Synthesis and evaluation of dual-targeting uPA/NHE1 inhibitors from the K+-sparring diuretic amiloride

Benjamin John Buckley
University of Wollongong

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Synthesis and evaluation of dual-targeting uPA/NHE1 inhibitors from the $K^+$-sparing diuretic amiloride

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by

Benjamin John Buckley
Bachelor of Biotechnology (Advanced, Hons I)

under the supervision of

Prof. Marie Ranson and Assoc. Prof. Michael Kelso

School of Biological Sciences
Illawarra Health and Medical Research Institute

2017
DECLARATION

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfillment of the degree of Doctor of Philosophy. It does not include any material published by another person except where due reference is made in the text. The experimental work described in this thesis is original work and has not been submitted for a degree at any other university.

Benjamin John Buckley,

13th April 2017
ACKNOWLEDGEMENTS

‘Work is love made visible’.

-Kahlil Gibran.

‘Some may never live, but the crazy never die’.

-H.S.T.

Well, it’s been a wild ride. Marie, I am indebted to you. Your care and guidance have made this a thoroughly enriching experience. Your support through the lumps and bumps made it all livable (and lovable). Your dedication to inclusiveness and the promotion of your students at every possibility is truly admirable (and very much appreciated). Thank you, it’s been allot of fun!

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CONTRIBUTIONS

Unless otherwise stated all work presented herein was performed by B. Buckley. Work contributed by others is acknowledged in the following table.

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<th>Section (percentage contribution)</th>
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<tr>
<td>Dr. Simon Cook</td>
<td>Performed TLSP selectivity experiments for compound 42.</td>
<td>Chapter 2, Section 2.3, Figure 2.10, p. 79 (100%). Table 2.4, p. 80 (33%).</td>
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<td>Dr. Karen Fildes</td>
<td>Performed TLSP selectivity experiments for 43 and 49.</td>
<td>Chapter 2, Section 2.3, Table 2.4, p. 80 (66%).</td>
</tr>
<tr>
<td>Dr. Longuang Jiang</td>
<td>Performed crystallization and X-ray diffraction experiments and solved X-ray crystal structures for all compounds. All interpretation and molecular graphics were produced by B. Buckley.</td>
<td>Chapter 2, Figure 2.1, p. 56. Section 2.4, p. 86 Chapter 3 p. 101-118.</td>
</tr>
<tr>
<td>Mrs. Elahe Minaei</td>
<td>Performed vinblastine and paclitaxel combination experiments. Experimental design, graphing and data interpretation performed by B. Buckley.</td>
<td>Chapter 4, Section 4.4, p 142-153.</td>
</tr>
<tr>
<td>Miss Jodie Wilkinson</td>
<td>Performed cuvette-based NHE1 inhibition assays.</td>
<td>Chapter 5, Section 5.3, Figure 5.4, p. 164 (33%). Section 5.3, Table 5.3, p. 166 (60%).</td>
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**Journal articles**


**Manuscripts in preparation**


Presentations


4. Developing potent uPA/NHE1 inhibitors with high selectivity over related TLSPs from the clinical drug amiloride, 9th General Meeting of the International Proteolysis Society Early Career Researcher Meeting, Penang, Malaysia, October 2nd and 3rd 2015. Invited presentation.

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Patents

ABSTRACT

Identification of proteins whose expression and/or activities are upregulated in clinical neoplasias provides insight into the mechanisms underlying carcinogenic transformation and malignancy. Validation of such proteins as biomarkers through correlation to patient outcomes can provide novel targets for the development of selective anticancer therapies. Therapeutics that target phenotypic alterations common to a variety of cancers could have a broad impact on the treatment of cancer as a whole. Increased invasive capability accompanying upregulated proteolytic activity is widely recognized as a critical determinant of metastatic potential and is commonly observed in progressive disease. The urokinase plasminogen activator (uPA) is a key contributor to cell invasiveness through the activation of plasminogen and downstream proteases. These proteolytic actions endow cancerous cells with the ability to degrade extracellular matrix and basement membrane components, facilitating their invasive spread. uPA and its cognate cell-surface receptor (uPAR) are of prognostic relevance in a variety of aggressive cancers where their overexpression predicts adverse patient outcomes and metastatic potential.

A second phenotype observed in virtually all cancers is the reversal of transmembrane pH gradients relative to healthy tissues. Alkalization of the intracellular pH ($p$H$_i$) and the concomitant acidification of the extracellular space create a permissive environment that promotes further dysregulated growth, insensitivity to apoptotic checkpoints and increased motility and invasiveness that propel transformed cells towards malignancy and metastasis. The Na$^+$/H$^+$ exchanger isoform 1 (NHE1) is upregulated in a wide variety of tumours and is a primary contributor to the cancer-specific reversal of transmembrane pH gradients. Hyperactivation of NHE1 is an early event in oncogene-driven carcinogenesis and has been shown to be essential for maintenance of transformed phenotypes and growth of tumour
xenografts in mice. Amiloride, a commonly prescribed oral potassium-sparing diuretic, presents a unique opportunity for medicinal chemistry optimization into dual-targeting uPA/NHE1 inhibitors as anticancer therapeutics as it is a moderate inhibitor of both targets and shows robust anticancer side activities in vivo.

