2011

Synthesis and biological evaluation of plasminogen activation inhibitors as antitumour/antimetastasis agents

Hayden Matthews

University of Wollongong, hm96@uowmail.edu.au

Recommended Citation
NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.
Synthesis and Biological Evaluation of Plasminogen Activation Inhibitors as Antitumour/Antimetastasis Agents.

A thesis submitted in fulfilment of the requirements for the award of the degree Doctor of Philosophy from University of Wollongong by Hayden Matthews

Bachelor of Medicinal Chemistry (Advanced, Honours I) School of Chemistry

2011
Declaration

I, Hayden Matthews, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The work has not been submitted for qualification at any other academic institution.

Hayden Matthews

11th March 2011
Acknowledgements

Firstly, I would like to express my gratitude to my supervisors, with whom I shared my PhD experience in what was such an enjoyable and rewarding team effort.

To Michael: Thankyou so much for all the time and effort you have invested to make my PhD a success. You have certainly been the driving force behind my enthusiasm for Medicinal Chemistry and I have been very fortunate to have a supervisor so approachable, encouraging and generous with his time. Thanks for your invaluable advice and insight into all aspects of my project, for developing my skills as a medicinal chemist and for your friendship.

To Marie: When I started this project I didn’t expect to be spending so much time in a molecular biology lab, but I couldn’t imagine my PhD without such a valuable experience. I truly appreciate your time and patience in teaching me so many molecular biology techniques from scratch. Thanks for your advice and enthusiasm in the preparation of our manuscripts and for making me feel a welcome member of your research group.

To my collaborators Melissa De Souza and Sarah Vella, thanks for sharing your honours projects with me, contributing so much to the success of my PhD and for your enjoyable company during so many long days in the lab.

To Joey, Celine, Jodi and Bharat, thanks for taking time to time to teach me new skills and for your advice and encouragement in the lab. Thanks also to the other members of the
Kelso and Ranson groups: Anthony, Naveen, Ardesir, Ghazala, Pichit, Nick, Kara, Blake, Sergei, Spencer and Vineesh; for your camaraderie and encouragement.

Thanks to all the tech staff that contributed to my work, in particular to; Roza for her always lively and amusing conversations while we taught undergrads; Wilford for his valued NMR advice; Steve Cooper for being the nicest guy in the building, always happy to help with all things mechanical; and Peter Sara for keeping all our electronics operational.

Finally, I’d like to thank my family and friends for all their love and support. Thanks Mum, Dad and Lana; Bronwyn, Paul and John; and, of course, thanks Michelle for sharing your love and patience every day.
Publications Arising from this Thesis


Abstract

The altered protein expression and activity that promotes changes in cancer cells can provide drug targets for tumour-selective chemotherapy. Typically these treatments are devised based on biomarkers that have been identified as indicators of tumour grade and patient prognosis. Acquisition of enhanced proteolytic capability and invasive potential is widely recognised as the final breach of normal cellular behaviour and one which promotes tumour metastasis, and one of the key mediators is the urokinase plasminogen activation system. The system incorporates the serine protease urokinase plasminogen activator (uPA) and its cognate receptor uPAR, the upregulation of which results in the enhancement of tumour proteolytic and migratory capability. Both uPA and uPAR can be targeted with peptides and small molecules to effect a decrease in the metastatic ability of tumour cells.

Another key protein in tumour development is the sodium hydrogen exchanger (NHE). NHEs are integral membrane proteins that exchange one intracellular proton for one extracellular sodium ion. Nine mammalian NHE isoforms (NHE1-NHE9) are known and several have been shown to be upregulated and/or activated in tumours, regulating intracellular pH and cell volume as well as establishing the well characterised low extracellular pH of solid tumours. Conveniently, clinically-used and orally administered potassium-sparing diuretic amiloride has been shown to inhibit both the proteolytic activity of uPA and the ion exchange of NHE1, providing an excellent lead for anticancer drug development.
Section 1 explores structure-activity relationships of amiloride analogues as inhibitors of uPA. Initially, analogues substituted at the 5-position were targeted for synthesis from precursor 13, an acylguanidine derivative of the commercially available methyl ester 14. Other analogues targeted were 6-substituted derivatives which were to be accessed from 6-iodoester 57. Alternative acylguanidines, synthesised through guanidinylation of methyl ester precursors, were investigated to characterise the binding contribution of the acylguanidine unit of amiloride and to possibly identify new acylguanidine cores. Other analogues in which the acylguanidine was substituted with isosteric groups, or where exocyclic amino groups were substituted for hydrogen, were synthesised in order to establish the importance of these substituents for inhibitor binding. Overall, analogues with substituents at the 5-position were generally as potent or only slightly less potent than amiloride. Further analogues substituted at the 6-position with bulkier groups (i.e. 15, 58, 63 and 64) showed improved potency. Indeed 15 and 64 were the most potent uPA inhibitors identified in this study (6-fold increase in potency relative to amiloride). It was found that simple arylacylguanidines are not useful as uPA inhibitors and should not be pursued as alternative uPA inhibitor scaffolds, while analogues lacking either the 3- or 5-amino substituent were found to be inactive, demonstrating the importance of these groups. Compounds 11 and 12 were of particular interest in the study as they are reported as potent inhibitors of NHE-1 (11 $K_i = 30$ nM, 12 $K_i = 13$ nM). The two analogues produced 2-fold improvements in potency relative to amiloride against uPA. Compounds 15, 27 and 64 were the most potent inhibitors identified, suggesting that analogues containing double modifications at the 5- and 6-positions should be investigated further.
Section 2 investigates two highly potent non-peptidic antagonists 83 and 84 (IC$_{50}$ 83 = 0.8 nM, IC$_{50}$ 84 = 33 nM) of the uPA:uPAR interaction that represent useful pharmacological tools for studying the plasminogen activation system and the effects that small molecule uPAR antagonists can have as antitumour/antimetastasis agents. Antagonists 83 and 84 were prepared by new solution phase and mixed solid/solution phase syntheses, respectively. The activities of 83 and 84 were assessed in semi-quantitative competition flow cytometry assays and quantitative cell-based uPA activity assays that employ HMW-uPA as the competing ligand. Compounds 83 and 84 were found to be poor antagonists of the uPA:uPAR interaction in physiologically relevant cellular systems and are therefore not useful as small molecule probes for pharmacological investigations of the plasminogen activation system. The work highlights the importance of using HMW-uPA as the competing ligand in competition experiments aimed at measuring the potency of small molecule uPAR antagonists as potential pharmacological tools or anticancer drug leads.
Abbreviations

ADP  Adenosine 5'-diphosphate
Alexa-HMW-uPA  Alexa-488 labelled high molecular weight uPA
AMC  7-amino-4-methylcoumarin
ASIC  Acid-sensing ion channel
ATF  Amino terminal fragment
Boc₂O  tert-butyloxycarbonyl anhydride
BSA  Bovine serum albumin
Cdk  Cyclin-dependent kinase
DMSO  Dimethyl sulfoxide
cDNA  Complementary DNA
CENP-E  Centromeric binding protein E
CENP-F  Centromeric binding protein F
DCM  Dichloromethane
DIPEA  N,N-diisopropylethylamine
DMAP  4-((N,N-dimethylamino)pyridine
DMF  N,N-dimethylformamide
DNA  Deoxyribonucleic acid
dppe  1,2-Bis(diphenylophosphino)ethane
ECM  Extracellular matrix
EGF  Epidermal growth factor
EGFR  EGF receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EI</td>
<td>Electron ionisation</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-((N-ethyl-N-isopropyl)amiloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Electrospray</td>
</tr>
<tr>
<td>Et$_2$O</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>F1k-1/KDR/VEGF-2</td>
<td>Vascular endothelial growth factor receptor type 2</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen binding</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTI</td>
<td>Farnesyltransferase inhibitor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-benzotriazole-N,N,N′,N′-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMA</td>
<td>5-((N,N-hexamethylene)amiloride</td>
</tr>
<tr>
<td>HMW-uPA</td>
<td>High Molecular Weight uPA</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectrum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>iPrOH</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>i-uPA</td>
<td>Catalytically inactivated high molecular weight uPA</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducers and activators of transcription</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
</tr>
<tr>
<td>LXA4R</td>
<td>Lipoxin A4 receptor</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteases</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NCS</td>
<td>N-chlorosuccinimide</td>
</tr>
<tr>
<td>NHE</td>
<td>Sodium-Hydrogen exchanger</td>
</tr>
<tr>
<td>NIS</td>
<td>N-iodosuccinimide</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpyrrolidone</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>p53</td>
<td>p53 tumour suppressor protein</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>pH_e</td>
<td>Extracellular pH</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>rp</td>
<td>Reverse Phase</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-Activity Relationship</td>
</tr>
<tr>
<td>SMB</td>
<td>Somatomedin B Domain</td>
</tr>
<tr>
<td>SOSA</td>
<td>Selective-Optimisation of Side Activity</td>
</tr>
<tr>
<td>suPAR</td>
<td>GPI-cleaved soluble uPAR</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>uPAS</td>
<td>Urokinase plasminogen activation system</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Table of Contents

Declaration ii

Acknowledgements iii

Publications Arising from this Thesis v

Abstract iv

Abbreviations ix

Table of Contents xiv

List of Figures xix

List of Tables xxii

List of Schemes xxii

Section 1: Structure-Activity Relationships of Amiloride Analogues as Inhibitors of the Urokinase Plasminogen Activator (uPA) 1

Chapter 1: Introduction 2

1.1 Carcinogenesis and Cancer 2

1.2 Overview of Targeted Therapeutics 5

1.3 Tumour Metastasis 10

1.4 The Urokinase Plasminogen Activation System (uPAS) and Metastasis 13

1.5 Reported uPA Inhibitors 16

1.6 Binding Interactions Between uPA Inhibitors and uPA 18

1.7 Amiloride as an Antitumour/Antimetastasis Agent 20
# 1.7.1 Antitumour/Antimetastasis Effects of Amiloride in Experimental Models

21

# 1.7.2 Antitumour/Antimetastasis Effects of Amiloride Mediated Through Inhibition of Sodium-Hydrogen Exchangers (NHEs)

24

# 1.7.3 Antitumour/Antimetastasis Effects of Amiloride Mediated Through Inhibition of uPA

29

# 1.7.4 Amiloride Analogues as Potential Antitumour/Antimetastasis Drugs

30

### 1.8 Aims of Section 1

35

#### 1.8.1 Binding Interactions Between Amiloride and uPA

36

#### 1.8.2 Specific Aims

38

---

### Chapter 2: Synthesis of Amiloride Analogues

40

#### 2.1 Synthetic Strategies Towards 5- and 6-Substituted Analogues

40

#### 2.2 Synthesis of 5-Substituted Analogues

41

#### 2.3 Synthesis of 6-Substituted Analogues

47

#### 2.4 Synthesis of Acylguanidines with Varied Aryl Cores

49

#### 2.5 Synthesis of Analogues Containing Acylguanidine Isosteres

50

#### 2.6 Synthesis of Analogues Lacking 3- and 5-Exocyclic Amines

50

#### 2.7 Unsuccessful Syntheses

51

---

### Chapter 3: Evaluation of Amiloride Analogues as uPA Inhibitors

54

#### 3.1 uPA Inhibition Assay

54

#### 3.2 Analogues with C5 Variations

55

#### 3.3 Analogues with C6 Variations

59
6.1 Synthetic Strategies

6.2 Solution-Phase Synthesis of uPAR Antagonist 83

6.3 Mixed Solution/Solid-Phase Synthesis of uPAR Antagonist 84

Chapter 7: Evaluation of 83 and 84 as uPAR Antagonists

7.1 Characterisation of (a) Alexa-HMW-uPA Binding to Cell Surface uPAR Receptors and (b) uPAR-Bound HMW-uPA Activity in U-937 Cells

7.2 Evaluation of 83 and 84 as Antagonists of the HMW-uPA:uPAR Interaction in U-937 Cells by Flow Cytometry

7.3 Evaluation of 83 and 84 as Antagonists of the HMW-uPA:uPAR Interaction in U-937 Cells by Quantitative Fluorometric Activity Assays

7.4 Discussion

7.5 Conclusions and Future Directions

Chapter 8: Section 2 Experimental and References

8.1 Synthesis - General

8.2 Characterisation

8.3 Biological Evaluation - General

8.4 Cell Culture

8.5 PMA Treatment

8.6 Preparation of i-uPA

8.7 IC$_{50}$ Determinations

8.8 References
9. Appendix

uPA Enzyme Inhibition Assay Protocol

Fluorostar Optima uPA:uPAR Antagonist Assay Protocol
List of Figures

Figure 1.1. Representation of breaches in normal cellular physiology that occur during carcinogenesis. 4

Figure 1.2. (a) Major steps required for tumour metastasis. (b) Representation of a mosaic tumour blood vessel where tumour cells form part of the vessel wall. 11

Figure 1.3. The urokinase plasminogen activation system (uPAS) and its role in ECM degradation. 14

Figure 1.4. Reported uPA inhibitors. 16

Figure 1.5. Chemical structure of the K+-sparing diuretic amiloride. 20

Figure 1.6. Amiloride analogues EIPA and HMA. 26

Figure 1.7. Summary of the antitumour/antimetastasis effects of amiloride arising through inhibition of sodium hydrogen exchangers (NHE) and uPA. 35

Figure 1.8. A: X-ray crystal structure of amiloride bound to uPA highlighting the binding site. B: Summary of binding interactions between amiloride, uPA and a sulphate ion bound in the oxyanion hole. 37

Figure 1.9. Summary of amiloride analogues targeted in the study and strategies used in their syntheses. 39

Figure 3.1. Summary of 96-well plate assay used to measure the potency of uPA inhibitors. 55

Figure 5.1. Schematic representation of mature uPAR showing primary structure and folding topology of individual domains. 148

Figure 5.2. Summary of the major non-proteolytic functions of uPAR. 151

Figure 5.3. Structure of pro-uPA (prior to activation by cleavage of the Lys158-Ile159 peptide bond). 153

Figure 5.4. Left: Amino acid sequence of the uPAR-binding ω-loop (residues 19-31) of the uPA EGF-like domain. Right: Cyclic peptide antagonist derived from the ω-loop which incorporates a disulfide between cysteines 19 and 31. 155
**Figure 5.5.** Examples of reported non-peptidic small-molecule antagonists of the uPA-uPAR interaction.

**Figure 5.6.** Crystal structures of uPAR in complex with ATF (A, B) or peptide antagonist AE147 (C, D).

**Figure 5.7.** Radioligand-binding assay used to determine IC\textsubscript{50} values of some uPAR antagonists including phage display peptides and small molecule antagonist \textbf{84}.

**Figure 5.8.** Radioligand-binding assay used to determine IC\textsubscript{50} values for small molecule uPAR antagonist \textbf{83}.

**Figure 6.1.** 2D NMR highlighting the rotomeric nature of \textbf{84}.

**Figure 7.1.** Summary of cell-based flow cytometric and fluorescence activity assays used to characterise the uPAR antagonist potencies of \textbf{83} and \textbf{84}.

**Figure 7.2.** (A) Characterisation of Alexa-HMW-uPA binding to uPAR receptors on U-937 cells by flow cytometry. (B) Characterisation of uPAR-bound HMW-uPA activity in U-937 cells.

**Figure 7.3.** Dual-colour competition flow cytometry assay of \textbf{83} and \textbf{84} as antagonists of the HMW-uPA:uPAR interaction in U-937 cells.

**Figure 7.4.** Fluorescence assays of cell surface-bound HMW-uPA activity in PMA-stimulated U-937 cells.
List of Tables

**Table 1.1.** Targeting anticancer therapeutics towards the six breaches in normal cellular physiology. 6

**Table 1.2.** Antitumour and antimetastasis effects of amiloride in rodent tumour models. 22

**Table 1.3.** The NHE-inhibitory potencies of amiloride and selected analogues. 32

**Table 2.1.** Table summarising 5-substituted analogues produced by nucleophilic aromatic substitution reactions of amines with 5,6-dichloropyrazine precursor 13. 43

**Table 2.2.** Synthesis of amiloride analogues modified at the 6-position and in some cases the 5- and 6-positions. 48

**Table 2.3.** Synthesis of acylguanidines with varied aryl cores. 49

**Table 3.1.** uPA inhibitory potencies of 5-substituted amiloride analogues. 57

**Table 3.2.** uPA inhibitory potencies of 6-substituted analogues. 60

**Table 3.3.** uPA inhibitory potencies of acylguanidines with varied aryl cores. 62

**Table 3.4.** Inhibitory potencies of analogues containing acylguanidine isosteres. 63

**Table 3.5.** uPA inhibitory potencies of analogues lacking 3- and 5-exocyclic amines. 64
List of Schemes

**Scheme 2.1.** Synthetic strategies employed in the preparation of 5- and 6-substituted amiloride analogues.

**Scheme 2.2.** Guanidinylation of 14 to afford key precursor 13.

**Scheme 2.3.** Synthesis of non-commercially available amines.

**Scheme 2.4.** Hydrolysis of carboxylate and phosphonate esters 16, 17, 19 and 20 to afford 51-55. Oxidation of thioester 35 to the corresponding sulfone 56 with Oxone (potassium peroxymonosulfate) and synthesis of heterocycle 27.

**Scheme 2.5.** Synthesis of 15, 40, 41, 57 and 58.

**Scheme 2.6.** Synthesis of amiloride analogues 74 and 75 which incorporate acylguanidine isosteres.

**Scheme 2.7.** Synthesis of 5- and 3-deamino amiloride analogues 80 and 82.

**Scheme 2.8.** Summary of unsuccessful syntheses.

**Scheme 6.1.** Mechanism of I$_2$-catalysed activation of Boc$_2$O useful in the Boc-protection of unreactive amines.

**Scheme 6.2.** Solution-phase synthesis of uPAR antagonist 83.

**Scheme 6.3.** Mixed Solid/Solution-phase synthesis of uPAR antagonist 84.
Section 1:

Structure-Activity Relationships of Amiloride Analogues as Inhibitors of the Urokinase Plasminogen Activator (uPA)
Chapter 1: Introduction

1.1 Carcinogenesis and Cancer

In 1971 when then US President Nixon “declared war on cancer”, leading to the establishment of the National Cancer Act (1971), our understanding of cancer was far removed from the multifaceted interpretation we have today. For several decades the popular misconception was that carcinoma is primarily a result of excessive cell proliferation arising from a change in a single cell that allows it to divide infinitely. Strategies for cancer treatment for a long time mirrored this perception with what today seems a naïve focus on drugs that attack the machinery of DNA replication and cell division.\(^1\) It is now apparent that these strategies are rather simplistic and don’t adequately address the heterogeneity of progressing tumours. An attitude change in the 1990s and advances in our molecular understanding of cancer have led to an acceptance that tumours are derived from a population of cells in various stages of a process akin to Darwinian evolution.\(^2\)

Carcinogenesis involves a discrete succession of genetic changes with each change conferring an advantage over neighbouring cells in a progression toward malignancy. Alterations in cellular physiology arise from breaches of a multilayered anticancer regulatory system that dictates normal cellular behaviour. While there are a wide variety of manifestations of cancer, there appears to be six key breaches that are common to most human tumours.\(^2\) These include:
• Self-sufficiency in growth signals
• Insensitivity to growth regulators
• Evasion of apoptosis
• Unchecked replication with limitless potential
• Sustained angiogenic capability
• Superior tissue invasion and metastatic capability

Acquisition of genomic changes in cancer cells occurs at a much greater frequency than the comparatively rare occurrence of mutations in non-tumour cells. In fact, the number of mutations required to achieve breaches of regular cellular behaviour are considered unlikely to occur in a human lifetime. The comparatively rapid accumulation of mutations seen in carcinogenesis appears to stem from damage to members of a complex system of genomic repair proteins. A central member in this system, the p53 tumour suppressor protein, is mutated or downregulated in a majority of human cancers. p53 functions to arrest the cell cycle when significant DNA damage is detected, triggering repair or apoptosis.³ The genomic instability that occurs as a result of a loss of function in central genomic caretakers allows rapid accumulation of mutations. The chronological order in which the six hallmark capabilities of tumour cells are attained can vary and the scale of changes can differ for individual mutational steps. For instance, certain genetic lesions confer several breaches in regular cell behaviour while others confer only a partial breach. This is illustrated in Figure 1.1.
Figure 1.1 Representation of breaches in normal cellular physiology that occur during carcinogenesis. Partial acquisition of an acquired capability is designated by partially coloured boxes. Several capabilities acquired simultaneously are represented by multiple symbols in a box. Adapted from Reference 2.

The altered protein expression and activity that promotes changes in cancer cells provides many potential anticancer drug targets. The changes can be tumour-type and patient-specific allowing for individualised or tailored treatments. Typically
these treatments are centred around biomarkers that have been identified as indicators of tumour grade and patient prognosis.⁴,⁵

### 1.2 Overview of Targeted Therapeutics

As recently as 1996 medicinal chemistry textbooks described chemotherapy drugs as agents that target cell division, such as DNA alkylators, antimetabolites and tubulin-binding drugs.⁶ In more recent times, tailoring of treatments towards specific malignant phenotypes arising from breaches in normal cellular physiology has emerged as a powerful complement to established surgical, hormonal and/or cytotoxic therapies. The last decade has seen a rapid expansion of the literature surrounding targeted therapeutics and several have been approved for clinical use.⁷,⁸ Table 1.1 lists some examples of strategies for targeting therapeutics towards the breaches of normal cellular physiology.

The most common targeted therapeutics in clinical use are monoclonal antibodies (e.g. trastuzumab) and small molecule inhibitors (e.g. erlotinib and imatinib⁸⁻¹¹) that target the receptor tyrosine kinase HER-2/neu, but several other promising strategies are being investigated.
Table 1.1 Targeting anticancer therapeutics towards the six breaches in normal cellular physiology.

<table>
<thead>
<tr>
<th>Acquired Capability</th>
<th>Example/s of Mechanism</th>
<th>Targeting Strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-sufficiency in growth signals</td>
<td>EGF and HER-2/neu receptor overexpression</td>
<td>Monoclonal antibodies, tyrosine kinase inhibitors</td>
</tr>
<tr>
<td></td>
<td>Mutation in the Ras protein</td>
<td>Farnesyltransferase inhibitors</td>
</tr>
<tr>
<td>Insensitivity to growth regulators</td>
<td>Loss of TGFβ responses leading to Cdk activation</td>
<td>Small molecule Cdk inhibitors</td>
</tr>
<tr>
<td>Evasion of apoptosis</td>
<td>Mutation of p53 tumour suppressor protein</td>
<td>Adenovirus affecting cells with mutant p53 only</td>
</tr>
<tr>
<td></td>
<td>Bcl-2 overexpression</td>
<td>Antisense Bcl-2 suppression</td>
</tr>
<tr>
<td>Unchecked and limitless replication</td>
<td>Telomerase expression</td>
<td>Telomerase inhibitors</td>
</tr>
<tr>
<td>Sustained angiogenic capability</td>
<td>Vascular Endothelial Growth Factor (VEGF) upregulation via activation of ras oncogene</td>
<td>VEGF signaling blockers</td>
</tr>
<tr>
<td>Superior tissue invasion and metastatic capability</td>
<td>Shift in expression of integrin subtypes</td>
<td>Specific integrin antagonists</td>
</tr>
<tr>
<td></td>
<td>Matrix Metalloprotease (MMP) overexpression</td>
<td>MMP inhibitors</td>
</tr>
</tbody>
</table>

The following list is not exhaustive but gives an indication of the current directions of targeted chemotherapy research and the variety of strategies being investigated:

- **Farnesyltransferase inhibitors (FTIs):** Small molecule enzyme inhibitors of mutant Ras proteins that are observed in ~30% of human cancers. The structurally altered Ras protein is constitutively activated and sits in a mitogenic signaling cascade downstream from epidermal growth factor
receptor (EGFR) tyrosine kinases. The activity of Ras is dependent on farnesylation, which serves to localise the protein to the cell membrane. FTIs (e.g. tipifarnib and lonafarnib) are able to block farnesylation. They are thought to elicit some of their antitumour activity by blocking farnesylation of other components of cell growth pathways (e.g. Rheb) and proteins involved in cytoskeletal rearrangements (e.g. RhoB, CENP-E and CENP-F).

- **Cyclin-dependent kinase (Cdk) inhibitors**: Small molecule enzyme inhibitors that prevent aberrant protein phosphorylation by Cdks. The primary substrates of Cdks are members of the retinoblastoma family of proteins (pRb). These proteins tightly regulate transcription factors controlling the expression of genes involved in cell cycle progression. Constitutive phosphorylation of pRb by Cdks triggers proliferation in tumour cells and occurs when the signaling cascade headed by the anti-growth protein transforming growth factor β (TGFβ) is disrupted in a variety of ways, including downregulation/mutation of TGFβ receptors, mutation or elimination of TGFβ signal transducers or loss of Cdk response to signal transducers. By disrupting pRb phosphorylation, Cdk inhibitors arrest cell cycle progression and induce apoptosis. Several inhibitors with differing Cdk subtype selectivities are in clinical trials (e.g. Flavopiridol and Roscovitine).

- **Bcl-2 modulators**: Small molecules and antisense oligonucleotides that inhibit the anti-apoptotic activity of the Bcl-2 family of proteins. Upregulation of anti-apoptotic proteins Bcl-2, Bcl-XL, Bcl-w, BFL1, Bcl-B and
MCL1 occurs via chromosomal translocation in many cancers and enhances the survival of cells. Antisense oligonucleotides have reached phase III trials as Bcl-2 suppressors but are yet to receive FDA approval. Several small molecule Bcl-2 inhibitors (e.g. R-(-)-gossypol, Obatoclax) are in Phase II clinical trials.\textsuperscript{16}

- **Telomerase Inhibitors:** Small molecule inhibitors of the telomerase reverse transcriptase (TERT) catalytic subunit. Telomerase expression varies from low/no expression in differentiated somatic cells to high activity in germline stem cells. This activity is essential for the maintenance of stem cell immortality and is hijacked by cancer cells when the TGF\β regulatory pathway is disrupted as part of the tumourigenic process.\textsuperscript{17} Small synthetic TERT inhibitors and template antagonists such as BIBR1532 and the oligonucleotide-lipid conjugate imetelstat (GRN163L) have shown promising activities, and Imetelstat is currently in early clinical trials.\textsuperscript{18-20} Indirect approaches for inhibiting telomerase also appear promising (e.g. inhibitors of poly(ADP-ribose) polymerases such as tankyrase I).\textsuperscript{18}

- **Vascular Endothelial Growth Factor (VEGF) Signal Blockers:** Small molecule inhibitors and antibodies that block the angiogenic signaling at VEGF receptors. Members of the VEGF family, such as VEGF, fibroblast growth factors (FGFs) and epidermal growth factor (EGF) are the primary stimulators of angiogenesis in cancer.\textsuperscript{21} These growth factors are often under the control of oncogenes such as \textit{ras}. Many tyrosine kinase inhibitors that block growth signaling (e.g. imatinib) also block angiogenesis by
inhibiting receptor tyrosine kinases such as the VEGF receptor. Some angiogenesis-specific small molecule growth factor receptor inhibitors have undergone clinical trials, including SU5416, a F1k-1/KDR/VEGF-2 receptor inhibitor, and SU6668, a broad-spectrum inhibitor of FGF, VEGF and platelet-derived growth factor receptor signaling. Sunitinib is an analogue of SU6668 and is one of only two receptor tyrosine kinase inhibitors approved for clinical use, the other being the structurally-unrelated Sorafenib. An alternative method for inhibiting angiogenic growth factor signaling uses immunotherapy directed against specific growth factors. One example in clinical use employs a humanised monoclonal antibody to target VEGF (Avastin).

- **Matrix Metalloprotease (MMP) Inhibitors** Expression of MMPs is upregulated in many cancers. These enzymes degrade the extracellular matrix (ECM) promoting cellular migration and exposing latent growth factors embedded within the matrix. MMP inhibitors generally target more than one type of MMP. For example, the promising natural product neovastat acts as a broad spectrum MMP inhibitor. More selective are the MMP-2/MMP-9 inhibitor BMS275291 and the specific MMP-9 inhibitor PCK3145. Several non-selective MMP inhibitors have reached clinical trials.
1.3 Tumour Metastasis

In patients with localised tumours, the likelihood of mortality following surgical removal and/or noninvasive therapeutic intervention is low. However, when primary tumours progress to a metastatic state, spreading to other organs and developing into secondary tumours, prognosis for patients is dramatically worsened.1,25

For a tumour to successfully metastasise, cells must gain motility, detach from tumour masses and subsequently degrade basement membranes and ECM in order to come into contact with the vasculature or lymph vessels (Figure 1.2a).25,26 Once in the bloodstream, tumour cells can attach to the endothelium at a distant site, commonly in highly vascularised tissues like the liver or lung. Extravasation from blood vessels (which requires relatively little proteolytic effort) then occurs allowing tumour cells to establish secondary metastases.27 Intravasation from primary tumour sites into blood vessels is generally the rate-limiting step in metastasis with the structurally and functionally abnormal vessels present in tumours often assisting this process due to their high permeability.23,28-31 Mosaic vessels, where tumour cells themselves contribute to 25% of the vessel perimeter, are often a common feature of the tumour vasculature (Figure 1.2b).

Acquisition of enhanced proteolytic capability and invasive potential is widely recognised as the final breach of normal cellular behaviour and one which
promotes tumour metastasis. Expression and excretion of extracellular proteases, such as MMPs, plasminogen (plasmin), cathepsins, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), is often observed in metastatic tumours.

**Figure 1.2.** (a) Major steps required for tumour metastasis: (1) Basement membrane breakdown and release of cells from the primary tumour; (2) Stromal invasion; (3) Intravasation; (4) Vascular transport; (5) Arrest on capillary endothelium; (6) Extravasation; (7) Secondary tumour establishment. (b) Representation of a mosaic tumour blood vessel where tumour cells form part of the vessel wall. Figures (a) and (b) adapted from references 26 and 23, respectively.

The stromal cells surrounding the tumour are often the source of proteases rather than the tumour cells themselves. Upregulation of these proteases occurs through a variety of signaling pathways, including classical growth factor stimulated mitogenic pathways, intracellular protein kinase C activation and mutations in p53.
Exposure of growth factors sequestered in the ECM during matrix remodelling serves to stimulate angiogenesis, cell growth and protease expression.\textsuperscript{37-39}

Rapid growth and turnover of cells coupled with aberrant vascularisation and nutrient deprivation makes solid tumours produce energy through glycolysis. This leads to an initial buildup of acidic byproducts in the cytosol but activation of the sodium hydrogen exchanger (NHE) (see chapter 1.7.2) counters the buildup of intracellular protons by displacing them to the extracellular environment.\textsuperscript{40,41} The modified transmembrane pH and acidic tumour microenvironment leads to activation of matrix-degrading proteases (e.g. cathepsin B),\textsuperscript{42} that promote metastasis.

Cell-cell signaling mediated through direct contact between a variety of integrins, focal adhesion kinase (FAK), the uPA receptor (uPAR) and a large array of other cell-surface proteins is important for metastasis.\textsuperscript{43,44} Altered integrin expression in metastatic cells allows for interactions with elements of the ECM that stimulate migration and enhance receptor expression. Overexpression of the $\alpha3\beta1$ integrin results in altered cell binding to the ECM proteins fibronectin, collagens and laminins. In particular, interaction of $\alpha3\beta1$ with the scatter factor laminin-5 promotes migration and reduces cell-cell adhesion.\textsuperscript{45,46} Laminin-5 is coexpressed with uPAR by migrating tumour cells and together these can drive invasion.\textsuperscript{47} uPAR is an important binding partner for integrins (see Section 2), with the complex
serving to transduce mitogenic signals through the FAK or extracellular regulated kinase (ERK) pathways.\textsuperscript{48-50}

1.4 The Urokinase Plasminogen Activation System (uPAS) and Metastasis

The urokinase plasminogen activation system (uPAS) is responsible for an extracellular proteolytic cascade that is fundamentally involved in regulation of macrophage migration, pericellular proteolysis in cell motility, adhesion, and proliferation.\textsuperscript{32,51} Components of the system include the serine protease uPA (and its zymogen pro-uPA), its cognate receptor uPAR, and several endogenous uPA inhibitors (i.e. plasminogen activator inhibitors PAI-1, PAI-2 and PAI-3).\textsuperscript{52-56}

To initiate basement membrane and ECM degradation during cell migration, uPAR colocalizes with integrins in caveolin and/or ganglioside-rich lipid rafts at the leading edge of migrating cells.\textsuperscript{57-61} Plasminogen associates with the cell surface by binding to a number of receptors, such as integrin $\alpha$IIb$\beta$3, annexin II and actin microfilaments, via the lysine-binding motifs in its five kringle domains.\textsuperscript{62-64} Cell-surface binding by plasminogen induces a conformational change to an open form that is more susceptible to activation. The low but detectable intrinsic proteolytic activity of pro-uPA is thought to be responsible for cleavage of plasminogen to reveal the active protease plasmin. In a positive feedback loop, plasmin then converts receptor-bound pro-uPA to active uPA.\textsuperscript{65} Activation of pro-uPA is also
effected, at least in vitro, by cathepsin B, cathepsin L and kallikrein, all of which are upregulated in tumour microenvironments. Receptor-bound uPA can convert plasminogen to plasmin more efficiently than unbound uPA, and can localise plasmin activity to the leading edge of migrating cells. Plasmin in the serum or plasma is inhibited by α2-antiplasmin and to a lesser extent by α2-macroglobulin. Cell-surface bound plasmin associated with components of the uPAS is protected from inhibition by these endogenous inhibitors. Plasmin cleaves ECM structural proteins such as fibrin, fibronectin, tenascin, aggrecan, proteoglycans and laminin, and activates zymogens of MMPs 1, 2, 3, 9 and 14. The activated MMPs degrade a broad-spectrum of matrix components. A summary of the key components of the uPAS is provided in Figure 1.3.

Figure 1.3. The urokinase plasminogen activation system (uPAS) and its role in ECM degradation. G = growth factor domain, K = kringle domain, SP = serine protease domain. The three domains of uPAR are labelled 1, 2 and 3 (See Section 2, 5.1 and 5.2 for descriptions of the three uPAR domains and three uPA domains, respectively). Solid filled arrows indicate stimulation. Unfilled arrows indicate inhibition. Circles with open sectors represent active proteases. Adapted from Reference 80.
Members of the uPAS have valuable prognostic significance in breast, colorectal, gastric, ovarian and renal cancer, where elevated levels of uPA, PAI-1 and uPAR all predict adverse patient outcomes. Elevated uPA and PAI-1 levels in breast cancer are in fact more reliable predictors of tumour progression than patient age, tumour grade and size, estrogen receptor, progesterone receptor, HER-2/neu and p53 status. Several Level 1 evidence studies have confirmed the prognostic value of uPA/PAI-1 as candidate markers for routine clinical testing in breast cancer.

Numerous studies have revealed that inhibition of the hydrolytic activity of uPA expressed in tumour tissues has an inhibitory effect on metastasis. Experiments with gene-knockout mice have shown that lack of uPA or uPAR does not compromise development or fertility, and does not impact on fibrinolytic ability. This is thought to be due to the overlap of function with the haemostatic plasminogen activator tPA. uPA or uPAR knockout mice also show a propensity to resist metastasis of induced tumours or xenografts. Plasminogen gene-knockout mice, on the other hand, develop spontaneous thrombotic lesions, have impaired wound healing and show signs of fibrin deposition and severe developmental impairment. This suggests that inhibiting uPA activity with therapeutics should not produce major detrimental side effects making it an attractive anticancer drug target. Accordingly, several classes of uPA inhibitors have been reported and selected analogues have undergone preclinical evaluation as non-cytotoxic antitumour/antimetastasis drugs.
1.5 Reported uPA Inhibitors

The uPA enzyme contains an inhibitor-binding pocket (S1 pocket) that overlaps to a large extent with the active site (See Section 2, Chapter 5.2 for a detailed description of uPA’s structure).\(^{105}\) Many of the known potent uPA inhibitors \((K_i < 1 \mu\text{M})\) share structural similarities, including an arginine-mimetic amidine or guanidine group that carries a positive charge at physiological pH. This group is essential for making a strong salt bridge contact with the negatively-charged Asp189 located at the base of the S1 binding pocket.\(^{106}\)

![Inhibitor Structures](attachment:inhibitor_structures.png)

**Figure 1.4.** Reported uPA inhibitors.
In 1965 it was reported that uPA is inhibited by ε-aminocaproic acid, but there was little interest in the development of uPA inhibitors until the late 1970s. Early synthetic uPA inhibitors were simple aryl amidines (e.g. benzamidine) and arginine- or lysine-like amino acid derivatives, such as esters of aminocaproic acid (e.g. 1). $K_i$ values as low as 300 nM were reported for these compounds. Cyclic peptide inhibitors such as 2, containing arginine or lysine, were reported as uPA inhibitors in 1993. The dimethylsulfonium ion acts to alkylate uPA catalytic Ser195 making these inhibitors irreversible ($2 \ K_i = 41 \text{ nM}$). Potent aryl amidine inhibitors based on benzo[b]thiophene scaffolds (e.g. B623, 3) have shown $K_i$ values as low as 70 nM. Naphthamidine-based uPA inhibitors with sub-nanomolar potency were reported in 2000 (e.g. 4, $K_i = 0.64 \text{ nM}$). Carbonate or hydroxyamidino prodrugs (e.g. 5) of peptidomimetic uPA inhibitors were recently reported and showed high potency. A series of uPA inhibitors bear an isoquinolyl guanidine scaffold with sulfonamides at the 7-position. The best compound 6 inhibited uPA with a $K_i$ of 10 nM but, importantly, exhibited 1000-fold selectivity for uPA over tPA and plasmin.

