the #3934 antibody (5 µg/ml) and data shown is the MFI ± SEM (n = 4). In order to measure cell-surface uPAR, only values from viable cells (excluded PI) were used and the asterisks denote statistically significant differences between cell-surface uPAR levels in comparison to the DNA control treatment (p < 0.05, n = 4).
The expression of uPAR at 48 h on MDA-MB-231 cells transfected at each of the pcDNA3/uPAR- plasmid concentration was significantly lower from control cells ($p < 0.05$, $n = 4$) (Figure 17 – B). The maximal decrease in uPAR expression was observed using 1.5 $\mu$g pcDNA3/uPAR- plasmid and 3 $\mu$l reagent (62% decrease compared to control MDA-MB-231 cells; $p < 0.05$, $n = 4$). uPAR expression on cells transfected with 1 $\mu$g of pcDNA3/uPAR- plasmid and 2 $\mu$l reagent was higher at 48 h than that at 24 h (compare Figure 17 – A and B). The decrease in uPAR expression using the 1:2 plasmid DNA ($\mu$g) to Lipofectamine™-2000 ($\mu$l) ratio was not associated with significant cell death as assessed by PI incorporation (Figure 18).

Figure 18: Viability of MDA-MB-231 cells transfected with increasing pcDNA3/uPAR-: Lipofectamine™-2000 amounts

Cell populations were identically treated during the transfection procedure described earlier (Figure 17 – B). Cells were either incubated (A) in the absence of plasmid DNA and Lipofectamine™-2000 (LF) reagent, or with (B) 1.0 $\mu$g DNA: 2 $\mu$l LF, (C) 1.5 $\mu$g DNA: 3 $\mu$l LF, (D) 2 $\mu$g DNA: 4 $\mu$l LF, (E) 2.5 $\mu$g DNA: 5 $\mu$l LF or (F) reagent (5 $\mu$l) alone. Viability was assessed by the exclusion of PI dye (R1 gate).
3.3.A.2. TRANSIENT TRANSFECTION OF T-47D CELLS

Adopting the transient transfection procedure optimised for MDA-MB-231 cells, uPAR expression in T-47D cells was significantly increased using different amounts and ratios of pcDNA3/uPAR+ plasmid: Lipofectamine™-2000 reagent compared to cells treated with Lipofectamine™-2000 reagent alone (Figure 19, *p < 0.05, n > 3). Transfection of T-47D cells with 1.5 µg pcDNA3/uPAR+ and 4.5 µl Lipofectamine™-2000 resulted in 2.0-fold increase in cell surface uPAR compared to cells treated with Lipofectamine™-2000 alone. Consistent high transfection efficiency and cell viability were obtained when 5 x 10⁵ of T-47D cells were transfected using 2 µg of pcDNA3/uPAR+ and 6 µl of Lipofectamine™-2000 reagent (data not shown).

![Figure 19: Transfection of T-47D cells with different amounts of pcDNA3/uPAR+: Lipofectamine™-2000 complexes](image)

T-47D cells were used to inoculate 6-wells plate at 5 x 10⁵ cells per well in 2 ml of RPMI-1640 + 5 % FCS the day before transfection procedure. Cells were transfected according to optimised protocol using specified pcDNA3/uPAR+ plasmid (µg): of LF reagent (µl). 48 h past transfection, cells were detached and assayed for uPAR expression using the MAB807 anti-uPAR monoclonal antibody (10 µg/ml). Data shown is the MFI ± SEM (n ≥ 3) for cell-surface uPAR detection. Asterisks mark significantly different uPAR levels in comparison to control treatment (no DNA control, 0 µg DNA: 4.5 µl LF) (*p < 0.05, n ≥ 3).
Given that conditions were optimised in terms of cell-viability and uPAR expression, mock transfections were also performed using the a 1:3 ratio (2 µg: 6 µl) of pcDNA3 plasmid: Lipofectamine™-2000 reagent for 48 h incubation in order to determine whether the increase of cell-surface uPAR was specifically due to the presence of uPAR cDNA expression. As shown in Figure 20 - A, transfection of T-47D cells with pcDNA3 plasmid did not affect cell-surface uPAR levels at all. In contrast, transfection of these cells with pcDNA3/uPAR+ plasmid significantly increased cell-surface uPAR compared to both control and mock-transfected T-47D cells ($p < 0.05$, $n = 3$) as detected by flow cytometry (Figure 20 – A) and confocal microscopy (Figure 20 – B).

3.3.B. THE EFFECT OF UPAR OVEREXPRESSION

The effect of increasing uPAR expression via transfection was investigated with respect to cell-surface expression of the components of the plasminogen activation pathway and cell morphology. Flow cytometry and confocal microscopy techniques (see Section 2.2.A.) were used to compare control and transfected cells.

3.3.B.1. CELL-SURFACE ANTIGEN CHANGES

The upregulation of uPAR on the surface of T-47D cells was found to be associated with significantly increased levels of endogenous cell-surface uPA and increased lysine-dependent binding of plasminogen (Table II). The values were obtained from five different transfection experiments performed independently using different batches of pcDNA3/uPAR+ plasmid.
Figure 20: Increased cell-surface uPAR is due transfection with uPAR cDNA

(A) Cell-surface uPAR level was determined on DNA only control (2 µg pcDNA3/uPAR+: 0 µl LF, clear bars), mock-transfected (2 µg pcDNA3: 6 µl LF, light grey) and pcDNA3/uPAR+-transfected (2 µg pcDNA3/uPAR+: 6 µl LF, dark grey) T-47D cells using the MAB807 antibody. Data presented are the MFI ± SEM (n = 3) and the viability of cell population in all assays was higher than 70%. The levels on pcDNA3/uPAR+-transfected cells were significantly different compared to both control and mock-transfected cells (p < 0.05). (B) Mock-transfected (I and II) and pcDNA3/uPAR+-transfected (III – VI) T-47D cells were analysed by confocal microscopy. Mock- and pcDNA3/uPAR+-transfected cells were incubated with polyclonal anti-uPAR antibody (I - IV, 20 µg/ml). pcDNA3/uPAR+-transfected cells were alternatively incubated with isotype control (rabbit serum, V and VI, 20 µg/ml). Cells were then incubated with anti-rabbit IgG FITC-conjugated antibody, washed and analysed by confocal microscopy. Top panels are transmission images of basal sections and the scale bars represent 10 µm of actual length. Bottom panels are the corresponding fluorescent images. Data shown in (A) and (B) are representative data of at least two independent assays.
T-47D cells were transfected according to the optimised transfection protocol where $5 \times 10^5$ cells were seeded in 2 ml of RPMI-1640 + 5 % FCS per well in 6-wells plate, incubated overnight, washed and incubated for 24 h with transfection solution containing preformed complexes (20 min at RT) of 2 µg pcDNA3/uPAR+ and 6 µl Lipofectamine™-2000 in 200 µl RPMI-1640 then 300 µl RPMI-1640 + 5 % FCS was added. Cells incubated with the 500 µl transfection solution were supplemented with 2 ml or RPMI-1640 + 5 % FCS and incubated for another 24 h before flow cytometry assays using MAB807 anti-uPAR and #392 anti-uPA antibodies for cell-surface uPAR and uPA, respectively. Lysine-dependent plasminogen binding was also determined by flow cytometry using plasminogen-FITC incubated with cells in the absence or presence of TA. Controls were either no treatment, Lipofectamine™-2000 reagent alone or mock-transfected (2 µg pcDNA3 complexed with 6 µl Lipofectamine™-2000) which showed no difference in uPAR expression. Values presented are the mean fold-increase of cell-surface parameter on transfected cells compared to control T-47D cells. There was a significant difference between the levels of each parameter on control and pcDNA3/uPAR+-transfected T-47D cells in all cases ($p < 0.05$, $n \geq 3$). Values shown were obtained from viable cells only (excluded PI) and thus represent cell-surface levels.

A linear correlation ($r^2 = 0.95$) described the relationship between the fold-increase of endogenous cell-surface uPA and the fold-increase of cell-surface uPAR shown in Table II (Figure 21). This indicated that the significant increase in cell-surface expression of uPAR on T-47D cells was directly associated with increased uPA levels on the cell-surface. Cell-surface uPA completely co-localised with cell-surface uPAR on pcDNA3/uPAR+-transfected T-47D cells (Figure 22), emphasising the role of uPAR to localise uPA on the cell-surface. Nevertheless, areas of unoccupied uPAR were evident in the overlays, indicating incomplete occupancy of cell-surface uPAR.
As suggested in Table II, the increase of lysine-dependent binding of plasminogen, whilst significant, failed to directly correlate to either the increase of cell-surface uPAR or the resulting increase of endogenous cell-surface uPA (data not shown). Mock-transfected T-47D cells did not show any significant changes in cell-surface uPA and lysine-dependent plasminogen binding in comparison to untreated T-47D cells ($p < 0.05$, $n = 3$) (data not shown).

![Figure 21: Correlation between increased surface uPAR and uPA on transfected T-47D cells](image)

The fold-increase of cell-surface uPA on transfected T-47D cells compared to control cells was directly correlated to the fold-increase of cell-surface uPAR. Data points are the mean of triplicate determination for both control and transfected T-47D cells. Error bars are the SEM and data were obtained from independent assays performed at different times. The bold line represents the linear correlation with a regression ($r^2$) of 0.95 and the dotted lines are the 95% confidence interval of the linear correlation.
Figure 22: Distribution of cell-surface uPA and uPAR on T-47D cells transfected with uPAR cDNA

Transiently transfected T-47D cells were stained with mouse anti-human B-chain uPA then goat anti-mouse IgG Cy3-conjugated antibody. Cells were washed and then incubated with rabbit anti-human uPAR polyclonal antibody (provided by David Croucher, University of Wollongong) then goat anti-rabbit IgG FITC-conjugated antibody. There was no leakage between the FITC and Cy3 channels and addition of secondary antibodies alone did not produce any staining (data not shown). Furthermore, according to manufacturer’s specifications, the goat anti-mouse IgG Cy3-conjugated and the goat anti-rabbit IgG FITC conjugated antibodies do not show cross reactivity. Co-localisation of uPAR (green, panel II) and uPA (blue, panel III) is shown in the overlay and appear in cyan colour (panel IV). The image shown is a representative sample obtained from basal sections and the scale bar in the transmission image (panel I) represents 10 µm.

The optimised transfection procedure was utilised to modulate uPAR expression on MCF-7 cells. This cell line did not resist manipulation as cell viability was generally low when cells were assayed by flow cytometry (50 – 70 % viability). This reduced viability was consistently observed with experiments on both control and transfected MCF-7 cells. Despite this resistance to manipulation, a small but significant increase in uPAR expression was observed on transfected MCF-7 cells overexpression which was also associated with small
but significant increase in cell-surface uPA (Figure 23). However, this was not accompanied by a significant increase in the lysine-dependent binding of plasminogen possibly due to the low increase in cell-surface uPAR/uPA on MCF-7 cells or that the overexpression of uPAR/uPA on T-47D and MCF-7 cells resulted in different effects on the binding of plasminogen to the cell-surface.

![Graph](https://via.placeholder.com/150)

**Figure 23: The effect of uPAR overexpression on MCF-7 cells**
MCF-7 cells were transfected with pcDNA3/uPAR plasmid and cell-surface uPAR (#3934 antibody), uPA (#392 antibody) and lysine-dependent plasminogen binding were compared between DNA only control (empty bars) and transfected (grey bars) cells. Data are the MFI ± SEM (n = 3) and cell viability in all assays ranged between 65 – 75 %. Only the lysine-dependent plasminogen binding was not significantly different between control and transfected cells \(p > 0.05\). Data presented are from one representative experiment and similar data were observed in other experiments.

### 3.3.B.2. SPATIAL AND MORPHOLOGICAL CHANGES

Control and T-47D cells transiently transfected with pcDNA3/uPAR plasmid were analysed for the localisation of uPAR, uPA and plasminogen binding on the cell-surface by confocal microscopy. Cell-surface uPA and plasminogen binding on control T-47D cells was very low (Figure 24 – A) compared to pcDNA3/uPAR+-transfected T-47D cells (Figure 24 – B and C).
Plasminogen binding was greatly reduced in the presence of the lysine analogue TA (Figure 24 – D), indicating that lysine-dependent binding dominated the total plasminogen binding to transfected cells. Thus, confocal microscopy analysis emphasised the success of the transfection procedure and showed that endogenous cell-surface uPA levels and plasminogen binding were increased in association with overexpression of uPAR on these cells.

Control incubations with antibody isotypes (e.g. Figure 24 – E), secondary antibodies alone (data not shown) or in the presence of TA (Figure 24 – D) confirmed the lack of signal leakage between the different channels used for the detection of the different fluorochrome-emissions by the confocal microscope. Therefore, overlays of images were a bona fide evidence of co-localisation not artefacts of staining procedure. uPA and plasminogen binding had a higher distribution on the cell surface at basal and transverse sections (Figure 24) compared to the apical surfaces (data not shown) of attached transfected T-47D cells. Plasminogen binding and uPA displayed a punctuate distribution and co-localised almost completely on transfected T-47D cells. Nevertheless, small areas on the cell surface of the transfected cells did not bind plasminogen although uPA appeared in these areas.

Control T-47D cells displayed a general cuboidal (spherical) shape as seen in the transmission images in Figure 24. The overexpression of uPAR affected the morphology of these cells where transfected cells appeared to display a more elongated (fibroblastic) shape compared to control cells. This was seen in most transmission images of transfected T-47D cells; however it was most apparent in the transmission images displayed in Figure 24 – B and C compared to Figure 24 - A.
Continues …
Figure 24: Confocal microscopy of control and T-47D cells transiently transfected with pcDNA3/uPAR⁺ plasmid

T-47D cells cultured on glass slide cover-slips were transfected as per optimised protocol and subjected to double staining procedure then analysed by confocal microscopy. Cells in Panels I - IV were incubated with pcDNA3/uPAR⁺ plasmid only (control T-47D cells) whereas in Panels V - XX were incubated with pcDNA3/uPAR⁺: Lipofectamine™-2000 complexes (pcDNA3/uPAR⁺-transfected T-47D cells). Cells in Panels I – XII were incubated with plasminogen-Cy5, washed and incubated with anti-B-chain uPA monoclonal antibody (#394, 20 µg/ml) then with anti-mouse IgG FITC-conjugated antibody. Cells in Panels XIII- XVI were incubated with plasminogen-Cy5 in the presence of TA, washed and incubated with anti-B-chain uPA monoclonal antibody (#394, 20 µg/ml) then with anti-mouse IgG FITC-conjugated antibody. Cells in panels XVII – XX were incubated with plasminogen-Cy5 then with mouse isotype IgG1 followed with anti-mouse IgG FITC-conjugated antibody. The lack of signal in XV when cells incubated with TA compared to VII and XI when cells incubated in the absence of TA indicate that plasminogen binding is completely lysine dependent on these cells and that the signal from uPA staining (green, VI, X and XIV) did not leak into the plasminogen channel (red, VII, XI and XV). On the other hand, the lack of signal in XVII indicates that staining with anti-uPA antibody is specific (no staining seen with isotype) and that the signal from plasminogen-Cy5 (VII, XI, and XIX) does not leak into the uPA channel (VI, X, and XVIII). Images in I, V, IX, XIII and XVII are transmission images and the scale bars represent 10 µm of actual length. Overlays of uPA and plasminogen staining are shown in IV, VIII, XII, XVI and XX and co-localisation appears in orange/yellow. All images shown in Figure were obtained from basal sections of the attached cells (i.e. sections at the site of attachments to the cover slip) except for Panels IX - XII. Images in Panels IX - XII were obtained from transverse section (i.e. section between the basal [attachment site] and apical [opposite side] sections of attached cells). Transverse sections were selected where the cells are largest and the membrane is highest in focus. Images shown in the Figure are representative samples of several assays each performed in replicates (> 3 images per assay).
3.3.C. COMPARISON BETWEEN T-47D AND MDA-MB-231 CELLS

The previous results showed that the overexpression of uPAR resulted in an increase of uPA and lysine-dependent plasminogen binding on the surface of T-47D cells (Table II and Figure 24). Taken together, the overexpression of uPAR/uPA and increased plasminogen binding on T-47D cells appeared to manifest a more metastatic phenotype (Ranson et al., 1998). To further characterise the effect of uPAR-overexpression on T-47D cells, these cells were directly compared to the metastatic MDA-MB-231 cells.

3.3.C.1. CO-LOCALISATION OF uPA AND PLASMINOGEN

The lysine-dependent binding of plasminogen on the surface MDA-MB-231 cells was independent from the pattern of uPA distribution at both the basal (Figure 25) and transverse sections (data not shown). Cell-surface uPA displayed a punctuate distribution while diffused plasminogen binding was observed on MDA-MB-231 cells. These patterns were previously reported on this cell line by Andronicos and Ranson (2001). In comparison to MDA-MB-231 cells, transiently uPAR-overexpressing T-47D cells displayed punctuate distribution and very high co-localisation of both uPA and plasminogen (Figure 24). Cell-surface uPA and plasminogen binding on MDA-MB-231 cells was more than 5- to 10-fold higher than that on uPAR-overexpressing T-47D cells. This indicated that MDA-MB-231 cells still showed a more malignant phenotype, however, in order to allow spatial comparison, the signals detected in Figure 24 and Figure 25 were normalised to the same level.

3.3.C.2. THE EFFECT OF EXOGENOUS uPA BINDING ON PLASMINOGEN BINDING

Incubation of MDA-MB-231 cells with exogenous uPA showed a significant increase (1.5-fold) of cell-surface uPA compared to control cells ($p < 0.05$, $n = 3$, Figure 26 - A). This indicated that majority, but not all, of cell-
Surface uPAR was occupied with uPA (69% in the representative Figure 26 - A) prior to addition of exogenous uPA. The level of uPA and the occupancy of uPAR on the surface of MDA-MB-231 cells were much higher than control or transfected T-47D cells. Furthermore, in contrast to T-47D cells (Figure 26 - B), lysine-dependent binding of plasminogen on MDA-MB-231 cells was not significantly different before and after incubation with exogenous uPA ($p > 0.05$, n = 3, Figure 26 - A).

The incubation of T-47D cells with exogenous uPA showed a significant increase (2.3-fold) of cell-surface uPA compared to control T-47D cells ($p < 0.05$, n = 3, Figure 26 - B). This indicated that only a fraction (44% in the representative Figure 26 - B) of cell-surface uPAR was occupied with uPA prior to addition of exogenous uPA. Lysine-dependent binding of plasminogen also significantly increased (2.2-fold) on cells incubated with exogenous uPA compared to control T-47D cells ($p < 0.05$, n = 3, Figure 26 - B). It should be noted that uPAR occupancy was not significantly different between transfected and control T-47D cells, 44 ± 6% and 36 ± 2% respectively ($p > 0.05$ from experiments repeated 3 times in triplicate, data not shown).

### 3.3.C.3. Effect of uPA or uPAR Blocking on Plasminogen Binding

Lysine-dependent plasminogen binding was not significantly different between cells blocked with anti-uPA or anti-uPAR antibodies and cells blocked with isotype antibody ($p > 0.05$, n = 3 in two identical assays) (data not shown). This indicated that blocking of uPA or uPAR on transfected T-47D cells did not specifically affect plasminogen binding. The lysine-dependent binding of plasminogen to the invasive MDA-MB-231 cells also was not significantly affected by conditions leading to the blocking of uPA or uPAR ($p > 0.05$, n = 3 in two identical assays) (data not shown).
Cells cultured on glass slide cover-slips were subjected to double staining procedure, mounted onto glass slides and analysed immediately by confocal microscope. Scale bars in all images represent 20 µm and only images from basal sections are presented. **Top panel:** Cells incubated with plasminogen-Cy5 followed by indirect immunostaining for uPA using anti-B-chain uPA antibody (#394) then by rabbit anti-mouse IgG FITC conjugated antibody. **Bottom panel:** Cells incubated with plasminogen-Cy5 in the presence of 5 mM TA followed by uPA staining. The lack of signal in the plasminogen channel when cells incubated in the presence of TA indicate that plasminogen binding was lysine-dependent and that signal from uPA staining did not leak into this channel.
Figure 26: Effect of exogenous uPA on plasminogen binding to the cell-surface

Cell-surface uPA (using the #394 antibody) and lysine-dependent binding of plasminogen (using plasminogen-FITC) to (A) MDA-MB-231 cells and (B) T-47D cells were determined by flow cytometry before and after the incubation of cells with exogenous uPA (50 nM). Data presented are the MFI ± SEM (n = 3) and cell-viability was higher than 70 % and 90 % in all assays for T-47D and MDA-MB-231 cells, respectively. One asterisk denotes significant differences in cell-surface uPA level before and after incubation with exogenous uPA (p < 0.05). Two asterisks denote significant differences in the lysine-dependent binding of plasminogen to the cell-surface before and after incubation with exogenous uPA (p < 0.05).
3.4. DISCUSSION

A transient transfection procedure was successfully optimised using the Lipofectamine™-2000 transfection reagent and uPAR cDNA for the modulation of cell-surface expression of uPAR in breast cancer cell lines. Consistent significant increases (1.5- to 3-fold) of cell-surface uPAR were observed by transfection of T-47D cells with pcDNA3/uPAR+ plasmid using Lipofectamine™-2000 reagent.