5-substituted amiloride analogues are known to show greatly improved NHE1 potency relative to amiloride (e.g. 5-(N,N-hexamethylene)amiloride 27 (HMA) >520-fold) whilst exhibiting reduced activity against epithelial sodium channels (ENaCs), the target responsible for the diuretic and antkalliuretic properties of the parent drug. This PhD project explored structure-activity relationships (SAR) of 6-HMA analogues as inhibitors of uPA and NHE1, with the overall aim of identifying 6-HMA analogues with high (nM) potency against both targets.

Significant increases in uPA inhibitory activity were achieved through addition of various heteroaryl substituents at the 6-position of HMA (Chapter 2). Two structurally distinct lead scaffolds with divergent physicochemical properties were discovered that showed excellent inhibitory potency against uPA (43 IC_{50} = 143 nM, 50 IC_{50} = 69 nM). X-ray crystallographic analysis (Chapter 3) revealed that the potency gains were due to increased binding interactions in the uPA S1β subsite. Biochemical assays demonstrated a high degree of selectivity for uPA over closely related trypsin-like serine proteases (Chapter 4). In vitro assays revealed a diverse spread for cytotoxic activities of the lead inhibitors across a variety of cell lines.

A novel fluorescence plate reader assay was developed for measuring NHE1 inhibition and was used to characterize SAR around the HMA scaffold (Chapter 5). In general, introduction of (hetero)aryl substituents at the 6-position did not decrease potency of HMA analogues against
NHE1, and in some cases yielded potent uPA/NHE1 dual-targeting compounds (48 uPA IC₅₀ = 175 nM; NHE1 IC₅₀ = 397 nM), along with uPA-selective inhibitors (49 uPA IC₅₀ = 86 nM; NHE1 IC₅₀ = 21 μM).

Research performed under contract by Charles River Laboratories using a selection of 6-HMA analogues and HMA revealed the absence of activity against ENaCs, the diuretic and antikeliuretic target of amiloride.

In summary, this work reports the discovery of potent, selective and non-toxic uPA/NHE1 dual targeting 6-HMA analogues suitable for entry into in vivo anticancer models. Further work should involve determining the absence of diuretic/anti-kaliuretic effects in mice to ensure the successful loss of these activities.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ADMET</td>
<td>Absorption/distribution/metabolism/excretion/toxicity</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>AML</td>
<td>Amiloride</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATF</td>
<td>Amino terminal fragment</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid-sensing ion channel</td>
</tr>
<tr>
<td>AUD</td>
<td>Australian dollar</td>
</tr>
<tr>
<td>BCECF-AM</td>
<td>2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluoresceinacetoxymethyl ester</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-dependent aspartate-directed protease</td>
</tr>
<tr>
<td>CI</td>
<td>Cell index</td>
</tr>
<tr>
<td>CRP</td>
<td>Cariporide</td>
</tr>
<tr>
<td>Cyt-c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DPP</td>
<td>Dipeptidyl peptidase</td>
</tr>
<tr>
<td>DMA</td>
<td>5-(N,N-dimethyl)amiloride</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECS</td>
<td>Extracellular space</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>EIPA</td>
<td>5-(N-ethyl-N-isopropyl)amiloride</td>
</tr>
<tr>
<td>Em</td>
<td>Emission</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin/radixin/moesin family</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>Ex</td>
<td>Excitation</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FIGO</td>
<td>International federation of gynaecology and obstetrics</td>
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<tr>
<td>FJIRSM</td>
<td>Fujian Institute of Research into the Structure of Matter</td>
</tr>
<tr>
<td>$F_{oral}$</td>
<td>% oral bioavailability</td>
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<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HAC</td>
<td>Heavy atom count</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney (cells)</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>HMA</td>
<td>5-(N,N-hexamethylene)amiloride</td>
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<td>HMW</td>
<td>High molecular weight</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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HuPA  Human urokinase plasminogen activator
HUVEC  human umbilical vein endothelial cells
$K_i$  Inhibitory constant
$K_m$  Michaelis constant
KO  Knockout
LDH  Lactate dehydrogenase
LDLR  Low density lipoprotein
LE  Ligand efficiency
LLE  Lipophilic ligand efficiency
MCT  Monocarboxylate transporter
MDR  Multidrug resistant
MIBA  5-\((N\text{-methyl-N-isobutyl})amiloride\)
MIC  Minimum inhibitory concentration
MMP  Matrix metalloproteinase
MS  Mass spectrometry
MTS  3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MuPA  Murine urokinase plasminogen activator
MW  Molecular weight
NCE  New chemical entity
NCX  Na\(^+\)/Ca\(^{2+}\) exchanger
NHE  Na\(^+\)/H\(^+\) exchanger
NMR  Nuclear magnetic resonance
NSCLC  Non-small cell lung carcinoma
OxPhos  Oxidative phosphorylation
PBMC  Peripheral blood mononuclear cells
Pac  Paclitaxel
PAI  Plasminogen activator inhibitor
PAS  Plasminogen activation system
PDB  Protein data bank
$pHe$  Extracellular pH
$pHi$  Intracellular pH
PSA  Polar surface area
RFU  Relative fluorescence units
RM  Reaction mixture
RNA  Ribonucleic acid
rpm  Revolutions per minute
RP-HPLC  Reverse-phase high performance liquid chromatography
RT  Room temperature
SAR  Structure-activity relationship(s)
SEM  Standard error of the mean
Serpin  Serine protease inhibitor
SM  Starting material
SOSA  Selective optimization of side-activity
TDR  Totally drug resistant
TFA  Trifluoroacetic acid
TIF  Telomerase-immortalized fibroblasts
TLC  Thin layer chromatography
TLSP  Trypsin-like serine protease
TM  Transmembrane
<table>
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<th>Symbol</th>
<th>Term</th>
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<tr>
<td>$t_{1/2}$</td>
<td>Plasma half-life</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>tPSA</td>
<td>Topographical polar surface area</td>
</tr>
<tr>
<td>UOW</td>
<td>University of Wollongong</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAS</td>
<td>Urokinase plasminogen activation system</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>USD</td>
<td>United States dollar</td>
</tr>
<tr>
<td>$V_0$</td>
<td>Initial rate of enzymatic reaction</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Vin</td>
<td>Vinblastine</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal rate of enzymatic reaction</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
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CHAPTER 1: Introduction