Attempts to address the poor bioavailability of many reported uPA inhibitors led to the discovery of isocoumarin derivatives (e.g. 7), which use a thiourea to fill uPA’s Asp189-containing S1 pocket. Other studies aimed at improving oral bioavailability recently led to non-amidine containing compounds like mexiletine derivative 8, which shows reasonable uPA selectivity and good potency ($IC_{50} = 72 \text{ nM}$).
The only uPA inhibitor to undergo clinical trials to date is an orally-active N-hydroxyamidine prodrug form of WX-UK1 (Mesupron, $K_i = 410$ nM). This compound shows low selectivity for uPA over related proteases but showed effective antitumour activity in animals and has completed phase II clinical trials, where it is being investigated for use in combination with gemcitabine in late stage pancreatic cancer patients. It is also in phase II trials for treatment of HER2-negative breast cancer patients in combination with capecitabine. Trials to date have shown that the drug is well tolerated and combination therapy with gemcitabine gave an impressive one year progression-free survival rate improvement of 66% and a one year overall survival rate improvement of 49%. The outcome of pivotal phase III trials will validate (or otherwise) the efficacy of uPA-targeted compounds against cancer.

1.6 Binding Interactions Between uPA Inhibitors and uPA

Several co-crystal structures of uPA complexed with inhibitors are available making drug design based on the enzyme’s three-dimensional structure possible. A net positive charge at physiological pH is an essential feature in all uPA inhibitors as this makes the crucial salt-bridge interaction with Asp189 at the base of the S1 pocket (See Figure 1.8, Page 38). Some inhibitors extend out from the S1 site to other pockets in the uPA substrate-binding groove making contacts with residues of the catalytic triad (His57, Asp102 and Ser195). The S1 site is bounded by residues Lys143, Ser146, Asp189, Ser190, disulfide Cys191-Cys220, Gln192, Asp194, Ser195,
Val213, Ser214, Gly218 and Tyr228. A nearby hydrophobic subsite designated S1β is bounded by residues Gly218, Ser146, disulfide Cys191-Cys220 and parts of Lys143 and Gln192. Inhibitors bearing substituents that occupy the S1β subsite tend to have higher selectivity for uPA over other trypsin-like serine proteases.

Selectivity over other serine proteases which replace the Ser190 of uPA with alanine (e.g. tPA) is achieved with inhibitors that displace a water molecule that participates in a hydrogen bond network in the S1 site of Ala190 proteases. The water molecule compensates for the lack of a hydrogen bonding partner at position 190 and its displacement creates a hydrogen bond deficit that significantly amplifies specificity. The S2, S3 and S4 sites of uPA are reduced in comparison with other serine proteases due to a two-residue insertion at His99. Improving contacts with Asp60A, a residue unique to uPA through a single-residue insertion, leads to inhibitors with higher uPA selectivity.

Crystal structures of uPA and related serine proteases often show a sulfate ion (arising from the crystallisation buffer) bound in the active site. The sulfate makes contacts with Ser195Oγ, Gly193N and His57Ne2. This region, which is common to all serine proteases and is referred to as the oxyanion hole, can accommodate Zn²⁺, water molecules or sulfate ions and is commonly targeted by serine protease inhibitors.
1.7 Amiloride as an Antitumour/Antimetastasis Agent

Amiloride.HCl 10 is an orally administered potassium-sparing diuretic that has been used clinically for several decades. Its principal clinical use is, ironically, not as a diuretic per se, because its diuretic effects are relatively mild. It is instead more commonly used in combination with thiazide (e.g. hydrochlorothiazide) or loop diuretics (e.g. frusemide) as an antikaliuretic in patients at risk of hypokalaemia during long term treatment of hepatic cirrhosis and heart failure. It is also frequently used in combination with other diuretics to control K⁺-levels when treating hypertension.¹³⁹ The drug is usually well tolerated at normal doses, shows low incidence of side effects and is contraindicated only in patients displaying impaired renal function, hyperkalaemia or acidosis. Hyperkalaemia and associated arrhythmias are amiloride’s most serious side effects but when used in combination with thiazide diuretics the incidence of these is less than 1-2%.¹⁴⁰

![Chemical structure of the K⁺-sparing diuretic amiloride.](image)

**Figure 1.5.** Chemical structure of the K⁺-sparing diuretic amiloride.

After its discovery amiloride found other important non-clinical uses as a pharmacological tool for probing sodium transport in many types of tissues and cells – uses which continue to this day.¹⁴¹ Multiple studies over the past 3 decades have demonstrated that amiloride also possesses significant antitumour and
antimetastasis activities in biochemical, cellular and animal tumour models. The following sections summarise these reported properties of amiloride and discuss its purported mechanisms of action.

1.7.1 Antitumour/Antimetastasis Effects of Amiloride in Experimental Models

Reports of in vivo antitumour properties for amiloride first appeared in 1983 when it was found that the drug inhibited H6 hepatoma growth and DMA/ J mammary adenocarcinoma growth in a dose-dependent fashion in Male A/ J mice. Multiple injections of 1 mg/kg were shown to inhibit tumour growth and tumour cell proliferation and the effects correlated with significant decreases in tumour cell sodium content. Since this initial report several studies have shown that amiloride inhibits the formation of mutagen-induced lesions in animals, decreases the size of tumour allografts and xenografts and suppresses metastases. These effects are summarised in Table 1.2.
Table 1.2. Antitumour and antimetastasis effects of amiloride in rodent tumour models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Drug started at time of tumour inoculation/ Mutagenesis</th>
<th>Amiloride dose and route of administration</th>
<th>Schedule</th>
<th>Outcomes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinogenesis in rats</td>
<td>Mutagen induced pancreatic lesions</td>
<td>Yes</td>
<td>5 mg/kg, s.c.</td>
<td>Every other day for 62 weeks</td>
<td>↓ Number and size of ATPase-positive lesions</td>
</tr>
<tr>
<td></td>
<td>Mutagen induced hepatic lesions</td>
<td>Yes</td>
<td>5 mg/kg, s.c.</td>
<td>Every other day for 16 weeks</td>
<td>↓ Number and size of lesions</td>
</tr>
<tr>
<td></td>
<td>Mutagen induced gastric cancer</td>
<td>No, 25 week lead time</td>
<td>5 mg/kg, s.c.</td>
<td>Every other day for 25 weeks</td>
<td>↓ incidence antral mucosa proliferation - no effect</td>
</tr>
<tr>
<td></td>
<td>Mutagen induced colon cancer</td>
<td>Yes</td>
<td>7.5 mg/kg, s.c.</td>
<td>Daily for 35 weeks</td>
<td>↓ incidence tumour cell proliferation - no effect</td>
</tr>
<tr>
<td></td>
<td>Mutagen induced intestinal cancer with Bombesin-enhanced peritoneal metastases</td>
<td>Yes</td>
<td>5.0 mg/kg plus 40 μg/kg bombesin, s.c.</td>
<td>Every other day for 45 weeks</td>
<td>↓ incidence of peritoneal metastases</td>
</tr>
<tr>
<td>Primary tumour allografts in mice</td>
<td>H6 hepatoma or DMA/J mammary, non-orthotopic</td>
<td>Yes</td>
<td>1 mg/kg i.v. - site of injection not specified</td>
<td>Every 8 h for ~5 days</td>
<td>↓ Tumour growth Tumour cell proliferation Tumour cell Na content</td>
</tr>
<tr>
<td>Established primary tumour xenografts, mice</td>
<td>DU145 or LnCaP human prostate, non-orthotopic</td>
<td>No, 24 day lead time</td>
<td>200 mg/kg/day, oral</td>
<td>ad libitum for 16 days</td>
<td>↓ Tumour growth toxic effects observed</td>
</tr>
<tr>
<td>Primary tumour implant in rats</td>
<td>R3327-AT3 rat prostate, non-orthotopic</td>
<td>N/S³</td>
<td>0.25 mg/kg/day, oral</td>
<td>ad libitum for 3 weeks</td>
<td>- no effect on tumour growth - no metastases to lymph nodes by visual inspection</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------------</td>
<td>------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Primary tumour allograft with metastases to lungs</td>
<td>MATB rat mammary, orthotopic</td>
<td>Yes</td>
<td>20 mg/kg/day, oral</td>
<td>ad libitum for 28 days</td>
<td> Number of pulmonary metastatic foci - no effect on primary tumours - no effect</td>
</tr>
<tr>
<td>Experimental lung metastasis allograft in rats</td>
<td>R3230 AC rat mammary</td>
<td>Yes</td>
<td>0.3 mg/kg/day, oral</td>
<td>ad libitum for 24 days</td>
<td>- Complete elimination of lung metastases</td>
</tr>
<tr>
<td>MATB 13762 rat mammary</td>
<td>No, 7 days prior to inoculation Yes No, 3 day lead time</td>
<td>10 or 0.3 mg/kg/day, oral</td>
<td>ad libitum for 17 days</td>
<td> Number of pulmonary metastatic foci, dose-dependent  Number of pulmonary metastatic foci - no differences between dose schedules</td>
<td></td>
</tr>
<tr>
<td>MATB rat mammary</td>
<td>No, 7 days prior to inoculation Yes No, 3 day lead time</td>
<td>20 mg/kg/day, via osmotic pump implanted i.p.</td>
<td>Over 17 days</td>
<td> Number of pulmonary metastatic foci but no differences between dose schedules</td>
<td></td>
</tr>
</tbody>
</table>

¹ Compared to controls
² All oral administrations were via drinking water
³ Not specified
1.7.2 Antitumour/Antimetastasis Effects of Amiloride Mediated Through Inhibition of Sodium-Hydrogen Exchangers (NHEs)

It is well established that the clinical diuretic effects of amiloride arise through blockade of epithelial sodium channels (ENaCs) in cells of the distal nephron. However, multiple studies have shown that amiloride also blocks NHEs (also referred to as $\text{Na}^+$/H$^+$ antiporters).$^{154}$ NHEs are integral membrane proteins that exchange one intracellular proton for one extracellular sodium ion. Nine mammalian NHE isoforms (NHE1-NHE9) are known and several are upregulated and/or activated in tumours.$^{154,155}$ Inhibitory effects of amiloride on NHEs, particularly NHE1, are thought to be responsible, at least in part, for its anticancer effects.

NHE1 is a primary cellular housekeeping $\text{Na}^+$/H$^+$ antiporter and is present in the membranes of many types of cells. It serves to regulate intracellular pH ($\text{pH}_i$) and cell volume and is involved in changing the growth and functional states of cells. Upon activation, NHE1 activity reverses the transmembrane pH gradient in transformed and malignant cells by lowering the extracellular pH ($\text{pH}_e$). This occurs mainly in solid tumours because, as mentioned previously (Section 1.3), poor vascularisation promotes regions of hypoxia where cells metabolise glucose predominantly via anaerobic glycolysis. The resulting acidic metabolites (e.g. lactic acid) initially lower $\text{pH}_i$ but activation of NHE1 activity quickly transfers the
increased intracellular $[\text{H}^+]$ to the extracellular environment to establish the well characterised low pH$_e$ of solid tumours (pH$_e$ = 6.8 vs pH$_e$ = 7.2 in normal cells).\textsuperscript{156}

The switch to acidic pH$_e$ is thought to be a key step in oncogenic transformation and necessary for tumour cells to maintain the transformed phenotype. For example, it has been shown that tumour cells deficient in NHEs fail to grow tumours or show very slow growth when implanted into immunocompromised mice.\textsuperscript{157} The transmembrane pH reversal is also thought to be a key driver of invasion and metastasis\textsuperscript{158-160} since low pH$_e$ activates ECM-degrading enzymes (cysteine proteases, e.g. cathepsin B), hyaluronidase 2 and pro-angiogenic factors.\textsuperscript{42,161} As examples, increases in lysosomal trafficking to cell surfaces and exocytosis of matrix-degrading proteases have been shown to occur in prostate cancer cells at low pH$_e$,\textsuperscript{162} and heightened levels of the pro-angiogenic factor VEGF have been observed in human myeloid K562 cells.\textsuperscript{163} The requirement that tumour cells maintain acidic extracellular environments through upregulation of NHE activity and observations that decreased pH$_e$ promotes invasion and metastasis are increasingly being recognised as two general hallmarks of cancer.\textsuperscript{164} Accordingly, inhibition of NHEs is emerging as an exciting new anticancer therapeutic strategy.\textsuperscript{165,166}

Quantitative measurements of NHE-dependent Na$^+$ uptake into chick skeletal muscle cells showed that amiloride is a moderate NHE inhibitor (reduces cellular
Na⁺ uptake by 50% (K₀.₅) at a concentration of 7 μM.¹⁶⁷ Amiloride inhibits NHE1 by binding to a site surrounding Glu346 located in its extracellular domain – the same site responsible for Na⁺ binding.¹⁶⁸ NHE inhibition by amiloride or its more potent analogue 5-(N-ethyl-N-isopropyl)-amiloride ¹₁¹ (EIPA; 223 x more potent than amiloride)¹⁷⁰ has been shown to impair DNA replication, inhibit proliferation and migration and promote apoptosis in smooth muscle cells.¹⁶⁷,¹⁶⁹,¹⁷⁰ Another amiloride analogue 5-(N,N-hexamethylene)amiloride ¹₂ (HMA, 524 x more potent than amiloride)¹⁷⁰ impairs the growth and viability of human and rat hepatocarcinoma cells, possibly through a lowering of reduced glutathione levels and loss of lysosomal integrity.¹⁷¹

**Figure 1.6.** Amiloride analogues EIPA and HMA.

A recent report showed that NHE1 inhibition by EIPA reduces hepatocellular carcinoma invasion and motility.¹⁵⁸ NHE-mediated reductions in pHᵢ induced by liver tumour promoting xenobiotics can increase DNA synthesis and transcription in hepatocytes, suggesting that NHEs are key drivers of hepatocyte proliferation. Inhibition of NHEs by amiloride, EIPA or HMA has been shown to reduce pHᵢ and suppress these proliferative effects.¹⁷²
Inhibition of NHEs by amiloride can produce tumour-selective effects. It has been found that altered proton gradients established through upregulated NHE activity sensitise tumour cells much more than normal cells to intracellular acid damage when NHEs are inhibited.\textsuperscript{156} It is thought that low pH\textsubscript{e} of tumours also potentiates amiloride’s tumour selectivity by increasing the active NHE-inhibiting protonated acylguanidinium form of the drug in the local tumour environment.\textsuperscript{168} Some reports indicate that NHE inhibition and cytoplasmic acidification with amiloride and its analogues increases thermosensitivity of tumour cells relative to normal cells.\textsuperscript{173-175}

When tested against SCK mammary adenocarcinoma cells, amiloride, EIPA and HMA each showed enhanced tumour cell killing at 43 °C in pH 6.6 media.\textsuperscript{176} It has been proposed that hyperthermic treatments with such agents might be worth trialling against poorly responding malignant gliomas since C6 glioma cells exposed to amiloride show enhanced thermosensitivity at clinically achievable temperatures (42.0 – 42.5 °C).\textsuperscript{177} However, the fact that the acylguanidine carries a charge at physiological pH makes it unlikely to cross the blood brain barrier and administration would require either direct physical injection in the cerebrospinal fluid or masking of the acylguanidine as a prodrug.

Recent findings indicate that NHE-induced reductions in pH\textsubscript{e} can give rise to tumour-selective activation of acid sensing ion channels (ASICs).\textsuperscript{178} ASICs, which belong to the ENaC superfamily, are predominantly found in neurons but they are also being discovered in other cell types.\textsuperscript{178,179} While their normal physiological
functions are still being elucidated one possibility is that that functional expression of ASICs may help tumours adapt to the low pH environments created by increased NHE activity.\(^{180}\) An important contributor to the migratory capacity of metastatic tumour cells is their ability to alter size and shape through volume regulation. Constitutively active ASICs function to regulate volume changes in human glioma cells\(^{181}\) and it is postulated that this may contribute to their high invasiveness.\(^{179,182}\) Amiloride inhibition of ASICs has been shown to render glioma cells unable to volume regulate and to reduce their migratory capacity.\(^{179,181}\)

Leukemic and normal hematopoietic cells exhibit different sensitivities to NHE inhibition by amiloride and its analogues.\(^{183}\) While treatment with HMA induces apoptosis in more than 90% of leukemic cells, the presence of the drug does not affect the viability of normal hematopoietic cells. It is suggested that this effect probably arises because NHE1 is constitutively active in proliferating leukemic cells but not normal hematopoietic cells.\(^{183}\) Tumour-cell selective apoptosis arising through NHE-inhibition may in fact be a more general phenomenon, possibly driven by enhancements in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) activity and heightened inhibition of cell survival-promoting kinases at lowered pH.\(^{184}\) Tumour cell-selective TRAIL-mediated pro-apoptotic responses to amiloride appear to parallel the signaling events observed with the HER-2/neu antibody trastuzumab (Herceptin).\(^{185}\)
1.7.3 Antitumour/Antimetastasis Effects of Amiloride Mediated through Inhibition of uPA

While much evidence supports inhibition of NHEs as a key mechanism responsible for amiloride’s anticancer properties it is clear that some of the effects, particularly those involving metastasis suppression, may arise either independently or synergistically through inhibition of the uPAS. Amiloride is a moderately potent, inhibitor of uPA ($K_i = 7\mu M$)\textsuperscript{186} that binds reversibly to its active site.\textsuperscript{130} Several reports have shown that inhibition of uPA by amiloride closely correlates with anticancer effects. PC-3 prostate cancer cells displaying amplified expression of the uPA gene and increased invasiveness are more sensitive to amiloride’s effects than cells which do not have the amplification (DU145 or LNCaP).\textsuperscript{187} Experiments comparing rats bearing fast growing highly metastatic R3327-AT3 prostatic tumours, which exhibit high uPA activity, with others bearing slower growing non-metastatic G3327-G tumours and low uPA activity, showed that amiloride treatment reduces uPA activity and affords protection from secondary lymph metastases.\textsuperscript{149} UCT-2 transitional cells are an invasive urothelial cancer cell line that shows high levels of uPA mRNA expression as well as high uPA protein content and activity. These parameters are all low in the non-invasive UCT01 cells. Treatment \textit{in vitro} with amiloride was shown to significantly inhibit plasmin formation and invasiveness in both cell types.\textsuperscript{188} Suppression of experimentally induced metastasis of MTAB rat mammary cancer cells has been correlated to inhibition of uPA.\textsuperscript{152}
Clear demonstration of antitumour and antimetastasis effects in multiple biochemical, cellular and in vivo animal models of cancer would normally provide very strong support for examining a compound such as amiloride in anticancer clinical trials. Oral delivery of the drug along with its longstanding use in humans and well known safety profile should make it a particularly attractive candidate. Additionally, epidemiological studies into the survival rates of cancer patients who have coincidentally been co-prescribed amiloride for separate conditions would normally also be of significant interest. It is therefore somewhat surprising that there is only one report in which the drug has undergone any form of clinical evaluation in the cancer setting, either as a standalone agent or in combination with other chemotherapeutics. In that report, no significant difference in cancer risk was found in old patients with hypertension on a fixed-ratio of hydrochlorothiazide 25 mg plus low dose amiloride (2.5 mg) verses controls over a five year period.\textsuperscript{189}

There are two important issues which may complicate and ultimately preclude successful anticancer outcomes with amiloride in humans. Firstly, patients taking amiloride at the maximum recommended daily dosage (20 mg/day in a single or divided doses)\textsuperscript{140} would require careful electrolyte monitoring over an extended period in order to reduce the risks of hyperkalaemia and cardiac arrhythmias. In fact, amiloride administered at this high dosage would in all likelihood need to be
co-administered with a kaliuretic agent to minimise these risks. Secondly, as discussed above, amiloride’s anticancer properties appear to arise principally through its effects on NHEs and uPA; targets for which it shows only moderate in vitro potency. It is therefore reasonable to expect that administration of 20 mg/day might not yield plasma concentrations high enough to elicit useful anticancer effects in humans. Compounding this would be the fact that amiloride acts as a reversible competitive antagonist of Na⁺ at NHEs. An abundance of extracellular Na⁺ ions means that very high local concentrations of amiloride would therefore need to be achieved in tumours in order to inhibit NHE function.

An alternative option might be to identify an amiloride analogue which shows higher potency against NHE1 with a concomitant reduction in diuretic and antikaliuretic effects. Modifying an existing drug to produce a new drug for a different disease indication (while losing its original pharmacological effects) is known as the Selective Optimisation of a Side Activity (SOSA) approach, and is popular in modern drug discovery. Much is known about the structure-activity relationships (SARs) of amiloride analogues and their relative potencies against NHE1 (See Table 1.3). Analogues bearing substituents at the 5-position, such as EIPA and HMA, can be 500x more potent NHE inhibitors than amiloride (Table 1.3). Importantly, 5-substituted
analogues can also show reduced effects on urinary electrolytes. For example, HMA effects on urinary Na⁺/K⁺ ratios are 80-fold less than those of amiloride.¹⁹¹

Table 1.3. NHE-inhibitory potencies of amiloride and selected analogues.¹⁶⁷,¹⁷⁰,¹⁹³ \( K_i \) values determined using Rat NHE1 are indicated by italics.¹⁹³ Numbers in brackets indicate potency relative to amiloride, determined in human neutrophils.¹⁷⁰ PSA is a platelet-swelling assay where the swelling of human platelets is induced by incubation in an acidic (pH 6.7) buffer.¹⁹² The optical density of platelet solutions is measured over time to determine degree of platelet swelling (corresponding to inhibition of NHE). Results from NHE inhibition assays are heavily dependent upon several factors including pH, which affects the proportion of amiloride analogue molecules in the active charged form, and extracellular Na⁺ concentration, which decreases the apparent affinity of amiloride analogues as it increases due to competitive binding.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NHE1 Inhibition IC₅₀ or ( K_i ) (µM)</th>
<th>PSA IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Amiloride)</td>
<td>83.8, (1)</td>
<td>100.1</td>
</tr>
<tr>
<td>(HMA)</td>
<td>0.013, (524)</td>
<td>3.3</td>
</tr>
<tr>
<td>(EIPA)</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>(190)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(133)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(125)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chemical Structure</td>
<td>Chlorine (Cl)</td>
<td>Assay Value</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td><img src="image1.png" alt="Chemical Structure 1" /></td>
<td>Cl</td>
<td>(123)</td>
</tr>
<tr>
<td><img src="image2.png" alt="Chemical Structure 2" /></td>
<td>Cl</td>
<td>(102)</td>
</tr>
<tr>
<td><img src="image3.png" alt="Chemical Structure 3" /></td>
<td>Cl</td>
<td>(62)</td>
</tr>
<tr>
<td><img src="image4.png" alt="Chemical Structure 4" /></td>
<td>Cl</td>
<td>(61)</td>
</tr>
<tr>
<td><img src="image5.png" alt="Chemical Structure 5" /></td>
<td>Cl</td>
<td>(29)</td>
</tr>
<tr>
<td><img src="image6.png" alt="Chemical Structure 6" /></td>
<td>Cl</td>
<td>(26)</td>
</tr>
<tr>
<td><img src="image7.png" alt="Chemical Structure 7" /></td>
<td>Cl</td>
<td>(26)</td>
</tr>
<tr>
<td><img src="image8.png" alt="Chemical Structure 8" /></td>
<td>Cl</td>
<td>(22)</td>
</tr>
<tr>
<td><img src="image9.png" alt="Chemical Structure 9" /></td>
<td>Cl</td>
<td>(13)</td>
</tr>
<tr>
<td><img src="image10.png" alt="Chemical Structure 10" /></td>
<td>Cl</td>
<td>(13)</td>
</tr>
<tr>
<td><img src="image11.png" alt="Chemical Structure 11" /></td>
<td>Cl</td>
<td>0.023, (12)</td>
</tr>
<tr>
<td><img src="image12.png" alt="Chemical Structure 12" /></td>
<td>Cl</td>
<td>(10)</td>
</tr>
<tr>
<td><img src="image13.png" alt="Chemical Structure 13" /></td>
<td>Cl</td>
<td>(9.6)</td>
</tr>
<tr>
<td><img src="image14.png" alt="Chemical Structure 14" /></td>
<td>Cl</td>
<td>(9)</td>
</tr>
<tr>
<td><img src="image15.png" alt="Chemical Structure 15" /></td>
<td>Cl</td>
<td>(8.7)</td>
</tr>
<tr>
<td><img src="image16.png" alt="Chemical Structure 16" /></td>
<td>Cl</td>
<td>(7.4)</td>
</tr>
<tr>
<td><img src="image17.png" alt="Chemical Structure 17" /></td>
<td>Cl</td>
<td>(7.4)</td>
</tr>
<tr>
<td><img src="image18.png" alt="Chemical Structure 18" /></td>
<td>Cl</td>
<td>(6.6)</td>
</tr>
<tr>
<td><img src="image19.png" alt="Chemical Structure 19" /></td>
<td>Cl</td>
<td>(6.3)</td>
</tr>
<tr>
<td><img src="image20.png" alt="Chemical Structure 20" /></td>
<td>Cl</td>
<td>(5.1)</td>
</tr>
<tr>
<td><img src="image21.png" alt="Chemical Structure 21" /></td>
<td>Cl</td>
<td>(4.1)</td>
</tr>
<tr>
<td><img src="image22.png" alt="Chemical Structure 22" /></td>
<td>Cl</td>
<td>(3.6)</td>
</tr>
<tr>
<td><img src="image23.png" alt="Chemical Structure 23" /></td>
<td>Cl</td>
<td>(3.3)</td>
</tr>
<tr>
<td><img src="image24.png" alt="Chemical Structure 24" /></td>
<td>Cl</td>
<td>0.1*</td>
</tr>
<tr>
<td><img src="image25.png" alt="Chemical Structure 25" /></td>
<td>Cl</td>
<td>0.1*</td>
</tr>
<tr>
<td><img src="image26.png" alt="Chemical Structure 26" /></td>
<td>Cl</td>
<td>0.2*</td>
</tr>
<tr>
<td>Structure</td>
<td>Inhibitor</td>
<td>$K_0.5$ Value</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
</tr>
<tr>
<td><img src="image1" alt="Structure" /></td>
<td>Cl</td>
<td>0.3*</td>
</tr>
<tr>
<td><img src="image2" alt="Structure" /></td>
<td>Cl</td>
<td>0.3*</td>
</tr>
<tr>
<td><img src="image3" alt="Structure" /></td>
<td>Cl</td>
<td>1*</td>
</tr>
<tr>
<td><img src="image4" alt="Structure" /></td>
<td>Cl</td>
<td>2*</td>
</tr>
<tr>
<td><img src="image5" alt="Structure" /></td>
<td>Cl</td>
<td>2.3*</td>
</tr>
<tr>
<td><img src="image6" alt="Structure" /></td>
<td>Cl</td>
<td>3*</td>
</tr>
<tr>
<td><img src="image7" alt="Structure" /></td>
<td>Cl</td>
<td>3*</td>
</tr>
<tr>
<td><img src="image8" alt="Structure" /></td>
<td>Cl</td>
<td>5*</td>
</tr>
<tr>
<td><img src="image9" alt="Structure" /></td>
<td>I</td>
<td>(4.6)</td>
</tr>
<tr>
<td><img src="image10" alt="Structure" /></td>
<td>Br</td>
<td>(1.9)</td>
</tr>
<tr>
<td><img src="image11" alt="Structure" /></td>
<td>H</td>
<td>(1.7)</td>
</tr>
<tr>
<td><img src="image12" alt="Structure" /></td>
<td>Br</td>
<td>(566)</td>
</tr>
<tr>
<td><img src="image13" alt="Structure" /></td>
<td>I</td>
<td>(313)</td>
</tr>
<tr>
<td><img src="image14" alt="Structure" /></td>
<td>I</td>
<td>(68)</td>
</tr>
<tr>
<td><img src="image15" alt="Structure" /></td>
<td>Cl</td>
<td>(21.2)</td>
</tr>
</tbody>
</table>

* = $K_0.5$ value determined when amiloride inhibition of NHE was thought to be non-competitive.

Given the central role of the uPAS in cancer invasion and metastasis it is reasonable to speculate that the ideal amiloride analogue for use as an anticancer agent might be a dual-action compound that potently inhibits both uPA and NHE1. The strategy of intentionally optimising inhibitors for potent interactions with two independent targets is called polypharmacology and is receiving increasing interest in drug discovery. Figure 1.7 summarises the combined effects of amiloride on NHEs and the uPAS and illustrates why a potent dual NHE/uPA inhibitor could represent an attractive anticancer/antimetastasis. There is currently no uPA inhibitory data reported for any amiloride analogues.
Figure 1.7. Summary of the antitumour/antimetastasis effects of amiloride arising through inhibition of sodium hydrogen exchangers (NHE) and uPA. A possible link exists between NHE1 activation and activation of the uPAS. 195

1.8 Aims of Section 1

The development of orally active therapeutics that could provide prophylaxis against the progression of primary tumours toward metastasis is a major challenge in modern cancer treatment. 196 Moreover, there is a need for a broader range of therapeutics that can be drawn upon to target the vast range of cancer phenotypes and the differing stages of carcinogenesis. uPA appears as an attractive drug target for this purpose and several uPA inhibitors have been reported, including one currently entering Phase III clinical trials. 128 Part of the problem with uPA inhibitors to date has been that most incorporate highly basic amidine or guanidine groups which are well known to confer poor oral bioavailability and pharmacokinetics. 104,116,134 Although only moderately potent, amiloride represents an attractive lead for the development into a uPA inhibitor because it uses an
acylguanidine group to make the key salt-bridge interaction with Asp189. Acylguanidines \((pK_a = 8.8)\)\(^{170}\) are much less basic than arylamidines \((pK_a \sim 10.2)\)\(^{116}\) and arylguanidines \((pK_a \sim 11)\) and generally show superior ‘drug like’ properties \(i.e.\) high water solubility and oral bioavailability).\(^{135}\) This is clearly evidenced by the use of amiloride as an oral K\(^+\)-sparing diuretic. Another factor contributing to interest in amiloride as a lead uPA inhibitor is its specificity for uPA over related serine proteases \(i.e.\) tPA, plasmin, thrombin and kallikrein), which are required for normal physiological processes.\(^{186}\) If an acylguanidine-containing amiloride analogue could be identified that potently and specifically inhibits uPA, such a compound should have excellent drug-like properties.

The antitumour and antimetastasis activities of amiloride appear to arise through mild inhibition of both NHE1 and uPA. Identification of an amiloride analogue \(using\) the SOSA approach \(that\) potently inhibits both NHE1 and uPA may provide a compound with potentially outstanding anticancer effects. In order to advance towards such a compound, the uPA inhibitory potencies of a variety of different amiloride analogues must first be characterised.

1.8.1 Binding Interactions Between Amiloride and uPA

A reported amiloride:uPA co-crystal structure shows that amiloride binds in the S1 and S1\(\beta\) sites of uPA by making multiple hydrogen bonds and van der Waals
contacts (Figure 1.8). Interactions between the protonated acylguanidine of amiloride and Asp189 create the essential salt bridge discussed previously (Chapter 1.6). Ser190 forms hydrogen bonds to the carbonyl oxygen and a terminal nitrogen of the acylguanidine.

Figure 1.8. A: X-ray crystal structure of amiloride bound to uPA highlighting the binding site. B: Summary of binding interactions between amiloride, uPA and a sulfate ion bound in the oxyanion hole.
The Gly218 carbonyl oxygen forms a hydrogen bond to a terminal guanidine nitrogen. Ser195Oγ is hydrogen bonded to the amino group at the pyrazine 3-position, while the amino group at position 5 forms a hydrogen bond to a sulfate ion bound in the oxyanion hole. The chlorine atom at pyrazine position 6 participates in hydrophobic interactions with residues Gln192, Gly216, Gly218 and disulfide Cys191-Cys220 and lies partially within the S1β subsite.¹³⁰

1.8.2 Specific Aims

The overall aim of Section 1 was to explore SARs of amiloride analogues as inhibitors of uPA and hopefully identify more potent compounds.

The structural features of amiloride analogues investigated are summarised in Figure 1.9. 96-well plate colorimetric uPA assays were used to characterise the uPA inhibitory potency of all inhibitors. Initially, analogues substituted at the 5-position were targeted for synthesis from a precursor 13, an acyl guanidine derivative of the commercially available methyl ester 14. Other analogues targeted were 6-substituted derivatives which were to be accessed from 6-iodoamiloride 15. Alternative acylguanidines, synthesised through guanidinylation of methyl ester precursors, were investigated to characterise the binding contribution of the acylguanidine unit of amiloride and to possibly identify new acylguanidine cores. Other analogues in which the acylguanidine was substituted with isosteric groups,
or where exocyclic amino groups were substituted for hydrogen, were synthesised in order to establish the importance of these substituents for inhibitor binding.

**Figure 1.9.** Summary of amiloride analogues targeted in the study and strategies used in their syntheses.
Chapter 2: Synthesis of Amiloride Analogues

2.1 Synthetic Strategies Towards 5- and 6-Substituted Analogues

Nucleophilic substitution reactions of various amines with the 5,6-dichloro-2-pyrazine acylguanidine 13, or in one case the methyl ester 14, were used to access 5-substituted analogues (Scheme 2.1). Direct amine substitution of acylguanidine 13 afforded 11, 12, 16-37 (Method A) while 5-substitution of methyl ester 14 followed by guanidinylation (Method B) provided 39.