The increase of cell-surface uPAR on T-47D cells was associated with increased cell-surface levels of uPA and plasminogen binding. Mock transfection, using null pcDNA3 plasmid, confirmed that this increase of cell-surface markers was specifically due to transfection of these cells with the uPAR cDNA. The increase of cell-surface uPA and plasminogen binding observed may be due to concurrent increased secretion of uPA and overexpression of plasminogen receptors with the overexpression of uPAR in T-47D cells. The direct correlation between the increase of cell-surface uPA and the increase of cell-surface uPAR on transfected T-47D cells supported this. Kariko et al. (1993) reported that transfection of human osteosarcoma cell line (HOS) with uPAR cDNA showed a 2-fold increase in the surface expression of uPAR associated with 2-fold increase in receptor-bound uPA-mediated plasmin generation. In contrasting experiments, antisense inhibition of the cell surface expression of uPAR not only suppresses ERK/MAPK signalling activity but was also associated with inhibition of adhesion, suppression of uPA secretion and inhibition of pro-MMP-9 secretion (Ahmed et al., 2003a). In fact, proteomic analysis of uPAR-antisense-clones compared to wild-type and mock-transfected control colon cancer cell line (HCT116) cells shows the loss of approximately 200 proteins and quantitative differences in the expression of 141 other proteins (Ahmed et al., 2003b). Thus, evidence in the literature supports
the notion that transfection of T-47D cells with uPAR cDNA may have affected the secretion of uPA and expression of plasminogen receptors on the cell surface along with that of cell-surface uPAR.

The low occupancy (44%) of cell-surface uPAR on control untransfected T-47D cells indicated that free uPAR was available for uPA binding prior to transfection. The increase of cell-surface uPA on transfected T-47D cells is due to concurrent upregulation of uPA expression and secretion in these cells, which allowed more uPA to the increased cell-surface uPAR. Interestingly, despite the increase of both cell-surface uPA and uPAR, overall uPAR occupancy on transfected T-47D cells (~ 50%) was not significantly different from that on control T-47D cells. This was also reflected by the fact that there was a direct correlation between the increase of cell-surface levels of uPAR and uPA, thus the occupancy rates of uPAR on the cell-surface was not affected. In other words, these cells appear to dynamically maintain a proportion of receptor unoccupied despite transfection procedures. The significance of maintenance of unoccupied receptors on these cells prior to and after transfection may be related to the newly characterised functions of unoccupied uPAR. The clustering of unoccupied uPAR has been implicated in cell signalling leading to changes in adhesion and migration (Koshelnick et al., 1997; Sitrin et al., 2000; Gellert et al., 2003; Sitrin et al., 2004) via association with lipid rafts or caveolae (Wei et al., 1999) and integrins (Tarui et al., 2001). Since the binding of uPA restricts the lateral mobility of uPAR (Myöhänen et al., 1993), reserving unoccupied receptor may be a tactic used by cells to allow the movement of uPAR to focal adhesion contact points where it may cluster and accumulate with caveolae (Wei et al., 1999) and integrins before being occupied by uPA and being restricted to these areas.
While there was not a direct correlation, the increase in uPAR/uPA on transfected T-47D cells corresponded to an increase in plasminogen binding capacity. Furthermore, plasminogen binding was co-localised with cell-surface uPA when analysed by confocal microscopy. On both MDA-MB-231 and uPAR-overexpressing T-47D cells, uPA preferentially distributed to basal sections of attached cells. This was in agreement with previous studies which documented the high concentration of uPA/uPAR at focal adhesion sites (see review by Andreasen et al., 1997). Areas of co-localisation of uPA and plasminogen were confirmed on cell-surface of MDA-MB-231 cells as previously shown by Andronicos and Ranson (2001). However, the overall distribution of plasminogen was different on transfected T-47D cells compared to MDA-MB-231 cells. MDA-MB-231 cells showed a broader diffuse staining for plasminogen and not all co-localised with uPA (as per Andronicos and Ranson, 2001). The large colocalisation of uPA and plasminogen on transfected T-47D cells in addition to the increased plasminogen binding observed on control T-47D cells when incubated with exogenous uPA suggests that uPA may act as a receptor for plasminogen. In support of this suggestion, a novel non-active-site interaction between uPA and plasminogen is necessary for the assembly and efficiency of cell-surface plasminogen activation complexes (Ellis et al., 1999). The inability to reduce the binding of glu-plasminogen when T-47D cells where blocked with anti B-chain uPA monoclonal antibody confirmed that bound plasminogen was independent of the active site of uPA. Similarly, this antibody did not alter the co-localisation of glu-plasminogen and uPA detected by confocal microscopy of MDA-MB-231 cells (Andronicos and Ranson, 2001). The anti A-chain uPA monoclonal antibody also did not affect plasminogen binding on transfected T-47D cells or MDA-MB-231 cells. This antibody is raised against a different epitope in the A-chain of uPA deduced by Ellis et al. (1999) for the
binding of glu-plasminogen, thus the possibility of uPA acting as a site for plasminogen binding cannot be confidently excluded.

All together, the underlying reason for the co-localisation of glu-plasminogen and uPA on the cell surface is to efficiently generate plasmin on the cell surface (Andronicos and Ranson, 2001). However, other potential receptors responsible for the co-localisation of plasminogen at close proximity to cell-surface uPA are not known. Furthermore, comparison of transfected T-47D cells to transfected MCF-7 cells complicates the scenario since both transfected cell-lines displayed increased cell-surface uPAR and uPA, whereas only T-47D cells showed increased plasminogen binding upon transfection with uPAR cDNA. Similarly, while plasminogen binding increased on T-47D cells incubated with exogenous uPA, plasminogen binding was not affected on MDA-MB-231 after the binding of exogenous uPA to these cells. These differences may reflect cell line differences related to the expression of different plasminogen receptors.

In order to provide an explanation to these findings, two models are proposed. First, intracellular signals initiated from increased uPAR and/or increased uPA binding to uPAR may increase the expression of plasminogen receptors on the cell surface. Intracellular signalling pathways initiated by uPAR/uPA are indicated to regulate the proteolytic as well as the non-proteolytic functions of the urokinase plasminogen pathway such as cellular adhesion, chemotaxis, cell proliferation, expression, differentiation and endocytosis (see reviews by Conese and Blasi, 1995; Andreasen et al., 1997; Chapman, 1997; Dear and Medcalf, 1998; Konakova et al., 1998; Preissner et al., 2000; Blasi and Carmeliet, 2002). Increased plasmin generation, which would require increased plasminogen binding, has been described for uPAR cDNA transfected human osteosarcoma (HOS) cells (Kariko et al., 1993). In the case of control untransfected T-47D cells incubated with exogenous uPA,
intracellular signals are initiated simply due to the binding of uPA to unoccupied cell-surface uPAR (Figure 27). Sufficient evidence exists to suggest that intracellular signalling due to uPAR/uPA overexpression and/or the addition of exogenous uPA was responsible for increased plasminogen binding in T-47D cells. This appeared not to be the case for transfected MCF-7 cells possibly due to inefficient increase in cell-surface uPAR/uPA.

The second proposed model which may explain the increased plasminogen binding on uPAR/uPA overexpressing T-47D cells or uPA-incubated control T-47D cells may relate to proteolytic activity on the cell surface (Figure 27). The increased proteolytic activity of plasmin on the cell-surface may have caused the exposure of new lysine residues nearby and resulted in increased plasminogen binding and the apparent co-localisation of uPA and plasminogen on transfected T-47D cells. This was not observed on MDA-MB-231 cells incubated with exogenous uPA since the cell-surface may have been already maximally activated for plasminogen binding on these invasive cells. In the case of transfected MCF-7 cells the small increase in cell-surface uPAR/uPA may have not substantially increased plasmin activity on the cell-surface. Candidates receptors for plasminogen include actin, annexin II, cytokeratin 8, α-enolase, megalin and tetranectin (reviewed by Félez, 1998; Ranson and Andronicos, 2003). While only cytokeratin 8 and α-enolase display a C-terminal lysine residue required for the lysine-dependent binding of glu-plasminogen, the other plasminogen receptors may be considered as latent receptors which require plasmin modification for glu-plasminogen binding (Félez, 1998; Ranson and Andronicos, 2003). Thus, increased cell-surface uPA on transfected T-47D cells and control T-47D cells incubated with exogenous uPA as well as increased plasminogen receptors may result in increased plasmin activity which may in turn activate latent plasminogen receptors.
Finally, the overexpression of uPAR affected the morphology of transfected T-47D cells where the cells adopted a less epithelial (round) shape and more fibroblastic (elongated) shape, similar to MDA-MB-231 cells, in comparison to control T-47D cells. This observation is supported by data showing that, in contrast, antisense uPAR-transfected human glioma cell line (SNB19) adopt larger and more round morphology, fail to form an organised actin cytoskeleton and do not spread efficiently when plated over ECM substrates in comparison to control cells (Chintala et al., 1997). Thus, modulation of uPAR-expression appears to affect cell differentiation/expression and morphology relating to the malignant phenotype of cancer cells.

In summary, transient overexpression of uPAR cDNA in T-47D cells affected the level of cell-surface uPA and plasminogen binding. Transient uPAR-overexpressing T-47D cells, as discussed in the next chapter were used to assess PAI-2 binding in comparison to other breast cancer cell lines. uPAR-overexpressing T-47D cells were also useful to elucidate some molecular details regarding the effect of uPAR expression on invasiveness. Increased lysine dependent plasminogen binding due to increased cell-surface uPA on uPAR-overexpressing T-47D cells or control T-47D cells incubated with exogenous uPA suggests a role for uPA/uPAR in plasminogen binding. Two models for this effect were proposed, illustrated in Figure 27, which describe possible molecular details for increased invasiveness due to uPAR overexpression however; future work is required to provide definitive evidence for these conclusions or other explanations.
Figure 27: Proposed model for increased plasminogen binding due to increased cell-surface uPA/uPAR.
Chapter 4: KINETIC ANALYSIS OF uPA:PAI-2 COMPLEX FORMATION AND SUBSEQUENT INTERNALISATION BY BREAST CARCINOMA CELL LINES

4.1. INTRODUCTION

Binding of uPA to its receptor significantly increases the rate of uPA-mediated plasminogen activation 
\textit{in vitro} (Ellis \textit{et al.}, 1991) and \textit{in vivo} (Quax \textit{et al.}, 1991b). The co-localisation of plasminogen with uPA at cell surfaces provides a mechanism for accelerated plasminogen activation by cells (Andronicos and Ranson, 2001). Receptor-bound plasmin is protected from inactivation by circulating inhibitors (Hall \textit{et al.}, 1991) and thus promotes the remodelling of the local extracellular environment by either directly degrading basement membranes and extracellular matrix (ECM) or by activating pro-metalloproteases (Andreasen \textit{et al.}, 1997). Plasmin is also responsible for the release of latent growth/angiogenic factors from ECM (Bass and Ellis, 2002; Rakic \textit{et al.}, 2003).

The proteolytic activity of soluble and uPAR-bound uPA is efficiently inhibited by PAI-1 and PAI-2 (see Section 1.4.D. for details, Cubellis \textit{et al.}, 1989; Ellis \textit{et al.}, 1990; Pöllänen \textit{et al.}, 1990). The crystal structures and inhibitory mechanism of both PAI-1 (Nar \textit{et al.}, 2000) and PAI-2 (Jankova \textit{et al.}, 2001; Saunders \textit{et al.}, 2001) are well defined (see Section 1.4.D.1). Both act as classic serpins and form SDS-stable 1:1 stoichiometric complexes with the catalytic site on their target protease (Kruithof \textit{et al.}, 1995; Huntington \textit{et al.}, 2000; Nar \textit{et al.}, 2000; Jankova \textit{et al.}, 2001; Saunders \textit{et al.}, 2001).
Although the PAI-2 inhibitory mechanism is well characterised in vitro, to date only indirect quantitative analyses of cell-bound uPA inhibition by PAI-2 have been made by monitoring plasminogen activation using a coupled colorimetric assay (Ellis et al., 1990; McGowan et al., 2000). Using such an assay, Ellis et al. (1990) derived the second order association rate constant value of $3.3 \times 10^5$ M$^{-1}$s$^{-1}$ on U937 cells, thus were the first to describe the rapid and efficient inhibition of cell surface uPAR-bound uPA by PAI-2. However, insights into the serpin inhibitory mechanism gained since this study was published (Kruithof et al., 1995; Jankova et al., 2001; Saunders et al., 2001) cast some doubt on the parameters used to measure inhibition. Silverman (1996) states that as serpins are ‘mechanism-based enzyme inactivators’, in that they irreversibly inhibit their target protease by forming a covalent bond with the protease (Huntington et al., 2000), the two principal parameters for describing this class of inhibitors are the inactivation rate constant ($k_{\text{inact}}$) and the inactivation constant ($K_i$). As PAI-2 has been shown to be a classic serpin (Jankova et al., 2001; Saunders et al., 2001), $k_{\text{inact}}$ and $K_i$ are more appropriate parameters for measuring inhibition by PAI-2. The irreversible formation of the complex between PAI-2 and uPA reflects the complete inhibition of uPA activity. Therefore, the level of PAI-2 binding to cells would provide a direct measure for the inhibition of cell-surface uPA. While Estreicher et al. (1990) investigated the kinetics of complex formation between receptor-bound uPA on the THP-1 monocytic cell line and PAI-2, this was a semi-quantitative and indirect analysis of PAI-2 binding as $^{125}$I-uPA was used as the means of detection. Thus, the inactivation constants for PAI-2 inhibition of uPA ($k_{\text{inact}}$ and/or $K_i$) have not been previously reported.

There is also very little direct data regarding the fate of PAI-2 after inactivation of uPAR-bound uPA. In contrast, PAI-1, either added to uPAR-
bound uPA or as a preformed complex with uPA, has been definitively shown to induce the rapid cellular internalisation and degradation of the complex. This internalisation process requires uPAR and one or more of the members of the low density lipoprotein receptor (LDLR) family of endocytosis receptors such as \( \alpha_2 \)-macroglobulin receptor/low-density lipoprotein receptor-related protein (\( \alpha_2 \)MR/LRP) (see reviews by Andreasen et al., 1997; Loskutoff et al., 1999). Estreicher et al. (1990) reported that \(^{125}\text{I}-\text{uPA:PAI-2}\) complexes are rapidly cleared from the cell surface of THP-1 cells following the formation of a cell-surface bound 70 kDa degradation intermediate. The identity of the cleavage site or product was not ascertained. Jensen et al. (1990) reported that PAI-2 mediated about 40 % degradation of uPAR-bound \(^{125}\text{I}-\text{uPA}\) on human choriocarcinoma cells. Both authors speculated that like PAI-1, PAI-2 mediates the endocytosis of uPAR-bound uPA whereupon both uPA and PAI-2 are degraded in lysosomes. However, neither study provided data supporting these claims. Ragno et al. (1995), also using THP-1 cells, found that the \(^{125}\text{I}-\text{uPA:PAI-2}\) complex is cleaved into a 70 kDa fragment (comprising the uPA B chain complexed to PAI-2) as well as a 22 kDa fragment (mostly uPA A chain) after binding to uPAR. These authors concluded that neither the 22 nor 70 kDa fragments are internalised but rather that the 70 kDa fragment is released into the cell medium while the 22 kDa fragment remains cell-surface bound. It should be noted that a 70 kDa fragment (Saunders et al., 1998: See Figure 5) as well as a 22 kDa fragment (Croucher et al., unpublished data) can also be obtained in solution by incubating excess 55 kDa uPA with PAI-2. This suggests that the intermediary formation of these fragments may not necessarily be a step related to uPAR binding of uPA:PAI-2 but to an undefined cleavage step in solution. Despite these apparent contradictions and lack of any direct evidence, it has become accepted in the literature that PAI-2, unlike PAI-1 and other
serpins, is not internalised after inhibiting uPAR-bound uPA on cells (Andreasen et al., 1997; Andreasen et al., 2000; Schmitt et al., 2000).

4.1.A. RATIONALE AND AIMS

The inhibition kinetics of PAI-2 towards cell-surface uPA were measured using coupled assays which utilised uPA-mediated plasmin generation as an indicator of PAI-2 inhibition of uPA. The aim of this chapter was to develop binding assays to measure uPA:PAI-2 complex formation on the cell surface which would directly reflect PAI-2 inhibitory kinetics on the cell-surface. Due to the controversial fate of cell-bound uPA:PAI-2 complexes, it was also the aim to provide definitive evidence for the fate of these complexes in breast carcinoma cell lines.

4.2. METHODS

4.2.A. PREPARATION OF $^{125}$I-PAI-2 FOR RADIOLIGAND BINDING STUDIES

Radio-iodination of PAI-2 was performed as per manufacturer's instructions (Pierce Chem. Co., IL, USA) and as previously described (Hang et al., 1998). Briefly three iodo-beads were washed three times with 3 ml of 1 x PBS (pH 7.2) at room temperature and dried on Whatman #1 filter paper. Washed beads were transferred to a reaction tube containing 0.5 milli-Curies (mCi) of iodine-125 radioisotope ($^{125}$I) diluted in a final 500 µl volume of 1 x PBS. After 5 min incubation at room temperature, 50 µg of PAI-2 was added to the reaction vessel, mixed gently and incubated 15 min at room temperature.

Radiolabelled PAI-2 was immediately separated from free $^{125}$I by size exclusion chromatography where PD-10 columns were equilibrated with 15 ml of freshly prepared elution buffer [1 x PBS, 0.1 % w/v BSA, pH 7.4]. The
Radiolabelling reaction was allowed to pass through the column followed by two 0.2 ml aliquots of elution buffer. Ten to twelve 0.5 ml-fractions of the eluant were collected during the continuous addition of elution buffer. Aliquots (10 µl) from eluted fractions were counted using normalised\footnote{See Appendix 1 (Section 7.1.) for normalisation procedure of the Cobra\textsuperscript{®} \(\gamma\)-counter} Cobra 5005R gamma (\(\gamma\))-counter (Packard Instruments Co., CT, USA). The fraction(s) corresponding to the first high radioactivity peak were collected. This represented radiolabelled PAI-2 (Figure 28) and was stored at 4\(^\circ\)C in dark until further use for up to 14 days.

\textbf{Figure 28: Representative purification profile of ¹²⁵I-PAI-2}

Radiolabelling and purification of ¹²⁵I-PAI-2 were performed as described earlier. The radioactivity of 10 µl aliquots from the 0.5 ml fractions collected was measured and plotted (Y-axis) against the elution volume required to obtain that fraction (X-axis). The first peak (i.e. fraction 4) represents purified ¹²⁵I-PAI-2 whereas the second peak represents free ¹²⁵I.
The percentage of free $^{125}$I in the purified $^{125}$I-PAI-2 solution was determined by instant thin layer chromatography (Section 2.2.B.5.). The purity of $^{125}$I-PAI-2 was generally more than 95 % ($97 \pm 1 \%, n = 4$) indicating that the majority of free $^{125}$I was removed from $^{125}$I-PAI-2.

4.2.A.1. Determination of Specific Radioactivity

The specific radioactivity of $^{125}$I-PAI-2 was determined using two methods; autoradiography of SDS-PAGE fractionation and utilising the data from PD-10 column purification (Bolton, 1977). This was done due to the contaminating presence of BSA in the preparations as 0.1 % BSA (fraction V§) was used in the PD-10 column purification.

In the first method, both $^{125}$I-PAI-2 and $^{125}$I-uPA:PAI-2 complexes were fractionated on the same gel as PAI-2 standards of known protein amounts (Figure 29 – A). Autoradiogram of this showed that all of the $^{125}$I-PAI-2 was complexed with uPA, thus the 98 kDa band (Lane 2 Figure 29 – B) contains all the $^{125}$I-PAI-2 added to the reaction. This indicated that some of the protein in the 47 kDa band (Lane 1 Figure 29 – A) was due to the contaminating presence of BSA which mostly fractionated at around this molecular weight (data not shown). Since uPA:PAI-2 form 1:1 complexes, then 50 % of the protein in the 98 kDa band (Lane 2 Figure 29 – A) is due to PAI-2. The amount of $^{125}$I-PAI-2 in the aliquot (10 µl) used was interpolated by comparison of the intensity of the 98 kDa band (Lane 2 Figure 29 – A) to the standard curve constructed for the relationship between amounts of PAI-2 protein standards (Lanes 3 – 6 Figure 29 – A) and staining intensity (data not shown).