Scheme 2.1. Strategies employed in the synthesis of 5- and 6-substituted amiloride analogues: (1) Substitution of 5,6-dichloropyrazine-2-acylguanidine 13 (Method A) or methyl ester 14 (Method B) with primary or secondary amines afforded 5-substituted analogues (following guanidinylation in the case of 39); (2) Hydrodehalogenation of 10 (amiloride) or its methyl ester equivalent 42 and subsequent functionalisation/and guanidinylation to give 6-substituted analogues. 191
Similar approaches were employed previously in the synthesis of amiloride and several of its analogues, including some prepared here.$^{191}$ Inhibitors containing variations at the pyrazine 6-position were prepared by functionalisation of the pyrazine acylguanidine 40, accessed through hydrodehalogenation of 10 (amiloride), or from methyl ester 41 (accessed from 42 by hydrodehalogenation) with subsequent functionalisation and final guanidinylation.$^{191}$

### 2.2 Synthesis of 5-Substituted Analogues

The common precursor for the majority of 5-substituted analogues, 13, was produced from commercially available pyrazine methyl ester 14 by guanidinylation with free base guanidine, generated in situ from guanidine.HCl in the presence of Na in isopropanol (iPrOH) (Scheme 2.2).$^{191}$

![Scheme 2.2. Guanidinylation of 14 to afford key precursor 13.](image_url)

Nucleophilic aromatic substitution of 13 (or its precursor methyl ester 14) with primary or secondary amines occurs preferentially at the 5-position due to the extra resonance stabilisation of the transition state provided by the carbonyl group
positioned para to the 5-chloro group. Table 2.1 summarises the syntheses of 5-substituted amiloride analogues.
Table 2.1. Table summarising the syntheses of 5-substituted amiloride analogues produced by nucleophilic aromatic substitution reactions of amines with 5,6-dichloropyrazine precursor 13.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amine</th>
<th>Yield, %</th>
<th>Lit. Yield, %</th>
<th>Reference</th>
<th>Literature Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td></td>
<td>82</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>51</td>
<td></td>
<td>77</td>
<td>45 (two-step)**</td>
<td>197</td>
<td>c</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>69</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>52</td>
<td></td>
<td>83</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>53</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>54</td>
<td></td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>55</td>
<td></td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>63</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>61</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>74</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>88</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>27*</td>
<td></td>
<td>83</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
</tbody>
</table>
Table 2.1 (cont.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amine</th>
<th>Yield, %</th>
<th>Lit. Yield, %</th>
<th>Reference</th>
<th>Literature Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>(\text{NH}_2)</td>
<td>78</td>
<td>75</td>
<td>191</td>
<td>b</td>
</tr>
<tr>
<td>12</td>
<td>(\text{NH}_2)</td>
<td>26</td>
<td>37 (two-step)**</td>
<td>191</td>
<td>b</td>
</tr>
<tr>
<td>28</td>
<td>(\text{NH}_2)</td>
<td>97</td>
<td>28 (two-step)**</td>
<td>191</td>
<td>b</td>
</tr>
<tr>
<td>29</td>
<td>(\text{NH}_2)</td>
<td>63</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>30</td>
<td>(\text{NH}_2)</td>
<td>77</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>31</td>
<td>(\text{NH}_2)</td>
<td>81</td>
<td>44 (two-step)**</td>
<td>197</td>
<td>b</td>
</tr>
<tr>
<td>32</td>
<td>(\text{NH}_2)</td>
<td>78</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>33</td>
<td>(\text{NH}_2)</td>
<td>84</td>
<td>34 (two-step)**</td>
<td>197</td>
<td>b</td>
</tr>
<tr>
<td>34</td>
<td>(\text{NH}_2)</td>
<td>69</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>35</td>
<td>(\text{NH}_2)</td>
<td>92</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>36</td>
<td>(\text{NH}_2)</td>
<td>90</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>37</td>
<td>(\text{NH}_2)</td>
<td>78</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>39†</td>
<td>(\text{NH}_2)</td>
<td>52</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
</tbody>
</table>

*The literature status for each compound is indicated by: a = novel compound, b = published compound with limited/no characterisation, or c = fully characterised published compound. Reaction yields are provided along with literature yields where applicable. Full characterisation has been provided for all novel compounds and compounds with limited or no characterisation.
**The available literature yield is given for a two-step reaction where 14 is substituted at the 5-position and subsequently guanidylated. †Synthesised by Method B (see text). ‡Expected product not formed (see text and Scheme 2.4).

Amines used in the synthesis of 11, 12, 16-20, 22, 24, 26-33, 35, 36 and 39 were all commercially available, but all others required synthesis. Diethyl phosphorohydrazidate 43, 1-(2-aminoethyl)pyridinium bromide 44 and 2-(benzyl(methyl)amino)ethylamine 46 (used in the synthesis of 21, 34 and 37, respectively) were each prepared by literature methods (Scheme 2.3). 198-200

Scheme 2.3. Syntheses of non-commercially available amines. 198-200

Tetrazolylalkylamines 47 and 48 (used in the synthesis of 23 and 25) were prepared from their respective commercially available chlorides (49 and 50), either by conversion to the azide followed by triphenylphosphine-mediated reduction (48) or by substitution with ammonia (47) (Scheme 2.3).
Esters 16 and 17 were hydrolysed under basic conditions (K₂CO₃/MeOH/H₂O) to yield carboxylic acids 51 and 52, respectively in 77 and 83% yields. Phosphonate esters 19 and 20 were fully or partially hydrolysed using trimethylsilyl chloride to afford 53, 54 and 55 in 75, 22 and 32% yields, respectively. The phosphonate acylguanidines were highly insoluble and gave poor ¹H and ¹³C NMR spectra. Oxidation of thioether 35 with Oxone yielded sulfone 56 in 90% yield (Scheme 2.4).

Scheme 2.4. Hydrolysis of carboxylate and phosphonate esters 16, 17, 19 and 20 to afford 51-55. Oxidation of thioester 35 to the corresponding sulfone 56 with Oxone (potassium peroxymonosulfate) and synthesis of heterocycle 27.
An attempt to produce 5-(N,N’-dimethylethene-1,2-diamino)-amiloride by reaction of 13 with N,N’-dimethylethanediamine (Table 2.1) instead resulted in formation of the novel heterocycle 27 (Scheme 2.4).

2.3 Synthesis of 6-Substituted Analogues

Hydrodehalogenation according to the literature method (H₂, Pd/C,MgO)₁⁹¹ of 10 (amiloride) or its methyl ester precursor 42 yielded the dechlorinated compounds 40 and 41, respectively. Halogenation of 40 or 41 using N-bromosuccinimide (NBS) or N-iodosuccinimide (NIS) yielded aryl halides 15, 57 and 58 (Scheme 2.5). Inhibitors 59 and 60 were produced by hydrodehalogenation (H₂, Pd/C,MgO) of 28 and 33.

Scheme 2.5. Synthesis of 15, 40, 41, 57 and 58.

Stille and Sonogashira couplings were performed under standard conditions on iodide 57 with phenyltributylstannane and phenylacetylene to afford methyl ester precursors (61 and 62), respectively. Subsequent guanidinylation afforded the 6-substituted amiloride analogues 63 and 64 (Table 2.2).
Table 2.2. Synthesis of amiloride analogues modified at the 6-position and in some cases the 5- and 6-positions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield, %</th>
<th>Lit. Yield, %</th>
<th>Reference</th>
<th>Literature Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>H</td>
<td>59</td>
<td>NS</td>
<td>191</td>
<td>c</td>
</tr>
<tr>
<td>59</td>
<td></td>
<td>78</td>
<td>NS</td>
<td>191</td>
<td>b</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>81</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>Yield, %</th>
<th>Lit. Yield, %</th>
<th>Reference</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>Br</td>
<td>81</td>
<td>NS</td>
<td>191</td>
<td>c</td>
</tr>
<tr>
<td>15</td>
<td>I</td>
<td>72</td>
<td>NS</td>
<td>191</td>
<td>c</td>
</tr>
</tbody>
</table>

(a) Stille
Pd(PPh₃)₄, PhSnBu₃, Cul
(b) Sonogashira
Pd(PPh₃)₂Cl₂, Phenylacetylene, Cul, DIPEA

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield, %</th>
<th>Lit. Yield, %</th>
<th>Reference</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td></td>
<td>72</td>
<td>NS</td>
<td>201</td>
<td>b</td>
</tr>
<tr>
<td>64</td>
<td></td>
<td>81</td>
<td>NS</td>
<td>202</td>
<td>b</td>
</tr>
</tbody>
</table>

*See Table 2.1 for definitions of a-c. NS = not supplied.
2.4 Synthesis of Acylguanidines with Varied Aryl Cores

A range of aryl acylguanidines 67-73 with various aryl cores were accessed via guanidinylation of their corresponding methyl esters (most were commercially available) by the method described previously (Scheme 2.2). Methyl esters that were not commercially available were synthesised from their commercially available carboxylic acids by methylation using CH₃I/C₅H₅N in N,N-dimethylformamide (DMF) (65 and 66, ester precursors to 71 and 72, respectively). The arylacylguanidines synthesised are shown in Table 2.3.

Table 2.3. Synthesis of acylguanidines with varied aryl cores.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield, %</th>
<th>Lit. Yield, %</th>
<th>Reference</th>
<th>Literature Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td></td>
<td>89</td>
<td>35</td>
<td>203</td>
<td>b</td>
</tr>
<tr>
<td>68</td>
<td></td>
<td>42</td>
<td>19</td>
<td>191</td>
<td>b</td>
</tr>
<tr>
<td>69</td>
<td></td>
<td>27</td>
<td>NS</td>
<td>204</td>
<td>b</td>
</tr>
<tr>
<td>70</td>
<td></td>
<td>56</td>
<td>NS</td>
<td>205</td>
<td>b</td>
</tr>
<tr>
<td>71</td>
<td></td>
<td>27</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>62</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
<tr>
<td>73</td>
<td></td>
<td>93</td>
<td>-</td>
<td>-</td>
<td>a</td>
</tr>
</tbody>
</table>

*See Table 2.1 for definitions of a and b. NS = not supplied.
2.5 Synthesis of Analogues Containing Acylguanidine Isosteres

Two analogues were synthesised containing the acylguanidine isosteric replacements, 3-amino-1,2,4-oxadiazole 74 and acylhydrazide 75. 74 was accessed by heating hydroxyguanidine 77 (prepared by a known procedure)\(^{206}\) with methyl ester 42 in the presence of Na, according to the literature method.\(^{207}\) Acylhydrazide 75 was prepared by reaction of 42 with hydrazine hydrate (Scheme 2.6).

![Scheme 2.6. Synthesis of amiloride analogues 74 and 75 which incorporate acylguanidine isosteres.](image)

2.6 Synthesis of Analogues Lacking 3- and 5- Exocyclic Amines

Amiloride analogues 82 and 80, lacking amino groups at the 3- and 5-positions, respectively, were synthesised. Chlorination of the commercially available pyrazine methyl ester 78 using N-chlorosuccinimide (NCS) followed by guanidinylation afforded 80. Guanidinylation of the commercially available methyl ester 81 afforded compound 82 (Scheme 2.7). The chloro analogue of 81 was not commercially
available so the direct amiloride analogue of 82 (**i.e.** replace the 6-Bromo group with 6-Chloro) was not pursued.

![Chemical Structure](image)

**Scheme 2.7.** Synthesis of 5- and 3-deamino amiloride analogues 80 and 82.

### 2.7 Unsuccessful Syntheses

Attempts to substitute 13 with primary and secondary amines were generally successful with the exception of aminomethylsulfonic acid (Scheme 2.8, Entry 1) and 2-aminoethylphosphonic acid (Scheme 2.8, Entry 2). An attempt to deprotect 21 with trimethylsilyl chloride to give the phosphonate was unsuccessful leading to formation of multiple products which were not pursued for characterisation (Scheme 2.8, Entry 3).

Several methods were explored for producing amiloride analogues with a range of variations at the 6-position.
<table>
<thead>
<tr>
<th>Entry</th>
<th>Unsuccessful Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Reaction 1" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Reaction 2" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Reaction 3" /></td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Reaction 4" /></td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Reaction 5" /></td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Reaction 6" /></td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Reaction 7" /></td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Reaction 8" /></td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Reaction 9" /></td>
</tr>
</tbody>
</table>

**Scheme 2.8.** Summary of unsuccessful syntheses.
Initially, a Pd-catalysed Suzuki coupling between the 6-chloro methyl ester 42 and phenylboronic acid was attempted using Pd(OAc)$_2$ as catalyst and 1,2-Bis(diphenyphosphino)ethane (dppe) as ligand (Scheme 2.8, Entry 4). With minimal product resulting, the Suzuki reaction was abandoned in favour of Stille couplings. Stille coupling between aryl iodide 57 and phenyltributylstannane was successful (Table 2.2), however attempts to couple 57 or acylguanidine 15 with 2-(tributylstanny1)pyrimidine using DMF or dioxane as solvent and Pd(PPh$_3$)$_4$ or Pd(PPh$_3$)$_2$Cl$_2$ as catalyst all failed (Scheme 2.8, Entry 5). Attempts to substitute 2-aminopyrimidine in a Buchwald-Hartwig amination reaction with iodide 57 using Pd$_2$dba$_3$ as catalyst, BINAP as ligand and sodium tert-butoxide as base yielded only starting material (Scheme 2.8, Entry 6). Attempts to fluorinate 40 or 41 with Selectfluor to generate 6-fluoroamiloride were unsuccessful, with decomposition of the compounds to multiple products observed (Scheme 2.8, Entry 7).

Attempted partial hydrogenation (2 atm. H$_2$) of the alkyne 62 to give the cis alkene using Lindlar catalyst (Pd/CaCO$_3$) or Pd/BaSO$_4$ deactivated with quinoline failed to give the desired product regardless of solvent (MeOH or Tetrahydrofuran (THF)/Hexane) or reaction time (Scheme 2.8, Entry 8). Attempts to fully hydrogenate (2 atm. H$_2$) the alkyne 62 to the alkane derivative returned only starting material (Scheme 2.8, Entry 9).
Chapter 3: Evaluation of Amiloride Analogues as uPA Inhibitors

3.1 uPA Inhibition Assay

The synthesised amiloride analogues were evaluated for uPA inhibitory potency using an *in vitro* enzyme assay (96-well plate format). Compounds were dissolved in dimethyl sulfoxide (DMSO) to create 20 mM stock solutions which were diluted in series in the assay plates to give final DMSO concentrations less than 2%. Two compounds were assayed per plate with triplicate measurements taken for each inhibitor concentration. Assay blanks (no enzyme) were included to account for the colour of some inhibitors. An amiloride dilution series was included in all assays as a positive control. A commercially available colorimetric uPA substrate (Spectrozyme UK, American Diagnostica Inc.) was in the assays used along with active high molecular weight uPA (HMW-uPA). Plates were incubated at 37 °C in a plate reader and the absorbance read at 405 nm over time. Absorbance values were recorded for a time point taken from the linear region of plots of absorbance vs time. IC\(_{50}\) values were calculated from sigmoidal dose response curves of absorbance vs log[inhibitor] using GraphPad Prism V. 5.01 software. A graphical summary of the assay is provided in Figure 3.1 (also see Chapter 9. Appendix for the assay protocol).
Figure 3.1. Summary of 96-well microtitre plate assay used to measure the potency of uPA inhibitors. Cleavage of the uPA substrate Spectrozyme UK releases coloured p-nitroaniline which absorbs at 405 nm.

3.2 Analogues with C5 Variations

This study focused heavily on exploring amiloride analogues carrying various substituted amines at C5 due to their ease of synthesis and because many 5-substituted analogues are known to show greatly reduced diuretic effects. Applying the SOSA approach successfully to create an amiloride analogue with higher uPA inhibitory potency useful as an anticancer agent will require that the
eventual compound shows minimal effects on diuresis (K+ levels in particular). The amiloride/uPA X-ray co-crystal structure (Figure 1.8) indicated that substituted amines could extend away from the pyrazine ring towards the oxyanion hole being occupied by an adventitious sulfate ion. Analogues incorporating negatively charged groups (e.g. carboxylates 51 and 52, sulfonate 18, tetrazoles 23 and 25, and phosphonates 53-55) were explored as it was rationalised that these groups could potentially act as sulfate mimetics and pick up favourable contacts in the oxyanion hole. This hypothesis proved to be incorrect as all inhibitors bearing negatively charged substituents showed dramatically reduced potency or total loss of activity. Uncharged synthetic precursors of these inhibitors (16, 17, 19-22, 24) universally showed less dramatic losses in potency. Only the diethylphosphorohydrazidate 21 retained the potency of amiloride (IC50 = 11 µM in our assay).

Other 5-substituted amiloride analogues were chosen in order to explore a cross-section of inhibitor structures containing aromatic rings, basic (or quaternary) amines bearing positive charges, and sulfur. Benzylic extensions (28-32) were well tolerated and produced slight (up to 2-fold) increases in potency (p-fluorobenzyl 32, IC50 = 6 µM). Of the three analogues substituted with pyridylmethanes (29-31) only the p-substituted pyridine 30 (IC50 = 6 µM) showed increased potency relative to amiloride. Extension of the benzylamine substituent to a phenylethylamine caused a 2-fold drop in potency (33 vs 28).
### Table 3.1. uPA inhibitory potencies of 5-substituted amiloride analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$, μM</th>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>H</td>
<td>11</td>
<td>30</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>O</td>
<td>26</td>
<td>31</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>51</td>
<td>HO</td>
<td>110</td>
<td>32</td>
<td>F</td>
<td>6</td>
</tr>
<tr>
<td>17</td>
<td>EtO</td>
<td>25</td>
<td>33</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>52</td>
<td>HO</td>
<td>102</td>
<td>26</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>18</td>
<td>HO$^-$</td>
<td>&gt;1000</td>
<td>34</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>19</td>
<td>EtO, EtO</td>
<td>37</td>
<td>37</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>53</td>
<td>HO, OH, Oh</td>
<td>&gt;1000</td>
<td>39</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>20</td>
<td>EtO$^-$</td>
<td>44</td>
<td>36</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>54</td>
<td>HO$^-$</td>
<td>&gt;1000</td>
<td>35</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>55</td>
<td>EtO$^-$</td>
<td>&gt;1000</td>
<td>56</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>21</td>
<td>EtO, EtO, N</td>
<td>10</td>
<td>27</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>50</td>
<td>11</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>177</td>
<td>12</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5-substituted analogues containing $2^\circ$, $3^\circ$ or $4^\circ$ nitrogens capable of sustaining a positive charge at physiological pH were investigated to determine whether the charged groups lead to potency increases. Analogues 26 and 37 bearing $3^\circ$ amino groups showed only slightly reduced potency, while 34 (quaternary pyridinium) and 39 ($2^\circ$ amine) were $\sim$3-fold less potent than amiloride. It is concluded that positive charges in C5 substituents do not confer increased potency but they are largely tolerated and produce only minor losses in potency.

Analogues 35, 36 and 56, which included sulfur in the C5-substituent, were studied because some potent uPA inhibitors (e.g. 6 and 9, Figure 1.4) position a sulfonamide in the oxyanion hole. The sulfone 56 was found to be slightly more potent than its corresponding sulfide 35, but both compounds were slightly less potent than amiloride. Sulfonamide 36 was more than 3-fold less potent.

Three amiloride analogues bearing $3^\circ$ amines at the 5-position were investigated. 11 and 12 were of great interest because they are exceptionally potent inhibitors of NHE1 (Chapter 1.7.2). Both of these compounds showed 2-fold improvements in potency relative to amiloride. The 5,6-disubstituted heterocycle 27 (synthesised by accident – see Scheme 2.5) showed a 4-fold improvement in potency, indicating that disubstitution of the 5- and 6-positions might be worth exploring further as a means of increasing potency.
The overall trend indicated that shorter 5-substituted analogues tend to improve inhibitor potency while the more extended substituents produce no improvement or a reduction in potency. Examining the X-ray co-crystal structure of amiloride bound to uPA (Figure 1.8) suggests that 5-substituted analogues should place the substituent in the oxyanion hole, with longer substituents extending out further, probably towards solvent. The finding that only modest increases (up to 4-fold) and relatively minor reductions (except for the negatively charged analogues) in potency are observed with this diverse range of analogues suggests that only non-specific contacts are being made between the 5-substituents and the enzyme. The flat SAR trend would also be compatible with these substituents significantly interacting with the surrounding solvent.

3.3 Analogues with C6 Variations

The amiloride:uPA X-ray co-crystal structure (Figure 1.8) indicates that the chloro substituent at the amiloride C6-position projects toward and partially occupies the S1β site within the uPA active site. A variety of 6-substituted amiloride analogues which remove or replace the chloro group were investigated to explore the SAR at the S1β site (Table 3.2).

Compounds 40, 59 and 60 (i.e. dechloro analogues of 10, 28 and 33, respectively) were used to explore the contribution of the chloro group to potency. The dechloro
compounds all showed reduced potency (~2-7.5 fold) compared to their 6-chloro precursors (Table 3.2), confirming the favourable interaction of the chloro group with the S1β site.

The effect of larger halogens at the 6-position was also investigated. Relative to amiloride, bromide 58 and iodide 15 produced 2- and 5-fold improvements in potency, respectively, suggesting that the larger halogens more favourably occupy the S1β site.

**Table 3.2.** uPA inhibitory potencies of 6-substituted analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>H</td>
<td>19</td>
</tr>
<tr>
<td>59</td>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>120</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>IC50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>Br</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>I</td>
<td>2</td>
</tr>
<tr>
<td>63</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
uPA-inhibitor co-crystal structures have shown that the S1β site can accommodate an aryl ring\textsuperscript{116} leading us to explore amiloride analogues 63 and 64. The two compounds showed equal or higher potency than amiloride, with the IC\textsubscript{50} of 64 (2 μM) being equivalent to that observed for iodide 15, the most potent inhibitor identified in this study. Although only a preliminary exploration of C6 variations the promising results suggest that further examination of this position should be explored in searching for higher potency uPA inhibitors.

### 3.4 Acylguanidines with Varied Aryl Cores

The salt-bridge contact between the positively charged acylguanidine of amiloride and Asp189 of uPA is an essential interaction that presumably contributes significantly to the binding energy. To investigate the possibility that this interaction is responsible for the majority of amiloride’s affinity for uPA, with the other substituents playing only a minor role, a series of acylguanidines with varied aryl cores were studied. As shown in Table 3.3, all acylguanidines were essentially inactive with IC\textsubscript{50} values above 1 mM (except 72, IC\textsubscript{50} = 222 μM). It is significant that even pyrazine-2-acylguanidine 70 was inactive as this is the core structure of amiloride. These findings are evidence that the arylacylguanidine core of amiloride is not alone responsible for the bulk amiloride’s uPA binding affinity. The 3- and 5-amino and 6-chloro groups present on amiloride must clearly be contributing significantly to affinity. The slightly improved potency of the polysubstituted
benzoylguanidine 72 relative to the unsubstituted benzoylguanidine 67 supports this conjecture (Table 3.3).

**Table 3.3.** uPA inhibitory potencies of acylguanidines with varied aryl cores.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC₅₀, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td><img src="compound67.png" alt="Image" /></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>68</td>
<td><img src="compound68.png" alt="Image" /></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>69</td>
<td><img src="compound69.png" alt="Image" /></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>70</td>
<td><img src="compound70.png" alt="Image" /></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>71</td>
<td><img src="compound71.png" alt="Image" /></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>72</td>
<td><img src="compound72.png" alt="Image" /></td>
<td>222</td>
</tr>
<tr>
<td>73</td>
<td><img src="compound73.png" alt="Image" /></td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

3.5 Analogues Containing Acylguanidine Isosteres

Amiloride analogues featuring the acylguanidine isosteres 3-amino-1,2,4-oxadiazole 74 and acylhydrazide 75 were examined to further explore the importance of the acylguanidine group for uPA binding. Both analogues were found to be inactive (IC₅₀ >1000 µM, Table 3.4). The isosteres present in 74 and 75 are less basic (although acylhydrazides are amphoteric, with pKₐ₁ = 3.62-3.64 and pKₐ₂ = 13.86-13.91) than
the acylguanidine which would reduce the population of these analogues existing in
the positively-charged state at physiological pH. Loss of the charge would weaken
the salt bridge interaction with Asp189 in the S1 pocket and explain the loss of
potency.

Table 3.4. Inhibitory potencies of analogues containing acylguanidine isosteres.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50}, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>75</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

3.6 Analogues Lacking 3- and 5-Exocyclic Amines

The contribution of individual exocyclic amino groups at the 3- and 5-positions of
amiloride was investigated with analogues 80 and 82. It was found that removal of
either amine resulted in total loss of potency relative to amiloride, or in the case of
82, relative to the 6-bromo amiloride analogue 58. It was concluded that both
amino groups must therefore contribute significantly to binding. Loss of activity
after removal of the 3-amino group is most likely a result of losing a hydrogen bond
between this amine and the OH group of the catalytic Ser195 residue (see Figure
1.8 B). Explaining the loss of potency after removing the 5-amino group is more
difficult as this group forms a hydrogen bond to the adventitious sulfate ion and not
the enzyme itself. Loss of such a hydrogen bond would not be expected to totally
abrogate potency. The finding that $3^o$ amines at the 5-position can produce increased potency (*i.e.* compounds 11 and 12) indicates that the loss of potency with 80 is not caused by loss of a hydrogen bond to the enzyme where the 5-NH$_2$ group is acting as a hydrogen bond donor.

**Table 3.5.** uPA inhibitory potencies of analogues lacking 3- and 5-exocyclic amines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$, $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>82</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

3.7 Conclusions and Future Directions

Amiloride shows promising anticancer properties that appear to arise from its effects on NHE1 and uPA. The *in vitro* potency of the drug against these two targets is only moderate so it is conceivable that an analogue with higher potency at both targets would make for a superior anticancer drug. The NHE inhibitory potency of several amiloride analogues has been reported and some show greatly increased potency relative to amiloride (Table 1.3), including the well studied compounds EIPA 11 and HMA 12. In order to advance towards the goal of a potent dual-action
NHE1/uPA inhibitor it was first necessary to characterise the potency of amiloride analogues as uPA inhibitors, since this information has not been reported.

Section 1 of the thesis aimed to synthesise a diverse range of amiloride analogues, develop SARs and potentially identify a uPA inhibitor with nanomolar potency. If such an inhibitor were to be identified and subsequently found to show high NHE1 inhibitory potency it could be advanced to further pre-clinical evaluation as a dual action NHE/uPA inhibiting anticancer drug.

It was proposed that amiloride analogues incorporating a negatively charged sulfate mimetic at C5 could access the oxyanion hole of uPA leading to enhanced binding affinity. Surprisingly, analogues bearing negatively-charged groups all showed greatly reduced potency relative to amiloride. Ironically, the uncharged synthetic precursors of these analogues all showed far lower reductions in potency. Investigation of alkylaryl extensions at C5 were more fruitful, with several analogues (i.e. 28, 30 and 32) showing slightly improved potency. Overall, substituents at the 5-position were generally well tolerated with inhibitors being as potent as, or only slightly less potent than amiloride. While exploration of these analogues did not yield an inhibitor with nanomolar potency as hoped, it succeeded in showing that substitution at the 5-position is at least well tolerated. Amiloride analogues substituted at the 5-position are also known to often show reduced diuretic effects. If a high potency uPA inhibitor is eventually identified, for example, through exploration of the 6-position, and appears as a promising lead, it
will be useful to know that the 5-position can be varied to reduce diuretic effects and potentially to modulate physiochemical properties.

Removal of the 6-chloro was investigated with three analogues 40, 59 and 60. When compared with their 6-chloro counterparts, the dechloro compounds all showed significantly reduced potency indicating that placing a group into the S1β pocket of uPA is important. Other analogues substituted at the 6-position with bulkier groups (i.e. 15, 58, 63 and 64) showed improved potency, with 15 and 64 being the most potent inhibitors identified in the study (6-fold increase in potency relative to amiloride).

Other analogues investigated included a variety of simplified aryl acylguanidines and analogues containing acylguanidine isosteres. It was found that simple arylacylguanidines are poor uPA inhibitors and should not be pursued further as alternative uPA inhibitor lead scaffolds. Analogues bearing acylguanidine isosteres were found to be totally inactive indicating that the acylguanidine group should be retained. Analogues lacking either the 3- or 5-amino substituent were used to establish the importance of these substituents for inhibitor binding. Both analogues were found to be inactive demonstrating the importance of these groups.

Compounds 11 and 12 were of particular interest in the study since they are reported as potent inhibitors of NHE-1 (11 $K_i = 30$ nM, 12 $K_i = 13$ nM, see Section 1.7.2 and Table 1.3). The two analogues showed 2-fold improvements in
potency relative to amiloride. While not large increases in potency this confirms that 5-substituted amiloride analogues with very high potency against NHE-1 do not lose potency against uPA relative to the unsubstituted amiloride. Compounds 15, 27 and 64 were the most potent inhibitors identified, suggesting that analogues containing double modifications at the 5- and 6-positions should be investigated further. For example, analogues of 11 and 12 that replace the 6-chloro group with bulkier groups capable of making more favourable contacts in uPA’s S1β site may provide more potent uPA inhibitors that also retain potency against NHE1. It is important to note that 6-Bromo-12 and 6-Iodo-11 are 566- and 313-fold more potent NHE1 inhibitors than amiloride and exhibit 1.08- and 1.40-fold higher potency than their 6-chloro counterparts. The current study has shown that changing to the 6-Bromo or 6-Iodo derivatives of amiloride enhances uPA inhibitory potency. On this basis, 6-Bromo-12 and 6-Iodo-11 should be tested as uPA inhibitors, as should other derivatives of 11 and 12 that carry bulky C6-substituents.

The search for dual-action NHE-1/uPA inhibitors could be extended beyond amiloride analogues. Several structurally diverse acylguanidines have been reported as highly potent and selective NHE1 inhibitors, with several being up to 3 orders of magnitude more potent than amiloride (e.g. eniporide). Most interest in these compounds has focussed on their potential use as selective NHE1 inhibitors for the prevention of cardiac damage in ischemia–reperfusion episodes. Examination of the inhibitory effects on uPA of the more potent NHE1 inhibitors may yield a promising dual-action anticancer lead. Most of the highly potent uPA
inhibitors unrelated to amiloride (see Figure 1.4 for examples) haven’t yet been cross-screened for NHE1 inhibitory effects. Screening of these compounds against NHE1 using a platelet-swelling assay, $^{22}\text{Na}^+$ uptake assay or similar might similarly yield an attractive dual action uPA/NHE1 inhibitor worth investigating in the cancer setting.
Chapter 4: Section 1 Experimental and References

4.1 Synthesis - General

Reagents and solvents were utilised without further purification unless stated henceforth. Dichloromethane (DCM), DMF, toluene and diethyl ether (Et₂O) were used directly from a PureSolv Solvent purification system. N, N-diisopropylethylamine (DIPEA) and triethylamine (TEA) were distilled from KOH and stored over KOH pellets. PrOH was distilled from BaO and stored over 4 Å molecular sieves. THF was purified by drying over KOH before distillation from sodium benzophenone ketyl. NCS was purified by recrystallisation from acetic acid, followed by rinsing with water and drying under high vacuum. NBS was purified by recrystallisation from water followed by drying under high vacuum. NIS was purified by recrystallisation from dioxane. Tetrakis(triphenylphosphine)palladium (0) was purified by washing with methanol and drying under high vacuum prior to use.²⁰⁸

Diethyl(aminomethyl)phosphonate oxalate and diethyl(aminoethyl)phosphonate oxalate were purchased from Acros Organics. Methyl 5-amino-6-bromopyrazine-2-carboxylate was purchased from Toronto Research Chemicals. 5-(2-chloroethyl)-1H-tetrazole was purchased from Waterstone Technology. 5-chloromethyl-1H-tetrazole was purchased from 3B Pharmachem (Wuhan). 4-fluorobenzylamine was purchased from Alfa Aesar. All other reagents were purchased from Sigma Aldrich.

Compounds were weighed using a Sartorius Extend 220g balance. Solvent was removed under reduced pressure (in vacuo) at 60 °C with a Büchi rotary evaporator.
attached to a vacuubrand CVC2 pump. Solvent residues were removed \textit{in vacuo}
using a Javac Vector RD-90 double stage high vacuum pump. Fractions collected from preparative HPLC purification were concentrated by freeze drying using a Christ Alpha 1-4 LOC-1M condenser attached to an Alcatel Pascal 2015 SD high vacuum pump, or a Christ Alpha 1-2 LD \textit{plus} condenser attached to a vacuubrand R@ 2.5 pump. Analytical thin layer chromatography (TLC) was conducted on Merck 0.2 mm silica gel 60 F$_{254}$ coated aluminium plates. Compounds on TLC plates were detected by UV (\(\lambda\) 254 nm). Preparative TLC was conducted using Whatman Partisil 1 mm silica gel 60 PK6F glass plates. Column chromatography was performed under ‘flash’ conditions on Merck silica gel 60 (230-400 mesh). Pressurised hydrogenation reactions were performed using a 3900 series Parr Hydrogenation apparatus. All compounds were purified to > 95% purity for testing using a Waters PLC/DSC Prep LC150 System with detection at 254 nm. Purifications were performed using gradient elutions with solvents A (100% H$_2$O, 0.1% HCl) and B (90% CH$_3$CN, 10% H$_2$O, 0.1% HCl) on a Sunfire$^{\text{TM}}$ PREP C18 OBD$^{\text{TM}}$ (5 \(\mu\)M) steel jacketed column run at 20 mL.min$^{-1}$. Analytical HPLC analyses were performed using a Shimadzu CLASS-LC10 VP HPLC system with detection at 254 nm. The analyses were performed using gradient elutions with solvents A and B on a Phenomenex Luna 5 \(\mu\)M C18 column run at 1 mL.min$^{-1}$ with detection at 254 nm.
4.2 Compound Characterisation

Low resolution electron impact (EI⁺) mass spectra were obtained on a Shimadzu QP-5000 mass spectrometer by direct insertion with a 70 eV electron beam. Low resolution electrospray (ES⁺) mass spectra were obtained on a micromass Z-path (LCZ) platform spectrometer. EI High Resolution Mass Spectra (HRMS) were obtained on a Fisons/VG Autospec spectrometer using perfluorokerosene as internal standard. ES HRMS were obtained on a Waters QTof Ultima spectrometer using polyethylene glycol or polypropylene glycol as internal standard.

Proton, carbon and two-dimensional NMR experiments were performed using Varian Mercury 300 MHz, Varian Inova 500 MHz or Varian Premium Shielded 500 MHz spectrometers at 25 °C. Chemical shifts are reported in δ values (ppm) relative to internal TMS or solvent. The abbreviations Ar = aryl, s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet and bs = broad singlet are used throughout. Many compounds in this study contain several exchangeable protons bound to nitrogens. The ¹H chemical shifts of these protons were highly temperature and concentration dependent.

Compounds are named according to IUPAC convention. Previously unreported compounds are indicated with the letter: a as a superscript following the compound name. Names of previously reported compounds with limited spectral data available are indicated with the letter: b and a number for the reference is given.
Previously reported compounds that have been fully characterised are indicated with the letter: c and a number for the reference is given.

4.2.1 General Methods for the Synthesis of 5-Substituted Analogues:

**General Method A:** 13 and the amine (2 mole equivalents) were dissolved in DMF and stirred at 100 °C for 1 h before the solvent was removed in vacuo and the product purified by rp-HPLC.

**General Method B:** 13 and the amine (2 mole equivalents) were dissolved in DMF and heated to 100 °C. DIPEA (7 mole equivalents) was then added and the solution stirred for 2 h before the solvent was removed in vacuo and the product purified by rp-HPLC.

**General Method C:** 13 and the primary or secondary amine (2 mole equivalents) were dissolved in DMF and heated to 100 °C. TEA (4 mole equivalents) was then added and the solution stirred for 1 h before the solvent was removed in vacuo and the product purified by rp-HPLC.

5-(N-ethyl-N-isopropylamino)-3-amino-N-carbamidoyl-6-chloropyrazine-2-carboxamide hydrochloride (11)b,191

The compound was produced by General Method A using N-ethylisopropylamine (85 µL, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by
preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 23.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 11 as a bright yellow solid (92 mg, 78% after HPLC): \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 10.66 (s, 1H, Amide NH), 8.61 (s, 2H, Guanidine NH\(_2\)), 8.43 (s, 2H, Guanidine NH\(_2\), 7.44 (bs, 2H, Ar-NH\(_2\)), 4.66 (sep, \(J = 6.5\) Hz, 1H, Ar-\(\text{NCH(CH}_3)_2\)), 3.54 (q, \(J = 7.0\) Hz, 2H, Ar-\(\text{NCH}_2\text{CH}_3\)), 1.22 (d, \(J = 6.5\) Hz, 6H, Ar-\(\text{NCH}(\text{CH}_3)_2\)), 1.11 (t, \(J = 7.0\) Hz, 3H, Ar-\(\text{NCH}_2\text{CH}_3\)); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 165.2 (C=O), 155.0 (Guanidine C), 153.8 (ArC5), 153.7 (ArC3), 120.5 (ArC6), 110.5 (ArC2), 50.7 (Ar-\(\text{NCH(CH}_3)_2\)), 37.7 (Ar-\(\text{NCH}_2\text{CH}_3\)), 20.1 (Ar-\(\text{NCH}(\text{CH}_3)_2\)), 15.1 (Ar-\(\text{NCH}_2\text{CH}_3\) ); ES TOF MS m/z (M)\(^+\) 300; Anal. for C\(_{11}\)H\(_{19}\)N\(_7\)OCl: Calc. Mass 300.1340. Found 300.1346.

3-amino-5-(azepan-1-yl)-N-carbamidoyl-6-chloropyrazine-2-carboxamide hydrochloride (12)\(^b,191\)

The compound was produced by General Method A using hexamethyleneimine (40 \(\mu\)L, 0.35 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 23.4 min). The collected fractions containing purified...
product were concentrated by freeze-drying to yield 12 as a yellow solid (16 mg, 26% after HPLC): \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 8.71 (s, 2H, Guanidine NH\(_2\)), 8.68 (s, 2H, Guanidine NH\(_2\)), 7.40 (bs, 2H, Ar-NH\(_2\)), 3.82 (t, \(J = 5.5\) Hz, 4H, C\(_2'\)H\(_2\) and C\(_7'\)H\(_2\)), 1.76 (m, 4H, C\(_3'\)H\(_2\) and C\(_6'\)H\(_2\)), 1.50 (m, 4H, C\(_4'\)H\(_2\) and C\(_5'\)H\(_2\)); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 165.1 (Amide C=O), 155.2 (Guanidine C), 153.7 (ArC5), 153.0 (ArC3), 118.2 (ArC6), 109.9 (ArC2), 50.7 (C\(_2'\) and C\(_7'\)), 27.7 (C\(_3'\) and C\(_6'\)), 26.0 (C\(_4'\) and C\(_5'\)); ES TOF MS \(m/z\) (M)\(^+\) 312; Anal. for C\(_{12}\)H\(_{19}\)N\(_7\)OCl: Calc. Mass 312.1340. Found 312.1332.

3-amino-N-carbamimidoyl-5,6-dichloropyrazine-2-carboxamide hydrochloride (13)

Sodium (920 mg, 40.02 mmol) was added to dry \(^1\)PrOH (50 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (3.85 g, 40.30 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with methyl 3-amino-5,6-dichloropyrazine-2-carboxylate (4.44 g, 19.00 mmol). The mixture was heated at reflux for 30 min before cooling to 10 °C. The solid that formed was collected and dissolved in water (50 mL) with 6 M HCl (3 mL) added at 70 °C. The solution was cooled to r.t. and crystals were collected in several crops, yielding 13 as bright orange crystals (4.558 g, 84%): \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 11.22 (s, 1H,
Amide NH), 8.89 (s, 2H, Guanidine NH$_2$), 8.73 (s, 2H, Guanidine NH$_2$), 8.41 (bs, 1H, Ar-NH), 7.68 (bs, 1H, Ar-NH); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 165.2 (Amide C=O), 154.9 (Guanidine C), 153.8 (ArC3), 149.3 (ArC5), 128.6 (ArC6), 120.0 (ArC2); ES TOF MS m/z (M)$^+$ 249; Anal. for C$_6$H$_7$N$_6$OCl$_2$: Calc. Mass 249.0058. Found 249.0025.

3,5-diamino-N-carbamimidoyl-6-iodopyrazine-2-carboxamide hydrochloride (15)$^c$

![Image](image)

40 (100 mg, 0.43 mmol) and NIS (107 mg, 0.48 mmol) were dissolved in dry DMF (5 mL) and stirred at 80 °C for 1 h. The solution was then diluted with water (20 mL) and concentrated in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 15.1 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 15 as a yellow solid (111 mg, 72% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 10.55, 8.67, 8.54, 7.35; $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 165.3, 157.0, 155.6, 155.1, 113.2, 88.0; ES TOF MS m/z (M)$^+$ 321; Anal. for C$_6$H$_9$N$_7$O$I$: Calc. Mass 321.9913. Found 321.9880.

3-amino-N-carbamimidoyl-6-chloro-5-(2-methoxy-2-oxoethylamino)pyrazine-2-carboxamide hydrochloride (16)$^b$
The compound was produced by General Method A using glycine methyl ester (69 mg, 0.77 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 24.1 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 16 as a light yellow solid (97 mg, 82% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 10.70 (s, 1H, Amide NH), 8.64 (s, 4H, 2 x Guanidine NH$_2$), 8.26 (t, $J$ = 4.5 Hz, 1H, Ar-NH-R), 7.55 (bs, 2H, Ar-NH$_2$), 4.13 (d, $J$ = 4.5 Hz, 2H, NH-CH$_2$), 3.65 (s, 3H, OCH$_3$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 169.6 (Ester C=O), 165.3 (Amide C=O), 155.4 (Guanidine C), 155.2 (ArC3), 152.1 (ArC5), 119.7 (ArC6), 109.2 (ArC2), 52.0 (OCH$_3$), 42.4 (NHCH$_2$); ES TOF MS $m/z$ (M)$^+$ 302; Anal. for C$_9$H$_{13}$N$_7$O$_3$Cl: Calc. Mass 302.0768. Found 302.0779.

3-amino-$N$-carbamimidoyl-6-chloro-5-(3-ethoxy-3-oxopropylamino)pyrazine-2-carboxamide hydrochloride (17)$^a$

The compound was produced by General Method B using $\beta$-alanine methyl ester hydrochloride (108 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 26.3 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 17 as a light brown solid (88 mg, 69% after HPLC): mp 280-
282 °C (dec); \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 10.65 (s, 1H, Amide NH), 8.65 (s, 4H, 2 x Guanidine NH\(_2\)), 7.93 (t, \(J = 6.0\) Hz, 1H, Ar-NH-R), 7.56 (bs, 2H, Ar-NH\(_2\)), 4.04 (q, \(J = 7.0\) Hz, 2H, OCH\(_2\)CH\(_3\)), 3.60 (dt, \(J = 6.0, 7.0\) Hz, 2H, NH-C\(_2\)H\(_2\)), 2.65 (t, \(J = 7.0\) Hz, 2H, NHCH\(_2\)CH\(_2\)), 1.15 (t, \(J = 7.0\) Hz, 3H, OCH\(_2\)CH\(_3\)), \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 171.2 (Ester C=O), 165.3 (Amide C=O), 155.7 (Guanidine C), 155.3 (ArC3), 152.1 (ArC5), 120.1 (ArC6), 108.5 (ArC2), 60.0 (OCH\(_2\)CH\(_3\)), 37.0 (NH-C\(_2\)H\(_2\)), 32.8 (NHCH\(_2\)C\(_2\)H\(_2\)), 14.1 (OCH\(_2\)CH\(_3\)); ES TOF MS \(m/z\) (M)\(^+\) 330; Anal. for C\(_{11}\)H\(_{17}\)N\(_7\)O\(_3\)Cl: Calc. Mass 330.1081. Found 330.1074.

2-[6-amino-5-(carbamidoylcarbamoyl)-3-chloropyrazin-2-ylamino]ethylsulfonic acid hydrochloride (18)

The compound was produced by General Method C using taurine (88 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins with eluent containing 0.1% HCl (t, 23.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 18 as a white solid (63 mg, 48% after HPLC): mp >300 °C (dec); \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 10.57 (s, 1H, CH\(_2\)SO\(_3\)H), 8.55 (s, 2H, Guanidine NH\(_2\)), 8.32 (s, 2H, Guanidine NH\(_2\)), 8.12 (t, \(J = 5.0\) Hz, 1H, Ar-NH-R), 7.58 (bs, 2H, Ar-NH\(_2\)), 3.65 (dt, \(J = 5.5, 6.0\) Hz, 2H, NHCH\(_2\)H), 2.77 (t, \(J = 6.5\) Hz, 2H, CH\(_2\)SO\(_3\)H); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 165.3 (Amide C=O), 155.9 (Guanidine C), 155.0 (ArC3), 151.8
(ArC5), 120.3 (ArC6), 108.2 (ArC2), 49.0 (CH₂SO₃H), 37.8 (NHCH₂); ES TOF MS m/z (M)⁺ 338; Anal. for C₈H₁₃N₇O₄ClS: Calc. Mass 338.0438. Found 338.0448.

diethyl [6-amino-5-(carbamimidoylcarbamoyl)-3-chloropyrazin-2-ylamino]methylphosphonate hydrochloride (19)ᵃ

The compound was produced by General Method C using diethyl (aminomethyl)phosphonate oxalate (180 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 21.8 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 19 as a hygroscopic yellow solid (66 mg, 45% after HPLC): ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.67 (s, 1H, Amide NH), 8.64 (s, 4H, 2x Guanidine NH₂), 8.10 (t, J = 6.0 Hz, 1H, Ar-NH-R), 7.55 (bs, 2H, Ar-NH₂), 4.01 (dq, J = 7.0, ³¹P J = 7.0 Hz, 4H, P(OCH₂CH₃)₂), 3.86 (dd, J = 6.0, ³¹P J = 11.0 Hz, 2H, P-CH₂-NH), 1.14 (t, J = 7.0 Hz, 6H, P(OCH₂CH₃)₂); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 165.2 (Amide C=O), 155.3 (Guanidine C), 155.2 (ArC5), 151.6 (ArC3), 119.8 (ArC6), 109.0 (ArC2), 61.9 (d, ³¹P J = 5.5 Hz, P(OCH₂CH₃)₂), 40.6 (d, ³¹P J = 41.0 Hz, P-CH₂-NH), 16.2 (d, ³¹P J = 6.5 Hz, P(OCH₂CH₃)₂); ES TOF MS m/z (M)⁺ 380; Anal. for C₁₄H₂₀N₇O₄ClP: Calc. Mass 380.1003. Found 380.0993.
diethyl 2-[6-amino-5-(carbamidoylcarbamoyl)-3-chloropyrazin-2-ylamino]ethylphosphonate hydrochloride (20)\textsuperscript{a}

![Chemical Structure]

The compound was produced by General Method C using diethyl (aminoethyl)phosphonate oxalate (190 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 24.3 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 20 as a hygroscopic light yellow solid (80 mg, 53% after HPLC): \(^1\text{H} \text{NMR} \) (500 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \) 10.66 (s, 1H, Amide NH), 8.67 (s, 4H, 2 x Guanidine NH\textsubscript{2}), 7.95 (m, 1H, Ar-NH-R), 7.53 (bs, 2H, Ar-NH\textsubscript{2}), 3.98 (m, 4H, P(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}), 3.56 (m, 2H, NHCH\textsubscript{2}), 2.15 (m, 2H, CH\textsubscript{2}P(OR)\textsubscript{2}), 1.21 (t, \( J = 6.5 \) Hz, 6H, P(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}); \(^{13}\text{C} \text{NMR} \) (125 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \) 165.3 (Amide C=O), 155.6 (Guanidine C), 155.2 (ArC5), 151.9 (ArC3), 120.1 (ArC6), 108.4 (ArC2), 61.2 (d, \( ^{31}\text{P} \) \( J = 6.5 \) Hz, P(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}), 35.3 (NHCH\textsubscript{2}), 24.2 (d, \( ^{31}\text{P} \) \( J = 135.1 \) Hz, CH\textsubscript{2}P(OR)\textsubscript{2}), 16.3 (d, \( ^{31}\text{P} \) \( J = 5.5 \) Hz, P(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}); ES TOF MS \textit{m/z} (M)+ 394; Anal. for C\textsubscript{12}H\textsubscript{22}N\textsubscript{7}O\textsubscript{4}ClP: Calc. Mass 394.1159. Found 394.1154.
diethyl N’-[6-amino-5-(carbamimidoylcarbamoyl)-3-chloropyrazin-2-
yl]phosphorohydrazidate hydrochloride (21)\textsuperscript{a}

13 (50 mg, 0.18 mmol) and diethyl phosphorohydrazidate (59 µL, 0.35 mmol) were dissolved in DMF (5 mL) and heated to 50 °C. DIPEA (214 µL, 1.23 mmol) was added and the solution was stirred for 1 h before the solvent was removed \textit{in vacuo}. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins \((t, 21.7\text{ min})\). The collected fractions containing purified product were concentrated by freeze-drying to yield 21 as a hygroscopic dark orange solid (18 mg, 24% after HPLC): \(^{13}\text{C}\) NMR (125 MHz, 
\((\text{CD}_3)_2\text{SO}) \delta 165.2 (\text{C}=\text{O}), 155.2 (\text{Guanidine C}), 155.1 (\text{ArC5}), 150.9 (\text{ArC3}), 118.4 (\text{ArC6}), 109.3 (\text{ArC2}), 62.0 (d, \(^{31}\text{P}\) \(J = 5.5\text{ Hz}\), \(\text{P(OCH}_2\text{CH}_3)_2\)), 16.1 (d, \(^{31}\text{P}\) \(J = 6.5\text{ Hz}\), \(\text{P(OCH}_2\text{CH}_3)_2\)); ES TOF MS \(m/z\) (M)\(^+\) 381.

3-amino-N-carbamimidoyl-6-chloro-5-(cyanomethylamino)pyrazine-2-
carboxamide hydrochloride (22)\textsuperscript{a}

The compound was produced by General Method C using aminoacetonitrile hydrochloride (130 mg, 1.40 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins \((t, 21.6\text{ min})\). The collected fractions
containing purified product were concentrated by freeze-drying to yield 22 as a light yellow solid (124 mg, 58% after HPLC): \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 10.76 (s, 1H, Amide NH), 8.64 (s, 4H, 2x Guanidine NH\(_2\)), 8.52 (t, \(J = 5.5\) Hz, 1H, Ar-NH-R), 7.78 (bs, 1H, Ar-NH), 7.63 (bs, 1H, Ar-NH), 4.37 (d, \(J = 5.5\) Hz, 2H, CH\(_2\)CN); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 165.4 (Amide C=O), 155.2 (Guanidine C), 155.1 (ArC5), 151.5 (ArC3), 119.6 (ArC6), 117.1 (CH\(_2\)CN), 110.0 (ArC2), 29.4 (CH\(_2\)CN); ES TOF MS m/z (M)+ 269; Anal. for C\(_8\)H\(_{10}\)N\(_3\)OCl: Calc. Mass 269.0666. Found 269.0642.

5-[[1H-tetrazol-5-yl]methyl]amino]-3-amino-N-carbamimidoyl-6-chloropyrazine-2-carboxamide hydrochloride (23)\(^b\)

The compound was produced by General Method B using 47 (95 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (\(t = 20.9\) min). The collected fractions containing purified product were concentrated by freeze-drying to yield 23 as a light yellow solid (77 mg, 63% after HPLC): \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 10.69 (s, 1H, Amide NH), 8.61 (s, 4H, 2x Guanidine NH\(_2\)), 8.55 (m, 1H, Ar-NH-R), 7.60 (bs, 2H, Ar-NH\(_2\)), 4.84 (d, \(J = 5.5\) Hz, 2H, NH-CH\(_2\)); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 165.3 (Amide C=O), 155.4 (Guanidine C), 155.4 (ArC5'), 155.1 (ArC3), 152.1 (ArC5), 120.1 (ArC6), 109.2 (ArC2), 34.7 (NHCH\(_2\)); ES TOF MS m/z (M)+ 312; Anal. for C\(_8\)H\(_{11}\)N\(_3\)OCl: Calc. Mass 312.0837. Found 312.0845.
The compound was produced by General Method C using 3-aminopropionitrile fumarate (180 mg, 1.40 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 22.1 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 24 as a light orange solid (136 mg, 61% after HPLC): mp 274-276 °C (dec); 1H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.70 (s, 1H, Amide NH), 8.68 (s, 4H, 2x Guanidine NH$_2$), 8.22 (t, $J = 5.5$ Hz, Ar-NH-R), 7.44 (bs, 2H, Ar-NH$_2$), 3.62 (dt, $J = 6.0$, 6.0 Hz, 2H, NH-$\text{C}_2$H$_2$), 2.89 (t, $J = 6.0$ Hz, 2H, CH$_2$CN); 13C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.3 (Amide C=O), 155.4 (ArC5), 155.3 (Guanidine C), 152.1 (ArC3), 119.9 (ArC6), 119.1 (CH$_2$CN), 109.0 (ArC2), 37.0 (NH-$\text{CH}_2$), 16.6 (CH$_2$CN); ES TOF MS $m/z$ (M)$^+$ 283; Anal. for C$_9$H$_{12}$N$_8$OCl: Calc. Mass 283.0823. Found 283.0815.

The compound was produced by General Method B using 48 (80 mg, 0.71 mmol). The residue was taken up in DMSO (2 mL) for
purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 14.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 25 as a bright yellow solid (94 mg, 74% after HPLC): ^1^H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.64 (s, 1H, Amide NH), 8.61 (s, 2H, Guanidine NH$_2$), 8.50 (s, 2H, Guanidine NH$_2$), 8.07 (m, 1H, Ar-NH-), 7.55 (bs, 2H, Ar-NH$_2$), 3.74 (m, J = 4.5 Hz, 2H, NH-CH$_2$), 3.22 (m, 2H, NH-CH$_2$CH$_2$); ^1^C NMR (125 MHz, CD$_3$OD) δ 166.9 (Amide C=O), 157.4 (Guanidine C), 157.0 (ArC5), 155.0 (ArC5'), 154.1 (ArC3), 122.3 (ArC6), 110.1 (ArC2), 40.3 (N(CH$_2$)$_2$), 23.9 (NHCH$_2$CH$_2$); ES TOF MS m/z (M)$^+$ 326; Anal. for C$_9$H$_{13}$N$_{11}$OCl: Calc. Mass 326.0993. Found 326.1002.

3-amino-N-carbamimidoyl-6-chloro-5-[2-(diethylamino)ethylamino]pyrazine-2-carboxamide hydrochloride (26)$^\text{a}$

The compound was produced by General Method A using N,N-diethylethylenediamine (49 µL, 0.35 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 21.8 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 26 as a yellow solid (58 mg, 88% after HPLC): ^1^H NMR (500 MHz, CD$_3$OD) δ 3.91 (m, 2H, Ar-NH-CH$_2$), 3.44 (m, 2H, Ar-NH-CH$_2$CH$_2$), 3.34 (m, 4H, N(CH$_2$CH$_3$)$_2$), 1.37 (m, 6H, N(CH$_2$CH$_3$)$_2$); ^1^C NMR (125 MHz, CD$_3$OD) δ 166.8 (C=O), 157.0 (Guanidine C), 156.8 (ArC5), 154.1 (ArC3), 122.3 (ArC6), 110.5 (ArC2), 51.6 (Ar-NH-CH$_2$CH$_2$), 49.4
(\text{N(CH}_2\text{CH}_3)_2\text{)}, \ 37.4 \ (\text{Ar-NH-CH}_2\text{)}, \ 9.5 \ (\text{N(CH}_2\text{CH}_3)_2\text{}); \ ES \ TOF \ MS \ m/z \ (M)^+ \ 329; \ Anal. \ for \ \text{C}_{12}\text{H}_{22}\text{N}_8\text{OCl}: \ Calc. \ Mass \ 329.1605. \ Found \ 329.1621.

3-amino-N-carbamidoyl-5,8-dimethyl-5,6,7,8-
tetrahydropyrazino[2,3b]pyrazine-2-carboxamide hydrochloride (27)\textsuperscript{a}

The compound was produced by General Method A using \textit{N,N’}-dimethylethylenediamine (75 µL, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (\textit{t}, 16.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 27 as an orange solid (87 mg, 83% after HPLC): mp 286-288 °C (dec); \textsuperscript{1}H NMR (500 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \) 10.17 (s, 1H, Amide NH), 9.14 (bs, 2H, Guanidine NH\textsubscript{2}), 8.40 (Guanidine NH\textsubscript{2}), 5.14 (bs, ArNH\textsubscript{2}), 3.58 (s, 2H, C7H\textsubscript{2}), 3.27 (s, 2H, C8H\textsubscript{2}), 3.10 (s, 3H, ArC5-N(CH\textsubscript{3})), 3.01 (s, 3H, ArC10-N(CH\textsubscript{3})); \textsuperscript{13}C NMR (125 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \) 165.4 (C=O), 155.3 (Guanidine C), 152.4 (ArC5), 146.9 (ArC3), 135.6 (ArC10), 103.1 (ArC2), 47.4 (C7), 45.8 (C8), 37.0 (ArC5-N(CH\textsubscript{3})), 35.9 (ArC9-N(CH\textsubscript{3})); ES TOF MS \textit{m/z} (\textit{M})^+ \ 265; Anal. for \text{C}_{10}\text{H}_{17}\text{N}_8\text{O}: \ Calc. \ Mass \ 265.1525. \ Found \ 265.1644.
3-amino-5-(benzylamino)-N-carbamimidoyl-6-chloropyrazine-2-carboxamide hydrochloride (28)\textsuperscript{b,197}

The compound was produced by General Method A using benzylamine (153 µL, 1.40 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 17.4 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 28 as a light orange solid (242 mg, 97% after HPLC): mp 200-202 °C; \textsuperscript{1}H NMR (500 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \)

10.65 (s, 1H, Amide NH), 8.64 (s, 4H, 2 x Guanidine NH\textsubscript{2}), 8.53 (s, 1H, Ar-NH-R), 7.50 (bs, 2H, Ar-NH\textsubscript{2}), 7.34 (m, 2H, ArH3' and ArH5'), 7.29 (m, 2H, ArH2' and ArH6'), 7.22 (m, 1H, ArH4'), 4.59 (s, 2H, Ar-NH-CH\textsubscript{2}); \textsuperscript{13}C NMR (125 MHz, (CD\textsubscript{3})\textsubcript{2}SO) \( \delta \)

165.0 (C=O), 155.4 (Guanidine C), 155.0 (ArC5), 151.9 (ArC3), 138.3 (ArC1'), 128.1 (ArC3' and ArC5'), 127.4 (ArC2' and ArC6'), 126.8 (ArC4'), 119.8 (ArC6), 108.4 (ArC2), 43.7 (Ar-NH-CH\textsubscript{2}); ES TOF MS \( m/z \) (M)\textsuperscript{+} 320; Anal. for C\textsubscript{13}H\textsubscript{15}N\textsubscript{7}OCl: Calc. Mass 320.1027. Found 320.1021.

3-amino-N-carbamimidoyl-6-chloro-5-(2-pyridylmethylamino)pyrazine-2-carboxamide hydrochloride (29)\textsuperscript{a}

The compound was produced by General Method A using 2-(aminomethyl)pyridine (72 µL, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 12.6 min).
The collected fractions containing purified product were concentrated by freeze-drying to yield 29 as a light brown solid (79 mg, 63% after HPLC): $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 10.73 (s, 1H, Amide NH), 8.82 (m, 1H, ArH$^4'$), 8.76 (s, 1H, Ar-NH-R), 8.68 (s, 4H, 2 x Guanidine NH$_2$), 8.53 (m, 1H, ArH$^6'$), 8.03 (d, $J = 7.0$ Hz, 1H, ArH$^3'$), 7.94 (m, 1H, ArH$^5'$), 4.93 (d, $J = 4.0$ Hz, 2H, Ar-NH-CH$_2$); $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.3 (C=O), 155.2 (Guanidine C), 155.2 (ArC5), 152.9 (ArC2'), 151.9 (ArC3), 146.3 (ArC6'), 141.1 (ArC4'), 126.0 (ArC3'), 125.7 (ArC5'), 120.0 (ArC6), 109.5 (ArC2), 42.2 (Ar-NH-CH$_2$); ES TOF MS $m/z$ (M)$^+$ 321; Anal. for C$_{12}$H$_{14}$N$_8$OCl: Calc. Mass 321.09797. Found 321.0995.

3-amino-N-carbamimidoyl-6-chloro-5-(3-pyridylmethylamino)pyrazine-2-carboxamide hydrochloride (30)*

The compound was produced by *General Method A* using 3-(aminomethyl)pyridine (71 μL, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 13.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 30 as a light brown solid (96 mg, 77% after HPLC): $^1$H NMR (500 MHz, CD$_3$SO) $\delta$ 10.68 (s, 1H, Amide NH), 9.04 (s, 1H, ArH$^2'$), 8.82 (s, 1H, ArH$^6'$), 8.73 (m, 1H, Ar-NH-R), 8.65
(s, 4H, 2 x Guanidine NH₂), 8.02 (m, 1H, ArH4’), 7.65 (bs, 2H, Ar-NH₂), 7.52 (m, 1H, ArH5’), 4.70 (d, J = 5.0 Hz, 2H, Ar-NH-CH₂); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 165.3 (C=O), 155.4 (Guanidine C), 155.2 (ArC5), 151.9 (ArC3), 145.3 (ArC2’), 141.4 (ArC6’), 140.5 (ArC4’), 138.4 (ArC3’), 126.9 (ArC5’), 120.1 (ArC6), 109.1 (ArC2), 41.5 (Ar-NH-CH₂); ES TOF MS m/z (M)⁺ 321; Anal. for C₁₂H₁₄N₈OCl: Calc. Mass 321.0979. Found 321.0980.

3-amino-N-carbamimidoyl-6-chloro-5-(4-pyridylmethylamino)pyrazine-2-carboxamide hydrochloride (31)ᵇ,¹⁹⁷

The compound was produced by General Method A using 4-(aminomethyl)pyridine (71 µL, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 13.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 31 as an orange solid (101 mg, 81% after HPLC): ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.72 (s, 1H, Amide NH), 8.84 (m, 2H, ArH2’ and ArH6’), 8.79 (m, 1H, Ar-NH-R), 8.70 (s, 4H, 2 x Guanidine NH₂), 8.04 (m, 2H, ArH3’ and ArH5’), 7.45 (bs, 2H, Ar-NH₂), 4.81 (m, 2H, Ar-NH-CH₂); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 165.3 (C=O), 159.4 (ArC4’), 155.3 (Guanidine C), 155.2 (ArC5), 152.0 (ArC3), 140.9 (ArC2’ and ArC6’), 125.6 (ArC3’ and ArC5’), 120.1 (ArC6), 109.3 (ArC2), 44.0 (Ar-NH-CH₂); ES TOF MS m/z (M)⁺ 321; Anal. for C₁₂H₁₄N₈OCl: Calc. Mass 321.0979. Found 321.0972.
3-amino-N-carbamimidoyl-6-chloro-5-(4-fluorobenzyl)aminopyrazine-2-carboxamide hydrochloride (32)\textsuperscript{a}

The compound was produced by General Method A using 4-fluorobenzylamine (88 \( \mu \)L, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0\% to 100\% B over 30 mins (t, 26.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 32 as a light yellow solid (102 mg, 78\% after HPLC): \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) δ 10.65 (s, 1H, Amide NH), 8.65 (s, 4H, 2 x Guanidine NH\(_2\)), 8.55 (s, 1H, Ar-NH-R), 7.53 (bs, 2H, Ar-NH\(_2\)), 7.41 (m, 2H, ArH2’ and ArH6’), 7.10 (m, 2H, ArH3’ and ArH5’), 4.55 (m, 2H, Ar-NH-C\(_2\)H\(_2\)); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) δ 165.2 (C=O), 161.2 (d, \(^{19}\)F J = 242 Hz, ArC4’), 155.6 (Guanidine C\(_6\)), 155.2 (ArC5), 152.0 (ArC3), 134.7 (ArC1’), 129.8 (d, \(^{19}\)F J = 8.0 Hz, ArC2’ and ArC6’), 120.0 (ArC6), 114.9 (d, \(^{19}\)F J = 21 Hz, ArC3’ and ArC5’), 108.6 (ArC2), 43.2 (Ar-NH-C\(_2\)H\(_2\)); ES TOF MS m/z (M)\(^+\) 338; Anal. for C\(_{13}\)H\(_{14}\)N\(_3\)OClF: Calc. Mass 338.0927. Found 338.0996.

3-amino-N-carbamimidoyl-6-chloro-5-(2-phenylethylamino)pyrazine-2-carboxamide hydrochloride (33)\textsuperscript{b,197}

The compound was produced by General Method A using 2-phenylethylamine (176 \( \mu \)L, 1.40 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0\% to 100\% B over 30 mins (t, 18.5 min).
The collected fractions containing purified product were concentrated by freeze-drying to yield 33 as a light orange solid (218 mg, 84% after HPLC): mp 128-130 °C; $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.63 (s, 1H, Amide NH), 8.64 (s, 2H, Guanidine NH$_2$), 8.60 (s, 2H, Guanidine NH$_2$), 8.02 (s, 1H, Ar-NH-R), 7.56 (bs, 2H, Ar-NH$_2$), 7.26 (m, 4H, ArH$_2$’ and ArH6’), 7.19 (t, $J = 6.5$ Hz, 1H, ArH4’), 3.56 (m, 2H, Ar-NH-C$_2$H$_2$), 2.87 (t, $J = 7.5$ Hz, 2H, NH-CH$_2$-CH$_2$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.2 (C=O), 155.7 (Guanidine C), 155.2 (ArC3), 152.0 (ArC5), 139.0 (ArC1’), 128.8 (ArC3’ and ArC5’), 128.4 (ArC2’ and ArC6’), 126.3 (ArC4’), 120.0 (ArC6), 108.2 (ArC2), 42.5 (Ar-NH-CH$_2$), 34.1 (NH-CH$_2$-CH$_2$); ES TOF MS m/z (M)$^+$ 334; Anal. for C$_{14}$H$_{17}$N$_7$OCl: Calc. Mass 334.1183. Found 334.1186.

1-[2-[6-amino-5-(carbamimidoylcarbamoyl)-3-chloropyrazin-2-ylamino]ethyl]pyridinium chloride (34)$^a$

The compound was produced by General Method A using 44 (132 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins ($t$, 14.6 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 34 as a light orange solid (90 mg, 69% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.70 (s, 1H, Amide NH), 9.13 (d, $J = 4.0$ Hz, 2H, ArH2 and
3-amino-5-[2-(benzylthio)ethylamino]-N-carbamidimoyl-6-chloropyrazine-2-carboxamide hydrochloride (35)

The compound was produced by General Method B using S-benzylcysteamine hydrochloride (143 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 23.6 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 35 as a yellow solid (134 mg, 92% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 10.64 (s, 1H, Amide NH), 8.63 (s, 2H, Guanidine NH$_2$), 8.56 (s, 2H, Guanidine NH$_2$), 8.04 (t, $J = 5.5$ Hz, 1H, Ar-NH-R), 7.54 (s, 2H, Ar-NH$_2$), 7.30 (m, 4H, ArH2’, ArH3’, ArH5’ and ArH6’), 7.21 (t, $J = 7.0$ Hz, 1H, ArH4’), 3.78 (s, 2H, S-CH$_2$-Ph), 3.58 (dt, $J = 6.5$, 7.0 Hz, 2H, Ar-NH-CH$_2$), 2.62 (t, $J = 7.0$ Hz, 2H, CH$_2$-CH$_2$-S); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 165.2 (C=O), 155.6 (Guanidine C), 155.2 (ArC5), 152.0

...
(ArC3), 138.5 (ArC1’), 128.9 (ArC2’ and ArC6’), 128.3 (ArC3’ and ArC5’), 126.8 (ArC4’), 120.0 (ArC6), 108.5 (ArC2), 40.1 (Ar-NH-CH$_2$), 34.8 (S-CH$_2$-Ph), 29.3 (CH$_2$-CH$_2$-S); ES TOF MS m/z (M)$^+$ 380; Anal. for C$_{15}$H$_{19}$N$_7$OSCl: Calc. Mass 380.1060. Found 380.1060.

3-amino-N-carbamimidoyl-6-chloro-5-[2-(4-methylphenylsulfonamido)ethylamino]pyrazine-2-carboxamide hydrochloride (36)$^a$

The compound was produced by General Method A using N-tosylethylenediamine (150 mg, 0.70 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 20.3 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 36 as a yellow solid (144 mg, 89% after HPLC): mp 178-180 °C; $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.61 (s, 1H, Amide NH), 8.60 (s, 2H, Guanidine NH$_2$), 8.43 (s, 2H, Guanidine NH$_2$), 7.74 (t, J = 5.5 Hz, 1H, Ar-NH-R), 7.65 (t, J = 5.0 Hz, 1H, NH-SO$_2$-Ar), 7.62 (d, J = 8.5 Hz, ArH2’ and ArH6’), 7.47 (bs, 2H, Ar-NH$_2$), 7.28 (d, J = 8.0 Hz, 2H, ArH3’ and ArH5’), 3.42 (dt, J = 5.5, 6.5 Hz, 2H, Ar-NH-CH$_2$), 2.99 (dt, J = 5.5, 5.5 Hz, 2H, SO$_2$-NH-CH$_2$), 2.30 (s, 3H, Ar-CH$_3$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.3 (C=O), 155.6 (Guanidine C), 155.1 (ArC5), 152.0 (ArC3), 142.5 (ArC4’), 137.3 (ArC1’), 129.5 (ArC3’ and ArC5’), 126.5 (ArC2’ and

91
ArC6'), 120.3 (ArC6), 108.4 (ArC2), 40.7 (Ar-NH-CH2), 40.5 (SO2-NH-CH2), 20.9 (Ar-CH3); ES TOF MS m/z (M)+ 427; Anal. for C25H20N8O3SCl: Calc. Mass 427.1068. Found 427.1082.

3-amino-5-[2-[benzyl(methyl) amino]ethylamino]-N-carbamidimoyl-6-chloropyrazine-2-carboxamide hydrochloride (37)\textsuperscript{a}

The compound was produced by General Method A using 46 (81 mg, 0.49 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t<sub>r</sub> 15.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 37 as a yellow solid (81 mg, 78% after HPLC): \(^1\)H NMR (500 MHz, (CD$_3$)$_2$SO) δ 11.05 (s, 1H, Bz-NH(CH$_3$)-R), 10.68 (s, 1H, Amide NH), 8.67 (s, 2H, Guanidine NH$_2$), 8.64 (s, 2H, Guanidine NH$_2$), 8.18 (t, J = 5.5 Hz, 1H, Ar-NH-CH$_2$), 7.60 (m, 2H, ArH3' and ArH5'), 7.54 (bs, 2H, Ar-NH$_2$), 7.36 (m, 3H, ArH2', ArH4' and ArH6'), 4.42 (dd, J = 5.0, 13.0 Hz, 1H, Ph-CH-NH(CH$_3$)-R), 4.33 (dd, J = 5.0, 13.0 Hz, 1H, Ph-CH-NH(CH$_3$)-R), 3.75 (m, 2H, Ar-NH-CH$_2$), 3.29 (m, 1H, Ar-NH-CH$_2$-CH), 3.21 (m, 1H, Ar-NH-CH$_2$-CH), 2.76 (d, J = 4.0 Hz, 3H, R-NH(CH$_3$)-Bz); \(^13\)C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.3 (C=O), 155.4 (Guanidine C), 155.2 (ArC5), 152.0 (ArC3), 131.2 (ArC2' and ArC6'), 130.2 (ArC1'), 129.3 (ArC4'), 128.7 (ArC3' and ArC5'), 120.4 (ArC6), 108.8 (ArC2), 58.5 (RNH(CH$_3$)-
methyl 5-(2-(benzylamino)ethylamino)-3-amino-6-chloropyrazine-2-carboxylate

(38)³

Methyl 3-amino-5,6-dichloropyrazine-2-carboxylate (100 mg, 0.45 mmol) and N-benzylethlenediamine (130 μL, 0.9 mmol) were heated at 100 °C for 1h before the solvent was removed in vacuo and the residue purified by column chromatography in a 10 mm diameter column with a silica height of 150 mm. The eluent used was a mixture of petroleum spirit (B.R. 40-60 °C) and EtOAc. The fraction containing the desired product was isolated and concentrated in vacuo to yield 38 as light yellow crystals (128 mg, 85%): ¹H NMR (500 MHz, CD₃OD) δ 7.31-7.20 (m, 5H, ArH₂-6’), 3.78 (m, 5H, COOCH₃ and NHCH₂Ph), 3.57 (t, J = 5.5 Hz, 2H, Ar-NHCH₂), 2.85 (m, 2H, Ar-NHCH₂CH₂); ¹³C NMR (125 MHz, CD₂OD) δ 167.5 (C=O), 157.1 (ArC₅), 153.3 (ArC₃), 139.7 (ArC¹’), 129.5 (ArC³’ and ArC₅’), 129.5 (ArC²’ and ArC₆’), 128.3 (ArC⁴’), 122.0 (ArC₆), 110.3 (ArC₂), 54.0 (NHCH₂Ph), 52.0 (COOCH₃), 48.3 (Ar-NHCH₂), 41.1 (Ar-NHCH₂CH₂); ES TOF MS m/z (M)⁺
3-amino-5-[2-(benzylamino)ethylamino]-N-carbamimoyl-6-chloropyrazine-2-carboxamide hydrochloride (39)*

Method 1:

The compound was produced by General Method A using N-benzylethylenediamine (90 µL, 0.49 mmol). The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 17.4 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 39 as a yellow solid (28 mg, 28% after HPLC).

Method 2:

Sodium (9 mg, 0.39 mmol) was added to dry iPrOH (1 mL) and stirred under N₂ gas until the reaction had slowed. Guanidine hydrochloride (37 mg, 0.39 mmol) was added and the solution stirred until the sodium was completely dissolved. The resultant sodium chloride was filtered off and the filtrate was combined with 38 (65 mg, 0.19 mmol). The mixture was heated at reflux for 30 min before cooling and concentrating in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 17.4 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 39 as a yellow solid (39 mg, 52% after HPLC): ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.64 (s, 1H, Amide NH), 9.64 (s, 1H, NH-Bz), 8.64 (s, 4H, 2 x Guanidine
NH$_2$), 8.09 (s, 1H, Ar-NH-R), 7.57 (m, 2H, ArH3’ and ArH5’), 7.33-7.22 (m, 3H, ArH2’, ArH4’ and ArH6’), 5.98 (bs, 2H, Ar-NH$_2$), 4.14 (m, 2H, NHCH$_2$Ph), 3.70 (m, 2H, Ar-NHCH$_2$), 3.11 (m, 2H, Ar-NHCH$_2$CH$_2$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 165.3 (C=O), 155.5 (Guanidine C), 155.3 (ArC5), 152.5 (ArC3), 132.0 (ArC1’), 130.2 (ArC3’ and ArC5’), 130.1 (ArC2’ and ArC6’), 128.6 (ArC4’), 120.6 (ArC6), 108.7 (ArC2), 49.7 (NHCH$_2$Ph), 45.2 (Ar-NHCH$_2$), 37.3 (Ar-NHCH$_2$CH$_2$); ES TOF MS m/z (M)$^+$ 363; Anal. for C$_{15}$H$_{20}$N$_8$OCl: Calc. Mass 363.149. Found 363.1440.