§ BSA was used in PD-10 purification in order to minimise the non-specific binding of the small amounts of PAI-2 used to the column bed or plastic.
Figure 29: Representative SDS-PAGE and autoradiogram of \(^{125}\text{I}\)-PAI-2

\(^{125}\text{I}\)-uPA:PAI-2 complexes were generated as described in Section 2.2.B.4 except that a molar excess of uPA was used. (A) Samples were fractionated by reducing 12 % SDS-PAGE, (B) the gel was exposed to phosphor screen for 3 h and the screen was scanned, then the gel was stained with Coomassie blue, destained and documented. **Lane 1.** \(^{125}\text{I}\)-PAI-2, **lane 2.** \(^{125}\text{I}\)-uPA:PAI-2. Equivalent amount of \(^{125}\text{I}\)-PAI-2 (10 µl) was used for lanes 1 and 2. **Lanes 3 – 6:** PAI-2 standard solutions - 0.1, 0.4, 0.8 and 1.0 µg respectively. The position and size of bands (in kDa) of the molecular weight marker are shown at the right. SDS-PAGE and autoradiography produced the amount of \(^{125}\text{I}\)-uPA:PAI-2 complex (98 kDa). This was converted to the amount of \(^{125}\text{I}\)-PAI-2 using molar equivalence since 1 mole of complex is equivalent to 1 mole of PAI-2.

The second method for determination of protein concentration utilised the data from PD-10 column purification (Bolton, 1977). Briefly, the difference between the total radioactivity used and the activity eluted as free \(^{125}\text{I}\) was
calculated to represent PAI-2-bound radioactivity (data not shown). The radioactivity of the selected purified fraction over the total bound radioactivity represented the percentage recovery of protein in the purified fraction (e.g. 6.3% for preparation shown in Figure 28). The amount of PAI-2 in the purified fractions was calculated by the percentage recovery and the total amount of PAI-2 used (6.3 µg for preparation in Figure 28). The estimated protein concentration of ¹²⁵I-PAI-2 preparations was almost identical using the two different methods and the average was used (Table III). Thus, specific radioactivity (s.a.) could be calculated and this was based on the average protein concentration derived using the above two methods, radioactivity in each sample and the purity of ¹²⁵I-PAI-2 preparations (Table III).

4.2.B. PREPARATION OF PAI-2-CY5 FOR INTERNALISATION STUDIES

PAI-2 was labelled with Cy5 fluorochromes as per manufacturer’s instructions of the Cy5 mono-reactive dye pack (Amersham Biosciences, Uppsala, Sweden). PAI-2-Cy5 was separated from free Cy5 by size-exclusion chromatography (see Section 4.2.B.1.); however BSA was not included in the equilibrium or elution buffer since large amounts of PAI-2 were used for the conjugation reactions. Purified PAI-2-Cy5 was stored in the dark at 4°C until further use. The protein concentration and the dye/protein ratio (D/P) for PAI-2-Cy5 preparation were calculated as per manufacturer instructions**. PAI-2-Cy5 retained the ability to form SDS-stable complexes with uPA (data not shown).

** D/P = [Cy5]/[PAI-2] = (A₆₅₀ / εCy5)/(A₂₈₀ - (0.05 x A₆₅₀) / εPAI-2)

Concentration is in molar units, A: is the absorbance and ε is the extinction coefficient (M⁻¹ cm⁻¹)
Table III: Properties of the purified $^{125}$I-PAI-2 preparations

<table>
<thead>
<tr>
<th>$^{125}$I-PAI-2 Preparation</th>
<th>PAI-2 Concentration (ng/µl)$^a$</th>
<th>PAI-2 Concentration (ng/µl)$^b$</th>
<th>Radioactivity (cpm/µl)</th>
<th>% Purity</th>
<th>Specific radioactivity (cpm/µg)</th>
<th>Specific radioactivity (cpm/nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>14</td>
<td>$1.44 \times 10^5$</td>
<td>99.4%</td>
<td>$1.28 \times 10^7$</td>
<td>$6.01 \times 10^8$</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1.6</td>
<td>$5.03 \times 10^4$</td>
<td>96.3%</td>
<td>$4.07 \times 10^7$</td>
<td>$1.91 \times 10^9$</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>2.1</td>
<td>$7.20 \times 10^4$</td>
<td>96.7%</td>
<td>$4.35 \times 10^7$</td>
<td>$2.05 \times 10^9$</td>
</tr>
</tbody>
</table>

PAI-2 concentration was determined by (a) SDS-PAGE and autoradiography method or (b) PD-10 column-based method. Radioactivity was determined by counting the radioactivity of a known volume of $^{125}$I-PAI-2 solution. The specific radioactivity was calculated as the radioactivity concentration (corrected to 80% counting efficiency of detector) multiplied by the percentage purity (bound activity) and divided by the average protein concentration. The specific activity was converted from units of cpm/µg to cpm/nmole using the molecular weight of PAI-2.
4.2.C. Radioligand binding studies using $^{125}$I-PAI-2

The formation of complexes between $^{125}$I-PAI-2 and cell-surface uPA in terms of PAI-2 binding reflects the inhibition of uPA activity and can be used to analyse inhibitory kinetics of PAI-2 at the cell surface. Radioligand binding studies were performed using $^{125}$I-PAI-2 of known concentration and specific activity. Metastatic and non-metastatic breast carcinoma cell lines were prepared and resuspended in binding buffer as described earlier (Section 2.2.A.1.).

Initial binding experiments were performed in order to estimate the time required for saturation binding and establish a linear correlation between binding and cell number. Cells were incubated at 4°C with $^{125}$I-PAI-2 ($\sim 5 \times 10^5$ cpm/ml) in the presence or absence of unlabelled PAI-2. Triplicate aliquots (200 µl) were removed at the specified time point and cells were processed for radioactivity counting as described below. The relation between cell number and binding of $^{125}$I-PAI-2 was established by the incubation of increasing cell numbers at 4°C for the time required to reach saturation with $5 \times 10^5$ cpm/ml of $^{125}$I-PAI-2 in the absence or presence of unlabelled PAI-2. The amount of PAI-2 bound was plotted against the number of cells. Further binding experiments were performed using the time required to reach saturation and number of cells which falls in a linear correlation in the relationship between the amounts of bound PAI-2.

Generally, after binding of $^{125}$I-PAI-2 to cells, cells were processed for radioactivity counting and analysis as follows. Cells were washed using ice-cold 1 x PBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$, lysed using 200 µl of 0.1 M NaOH and counted using the $\gamma$-counter normalised for $^{125}$I counting. The total binding of PAI-2 was measured as the binding of $^{125}$I-PAI-2 in the absence of any
inhibitors, whereas the non-specific binding was measured as the binding of $^{125}$I-PAI-2 to cells in the presence of 100-fold molar excess of unlabelled PAI-2. The specific binding in all assays was calculated as the difference between the total and non-specific binding. Binding was initially measured in units of radioactivity relative to the number of cells used (i.e. cpm per million cells). This binding was converted to be expressed in units of femtomole (fmole) per million cells using the specific activity of $^{125}$I-PAI-2 used (cpm/mole). Data from all binding experiments was analysed using the functions specified in the radioligand binding analysis software, GraphPad® prism (v 3.03).

4.2.D. INTERNALISATION STUDIES USING PAI-2-CY5

PAI-2-Cy5 and confocal microscopy were used to detect the fate of PAI-2 on MDA-MB-231 and T-47D cells either detached or attached to cover slips. For detached cells, cells were prepared as described earlier (Section 2.2.A.1.), washed and resuspended in ice-cold phenol red-free binding buffer at 1 x 10⁶ cells/ml. The cell solution was adjusted to 37°C and PAI-2-Cy5 was added at a final concentration of 100 nM. Samples were removed at specified time points and diluted 1:25 in ice-cold phenol red-free binding buffer in order to stop the reaction. Samples were centrifuged at 652 x g for 3 min at 4°C and pellets were washed with ice-cold PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ and analysed immediately by confocal microscopy (as described in Section 2.2.A.3.). For attached cells, cells grown for 48 h on cover slips in 6-wells plates were washed and activated with plasminogen (5 µg/ml in RPMI-1640 for 5 min at RT). Cells were washed four times using PBS containing 1 mM CaCl₂ and 1 mM MgCl₂, temperature was adjusted to 37°C and pre-warmed phenol red-free binding buffer containing 100 nM PAI-2-Cy5 was added. At specified time points, ice-cold phenol red-free binding buffer was added to dilute PAI-2-Cy5
solution then the total volume was immediately removed. Cells were washed gently 5 times with ice-cold 1 x PBS containing 1 mM CaCl₂ and 1 mM MgCl₂, cover slip mounted on glass slide and analysed immediately by confocal microscopy. In addition, internalisation was compared between cells before and after blocking of uPA by pre-incubation of cells with 20 µg/ml anti-B-chain uPA monoclonal antibody at 4°C. Cells pre-incubated with exogenous uPA (100 nM at 4°C for 30 min) were also compared to control cells.

Endosomal and lysosomal markers conjugated with fluorochromes were utilised in order to detect whether PAI-2-Cy5 localised into these organelles. Cells were incubated for 20 min in ice-cold phenol red-free binding buffer containing 100 nM of PAI-2-Cy5 and 50 µg/ml transferrin-Alexa488 (endosomal marker Van Dam et al., 2002). Cells were washed with PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ at room temperature and then resuspended in 37°C-prewarmed phenol red-free binding buffer containing 75 nM LysoTracker® Yellow DND-68 (lysosomal marker). Samples were removed at specified time points, washed with ice-cold PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ and analysed immediately by confocal microscopy.

In order to confirm lysosomal degradation of any internalised uPA:PAI-2 complexes, lysates of MDA-MB-231 cells pre-incubated with 100 nM PAI-2-Cy5 at 37°C for specified periods of time were fractionated by 12 % SDS-PAGE under reducing conditions. The resulting gel was scanned using the red fluorescence scanner then Coomassie blue stained (as described in Sections 2.2.B.2. and 2.2.B.3.) The acquired Cy5 fluoro-image was superimposed onto the Coomassie blue stained gel and the molecular weight of the observed bands in the Cy5 scan was calculated using the standard molecular weight marker in the Coomassie blue stained gel.
4.2.E. Effect of PAI-2 on cell-surface uPA/uPAR levels

The effect of PAI-2 binding to cell-surface uPA on uPA/uPAR was analysed by flow cytometry. MDA-MB-231 cells pre-activated with plasminogen were incubated in the absence or presence of 100 nM of PAI-2 in binding buffer at 37°C. At specified time points, samples were removed, washed and then subjected to indirect immunofluorescence detection of cell-surface uPA and uPAR as described earlier (Section 2.2.A.2.). Anti human A-chain uPA monoclonal antibody was used since PAI-2 inhibits uPA by interaction at the uPA B-chain which may sterically hinder anti-B chain antibody access hence inhibit detection by flow cytometry. In order to test dose-dependency of the effect of PAI-2 on cell-surface uPA, this assay was performed using different concentrations of PAI-2 (1, 10 and 100 nM).

The effect of PAI-2 on the total capacity of cells to bind uPA was assayed by incubation of cells with 100 nM of PAI-2 at 37°C and samples were removed at specified time points. The cells were adjusted to 4°C to inhibit any further possible endocytosis then incubated in the absence (endogenous uPA) or presence of 100 nM exogenous uPA (total maximal uPA) for 30 min on ice prior to analysis of cell-surface uPA by flow cytometry. The difference between the total maximal uPA and the endogenous uPA levels was used to represent the binding of exogenous uPA (i.e. unoccupied uPAR) under the effect PAI-2 internalisation over time.

Finally, the effect of uPAR occupancy on the effect of PAI-2 on cell-surface uPA was assayed on cells pre-incubated with 100 nM of exogenous uPA in binding buffer at 4°C (i.e. saturated cells) then incubated with 100 nM of PAI-2 in binding buffer at 37°C. Samples were removed and washed and cell-surface uPA level was measured by flow cytometry.
4.3. RESULTS

4.3.A. QUANTIFICATION OF $^{125}$I-PAI-2 BINDING TO CELL-SURFACE uPAR-BOUND uPA

4.3.A.1. JUSTIFICATION FOR PERFORMING BINDING EXPERIMENTS AT 4°C

The use of uPA colorimetric substrates was not suitable to assay cell-surface uPA activity since cell-viability was greatly reduced (> 90 % dead) within few minutes of incubation (data not shown). To the best of my knowledge, uPA:PAI-2 complex formation at 4°C over short periods of time has not been shown previously. Since cell binding experiments with $^{125}$I-PAI-2 required incubation at 4°C to prevent possible endocytosis, confirmation of complex formation at 4°C was essential in order to reflect complete inhibition of uPA activity. Incubation of molar excess of PAI-2 with uPA in fluid phase, to mimic cell binding studies where saturating amount of PAI-2 would be added to cells, showed that SDS-stable complexes between PAI-2 and uPA formed after 15 min at 4°C (Figure 30). The conversion of the synthetic colorimetric uPA substrate, Spectrozyme®-UK, was entirely inhibited when equimolar amounts of PAI-2 were incubated for 15 min with uPA in solution at 4°C (data not shown). Thus, complex formation between $^{125}$I-PAI-2 and cell-surface uPA at 4°C should occur and would reflect the inhibition of uPA activity.

Initial binding experiments showed that complex formation on the metastatic MDA-MB-231 cells reached equilibrium by 30 min of incubation at 4°C using 1.5 nM $^{125}$I-PAI-2 (data not shown). As only a small fraction of the total $^{125}$I-PAI-2 added was bound to cells, this indicated that PAI-2 ligand was not depleted in the assay (Motulsky and Christopoulos, 2004). Using this concentration of $^{125}$I-PAI-2 and 2 h incubation time, a linear correlation was established between the amount of PAI-2 bound and cell concentration (1 – 5 x 10⁶ cells/ml) (data not shown). Thus, subsequent experiments were performed
using ~ 1.5 nM $^{125}$I-PAI-2 and 1 - 4 x $10^6$ cells/ml and 1 - 2 h incubation. Furthermore, $^{125}$I-PAI-2 binding assays did not affect cell viability as judged by trypan blue staining compared to control cells (incubated in the absence of PAI-2) performed on samples from each individual assay (data not shown).

![Figure 30: Formation of uPA:PAI-2 complexes at 4°C in fluid phase](image)

A 4-fold molar excess of PAI-2 was incubated with uPA (containing both high [55 kDa] and low [33 kDa] molecular weight forms) at 4°C. Equal aliquots were removed after 15 min (Lane 3), 30 min (Lane 4), 45 min (Lane 5), 60 min (Lane 6) and 120 min (Lane 7) and fractionated by reducing 12 % SDS-PAGE. PAI-2 alone (4 µg, Lane 2), uPA alone (1 µg, Lane 1) and 4-fold molar excess PAI-2 incubated with uPA for 120 min at 37°C (Lane 8) were also fractionated as controls.

A series of $^{125}$I-PAI-2 binding experiments confirmed that PAI-2 binding capacity is directly related to the amount of cell-surface uPA which indicate that $^{125}$I-PAI-2 binding to the cell-surface was reflective of uPA inhibition and that uPA is the only specific cell-surface protease inhibited by PAI-2 on human breast carcinoma cell lines. First, the specific $^{125}$I-PAI-2 binding on high uPA expressing MDA-MB-231 cells was significantly higher (~ 10-fold) than that on low uPA-expressing MCF-7 and T-47D cells ($p < 0.05$ n =3, Figure 31). Furthermore, for each cell line PAI-2 binding was significantly enhanced by plasminogen pre-treatment (to activate any receptor-bound pro-uPA) (Figure
These data also indicated that a proportion of the total endogenous cell surface uPA was in the pro-uPA form.

Figure 31: $^{125}$I-PAI-2 binding to metastatic and non-metastatic breast cancer cells
MDA-MB-231, MCF-7 and T-47D cells were incubated in the absence (clear bars) or presence (filled bars) of 5 µg/ml plasminogen at room temperature. Cells were washed and incubated with 1.5 nM $^{125}$I-PAI-2 at 4°C for 2 h in the absence (total) or presence (non-specific) of 150 nM of unlabelled PAI-2. Specific binding was calculated and data presented are the mean ± SEM (n = 3). One asterisk denotes significant difference in PAI-2 binding to the same cell line under different treatment, whereas two asterisks denote significant difference between different cell lines subjected to the same treatment ($p < 0.05$).

Secondly, when cell-surface uPA was either pre-blocked using a neutralizing monoclonal antibody, or a uPA specific active-site alkylating agent (EGR-CMK) or acid-stripped prior to $^{125}$I-PAI-2 incubation, the total $^{125}$I-PAI-2 binding to MDA-MB-231 cells was reduced to the same levels obtained in the presence of excess unlabelled PAI-2 (i.e. non-specific binding, approximately 25% of total binding) (Figure 32). These data indicate that all of the specific binding of PAI-2 to these cells can be attributed to cell surface uPAR-bound uPA and that there may be some non-specific binding sites for PAI-2. Similar results...
were obtained using HCT116 colorectal (Hang et al., 1998) and PC-3 prostate (Al-Ejeh et al., 2004) carcinoma cells. This indicates that these treatments sufficiently decreased binding to non-specific binding levels and indicate that uPA is the only specific target for PAI-2 on breast, colorectal and prostate carcinoma cells. When surface-stripped cells were then incubated with exogenous uPA prior to $^{125}$I-PAI-2 incubation, total $^{125}$I-PAI-2 binding was enhanced by 2.3-fold compared to control cells (Figure 32). These data also suggest the presence of unoccupied uPAR sites on non-treated cells.

![Figure 32: $^{125}$I-PAI-2 binding to MDA-MB-231 cells under different treatments](image)

MDA-MB-231 cells pre-activated with plasminogen were incubated in the absence (control) or presence of anti-B-chain uPA (10 µg/ml of the #394 monoclonal antibody) or the active-site alkylating agent (EGR-CMK, 0.5 mM) for 30 min at 4°C. Other cells were incubated for 5 min in acid wash solution (acid strip: 100 mM NaCl, 50 mM glycine, pH 2.8, Stoppelli et al., 1986). All cells were diluted 1:10 in binding buffer to neutralise the incubation condition and washed with PBS. Stripped cells were incubated in the absence of presence of 100 nM uPA for 30 min at 4°C and then washed. Treated cells were incubated with 1.5 nM $^{125}$I-PAI-2 for 2 h at 4°C whereas control cells were similarly incubated with $^{125}$I-PAI-2, however in the absence (control) or presence of 150 nM unlabelled PAI-2 (excess unlabelled). Cells were washed and lysed for radioactivity counting. Asterisks denote significant differences in amounts of PAI-2 bound compared to control MDA-MB-231 cells ($p < 0.05, n = 3$).
Cell-surface endogenous uPA levels were significantly enhanced (2-fold, Figure 33 – A) on uPAR overexpressing T-47D cells (2-fold increase in uPAR, data not shown) compared to controls (i.e. untreated or mock-transfected cells, as described in Section 3.3.B.1). Concomitantly, a significant increase in PAI-2 binding capacity was also observed on uPAR-transfected T-47D cells compared to control T-47D cells ($p < 0.05$ $n = 3$, Figure 33 – B).

**Figure 33: Specific binding of $^{125}$I-PAI-2 to control and transiently uPAR-overexpressing T-47D cells**

T-47D cells were transiently transfected with pcDNA3/uPAR$^+$ plasmid as per optimised method (Section 3.3.A.). (A) Control and transfected T-47D cells (T-47D/uPAR$^+$) were assayed for uPA expression by flow cytometry using the #394 anti-uPA monoclonal antibody. Data are presented as the mean fluorescence intensity (MFI) ± SEM ($n = 3$). (B) Control and transfected T-47D cells were incubated with 1.5 nM $^{125}$I-PAI-2 in the absence or presence of 150 nM of unlabelled PAI-2. The specific binding was calculated and data presented are the mean ± SEM ($n = 3$). Asterisks denote significant differences between control and transfected T-47D cells ($p < 0.05$). Transfected cells showed 2-fold increase in cell-surface uPAR (data not shown).
Finally, when simultaneous assays for $^{125}$I-PAI-2 binding and immunofluorescent detection of uPA were performed, a linear correlation was observed between PAI-2 binding capacity and cell-surface uPA levels on the various cancer cell lines (Figure 34). This was expected since PAI-2 complexes to uPA at 1:1 molar stoichiometric ratio.