3,5-diamino-N-carbamimidoylpyrazine-2-carboxamide hydrochloride (40)$^{191}$

3,5-diamino-N-carbamimidoyl-6-chloropyrazine-2-carboxamide hydrochloride (300 mg, 1.13 mmol), Pd/C with 10% loading (122 mg) and MgO (84 mg, 2.08 mmol) were combined in MeOH (6 mL). The flask was saturated with H$_2$ from a balloon, using a 3-way tap connected to a vacuum pump that allowed for several repetitions of evacuation and refilling. The hydrogenation was stirred for 2 days before filtration using a 0.45 micron syringe filter and concentration in vacuo. The residue was taken up in 2 mL DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 9.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 40 as a light yellow solid (154 mg, 59% after HPLC): mp 260-262 °C (dec); $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 10.97, 8.72, 7.41, 6.72; $^{13}$C NMR
methyl 3,5-diaminopyrazine-2-carboxylate (41)$^b,191$

\[
\text{H}_2\text{N}^+\text{N}^-\text{NH}_2\]

Palladium (10%) on activated charcoal (200 mg, 0.19 mmol), methyl 3,5-diamino-6-chloropyrazine-2-carboxylate (1 g, 4.94 mmol) and MgO (230 mg, 5.71 mmol) were combined in dry MeOH (20 mL) in a Parr Hydrogenator flask. The mixture was degassed in vacuo before being subjected to H$_2$ gas at 50 psi with shaking for 3 days. The mixture was then combined with DMF (20 mL) before being filtered with a 0.45 μM syringe filter and concentrated in vacuo, yielding 41 as a yellow solid (814 mg, 98%): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 7.25 (bs, 4H, 2 x NH$_2$), 7.10 (s, 1H, ArH6), 3.71 (3H, COOC$_3$H$_7$): $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.8 (C=O), 155.8 (ArC5), 153.2 (ArC3), 119.2 (ArC6), 109.4 (ArC6), 51.2 (COOCH$_3$); ES TOF MS m/z (M + H)$^+$ 169; Anal. for C$_6$H$_9$N$_4$O$_2$: Calc. Mass 169.0726. Found 169.0742.

diethyl phosphorohythrazidate (43)$^c,198$

Hydrazine hydrate 80% solution (24.3 mL, 0.40 mol) was added dropwise to a stirred solution of anhydrous K$_2$CO$_3$ (41.163 g, 0.30 mol) and triethylbenzylammonium chloride (456 mg, 2.00 mmol) in a 4:7 mixture of carbon tetrachloride and DCM (100 mL). A solution of diethyl
phosphite (2.574 mL, 0.02 mol) in DCM (10 mL) was added and the solution stirred for 6 h before the K$_2$CO$_3$ was filtered off and rinsed with DCM. The filtrate was concentrated in vacuo to yield a liquid product that was redissolved in chloroform (10 mL) and filtered to remove remaining triethylbenzylammonium chloride. Concentration of the filtrate in vacuo yielded 43 as a viscous pale yellow liquid (3.026 g, 90%): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 5.28, 4.11, 3.48, 1.35; $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 62.5, 16.0; ES TOF MS m/z (MH)$^+$ 169; Anal. for C$_4$H$_{14}$N$_2$O$_3$P: Calc. Mass 169.0742. Found 169.0733.

1-(2-aminoethyl)pyridinium bromide (44)$^{199}$

![Structure of 44](image)

2-bromoethylamine hydrobromide (2.049 g, 10 mmol) was heated at reflux in dry pyridine (5 mL) for 1 day before excess pyridine was removed using N$_2$-assisted evaporation. The residue was dissolved in water (10 mL) and the pH adjusted to 8 with the addition of KOH pellets. The solution was extracted with EtOAc (2 x 10 mL) and the aqueous layer concentrated in vacuo. The residue was redissolved in a small amount of chloroform and acetonitrile before being concentrated in vacuo to yield 44 as a viscous brown liquid (1.746 g, 86%): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 9.13, 8.60, 8.14, 4.63, 3.01, 2.39; $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 145.2, 145.0, 127.4, 62.8, 42.2; ES TOF MS m/z (M)$^+$ 123; Anal. for C$_7$H$_{11}$N$_2$: Calc. Mass 123.0922. Found 123.0929.
2-[benzyl(methyl)amino]acetonitrile (45)^c,200

Chloroacetonitrile (3.797 mL, 60 mmol) and TEA (8.363 mL, 60 mmol) were combined in toluene (30 mL). A solution of N-benzylmethylamine (6.432 mL, 50 mmol) in toluene (10 mL) was added and the mixture stirred at 100 °C for 2 h. Water (40 mL) was added and the solution extracted with EtOAc (3 x 30 mL). The combined organic extracts were dried with MgSO₄ and concentrated in vacuo to yield 45 as a yellow liquid (8.011 g, 100%): \(^1\)H NMR (500 MHz, CDCl₃) δ 7.30, 3.56, 3.38, 2.39; \(^{13}\)C NMR (125 MHz, CDCl₃) δ 136.8, 128.7, 128.4, 127.5, 114.3, 59.9, 43.9, 42.0; EI MS m/z (M)\(^+\) 160; Anal. for C₁₀H₁₂N₂: Calc. Mass 160.100048. Found 160.100364. Comparison \(^1\)H NMR (200 MHz CDCl₃) δ 7.2, 3.5, 3.3, 2.3.200

2-[benzyl(methyl)amino]ethylamine (46)^c,200

To a suspension of LiAlH₄ (570 mg, 15 mmol) in dry THF (15 mL) under N₂ was added 45 (1.602 g, 10 mmol) in dry THF (5 mL). The mixture was stirred at 0 °C for 1 h before water (10 mL) was added and the solution extracted with EtOAc (3 x 10 mL). The combined organic extracts were dried with MgSO₄ and concentrated in vacuo to yield 46 as a yellow liquid (1.544 g, 94%): \(^1\)H NMR (500 MHz, CDCl₃) δ 7.30, 3.50, 2.77, 2.44, 2.19, 1.44; \(^{13}\)C NMR (125 MHz, CDCl₃) δ 139.1, 128.9, 128.0, 126.8, 62.5, 60.0, 42.0, 39.4; EI MS m/z (M)\(^+\) 164; Anal. for C₁₀H₁₆N₂: Calc. Mass 164.131349. Found 164.133878. Comparison \(^1\)H NMR (200 MHz CDCl₃) δ 7.2, 3.5, 2.8, 2.4, 2.2.200
(1H-tetrazol-5-yl)methanamine hydrochloride (47)

5-(chloromethyl)-1H-tetrazole (2 g, 16.87 mmol) was dissolved in water (100 mL) and transferred to a self-equilibrating dropping funnel. The funnel was fitted to a flask containing 28% aqueous ammonia (200 mL) stirred at 70 °C. The solution in the dropping funnel was added dropwise to the stirred solution before N₂ was bubbled through for 1 day to displace remaining ammonia. The solvent was removed in vacuo and the residue triturated with 1 M HCl in Et₂O and recrystallised from ethanol/water/Et₂O. The crystals were collected and dried in vacuo to yield 47 as colourless crystals (869 mg, 52%). ¹H NMR (500 MHz, (CD₃)₂SO) δ 7.60, 4.16, 3.82; ¹³C NMR (125 MHz, (CD₃)₂SO) δ 155.1, 34.3; EI MS m/z (M)⁺ 99; Anal. for C₂H₅N₅: Calc. Mass 99.054495. Found 99.054537.

2-(1H-tetrazol-5-yl)ethanamine (48)

5-(2-chloroethyl)-1H-tetrazole (1.326 g, 10.00 mmol) and NaN₃ (1.301 g, 20.00 mmol) were dissolved in DMF (25 mL) and stirred at 60 °C for 12 h before the solvent was removed in vacuo. The residue was covered with THF (5 mL) and water was added dropwise until a solution resulted. Triphenylphosphine (2.62 g, 10.00 mmol) was added and the solution was stirred for 24 h before 1 M HCl (20 mL) was added and the THF removed in vacuo. The
remaining aqueous solution was concentrated by freeze drying before being taken up into MeOH and filtered to remove NaCl. The filtrate was concentrated in vacuo, taken up in water and subjected to continuous extraction with Et₂O for 2 days to remove triphenylphosphine oxide. The aqueous solution was concentrated by freeze drying to yield 48 as pale yellow crystals (837 mg, 74%): ¹H NMR (500 MHz, CD₃OD) δ 3.49 (t, J = 6.0 Hz, 2H, NH₂-C₆H₂), 3.42 (t, J = 7.0 Hz, 2H, Ar-CH₂); ¹³C NMR (125 MHz, CD₃OD) δ 155.0 (ArC₅), 38.1 (NH₂-C₆H₂), 22.7 (Ar-CH₂); EI MS m/z (M)⁺ 113; Anal. for C₃H₇N₅: Calc. Mass 113.070145. Found 113.069743.

2-[6-amino-5-(carbamidoylcarbamoyl)-3-chloropyrazin-2-ylamino]acetic acid hydrochloride (51)³

16 (32 mg, 0.09 mmol) was dissolved in a mixture of MeOH (5 mL) and water (2 mL). K₂CO₃ (131 mg, 0.95 mmol) was added to the stirred solution and the mixture stirred for 4 h before the solvents were removed in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 22.4 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 51 as a light brown solid (22 mg, 77% after HPLC): ¹H NMR (500 MHz, (CD₃)₂SO) δ 12.80, 10.68, 8.63, 8.12, 7.55, 4.03; ¹³C NMR (125 MHz, (CD₃)₂SO) δ 170.4, 165.3, 155.5, 155.1, 152.1, 119.9, 109.0, 42.5; ES TOF MS m/z (M)⁺ 288; Anal. for C₈H₁₁N₇O₃Cl: Calc. Mass 288.0612. Found 288.0611.
3-[6-amino-5-(carbamidoylcarbamyl)-3-chloropyrazin-2-ylamino]propanoic acid hydrochloride (52)\textsuperscript{a}

\[ \text{52} \]

17 (40 mg, 0.11 mmol) was dissolved in a mixture of MeOH (5 mL) and water (2 mL). K\textsubscript{2}CO\textsubscript{3} (151 mg, 1.09 mmol) was added to the stirred solution and the mixture stirred for 1 h before the solvents were removed \textit{in vacuo}. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0\% to 100\% B over 30 mins (\( t_r \) 22.0 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 52 as a light brown solid (31 mg, 83\% after HPLC): mp >300 °C (dec); \(^1\)H NMR (500 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \) 8.62 (s, 4H, 2 x Guanidine NH\textsubscript{2}), 7.90 (m, 1H, Ar-NH-R), 7.57 (bs, 2H, Ar-NH\textsubscript{2}), 3.57 (m, 2H, NH-CH\textsubscript{2}), 2.59 (m, 2H, NHCH\textsubscript{2}CH\textsubscript{2}); \(^{13}\)C NMR (125 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \( \delta \) 172.8 (Acid C=O), 165.3 (Amide C=O), 155.7 (Guanidine C), 155.2 (ArC3), 152.1 (ArC5), 120.1 (ArC6), 108.4 (ArC2), 37.1 (NHCH\textsubscript{2}), 32.8 (NHCH\textsubscript{2}CH\textsubscript{2}); ES TOF MS \( m/z \) (M)\(^+\) 302; Anal. for C\textsubscript{8}H\textsubscript{13}N\textsubscript{7}O\textsubscript{3}Cl: Calc. Mass 302.0768. Found 302.0765.
6-amino-5-(carbamidoylcarbamoyl)-3-chloropyrazin-2-ylaminomethylphosphonic acid (53)\(^a\)

19 (65 mg, 0.16 mmol) was dissolved in dry DMF (1 mL) in a vial with screw cap. Chlorotrimethylsilane (20 \(\mu\)L, 0.16 mmol) was added dropwise to the stirred solution. The vial was capped tightly and the stirred solution heated to 130 \(^o\)C for 2 h. The vial was allowed to cool and the solvent removed by \(\text{N}_2\)-assisted evaporation. The residue was dissolved in 0.1 M NaOH (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins with HCl omitted from solvents \((t_r\ 7.6\ \text{min})\). The collected fractions containing purified product rapidly developed crystals that were collected and dried in vacuo to yield 53 as light yellow crystals (39 mg, 75% after HPLC): Due to a lack of solubility in suitable solvents, NMR data was not obtainable; ES TOF MS \(m/z\) \((\text{M-H})^-\) 322.

2-[6-amino-5-(carbamidoylcarbamoyl)-3-chloropyrazin-2-yl]aminoethylphosphonic acid (54)\(^a\)

ethyl hydrogen 2-[6-amino-5-(carbamidoylcarbamoyl)-3-chloropyrazin-2-ylamino]ethylphosphonate (55)\(^a\)
20 (50 mg, 0.12 mmol) was dissolved in dry DMF (1 mL) in a vial with screw cap. Chlorotrimethylsilane (40 µL, 0.32 mmol) was added dropwise to the stirred solution. The vial was capped tightly and the stirred solution heated to 130 °C for 4 h. The vial was allowed to cool and the solvent removed by N₂-assisted evaporation. The residue was dissolved in 0.1 M NaOH (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins with HCl omitted from solvents (t, 54: 10.3 min, 55: 12.1 min). The collected fractions containing purified product rapidly developed crystals that were collected and dried in vacuo to yield 54 as white crystals (10 mg, 22% after HPLC) and 55 as light brown crystals (15 mg, 32% after HPLC): Due to a lack of solubility in suitable solvents, NMR data was not obtainable; 54 mp >300 °C (dec); ES TOF MS m/z (MH)+ 338; Anal. for C₈H₁₄N₇O₄PCl: Calc. Mass 338.0533. Found 338.0523; 55 ES TOF MS m/z (M)+ 366.

3-amino-5-[2-(benzylsulfonyl)ethylamino]-N-carbamimidoyl-6-chloropyrazine-2-carboxamide hydrochloride (56)\(^a\)

35 (10 mg, 0.024 mmol) and Oxone® (30 mg, 0.049 mmol) were combined in water (1 mL) and stirred as acetonitrile was added dropwise to obtain a homogeneous solution. The solution was stirred for 24 h before the solvent was
removed in vacuo. The residue was taken up in DMSO (1 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 20.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 56 as a light brown solid (10 mg, 90% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.65 (s, 1H, Amide NH), 8.60 (s, 2H, Guanidine NH$_2$), 8.45 (s, 2H, Guanidine NH$_2$), 8.06 (t, J = 6.0 Hz, 1H, Ar-NH-R), 7.60 (bs, 2H, Ar-NH$_2$), 7.39 (m, 5H, ArH2’, ArH3’, ArH4’, ArH5’ and ArH6’), 4.56 (s, 2H, SO$_2$-CH$_2$-Ph), 3.81 (dt, J = 6.0, 7.0 Hz, 2H, Ar-NH-CH$_2$), 3.40 (t, J = 7.0 Hz, 2H, CH$_2$-CH$_2$-SO$_2$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.3 (C=O), 155.6 (Guanidine C), 155.1 (ArC5), 152.0 (ArC3), 131.2 (ArC2’ and ArC6’), 128.5 (ArC4’), 128.5 (ArC3’ and ArC5’), 128.3 (ArC1’), 120.1 (ArC6), 108.8 (ArC2), 58.2 (SO$_2$-CH$_2$-Ph), 49.7 (CH$_2$-CH$_2$-SO$_2$), 34.5 (Ar-NH-CH$_2$); ES TOF MS m/z (M)$^+$ 412; Anal. for C$_{15}$H$_{19}$N$_7$O$_3$S: Calc. Mass 412.0959. Found 412.0954.

methyl 3,5-diamino-6-iodopyrazine-2-carboxylate (57)$^h$$^{191}$

41 (242 mg, 1.44 mmol) and NIS (356 mg, 1.58 mmol) were dissolved in dry DMF (10 mL) and heated at 80 °C with stirring for 1 h before the solvent was removed in vacuo. The residue was purified by column chromatography to yield 57 as a bright yellow solid (419 mg, 99%): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 7.01 (bs, 4H, 2 x Ar-NH$_2$), 3.71 (s, 3H, COOCH$_3$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.9 (C=O), 156.0 (ArC5), 155.6 (ArC3), 155.1 (ArC5), 152.0 (ArC3), 131.2 (ArC2’ and ArC6’), 128.5 (ArC4’), 128.5 (ArC3’ and ArC5’), 128.3 (ArC1’), 120.1 (ArC6), 108.8 (ArC2), 58.2 (SO$_2$-CH$_2$-Ph), 49.7 (CH$_2$-CH$_2$-SO$_2$), 34.5 (Ar-NH-CH$_2$); ES TOF MS m/z (M)$^+$ 412; Anal. for C$_{15}$H$_{19}$N$_7$O$_3$S: Calc. Mass 412.0959. Found 412.0954.
113.5 (ArC2), 87.1 (ArC6), 51.3 (COOCH$_3$); ES TOF MS $m/z$ (M + H)$^+$ 294; Anal. for C$_6$H$_8$N$_4$O$_2$I: Calc. Mass 294.9692. Found 294.9678.

**3,5-diamino-6-bromo-$N$-carbamimidoylpyrazine-2-carboxamide hydrochloride**

(58)$^c$$^{191}$

\[
\text{Br}_6\text{N}^1\text{N}^2\text{N}^3\text{N}^4\text{N}^5\text{H}_2\text{N}^3\text{NH}_2
\]

\[
\text{Cl}\oplus\text{NH}_2\text{NH}_2
\]

40 (100 mg, 0.43 mmol) and NBS (85 mg, 0.48 mmol) were dissolved in dry DMF (5 mL) and stirred at 80 °C for 1 h. The solution was then diluted with water (20 mL) and concentrated *in vacuo*. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t: 15.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 58 as an orange solid (108 mg, 81% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 10.58, 8.62, 8.48, 7.40; $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 165.2, 155.9, 155.1, 155.1, 110.7, 110.7; ES TOF MS $m/z$ (M)$^+$ 274; Anal. for C$_6$H$_9$N$_7$OBr: Calc. Mass 274.0052. Found 274.0050.
3-amino-5-(benzylamino)-N-carbamidoylpyrazine-2-carboxamide hydrochloride (59)b,191

28 (100 mg, 0.28 mmol), Pd/C with 10% loading (30 mg) and MgO (15 mg, 0.37 mmol) were combined in MeOH (2 mL). The flask was saturated with H₂ from a balloon, using a 3-way tap connected to a vacuum pump that allowed for several repetitions of evacuation and refilling. The hydrogenation was stirred for 3 days before filtration using a 0.45 micron syringe filter and concentration in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t_r 19.3 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 59 as a light yellow solid (70 mg, 78% after HPLC): mp 192-194 °C; 1H NMR (500 MHz, CD₃OD) δ 7.54 (s, 1H, ArH6), 7.42 (m, 2H, ArH3’ and ArH5’), 7.37 (m, 2H, ArH2’ and ArH6’), 7.31 (m, 1H, ArH4’), 4.71 (s, 2H, Ar-NH-CH₂); 13C NMR (125 MHz, CD₃OD) δ 167.1 (C=O), 156.7 (Guanidine C), 154.2 (ArC5), 154.0 (ArC3), 137.8 (ArC1’), 129.8 (ArC3’ and ArC5’), 129.2 (ArC2’ and ArC6’), 128.8 (ArC4’), 124.7 (ArC6), 111.1 (ArC2), 46.7 (Ar-NH-CH₂); ES TOF MS m/z (M)+ 286; Anal. for C₁₃H₁₆N₇O: Calc. Mass 286.1416. Found 286.1436.
3-amino-N-carbamimidoyl-5-(2-phenylethlamino)pyrazine-2-carboxamide hydrochloride (60)*

33 (100 mg, 0.27 mmol), Pd/C with 10% loading (30 mg) and MgO (15 mg, 0.37 mmol) were combined in MeOH (2 mL). The flask was saturated with H₂ from a balloon, using a 3-way tap connected to a vacuum pump that allowed for several repetitions of evacuation and refilling. The hydrogenation was stirred for 3 days before filtration using a 0.45 micron syringe filter and concentration in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 20.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 60 as a light yellow solid (73 mg, 81% after HPLC): ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.92 (s, 1H, Amide NH), 8.61 (s, 2H, Guanidine NH₂), 8.56 (s, 2H, Guanidine NH₂), 7.39 (s, 1H, ArH₆), 7.26 (m, 4H, ArH2 (ArH3 and ArH6), 7.19 (t, J = 3.5 Hz, 1H, ArH4’), 3.54 (m, 2H, Ar-NH-CH₂), 2.85 (t, J = 6.5 Hz, 2H, NH-CH₂-CH₂); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 166.1 (C=O), 156.4 (Guanidine C), 155.9 (ArC3), 155.2 (ArC5), 139.2 (ArC1’), 128.7 (ArC3’ and ArC5’), 128.3 (ArC2’ and ArC6’), 126.2 (ArC4’), 123.6 (ArC6), 108.8 (ArC2), 41.8 (Ar-NH-CH₂), 34.3 (NH-CH₂-CH₂); ES TOF MS m/z (M)+ 300; Anal. for C₁₄H₁₈N₇O: Calc. Mass 300.1573. Found 300.1565.
methyl 3,5-diamino-6-phenylpyrazine-2-carboxylate (61)\textsuperscript{a}

\[
\begin{align*}
\text{57} & \quad (50 \text{ mg, } 170 \text{ \(\mu\text{mol})} \quad \text{was combined with} \\
& \quad \text{tetrakis(triphenylphosphine)palladium(0) (10 mg, 8.7 \text{ \(\mu\text{mol}}, \\
& \quad \text{phenyltributylstannane (94 mg, 256 \text{ \(\mu\text{mol})} \quad \text{and CuI (32 mg,} \\
& \quad 170 \text{ \(\mu\text{mol}) \quad \text{in dry DMF (5 mL). The stirred solution was heated at 80} \text{ \(^\circ\text{C for 1 h} \\
& \quad \text{before the solvent was removed \textit{in vacuo}. Water (20 mL) was added and the} \\
& \quad \text{solution extracted with EtOAc (3 x 20 mL). The organic extracts were dried over} \\
& \quad \text{MgSO}_4 \quad \text{and concentrated \textit{in vacuo} before being purified by column chromatography} \\
& \quad \text{to yield 61 as a yellow solid (42 mg, 99\%).} \quad \text{\(^1\text{H NMR (500 MHz, (CD}_3\text{)}_2\text{SO) \quad \delta 7.52 (d,} \\
& \quad \text{J} = \text{7.5 Hz, 2H, ArH2' and ArH6'), 7.43 (t,} \quad \text{J} = \text{7.5 Hz, 2H, ArH3' and ArH5'), 7.36 (t,} \\
& \quad \text{J} = \text{7.5 Hz, 1H, ArH4'), 7.04 (bs,} \quad \text{2H, ArNH2), 6.63 (bs,} \quad \text{2H, ArNH2), 3.72 (s,} \\
& \quad \text{3H, COOCH}_3); \quad \text{\(^{13}\text{C NMR (125 MHz, (CD}_3\text{)}_2\text{SO) \quad \delta 166.9 (\text{C=O),} \\
& \quad \text{155.6 (ArC5), 154.1 (ArC3), 137.2 (ArC1'),} \\
& \quad \text{129.7 (ArC6), 128.5 (ArC3' and ArC5'), 128.1 (ArC2' and ArC6'), 127.6 (ArC4'),} \\
& \quad \text{111.2 (ArC2), 51.1 (COOCH}_3); \quad \text{ES TOF MS m/z (M + H)} \quad \text{+ 245}; \quad \text{Anal. for C}_{12}\text{H}_{13}\text{N}_4\text{O}_2:} \\
& \quad \text{Calc. Mass 245.1039. Found 245.1027.}
\end{align*}
\]

methyl 3,5-diamino-6-(phenylethynyl)pyrazine-2-carboxylate (62)\textsuperscript{c,202}

\[
\begin{align*}
\text{57} & \quad (50 \text{ mg, } 170 \text{ \(\mu\text{mol}) \quad \text{was combined with} \\
& \quad \text{Pd(PPh}_3\text{)_2Cl}_2 \\
& \quad (12 \text{ mg, 17 \(\mu\text{mol}) \quad \text{in dry DMF (3 mL) and stirred at 80} \\
& \quad \text{\(^\circ\text{C. In a second flask phenylacetylene (28 \text{ \(\mu\text{L}} \quad \text{255} \\
& \quad \text{\(\mu\text{mol}), CuI (48 mg, 255 \text{ \(\mu\text{mol}) \quad \text{and DIPEA (47 \text{ \(\mu\text{L}}, 272} \\
& \quad \text{\(\mu\text{mol) \quad \text{in dry DMF (2 mL) was stirred at 80} \text{ \(\circ\text{C. After 10 min, the two flasks were}}}
\]

108
combined and the reaction stirred for 1 h before concentration *in vacuo*. The residue was taken up in water (20 mL) and extracted with EtOAc (3 x 20 mL) before the combined organic extracts were dried over MgSO₄ and concentrated *in vacuo*. The residue was purified by column chromatography, yielding 62 as a dark yellow solid (26 mg, 57%): ¹H NMR (500 MHz, (CD₃)₂SO) δ 7.67, 7.41, 7.39, 7.10, 3.75; ¹³C NMR (125 MHz, (CD₃)₂SO) δ 166.1, 156.7, 155.4, 131.4, 128.6, 128.5, 122.2, 112.8, 112.5, 91.5, 85.2, 51.2; ES TOF MS *m/z* (M + H)⁺ 269; Anal. for C₁₄H₁₃N₄O₂: Calc. Mass 269.1039. Found 269.0999.

3,5-diamino-N-carbamimidoyl-6-phenylpyrazine-2-carboxamide hydrochloride (63)b. ²⁰₁

Sodium (7.9 mg, 0.34 mmol) was added to dry ¹PrOH (0.5 mL) and stirred under N₂ gas until oxidation had slowed. Guanidine hydrochloride (32.8 mg, 0.34 mmol) was added and the solution stirred until the sodium was completely dissolved. The resultant sodium chloride was filtered off and the filtrate was combined with 61 (42 mg, 0.17 mmol). The mixture was heated at reflux for 30 min before cooling and concentrating *in vacuo*. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (*t* 19.1 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 63 as a yellow solid (38 mg, 72% after HPLC): ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.54 (s, 1H, Amide NH), 8.77 (s,
2H, Guanidine N\(\text{H}_2\)), 8.42 (s, 2H, Guanidine N\(\text{H}_2\)), 7.65 (d, \(J = 7.5\) Hz; 2H, ArH\(2'\) and ArH\(6'\)), 7.47 (t, \(J = 7.5\) Hz, 2H, ArH\(3'\) and ArH\(5'\)), 7.41 (t, \(J = 7.5\) Hz, 1H, ArH\(4'\)), 7.08 (bs, 4H, 2 x ArN\(\text{H}_2\)); \(^{13}\)C NMR (125 MHz, (CD\(\text{3}\))\(\text{2}\)SO) \(\delta\) 166.1 (C\(=\)O), 155.8 (Guanidine C), 155.5 (ArC\(5\)), 155.1 (ArC\(3\)), 136.0 (ArC\(1'\)), 130.5 (ArC\(6\)), 128.6 (ArC\(3'\) and ArC\(5'\)), 128.5 (ArC\(2'\) and ArC\(6'\)), 128.2 (ArC\(4'\)), 110.1 (ArC\(2\)); ES TOF MS \(m/z\) (M)\(^+\) 272; Anal. for C\(_{12}\)H\(_{14}\)N\(_7\)O: Calc. Mass 272.1260. Found 272.1273.

3,5-diamino-\(\text{N}\)-carbamimidoyl-6-(phenylethynyl)pyrazine-2-carboxamide hydrochloride (64)b,\(^{202}\)

Sodium (4.4 mg, 0.19 mmol) was added to dry \(^{t}\)PrOH (0.5 mL) and stirred under N\(_2\) gas until oxidation had slowed. Guanidine hydrochloride (18.4 mg, 0.19 mmol) was added and the solution stirred until the sodium was completely dissolved. The resultant sodium chloride was filtered off and the filtrate was combined with 62 (26 mg, 0.097 mmol). The mixture was heated at reflux for 30 min before cooling and concentrating \textit{in vacuo}. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (\(t\), 20.8 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 64 as a yellow solid (26 mg, 81% after HPLC): \(^1\)H NMR (500 MHz, (CD\(\text{3}\))\(\text{2}\)SO) \(\delta\) 10.76 (s, 1H, Amide NH), 8.61 (s, 2H, Guanidine N\(\text{H}_2\)), 8.35 (s, 2H, Guanidine N\(\text{H}_2\)), 7.67 (m, 2H, ArH\(3'\) and ArH\(5'\)), 7.56 (m, 1H, ArH\(4'\)), 7.43 (m, 2H, ArH\(2'\) and ArH\(6'\)); \(^{13}\)C NMR (125 MHz, (CD\(\text{3}\))\(\text{2}\)SO) \(\delta\) 166.1 (C\(=\)O), 155.8 (Guanidine C), 155.5 (ArC\(5\)), 155.1 (ArC\(3\)), 136.0 (ArC\(1'\)), 130.5 (ArC\(6\)), 128.6 (ArC\(3'\) and ArC\(5'\)), 128.5 (ArC\(2'\) and ArC\(6'\)), 128.2 (ArC\(4'\)), 110.1 (ArC\(2\)); ES TOF MS \(m/z\) (M)\(^+\) 272; Anal. for C\(_{12}\)H\(_{14}\)N\(_7\)O: Calc. Mass 272.1260. Found 272.1273.
MHz, (CD$_3$)$_2$SO $\delta$ 165.5 (C=O), 157.6 (Guanidine $\equiv$), 155.5 (ArC5), 155.0 (ArC3), 131.8 (ArC2’ and ArC6’), 128.7 (ArC4’), 128.5 (ArC3’ and ArC5’), 121.8 (ArC1’), 113.3 (ArC2), 111.7 (ArC6), 92.8 (Ar-C$\equiv$C-Ar’), 84.5 (Ar-C$\equiv$C-Ar’); ES TOF MS m/z (M)$^+$ 296; Anal. for C$_{14}$H$_{14}$N$_7$O: Calc. Mass 296.1260. Found 296.1230.

methyl 2,3-dichloropyridine-5-carboxylate (65)$^b$

5,6-dichloronicotinic acid (4.22g, 21.98 mmol), Cs$_2$CO$_3$ (21.484g, 65.94 mmol) and iodomethane (4.12 mL, 66.15 mmol) were dissolved in dry DMF (70 mL) and stirred for 24 h. Water (120 mL) was added and the mixture stirred for 5 min before the precipitate was filtered out, washed with water and dissolved in EtOAc (120 mL). The solution was washed with sat. NaHCO$_3$ (2 x 80 mL) and brine (80 mL) before being dried over MgSO$_4$, filtered and concentrated in vacuo to yield 65 as colourless crystals (3.034 g, 67%):

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.84 (s, 1H, ArH2), 8.32 (s, 1H, ArH4), 3.94 (s, 3H, COOC$_3$H$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 164.0 (C=O), 153.4 (ArC6), 148.3 (ArC2), 139.5 (ArC4), 130.9 (ArC5), 126.2 (ArC3), 53.0 (COOC$_3$H$_3$); EI MS m/z (M)$^+$ 204.

methyl 4-amino-5-chloro-2-methoxybenzoate (66)$^c$

4-amino-5-chloro-2-methoxybenzoic acid (200 mg, 0.93 mmol), Cs$_2$CO$_3$ (970 mg, 2.98 mmol) and iodomethane (186 $\mu$L, 2.98 mmol) were stirred at r.t. in DMF (5 mL) for 24 h. The
mixture was diluted with water (20 mL) and stirred for 5 min before extraction with EtOAc (4 x 10 mL). The organic extracts were dried with MgSO₄ and concentrated in vacuo to yield 66 as pale brown crystals (110 mg, 55%): mp 116-118 °C; ¹H NMR (500 MHz, CDCl₃) δ 7.82, 6.29, 4.94, 3.82, 3.82; ¹³C NMR (125 MHz, CDCl₃) δ 165.1, 160.1, 147.8, 133.3, 109.9, 109.6, 98.2, 56.1, 51.6; ES TOF MS m/z (M + H)⁺ 216; Anal. for C₉H₁₁NO₃Cl: Calc. Mass 216.0427. Found 216.0354.

**N-carbamimidoylbenzamide hydrochloride (67)**

Sodium (250 mg, 10.88 mmol) was added to dry iPrOH (20 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (1.04 g, 10.88 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with methyl benzoate (1.697 mL, 13.64 mmol). The mixture was heated at reflux for 18 h before concentrating in vacuo. The residue was taken up in 1 M HCl (25 mL) and extracted with EtOAc (2 x 25 mL). The aqueous extract was filtered and 5 M NaOH (15 mL) was added before extraction with EtOAc (3 x 25 mL). The combined EtOAc extracts from the basic solution were concentrated in vacuo, yielding 67 as colourless crystals (2.424 g, 89%). A small sample of the product (50 mg) was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (tᵣ, 22.4 min). The collected fractions containing purified product were concentrated by freeze-
drying to yield the hydrochloride salt for testing and characterisation: mp 206-208 °C; \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 12.12 (s, 1H, Amide NH), 8.83 (s, 2H, Guanidine NH\(_2\)), 8.63 (s, 2H, Guanidine NH\(_2\)), 8.16 (d, \(J = 7.0\) Hz, 2H, ArH2 and ArH6), 7.71 (t, \(J = 6.5\) Hz, 1H, ArH4), 7.58 (d, \(J = 7.0\) Hz, 2H, ArH3 and ArH5); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 167.4 (Amide C=O), 155.6 (Guanidine C), 133.9 (ArC4), 131.0 (ArC1), 128.8 (ArC3 and ArC5), 128.4 (ArC2 and ArC6); ES TOF MS \(m/z\) (M)\(^+\) 164; Anal. for C\(_8\)H\(_{10}\)N\(_3\)O: Calc. Mass 164.0824. Found 164.0821.

**\(N\)-carbamimidoylpyridine-3-carboxamide hydrochloride (68)**

Sodium (335 mg, 14.57 mmol) was added to dry iPrOH (21 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (1.392 g, 14.57 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with methyl picolinate (1 g, 7.29 mmol). The mixture was heated at reflux for 20 min before concentration *in vacuo*. The residue was triturated with 1 M HCl in Et\(_2\)O (10 mL) and recrystallised from water/MeOH to yield 68 as light yellow crystals (614 mg, 42%). A small sample of the product (50 mg) was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 5.9 min). The collected fractions containing purified product were concentrated by freeze-drying to yield the hydrochloride salt for testing and characterisation: mp 256-258 °C; \(^1\)H NMR (500
MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 12.70 (bs, 1H, Amide NH), 9.37 (s, 1H, ArH2), 8.96 (d, J = 4.5 Hz, 1H, ArH6), 8.83 (s, 2H, Guanidine NH<sub>2</sub>), 8.79 (d, J = 7.5, 1H, ArH4), 8.79 (s, 2H, Guanidine NH<sub>2</sub>), 7.85 (t, J = 7.0 Hz, 1H, ArH5); <sup>13</sup>C NMR (125 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 165.3 (C=O), 155.5 (Guanidine C), 150.9 (ArC6), 146.9 (ArC2), 139.5 (ArC4), 128.6 (ArC3), 125.2 (ArC5); ES TOF MS m/z (M)<sup>+</sup> 165; Anal. for C<sub>7</sub>H<sub>9</sub>N<sub>4</sub>: Calc. Mass 165.0776. Found 165.0805.