![Figure 34: Correlation between cell-surface uPA and $^{125}$I-PAI-2 binding](image)

The specific $^{125}$I-PAI-2 binding on cultured breast cancer cells was plotted against the cell-surface uPA simultaneously assayed for the same cell line. Data are presented as mean ± SEM ($n = 3$) for PAI-2 binding and MFI ± SEM ($n = 3$) for cell-surface uPA level. The $r^2$ for the linear correlation was 0.99 and the data did not deviate from the fitted line as judged by runs test (degrees of freedom = 28). Asterisks mark significant differences in cell-surface uPA and PAI-2 binding level compared between the different cell lines used. There was no significant difference between MCF-7 and T-47D cells for cell-surface uPA or PAI-2 binding level.
4.3.A.2. PAI-2 INHIBITION PARAMETERS AT THE CELL SURFACE

Saturation assays showed that PAI-2 bound in a saturable and specific manner to MDA-MB-231 cells (representative saturation curve shown in Figure 35). As expected, saturation binding best fitted a single-binding site model since PAI-2 inhibits uPA by forming an irreversible 1:1 stoichiometric covalent complex with the protease. The maximal amount of PAI-2 complexed with uPA and the concentration PAI-2 required for half-inactivation†† (inhibition constant, \(K_I\)) of uPA on the surface MDA-MB-231 cells were calculated by the fitted model from three separate experiments and summarised in Table IV. The average maximal binding was calculated to be 5.0 ± 0.2 fmoles of \(^{125}\text{I}-\text{PAI-2}\) per million cells and the average \(K_I\) was 81 ± 9 pM.

Maximal binding of PAI-2 was significantly different between one of the assays compared to the other two assays (5.9 ± 0.2 compared to 4.6 ± 0.1 and 4.5 ± 0.1 fmoles per million cells, \(p < 0.05\)). In assay 1, cells were cultured for 72 h compared to 48 h for assays 2 and 3. This is explained by the fact that cell-surface uPA levels are higher after 72 h in culture without passage compared to 48 h and that PAI-2 binding capacity is directly correlated to cell-surface uPA (Figure 34). Nevertheless, the \(K_I\) was not significantly different between the three assays \((p > 0.05)\), indicating that inhibition was mechanism-dependent rather than uPA-level dependent. As further proof to the independency of \(K_I\) from cell-surface uPA levels, saturation analysis of the non-metastatic, low cell-surface uPA expressing T-47D cells which bind less PAI-2 (Section 4.3.A.1) also produced a similar \(K_I\) to that reported for MDA-MB-231 cells (83 ± 3 pM, Figure 36).

†† \(K_I\) = concentration of PAI-2 required to reach half the maximal amount of PAI-2 complexed with cell-surface uPA. Since complex formation is reflective of inhibition then \(K_I\) = the concentration of PAI-2 required to inhibit half of cell-surface uPA (Silverman, 1996).
Figure 35: Saturation binding of $^{125}$I-PAI-2 to MDA-MB-231 cells at 4°C

$^{125}$I-PAI-2 was measured in the absence (▼, total binding) or presence (●, non-specific binding) of a 100-fold molar excess of unlabeled PAI-2 at each concentration. Specific binding (■) was calculated by the subtraction of non-specific binding from the total binding. Data was fitted best to a one binding site hyperbolic model ($r^2 = 0.97$) from which the concentration of $^{125}$I-PAI-2 required to achieve half-maximal binding ($K_I$) as well as the maximal binding was calculated. Irreversible binding of $^{125}$I-PAI-2 directly reflects the inactivation of uPAR-bound uPA. Each data point represents the mean ± standard error of the mean (SEM, n = 3).

Table IV: Inhibition parameters of $^{125}$I-PAI-2 binding to MDA-MB-231 cells

<table>
<thead>
<tr>
<th>Saturation Assay</th>
<th>$K_I$ (pM)</th>
<th>Maximal Binding (fmoles/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81 ± 4</td>
<td>5.9 ± 0.2*</td>
</tr>
<tr>
<td>2</td>
<td>83 ± 6</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>79 ± 5</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>81 ± 9</td>
<td>5.0 ± 0.2</td>
</tr>
</tbody>
</table>

Saturation assays were performed using three different preparations of $^{125}$I-PAI-2. Data were fitted to one-binding site hyperbolic function as per Figure 35. None of the data deviated from the fitted model ($r^2 = 0.93 - 0.97$) as judged by the runs test performed by GraphPad® (degrees of freedom = 18 – 21). The average $K_I$ and average maximal binding and their respective SEM from the three experiments are shown. Asterisk denote significant difference of maximal binding in assay 1 compared to assay 2 and 3 ($p < 0.05$, n = 20).
Figure 36: Saturation binding of ¹²⁵I-PAI-2 to T-47D cells at 4°C

T-47D cells (2 x 10⁶ cells/ml) pre-incubated with 5 µg/ml plasminogen (at room temperature for 5 min) were incubated in triplicate with increasing concentration of ¹²⁵I-PAI-2 in the absence or presence of 100-fold molar excess of unlabelled PAI-2. After 2 h incubation, cells washed with PBS and lysed for activity counting. The specific binding was calculated and data presented are the mean ± SEM. Data was fitted to one-binding site hyperbolic curve (r² = 0.95) and did not deviate from the fitted model as judged by the runs test (degrees of freedom = 17).

The kinetics of formation of ¹²⁵I-PAI-2 complexes with cell-surface uPA was assayed at both 4°C and 37°C (Figure 37). The rate of complex formation was reflective of the rate of inhibition (k_{inact}) of cell-surface uPA and was calculated to be 0.32 ± 0.03 and 0.48 ± 0.04 min⁻¹ at 4°C and 37°C, respectively. Based on the equation which describes the kinetics of mechanism-based enzyme inactivators# the time required to reach half inhibition (t₁/₂) was

# t₁/₂ = \frac{\ln 2}{k_{inact}} + \frac{\ln 2\cdot K_i}{k_{inact}\cdot [I]}  

Where t₁/₂ is the time for half inactivation, [I] concentration of the inactivator, K_i is the inactivation constant ([I] required for half inactivation) and k_{inact} is the rate of inactivation. The term inhibition is used here instead of inactivation to avoid confusion as PAI-2 is described as uPA inhibitor.
calculated to be $2.3 \pm 0.1$ and $1.5 \pm 0.1$ min at $4^\circ C$ and $37^\circ C$, respectively. These differences in $k_{\text{inact}}/t_{1/2}$ inhibition at $4^\circ C$ compared to $37^\circ C$ indicated that inhibition of cell-surface uPA was faster at $37^\circ C$ than that at $4^\circ C$. The maximal amount of bound $^{125}\text{I}-\text{PAI-2}$ were not significantly different between cells incubated at $4^\circ C$ and $37^\circ C$, indicating that the total amount of complexes formed was temperature independent.

![Figure 37: Kinetics of $^{125}\text{I}-\text{PAI-2}$ association to MDA-MB-231 cells](image)

MDA-MB-231 cells ($1 \times 10^6$ cells/ml) pre-activated with plasminogen were incubated with 1.5 nM of $^{125}\text{I}-\text{PAI-2}$ in the absence or presence of 150 nM unlabelled PAI-2 at $4^\circ C$ (○) and $37^\circ C$ (▲). At the specified time points aliquots were removed for radioactivity counting. The specific binding of $^{125}\text{I}-\text{PAI-2}$ (fmoles/10$^6$ million cells) was calculated and data presented are the mean ± SEM (n = 3). Data was fitted to one-phase exponential association function ($r^2 > 0.96$) and the curves did not deviate from the model. Only the rate of complex was found to be significantly different ($p < 0.05$).
4.3.B. THE FATE OF CELL-BOUND PAI-2

As shown in Figure 37, maximal PAI-2 binding to MDA-MB-231 cells occurs within 15 min at 4°C. As incubation at 4°C prevents any endocytosis that might occur, the majority of bound PAI-2 should be at the cell surface. This was confirmed using PAI-2-Cy5 as the ligand for analysis of PAI-2 binding by confocal microscopy (Figure 38). That binding was uPA-dependent was further confirmed by pre-blocking cells with anti-B-chain uPA monoclonal antibody (Figure 38). Pre-incubation of cells with exogenous 100 nM uPA, which saturates all cell-surface uPAR (Stillfried G., manuscript in preparation), lead to an increase in cell-surface associated PAI-2-Cy5 (Figure 38).

Confocal microscopy analysis of MDA-MB-231 cells incubated with PAI-2-Cy5 at 37°C showed that PAI-2 was mainly localised in intracellular vesicles after 15 min (Figure 39 - B). Internalisation of PAI-2-Cy5 at 37°C was specific and selective since incubation of cells with BSA-Cy5 did not show any cell-associated fluorescence (Figure 39 – C). Indeed, pre-blocking of cell-surface uPA on MDA-MB-231 cells with the anti-B chain uPA (#394) monoclonal antibody not only inhibited the binding of PAI-2 (see Figure 38) but also the internalisation of PAI-2-Cy5 (Figure 40). In contrast, when cells were pre-incubated with exogenous uPA in order to saturate cell-surface uPAR there was a significant increase in PAI-2-Cy5 detected in the cytoplasm (Figure 40). This suggests that PAI-2 not only binds in a uPAR-bound uPA-dependent manner (see Figure 38) but is also internalised via a uPA-dependent pathway.
Figure 38: Binding of PAI-2-Cy5 to MDA-MB-231 cells at 4°C

MDA-MB-231 cells (1 x 10^6 cells) pre-activated with plasminogen were incubated in the absence (control: I, V, and IX) or presence of 20 µg/ml mouse IgG1 (isotype: II, VI and X), 20 µg/ml anti-B-chain uPA (#394) monoclonal antibody (anti-uPA: III, VII and XI), or 100 nM of exogenous uPA for 30 min at 4°C (exogenous uPA: IV, VIII, and XII). Cells were washed and incubated with 5 µg/ml of PAI-2-Cy5 at 4°C for 15 min then washed with PBS and analysed immediately by confocal microscopy. Samples were analysed using the same settings for photomultiplier voltage and pinhole (2.8) except for cells incubated with exogenous uPA where the pinhole was reduced to half pinhole value (1.4) of other treatments due to the high intensity of the signal. Only a representative image is shown for each treatment selected from > 10 images obtained from assays (> 2) repeated independently. The scale bars in all transmission images (I – IV) and overlays of PAI-2-Cy5 signal with the transmission images (IX – XII) represent 10 µm and cell viability was higher than 95 % in all samples as determined by trypan blue stain.
MDA-MB-231 cells (1 x 10^6 cells/ml) pre-activated with plasminogen were incubated with 5 µg/ml of PAI-2-Cy5 at 37°C for (A) 0 min or (B) 15 min. (C) Cells pre-activated with plasminogen were also incubated with 5 µg/ml BSA-Cy5 at 37°C for 15 min. Aliquots were removed, washed with ice-cold PBS and analysed immediately by confocal microscopy. This assay was repeated twice using different cultures of cells and only a representative image is shown from > 10 images obtained in each assay. The scale bars in all transmission images represent 5 µm and cell viability was higher than 95 % in all samples as detected by trypan blue stain.

**Figure 39: Internalisation of PAI-2-Cy5 by MDA-MB-231 cells at 37°C**
MDA-MB-231 cells (1 x 10^6 cells) pre-activated with plasminogen were incubated in the absence (control) or presence of 20 µg/ml anti-B-chain uPA (#394) monoclonal antibody (anti-uPA) or 100 nM of exogenous uPA for 30 min at 4°C (exogenous uPA). Cells were washed and incubated 5 µg/ml of PAI-2-Cy5 for 15 min at 37°C. Samples were washed with ice-cold PBS and analysed immediately by confocal microscopy. Samples were analysed using the same settings for photomultiplier voltage and pinhole except for cells incubated with exogenous uPA where the pinhole was reduced to half the value for pinhole of other treatments due to the high intensity of the signal. The scale bars in all transmission images represent 5 µm and cell viability was higher than 95% in all samples as detected by trypan blue stain. Samples shown are from two representative experiments (n > 10 experiments).
4.3.B.1. Compartmentation and degradation of internalised PAI-2

Transferrin-Alexa488 (endosomal marker) and LysoTracker® Yellow DND-68 (lysosomal marker) were used for the visualisation of these compartments in T-47D and MDA-MB-231 cells incubated with PAI-2-Cy5. Co-localisation of the endosomal and lysosomal markers after 15 min at 37°C showed three different areas of compartmentation in T-47D cells (Figure 41-A). There were areas where only endosomal marker was observed and areas where only lysosomal marker was observed, explained by early endosomes and lysosomes, respectively. Finally, endosomes co-localised with lysosomes at some areas of the cytoplasm of these cells, suggesting the fusion of late endosomes and lysosomes at these areas. In the case of MDA-MB-231 cells, transferrin-Alexa488 always co-localised with LysoTracker® Yellow DND-68 after 15 min at 37°C (Figure 41-B). Since differentiation between these two markers was possible in the case of T-47D cells, the co-localisation of transferrin and LysoTracker® in MDA-MB-231 cells may be due to inability of these cells to shuttle transferrin-Alexa488 from endosomes to the cell exterior which is a requirement for detecting early endosomes (Van Dam et al., 2002). Thus, co-localisation of internalised PAI-2-Cy5 with transferrin-Alexa488 in MDA-MB-231 was not informative as endosomal compartmentation was not clear.

As shown in Figure 41-A, T-47D cells did not bind or internalise detectable amounts of PAI-2-Cy5 when analysed by confocal microscopy although PAI-2 binding was measured using 125I-PAI-2 on these cells (see Figure 36). This may be due to lower detection limit of fluorescent confocal microscopy compared to radio-detection methods in addition to the fact that T-47D cells express very little of uPA on the cell-surface. While a large proportion of internalised PAI-2-Cy5 co-localised with the lysosomal marker by 15 min of incubation at 37°C, a small amount of intracellular PAI-2-Cy5 was not localised
in lysosomes (Figure 41 – B). This non-lysosomal intracellular PAI-2-Cy5 at 15 min incubation was presumably in endosomes as after 45 min incubation all PAI-2-Cy5 localised in lysosomes (Figure 41 – C). The presumed endosomal and the evident lysosomal compartmentation of internalised PAI-2-Cy5 indicated the endocytosis of bound PAI-2 on metastatic MDA-MB-231 cells. As little PAI-2 bound to and is hence internalised by the non-metastatic low uPA-expressing T-47D cells, this further confirmed the uPAR-bound uPA-dependent pathway of PAI-2 internalisation.

Lysates of MDA-MB-231 cells incubated with PAI-2-Cy5 showed the presence of degradation fragments with a molecular weight less than 37 kDa the intensities of which increased over time (Lanes 3 - 5 Figure 42). The intensity of these degradation fragments was significantly lower for lysates prepared from MDA-MB-231 cells pre-treated with anti-B chain uPA monoclonal antibody before incubation with PAI-2-Cy5 (lane 2 in Figure 42). Thus, the presence of uPA:PAI-2 fluorescent degradation products further confirmed the localisation of internalised PAI-2-Cy5 in lysosomes.
A

Transferrin          LysoTracker           PAI-2-Cy5

Transferrin/LysoTracker overlay  transmission image  Triple overlay with transmission image

Figure continues next page...
C

Transferrin          LysoTracker          PAI-2-Cy5

Transferrin/PAI-2   LysoTracker/PAI-2   Triple overlay with
overlay              overlay          transmission image
Cells incubated with PAI-2-Cy5 and transferrin-Alexa488 for 20 min at 4°C were washed and incubated at 37°C with LysoTracker® Yellow DND-68. Cells were then washed at the specified times and analysed by confocal microscopy obtaining images sequentially using detectors for Cy5, Alexa488 and LysoTracker® fluorescence. (A) T-47D cells after 15 min incubation at 37°C, (B) MDA-MB-231 cells after 15 min incubation at 37°C and (C) MDA-MB-231 cells after 45 min incubation at 37°C. For (A) 6 panels are shown, which are Transferrin-Alexa488 (green), LysoTracker® (blue), PAI-2-Cy5 (red), Transferrin-Alexa488/LysoTracker® overlay, transmission image and Transferrin-Alexa488/LysoTracker®/PAI-2-Cy5 overlay superimposed on the transmission image (scale bars represents 10 µm). For each (B) and (C), 6 panels are shown, which are Transferrin-Alexa488 (green), LysoTracker® (blue), PAI-2-Cy5 (red), Transferrin-Alexa488/PAI-2-Cy5 overlay, LysoTracker®/PAI-2-Cy5 overlay and Transferrin-Alexa488/LysoTracker®/PAI-2-Cy5 overlay superimposed on the transmission image (scale bars represents 10 µm). Co-localisation of Transferrin-Alexa488 and PAI-2-Cy5 appears in yellow/orange, co-localisation of LysoTracker® and PAI-2-Cy5 appears in pink, co-localisation of Transferrin-Alexa488 and LysoTracker® appears in cyan and the co-localisation of Transferrin-Alexa488/LysoTracker®/PAI-2-Cy5 appears in white. Cell viability was higher then 95 % for all cells and only representative samples are shown.
Figure 42: Degradation of internalised PAI-2-Cy5
MDA-MB-231 cells pre-incubated with plasminogen were incubated with PAI-2-Cy5 at 37°C for 5 (Lane 3), 10 (Lane 4) and 15 (Lane 5) min prior to washing and lysing in reducing sample buffer and fractionation by 12% SDS-PAGE. MDA-MB-231 cells pre-incubated with plasminogen were pre-blocked with 10 µg/ml anti-B chain uPA monoclonal antibody for 30 min at 4°C prior to incubation with PAI-2-Cy5 for 15 min at 37°C (Lane 2) and PAI-2-Cy5 (0.33 µg, 7.5 fmoles) (Lane 1) were also fractionated as controls. (A) The SDS-PAGE was stained in Coomassie blue stain, destained and documented after (B) being scanned using the red fluorescence detector of the Storm® scanner. The Coomassie blue stained gel was superimposed on the fluorescence scan and the molecular weight marker bands (kDa) were marked on the scan.
4.3.C. FURTHER INSIGHTS INTO THE MECHANISM OF PAI-2 ENDOCYTOSIS

4.3.C.1. CLEARANCE OF CELL-SURFACE uPA

At 37°C in the absence of PAI-2, endogenous cell-surface uPA levels on MDA-MB-231 cells slowly decreased over the observation period (~10% loss by 60 min), indicating slow endogenous uPA clearance from the cell surface (Figure 43). A similarly slow turnover of cell-associated exogenous uPA has been shown for monocyctic cells that do not produce any detectable PAIs (THP-1 cells) (Estreicher et al., 1990). Since (1) endogenous cell surface PAIs are also not detectable on breast carcinoma cells used in this study (Gillian Stillfried, personal communications), (2) PAI-2 is internalised via a uPA/uPAR dependent pathway; and (3) PAIs affect cell-associated uPA clearance via uPAR endocytosis (Cubellis et al., 1990; Estreicher et al., 1990; Jensen et al., 1990), then exogenous PAI-2 should influence uPA disappearance. Indeed, the addition of 100 nM PAI-2 significantly accelerated the loss of cell surface endogenous uPA (Figure 43). This effect was most apparent within the first 10 min ($t_{1/2} = 1.14 \pm 0.06$ min in the presence of 100 nM PAI-2 vs. $t_{1/2} = 787 \pm 37$ min in the absence of PAI-2) and was dose-dependent ($t_{1/2} = 1.52 \pm 0.06$ and $3.07 \pm 0.04$ min in the presence of 10 and 1 nM PAI-2, respectively). The rate of PAI-2 mediated uPA loss from the cell surface was appreciably slower after 10 min in the presence of PAI-2 and, by 60 min in the presence of 100 nM PAI-2, approximately 30% of the total cell-surface endogenous uPA disappeared from the cell surface (Figure 43). This apparent stabilisation of cell-surface uPA levels at approximately 70 - 80% of initial cell-surface levels, even in the presence of high PAI-2 concentrations (i.e. 100 nM), appears to be at odds with the uPA-dependent endocytosis of the majority of cell-bound PAI-2 (refer to Figure 41). This suggested additional complex concurrent processes during endocytosis of the
uPA:PAI-2 complex involving uPAR occupancy levels, potential uPAR recycling and uPA secretion, which must lead to replenishment of cell-surface uPA, resulting in the apparent stabilisation of relatively high uPA levels.

![Graph showing PAI-2-mediated clearance of cell-surface uPA](image)

**Figure 43: PAI-2-mediated clearance of cell-surface uPA**
MDA-MB-231 cells pre-treated with plasminogen were incubated in the absence (●) or presence of 1 (■), 10 (▲) or 100 (▼) nM of PAI-2 at 37°C. Samples were removed at the specified time points, washed and analysed by flow cytometry for cell-surface uPA. Only fluorescence associated with viable cells was used and the data are displayed as the percentage of MFI over the maximal MFI observed at time zero (absence of PAI-2). Data points, % MFI ± SEM (n ≥ 3), were fitted to one-phase exponential decay curve and did not deviate from the fitted model as judged by runs test (degrees of freedom > 18).

### 4.3.C.2. EFFECT OF PAI-2 ON CELL-SURFACE uPAR

The effect of uPAR/uPA:PAI-2 complex formation and endocytosis on potential uPAR recycling and occupancy levels was assessed using two different methods. Firstly, endogenous levels of cell surface uPAR were directly measured by immunofluorescence and flow cytometry. During the first 10 min of incubation with PAI-2 the total cell-surface uPAR level decreased rapidly to ~40% of the maximal uPAR levels (i.e. at zero min in the presence of PAI-2).
(Figure 44). Between 10 and 15 min after incubation with PAI-2, uPAR cell-surface levels were restored to 86% of the maximal level.

![Figure 44: PAI-2-mediated clearance of cell-surface uPAR](image)

MDA-MB-231 cells pre-treated with plasminogen were incubated with 100 nM of PAI-2 at 37°C. At the specified time points cell-surface uPAR was measured using the #3934 antibody by flow cytometry. Only fluorescence associated with viable cells was used and the data are displayed as percentage of MFI over the maximal MFI observed at time zero (% MFI ± SEM, n = 3).

This result indicated that some uPAR recycling must take place. Given that not all uPAR disappeared from the cell surface, this further indicates that only a proportion of uPAR is occupied with endogenous uPA on this cell (refer to Figure 32). Thus, it appears that the effect of PAI-2 incubation is to cause internalisation of existing occupied uPAR and presumably recycling of unoccupied receptor to the cell surface. As suggested above, any uPA secretion that may occur as a result of complex endocytosis could bind to unoccupied uPAR (either pre-existing or recycled). Thus, the difference between the amount of endogenous uPA and the total amount of uPA the cells could potentially bind to the cell surface (i.e. measured after the addition of exogenous uPA) gives an
indication of the amount of unoccupied uPAR at any time after incubation with PAI-2. In agreement with previous experiments (refer to Figure 43), the endogenous cell-surface uPA level decreased by 30% by 10 min of incubation with 100 nM PAI-2, after which time it stabilised at this level (Figure 45 – A). Total uPA levels (endogenous uPA plus bound exogenous uPA) decreased to 65% by 10 min after incubation with PAI-2. However, unlike the endogenous uPA levels, total uPA levels were restored to 90% of the maximal levels (i.e. at zero min in the presence of PAI-2) between 10 and 30 min after incubation with PAI-2 (Figure 45 – B). Unoccupied uPAR was then calculated and represented graphically, and found to initially decrease by 40% in the first 10 min after incubation with PAI-2 (Figure 45 – C). By 30 min after incubation with PAI-2, unoccupied uPAR levels increased to 130% of that at time zero of incubation with PAI-2.

Assuming that unoccupied uPAR remains on the cell surface, the data suggests that while the majority of cell-surface uPAR/uPA:PAI-2 is internalised, cells may replenish cell-surface uPA from intracellular reservoir resulting in the decrease of unoccupied cell-surface uPAR and an overall stabilisation of cell surface uPA levels. After the first 10 min of internalisation, uPAR recycling may result in the observed restoration of cell-surface uPAR and the marked increase in unoccupied cell-surface uPAR between 10 and 30 min. Indeed, when unoccupied cell-surface uPAR was saturated with uPA by incubation with exogenous uPA prior to incubation with PAI-2, the clearance of cell-surface uPA was slower but greater percentage was cleared in comparison to previous uPA clearance data. As shown in Figure 46, cell-surface uPA continued to decrease rapidly after 10 min of incubation with PAI-2 (t1/2 = 9.1 ± 0.4 min). Stabilisation of cell-surface uPA occurred between 30 and 60 min where unoccupied uPAR would have been made available by recycling in order to bind secreted uPA.
Figure 45: Total cell-surface uPA capacity and endogenous cell-surface uPA during PAI-2 endocytosis

MDA-MB-231 cells pre-treated with plasminogen were incubated with 100 nM of PAI-2 at 37°C. At the specified time points samples were removed and processed as follows. (A) Cells were analysed for cell-surface uPA by flow cytometry (i.e. endogenous cell-surface uPA). (B) Cells were incubated with 100 nM exogenous uPA for 30 min at 4°C then analysed for cell-surface uPA by flow cytometry (i.e. total cell-surface uPA capacity). (C) The difference between B and A was calculated to represent the binding of exogenous uPA (i.e. unoccupied cell-surface uPAR). Only fluorescence associated with viable cells was used and the data are displayed as absolute MFI units ± SEM (n = 3).
Figure 46: The effect of uPAR saturation on PAI-2-mediated clearance of cell-surface uPA

MDA-MB-231 cells pre-treated with plasminogen were incubated with exogenous uPA (30 min at 4°C), washed then incubated with 100 nM of PAI-2 at 37°C. Samples were removed at the specified time points, washed and analysed by flow cytometry for cell-surface uPA. Only fluorescence associated with viable cells was used and the data are displayed as the percentage of MFI over the maximal MFI observed at time zero (absence of PAI-2). The MFI for cells incubated with exogenous uPA was 2-fold higher than normal cells indicating 50% of cell-surface uPAR was unoccupied (data not shown). Data points, % MFI ± SEM (n ≥ 3), were fitted to one-phase exponential decay curve and did not deviate from the fitted model as judged by runs test (degrees of freedom > 18).

4.4. DISCUSSION

The results presented in this chapter describe the irreversible inhibitory kinetics and fate of PAI-2 bound to the surface of human carcinoma cells and provides definitive evidence of uPAR-bound uPA mediated endocytosis of PAI-2. In order to investigate the cellular fate of PAI-2, it was essential to quantify the irreversible binding of PAI-2 to cell-surface uPAR-bound uPA and perform internalisation assays under saturating conditions. PAI-2 is known to be an efficient inhibitor of uPA activity (Ellis et al., 1990) and can form 1:1 SDS-stable complexes with ¹²⁵I-uPA on the cell surface (Estreicher et al., 1990). Therefore,
the amount of PAI-2 bound to cells and the concentration of PAI-2 required to saturate uPAR-bound uPA are directly reflective of the irreversible inhibition constant $K_i$. The very low $K_i$ value described here confirms the efficient and potent inhibition of cell surface uPAR-bound uPA by PAI-2 and falls in the range of the most potent antiviral drugs. For example, anti-HIV cyclic urea drugs, which are also mechanism-based protease inactivators, have $K_i$ values in the range of 18 - 88 pM (De Lucca et al., 1998). The $k_{\text{inact}}$ value measured for PAI-2 is in the range of typical reported values (0.001 – 1 min$^{-1}$) and describes a relatively rapid inhibitory mechanism (Silverman, 1996).

That uPAR-bound uPA is the only specific site for PAI-2 binding on human carcinoma cells is supported by the following evidence. Firstly, the one-binding site model best fitted the specific PAI-2 binding data thus indicating the presence of a single population of high-specificity target for PAI-2. Secondly, the specific binding of PAI-2 to cells on which the uPA active site was either specifically blocked or inactivated was completely inhibited. Thirdly, a linear correlation exists between cell-surface uPA levels and the PAI-2 binding capacity. Given that uPA was the only determinant for $^{125}$I-PAI-2 binding to breast cancer cells, the inhibition parameters derived in this study represents specific targeting of cell surface uPA by PAI-2. It should be noted, however, that a small proportion of the total binding could not be competed by excess PAI-2 (refer to Figure 32), suggesting the presence of non-specific, uPA-independent PAI-2 binding moiety/ies. The nature of this interaction has yet to be characterised.

The uPA-dependent binding of PAI-2 was also illustrated using PAI-2-Cy5 by confocal microscopy. At 4°C, PAI-2-Cy5 was localised at the cell-surface of MDA-MB-231 cells however, rapid and specific internalisation of PAI-2-Cy5 was observed when cells were incubated at 37°C. The rate of internalisation was
determined using a novel assay (developed by David Croucher, University of Wollongong) utilising the Alexa488/Alexa488 quenching antibody system and the human prostate carcinoma PC-3 cells (Al-Ejeh et al., 2004) and MDA-MB-231 cells (Unpublished manuscript by Amy Wyatt, School of Biological Sciences, University of Wollongong, 2003). Maximal PAI-2 internalisation by these cells was achieved over a similar time frame (within ~ 15 - 20 minutes) at 37°C to that described for ¹²⁵I-uPA:PAI-1 complex in murine LB6 clone 19 cells via the uPA/uPAR-mediated pathway (Nykjær et al., 1997). Internalised PAI-2 was localised in lysosomes with MDA-MB-231 cells and in both endosomes and lysosomes within the metastatic prostate cancer cells, PC-3 (Al-Ejeh et al., 2004). These results clearly illustrate that PAI-2 was localised in endosomes and lysosomes where PAI-2 (and presumably uPA) is degraded.

The internalisation process of PAI-2 appeared similar to that described for all other serpin:uPA complexes, whereupon the latter is degraded within lysosomes while uPAR is recycled back to the cell surface (Andreasen et al., 1997; Nykjær et al., 1997; Nykjær and Willnow, 2002; Strickland et al., 2002). Given that PAI-2 forms a stable irreversible complex with receptor-bound uPA and that PAI-2 internalisation occurs via the uPAR/uPA pathway as other serpin:uPA complexes, then PAI-2 should initiate the internalisation of uPA. Evidence for the PAI-2-mediated internalisation of cell-surface uPA was observed by the loss or clearance of uPA from the cell surface upon inhibition by PAI-2. In the absence of added PAI-2 the disappearance of cell surface uPAR-bound uPA levels was slow. The addition of PAI-2 initially greatly accelerated this process. Similar clearance characteristics were described for bound exogenous radiolabelled uPA in the absence and presence of PAI-2 using monocytic cells with low endogenous PAIs (Estreicher et al., 1990). The internalisation of the majority of uPA-occupied cell-surface uPAR within the first
10 min was in agreement with the internalisation of PAI-2. The reappearance of uPAR on the cell-surface after 10 min was in agreement with the previously reported recycling of uPAR after the internalisation of serpin:uPA complexes (Nykjær et al., 1997). Thus, results from this study are largely in agreement with previously published reports of uPA:PAI-1 internalisation. However, PAI-2-mediated clearance of uPA from the cell-surface stabilised at 20 - 30 % of the total uPA after the initial rapid clearance. This result seemed at odds since the majority of bound PAI-2 was internalised within 40 min incubation at 37°C whereas there was only an apparent 20 - 30 % decrease in cell-surface uPA during this time frame. The low uPA clearance in the presence of PAI-2 may be explained by concomitant cell surface uPA replenishment since the cells used in this study continuously express cell bound uPAR and secrete pro-uPA (Ranson et al., 1998; Andronicos and Ranson, 2001). If the initial internalisation of inhibited uPA is concomitantly counterbalanced by the secretion of pro-uPA, some of which ligands unoccupied uPAR originally present on the cells and/or recycled uPAR; then it is possible that secreted pro-uPA replenishes a proportion of these receptors. This suggestion was tested by assaying the binding of exogenous uPA to MDA-MB-231 cells incubated with PAI-2. The decreased binding of exogenous uPA overtime during uPAR/uPA:PAI-2 internalisation indicated that unoccupied uPAR was being masked from exogenous uPA. This provides preliminary evidence for the replenishment of cell-surface uPA during uPAR/uPA:PAI-2 internalisation. Thus, while the majority of uPAR/uPA:PAI-2 were internalised causing the observed internalisation of the majority of PAI-2 and uPAR within the first 10 min of incubation, secreted uPA liganded to previously unoccupied uPAR resulting in the observed decrease of unoccupied cell-surface uPAR levels and the stabilisation of uPA clearance. This was supported by the effect of uPAR-
saturation with uPA prior to the addition of PAI-2 where a larger decrease in cell-surface uPA (~ 60 %) was observed and stabilisation occurring between 30 and 60 min of internalisation where uPAR would be recycled to the cell surface and secreted uPA may ligand to these newly presented unoccupied receptors. The addition of PAI-2 to THP-1 cells preloaded with exogenous uPA resulted in similar effects on the rate of uPA disappearance with 30 – 40 % of the total cell-associated uPA also lost by 60 min at 37°C (Estreicher et al., 1990). While ~ 60 % exogenous radioactive uPA:PAI-1 complex is lost from the cell surface of U937 monocytic (Cubellis et al., 1990) or trophoblastic cells (Jensen et al., 1990) by 60 min, a similar biphasic pattern was also apparent. The higher percentage loss using PAI-1 may either reflect slightly different mechanisms between PAI-1 and PAI-2 and/or different cell types and/or methods of analysis.

Given the wide involvement of the LDLR family members in processes of endocytosis including that of uPA:PAI-1 (Nykjær and Willnow, 2002; Strickland et al., 2002), it is not surprising to find that this family is at least partially responsible for endocytosis of uPA:PAI-2 complexes (as indicated by RAP inhibition experiments performed by Croucher D. in Al-Ejeh et al., 2004). (RAP: receptor associated protein, inhibitor of LDLR endocytosis). Certain LDLR family members when associated with uPAR complexed with uPA:PAI-1 sustain the cell-signalling pathways that are activated via uPAR, resulting in enhanced uPA and uPAR levels among other effects (Nykjær and Willnow, 2002; Strickland et al., 2002). Thus, it is also conceivable that in the presence of PAI-2, LDLR-mediated signalling pathways would be activated and may also enhance the secretion of intracellular pro-uPA stores. This effect may take up to 10 min at 37°C thus accounting for the biphasic effect of PAI-2 on uPA clearance (i.e. rapid then slow clearance) observed. Assuming that a proportion of the newly liganded uPA stays in the pro-enzyme form despite the presence of any residual
cell bound plasmin, then only the two-chain uPA would be internalised by the PAI-2 still present in the cell environment.

Along with published data (Al-Ejeh et al., 2004), the results presented in this chapter allow the proposal of a model for the internalisation of PAI-2 (Figure 47). In this model, PAI-2 forms complexes with uPAR-bound uPA where uPAR occupancy is generally less than 70%. The internalisation of uPA:PAI-2 complexes occurs shortly after complex formation and endocytosis of uPAR/uPA:PAI-2 is mediated by a member of the low-density lipoprotein receptor (LDLR) gene family. During the internalisation of the majority of uPAR/uPA:PAI-2 complexes formed on the cell surface, a signal (possibly mediated by LDLR) initiates the secretion of uPA from an intracellular reservoir. The cell-surface is replenished with uPA, which leads to increased occupancy of uPAR. The uPA:PAI-2 complex is degraded in the lysosomes while uPAR reappears on the cell surface possibly via recycling along with the LDLR, as previously documented (Nykjær et al., 1997).

It is noteworthy that as in the case of T-47D cells (see Chapter 3), MDA-MB-231 cells showed preferential maintenance of partially unoccupied uPAR levels on the cell surface despite the continual secretion of uPA by these cells. As discussed in Chapter 3, it is possible that unoccupied uPAR may be essential for other uPAR-related functions such as uPA-independent signalling (Gellert et al., 2003) or cell-attachment to ECM via interaction of uPAR clusters with integrins and matrix proteins (Wei et al., 1999; Ossowski and Aguirre-Ghiso, 2000; Wei et al., 2001).
Figure 47: Mechanism proposal for the internalisation of uPAR/uPA:PAI-2 complexes
For simplicity plasminogen receptors are not shown.
In summary, this study, as published by Al-Ejeh et al. (2004), provides the first conclusive evidence for the internalisation of PAI-2 by human carcinoma cells, which represents an advance in the understanding of the regulation of cell-surface plasminogen activation. Together with the fact that PAI-2 binding capacity and internalisation is uPA-dependent, these results clearly indicate that PAI-2 would not only preferentially target uPA-expressing tumour cells by very efficiently and irreversibly inhibiting uPA, but would also efficiently deliver attached cytotoxins to the intracellular space. The endosomal/lysosomal localisation of PAI-2 further promotes the utility of α-emitting radionuclide-PAI-2 constructs (or any other PAI-2-cytotoxin construct) as a uPA-targeted anti-tumour drug (Kruithof et al., 1995; Hang et al., 1998; Li et al., 2002; Ranson et al., 2002; Allen et al., 2003). Furthermore, the quantification of PAI-2 binding capacity in terms of $K_i$ values is important for determining PAI-2 concentrations required for delivering sufficient amounts of cytotoxins to the target cell.
Chapter 5: Optimisation of Radiolabelling of PAI-2 with Therapeutic Radioisotopes

5.1. Introduction

The clinical significance of uPA as a marker of metastatic cancers has been established for several human cancers and the elevated levels of uPA in malignant tumours compared to normal tissue is commonly discussed as a therapeutic target (Duffy, 1993b; Duffy, 1996; Andreasen et al., 1997; Schmitt et al., 1997; Brunner et al., 1999; Duffy et al., 1999; Gershtein and Kushlinskii, 1999; Look and Foekens, 1999; Mazar et al., 1999). As illustrated in Table I (Chapter 1), there is conclusive evidence indicating the prognostic significance of uPA in various human cancers and, in the case of breast cancer, level-1 of evidence exists for this prognostic significance (Duffy, 2004). Identification of such a strong marker allows the development of therapy tactics which exploit the ability to target uPA.

Modulation of uPA expression and the use of antagonist of the uPA/uPAR binding have shown success in regulating cancer metastasis and invasion (see Section 1.7.). In the case of transfection methods, the therapeutic potential of such strategy in humans is limited due to the requirement of cancer cell-specific gene delivery methods. Thus antagonists of the uPA/uPAR binding seem more feasible for clinical applications. Although such pharmaceuticals may be administered to inhibit tumour growth, migration or differentiation, tactics such as cytotoxins or toxic radiation for the killing and clearance of tumour cells present more valuable curative advantages.
Radionuclides have been long used for medical applications. α-emitters emit high energy (6 - 9 MeV) over a short distance (40 to 80 µm) (Waldmann, 1991; Behr et al., 1998; Behr et al., 1999; Chatal and Hoefnagel, 1999; Griffiths et al., 1999), thus this energy is mainly deposited in the targeted cells and very few nuclear hits are required to kill these cells (Lloyd et al., 1979; Kassis et al., 1986). Monoclonal antibodies radiolabelled with α-emitting radionuclides (e.g. $^{213}\text{Bi}$ and $^{211}\text{At}$) are starting to show promise in radioimmunotherapy (Couturier et al., 1999; Nikula et al., 1999; McDevitt et al., 2000; Zalutsky and Vaidyanathan, 2000; McDevitt et al., 2001; Jurcic et al., 2002).

Several biological and biochemical characteristics of PAI-2 advocate the possible utilisation of this serpin as a cancer radiotherapeutic agent. Biological characteristics that favour PAI-2 for targeting uPA include: (1) PAI-2 inhibits uPA-mediated invasion of cancer cells in vitro (Kirchheimer and Remold, 1989; Brückner et al., 1992; Stahl and Mueller, 1994); (2) Exogenously added recombinant PAI-2 inhibits cancer invasion and metastasis in vivo (Baker et al., 1990a; Shinkfield et al., 1992; Montgomery et al., 1993; Reiter et al., 1993; Billström et al., 1994; Evans and Lin, 1995) and (3) PAI-2 preferentially accumulates in cancer tissue compared to normal tissue in tumour-bearing mice compared to mice without tumours (Hang et al., 1998). Taken together, and despite the presence of PAI-1 in malignant tumours, it is apparent that uPA is accessible to exogenously administered PAI-2 in several tumour types in vivo.

Biochemical characteristics which favour the use of PAI-2 for cancer therapy include, the specific and efficient inhibition of uPAR-bound uPA while reserving thrombosis as suggested by the low second order inhibition rate constant towards tPA and protection of fibrin-bound tPA from inhibition by PAI-2 (Kruithof et al., 1995). Secondly, PAI-2 is very stable in vitro (Ranson et al.,}
2002), unlike PAI-1 which is subject to oxidation (Baker et al., 1990b), implying that PAI-2 can be easily modified for radiolabelling. These characteristics of PAI-2 in addition to the recent documentation of the internalisation of uPA:PAI-2 complex (Al-Ejeh et al., 2004) encourage the use of PAI-2 for radiotherapy of human cancers.