**N-carbamimidoylpyridine-2-carboxamide hydrochloride (69)**

Sodium (335 mg, 14.57 mmol) was added to dry iPrOH (21 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (1.392 g, 14.57 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with methyl nicotinate (1 g, 7.29 mmol). The mixture was heated at reflux for 20 min before cooling to 10 °C. The crystals that formed were tritutated with 1 M HCl in Et<sub>2</sub>O (10 mL) and recrystallised from Et<sub>2</sub>O/MeOH to yield 69 as colourless crystals (395 mg, 27%). A small sample of the product (50 mg) was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 9.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield the hydrochloride salt for testing and characterisation: mp 246-248 °C (dec); <sup>1</sup>H NMR (500 MHz, (CD<sub>3</sub>)<sub>2</sub>S0) δ 11.73 (s, 1H, Amide NH), 9.00 (s, 2H, Guanidine NH<sub>2</sub>), 8.86 (s, 2H, Guanidine NH<sub>2</sub>),
$8.75 \ (d, \ J = 4.5 \ Hz, \ 1H, \ ArH6), \ 8.15 \ (d, \ J = 7.5 \ Hz, \ 1H, \ ArH3), \ 8.12 \ (t, \ J = 7.5 \ Hz, \ 1H, \ ArH4), \ 7.78 \ (t, \ J = 6.0 \ Hz, \ 1H, \ ArH5); \ ^{13}C \ NMR \ (125 \ MHz, \ (CD_{3})_{2}SO) \ \delta \ 165.2 \ (C=O), \ 155.2 \ (Guanidine \ C), \ 148.9 \ (ArC6), \ 146.7 \ (ArC2), \ 138.6 \ (ArC4), \ 128.8 \ (ArC5), \ 123.4 \ (ArC3); \ ES \ TOF \ MS \ m/z \ (M)^{+} \ 165; \ Anal. \ for \ C_{7}H_{9}N_{4}: \ Calc. \ Mass \ 165.0776. \ Found \ 165.0784.$

$N$-carbamimidoylpyrazine-2-carboxamide hydrochloride (70)$^{b}$

\[ \text{Sodium (70 mg, 3.04 mmol) was added to dry } \text{iPrOH (5 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (290 mg, 3.04 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with methyl 2-pyrazinecarboxylate (200 mg, 1.45 mmol). The mixture was heated at reflux for 1 h before concentration in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 10.4 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 70 as a white solid (164 mg, 56% after HPLC): \(^{1}H \ NMR \ (500 \ MHz, \ (CD_{3})_{2}SO) \ \delta \ 11.75 \ (s, \ 1H, \ Amide \ NH), \ 9.32 \ (s, \ 1H, \ ArH3), \ 9.03 \ (s, \ 1H, \ ArH6), \ 8.85 \ (s, \ 1H, \ ArH5), \ 8.85 \ (s, \ 2H, \ Guanidine \ NH_{2}), \ 8.74 \ (s, \ 2H, \ Guanidine \ NH_{2}); \ ^{13}C \ NMR \ (125 \ MHz, \ (CD_{3})_{2}SO) \ \delta \ 164.5 \ (C=O), \ 154.9 \ (Guanidine \ C), \ 144.9 \ (ArC5), \ 144.6 \ (ArC2), \ 143.6 \ (ArC6), \ 142.2 \ (ArC3); \ ES \ TOF \ MS \ m/z \ (M)^{+} \ 166; \ Anal. \ for \ C_{6}H_{8}N_{5}O: \ Calc. \ Mass \ 166.0729. \ Found \ 166.0737. \]
**N-carbamimidoyl-2,3-dichloropyridine-5-carboxamide hydrochloride (71)**

Sodium (700 mg, 30.57 mmol) was added to dry iPrOH (50 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (2.9 g, 30.36 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with 65 (3.132 g, 15.18 mmol). The mixture was heated at reflux for 20 min before cooling to 10 °C. The solid that formed was collected and recrystallised from Et₂O/MeOH, yielding 71 as a white solid (1.105 g, 27%). A small sample of the product (50 mg) was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 15.4 min). The collected fractions containing purified product were concentrated by freeze-drying to yield the hydrochloride salt for testing and characterisation: ¹H NMR (500 MHz, (CD₃)₂SO) δ 8.87 (s, 1H, ArH₂), 8.45 (s, 1H, ArH₄), 8.00 (bs, 4H, 2 x Guanidine NH₂); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 171.5 (C=O), 162.9 (Guanidine C), 149.0 (ArC₆), 147.9 (ArC₂), 138.8 (ArC₄), 135.2 (ArC₅), 128.7 (ArC₁); ES TOF MS m/z (M)+ 232; Anal. for C₇H₇N₄OCl₂: Calc. Mass 232.9997. Found 232.9996.

**4-amino-N-carbamimidoyl-5-chloro-2-methoxybenzamide hydrochloride (72)**

Sodium (11 mg, 0.46 mmol) was added to dry iPrOH (1 mL) and stirred under N₂ gas until oxidation had slowed. Guanidine hydrochloride (44 mg, 0.45 mmol) was added and the solution stirred until the sodium was completely dissolved. The resultant
sodium chloride was filtered off and the filtrate was combined with 66 (50 mg, 0.23 mmol). The mixture was heated at reflux for 2 h before cooling and concentrating in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 19.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 72 as a light yellow solid (40 mg, 62% after HPLC): $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 10.48 (s, 1H, Amide NH), 9.17 (bs, 2H, Guanidine NH$_2$), 8.53 (bs, 2H, Guanidine NH$_2$), 7.70 (s, 1H, ArH6), 6.54 (s, 1H, ArH3), 4.52 (bs, Ar-NH$_2$), 3.94 (s, 3H, Ar-OCH$_3$); $^{13}$C NMR (75 MHz, (CD$_3$)$_2$SO) $\delta$ 164.5 (C=O), 158.5 (ArC2), 155.0 (Guanidine C), 151.5 (ArC4), 132.3 (ArC6), 109.8 (ArC5), 106.2 (ArC1), 96.8 (ArC3), 56.5 (Ar-OCH$_3$); ES TOF MS m/z (M)$^+$ 243; Anal. for C$_9$H$_{12}$N$_4$O$_2$Cl: Calc. Mass 243.0649. Found 243.0595.

*N-carbamimidoylimidazole-4-carboxamide hydrochloride (73)*

Sodium (38 mg, 1.66 mmol) was added to dry iPrOH (2 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (159 mg, 1.66 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with methyl 4-imidazolecarboxylate (100 mg, 0.79 mmol). The mixture was heated at reflux for 1 h before concentration in vacuo. The residue
was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 3.2 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 73 as white crystals (139 mg, 93% after HPLC): $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.02 (s, 1H, ArH5), 7.80 (s, 1H, ArH2); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 162.5 (Amide C=O), 155.4 (Guanidine C), 137.2 (ArC4), 132.7 (ArC2), 124.3 (ArC5); ES TOF MS $m/z$ (M)$^+$ 154; Anal. for C$_5$H$_8$N$_5$O: Calc. Mass 154.0729. Found 154.0746.

3-({3-amino-1,2,4-oxadiazol-5-yl}-5-chloropyrazine-2,6-diamine (74)$^c$

Sodium (46 mg, 2.01 mmol) was added to a stirred solution of 77 (250 mg, 2.01 mmol) in dry EtOH (5 mL) under N$_2$ gas. Methyl 3,5-diamino-6-chloropyrazine-2-carboxylate (405 mg, 2.00 mmol) was added to the solution and the mixture heated to reflux for 4 h. After cooling, the solid products were collected and taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 18.5 min). The collected fractions containing purified product produced crystals that were collected and further purified by preparative TLC to yield 74 as light yellow crystals (178 mg, 39%): mp 188-190 °C (dec); $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) $\delta$ 7.28, 6.25; $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 170.3, 167.7, 153.2, 152.7, 120.0, 107.1; El MS $m/z$ (M)$^+$ 227; Anal. for C$_6$H$_6$N$_7$OCl: Calc. Mass 227.032236. Found 227.032248.
3,5-diamino-6-chloropyrazine-2-carboxyrazide hydrochloride (75)\textsuperscript{b,205}

\[
\begin{array}{c}
\text{Cl} \quad \text{Cl} \\
\text{N} \quad \text{N} \\
\text{N} \quad \text{N} \\
\text{H}_2\text{N} \quad \text{H}_2\text{N} \\
\text{O} \\
\end{array}
\]

methyl 3,5-diamino-6-chloropyrazine-2-carboxylate (100 mg, 0.49 mmol) and hydrazine hydrate solution (113 µL, 1 mmol) were heated at reflux in EtOH (2 mL) for 20 h before the solvent was removed in vacuo. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 7.3 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 75 as a white powder (95 mg, 81% after HPLC): \textsuperscript{1}H NMR (300 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta\) 10.44 (s, 1H, Amide NH), 6.45 (bs, 2 x Ar-NH\textsubscript{2}, CONH-NH\textsubscript{3}\textsuperscript{+}); \textsuperscript{13}C NMR (75 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta\) 164.9 (Amide C=O), 154.7 (ArC5), 153.5 (ArC3), 118.7 (ArC6), 109.7 (ArC2); ES TOF MS m/z (M)\textsuperscript{+} 203; Anal. for C\textsubscript{5}H\textsubscript{8}N\textsubscript{6}OCl: Calc. Mass 203.0448. Found 203.0437.

2-methylisothiouuronium sulfate (76)\textsuperscript{c}

\[
\begin{array}{c}
\text{N} \\
\text{S} \\
\text{H}_2\text{N} \\
1/2 \text{SO}_4^2- \\
\end{array}
\]

A solution of thiourea (1g, 13.1 mmol) in water (1 mL) was stirred at r.t. as dimethyl sulfate (907 mg, 7.2 mmol) was added from a dropping funnel over a period of 1.5 h. The temperature was then raised to reflux for 5 h before the reaction was cooled and \textsuperscript{1}PrOH (1.3 mL) added. The mixture was stirred for 1 h before the solid product was collected and washed with a mixture of water (3 mL) and \textsuperscript{1}PrOH (6 mL). The filtrate was dried in vacuo to yield 76 as white crystals (620 mg, 52%).
hydroxyguanidinium sulfate (77)\textsuperscript{c,206}

\[
\text{Ba(OH)}_2\cdot8\text{H}_2\text{O} \ (1.62 \text{ g, 5.14 mmol}) \text{ was dissolved in boiling water (10 mL), then chilled in an ice bath. Hydroxylammonium sulfate (840 mg, 10.2 mmol) was added, followed by 76 (950 mg, 6.83 mmol) and the solution stirred for 24 h before filtration. The filtrate was combined with acetone until crystals began to form. The crystals were collected and dried \textit{in vacuo} to yield 77 as colourless crystals (312 mg, 60\%): EI MS m/z (M)\textsuperscript{+} 75; Anal. for CH\textsubscript{5}N\textsubscript{3}O: Calc. Mass 75.043262. Found 75.042619.}
\]

methyl 3-amino-6-chloropyrazinecarboxylate (79)\textsuperscript{b,197}

methyl 3-aminopyrazine-2-carboxylate (1 g, 6.53 mmol) and NCS (0.95 g, 7.11 mmol) were dissolved in dry DMF (10 mL) and heated at 80 °C for 1 h before the reaction mixture was diluted with water (40 mL) and concentrated \textit{in vacuo}. The residue was purified by column chromatography in a 10 mm diameter column with a silica height of 150 mm. The eluent used was a mixture of petroleum spirit (B.R. 40-60 °C) and EtOAc. The fraction containing the desired product was isolated and concentrated \textit{in vacuo} to yield 79 as light yellow crystals (882 mg, 72\%): mp 144-146 °C; \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ 8.20 (s, 1H, ArH5), 3.96 (s, 3H, COOCH\textsubscript{3}); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) δ 166.0 (C=O), 154.4 (ArC3), 147.5 (ArC5), 135.2 (ArC6), 122.1 (ArC2), 52.9 (COOCH\textsubscript{3}); EI MS m/z (M)\textsuperscript{+} 187; Anal. for C\textsubscript{6}H\textsubscript{6}N\textsubscript{3}O\textsubscript{2}Cl: Calc. Mass 187.014854. Found 187.013961.
Sodium (290 mg, 12.6 mmol) was added to dry $^1$PrOH (20 mL) and stirred with gentle heating to allow for dissolution of the metal. Guanidine hydrochloride (1.204 g, 12.6 mmol) was added when hydrogen evolution had slowed and the solution was stirred until all sodium had reacted. The insoluble sodium chloride was filtered out of the solution and the filtrate combined with 79 (1.125 g, 6.00 mmol). The mixture was heated at reflux for 1 h before being left to cool and crystallise. The collected crystals were taken up in DMSO (10 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins ($t$, 13.8 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 80 as yellow crystals (1.311 g, 87% after HPLC): mp 216-218 °C (dec); $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 11.21 (s, 1H, Amide NH), 8.88 (s, 2H, Guanidine NH$_2$), 8.74 (s, 2H, Guanidine NH$_2$), 8.49 (s, 1H, ArH5), 7.79 (bs, 2H, ArNH$_2$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 165.9 (C=O), 154.9 (Guanidine C), 154.5 (ArC3), 149.5 (ArC5), 132.0 (ArC6), 120.2 (ArC2); ES TOF MS $m/z$ (M)$^+$ 215; Anal. for C$_6$H$_8$N$_6$OCl: Calc. Mass 215.0448. Found 215.0438.
5-amino-6-bromo-N-carbamimidoylpyrazine-2-carboxamide hydrochloride (82) 

Sodium (10 mg, 0.45 mmol) was added to dry \textsuperscript{1}PrOH (1 mL) and stirred under \textsubscript{2}N gas until oxidation had slowed. Guanidine hydrochloride (43 mg, 0.45 mmol) was added and the solution stirred until the sodium was completely dissolved. The resultant sodium chloride was filtered off and the filtrate was combined with methyl 5-amino-6-bromopyrazine-2-carboxylate (50 mg, 0.22 mmol). The mixture was heated at reflux for 2 h before cooling and concentrating \textit{in vacuo}. The residue was taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0\% to 100\% B over 30 mins (\textit{t}, 15.6 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 82 as a light yellow solid (18 mg, 28\% after HPLC): \textsuperscript{1}H NMR (500 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta\) 11.05 (s, 1H, Amide NH), 8.77 (s, 2H, Guanidine NH\textsubscript{2}), 8.72 (s, 2H, Guanidine NH\textsubscript{2}), 8.66 (s, 1H, ArH3); \textsuperscript{13}C NMR (125 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta\) 163.5 (C=O), 156.3 (Guanidine C), 155.2 (ArC5), 144.2 (ArC3), 129.9 (ArC1), 123.2 (ArC6); ES TOF MS \(m/z\) (\(M^+\)) 258; Anal. for C\textsubscript{6}H\textsubscript{8}N\textsubscript{6}OBr: Calc. Mass 258.9943. Found 258.9913.

4.3 Biological Testing General Notes

UV-Vis absorption data for 96-well assay plates was recorded using a Molecular Devices Spectramax Plus 384 plate reader. (Software: Softmax Pro version 1.17, 2004). Amiloride hydrochloride was purchased from Oakwood Products.
4.4 Enzyme Assays

Urokinase inhibition assays were performed at 37 °C using HMW-uPA at a final assay concentration of 5 nM in a buffer containing 50 mM Tris/200 nM aprotinin /0.01% Tween-20, pH 8.8. Spectrozyme uPA was added to a final concentration of 0.125 mM. Serial dilutions of inhibitors were set up in 96-well plates prior to addition of Spectrozyme uPA and HMW-uPA. The plates were analysed with a Spectramax Plus 384 Molecular Devices plate reader for colour development at 405 nM. The IC\textsubscript{50} values were obtained by conducting a log transformation of the inhibitor concentrations. Data was normalised to a common scale, where 100% activity was equal to maximal uPA activity, indicated by the control samples containing HMW-uPA and substrate, but no inhibitor. Values were calculated from logarithmic sigmoidal dose response curves using the variable slope parameter, generated from GraphPad Prism V. 5.01 software (GraphPad Software Inc.).
4.5 References


63. Ranson, M.; Andronicos, N. M. Plasminogen Binding and Cancer; Promises and Pitfalls. *Front. Biosci.* 2003, 8, s294-304.


112. Towle, M. J.; Lee, A.; Maduakor, E. C.; Schwartz, E.; Bridges, A. J.; Littlefield, B. A. Inhibition of Urokinase by 4-substituted Benzo[b]thiophene-2-


Micromedex® Healthcare Series, Drugdex® Evaluations, Amiloride (Online); Thomson Reuters; 2009.


lishi, H.; Tatsuta, M.; Baba, M.; Yano, H.; Uehara, H.; Nakaizumi A. Suppression by amiloride of bombesin-enhanced peritoneal metastasis of


169. Chen, Y.-X.; O’Brien, E. R. Ethyl isopropyl amiloride inhibits smooth muscle cell proliferation and migration by inducing apoptosis and antagonising


204. Japanese Pat. 41003742 B4, **1966**.


Section 2:

Synthesis and Biological Evaluation of

Urokinase Plasminogen Activator

Receptor (uPAR) Antagonists
Chapter 5: Introduction

5.1 The Urokinase Plasminogen Activator Receptor (uPAR) in Cancer

uPAR is an integral member of the plasminogen activation system (Chapter 1.4) which, in addition its role in matrix proteolysis, is involved in a complex interplay of extracellular interactions, acting as both an indirect signal transducer and an external adhesion receptor. Three internally repeated sequence motifs form three homologous domains in uPAR. The three repeats show surprisingly little sequence identity (<20%) but are recognisable through the repeating disulfide bond architecture and a conserved asparagine immediately preceding the C-terminal cysteine of each domain. Several other single-domain proteins incorporate the consensus sequence of the individual uPAR domains, including human glycosylphosphatidylinositol (GPI)-anchored proteins (e.g. Ly-6 antigens), human transmembrane proteins (e.g. TGF-β receptors) and the secreted snake α-neurotoxins α-bungarotoxin (Banded Krait) and Toxin α (Spitting Cobra).

Five potential sites have been identified for N-linked glycosylation in human uPAR, although only the first four sites actually carry heterogeneous N-linked carbohydrates. The linker region between domains I and II is susceptible to proteolytic cleavage by a number of proteases, including uPA and trypsin. Removal of Domain I by proteolysis abolishes the binding affinity of the receptor for uPA and exposes a chemotactic sequence that binds transmembrane receptor
partners (discussed below). uPAR is not a transmembrane protein itself but contains a GPI unit anchoring it to the plasma membrane, generally in caveolin-dominated membrane rafts.\textsuperscript{215,216} The receptor thus lacks a cytosolic domain and must recruit integral membrane protein partners to invoke intracellular signaling responses. These responses are typically mediated through the mitogen-activated protein kinase and JAK/STAT signal transduction pathways.\textsuperscript{217-219}

A signal transduction pathway that has been extensively characterised involves the interaction between uPAR and the G-protein-coupled lipoxin A4 receptor (LXA4R).\textsuperscript{220} Binding of uPA induces a conformational change in uPAR which exposes an epitope that binds LXA4R with high affinity. Cleavage of the linker between domains 1 and 2 in uPAR by plasmin, MMP-12 or uPA mimics uPA binding as it also exposes the epitope and stimulates binding to LXA4R.\textsuperscript{214,221} Binding of LXA4R to uPAR induces a conformational change in LXA4R’s associated G-protein that activates p56/p59\textsuperscript{hck} tyrosine kinase, which provides uPAR/uPA-expressing cells with a proliferative advantage.\textsuperscript{222-224}
Figure 5.1. Schematic representation of mature uPAR showing primary structure and folding topology of individual domains. Disulfide connections are denoted by black linkers; diamonds denote sites of N-linked glycan attachment. Linker regions sensitive to proteolysis are marked with arrows (Tryp = Trypsin cleavage site, Chym = Chymotrypsin cleavage site, PI-PLC = glycosylphosphatidylinositol specific phospholipase C). Other protease cleavage sites (Pepsin, uPA, MMP-12) are indicated.

Another signaling response involves the interaction of uPAR with L-selectins, which occurs via L-selectin’s carbohydrate binding domain. The resulting functional signaling complexes upregulate phosphoinositide hydrolysis and mobilise intracellular Ca\(^{2+}\). Association of uPAR with L-selectin may influence rolling adhesion of circulating tumour cells to the vascular endothelium which is a requirement for extravasation in metastasis (see Figure 1.2).
The role of uPAR as a binding partner for integrins is complex and intimately linked to tumour progression.\textsuperscript{49,50,226} Several subtypes of integrins act in concert with uPAR and PAI-1 to regulate adhesion/detachment and signaling events in cellular migration. In particular, expression of the vitronectin-binding $\alpha v\beta 3$ integrin is closely coupled with uPAR expression in the coordination of adhesion-proteolysis cycles.\textsuperscript{227-229} Interaction of uPAR with $\alpha v\beta 3$ and another vitronectin binding integrin $\alpha v\beta 5$ appears to be uPA dependent, with uPA-uPAR co-localising with integrins in a vitronectin binding complex.\textsuperscript{230} The pro-migratory response to integrin-vitronectin binding appears to be governed by both the presence of uPAR and the conformational state of vitronectin, with different signaling pathways activated when $\alpha v\beta 5$ binds vitronectin unaccompanied.\textsuperscript{231-234} While uPAR is able to bind vitronectin unpartnered, binding is significantly enhanced through prior binding to uPA. Additionally, the uPAR and vitronectin-binding $\alpha_v$ integrins may be recruited, generating a multi-member binding complex.\textsuperscript{235,236}

PAI-1 appears to indirectly regulate the uPAR-vitronectin interaction. The uPA-PAI-1 complex makes a high-affinity interaction with the low density lipoprotein receptor (LDLR), which promotes endocytosis and receptor complex clearance and recycling.\textsuperscript{237} The cleared receptor is then unable to interact with vitronectin. The interaction with members of the LDLR family also enhances mitogenic signaling responses and subsequent cell proliferation.\textsuperscript{238} PAI-1 binds to the same amino-terminal somatomedin B (SMB) domain of vitronectin targeted by uPAR and may
directly antagonise the uPAR-vitronectin interaction.\textsuperscript{239} By binding the SMB domain of vitronectin, PAI-1 also hinders access to the adjacent RGD sequence on vitronectin that binds $\alpha_v$ integrins. The regulated shift in uPA:PAI-1 ratio is therefore thought to govern the migration of cells on vitronectin.\textsuperscript{240}

Integrin-mediated uPAR signal transduction occurs \textit{via} a complex interplay between uPAR, EGFR, $\alpha_5\beta_1$ integrin and fibronectin.\textsuperscript{209,241} Upregulation of uPAR-bound uPA enhances association of the complex with $\alpha_5\beta_1$ integrin, which in turn activates the integrin to bind fibronectin. Associated EGFR is then activated \textit{via} phosphorylation in a FAK-dependent ERK activation complex.\textsuperscript{217}

The $\alpha_M\beta_2$ (Mac-1) integrin expressed in leukocytes binds both uPAR and uPA at separate sites on the $\alpha_M$ subunit. It has been demonstrated that functional interaction of uPAR and Mac-1 significantly promotes leukocyte adhesion to fibrinogen and co-adhesion with vitronectin.\textsuperscript{242} Binding of uPA to uPAR considerably loosens the Mac-1-fibrinogen interaction due to direct competition for a shared Mac-1 binding site.\textsuperscript{243}
Figure 5.2. Summary of the major non-proteolytic functions of uPAR. Interaction of uPAR with integrins can activate intracellular effectors FAK and ERK. Complexes involving occupied or unoccupied uPAR, vitronectin and transmembrane proteins such as LXA4R or LDLRs can activate Rac to stimulate changes in cell morphology. Both pathways can lead to altered adhesion, migration and proliferation. SMB = somatomedin B domain, CD = central domain, HBD = heparin binding domain, G = growth factor domain, K = kringle domain, SP = serine protease domain. The three domains of uPAR are labelled 1, 2 and 3. I domains in the integrin α and β subunits are labelled (α-1 and β-1), P = 7-bladed β-propeller region. Adapted from Reference 80.

5.2 Antitumour/Antimetastasis Effects of uPAR Antagonists

The role of uPAR in multiple pathways implicated in cell adhesion, migration and tumour progression makes it a promising target for antitumour/antimetastasis therapeutics, and several anti-uPAR strategies have been explored.244 Adenovirus-mediated delivery of antisense uPAR was shown to reduce receptor expression and cause regression or reduction in the growth of glioma and non small-cell lung
cancers in mouse models.\textsuperscript{245-247} Transfection of tumour cells with antisense uPAR oligonucleotides \textit{in vitro} prior to inoculation in mice was shown to inhibit metastasis of epidermoid carcinoma, colon cancer, melanoma and prostate cancer.\textsuperscript{248-251} A third gene-silencing technique using siRNA to interfere with uPAR mRNA in a sequence-specific manner was shown to reduce metastasis of glioma cells in mice.\textsuperscript{252}

Another anti-uPAR strategy that has been explored is the use of antagonists of the interactions between uPAR and uPA, or between uPAR and its other binding partners. Antagonists that have been investigated include antibodies, peptides and small molecules. A polyclonal IgG against uPAR was shown to reduce metastasis and elicit necrotic tumour regression of mammary adenocarcinoma cells in a rat model.\textsuperscript{253} Antibodies identified by fragment antigen binding (Fab) phage display as antagonists of uPAR/uPA or uPAR/\(\beta1\) integrin binding were shown to significantly inhibit uPAR-mediated signal transduction and cell migration.\textsuperscript{254} The antibodies were also effective against a lung cancer cell line.
Figure 5.3. Structure of pro-uPA (prior to activation by cleavage of the Lys158-Ile159 peptide bond to provide the two-chain HMW-uPA). Highlighted are the Serine Protease Domain (Grey), Kringle Domain (Light Blue) and EGF-like Domain (Magenta). The combined Kringle and EGF-like domains constitute the uPA amino-terminal fragment (ATF). The connecting peptide region of the serine protease domain is shown in Green. Cysteine residues involved in disulfide linkages are indicated in Red, and the catalytic residues are highlighted in Blue. The uPAR-binding ω-loop lies between the indicated Cys19 and Cys31 residues.

Peptide fragments derived from the uPAR-binding regions of uPA (see Figure 5.3 for description of uPA structure) have been shown to be effective inhibitors of metastasis, with both inoculation and expression via transfection producing in vivo efficacy in mouse tumour models. The amino-terminal fragment (ATF) of uPA, which consists of the noncatalytic kringle and EGF-like domains, is responsible for the majority of interactions between uPA and uPAR. Chimeric IgG-ATF and IgG-EGF-
like domain have been shown to be effective uPAR antagonists that suppress metastasis and primary tumour establishment of melanoma and colon carcinomas in mouse models.\textsuperscript{255,256} Substitution of a nonessential loop of the cysteine protease inhibitor cystatin with residues 19-31 of the uPA EGF-like domain afforded a bifunctional inhibitor of both uPAR-uPA binding and cathepsin activity \textit{in vitro}.\textsuperscript{257} Adenovirus-mediated delivery or cDNA transfection of cancer cells such that they express the ATF suppresses neovascularisation and metastasis of breast tumours, lung carcinomas and glioblastomas in mouse models.\textsuperscript{258-261}

A few short peptide uPAR antagonists have been described with the most well studied being peptides that mimic the uPAR-binding ω-loop (residues 19-31) of the uPA EGF-like domain (Figure 5.4).\textsuperscript{80,244,262} Some of these cyclic peptides were developed using affinity maturation techniques leading to very potent antagonists with IC\textsubscript{50}'s as low as 40 nM.\textsuperscript{263,264} Related truncated cyclopeptides with similar potency containing a norleucine at residue 23 and a D-Cys at residue 21 were developed to resist proteolytic cleavage.\textsuperscript{265} These compounds have been shown to reduce growth and metastasis of human ovary adenocarcinomas in mouse models.\textsuperscript{266} A capped octapeptide known as Å6 was developed as an antagonist of a secondary uPAR-binding sequence of uPA that encompasses residues 136-143 of the serine protease domain, a motif known as the connecting peptide region. The peptide inhibits matrigel invasion of breast cancer cells with IC\textsubscript{50} values between 5 and 25 μM and inhibits metastasis of breast cancer or glioblastoma cells in mouse
models. The A6 peptide may also block the interaction of uPAR with partner transmembrane proteins as its sequence is derived from the serine protease domain of uPA recognised as an integrin binding site.

Figure 5.4. **Left:** Amino acid sequence of the uPAR-binding ω-loop (residues 19-31) of the uPA EGF-like domain. **Right:** Cyclic peptide antagonist derived from the ω-loop which incorporates a disulfide between cysteines 19 and 31. Light circles denote residues essential to uPAR binding, bold circles denote nonessential residues. Adapted from Reference 263.

A series of uPAR antagonist peptides that bear considerably less sequence correspondence with uPA were identified using bacteriophage peptide display and affinity maturation by solid-phase peptide combinatorial chemistry. The study led to the discovery of AE105 (D-Cha-F-s-r-Y-L-W-S, IC$_{50}$ = 20 nM; Cha: L-β-cyclohexylalanine). AE105 inhibits intravasation of epidermoid carcinoma cells in chorioallantoic membrane assays and a Cu$^{64}$-labelled version has been used
successfully for PET imaging of glioblastoma cells in mice.\textsuperscript{272} Peptides that mimic the uPAR chemotactic sequence S\textsuperscript{88}-R-S-R-Y\textsuperscript{92}, (e.g. pyro-Glu-R-E-R-Y-NH\textsubscript{2}) do not antagonise uPA binding but instead block uPAR interactions with its membrane partner proteins to inhibit downstream kinase activation.\textsuperscript{273}

A very limited number of small-molecule non-peptidic uPAR antagonists have been reported in patents only. The compounds show varying structural features but generally they contain several aromatic rings which presumably interact with the hydrophobic residues of uPAR’s uPA binding site (See Chapter 5.3).\textsuperscript{274} Peptidomimetics such as the substituted aminobiphenylcarboxylic acid derivative \textbf{83} (IC\textsubscript{50} 0.8 nM) and \textbf{84} (CHIR11509, IC\textsubscript{50} 33 nM) are particularly potent antagonists.\textsuperscript{275-277}

\begin{center}
\textbf{Figure 5.5.} Examples of reported non-peptidic small-molecule antagonists of the uPA-uPAR interaction.
\end{center}
Some porphyrins obtained from algal extracts have been identified as uPAR antagonists, as has mesoporphyrin IX (85), which antagonises uPA binding with an IC₅₀ of 0.23 μg.mL⁻¹ (376 nM).²⁷⁸ Hydroxycumarane derivatives related to 86 were initially developed as antihistaminic agents by F. Hoffmann-La Roche AG but were later found to be uPAR antagonists.²⁷⁹ The initial series of O-substituted hydroxycumaranes showed IC₅₀ values as low as 91 nM. A subsequent series gave IC₅₀ values as low as 80 nM (e.g. 86).²⁷⁹,²⁸⁰ Oligothiophene antagonists (e.g. 87) with IC₅₀ values of 2 μM have been reported.²⁸¹

5.3 Binding Interactions Between uPAR and Ligands

Understanding the nature of the binding interaction between uPA and uPAR is useful for developing uPAR antagonists. The important binding residues on both uPA and uPAR have been identified using several methods, including X-ray crystallography, alanine scanning mutagenesis and photoaffinity labeling.²⁸² Domain I, which forms the amino-terminus of uPAR, contains the majority of residues required for uPA binding, but other essential residues are also located in domains II and III.²¹¹,²⁸³ The uPA-binding residues of uPAR reside in a deep conical cavity (25 Å across and 14 Å deep) bounded by the three domains.²⁸⁴,²⁸⁵ The residues located in the small ω-loop of the uPA growth factor domain between Cys19 and Cys31 make strong interactions within this cavity.
Mutational analyses of the ATF have been used to identify residues that contribute to uPAR binding affinity. Systematic alanine-scanning of residues 20-30 of the ATF identified Lys23, Tyr24, Phe25, Ile28 and Trp30 as key binding determinants. Alanine-scanning mutagenesis of uPAR revealed that clusters of residues in all domains contribute to uPA binding. Alanine-scanning of domain I and surface plasmon resonance measurements revealed that Arg53, Leu55, Tyr57 and Leu66 are important for uPA-binding. Analysis of domain II identified a continuous sequence from Arg137-Arg145 as important contributors. A comprehensive scan of all three domains confirmed these observations and additionally identified Arg25, Thr27 and Thr51 in domain I and Asp102, Thr127, Asp140, Leu150 and Leu168 in domain II as important. No uPA-binding residues were identified in domain III. This may be because domain III acts to stabilise uPAR’s structure rather than bind to uPA.

Co-crystal structures of uPAR in complex with both uPA and antagonists have provided detailed information regarding interactions at the uPAR/ligand interface (Figure 5.6). Co-crystallisation of the ATF with soluble uPAR (and uPAR antibody ATN615) showed hydrogen bonds are formed between Thr8, Arg53, Glu68, Thr127 and His166 of the uPAR internal cavity and Ser21, Lys23, Tyr24, Ser26 and Gln40 of the ATF. Tyr24 of the ATF makes contacts with Arg53, His166 and Asp254 of uPAR domains I, II and III respectively. A hydrophobic interface occurs toward the cavity entrance between residues Phe25, Ile28 and Trp30 of the ATF ω-loop and Val29, Leu31, Leu40, Leu55 and Leu66 within β-strands 3, 5 and 6 of uPAR domain I.
A third contact interface occurs between the kringle domain of the ATF and domain I of uPAR.

Figure 5.6. Crystal structures of uPAR in complex with ATF (A, B) or peptide antagonist AE147 (C, D). Panel A: molecular surface representation of three domains of uPAR shows a slight constriction to accommodate the ATF (Cyan ribbon diagram). Panel B: close-up of the surface interactions between key residues of the kringle (H87) and growth factor (all other labelled residues) domains of uPA with the three domains of uPAR. Nitrogen atoms are blue, oxygens red and sulfur yellow. Panel C: molecular surface representation of uPAR bound to antagonist AE147 (Blue ribbon diagram). uPAR residues interacting with the antagonist are coloured either cyan (polar) or orange (hydrophobic). Panel D: rotation of the structure in Panel C highlighting the upper surface and binding cavity of uPAR. Note the uPAR structure is much less constricted when bound to AE147 (Panel C) compared to when bound to ATF (Panel A). Adapted from References 284 and 285.
Positioning of the linear peptide antagonist AE147 (K-S-D-Cha-F-s-k-Y-L-W-S-S-K) in the co-crystal structure with uPAR showed very similar binding interactions to those observed in the ATF:uPAR structure. The tryptophan of AE147 mimics Tyr24 of the ATF and makes similar contacts with uPAR residues His166 and Asp254. The Tyr, Cha and Phe residues of AE147 interact with the uPAR hydrophobic patch residues (Val29, Leu40, Leu55 and Leu66) in a similar manner to Phe25, Ile28 and Trp30 of the ATF. Examination of the two uPAR crystal structures (i.e. AE147:uPAR and ATF:uPAR) shows major differences in orientation of the respective uPAR domains, including a 20.5° rotation of domain I relative to the remaining domains and a shift of several β-strands of domains I and II away from the centre of the binding cavity. These changes serve to enlarge the binding cavity when the peptide antagonist is present. A different shift of two β-strands of domain II away from domain I occurs to accommodate the ATF. The domain I-domain III interface in the ATF-bound structure is much greater, with a larger number of hydrogen bonds formed.

Taken together, the different approaches for identifying residues responsible for uPAR-ligand binding have given us an excellent picture of the key interactions at the binding interface. The residues of uPA that are most important for a high affinity interaction with uPAR are Lys23, Tyr24, Phe25, Ile28 and Trp30. These residues all occur within the ω-loop of uPA, highlighting the importance of this sequence for uPAR binding. Tyr24 is particularly important as it interacts with all three uPAR domains. A large number of uPAR residues from all three domains are important for
ligand binding, however several stand out, including Val29, Leu40, Arg53, Leu55 and Leu66 of domain I; Thr127 and His166 of domain II; and Asp254 of domain III.

5.4 Methods for Evaluating Antagonists of the uPA:uPAR Interaction

A variety of techniques have been used to identify uPAR antagonists and evaluate their potencies. These include flow fluorocytometry, surface plasmon resonance, in vivo mouse models, ELISA and radioligand binding assays. Of these assays, the whole animal and in vitro cell assays that involve cell-surface-bound uPAR have greater physiological relevance than those that simply employ a purified soluble form of uPAR. The wealth of literature describing the complex interplay between uPAR and uPA and cell-surface proteins such as integrins and matrix elements suggests that the efficacy of uPAR antagonists as antimetastatic agents is likely to be influenced by how antagonists affect these additional interactions.\(^{243,254,291,292}\)

Studying putative antagonists in physiologically relevant assays is therefore considered vital. Nevertheless, several simplified assays have been used to identify some antagonists.