Previous studies describe the conjugation of PAI-2 to the metal chelator, cDTPA, followed by radiolabelling of PAI-2-DTTA product with the $\alpha$-emitter, $^{213}$Bismuth. In these ‘proof-of-principle’ studies, the resulting adduct (PAI-2-DTTA-$^{213}$Bismuth) shows preferential targeting of metastatic breast cancer cells in vitro (Ranson et al., 2002) and targets tumour xenografts and micrometastases in vivo (Li et al., 2002; Allen et al., 2003). The efficient cytotoxicity of PAI-2-DTTA-$^{213}$Bismuth is explained by the efficient binding and rapid internalisation of uPA:PAI-2 complexes after their formation on the cell surface (Chapter 4, Al-Ejeh et al., 2004). Bismuth-213 ($^{213}$Bi) is efficiently eluted from Actinium-225 ($^{225}$Ac) column (Li et al., 2002; Ranson et al., 2002). Due to $^{213}$Bi being eluted from the $^{225}$Ac column with 0.075 M HI, pH fluctuations were observed that affected the solubility PAI-2-DTTA.

**5.1.A. RATIONALE AND AIDS**

The aim of this chapter was to further characterise PAI-2-DTTA. Furthermore, mainly due to the short half-life of $^{213}$Bi (45 min), a quick, efficient, reproducible and reliable method for radiolabelling of PAI-2-DTTA with $^{213}$Bi needed to be developed for this alpha therapy tactic to be accessible. Thus, another aim of this chapter was to test buffers which prevent PAI-2-DTTA precipitation and optimisation of radiolabelling with $^{213}$Bi.
5.2. METHODS

5.2.A. CONJUGATION OF PAI-2 TO cDTPA

Generally, PAI-2 was reacted with a 50-fold molar excess of the metal chelators as described by Ranson et al. (2002). In a final 450 µl volume of 1x PBS (pH 7.2), 31 µl of freshly prepared anhydrous 25 mg/ml cDTPA in DMSO was reacted with 2 mg of PAI-2 at RT for 1 h with vigorous shaking. Conjugation was stopped using 50 µl 1 M Tris-HCl (pH 7.2) (10 % v/v final concentration). PAI-2-DTTA was separated from free chelator moieties (cDTPA) using 30-kDa cut-off microconcentrators. Briefly, reactions were placed in the top chamber of 30 kDa cut-off microconcentrator units and centrifuged at 7430 x g for 3 - 5 min. The retained volume was washed three times by centrifugation with 500 µl of 1x PBS (pH 7.2). The washes were discarded and the final retained volume was removed to a fresh tube. The microconcentrator filter was washed (no centrifugation) using 1x PBS (pH 7.2) and the washes were added to the fresh tube and stored at 4oC.

The BioRad® DC protein concentration assay kit was used to determine the concentration of conjugated PAI-2 against standard BSA and/or PAI-2 solutions (see Section 2.2.B.1.). The activity of PAI-2-DTTA conjugates was compared to unconjugated PAI-2 using gel shift and the Spectrozyme-UK® assays as described in Section 2.2.B.4.

5.2.B. ELECTROSPRAY IONISATION MASS SPECTROSCOPY

For electrospray ionisation mass spectroscopy (ESI-MS) of PAI-2 and PAI-2-DTTA, samples were washed five times by centrifugation at 7430 x g for 3 - 5 min with milliQ water using 30 kDa cut-off microconcentrators. The retained washed samples were finally prepared in milliQ water and diluted 1:1 in 50 % methanol: 10 % acetic acid (v/v) solution at a final protein concentrations in the
range of 1 - 10 µM. Samples were injected in the Q-TOF mass spectrometer (Department of Chemistry, University of Wollongong) and analysed using a 30 volts cone and 5000 resolution power. Data collected were analysed using the software provided with the mass spectrometry instrument.

**5.2.C. Radiolabelling of PAI-2-DTTA with $^{213}$Bismuth**

**5.2.C.1. Maintenance of $^{225}$Actinium Column**

The total amount of $^{225}$Ac (Oak Ridge National Laboratory, United States Department of Energy, USA) was dissolved in 300 µl of 0.1 M HNO$_3$ and the solution was loaded onto resin (AG$^\circledast$ 50W-X4, provided in column Oak Ridge National Laboratory, United States Department of Energy, USA) pre-equilibrated with 1 ml 0.1 M HNO$_3$. The column was washed with 250 µl of dH$_2$O. When not in use, the $^{225}$Ac column was stored in 0.1 M HNO$_3$ and washed with 1 ml of dH$_2$O prior to use. The $^{225}$Ac column was stripped and reloaded on a weekly basis. Stripping was performed by washing 3 times with 250 µl of 8 M HNO$_3$ in a fume hood. Washes were collected in a 20 ml glass vial, 15 µl of H$_2$O$_2$ was added and content was evaporated on a hot plate until dryness. The evaporation step was repeated by adding 25 µl of 8 M HNO$_3$ and 5 µl of H$_2$O$_2$. The final remaining volume was loaded onto a fresh resin as described above.

**5.2.C.2. Optimisation of Labelling PAI-2-DTTA with $^{213}$Bi ($\alpha$-PAI-2)**

$^{213}$Bi was eluted in a final solution containing 0.075 M HI and 1 x PBS as previously described (Ranson et al., 2002). The pH of the elution solution (0.075 M HI; 1 x PBS) only was measured using pH strips (0 - 14 pH range). In addition, the pH was measured for elution solution titrated with three different solutions - 1 M sodium hydroxide, 3 M Sodium acetate (pH 5.5) and 1.5 M citric acid (citrate, pH 5.5). The effect of control and titrated elution solution on the solubility of various concentrations of PAI-2-DTTA was assessed as follows.
After the addition of PAI-2-DTTA, these solutions were centrifuged at 7430 x g for 2 min in order to pellet any precipitated PAI-2-DTTA. Pellet formation was assessed in these solutions in comparison to incubations containing the elution solutions or PAI-2-DTTA alone.

The radioactivity of elutions was measured (in µCi) using the AtomLab 200 dose calibrator (regularly calibrated using a Caesium source) before and after pH adjustment. ITLC was performed before and after pH-adjustment as described earlier (Section 4.3.A.2.) and the strip sections were counted using a normalised γ-counter for 213Bi counting (15 – 350 keV window). Generally, PAI-2-DTTA was added to the pH-adjusted elution and ITLC was performed using 1 µl samples in selected mobile phases (specified for each experiment in Figure legends).

The radiolabelling of PAI-2-DTTA with 213Bi was investigated with respect to PAI-2-DTTA concentration, 213Bi concentration and time. All reactions were conducted in a final 150 µl volume and for 10 min at RT (unless specified otherwise) and stopped with 5 µl of 0.5 M EDTA. For all assays, ITLC performed using 213Bi alone incubated identically to radiolabelling reactions was used as a control. For the effect of protein concentration, increased concentrations of PAI-2-DTTA were incubated with a set concentration of 213Bi (200 µCi/ml) and 213Bi incorporation was determined by ITLC. The effect of increasing 213Bi concentration on the radiolabelling reaction was assayed using a set concentration of PAI-2-DTTA (100 µg/ml) and incorporation was measured. In order to determine the effect of reactants’ concentrations on the radiolabelling reaction, equal amounts of PAI-2-DTTA and 213Bi (maintained at µg: µCi ratio of 1:1) were incubated in different final volumes and incorporation was assayed using ITLC. The time course experiments were conducted using 30 µg PAI-2-DTTA and 30 µCi 213Bi in a final 150 µl volume and ITLC was performed at
specified time points. The zero time point was obtained by the addition of 30 µCi
\(^{213}\text{Bi}\) to PAI-2-DTTA solution containing 5 µl of 0.5 M EDTA and the sample was
used immediately for ITLC.

**5.2.D. STABILITY OF \(\alpha\)-PAI-2**

\(^{213}\text{Bi}\)-labelled PAI-2-DTTA (\(\alpha\)-PAI-2) was prepared at different specific
radioactivities (µCi/µg). Under identical radiolabelling conditions, PAI-2 (not
conjugated to cDTPA) was incubated with \(^{213}\text{Bi}\). Protein concentration in all
samples was 100 µg/ml and 20 µl aliquots from each reaction were incubated in
triplicate with 0, 0.09, 0.9 or 9 µg of hydrolysed DTPA in a final 110 µl volume of
RPMI-1640 containing 5 % FCS. Reactions were incubated at 37°C for 45 min
and ITLC was performed in order to assess the stability of incorporated \(^{213}\text{Bi}\) in
the presence of excess free DTPA and FCS.

**5.2.E. INTEGRITY OF \(\alpha\)-PAI-2**

Optimally radiolabelled PAI-2-DTTA was assessed for structural integrity
and uPA binding ability using SDS-PAGE (as described in Section 2.2.B.4.)
followed by autoradiograph. \(\alpha\)-PAI-2 prepared at different specific radioactivities
(µCi/µg) was incubated in the presence or absence of uPA at 37°C for 45 min.
Samples were fractionated by reducing 12 % SDS-PAGE (Section 2.2.B.2.) and
autoradiography was performed as described in Section 2.2.B.3.

**5.3. RESULTS**

**5.3.A. CONJUGATION OF cDTPA TO PAI-2**

cDTPA was conjugated to PAI-2 in several different reactions and the
protein was efficiently recovered (> 90 % recovery). The ability of PAI-2-DTTA to
form SDS-stable complexes with uPA was identical to that of free untreated
PAI-2 (Figure 48). PAI-2 (47 kDa) formed complexes of 80 and 98 kDa with low-
and high-molecular weight uPA subunits (33 kDa, 55 kDa), respectively. Similarly, PAI-2-DTTA (lane 4) formed stable complexes with similar sizes when incubated with uPA (Figure 48).

Figure 48: PAI-2-DTTA forms SDS-stable complexes with uPA
PAI-2 and PAI-2-DTTA (2 and 1 µg, respectively) were incubated in a final 20 µl volume of PBS in the presence or absence of uPA (1 µg) (low and high molecular weight subunits 33 and 55 kDa respectively) at 37°C for 90 min. At the end of the incubation, samples were fractioned using 12 % reducing SDS-PAGE. Sizes of the molecular weight marker bands in kDa are displayed on the left; lane 1: uPA; lane 2: PAI-2; lane 3: PAI-2 incubated with uPA; lane 4: PAI-2-DTTA; lane 5: PAI-2-DTTA incubated with uPA. Similar data were obtained for the other PAI-2-DTTA conjugates prepared and for PAI-2-DTTA conjugates when analysed 6 months after preparation.

Incubation of PAI-2 conjugates at 1:1 molar ratio with uPA completely inhibited uPA enzyme activity (Figure 49). A dose-dependent decrease in the inhibition of uPA activity was seen as the ratio of PAI-2 to uPA was decreased. There were no significant differences between the inhibitory activity of PAI-2-DTTA and PAI-2 (Figure 49).
Figure 49: PAI-2-DTTA inhibits uPA enzymatic activity
Active uPA (20 IU, ~ 0.33 µg) was incubated with equimolar amount of unconjugated PAI-2 (clear bars) and PAI-2-DTTA conjugate (filled bars). In addition conjugates were incubated at 0.5:1 and 0.1:1 molar ratio of PAI-2:uPA. BSA (negative control) was also incubated with uPA (no effect on activity, data not shown). uPA activity was measured using Spectrozyme®-UK assay (see Section 2.2.B.4). Data presented are the mean ± standard error of the mean (SEM, n = 6 replicates). Similar data were obtained for the other PAI-2-DTTA conjugates prepared.

When analysed by electrospray ionisation mass spectroscopy, the spectrum of free PAI-2 showed a single band with a molecular weight of 46844.8 ± 0.7 Da (data not shown). In comparison, spectra of PAI-2-DTTA conjugates displayed at least 2 additional peaks with higher molecular weights than that of free PAI-2 (Figure 50). The difference between these high molecular weight peaks and the PAI-2 peak was always a whole multiple of 429 Da (Figure 50 - inset). These increments corresponded to one partially hydrolysed cDTPA ion (373.35 Da) with a chelated iron atom (Fe 55.85 Da). The source of iron was expected to be from the stainless steel bar that the samples face before being sprayed in the electro-field (Mr Larry Hicks, personal communications). Thus, the mass spectroscopy data demonstrated that the PAI-2-DTTA preparations contain a mixture of several PAI-2-DTTA moieties. The percentage of free PAI-2

![Graph showing uPA Activity (IU) vs Molar ratio of PAI-2-DTTA to uPA](attachment://graph.png)
in all PAI-2-DTTA preparations was less than 10%. Mass spectroscopy analysis performed immediately or up to 5 months after the preparation of PAI-2-DTTA (stored in dark at 4°C) produced identical spectra for the same samples, indicating that PAI-2-DTTA was relatively stable (data not shown).

**Figure 50: Electrospray mass spectroscopy of PAI-2-DTTA**

Samples were washed, adjusted to obtain approximately 1-10 µM final concentration and analysed by Q-TOF ESI-mass spectrometer at 30 volts cone and 5000-resolution power. Spectrum of PAI-2 only showed one peak (data not shown). The spectrum shown is from one PAI-2-DTTA preparation and the numbers displayed on the peaks represent the mass in Da. Similar spectra were obtained for other PAI-2-DTTA conjugates; however the spectra varied in the number of peaks observed (data not shown). **Inset:** The difference between the PAI-2 peak and each PAI-2-DTTA peak observed in spectra (labelled as 1-5) was plotted against the peak number. Each increase in the molecular weight represented an increase by the exact molecular weight of hydrolysed cDTPA molecule with a chelated iron atom.
The number of cDTPA molecules conjugated to PAI-2 in different conjugation experiments varied although the same batch of PAI-2 was used. This variation was also observed between different batches of PAI-2. The number of cDTPA molecules conjugated to PAI-2 varied between different batches and within different reactions using the same batch of PAI-2. The minimum number of conjugated cDTPA molecules was 2, the maximum was 5 and the average was 3 - 4 molecules (n = 15, data not shown).

5.3.B. PREPARATION OF 213Bi ELUTION SOLUTION FOR RADIOLABELLING

The solution used for eluting 213Bi from the 225Ac column, 0.075 M HI/1 x PBS, had a pH of 0.5. PAI-2-DTTA precipitated when added to this solution at a concentration of 0.19 – 1.1 mg/ml, possibly due to the low pH. The elution solution could be titrated using 1 M sodium hydroxide, 6 M sodium acetate pH 5.5 or 1 M citrate pH 5.5 (Figure 51). Adjustment of pH of elution solution to pH 5 – 6 was essential since radiolabelling with 213Bi is optimal at this pH (McDevitt et al., 1999) and PAI-2-DTTA is expected to be soluble at this pH as the pI of PAI-2 is 5.5 (Kruithof et al., 1995).

Sodium hydroxide showed a steep titration curve at around pH 5 - 6, thus pH adjustment of the 213Bi-elution solution required gradual careful addition of sodium hydroxide. Although successful radiolabelling of PAI-2-DTTA with 213Bi was possible using this system (data not shown), the use of 1 M sodium hydroxide was aborted due to the time lost during pH adjustment and irreproducibility of this method because of the pH leap. Titration of the elution solution using a final concentration of sodium acetate between 0.2 - 0.6 M showed that a buffering region existed where the pH ranged between 4 and 5.5. Nevertheless, precipitation of PAI-2-DTTA was observed when added at different protein concentrations (0.2 – 1 mg/ml) to elution solution adjusted with various concentrations of sodium acetate (0.1 - 0.6 M) (data now shown).
Figure 51: Titration of the $^{213}$Bi elution solution

Elution solution (0.075 M HI/1 x PBS) was titrated with (A) 1 M sodium hydroxide, (B) 6 M sodium acetate, pH 5.5 or (C) 1 M citrate buffer, pH 5.5. The pH of the solution was measured and plotted against the final concentration (mM) of the titration buffer in the final total volume.
5.3.B.1. LABELLING OF PAI-2-DTTA WITH $^{213}$Bi USING CITRATE BUFFER

Incubation of PAI-2-DTTA at 1 mg/ml with elution solution adjusted with 0.09 – 0.13 M citrate showed that protein precipitation decreased as the final molar concentration of citrate increased (Table V – A). PAI-2-DTTA precipitation was not observed when the elution solution was buffered using 0.13 M citrate (final solution pH 5.5 – 6). Incubation of various concentrations of PAI-2-DTTA in elution solutions adjusted with 0.13 M final concentration of citrate buffer did not result in precipitation of the protein (Table V – B). Omission of PBS from the elution solution resulted in PAI-2-DTTA precipitation (pH 5.5, data not shown), the reason for this precipitation was not clear.

**Table V: Effect of citrate buffered elution on PAI-2-DTTA precipitation**

<table>
<thead>
<tr>
<th>Elution solution</th>
<th>Final [citrate] (M)</th>
<th>[PAI-2-DTTA] (mg/ml)</th>
<th>pH</th>
<th>Precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.075 M HI and 1 x PBS</td>
<td>0.09</td>
<td>1</td>
<td>3.5 - 4</td>
<td>Most (+++)</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>1</td>
<td>4 - 4.5</td>
<td>Less (+++)</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>1</td>
<td>4.5 - 5</td>
<td>Less (+)</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>1</td>
<td>5 - 5.5</td>
<td>Least (-)</td>
</tr>
<tr>
<td></td>
<td>0.13</td>
<td>1</td>
<td>5.5 - 6</td>
<td>No precipitation</td>
</tr>
</tbody>
</table>

(A) Elution solution was titrated with 1 M citrate buffer (pH 5.5); PAI-2-DTTA was added at 1 mg/ml and precipitation was assessed. (B) PAI-2-DTTA was incubated at different concentrations in elution solution buffered using 1 M citrate buffer (pH 5.5) at 0.13 M final concentration. The pH of the final solution was measured and protein precipitation was assessed.
The studies performed with citrate-buffered elution solution above did not contain $^{213}$Bi radionuclide. Nevertheless, as shown in Table VI, the presence of $^{213}$Bi at various concentrations did not affect the pH of the citrate-buffered solution. ITLC performed using 1 µl of citrate-buffered $^{213}$Bi elution showed that using 0.1 M citrate buffer as the mobile phase, 99 % of radiation moved to the solvent front (Table VII - A). As other buffers showed that significant level of radiation remained at the origin, these could not be used for assessing the percentage incorporation of $^{213}$Bi using ITLC. No precipitation of protein was observed when PAI-2-DTTA was incubated with buffered $^{213}$Bi solution (0.075 M HI/1 x PBS/0.13 M citrate) for 15 min. The incorporation of $^{213}$Bi when buffered $^{213}$Bi solution was incubated with PAI-2-DTTA at 1 µCi:1 µg ratio was 93 and 93.7 % at 1 and 5 min, respectively (Table VII – B).

<table>
<thead>
<tr>
<th>0.075 M HI (µl)</th>
<th>$^{213}$Bi in 0.075 M HI (µl)</th>
<th>10 x PBS (µl)</th>
<th>1 M citrate (µl)</th>
<th>Final volume (µl)</th>
<th>$[^{213}\text{Bi}]$ (µCi/µl)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>13</td>
<td>17</td>
<td>130</td>
<td>0</td>
<td>5.5-6</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>13</td>
<td>17</td>
<td>130</td>
<td>0.12</td>
<td>5.5-6</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>13</td>
<td>17</td>
<td>130</td>
<td>0.24</td>
<td>5.5-6</td>
</tr>
<tr>
<td>25</td>
<td>75</td>
<td>13</td>
<td>17</td>
<td>130</td>
<td>0.36</td>
<td>5.5-6</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>13</td>
<td>17</td>
<td>130</td>
<td>0.48</td>
<td>5.5-6</td>
</tr>
</tbody>
</table>

$^{213}$Bi was eluted using 250 µl of 0.15 M HI followed by 250 µl of dH₂O. Different volumes of $^{213}$Bi elution were mixed with different volumes of 0.075 M HI in a final volume of 100 µl. The pH was adjusted using 10 x PBS and 1 M citric acid solution (pH 5.5) and the pH of the final 130 µl volume was measured.
Table VII: Effect of different solvents on separation of free and PAI-2-DTTA-incorporated $^{213}$Bi by ITLC

A: Mobility of free $^{213}$Bi

<table>
<thead>
<tr>
<th>ITLC buffer</th>
<th>Radioactivity at origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M acetate</td>
<td>58 %</td>
</tr>
<tr>
<td>0.1 M citrate</td>
<td>1 %</td>
</tr>
<tr>
<td>1 x PBS</td>
<td>33 %</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>20 %</td>
</tr>
</tbody>
</table>

B: Mobility of PAI-2-DTTA-$^{213}$Bi

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$^{213}$Bi incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>93.0 %</td>
</tr>
<tr>
<td>5 min</td>
<td>93.7 %</td>
</tr>
</tbody>
</table>

(A) 2 µl of buffered $^{213}$Bi (0.075 M HI/1 x PBS/0.13 M citrate, pH 5.5 – 6) was spotted onto ITLC strips and chromatography was performed using the specified mobile phases. Radioactivity at the origin was calculated as the percentage of radioactivity at origin over the radioactivity at both the origin and solvent front. (B) ITLC was performed using 0.1 M citrate (pH 5.5) solution as the mobile phase at 1 and 5 min from the incubation of PAI-2-DTTA with buffered $^{213}$Bi at 1 µg: 1 µCi ratio at RT. The percentage of activity at the origin (% origin/[origin + solvent front]) was calculated as a measure of $^{213}$Bi incorporation.

5.3.C. OPTIMISATION OF RADIOLABELLING OF PAI-2-DTTA WITH $^{213}$Bi

(α-PAI-2)

Using the citrate buffering system for preparing a suitable $^{213}$Bi-elution solution, the radiolabelling of PAI-2-DTTA with $^{213}$Bi was optimised in terms of protein/radioactivity concentrations and time. High percentage incorporation of $^{213}$Bi (> 90 %) was obtained when PAI-2-DTTA was incubated with 213Bi to give specific radioactivity less than 10 µCi/µg (Figure 52 – A and B). In addition to the effect of the $^{213}$Bi: PAI-2-DTTA ratio, $^{213}$Bi percentage incorporation was also affected by the final concentrations of $^{213}$Bi and PAI-2-DTTA even when both reactants were maintained at 1 µCi: 1 µg ratio (Figure 52 – C).
Figure 52: Radiolabelling optimisation of PAI-2-DTTA with $^{213}$Bi

(A) Buffered $^{213}$Bi solution (200 µCi/ml) was incubated with increasing concentrations of PAI-2-DTTA for 10 min at room temperature. $^{213}$Bi incorporation was assessed by ITLC using 0.1 M citrate (pH 5.5) as mobile phase. (B) 100 µg/ml PAI-2-DTTA was incubated with increasing concentrations of buffered $^{213}$Bi and incorporation was assessed. (C) PAI-2-DTTA (7.25 µg) and buffered $^{213}$Bi (7.25 µCi) were incubated in different final volumes for 10 min. Data presented in Figure are the mean incorporation percentages ± SEM (n = 3). **Note:** the percentage $^{213}$Bi incorporation is the percentage of $^{213}$Bi incorporated into PAI-2-DTTA over the total $^{213}$Bi added.
At high final concentrations (200 µCi and 200 µg per ml), the radiolabelling of PAI-2-DTTA with $^{213}$Bi was rapid and reached maximal within the first 5 min of incubation (Figure 53). The incorporation of $^{213}$Bi into PAI-2-DTTA was solely due to the DTTA moiety as PAI-2 failed to incorporate any $^{213}$Bi in comparison to PAI-2-DTTA (Figure 54 – A). Incorporated $^{213}$Bi was strongly chelated since only 15 and 16 % of $^{213}$Bi was stripped from PAI-2-DTTA-$^{213}$Bi prepared at 1 and 5 µCi/µg, respectively, when challenged with excess amount (80 µg/ml) of free DTPA (Figure 54 – B).

![Figure 53: Kinetics of the PAI-2-DTTA $^{213}$Bi-labelling reaction](image)

PAI-2-DTTA (200 µg/ml) and buffered $^{213}$Bi (200 µCi/ml) were incubated at room temperature and at the specified time points the reaction was stopped by the addition of EDTA (20 mM final concentration). $^{213}$Bi incorporation was assessed using ITLC and data presented are the mean ± SEM (n = 3).
Figure 54: Specificity and stability of the $^{213}$Bi-labelling reaction

(A) PAI-2-DTTA or PAI-2 were incubated for 10 min at RT with buffered $^{213}$Bi at 1 or 5 µCi/µg in the same final volume. $^{213}$Bi incorporation was assessed and data presented are the mean incorporation ± SEM (n = 3). (B) PAI-2-DTTA labelled with buffered $^{213}$Bi at 1 (clear bars) and 5 (filled bars) µCi/µg were incubated at 37°C for 45 min in RPMI-1640 + 5 % FCS containing increasing concentrations of hydrolysed cDTPA (DTPA). $^{213}$Bi incorporation was assessed and data presented are the mean ± SEM (n = 3). There was no significant differences in the percentage of $^{213}$Bi remaining with the protein between the 1 and 5 µCi/µg α-PAI-2 preparations after challenge with all DTPA concentrations ($p > 0.05$, n = 3). However, challenge of both specific radioactivities with 80 µg/ml DTPA caused a small but significant loss of $^{213}$Bi label from the protein compared to unchallenged controls (*, $p < 0.05$, n = 3).
5.3.D. **Integrity of α-PAI-2**

α-PAI-2 prepared at specific radioactivities decreased the structural and functional integrity of PAI-2. The intensity of the band corresponding to PAI-2 (47 kDa) detected by SDS-PAGE decreased as the specific radioactivity of α-PAI-2 increased from 0.1 to 10 µCi/µg (Figure 55 – A). In agreement, autoradiography (Figure 55 – B) showed an increase in the intensity of possible radioactive degradation products at the bottom of the gel with increasing specific radioactivities of α-PAI-2. The intensity of the 47 kDa band in the autoradiogram increased with higher specific radioactivity of α-PAI-2 which reflects higher incorporation of $^{213}$Bi (lanes 2, 4, 6 and 8 in Figure 55 – B).

The ability of α-PAI-2 to form SDS-stable complexes with uPA was also affected by its specific radioactivity. α-PAI-2 prepared at 0.1 and 1 µCi/µg efficiently formed complexes with uPA as bands for free α-PAI-2 were not evident (lanes 3 and 5 compared with lanes 2 and 4, respectively, Figure 55 – B). In contrast, for the 5 and 10 µCi/µg α-PAI-2, large amounts of free α-PAI-2 were evident even after incubation with excess uPA (lanes 7 and 9 compared with lanes 6 and 8, respectively, Figure 55 – B). This indicated that although a fraction of 5 and 10 µCi/µg α-PAI-2 remained intact, only a fraction of this intact α-PAI-2 at these specific radioactivities were able to form SDS-stable complexes with uPA.
PAI-2-DTTA was radiolabelled with buffered $^{213}$Bi to produce $\alpha$-PAI-2 at specific radioactivities of 0.1, 1, 5 and 10 $\mu$Ci/$\mu$g. The protein concentration for all incubations was identical and the same volume from each specific radioactivity was incubated at 37°C for 60 min in the absence or presence of excess uPA containing high (55 kDa) and low (33 kDa) molecular weight uPA. Aliquots containing the same protein amounts were fractionated by 12% reducing SDS-PAGE. (A) The SDS-PAGE gel was stained in Coomassie blue stain solution, destained and documented after (B) exposure to phosphor screen for 3 h and scanning using the Storm® scanner. Lane 1: molecular weight marker. Lane 2: 0.1 $\mu$Ci/$\mu$g $\alpha$-PAI-2; lane 3: 0.1 $\mu$Ci/$\mu$g $\alpha$-PAI-2 + uPA; lane 4: 1 $\mu$Ci/$\mu$g $\alpha$-PAI-2; lane 5: 1 $\mu$Ci/$\mu$g $\alpha$-PAI-2 + uPA; lane 6: 5 $\mu$Ci/$\mu$g $\alpha$-PAI-2; lane 7: 5 $\mu$Ci/$\mu$g $\alpha$-PAI-2 + uPA; lane 8: 10 $\mu$Ci/$\mu$g $\alpha$-PAI-2; lane 9: 10 $\mu$Ci/$\mu$g $\alpha$-PAI-2 + uPA and lane 10: uPA.
5.3.E. CYTOTOXICITY OF $\alpha$-PAI-2

5.3.E.1. CYTOTOXICITY OF BUFFER-EXCHANGED $\alpha$-PAI-2

$\alpha$-PAI-2 prepared at 1, 5 and 10 $\mu$Ci/$\mu$g specific radioactivities were efficiently buffer exchanged from the labelling buffer into RPMI-1640 + 5 % FCS using 30 kDa cut-off microconcentrators (90 % of the initial activity recovered, data not shown). $\alpha$-PAI-2 under these conditions showed slight cytotoxicity to MDA-MB-231 cells (Figure 56 - A). At 3 $\mu$Ci/100 l dose of 1, 5 and 10 $\mu$Ci/$\mu$g $\alpha$-PAI-2, the percentage of surviving cells compared to control cells was 79.4 ± 0.5, 80.2 ± 0.2 and 83.6 ± 2.4 %, respectively. The cytotoxicity was not significantly different using the different specific activities of $\alpha$-PAI-2 ($p > 0.05$, n = 3). In a separate experiment, treatment of the MDA-MB-231 cells with buffer-exchanged 1 $\mu$Ci/$\mu$g $\alpha$-PAI-2 significantly decreased cell survival to 75.4 ± 1.3 % ($p < 0.05$, n = 5, Figure 56 - B). The survival of the non-invasive breast cancer cells, T-47D cells, was not significantly affected by the addition of this dose of washed $\alpha$-PAI-2 ($p > 0.05$, n = 5). This suggested that the cytotoxicity of $\alpha$-PAI-2 observed on MDA-MB-231 cells was due to high cell-surface uPA levels on these cells compared to T-47D cells. Despite the success of this assay, the cytotoxicity level of buffer-exchanged $\alpha$-PAI-2 was lower than that previously reported by Ranson et al. (2002). This may be explained by difference in the radiolabelling procedure (Ranson et al., 2002). Higher doses of $\alpha$-PAI-2 (＞3 $\mu$Ci/100 l) were not possible due to the dilution of radioactivity during buffer exchange technique, thus alternative procedures were investigated (Section 5.4.E.2.).
Figure 56: Cytotoxicity of buffer-exchanged α-PAI-2

(A) MDA-MB-231 cells cultured for 48 h were detached and incubated with 5 µg/ml plasminogen for 5 min at room temperature. Cells (2 x 10⁴) were washed and incubated overnight in triplicate 100 µl volumes of RPMI-1640 + 5 % FCS in the absence or presence of specified doses of buffer-exchanged α-PAI-2 prepared at 1 (clear bars), 5 (light grey) and 10 (dark grey) µCi/µg. Cells were washed and incubated with 100 µl of phenol-free binding buffer containing 5 % FCS and 20 µl of MTS/PMS substrate. The absorbance of the product (at 495 nm) was measured after 3 h incubation at 37°C, blanked against absorbance of solution in the absence of cells and cell survival was calculated as the percentage of absorbance of α-PAI-2-treated cells against control cells.

(B) MDA-MB-231 cells (clear bars) and T-47D cells (filed bars) were subjected to treatment with 3 µCi/100 µl dose of 1 µCi/µg α-PAI-2 as described for A and cell survival was calculated (mean percentage survival ± SEM, n = 5). Asterisk denotes significant difference between control and treated MD-MB-231 cells (p < 0.05). The treatment of T-47D cells did not significantly affect cell survival compared to control T-47D cells (p > 0.05, n = 5).
5.3.E.2. CYTOTOXICITY OF NON-BUFFER-EXCHANGED α-PAI-2

In order to avoid the time lost during the purification of α-PAI-2 due to the short half-life of $^{213}$Bi and to prevent the dilution caused by the purification procedure, non-buffer-exchanged α-PAI-2 preparations were investigated for use in cytotoxicity assays. Control experiments showed that the percentage of surviving cells decreased with increasing concentrations of buffered elution solution (0.075 M HI, 1 x PBS, 0.2 M citrate, pH 5.5 - 6) added to culture media in comparison to cells incubated with culture media only (Figure 57 - A). In contrast to the cytotoxic effect of the elution solution, increased cell proliferation was observed with increasing concentrations of unlabelled PAI-2-DTTA added to culture media (Figure 57 - B). Thus, in order to account for these confounding effects, control treatments were included in cytotoxicity assays which were identical to the α-PAI-2 treatments except for the absence of $^{213}$Bi.

In comparison to the control solutions described above, cytotoxicity of non-buffer-exchanged α-PAI-2 preparations on MDA-MB-231 cells depended on both the specific radioactivity and the dose of α-PAI-2 added. At all specific radioactivities, there was a dose-dependent, significant decrease in MDA-MB-231 cell-survival (Figure 58 – A). In comparison to MDA-MB-231 cells, T-47D cells resisted treatment with low doses (1 and 2 µCi/100 µl) at all specific radioactivities ($p < 0.05$, $n = 3$) (Figure 58 – B). The use of high doses (4 and 8 µCi) of all specific radioactivities did not show significant difference between the survival of T-47D and MDA-MB-231 cells ($p > 0.05$, $n = 3$). This indicated that high doses of α-PAI-2 sufficiently targeted both cell lines. The highest specific radioactivity of α-PAI-2 (10 µCi/µg) had the least cytotoxic effect on both MDA-MB-231 and T-47D cells. For example using 1 µCi/µg α-PAI-2, cells survival at each dose used significantly decreased compared to that using 5 or 10 µCi/µg α-PAI-2 (Figure 58 – A and B). This was in agreement with decreased integrity
and uPA-binding ability of α-PAI-2 with increasing specific radioactivity (refer to Figure 55).

Figure 57: Effect of elution solution and PAI-2-DTTA on cell survival

(A) MDA-MB-231 cells were incubated overnight in RPMI-1640 + 5 % FCS in the absence or presence of buffered elution solution (0.075 M HI/1 x PBS/0.2 M citrate, pH 6). Cell survival was measured after overnight incubation in comparison to control treatment (RPMI-1640 + 5 % FCS only) and the mean ± SEM (n = 3) was plotted against the concentration of buffered elution solution in the 100 µl volume (v/v %). (B) MDA-MB-231 cells were incubated with increasing concentrations of unlabelled PAI-2-DTTA. Cell survival was compared to control cells and plotted against the concentration of PAI-2-DTTA (expressed as µg/100 µl) and the data shown are the mean + SEM (n =3).
Figure 58: Optimised cytotoxicity of α-PAI-2 on MDA-MB-231 and T-47D cells
(A) MDA-MB-231 and (B) T-47D cells, cultured for 48 h, were detached and treated with the specified doses of α-PAI-2 prepared at 1 (■), 5 (●) and 10 (▲) μCi/µg. For each dose of the different specific radioactivities, the corresponding control treatment involved the addition of the same volume of buffered elution solution and the same amount of protein in the form of unlabelled PAI-2-DTTA. Survival was determined after overnight treatment compared to these controls and data presented are the mean ± SEM (n = 3). (A) Survival plots of MDA-MB-231 cells were fitted to one-phase exponential decay model. (B) Survival plots of T-47D cells were fitted to sigmoidal dose-response model. The sigmoidal shape indicated that T-47D cells were resistant to low doses of α-PAI-2; however toxicity was achieved at high doses. Models were selected according to the best fit and none of the data deviated from the fitted model as judged by runs test performed by GraphPad® software. The dotted lines represent the toxic dose (Do, 37 % survival). Data presented are representative from two independent assays.
5.3.E.3. PROLIFERATIVE CAPACITY OF CELLS SURVIVING α-PAI-2 EXPOSURE

After overnight treatment with 4 and 8 µCi/100 µl doses of 1 µCi/µg α-PAI-2, 62 ± 0.6 and 70 ± 0.9 % of the total cell population compared to control cells did not survive, respectively (Figure 59 – A and B). Whether the remaining, presumably not targeted, cells were healthy and able to proliferate was checked by examining cell survival 48 h after treatment with 1 µCi/µg α-PAI-2. Using both 4 and 8 µCi/100 µl doses of 1 µCi/µg α-PAI-2 the cell numbers were significantly recovered (to 92.1 ± 3.2 and 54.1 ± 1.5 % compared to controls, respectively) after 48 h compared to the overnight time point (Figure 59 – A and B). This indicated that the surviving remaining cells after overnight treatment with 1 µCi/µg α-PAI-2 retained their ability to proliferate. Cells treated with 4 and 8 µCi/100 µl doses of 5 and 10 µCi/µg α-PAI-2 showed less than 50 % recovery between overnight and at 48 h (Figure 59 – A and B). Thus, although 5 and 10 µCi/µg α-PAI-2 were less cytotoxic than 1 µCi/µg α-PAI-2 after overnight incubation (Figure 59, clear bars), these higher specific radioactivity preparations appeared to decrease the proliferative capacity of the remaining cells that survived after overnight treatment during 48 h incubation after treatment (Figure 59, filled bars). This suggests that since treatment with 1 µCi/µg α-PAI-2 appeared not to affect the proliferation of surviving cells then 1 µCi/µg α-PAI-2 caused minimal co-lateral cytotoxicity. That is surviving cells (untargeted) were not affected by 1 µCi/µg α-PAI-2 in the media, therefore proliferated normally.

5.3.E.4. EFFECT OF CELL ATTACHMENT ON α-PAI-2 CYTOTOXICITY

Attached MDA-MB-231 cells, treated identically to detached cells, were less sensitive to the cytotoxicity of α-PAI-2. Nevertheless, cell survival depended on the dose and specific radioactivity of α-PAI-2 (Figure 60 – A). This indicated
that attachment of MDA-MB-231 cells did not affect the targeting mechanism of 
\( \alpha \)-PAI-2; however, only a fraction of the administered dose may have targeted 
these cells. In agreement, higher levels of PAI-2-Cy5 bound to detached MDA-
MB-231 cells compared to attached cells (Figure 60 - B). This binding was 
similarly inhibited by blocking the B-chain of uPA (data not shown). Thus, the 
effect of cell attachment on the binding of PAI-2-Cy5 and the cytotoxicity of 
\( \alpha \)-PAI-2 may be due to the unavailability of uPAR-bound uPA which is involved 
in focal adhesion during cell attachment.

![Figure 59: Recovery of \( \alpha \)-PAI-2-treated MDA-MB-231 cells at 48 hour post treatment](image)

MDA-MB-231 cells cultured for 48 h were detached and treated with (A) 4 \( \mu \)Ci/100 \( \mu \)l and (B) 8 \( \mu \)Ci/100 \( \mu \)l doses of \( \alpha \)-PAI-2 prepared at 1, 5 and 10 \( \mu \)Ci/\( \mu \)g as described for 
Figure 58. For each dose of the different specific radioactivities, control treatments 
involved the addition of the same volume of buffered elution solution and unlabelled 
PAI-2-DTTA. The percentage of surviving cells compared to control treatments was 
determined after overnight incubation (clear bars) and 48 h past the treatment (filled 
bars). Data presented are the mean \( \pm \) SEM (n = 3). The number of cells in control 
treatments was approximately doubled between the overnight time point and the 48 h 
time point (data not shown).
**Figure 60: Effect of cell-attachment on ability of PAI-2 to access cell-surface uPA**

(A) Attached MDA-MB-231 cells (2 x 10^4 cells) cultured for 48 h were activated with plasminogen and treated with the specified doses of α-PAI-2 prepared at 1 (■), 5 (●) and 10 (▲) µCi/µg. Survival was determined compared to control treatments as described for Figure 58 and data presented are the mean ± SEM (n = 3). (B) Detached (left column) and attached (right column) MDA-MB-231 cells pre-activated with plasminogen were incubated with PAI-2-Cy5 for 5 min at 37°C, cells were washed and then analysed by confocal microscopy. Top row are the transmission images and the scale bars represent 10 µm. Bottom row are the Cy5 scan of the transmission images. One representative sample (n = 6) is shown for each treatment and pre-incubation of cells with anti-B-chain uPA monoclonal antibody (#394) completely inhibited PAI-2-Cy5 binding (data not shown).
5.4. DISCUSSION

The results of this chapter describe the successful efficient radiolabelling procedure of PAI-2-DTTA with the $\alpha$-emitter radioisotope, $^{213}$Bi. It was confirmed that conjugation of PAI-2 to the metal chelator, cDTPA, did not affect the binding ability or the inhibitory effect of PAI-2 towards uPA (see also Ranson et al., 2002). Mass spectroscopy confirmed the addition of a variable number of DTTA moieties to PAI-2 in the PAI-2-DTTA preparations. Ranson et al. (2002) also showed that conjugation with cDTPA did not affect PAI-2 function and their preparation contained a mixture of modified PAI-2 containing 1 – 4 DTTA molecules per PAI-2 molecule. Further investigation in this chapter showed that there was always a mixture of different stoichiometric ratios of PAI-2 to DTTA (i.e. PAI-2-DTTA, PAI-2-[DTTA]$_2$ ... PAI-2-[DTTA]$_n$) which varied between different reactions and within replicates of the same reaction. This variation may be due to complex reaction kinetics. These complicated reaction kinetics are beyond the scope of this study; however, they may be related to reaction conditions and sterical hindrance on the surface of PAI-2 despite the fact that only limited sites on the surface of PAI-2 are available for reaction. Thus, although the average number of DTTA molecules conjugated to PAI-2 was calculated as 3 - 4 molecules ($n = 15$), it is recommended that mass spectroscopy should be performed for each preparation due to the variation observed.