A radioligand binding assay of relevance to this thesis work used a GPI-cleaved soluble form of uPAR (suPAR) immobilised in 96-well plates to characterise the antagonist potencies of some phage-display antagonists and small-molecule synthetic antagonists (e.g. 84) described earlier.\(^{270,275}\) In this assay, ATF labelled with \(^{125}\)I was incubated with antagonists in suPAR-lined wells. Competitive uPAR
binding was then assessed using a $\gamma$-counter. The main features of this type of assay are summarised in Figure 5.7.

**Figure 5.7.** Radioligand-binding assay used to determine $IC_{50}$ values of some uPAR antagonists, including phage display peptides and small molecule antagonist **84**.

Several assays have been developed that make use of uPAR-expressing cancer cells. Flow cytometry approaches where cells are incubated with antagonists and FITC-uPA have been popular. In these systems, antagonism of the fluor-labelled uPA binding to uPAR is measured as reductions in fluorescence. Radioligand-binding assays with uPAR-expressing cells grown in a monolayer on 24-well plates have been reported. These are performed by incubating with candidate antagonists and $^{125}$I-labelled ATF before washing and determining bound $^{125}$I-ATF with a $\gamma$-
A variant of this assay was employed in the assessment of various peptidomimetic antagonists, including 83, which is the focus of this thesis work. In assaying 83, a 21-amino acid peptide corresponding to residues 12-32 of uPA (with an alanine substitution for Cys19) was radiolabelled with $^{125}$I at Tyr24. The radioligand was incubated with uPAR-expressing DU-145 prostate cancer cells and 83. The cells were washed and the amount of bound radioligand determined with a $\gamma$-counter. A summary of this type of assay is provided in Figure 5.8.

Figure 5.8. Radioligand-binding assay used to determine IC$_{50}$ values for small molecule uPAR antagonist 83.
5.5 Aims – Section 2

Binding of the urokinase-type plasminogen activator (uPA) to its cell-surface-bound receptor uPAR and upregulation of the plasminogen activation system correlates with increased metastasis and poor prognosis in several tumour types. An increasing body of evidence implicates uPA:uPAR binding not only in ECM proteolysis but also in pro-migratory signaling, adhesion, and the coordination of invasive activity.\textsuperscript{293-296} Disruptors of the uPA:uPAR interaction therefore represent promising antitumour/antimetastasis agents and several approaches have been explored for this purpose, including the use of small molecule antagonists. Two highly potent non-peptidic antagonists 83 and 84 (IC\textsubscript{50} 83 = 0.8 nM, IC\textsubscript{50} 84 = 33 nM) from the patent literature were reportedly identified using competition assays employing radiolabelled uPAR-binding uPA fragments and appear to be useful pharmacological tools for studying the plasminogen activation system and the effects that small molecule uPAR antagonists can have on metastasis.

Before proceeding to such studies we sought to confirm that 83 and 84 retain their potencies in physiologically relevant cell-based competition assays employing uPAR’s native binding partner HMW-uPA. These antagonists had previously been identified and their potencies measured using only simplified radioligand binding assays employing \textsuperscript{125}I-labelled uPA fragments as the competing ligands. The aims of this section were to:

1. Prepare antagonist 83 by a new solution phase synthesis.
2. Synthesise antagonist 84 by a new mixed solid/solution phase approach.

3. Assess the activities of 83 and 84 in semi-quantitative competition flow cytometry assays and quantitative cell-based uPA activity assays that employ HMW-uPA as the competing ligand.

Confirmation of high uPAR antagonist potencies in these physiologically relevant assays would support further use of 83 and 84 as biological probes for pharmacological investigations of the plasminogen activation system and its role in cancer.
Chapter 6: Synthesis of uPAR Antagonists 83 and 84

6.1 Synthetic Strategies

Syntheses of 83 and 84 had previously been reported in the patent literature but few experimental details and no compound characterisations were provided. Synthetic strategies related to (but different from) those reported in the patents were implemented here to produce the patented antagonists for full characterisation and biological evaluation.

The reported method for preparing 83 and various analogues made use of Fmoc-based solid phase synthesis protocols on Sasrin resin. An alternative solution-phase strategy using N-Boc and methyl ester protected amino acids was developed here to produce 83. The patented method for preparing the putative antagonist CHIR11509 84 made use of the solid phase submonomer strategy reported by Zuckermann et al for producing oligo-N-substituted glycine peptoids. The method involved coupling bromoacetic acid to Wang resin followed by halogen substitution with N-(4,4’-dimethoxybenzhydryl)glycinamide. Further manipulations followed by resin cleavage with 90% TFA (trifluoroacetic acid)/H₂O apparently afforded compound 84. It is clear, however, that this procedure would not yield 84 due to loss of the 4,4’-dimethoxybenzhydryl group during resin cleavage with TFA. A new unambiguous mixed solid/solution phase synthesis of 84 based on the submonomer method was therefore devised and implemented here.
6.2 Solution-Phase Synthesis of uPAR Antagonist 83

Synthesis of 83 began with Boc-protection of the commercially available 3’-aminobiphenyl-4-carboxylic acid. Initial attempts to produce N-Boc-3’-aminobiphenyl-4-carboxylic acid 89 from 88 using Boc-anhydride (Boc₂O) proved surprisingly difficult but it was found that very high yields (99%) of 89 could be obtained if catalytic I₂ was added to reactions.²⁹ It is likely that the molecular iodine coordinates to both carbonyl oxygens of Boc₂O as a Lewis acid activating it towards nucleophilic attack by the relatively unreactive aniline-type amine as shown in Scheme 6.1.

![Scheme 6.1. Mechanism of I₂-catalysed activation of Boc₂O useful in the Boc-protection of unreactive amines.](image)

Carboxylic acid 89 was coupled to L-Phe-OMe.HCl using O-benzotriazole-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and DIPEA in DMF to produce 90 in 79-88% yield. The N-Boc group was then removed from 90 by stirring with neat TFA in the presence of p-cresol as cation scavenger. The deprotected crude product was coupled to Boc-L-Trp-OH using HBTU and DIPEA in DMF to afford 91 in 53-65% yield. Boc-deprotection of 91 (neat TFA/p-cresol) followed by acetylation with
acetic anhydride and hydrolysis of the methyl ester (LiOH/THF/H₂O) provided 83 in 90-95% yield over the final three steps.

Scheme 6.2. Solution-phase synthesis of uPAR antagonist 83.

6.3 Mixed Solution/Solid-Phase Synthesis of uPAR Antagonist 84

Rink amide AM resin bearing an Fmoc-protected amine was deprotected using 20% piperidine in CH₂Cl₂. Bromoacetic acid was then coupled to the amino-functionalised resin using DCC and 4-(N,N-dimethylamino)pyridine (DMAP) in a mixture of N-methylpyrrolidone (NMP) and DCM. Displacement of the bromide with H₂N-Gly-O⁻Bu.HCl in DMSO at 45 °C afforded a secondary amine which was acylated with bromoacetic acid using the above method. Displacement of the newly introduced bromide with β-naphthylamine in DMSO at 45 °C, followed by coupling of the secondary amine to bromoacetic acid provided an α-bromocarbonyl which
was subsequently displaced with propargylamine (DMSO at 45 °C). The resin was then cleaved and the tert-butyl ester simultaneously removed using a mixture of TFA:triisopropylsilane (TIPS):H$_2$O to afford the free acid 93 (17%) after purification by preparative rp-HPLC. Solution phase amide coupling of 93 with 4,4’-dimethoxybenzhydrylamine using HBTU and DIPEA in DMF gave the target peptoid 84 (28%) after rp-HPLC.

**Scheme 6.3.** Mixed Solid/Solution-phase synthesis of uPAR antagonist 84. Reagents: a. Bromoacetic acid, DCC, DMAP, NMP/DCM (1:2).
Interestingly, two-dimensional NMR experiments in DMSO-<i>d</i>6 revealed that 84 exists as a slowly interconverting 1:1 mixture of cis/trans rotomers about the C(O)-N(β-naphthyl) tertiary amide, a 1:0.87 mixture of cis/trans rotomers about the sterically hindered secondary amide bond and a 1:0.95 mixture of cis/trans rotomers about the remaining tertiary amide (Figure 6.1).

**Figure 6.1.** 2D NMR highlighting the rotomeric nature of 84. Top: NOESY spectrum showing coupling between the two rotomeric forms of the secondary amide proton and glycine α-protons. Bottom: gHMBC coupling between the rotomer forms of three sets of methylene protons and amide carbonyls within 84.
Chapter 7: Evaluation of 83 and 84 as uPAR Antagonists

Phorbol 12-myristate-13-acetate (PMA) is known to induce high expression levels of unoccupied cell surface uPAR receptors in monocyte-like U-937 leukemia cells without a corresponding increase in uPA expression. Accordingly, PMA-stimulated U-937 cells were chosen for use in semi-quantitative competition flow cytometry assays where the binding of HMW-uPA labelled with the fluorescent probe Alexa-488 (Alexa-HMW-uPA) to cell surface uPAR receptors was measured in the presence/absence of 83 and 84. PMA-stimulated U-937 cells were similarly used in a sensitive and quantitative cell-based fluorometric uPA activity assay where the rate of conversion of a fluorogenic uPA-selective substrate by uPAR-bound HMW-uPA was measured in the presence/absence of 83 and 84. Competition between HMW-uPA and antagonists 83 and 84 for uPAR binding in this assay indirectly inhibits conversion of a uPA substrate, thereby allowing for determination of IC$_{50}$ values for uPA inhibition by antagonists. The major features of these assays are summarised in Figure 7.1.
**Figure 7.1.** Summary of cell-based flow cytometric and fluorescence activity assays used to characterise the uPAR antagonist potencies of 83 and 84. Cleavage of the uPA Fluorogenic Substrate III releases fluorescent 7-amino-4-methylcoumarin whose emission following 383 nm excitation is monitored at 455 nm.
7.1 Characterisation of (a) Alexa-HMW-uPA Binding to Cell Surface uPAR Receptors and (b) uPAR-Bound HMW-uPA Activity in U-937 Cells

Preliminary flow cytometry and cell surface bound uPA activity experiments were carried out by Melissa De Souza as part of an honours project in 2008. These experiments confirmed that PMA stimulated U-937 cells were suitable for use in cell-based uPAR antagonist assays employing HMW-uPA as the competing ligand. The flow cytometry experiments showed that binding of exogenous Alexa-HMW-uPA to uPAR receptors on PMA-stimulated U-937 cells occurs in a dose dependent manner (Figure 7.2A, closed circles) and that saturation of ~1 x 10^6 cells is achieved in the presence of ~40 nM Alexa-HMW-uPA or greater. Unstimulated U-937 cells showed no increases in fluorescence upon addition of increasing concentrations of Alexa-HMW-uPA (Figure 7.2A, unshaded circles) suggesting that the few uPAR receptors present on these cells were being pre-saturated by endogenous uPA prior to the addition of Alexa-HMW-uPA. 1 x 10^6 PMA-stimulated U-937 cells and 40 nM Alexa-HMW-uPA were thus chosen for use in competition flow cytometry assays carried out in the presence of 83 and 84.
Figure 7.2. (A) Characterisation of Alexa-HMW-uPA binding to uPAR receptors on U-937 cells by flow cytometry. Unstimulated (unshaded circles) and PMA stimulated (shaded circles) U-937 cells were incubated in the presence of increasing concentrations of Alexa-HMW-uPA and the geometric mean of cell-associated fluorescence measured using dual colour flow cytometry. (B) Characterisation of uPAR-bound HMW-uPA activity in U-937 cells. Increasing numbers of PMA stimulated U-937 cells were pre-incubated in the absence (unshaded squares) or presence (shaded squares) of 40 nM HMW-uPA and analysed for cell surface HMW-uPA activity using a fluorogenic uPA-selective substrate. Initial rates of increase in fluorescence per min were measured. Values shown represent the mean ± SD (n = 3) from representative experiments. Adapted from Reference 300.

Preliminary fluorometric investigations of cell surface-bound HMW-uPA activity in PMA stimulated U-937 cells were conducted using the fluorogenic uPA-selective substrate Z-Glu-Gly-Arg-AMC.HCl in the presence/absence of added exogenous...
HMW-uPA. Cell number-dependent fluorescence increases were observed in both cases confirming that uPA activity was due to cell-bound uPA. The low but measurable uPA activity observed in the absence of exogenous HMW-uPA (Figure 7.2B, unshaded squares) confirmed that PMA stimulated U-937 cells express low levels of endogenous receptor-bound uPA. The rate of increase in fluorescence was considerably higher (indicating higher cell-bound uPA activity) when 40 nM exogenous HMW-uPA was added (Figure 7.2B, shaded squares). When the assay was extended to a competition format to determine the IC_{50} values for uPA inhibition of 83 and 84, 40 nM exogenous HMW-uPA was added routinely to enhance cell surface-bound uPA activity and increase sensitivity. The above-mentioned experiments were also carried out by Melissa De Souza.

7.2 Evaluation of 83 and 84 as Antagonists of the HMW-uPA:uPAR Interaction in U-937 Cells by Flow Cytometry

U-937 cells in log phase growth were re-suspended in cold Phosphate-Buffered Saline (PBS)/Bovine Serum Albumin (BSA) at 1 x 10^6 cells.ml^{-1} and pre-incubated with varying concentrations of 83 and 84. The compounds were dissolved in DMSO and diluted with PBS to varying concentrations such that final addition of compounds to cells yielded DMSO concentrations of 2% in each sample. Cell viability was unaffected by this concentration of DMSO but control samples containing 2% DMSO without antagonists were included in all assays nonetheless. Visible inspection of assay wells showed no evidence of compound precipitation,
thus it is assumed that the given concentrations are accurate. Two positive controls known to antagonise the uPA:uPAR interaction were included to validate the assay. These were: (1) HMW-uPA which had been inactivated with Glu-Gly-Arg-chloromethyl ketone (i-uPA)\textsuperscript{302} and (2) mouse anti-human uPAR monoclonal antibody (MAb #3936, American Diagnostica Inc. CT, USA).\textsuperscript{303} Controls for the i-uPA and MAb #3936 assays included an appropriate dilution of vehicle (PBS). All samples were incubated on ice for 30 min before adding 40 nM Alexa-HMW-uPA. After 30 min incubations with Alexa-HMW-uPA on ice, cells were washed twice in ice-cold PBS/BSA by centrifugation (300 x g, 5 min 4\textdegree{}C) before final re-suspension in ice cold PBS containing 5 \(\mu\)g/ml propidium iodide. Viable (\textit{i.e.} propidium iodide negative) cells in the samples were analysed by dual colour flow cytometry as described in Ranson \textit{et al.}\textsuperscript{304}

The i-uPA positive control was found to strongly antagonise Alexa-HMW-uPA binding at concentrations of 10 nM (80\% reduction in binding) and 100 nM (90\% reduction in binding). The anti-uPAR MAb produced a 70\% reduction in binding when administered at 100 nM, which is in reasonable agreement with a previous finding that 133 nM concentrations of the antibody produce 50\% reductions in binding of HMW-uPA to uPAR receptors on glioblastoma cells.\textsuperscript{303}

Compound 83 produced no reduction of fluorescence relative to the negative control (no test compound) indicating that it does not antagonise HMW-uPA binding, even at the high concentrations used (10 \(\mu\)M and 100 \(\mu\)M) (Figure 7.3). The
same concentrations of 84 reduced the observed fluorescence relative to the negative control by approximately 60% (10 μM) and 50% (100 μM) (Figure 7.3). This indicated that while 84 antagonises HMW-uPA binding its effects are only weak. These results strongly contradict the reported antagonist potencies of 83 (IC₅₀ 0.8 nM) and 84 (IC₅₀ 33 nM).

**Figure 7.3.** Dual-colour competition flow cytometry assay of 83 and 84 as antagonists of the HMW-uPA:uPAR interaction in U-937 cells. The fluorescence is expressed as a percentage of the fluorescence observed in the presence of 40 nM Alexa-HMW-uPA alone (i.e. fluorescence arising from 100% receptor occupancy). Fluorescence decreases observed after pre-incubation with positive controls: (1) HMW-uPA inactivated with Glu-Gly-Arg-chloromethyl ketone (i-uPA; 10 nM and 100 nM) and (2) mouse anti-human uPAR monoclonal antibody (MAb #3936; 100 nM), are shown for comparison. Values represent means ± SD (n = 3) from representative experiments.
7.3 Evaluation of 83 and 84 as Antagonists of the HMW-uPA:uPAR Interaction in U-937 Cells by Quantitative Fluorometric Activity Assays

Fluorescence enzyme activity assays using PMA-stimulated U-937 cells and the uPA-selective fluorogenic HMW-uPA substrate Z-Gly-Gly-Arg-AMC (Figure 7.1) were used to quantify the effects of 83 and 84 on cell-surface-bound HMW-uPA activity.

U-937 cell surface-bound HMW-uPA activity was measured using a fluorogenic uPA substrate. Fluorescence observed in this assay is directly proportional to cell-bound HMW-uPA activity due to the high specificity of the substrate for HMW-uPA. The excitation wavelength range of the substrate is 365-380 nm and the emission wavelength range is 430-460 nm. PMA stimulated cells were prepared as described above and incubated with test compounds for 30 min at 4 °C, after which HMW-uPA (40 nM) was added and the cells incubated for a further 30 min at 4 °C. Cells were then washed twice by centrifugation with PBS at room temperature, transferred to a fluor plate and overlayed with an equivalent volume of buffer containing 1 mM Z-Gly-Gly-Arg-AMC to give a final concentration of 0.5 mM of the fluorogenic substrate. Fluorescence emission was measured immediately using an Fluorostar Optima instrument at 37 °C (BMG Labtech, Offenburg, Germany). Data was recorded at 30 sec intervals over a period of 40-50 min. Control samples were included to measure background fluorescence of cells, fluorogenic substrate and buffer. Another control containing no antagonist, HMW-uPA and fluorogenic substrate was used to measure the fluorescence at full receptor occupancy in the
presence of fluorogenic substrate. The background fluorescence was subtracted from each reading before statistical analysis. The rate of change in fluorescence $\text{min}^{-1}$ was calculated from two timepoints within the linear region of graphs of fluorescence vs time.

The control compound i-uPA showed potent inhibition of cell-bound HMW-uPA activity exhibiting an IC$_{50}$ value of 2.9 nM. In contrast, compounds 83 and 84 showed only very weak inhibitory effects with each showing IC$_{50}$ values greater than 100 $\mu$M. In agreement with the flow cytometry assays, compound 83 elicited virtually no reduction in fluorescent substrate cleavage relative to the negative control (no test compound) indicating that it does not antagonise HMW-uPA binding, even at the high concentrations used. The same concentrations of 84 only slightly reduced substrate cleavage relative to the negative control. These results are presented in Figure 7.4.
Figure 7.4. Fluorescence assays of cell surface-bound HMW-uPA activity in PMA-stimulated U-937 cells. Cells were pre-incubated for 30 mins with a range of concentrations of (A) i-uPA, (B) 83 or (C) 84 before adding 40 nM HMW-uPA and incubating for a further 30 mins. Cells were washed, fluorogenic uPA-specific substrate added and fluorescence measurements recorded. Initial rates of change in fluorescence after subtraction of background fluorescence (cells only) are presented as a percentage of control (no test compound added). Values represent means ± SD (n = 3) obtained from representative experiments.
7.4 Discussion

In addition to the hydrophobic cavity used to engage uPA there exists a second well-characterised protein-protein interaction site on uPAR which binds to the SMB domain of matrix embedded vitronectin.\textsuperscript{291} The uPAR:vitronectin interaction is implicated in cell migration processes such as, for example, cytoskeletal rearrangements and remodelling of focal adhesion sites.\textsuperscript{305,306} Importantly, occupancy of the hydrophobic binding site on uPAR by uPA leads to a 5-fold increase in the affinity of uPAR for vitronectin ($K_D \sim 1.9 \ \mu M \rightarrow K_D \sim 0.4 \ \mu M$)\textsuperscript{307,308}, suggesting that pre-formation of the uPA:uPAR complex may enhance uPAR:vitronectin adhesion \textit{in vivo}. It would therefore be of considerable interest to investigate the effects that small molecule uPA:uPAR antagonists have on the uPAR:vitronectin interaction.

Comparing X-ray crystal structures of uPA(ATF):uPAR:SMB ternary complexes\textsuperscript{291} with those of binary complexes of uPAR bound to a high affinity linear peptide antagonist AE147 (See Figure 5.6)\textsuperscript{285} revealed that significant conformational tightening of uPAR's structure occurs in the uPA(ATF):uPAR:SMB complex that is not observed in the uPAR:AE147 structure. Absence of uPAR conformational tightening upon binding of the potent yet much smaller peptidic antagonist AE147 suggests that small molecule antagonists of the uPA:uPAR interaction might potentially be better suited for development into antitumour/antimetastasis drugs than their larger protein-based counterparts as they may be less likely to promote the uPAR:vitronectin interaction.\textsuperscript{309}
Compounds 83 and 84 were reported in the patent literature as being highly potent uPAR receptor antagonists and seemingly represented excellent pharmacological tools for exploring the effects of uPAR antagonism on tumours and metastasis and for exploring the above vitronectin hypothesis. As a precautionary step before proceeding to these types of pharmacological investigations with 83 and 84 it was considered important to confirm that both compounds retained their high potencies in physiologically relevant cell-based competition assays where native HMW-uPA was used as the competing ligand. The potencies of 83 and 84 had only previously been measured using radioligand binding assays employing labelled uPAR-binding uPA fragments as the competing ligands. A semi-quantitative competition flow cytometry assay using uPAR-expressing U-937 cells and a quantitative U-937 cell-based fluorescence assay were used for this purpose. Access to 83 and 84 was afforded through two simple new syntheses.

Compound 83 was found to show no antagonist effects at 100 μM in the flow cytometry assay and only weak inhibition of cell-surface uPA activity in the fluorescence assay (IC50 > 100 μM). Compound 84 was shown to be a weak uPAR antagonist at concentrations of 10 μM and 100μM and a weak inhibitor of cell-surface uPA activity (IC50 > 100 μM). Nanomolar concentrations of positive controls (anti-uPAR MAb and chloromethylketone-inactivated HMW-uPA) antagonised HMW-uPA binding and inhibited cell surface uPA activity under the same assay.
conditions. These results are in direct contrast to the high antagonist potencies reported for 83 and 84.\textsuperscript{275,277}

One possible explanation for the conflicting results arises from the fact that the uPA fragments employed in previous measurements contained \textsuperscript{125}I-labelled Tyr24 residues. As stated previously (Section 5.3), Tyr24 is located at the tip of the uPA \( \omega \)-loop and is crucial for high affinity uPAR binding. Evidence shows that modification of Tyr24 with bulky groups (e.g. iodine) greatly reduces uPA’s affinity for uPAR.\textsuperscript{310,311} Given this and the fact that data comparing the uPAR binding affinity of the \textsuperscript{125}I-Tyr24-uPA fragments to their unlabelled counterparts appears not to have been reported, it appears that the previously reported IC\textsubscript{50} values for 83 and 84 are not meaningful.

7.5 Conclusions and Future Directions

Compounds 83 and 84 were found to be poor antagonists of the uPA:uPAR interaction in physiologically relevant cellular systems and are therefore not useful as small molecule probes for pharmacological investigations of the plasminogen activation system. The work highlights the importance of using HMW-uPA as the competing ligand in competition experiments aimed at measuring the potency of small molecule uPAR antagonists as potential pharmacological tools or anticancer drug leads. It also highlights the dangers of identifying false-positive lead uPAR antagonists if this precaution is not taken.
The SMB-binding site of uPAR may present a less hydrophobic and more "druggable" target for antagonism. The hydrophilic uPAR:SMB interface contains both acidic (Glu34) and basic (Arg30) residues. In fact, peptides have been identified using random bacteriophage display that act as vitronectin antagonists with IC_{50} values in the range of 0.5-2.0 μM. These peptides show some sequence similarity to the vitronectin SMB domain. Further investigation of vitronectin antagonists as antimetastatic agents is certainly warranted. Antagonism of uPAR-integrin interactions is another possibility, and proof for this concept was recently provided. Antibodies identified by Fab phage display antagonised uPAR/β1 integrin binding and demonstrated significant inhibition of uPAR-mediated signal transduction and cell migration. Recent research indicates that inhibition of the role of uPAR in the modulation of cell morphology through vitronectin and integrin interactions may produce antimetastatic effects with effects similar to inhibition of uPA activity and antagonism of the uPA:uPAR interaction.
Chapter 8: Section 2 Experimental and References

8.1 Synthesis - General

Reagents and solvents were used without further purification unless otherwise stated. The purification of most solvents and reagents is described in Chapter 4, Section 4.1. p-cresol was purified by recrystallisation from petroleum spirit (Boiling Range 40-60 °C).  

Di-p-anisylmethanamine was purchased from TCI Japan and 3’-aminobiphenyl-4-carboxylic acid from Parkway Scientific. Rink amide AM resin (200-400 mesh) was purchased from Novabiochem. Peptide synthesis-grade DCM and DMF were purchased from Auspep.

Most general purification processes and equipment used are described in Chapter 4, Section 4.1. Solid-Phase peptide synthesis was performed in a Peptides International water-jacketed reaction vessel using a St. John Associates 180° variable rate shaker. Compounds 83 and 84 were purified for testing using a Waters PLC/DSC Prep LC150 System with detection at 254 nm. Purifications were performed using gradient elutions with solvents A (100% H₂O, 0.1% HCl) and B (90% CH₃CN, 10% H₂O, 0.1% HCl). The column used was a Sunfire™ PREP C18 OBD™ (5 μM) steel jacketed column run at 20 mL.min⁻¹. Analytical HPLC analyses were performed using a Shimadzu CLASS-LC10 VP HPLC system with detection at 254 nm.
The analyses were performed using gradient elutions with solvents A and B on a Phenomenex Luna 5 µM C18 column run at 1 mL.min⁻¹.

8.2 Characterisation

Techniques applied in the characterisation of compounds are described in Chapter 4, Section 4.2. Compounds are named according to IUPAC convention. Previously unreported compounds are indicated with the symbol: a as a superscript following the compound name. Names of previously reported compounds with limited spectral data available are indicated with the symbol: b. Previously reported compounds that have been fully characterised are indicated with the symbol: c.

3’-(N-acetyl-L-tryptophyl-amino)(1,1’-biphenyl)-4-carbonyl-L-phenylalanine (83)ᵇ

92 (76 mg, 0.13 mmol) was covered with water (1 mL) and stirred while THF was added dropwise until a solution was obtained. LiOH monohydrate (32 mg, 0.76 mmol) was added and the solution stirred for 4 h before 2 M HCl (10 mL) was added. The suspension was extracted with EtOAc (3 x 10 mL) and the combined organic extracts were dried over MgSO₄. After filtration, the solution was concentrated in
vacuo and the residue taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t, 23.0 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 83 as a light brown solid (75 mg, 100%): mp 186-188 °C; $^1$H NMR (500 MHz, (CD$_3$)$_2$SO) δ 10.83 (s, 1H, Trp amide NH), 10.24 (s, 1H, Trp NH), 8.77 (d, J= 7.5 Hz, 1H, Phe amide NH), 8.25 (d, J = 7.5 Hz, 1H, Acetamide NH), 7.93 (s, 1H, ArH2’), 7.91 (d, J = 8.5 Hz, 2H, ArH3 and ArH5), 7.68 (d, J = 8.5 Hz, 2H, ArH2 and ArH6), 7.65 (m, 1H, ArH4’’’), 7.64 (m, 1H, ArH6’’’), 7.41 (m, 2H, ArH4’ and ArH5’), 7.33 (m, 3H, ArH7’’’), ArH2’ and ArH6’), 7.28 (t, J = 7.0 Hz, 2H, ArH3’ and ArH5’’’), 7.19 (m, 2H, ArH4” and ArH2’’’), 7.05 (t, J = 7.5 Hz, 1H, ArH6’’’), 6.97 (t, J = 7.5 Hz, 1H, ArH5’’’), 4.72 (ddd, J = 7.5, 8.5, 11.0 Hz, 1H, Phe α-H), 4.65 (bs, 1H, Trp α-H), 3.21 (dd, J = 8.5, 13.0 Hz, 1H, Phe β-H), 3.19 (dd, J = 5.5, 14.5 Hz, 1H, Trp β-H), 3.08 (dd, J = 11.0, 13.0 Hz, 1H, Phe β-H), 3.02 (dd, J = 8.5, 14.5 Hz, 1H, Trp β-H), 1.84 (s, 1H, Acetyl CH$_3$); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) δ 173.1 (COOH), 170.9 (Trp Amide C=O), 169.2 (Acetyl C=O), 165.9 (Phe Amide C=O), 142.8 (ArC1), 139.6 (ArC1’), 139.5 (ArC3’), 138.2 (ArC1’’), 136.0 (ArC7’’’a), 132.8 (ArC4), 129.3 (ArC5’), 129.0 (ArC2’ and ArC6’), 128.2 (ArC3’ and ArC5’’’), 128.0 (ArC3 and ArC5), 127.2 (ArC3’’a), 126.4 (ArC2 and ArC6), 126.3 (ArC4’), 123.6 (ArC2’’’), 121.9 (ArC4’), 120.8 (ArC6’’’), 119.1 (ArC6’), 118.5 (ArC4’’’), 118.2 (ArC5’’’), 117.8 (ArC2’), 111.2 (ArC7’’’), 109.9 (ArC3’’’), 54.3 (Phe α-C), 54.2 (Trp α-C), 36.2 (Phe β-C), 27.9 (Trp β-C), 22.5 (COCH$_3$); [α]$_D$ = -14.1$^o$ (c. 1.0, MeOH); HPLC (254 nm) t, 24.2 min, 100%; ES TOF MS m/z (MH)$^+$ 589; Anal. for C$_{35}$H$_{33}$N$_4$O$_5$: Calc. Mass 589.2451. Found 589.2467.
N-[2-amino-2-oxoethyl]-N-[2-[bis(4-methoxyphenyl)methylamino]-2-oxoethyl]-2-[N-[naphthalen-2-yl]-2-[prop-2-ynlamino]acetamido]acetamide hydrochloride (84)

93 (88 mg, 168 μmol), HBTU (82 mg, 216 μmol), DIPEA (137 μL, 786 μmol) and 1,1-di(p-anisyl)methylamine (94 mg, 386 μmol) were dissolved in DMF (1 mL) and stirred under N₂ gas for 24 h. The solvent was removed in vacuo and the residue was taken up in 2 mL DMSO for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins (t_r 24.7 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 84 as a light brown solid (32 mg, 28% after HPLC): mp >300 °C (d); ¹H NMR (500 MHz, (CD₃)₂SO) δ 9.65 and 9.10 (rotomer, d, J = 8.0 Hz, 1H, CONHCHAr₂); 8.16 (s, 1H, H₂NCO), 8.02 (m, 1H, ArH¹), 8.01 (m, 1H, ArH⁴), 7.98 (m, 2H, ArH⁷ and ArH⁸), 7.91 (m, 1H, ArH⁵), 7.70 (s, 1H, COCH₂NH₂⁺CH₂), 7.59 (m, 1H, ArH⁶), 7.54 and 7.50 (rotomer, d, J = 8.5 Hz, ArH³), 7.37 (s, 1H, H₂NCO), 7.20 (s, 1H, COCH₂NH₂⁺CH₂), 7.13 and 7.11 (rotomer, d, J = 8.5 Hz, 4H, ArH²’ and ArH⁶’), 6.81 and 6.77 (rotomer, d, J = 8.5 Hz, 4H, ArH³’ and ArH⁵’), 5.95 (m, 1H, CONHCHAr₂), 4.60 and 4.59 (rotomer, s, 2H, COCH₂N(R)-2-naphthyl), 4.21 and 4.03 (rotomer, s, 2H, NCH₂CONH), 4.11 and 3.90 (rotomer, s, 2H, H₂NCOCH₂), 3.80 (s, 2H, NH₂⁺CH₂C=CH), 3.77 (s, 2H, COCH₂NH₂⁺CH₂), 3.69 and 3.66 (rotomer, s, 6H, OCH₃), 3.58 (s, 1H, C≡CH); ¹³C NMR (125 MHz, (CD₃)₂SO)}
δ 171.0 and 170.9 (rotomer, CONH₂), 168.1 and 168.0 (rotomer, CONHCH₄Ar₂), 167.9 and 167.6 (rotomer, COCH₂N(R)-2-naphthyl), 165.0 (COCH₂NH₂CH₂), 158.1 (ArC₄'), 137.7 (ArC₂), 134.8 and 134.5 (rotomer, ArC₁'), 133.0 (ArC₈ₐ), 132.5 (ArC₄a), 129.8 and 129.8 (rotomer, ArC₄), 128.3 and 128.2 (rotomer ArC₂' and ArC₆'), 128.2 (ArC₅), 127.8 (ArC₇), 127.2 (ArC₆), 127.0 (ArC₈), 126.9 and 126.8 (rotomer, ArC₁), 125.6 and 125.5 (rotomer, ArC₃), 113.7 (ArC₃' and ArC₅'), 79.6 (C≡CH), 74.7 (C≡CH), 55.2 and 54.9 (rotomer CONHCH₄Ar₂), 55.1 and 55.1 (rotomer OCH₃), 52.1 and 52.0 (rotomer, CH₂CONHCH), 51.9 and 51.5 (rotomer, H₂NCOCH₂), 51.0 and 51.0 (COCH₂N(R)-2-naphthyl), 46.6 (COCH₂NH₂CH₂), 35.5 (NH₂CH₂C≡CH); ES TOF MS m/z (M)⁺ 636; Anal. for C₃₆H₃₈N₅O₆: Calc. Mass 636.2822. Found 636.2853.

3′-[[1,1-dimethylethoxy]carbonyl]amino][1,1′-biphenyl]-4-carboxylic acid (89)⁺

3′-aminobiphenyl-4-carboxylic acid (1.7 g, 6.8 mmol) was covered with dry MeOH (6.8 mL) and stirred. A solution of I₂ (170 mg, 0.68 mmol) and di-tert-butyl dicarbonate (1.48 g, 6.8 mmol) was formed in a separate vessel using a minimal volume of dry MeOH and added to the stirred mixture. DIPEA (1.186 mL, 6.8 mmol) was added dropwise to the stirred mixture, producing a solution that was stirred for 5 h and then concentrated. The residue was diluted in EtOAc (80 mL) and washed with a 1 M HCl solution (40 mL) and saturated Na₂S₂O₃ solution (3 x 40
mL). The washes were repeated, and all aqueous layers were combined and extracted with EtOAc (3 x 30 mL). After separation the combined organic layers dried over MgSO₄ and concentrated, yielding 89 as an off-white solid (2.109 g, 99%): mp 210-212 °C (dec); ¹H NMR (CD₃OD) δ 8.09 (d, J = 8.5 Hz, 2H, ArH3 and ArH5), 7.77 (t, J = 1.5 Hz, 1H, ArH2’), 7.71 (d, J = 8.5 Hz, 2H, ArH2 and ArH6), 7.43 (dt, J = 1.5, 8.0 Hz, 1H, ArH4’), 7.37 (t, J = 8.0 Hz, 1H, ArH5’), 7.32 (dt, J = 1.5, 8.0 Hz, 1H, ArH6’), 1.54 (s, 9H, OC(CH₃)₃); ¹³C NMR (CD₃OD) δ 175.4, (COOH), 155.4 (NHCOO), 144.1 (ArC1), 142.6 (ArC1’), 141.0 (ArC3’), 137.9 (ArC4), 130.8 (ArC3 and ArC5), 130.2 (ArC5’), 127.3 (ArC2 and ArC6), 122.4 (ArC6’), 119.0 (ArC4’), 118.4 (ArC2’), 81.0 (OC(CH₃)₃), 28.7, OC(CH₃)₃; ES TOF MS m/z (M)⁺ 312; Anal. for C₁₈H₁₈NO₄: Calc. Mass 312.1242. Found 312.1236.