Previous radiolabelling of PAI-2-DTTA with $^{213}$Bi showed that the percentage of incorporated radioactivity was greater than 90 % (Ranson et al., 2002). However, $\alpha$-PAI-2 needed to be purified from free $^{213}$Bi using size exclusion chromatography of the radiolabelling reaction which is a time-consuming process. Although Ranson et al. (2002) demonstrated the uPA-dependent cytotoxicity of their $\alpha$-PAI-2 preparation, more efficient, rapid and
reproducible radiolabelling protocol was needed considering the short-life of $^{213}$Bi and for potential clinical applications. The radiolabelling reaction of PAI-2-DTTA with $^{213}$Bi was first optimised in terms of the buffer used for this reaction. PAI-2-DTTA was readily precipitated when added to the solution used to elute $^{213}$Bi due to the very low pH of this solution (pH 0 – 1). Although sodium acetate was described to neutralise the elution solution (Ranson et al., 2002), it was found that PAI-2-DTTA precipitated even when the elution solution was adjusted to pH 5.5 using sodium acetate. The reason for the unreported precipitation in the α-PAI-2 prepared by Ranson et al. (2002) may be due to the very low protein concentrations used in their study (5 – 10 µg in a 550 µl typical elution volume). Furthermore, considering that the average activity eluted in 550 µl volume is higher than 500 µCi, the previous labelling involved the addition of 100 µCi of $^{213}$Bi per 1 µg of PAI-2-DTTA (Ranson et al., 2002). The authors described the preparation of 15 – 20 µCi/µg of α-PAI-2 after PD-10 column purification, indicating that only 10 – 15 % of added $^{213}$Bi was incorporated thus necessitating further purification. This inefficient radiolabelling (Ranson et al., 2002) and the precipitation of PAI-2-DTTA reported in this thesis when using sodium acetate as buffering system suggested that alternative methods were required to optimise the radiolabelling of PAI-2-DTTA.

The use of citric acid buffer (pH 5.5) showed that protein precipitation by the elution solution was prevented by the presence of 0.13 – 0.2 M final concentration of citrate. Initial radiolabelling reactions under citrate buffering system described an efficient incorporation of $^{213}$Bi (> 90 %) which was confidently assessed using 0.1 M citrate (pH 5.5) as the mobile phase for ITLC. Using these conditions for pH adjustment and ITLC, the radiolabelling of PAI-2-DTTA with $^{213}$Bi was optimised with respect to the ratio of radioactivity to protein, concentration of reactants and time. A standard operating procedure
(SOP) for pre-clinical analyses was developed for the radiolabelling reaction where $^{213}$Bi is eluted using 250 µl of freshly prepared 0.15 M HI and 250 µl of dH$_2$O into reaction tube containing 85 µl of 1.5 M citrate (pH 5.5) and 65 µl of 10 x PBS (pH 7.2) then PAI-2-DTTA is added and incubated for 5 – 10 min.

Using the developed SOP, PAI-2-DTTA was radiolabelled with $^{213}$Bi at different specific activities and $^{213}$Bi incorporation was shown to be specific and stable. There was a loss in the integrity and uPA-binding ability with increasing specific radioactivity of α-PAI-2 preparations. In agreement, higher specific radioactivity (5 and 10 µCi/µg) preparations of α-PAI-2 were less cytotoxic towards MDA-MB-231 and T-47D cells compared to lower specific radioactivity (1 µCi/µg) preparation. T-47D cells tolerated low doses (< 4 µCi/100 µl) of 1 µCi/µg α-PAI-2 compared to MDA-MB-231 cells. This indicated that cytotoxic effect of 1 µCi/µg α-PAI-2 was uPA-dependent at doses less than 4 µCi/100µl. However, doses higher than 4 µCi/100 µl of 1 µCi/µg α-PAI-2 efficiently reduced the survival of suspension cultures of both high and low uPA expressing MDA-MB-231 and T-47D cells, respectively. Cells significantly recovered when incubated for 48 h after treatment with all doses of 1 µCi/µg α-PAI-2. On the other hand, cells treated with 5 and 10 µCi/µg α-PAI-2 did not recover as efficiently at 48 h incubation after treatment. This suggested that although 5 and 10 µCi/µg α-PAI-2 showed less cytotoxic effect than 1 µCi/µg α-PAI-2, these high specific radioactivity preparations may have caused co-lateral damage to untargeted cells and affected their proliferation ability. Finally, attached MDA-MB-231 cells tolerated the same doses of α-PAI-2 (1, 2, 4 and 8 µCi/100 µl) at the different specific radioactivities (1, 5 and 10 µCi/µg) delivered to suspension MDA-MB-231 cells. This increased tolerance maybe explained by
less binding of PAI-2 to these cells when attached compared to when in suspension.

The results of this chapter are important for the preparation and assessment of the use of α-PAI-2 in pre-clinical in vivo trials. Based on these results, the 1 µCi/µg α-PAI-2 preparation was suggested for use for pre-clinical trials since: (1) this preparation did not affect the structural integrity of PAI-2 or its uPA binding ability, (2) 213Bi incorporation was highly stable, (3) the uPA-dependent cytotoxic effect of α-PAI-2 was highest on both detached and attached cells and (4) the 1 µCi/µg α-PAI-2 preparation appeared to cause the least co-lateral damage.

In conclusion, this chapter described the efficient conjugation of cDTPA to PAI-2 and methods required for characterisation of the final product of this reaction. Furthermore, the preparation of PAI-2-DTTA and its radiolabelling with 213Bi was optimised into a standard operating procedure, which was adopted for the preparation of 213Bi-PAI-2 for pre-clinical trials of radiotherapy using mouse models of human tumours (Stutchbury et al., manuscripts in preparation). These pre-clinical trials showed that mice tolerated buffered 213Bi elution and that 1 µCi/µg α-PAI-2 effectively reduced tumour growth and conferred a survival advantage (Stutchbury et al., manuscript in preparation).
Chapter 6: CONCLUSIONS AND FUTURE WORK

The kinetics of the inhibition of cell-surface uPA on breast cancer cells by PAI-2 were directly described using appropriate parameters ($K_i$ and $k_{\text{inact}}$) in this thesis as compared to the previous indirectly assayed second order rate constant (Ellis et al., 1990). uPA was identified as the major specific target for PAI-2 on the cell surface. The inhibition parameters measured in this thesis characterise PAI-2 as a very specific potent inhibitor of uPAR-bound uPA on the cell surface. Inhibition of cell-surface uPA by PAI-2 is rapidly followed by the endocytosis of PAI-2, uPA as well as uPAR which seems to mimic the previously reported internalisation of cell surface uPA/uPAR induced by other serpins (Andreasen et al., 1997). Data also indicate that uPAR may be recycled to the cell surface which is in agreement with previous findings using other serpins (Nykjær et al., 1997). Members of the LDLR family are involved in part in the internalisation of PAI-2 (Al-Ejeh et al., 2004). Despite these similarities between PAI-2 induced internalisation of uPAR/uPA:PAI-2 complexes and other uPAR/uPA:serpin complexes, data suggests that the mechanism of PAI-2 internalisation may be novel in terms of endocytosis mediators (Croucher D., personal communications). During the internalisation of uPAR/uPA:PAI-2, uPA may be replenished on the cell surface in the pro-enzyme form. These results lead to an entire new PhD project (undertaken by Croucher D., Dr. M. Ranson Laboratory, University of Wollongong) to provide definitive evidence for the suggested replenishment of cell-surface uPA and the recycling of uPAR as well as deducing the complete mechanism of uPAR/uPA:PAI-2 internalisation.

The evident internalisation and the perinuclear localisation of internalised PAI-2 provide a definitive biological rationale for the targeted alpha therapy being developed using this serpin (Li et al., 2002; Ranson et al., 2002;
Allen et al., 2003). The standard operating procedures for the preparation and
characterisation of α-PAI-2 described in this thesis were adopted by Dr Marie
Ranson and collaborators (University of Wollongong, Australia) in pre-clinical
development of α-PAI-2 for treatment of breast and prostate cancer. Given
further time and resources, separation of the different PAI-2-DTTA moieties in
the conjugation reactions by high-pressure liquid chromatography would have
been advantages for at least two reasons. First, such separation would result in
a more uniform solution of PAI-2-DTTA for clinical use. Secondly, as tryptic
digest and peptide mass finger printing using mass spectroscopy resulted in
very complex spectra (data not shown), separation of different PAI-2-DTTA
moieties before fingerprinting may simplify these results. This may be employed
in order to deduce the sites of cDTPA conjugation to PAI-2 and completely
characterise this promising cancer targeting vehicle.

Finally, transfection of the non-invasive breast cancer T-47D cells with
uPAR cDNA was also useful for the PAI-2 binding studies. Furthermore, the
overexpression of uPAR in the non-invasive T-47D cells caused an interesting
modulation of other components of the urokinase-type plasminogen activation
cascade. Using this technique, initial and preliminary explanations were
provided for elucidating links between the upregulation of uPAR expression and
the adoption of metastatic phenotype. Although such a link has been described
previously (e.g. Ranson et al., 1998), the findings in this thesis gave deeper
insights into the adoption of the malignant phenotype by cancer cells and raised
new questions which may be the subject for future research. The possibility that
the non-active site epitope on uPA suggested by Ellis et al. (1999) can act as a
receptor for plasminogen binding may be evaluated by developing antibodies to
this epitope. This would provide an explanation for increased plasminogen
binding when increasing cell-surface uPA and the co-localisation of
uPA/plasminogen on T-47D cells. Furthermore, the possible effect of uPAR/uPA-transient overexpression on the expression of plasminogen receptors may be characterised by proteomic comparison between control and transfected T-47D cells. The ability to knock out the expression of uPAR in the invasive MDA-MB-231 cells would provide useful material for comparison with uPAR-overexpressing T-47D cells, however, consistent preparation of uPAR-downregulated MDA-MB-231 cells in this study failed. Further work is required to prepare uPAR-MDA-MB-231 cells and new techniques such as small interfering RNA (siRNA) transfection technology would be recommended. siRNA-transfection is showing more success than cDNA transfection methods in silencing the expression of cellular components (Caplen and Mousses, 2003). In addition, the possibility that increased cell-surface plasmin activity by the addition of exogenous uPA or transient overexpression of uPAR/uPA may activate latent plasminogen receptors needs to be tested. It is well known that increased cell-surface uPAR/uPA leads to an increase in plasmin-dependent proteolysis and invasion both in vitro and in vivo and that plasmin generation is mediated by the proteolytic activity of uPA on receptor-bound plasminogen (see review by Andreasen et al., 1997). However, it is not clear whether this involves modulation of plasminogen receptors on the cell surface. Altogether, the future work suggested above may be incorporated into a PhD project aimed at understanding the molecular details of the increased plasminogen binding due increased of cell-surface uPAR/uPA.

In conclusion, this thesis described the direct inhibition parameters of cell-surface uPA by PAI-2 and the internalisation of PAI-2:uPA complexes formed on the cell-surface. This published study (Al-Ejeh et al., 2004) also provided a biological rationale for the use of PAI-2 as uPA-based anti-cancer therapy, one of which is the use of cytotoxic alpha radiation. Alpha-PAI-2
preparation was successfully optimised according to standard operating procedures and these procedures showed success in trials of radiotherapy using mouse models of human tumours (Stutchbury et al., manuscripts in preparation). Finally, in addition to their use in elucidating the binding of PAI-2, transiently uPAR-overexpressing cells provided preliminary insights into the relationship between metastatic phenotype and plasminogen binding (used in part in Stillfried G. et al., manuscript in preparation) and may be expanded to a new PhD thesis.
Chapter 7: APPENDICES

7.1. Appendix 1: Normalisation of Gamma-Counter

The radionuclide (2.5 µCi) was dissolved in 1 ml of PBS and 200 µl aliquots were transferred to 5 γ-counter tubes (0.5 µCi in 200 µl per tube). Tubes were placed in normalisation rack at positions 1, 5, 9, 13 and 17 to normalise the 5 detectors of the Cobra 5005R γ-counter using specified normalisation window for each radionuclide. Machine was normalised for ‘position 2’ which was ideal for counting 0.2 ml – 0.5 ml samples. The Chi-Square test produced after normalisation was used to judge whether detectors were normalised efficiently (Chi Square 99 % confidence range = 7.63 – 36.19). In the case where detectors were out of this range, normalisation was repeated and in some cases only the efficiently normalised detectors were selected for counting.

7.2. Appendix 2: Quantification and Purity of DNA Preparations

Agarose gel electrophoresis of plasmid DNA against known amounts molecular weight marker allowed the quantification of the amounts of DNA present in the sample. The amount of each band in the molecular weight marker, Figure 61, was calculated using the ratio of the band size to the size of λDNA (85500 bp) multiplied by the amount of marker used (180 ng). The amount of DNA in bands, for example lane 1 and 2 Figure 61, was calculated
based on the standard curve constructed for the relationship between staining intensity and the amount from the molecular weight marker. The amount of DNA in the original sample was calculated based on the amount measured in the loaded volume and considering the dilution factors. For the example shown, the average amount of DNA was 263 ng in the 10 µl loaded. Thus, the amount in the 25 µl total volume was 657.5 ng which was acquired using 0.5 µl of plasmid solution. The concentration of the original plasmid solution calculated using this method (1.32 µg/µl) was compared to value obtained by spectrophotometric method (Table VIII). The concentration of DNA in plasmid solution was calculated from the absorbance at 260 nm and found to be 1.38 µg/µl. The average concentration (1.35 ± 0.03 µg/µl) from the two methods was used to represent the concentration of plasmid solution. The ratio of the absorbance at 260 nm to that at 280 nm (2.0) indicated that the preparation was of high purity and very small amounts of protein was present in the sample.

<table>
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<tr>
<th>Measurement</th>
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<tr>
<td>$A_{260}$</td>
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<tr>
<td>$A_{280}$</td>
<td>0.069</td>
</tr>
<tr>
<td>$A_{260}/A_{280}$</td>
<td>2</td>
</tr>
<tr>
<td>DNA concentration</td>
<td>6.9 µg/ml</td>
</tr>
<tr>
<td>Concentration of stock</td>
<td>1.38 µg/µl</td>
</tr>
<tr>
<td>preparation</td>
<td></td>
</tr>
</tbody>
</table>

Plasmid was diluted 1:200 in dH$_2$O and the absorbance at 260 and 280 nm was measured using dH$_2$O as blank. DNA concentration was measured given that 0.1 absorbance = 50 µg/ml (Maniatis et al., 1989). The concentration of the stock was then measured by multiplication with the dilution factor (200). The purity of DNA sample was measured as the ratio of $A_{260nm}/A_{280nm}$ (Maniatis et al., 1989).
Figure 61: Quantification of DNA preparation

(A) pcDNA3/uPAR (0.5 µl) was diluted in a final 20 µl dH2O and mixed 5 µl of 5 x DNA loading dye. **Lane 1** and **2**: 10 µl of pcDNA3/uPAR solution and **lane 3**: 5 µl of 37 ng/µl of λDNA/Hind III marker. Electrophoresis was performed using 1.2 % agarose gel at 70 volts for 1 h and gel was stained in ethidium bromide solution, washed and documented. (B) Analysis of the gel image using GelPro™ Analyzer software. (C) Quantification of the size and amount of bands observed in lanes 1 and 2 using standard curves constructed from the molecular weight marker (lane 3). The molecular weight of the bands (mol.w.) is in base pairs unit and the amount is in ng.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Lane 1 mol.w.</th>
<th>Lane 2 mol.w.</th>
<th>Lane 3 mol.w.</th>
<th>Lane 3 amount</th>
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<tr>
<td>1</td>
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<td>9416</td>
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<td>35911</td>
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<td>3</td>
<td>6318</td>
<td>246.64</td>
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<td>273.61</td>
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<tr>
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<td></td>
<td>4361</td>
<td>16616</td>
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<td>Sum</td>
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<td>273.61</td>
<td></td>
<td>164.46</td>
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</table>

7.3. Appendix 3: uPA Activity Standard Curve

The inhibition of uPA activity due incubation of PAI-2 was assayed using a colorimetric assay which utilised synthetic substrate for uPA, Spectrozyme®-UK. The kinetics of substrate conversion to the coloured product was assayed at 405 nm for uPA pre-incubated with PAI-2 and compared to standard curve for
the relationship between the activity units of uPA (IU) and the rate of substrate conversion. The standard curve was constructed using serial 1:2 dilutions of 20 IU of uPA incubated with 2 mM of Spectrozyme®-UK. The change in absorbance at 405 nm over time was plotted against the activity units (Figure 62) and the linear correlation was used to interpolate the activity of uPA retained after incubation with PAI-2.

Figure 62: Standard curve for the rate of substrate conversion and uPA concentration

See method in Section 2.2.B.4. The rate of substrate conversion (change in absorbance over time) was plotted against the concentration of uPA and a linear correlation ($R^2 = 0.996$) was fitted to the data (mean ± SEM, n = 3).
7.4. Appendix 4: Buffers and Solutions

**Agarose gel**
1.2 % w/v agarose
dissolved in 1 x Tris-Borate-EDTA (TBE) buffer

**Binding buffer (Hank's buffered saline solution)**
9.5 g/l Hank’s powder
1 mM HEPES
0.1 % w/v bovine serum albumin (BSA)
1 mM CaCl₂
1 mM MgCl₂
pH 7.4
Filtered with 0.22 μm filters

* Phenol red-free binding buffer was prepared identically however using phenol red-free Hank's powder

**Coomassie blue stain**
0.1 % w/v Brilliant Blue R250
40 % v/v methanol
10 % v/v glacial acetic acid

**Coomassie blue destain**
40 % methanol
10% glacial acetic acid
60 % water

**Culture media**
10.4 g/l of RPMI-1640 powder (contains 2 mM glutamine)
2 g/l sodium bicarbonate
adjusted to pH 7.2
Filter-sterilised using 0.22 μm sterile filters units

**DNA precipitation buffer**
1.5 M sodium acetate pH 8
250 mM EDTA

**DNA sample buffer [5 x concentration]**
30 % glycerol
0.125 % w/v xylene cyanol
0.125 % w/v bromophenol blue

**Elution solution for ²¹³Bi**
250 μl of 0.15 M HI followed by 250 μl of H₂O (0.075 M HI)
Eluted into tube containing 50 μl of 10 x PBS (final 1 x PBS concentration)
Elution buffer for PD-10 columns
1 x PBS
0.1 % w/v BSA
pH 7.4

0.1 % gelatine buffer
0.1 g gelatine
150 mM NaCl
20 mM Tris-HCl
In 100 ml of dH₂O and adjusted to pH 8.4

Luria Broth (LB)
10 g/l trypton
5 g/l yeast extract
10 g/l NaCl

Mounting buffer
1 x PBS containing 10 % v/v glycerol

5 x non-reducing sample buffer
60 mM Tris-HCl
2 % w/v SDS
10 % v/v glycerol
0.01 % w/v bromophenol blue

* 5 x reducing sample buffer prepared as for non-reducing sample buffer but including 5 % w/v 2-β-mercaptoethanol

1 x Phosphate buffered saline (PBS)
8 g/l NaCl
0.2 g/l KCl
1.44 g/l Na₂HPO₄
0.24 g/l KH₂PO₄
pH adjusted to 7.4
Filtered with 0.22 μm filters
(10 x PBS was prepared by adding 10 x the concentrations above).
* Note: When 1 x PBS was used with cells (e.g. washing), MgCl₂ and CaCl₂ were added each at 1 mM final concentration.

1 x running buffer for SDS-PAGE
3.03 g/l Tris-HCl
14.4 g/l glycine
1 g/l SDS
**SDS-PAGE gels**

Resolving gel
12 % v/v of liquid 40 % acrylamide
0.1 % w/v SDS
375 mM Tris-HCl pH 8.8
0.1 % v/v TEMED
0.05 % w/v ammonium persulphate (APS)

Stacking gel
4 % v/v of liquid 40 % acrylamide
0.1 % w/v SDS
126 mM Tris-HCl pH 6.8
0.1 % v/v TEMED
0.05 % w/v APS

**SOC medium**

Sterile solution containing:
2 % w/v Bactotryptone
0.5 % w/v yeast extract
10 mM NaCl
2.5 mM KCl
20 mM MgCl₂
10 mM MgSO₄
20 mM Glucose

**TBE buffer**

10 x buffer prepared at:
108 g/l Tris-HCl
55 g/l boric acid
40 mM EDTA pH 8.0
diluted 1 in 10 for use at 1 x concentration

**TE buffer**

10 mM Tris-HCl, pH 7.4
1 mM EDTA

**Z-agar plates**

Sterile 1.5 % w/v agar in LB poured and set on Petri plates
**THESIS PUBLICATIONS**


Stillfried, G., Al-Ejeh, F. and Ranson, M. Manuscript in preparation.
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