3’-[[[1,1-dimethylethoxy)carbonyl]amino][1,1’-biphenyl]-4-carbonyl-L-phenylalanine methyl ester (90)⁹

89 (2.18 g, 6.96 mmol) was stirred in dry DMF (80 mL) along with HBTU (2.64 g, 6.96 mmol) and DIPEA (4.85 mL, 27.84 mmol). To the stirred solution was added L-phenylalanine methyl ester hydrochloride (3.0 g, 13.91 mmol). After stirring under N₂ gas for 24 h, the solution was concentrated before dilution in DCM (50 mL). The organic layer was washed with saturated NaHCO₃ solution (2 x 40 mL) and brine (2 x 40 mL) and dried over MgSO₄. The solution was
concentrated and purified by column chromatography in a 40 mm diameter column with a silica height of 220 mm. The eluent used was a 3:1 mixture of petroleum spirit (B.R. 40-60 °C) and EtOAc. The fraction containing the desired product was isolated and concentrated, yielding 90 as pale yellow crystals (2.907 g, 88%): mp 104-106 °C; ¹H NMR (CDCl₃) δ 7.75 (d, J = 8.0 Hz, 2H, ArH₃ and ArH₅), 7.69 (s, 1H, ArH₂′), 7.58 (d, J = 8.0 Hz, 2H, ArH₂ and ArH₆), 7.35 (d, J = 8.0 Hz, 1H, ArH₄′), 7.33 (t, J = 8.0 Hz, 1H, ArH₅′), 7.28 (d, J = 7.5 Hz, 2H, ArH₂” and ArH₆”), 7.24 (t, J = 7.5 Hz, 1H, Ar₄”), 7.23 (d, J = 8.0 Hz, 1H, ArH₆’), 7.15 (d, J = 7.5 Hz, 2H, Ar₃” and Ar₅”), 6.99 (s, 1H, Boc-NH), 6.75 (d, J = 7.5 Hz, 1H, Amide NH), 5.11 (dd, J = 5.5, 14.0 Hz, 1H, Phe α-H), 3.75 (s, 3H, -OC₃H₃), 3.30 (dd, J = 5.5, 14.0 Hz, 1H, Phe β-H), 3.22 (dd, J = 5.5, 14.0 Hz, 1H, Phe β-H), 1.52 (s, 1H, -C(CH₃)₃); ¹³C NMR (CDCl₃) δ 172.0 (Ester C=O), 166.6 (Amide C=O), 152.8 (Boc C=O), 144.1 (ArC₁), 140.4 (ArC₁”), 139.1 (ArC₁’), 135.8 (ArC₃’), 132.2 (ArC₄), 129.2 (ArC₅’), 129.1 (ArC₃” and ArC₅”), 128.4 (ArC₂” and ArC₆”), 127.3 (ArC₃ and ArC₅), 127.0 (ArC₂ and ArC₆), 126.9 (ArC₄”), 121.4 (ArC₆’), 118.0 (ArC₄’), 117.1 (ArC₂’), 80.2 (OC(CH₃)₃), 53.5 (Phe α-C), 52.2 (OC₃H₃), 37.6 (Phe β-C), 28.1 (OC(CH₃)₃); [α]D = -24.5° (c. 1.0, MeOH); HPLC (254 nm) tᵣ 29.5 min; ES TOF MS m/z (MH)+ 475; Anal. for C₂₈H₃₁N₂O₅: Calc. Mass 475.2233. Found 475.2240.

3’-[N-{[1,1-dimethylethoxy]carbonyl}-L-tryptophylamino][1,1’-biphenyl]-4-carbonyl-L-phenylalanine methyl ester (91)
90 (200 mg, 0.42 mmol) and p-cresol (91 mg, 0.84 mmol) were stirred with neat TFA (2 mL) under N₂ gas for 1 h. The TFA was removed using N₂-mediated evaporation and the remaining residue was triturated with Et₂O (3 x 5 mL). The TFA salt was then dissolved in DMF (10 mL). To the stirred solution was added a solution of HBTU (175 mg, 0.46 mmol), Boc-L-Trp-OH (320 mg, 1.05 mmol) and DIPEA (366 µL, 2.1 mmol) in minimal DMF. The solution was stirred for 72 h at r.t. before concentration in vacuo to yield a residue that was taken up in DCM (15 mL). This solution was extracted with 0.1 M HCl solution (2 x 10 mL), saturated NaHCO₃ solution (2 x 10 mL) and brine (2 x 10 mL). The organic layer was concentrated and purified by column chromatography in a 10 mm diameter column with a silica height of 150 mm. The eluent used was a 1:1 mixture of petroleum spirit (B.R. 40-60 °C) and EtOAc. The fraction containing the desired product was isolated and concentrated in vacuo to yield 91 as a white solid (180 mg, 65%): mp 168-170 °C; ¹H NMR (500 MHz, CDCl₃) δ 8.59 (s, 1H, Trp NH), 8.13 (bs, 1H, NH; 7.70, d, J = 7.5 Hz, 1H, ArH₄’’’), 7.66 (d, J = 8.0 Hz, 2H, ArH₃ and ArH₅), 7.41 (d, J = 7.5 Hz, 2H, ArH₂ and ArH₆), 7.38 (s, 1H, ArH₂’), 7.34 (d, J = 8.0 Hz, 1H, ArH₇’’’), 7.29 (m, 2H, ArH₃’ and ArH₅’), 7.27 (m, 1H, ArH₄’), 7.24 (t, J = 4.0 Hz, 1H, ArH₅’), 7.21 (m, 1H, ArH₄’), 7.19 (d, J = 8.0 Hz, 1H, ArH₆’), 7.18 (d, J = 8.0 Hz, 2H, ArH₂’’ and ArH₆’’’), 7.17 (m, 1H, ArH₆’’’), 7.09 (t, J = 7.5 Hz, 1H, ArH₅’’’), 7.01 (s, 1H, ArH₂’’’), 5.46 (s, 1H, Phe-NH), 5.10 (dd, J = 6.0, 13.5 Hz, 1H, Phe α-H), 4.70 (bs, 1H, Trp α-H), 3.76 (s, 3H,
3'-((N-acetyl-L-tryptophylamino)(1,1'-biphenyl)-4-carbonyl-L-phenylalanine methyl ester (92)\(^a\)

\[\text{OCH}_3\), 3.33 (bs, 1H, Trp \(\beta\-H\)), 3.30 (dd, \(J = 6.0, 13.5\ Hz, 1H, \text{Phe}\ \beta\-H\)), 3.23 (bs, 1H, Trp \(\beta\-H\)), 3.23 (dd, \(J = 6.0, 13.5\ Hz, 1H, \text{Phe}\ \beta\-H\)), 1.44 (s, 9H, \(\text{OCH}_3\))

\(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 172.6 (Ester \(\text{C}=\text{O}\)), 170.5 (Amide \(\text{C}=\text{O}\) at biphenyl 3'-position), 166.6 (Amide \(\text{C}=\text{O}\) at biphenyl 4-position), 155.9 (Boc \(\text{C}=\text{O}\)), 143.6 (ArC1), 140.4 (ArC1'), 137.8 (ArC3'), 136.3 (ArC7"a), 136.1 (ArC1"), 132.2 (ArC4), 130.5 (ArC5'), 129.2 (ArC2" and ArC6"), 128.6 (ArC3" and ArC5"), 127.4 (ArC3 and ArC5), 127.2 (ArC3"a), 127.1 (ArC4"), 127.0 (ArC2'), 123.4 (ArC2"'), 123.0 (ArC6'), 122.3 (ArC6"'), 119.9 (ArC5"'), 119.8 (ArC4'), 118.7 (ArC2 and ArC6), 118.7 (ArC4"'), 111.4 (ArC7"'), 110.3 (ArC3"'), 80.4 (OC(CH\(_3\)_3)), 55.7 (Trp \(\alpha\-C\)), 53.8 (Phe \(\alpha\-C\)), 52.5 (OCH\(_3\)), 37.7 (Phe \(\beta\-C\)), 28.7 (Trp \(\beta\-C\)), 28.3 (OC(CH\(_3\)_3)); \([\alpha]_D = -12.7^\circ\) (c. 3.0, MeOH); HPLC (254 nm) \(t\) 29.0 min; ES TOF MS \(m/z\) (MH\(^+\)) 661; Anal. for C\(_{39}\)H\(_{41}\)N\(_4\)O\(_6\): Calc. Mass 661.3041. Found 661.3026.

91 (88 mg, 0.13 mmol) and \(p\)-cresol (30 mg, 0.28 mmol) were stirred with neat TFA (2 mL) under N\(_2\) gas for 1 h. The TFA was removed using N\(_2\)-mediated evaporation and the remaining residue was triturated with Et\(_2\)O (3 x 5 mL). The TFA salt was then dissolved in DMF (5 mL). To the stirred solution were added Acetic Anhydride (26 \(\mu\)L, 0.28 mmol) and DIPEA (70 \(\mu\)L, 0.4 mmol) and the reaction stirred at r.t. under N\(_2\) gas for 30 min. The reaction was quenched with
saturated NaHCO$_3$ solution (40 mL) and stirred for 30 min. The resulting suspension was extracted with EtOAc (4 x 20 mL) and the combined organic layers washed with 0.1 M HCl solution (3 x 20 mL) before drying over MgSO$_4$. The solution was filtered and concentrated in vacuo to yield 92 as a light brown solid (74 mg, 95%): $^1$H NMR (300 MHz, (CD$_3$)$_2$SO) $\delta$ 10.81 (s, 1H, Trp amide NH), 10.21 (s, 1H, Trp NH), 8.91 (d, $J$= 8.0 Hz, 1H, Phe amide NH), 8.23 (d, $J$ = 8.0 Hz, 1H, Acetamide NH), 7.93 (s, 1H, ArH2’), 7.91 (d, $J$ = 8.5 Hz, 2H, ArH3 and ArH5), 7.68 (d, $J$ = 8.0 Hz, 2H, ArH2 and ArH6), 7.67 (m, 1H, ArH4’”), 7.64 (m, 1H, ArH6’), 7.41 (m, 2H, ArH4’ and ArH5’), 7.39 (m, 3H, ArH7’”, ArH2’” and ArH6’”), 7.30 (t, $J$ = 7.0 Hz, 2H, ArH3’ and ArH5’”), 7.19 (m, 2H, ArH4’” and ArH2’””), 7.05 (t, $J$ = 7.0 Hz, 1H, ArH6’”), 6.97 (t, $J$ = 7.0 Hz, 1H, ArH5’”), 4.71 (dt, $J$ = 7.0, 16.0 Hz, 1H, Phe $\alpha$-H), 4.68 (bs, 1H, Trp $\alpha$-H), 3.65 (s, 3H, OCH$_3$), 3.19 (dd, $J$ = 5.5, 14.0 Hz, 1H, Phe $\beta$-H), 3.14 (dd, $J$ = 5.0, 14. Hz, 1H, Trp $\beta$-H), 3.04 (dd, $J$ = 7.0, 15.5 Hz, 1H, Phe $\beta$-H), 3.01 (dd, $J$ = 8.5, 14.0 Hz, 1H, Trp $\beta$-H), 1.83 (s, 3H, Acetyl CH$_3$); $^{13}$C NMR (75 MHz, (CD$_3$)$_2$SO) $\delta$ 172.2 (COOCH$_3$), 171.0 (Trp Amide C=O), 169.3 (Acetyl C=O), 166.1 (Phe Amide C=O), 143.0 (ArC1), 139.6 (ArC1’), 139.6 (ArC3’), 137.7 (ArC1’), 136.0 (ArC7’’a), 132.6 (ArC4), 129.4 (ArC5’), 129.1 (ArC2” and ArC6’”), 128.3 (ArC3” and ArC5’”), 128.1 (ArC3 and ArC5), 127.3 (ArC3’’a), 126.5 (ArC2 and ArC6), 126.5 (ArC4’), 123.6 (ArC2’”), 122.0 (ArC4’), 120.9 (ArC6’”), 119.1 (ArC6’), 118.6 (ArC4’”), 118.2 (ArC5’’”), 117.9 (ArC2’), 111.3 (ArC7’”), 109.9 (ArC3’”), 54.3 (Phe $\alpha$-C), 54.3 (Trp $\alpha$-C), 52.0 (OCH$_3$), 36.2 (Phe $\beta$-C), 27.9 (Trp $\beta$-C), 22.5 (Acetyl CH$_3$); ES TOF MS m/z (MH)$^+$ 603; Anal. for C$_{36}$H$_{35}$N$_4$O$_5$: Calc. Mass 603.2607. Found 603.2621.
2-[N-[2-amino-2-oxoethyl]-2-[N-(naphthalen-2-yl)-2-(prop-2-ynylamino)acetamido] acetamido]acetic acid trifluoroacetate (93)\(^a\)

Rink resin (2 g, 1.24 mmol) with 0.63 mmol/g substitution was shaken in a 45 mL solid-phase peptide synthesis vessel with 20% piperidine in DCM (10 mL) for 20 min before draining and washing with DCM (3 x 15 mL) and DMF (2 x 15 mL). The resin was swollen with DCM (15 mL). Bromoacetic acid (700 mg, 5 mmol) was combined with DCC (1.032 g, 5 mmol) in NMP (5 mL) and DCM (10 mL). DMAP (36.6 mg, 0.3 mmol) was added to the drained resin followed by the bromoacetic acid solution. The mixture was shaken for 1 h at r.t. before draining and washing with DCM (3 x 15 mL), DMF (2 x 15 mL) and iPrOH (2 x 15 mL). The resin was then dried in vacuo. The dry loaded resin was swollen with DMSO (24 mL) and drained before a solution of glycine t-butyl ester hydrochloride (832 mg, 4.96 mmol) and DIPEA (1.728 mL, 9.92 mmol) in DMSO (24 mL) was added. The mixture was shaken at 45 °C for 4 h before draining and washing with DMF (6 x 36 mL) and DCM (6 x 36 mL). The resin was swollen with DCM (36 mL). Bromoacetic acid (700 mg, 5 mmol) was combined with DCC (1.032 g, 5 mmol) in NMP (5 mL) and DCM (10 mL). DMAP (36.6 mg, 0.3 mmol) was added to the drained resin followed by the bromoacetic acid solution. The mixture was shaken for 1 h at r.t. before draining and washing with DCM (6 x 36 mL) and DMF (6 x 36 mL). The resin was swollen with
DMSO (24 mL) and drained before a solution of β-naphthylamine (716 mg, 5 mmol) in DMSO (30 mL) was added. The mixture was shaken at 45 °C for 4 h before draining and washing with DMF (6 x 36 mL) and DCM (6 x 36 mL). The resin was swollen with DCM (36 mL). Bromoacetic acid (700 mg, 5 mmol) was combined with DCC (1.032 g, 5 mmol) in NMP (5 mL) and DCM (10 mL). DMAP (36.6 mg, 0.3 mmol) was added to the drained resin followed by the bromoacetic acid solution. The mixture was shaken for 1 h at r.t. before draining and washing with DCM (6 x 36 mL) and DMF (6 x 36 mL). The resin was swollen with DMSO (24 mL) and drained before a solution of 3-aminopropyne (272 mg, 4.96 mmol) in DMSO (30 mL) was added. The mixture was shaken at 45 °C for 4 h before draining and washing with DMF (6 x 36 mL) and DCM (6 x 36 mL). The resin was then dried in vacuo, combined with 95% TFA, 2.5% water, 2.5% TIPS (36 mL) and cleaved for 1 h at r.t. before being filtered into a collecting vessel. The filtrate was concentrated in vacuo and the residue taken up in DMSO (2 mL) for purification by preparative HPLC using a gradient from 0% to 100% B over 30 mins with eluent containing 0.1% TFA instead of HCl (t_r 18.5 min). The collected fractions containing purified product were concentrated by freeze-drying to yield 93 as a white powder (111 mg, 17% after HPLC): mp >300 °C (dec); 1H NMR (500 MHz, (CD_3)_2SO) δ 9.60 (bs, 1H, COOH), 8.05 (s, 1H, R-NH_2), 8.03 (m, 2H, ArH1 and ArH4), 8.01 (m, 2H, ArH7 and ArH8), 7.96 (m, 1H, ArH5), 7.73 (s, 1H, CH_2NH_2^+CH_2), 7.59 (t, J = 4.5 Hz, 1H, ArH6), 7.55 and 7.53 (rotomer, d, J = 1.5 Hz, 1H, ArH3), 7.34 (s, 1H, R-NH_2), 7.235 (s, 1H, CH_2NH_2^+CH_2), 4.64 and 4.63 (rotomer, s, 2H, COCH_2N(R)-2-naphthyl), 4.19 and 3.98 (rotomer, s, 2H, NCH_2COOH), 4.05 and 3.89 (rotomer, s, 2H, H_2NCOCH_2), 3.80 (s, 2H,
NH₂⁺CH₂C≡CH), 3.78 (s, 2H, COCH₂NH₂⁺CH₂), 3.57 (s, 1H, C≡CH); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 170.8 and 170.8 (rotomer, CONH₂), 170.5 and 170.1 (rotomer, COOH), 168.0 and 167.8 (rotomer, COCH₂N(R)-2-naphthyl), 165.0 (COCH₂NH₂⁺CH₂), 137.7 (ArC2), 133.0 (ArC8a), 132.5 (ArC4a), 129.8 (ArC4), 128.1 (ArC5), 127.7 (ArC7), 127.2 (ArC6), 127.0 (ArC8), 126.9 (ArC1), 125.6 (ArC3), 79.5 (C≡CH), 74.7 (C≡CH), 50.7 (H₂NCOCH₂), 50.2 and 49.8 (rotomer, COCH₂N(R)-2-naphthyl), 49.4 (CH₂COOH), 46.6 (COCH₂NH₂⁺CH₂), 35.4 (NH₂⁺CH₂C≡CH); ES TOF MS m/z (M)⁺ 411; Anal. for C₂₁H₂₃N₄O₅: Calc. Mass 411.1668. Found 411.1605.

8.3 Biological Evaluation - General

RPMI 1640 powder and foetal calf serum (FCS) used for cell culture experiments were purchased from Trace Bioscientific (NSW, Australia). PMA and protease free BSA were purchased from Sigma-Aldrich (USA). Mouse anti-human uPAR (#3936) monoclonal antibody and Spectrozyme uPA were obtained from American Diagnostica Inc (CT, USA). HMW-uPA fluorogenic substrate III (Z-Glu-Gly-Arg-AMC.HCl; Z = benzylxycarbonyl, AMC = amino-4-methylcoumarin) was purchased from Calbiochem, (USA). Alexa-uPA conjugate was constructed using the Alexa Fluor ® 488 labelling kit purchased from Molecular Probes Inc., USA. Incubation and wash buffers for all cell experiments consisted of Dulbecco’s phosphate buffered saline (PBS, 1mM CaCl₂, MgCl₂) and 0.1% BSA (pH 7.4). Previously described radioligand-binding assays showing nM potency of 83 and 84 also employed 0.1% BSA.²⁷⁰,²⁷⁵,²⁷⁷ Cells were maintained in a Heraeus Hera Cell incubator. Sterile
experiments were carried out in a Email Air Handling Biological Safety Cabinet. Centrifugation was performed with a Heraeus Megafuge 1.0R Centrifuge. Fluorescence assays were performed using a BMG Labtech FLUOstar Optima plate reader, Software version 1.32. UV-Vis assays were performed using a Molecular Devices Spectramax Plus 384 plate reader (Software: Softmax Pro version 1.17, 2004).

8.4 Cell Culture

Human monocyte-like U-937 cells were used for all assays (American Tissue Culture Centre, USA). Cells were maintained in culture at 37 °C in a humidified incubator containing 5% CO₂ (Thermo Scientific) using pre-warmed RPMI 1640 media supplemented with 2 mM L-glutamine and 5% foetal calf serum (FCS, heat inactivated). Cells were passaged every 3-4 days to maintain a population > 1 x 10⁶ cells.mL⁻¹.

8.5 PMA Treatment

Cells were diluted in RPMI 1640 + 5% FCS to 2 x 10⁵ cells.mL⁻¹ and cultured for 7−8 h at 37°C. Solutions of PMA in DMSO were added to the cells to provide final PMA concentrations of 100 nM (final DMSO concentrations < 0.01%). Cells were then incubated for a further 16 h to induce optimal levels of uPAR expression.
8.6 Preparation of i-uPA

100 µL HMW-uPA (1 mg.mL⁻¹, 19 µM in distilled water) was reacted with Glu-Gly-Arg-chloromethyl ketone 100 µL (1 mM in distilled water) for 24 h at 4 °C to form i-uPA. Absence of activity was confirmed by treating i-uPA (5 nM) with Spectrozyme uPA chromogenic substrate (0.125 mM in distilled water) and monitoring (Spectramax Plus 384: Molecular Devices plate reader) for absence of colour development at 405 nM.

8.7 IC₅₀ Determinations

The IC₅₀ values (concentration (nM) required to inhibit 50% of uPA activity) were obtained by conducting a log transformation of the inhibitor concentrations. Data was normalised to a common scale, where 100% activity was equal to maximal uPA activity, indicated by the control samples containing HMW-uPA and substrate, but no antagonist. Values were calculated from logarithmic sigmoidal dose response curves using the variable slope parameter, generated from GraphPad Prism V. 5.01 software (GraphPad Software Inc.).
8.8 References


217. Aguirre-Ghiso, J. A.; Estrada, Y.; Liu, D.; Ossowski, L. ERK\(^{MAPK}\) Activity as a Determinant of Tumor Growth and Dormancy; Regulation by p38\(^{SAPK}\). *Cancer Res.* 2003, 63, 1684-95.


272. Li, Z.-B.; Niu, G.; Wang, H.; He, L.; Yang, L.; Ploug, M.; Chen, X. Imaging of Urokinase-Type Plasminogen Activator Receptor Expression Using a $^{64}$Cu-


312. Fong, S.; Doyle, M. V.; Goodson, R. J.; Drummond, R. J.; Stratton, J. R.; McGuire, L.; Doyle, L. V.; Chapman, H. A.; Rosenberg, S. Random Peptide

9. Appendix

uPA Enzyme Inhibition Assay Protocol

Materials

High Molecular weight uPA (ADI No. 128, USA)
1. Stock dissolved in dH$_2$O to give ~100,000U/mL (approx. 1 mg/mL = approx 19 µM). Make 10 µL aliquots and freeze at -70°C.
2. To make working stock, dilute concentrated stock 1:1000 in ice-cold Tris buffer = 200 IU/mL. Keep on ice until required.
   a. For a full 96 well plate will need ~ 8.6 mL enzyme mix. Dilute 10 µL concentrated stock into 8.99 mL
   b. Final assay concentration will be ~25 U/mL (~ 5 nM) final concentration (max [E] to use).

Spectrozyme uPA substrate (ADI No 244)
1. Stock dissolved in dH$_2$O to 5.0 mM. Aliquot and freeze at -20°C.
2. Use at 0.125 mM (final concentration in wells) for lower potency inhibitors (1:40 dilution)
   a. This requires ~ 1 mL per full 96 well plate

Tris Buffer Components
1. Make up 1.0 M Tris base (pH 8.8) stock, autoclave
2. Tween-20 diluted 1 in 10.
3. Aprotinin (Sigma A-1153)
   a. 7TIU/mg = 7000KIU/mg
   b. Dissolve in dH$_2$O to give 10 mg/mL = 70000KIU/mL = concentrated stock. Freeze at -20°C in 0.2 mL aliquots (as a minimum) (so as to freeze/thaw conc. stocks once only for further dilutions)
   c. To make working stocks dilute 0.2 mL conc. stock with 1.2 mL dH$_2$O (1 in 7 dilution) to give 10,000KIU/mL stock (~1.5 mg/mL). Make 14 x 100 µL aliquots and freeze at -20°C.
4. Working buffer: 0.05M Tris (pH 8.8), 0.01%v/v Tween-20, 10 KIU/mL (0.2 μM) aprotinin
   a. To make up 100 mL buffer (keeps for 1 week refrigerated):
      i. Take 5 mL 1M Tris pH 8.8 stock
      ii. Take 1 mL diluted Tween-20
      iii. Take 0.1 mL 10,000KIU/mL aprotinin stock
      iv. Make up to 100 mL using autoclaved dH2O

Inhibitors
1. Make up as 20 mM stocks in appropriate solvent (see HM/MK)

Method
1. Pre-book and pre-warm to 37°C the Spectromax plate reader. Use kinetic setting at 405nm over half an hour with reads every 30 s.

2. On day of experiment add 100 μL Tris buffer into wells A1 – H12 of a 96-well plate. Make serial dilutions of inhibitors (Use 1 in 5 initially, then retry using 1 in 10 if IC50 is sub-micromolar) in triplicate in this plate as follows (this can all be done at room temp):

<table>
<thead>
<tr>
<th>Dilutions plate</th>
<th>Dilution series</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor A</td>
<td>Tris buffer (μL)</td>
</tr>
<tr>
<td></td>
<td>Take 10μL stock</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
</tr>
</tbody>
</table>

3. Combine 250 μL spectrozyme soln. with 250 μL buffer. Add 10 μL of 2.5mM Spectrozyme uPA to all assay wells in plate (non blanks). Add 10 μL buffer to all wells containing blanks. Work from B to H using multichannel pipette with 3 tips. Gently tap plate to mix.
3. Working quickly in the Spectromax room, add 90µL of uPA (50IU/mL) to all assay wells. Gently tap plate to mix. This should all be done within 5 min.

4. Read plate immediately in Spectromax at 37 °C.

\textbf{Analysis}

1. Check data, note a timepoint from linear part of the curve (10 minutes is usually a good point for this assay).

2. Print off plate assay data from Spectromax and paste in lab book as permanent record of raw data.

3. Export data from Spectromax as txt. Import txt data into Excel.

4. Subtract blanks (this can also be done in Spectromax before exporting data).

5. Open Graphpad,
   a. Under New table and Graph - Choose XY
   b. Under Choose a Graph - Select “Points only” graph
   c. Under Sub columns for replicates or error values - Enter 3 replicate values for Y and plot mean +/- SD (or SEM if you want that too)
   d. Create
6. Paste your three y data points per x (inhibitor concentration in nM) in the sub columns

<table>
<thead>
<tr>
<th>Table format: XY</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>A:Y1</td>
<td>A:Y2</td>
</tr>
<tr>
<td>X</td>
<td>[l] nM</td>
<td>A:Y1</td>
<td>A:Y2</td>
</tr>
<tr>
<td>1</td>
<td>200000.000</td>
<td>0.044133</td>
<td>0.040693</td>
</tr>
<tr>
<td>2</td>
<td>40000.000</td>
<td>0.084667</td>
<td>0.126767</td>
</tr>
<tr>
<td>3</td>
<td>8000.000</td>
<td>0.227500</td>
<td>0.239200</td>
</tr>
<tr>
<td>4</td>
<td>1600.000</td>
<td>0.395167</td>
<td>0.288667</td>
</tr>
<tr>
<td>5</td>
<td>320.000</td>
<td>0.302867</td>
<td>0.312267</td>
</tr>
<tr>
<td>6</td>
<td>64.000</td>
<td>0.309457</td>
<td>0.317167</td>
</tr>
<tr>
<td>7</td>
<td>1.000</td>
<td>0.307500</td>
<td>0.300800</td>
</tr>
</tbody>
</table>

7. Click on = Analyse
8. Click on Normalize under “Transform, Normalize...”
   a. Under “How is 0% defined?” – Click on Y = 0
   b. Under “How is 100% defined?” – Click on Last value in each data set
   c. Present results as percentages
   d. Click off “Create a new graph of the results”

<table>
<thead>
<tr>
<th>X</th>
<th>A:Y1</th>
<th>A:Y2</th>
<th>A:Y3</th>
<th>B:Y1</th>
<th>B:Y2</th>
<th>B:Y3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>27.645</td>
<td>30.947</td>
<td>41.441</td>
<td>34.048</td>
<td>34.048</td>
<td>36.729</td>
</tr>
<tr>
<td>3</td>
<td>74.371</td>
<td>77.934</td>
<td>78.196</td>
<td>63.120</td>
<td>67.834</td>
<td>71.337</td>
</tr>
<tr>
<td>4</td>
<td>96.491</td>
<td>96.916</td>
<td>94.366</td>
<td>91.244</td>
<td>96.033</td>
<td>57.556</td>
</tr>
<tr>
<td>5</td>
<td>99.009</td>
<td>104.991</td>
<td>102.061</td>
<td>98.892</td>
<td>101.537</td>
<td>107.896</td>
</tr>
<tr>
<td>6</td>
<td>101.165</td>
<td>103.683</td>
<td>103.586</td>
<td>99.375</td>
<td>103.270</td>
<td>109.888</td>
</tr>
<tr>
<td>7</td>
<td>100.523</td>
<td>98.333</td>
<td>101.144</td>
<td>95.425</td>
<td>100.071</td>
<td>104.503</td>
</tr>
</tbody>
</table>

9. Click on = Analyse
10. Click on Transform
    a. Under “Function List” – click on Standard Functions
    b. Then click on Transform X values using X=log[X] (this usually works best if your x values span over an order of magnitude). This means that all x values will have to be added into your XY table as greater than 1. The zero inhibitor will have to be added in as 1 (as log10 of 1 = 0).
    c. Click off “Create a new graph of the results”
11. Click on Analyse

12. Click on Non-linear regression under XY analyses (you can also do column stats and/or row means if you wish/need to)

   a. Choose Dose-response –Inhibition, then log(inhibitor) vs response

   b. Click on Constrain (at top of page) and set Top as a constant = 100

   c. Click on OK

Check the resulting graph. You can re-format the graph and do a lot of other things with the graph if required.

---

**Transform of Normalize of Data 1**

![Graph](image)

- Amiloride
- HM041
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amiloride</td>
<td>HMD41</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>1</td>
<td>log(inhibitor) vs. response</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Best-fit values</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Bottom</td>
<td>2.210</td>
</tr>
<tr>
<td>4</td>
<td>Top</td>
<td>= 100.0</td>
</tr>
<tr>
<td>5</td>
<td>LogIC50</td>
<td>4.348</td>
</tr>
<tr>
<td>6</td>
<td>IC50</td>
<td>22262</td>
</tr>
<tr>
<td>7</td>
<td>Span</td>
<td>= 97.79</td>
</tr>
<tr>
<td>8</td>
<td>Std. Error</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Bottom</td>
<td>3.216</td>
</tr>
<tr>
<td>10</td>
<td>LogIC50</td>
<td>0.05071</td>
</tr>
<tr>
<td>11</td>
<td>95% Confidence Intervals</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Bottom</td>
<td>-4.521 to 8.941</td>
</tr>
<tr>
<td>13</td>
<td>LogIC50</td>
<td>4.242 to 4.454</td>
</tr>
<tr>
<td>14</td>
<td>IC50</td>
<td>17450 to 23451</td>
</tr>
<tr>
<td>15</td>
<td>Goodness of Fit</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Degrees of Freedom</td>
<td>19</td>
</tr>
<tr>
<td>17</td>
<td>$R^2$</td>
<td>0.9877</td>
</tr>
<tr>
<td>18</td>
<td>Absolute Sum of Squares</td>
<td>300.4</td>
</tr>
<tr>
<td>19</td>
<td>Sy.x</td>
<td>3.976</td>
</tr>
<tr>
<td>20</td>
<td>Constraints</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Top</td>
<td>Top = 100.0</td>
</tr>
<tr>
<td>22</td>
<td>Number of points</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Analyzed</td>
<td>21</td>
</tr>
</tbody>
</table>
Fluorostar Optima uPA:uPAR Antagonist Assay Protocol

Materials

Mouse anti-human uPAR monoclonal antibody (3936) at 6897 nM.

U-937 Cells in RPMI 1640 + 5% FCS

Phorbol 12-myristate 13-acetate (PMA) 1 mg.mL\(^{-1}\)

Round Bottom microplate

Flat Bottom microplate

Black Fluor Plate

1x PBS

Alexa uPA

Propidium Iodide

High Molecular weight uPA (ADI No. 128, USA)
1. Stock dissolved in dH\(_2\)O to give ~100,000U/mL (approx. 1 mg/mL = approx 19 \(\mu\)M). Make 10 \(\mu\)L aliquots and freeze at -70\(^\circ\)C.
2. To make working stock, dilute concentrated stock to 16 nM in working buffer. Keep on ice until required.
   a. For a full 96 well plate you will need to combine 8.42 \(\mu\)L uPA stock with 9992 \(\mu\)L working buffer.
   b. Final assay concentration will be ~40 U/mL (~ 8 nM) final concentration (max [E] to use).

PBS Buffer Components
1. BSA 0.1%, MgCl\(_2\) and CaCl\(_2\).
2. Keep on ice.

Urokinase III Fluorogenic Substrate (Calbiochem No. 672159)
1. Stock dissolved in dH\(_2\)O to 5 mM.
2. To make working stock, dilute concentrated stock to 1 mM in PBS. Keep away from strong light.
   a. For a full 96 well plate you will need to combine 1 mL stock with 4 mL PBS.
   b. Final assay concentration will be 0.5 mM.

Antagonists
1. Make up stocks in appropriate solvent (see HM/MK)

Method
1. Perform a count with a haemocytometer to ensure there are enough (at least 5 million for a full 96 well plate) viable U937 cells available. Book Fluorostar and Flow Cytometer.
2. Stimulate uPAR expression using PMA at a final concentration of 100 nM at least 12h prior to harvesting. To achieve this, initially dilute 10 μL PMA stock solution in 990 μL RPMI and then add a volume of this solution to the tissue culture flask to achieve a 1 in 100 dilution.
3. Prepare antagonist dilution series (6 replicates) and antibody 3936 (133 nM in triplicate) in flat bottom 96 well plate just prior to harvesting cells. Keep on ice.
4. To confirm uPAR expression, harvest cells by centrifugation (300 x g, 5 min, 4 °C) and resuspend in working buffer to a concentration of 1 million cells per mL. Aliquot 100 000 cells in 6 wells of a U-bottom 96 well plate. Centrifuge and remove supernatant. Incubate 3 wells with 40 nM Alexa uPA in the dark on ice for 20 min. Incubate the 3 remaining wells with buffer only as an autofluorescence set.
5. Following incubation, centrifuge cells (300 x g, 5 min, 4 °C) to remove unbound reagents, discarding supernatant. Resuspend in PBS. Repeat and resuspend, adding propidium iodide to all samples prior to flow cytometer analysis.
**Assay**

1. Aliquot cells (50 µL.well⁻¹) into a round bottom microplate including wells for all antagonist dilutions as well as triplicate wells for the antibody and background (endogenous uPA) controls. Centrifuge (300 x g, 5 min, 4 °C) and remove supernatant. Transfer 50 µL of the antagonist dilution series and antibody triplicates from the flat bottom plate to the corresponding wells of the U-bottom plate containing cell pellets. Add 50 µL buffer to background control wells. Resuspend all cells and incubate on ice for 30 min.

2. Add 50 µL uPA solution to all assay wells and incubate on ice for 30 min. Prepare fluorostar settings, checking that reader configuration is fluorescence, reading plate from above. Go to test protocols and select “uPA fluor substrate”. Ensure plate layout matches assay plate and adjust number of flashes required (~20). Check timing and select ok when finished.

3. Remove unbound reagents by centrifugation (300 x g, 5 min, 4 °C). Resuspend cells in PBS. Repeat centrifugation at room temperature and resuspend cells in 50 µL PBS.

4. Transfer all cells to the corresponding wells of a black fluor plate. Rupture any bubbles that form during transfer. Examine round bottom plate to ensure all cells are transferred. Add 50 µL fluorometric substrate solution to all wells, open tray on plate reader and insert fluor plate.

5. Go to measure and select “uPA fluor substrate”. Click “gain adjustment” and adjust to 80% of the value obtained. Click “start run”.

**Analysis**

1. Check data, note a timepoint from linear part of the curve (10 minutes is usually a good point for this assay).

2. Export data into Excel.

3. Subtract autofluorescence control from all wells.

4. Open Graphpad,
   a. Under New table and Graph - Choose XY
b. Under Choose a Graph - Select “Points only” graph

c. Under Sub columns for replicates or error values - Enter 3 replicate values for Y and plot mean +/- SD (or SEM if you want that too)

d. Create

5. Paste your three y data points per x (inhibitor concentration in nM) in the sub columns

6. Click on = Analyse

7. Click on Normalize under “Transform, Normalize…”
   a. Under “How is 0% defined?” – Click on Y = 0
   b. Under “How is 100% defined?” – Click on Last value in each data set
   c. Present results as percentages
   d. Click off “Create a new graph of the results”

8. Click on = Analyse

9. Click on Transform
   a. Under “Function List” – click on Standard Functions
   b. Then click on Transform X values using X=log[X] (this usually works best if your x values span over an order of magnitude). This means that all x values will have to be added into your XY table as greater than 1. The zero inhibitor will have to be added in as 1 (as log10 of 1 = 0).
   c. Click off “Create a new graph of the results”

10. Click on = Analyse

11. Click on Non-linear regression under XY analyses (you can also do column stats and/or row means if you wish/need to)
   a. Choose Binding - competitive, then One site – Fit logIC50
   b. Click on Constrain (at top of page) and set Top as a constant = 100
   c. Click on OK

Check the resulting graph. You can re-format the graph and do a lot of other things with the graph if required.