1.1 Overview

Cancer is the leading cause of death in the developed world and the second largest cause of death in developing countries. Cancer occurs when cells progressively accumulate growth promoting attributes that confer a survival advantage over neighbouring cells, resulting in dysregulated and disordered proliferation. The reigning paradigm in cancer biology over the past 25 years describes cancer as a genetic disease arising from mutations in genomic sequences that cause activation of growth-promoting genes (oncogenes) or inactivation of growth regulators (tumour-suppressor genes). Increased understanding of non-genetic factors, including epigenetic alterations, heterotypic interactions between cancerous and stromal cells and dynamic interactions between cancer cells and the tumor metabolic environment, has challenged the framing of cancer as a purely genetic disorder. With this in mind, targeting phenotypes and processes common to the majority of cancers is an attractive approach towards developing new anticancer agents with broad utility. Drugs able to simultaneously inhibit multiple cancer-associated processes could dramatically improve cancer treatment.

The urokinase plasminogen activator (uPA) is a trypsin-like serine protease that is overexpressed in a wide variety of cancers and plays a key role in cell invasiveness and metastasis. uPA functions to activate plasminogen to plasmin and the subsequent activation of downstream proteases (e.g. pro-MMPs and pro-cathepsins) facilitates degradation and broad-scale remodeling of the extracellular matrix (ECM) and basement membrane (BM), serving to promote cancer cell invasiveness and ultimately metastatic spread.
One phenotype observed in virtually all cancers is reversal of the pH gradient that exists across the cell membrane. Healthy cells typically feature a cytosolic pH ($pH_i$) lower than that of their extracellular environment ($pH_e$). In cancer cells, the gradient is reversed, with the interior of cells becoming more alkaline than the immediate surroundings. Alkalization of the cytosol and concomitant acidification of the tumour microenvironment has been shown to be an early event in carcinogenesis and essential for development and maintenance of the cancerous state. A key contributor to transmembrane pH gradient reversal in cancer cells is the Na$^+$/H$^+$ exchanger isoform 1 (NHE1). NHE1 facilitates the isoelectronic exchange of intracellular H$^+$ for extracellular Na$^+$ in a 1:1 ratio. NHE1 becomes constitutively activated in neoplastic cells, resulting in acidification of the tumor microenvironment and alkalinization of the cytosol, which both contribute to carcinogenesis and metastasis. The lowered $pH_e$ of the tumour microenvironment decreases avidity of cell-extracellular matrix (ECM) interactions, activates acid-dependent proteases, including members of the urokinase plasminogen activation system (uPAS), matrix metalloproteinases (MMPs) and cysteine/aspartyl cathepsins.

This chapter reviews the role of uPA and NHE1 in carcinogenesis and metastasis and highlights the potential for dual-inhibition of both targets as a novel anticancer strategy.

### 1.2 Overview of cancer metastasis

Modern cancer chemotherapy suffers from an inability to successfully treat disseminated versus localized tumours. In general, patients with localized tumours have good long-term prognoses following surgical excision and adjuvant radio- or chemotherapy. However, the outlook for patients with metastatic disease is much less optimistic. Formation of
secondary tumours via metastatic spread is responsible for ~ 90% of cancer-associated deaths.\textsuperscript{6,15}

Metastasis is a multistep process where cancer cells disseminate via the circulatory and lymphatic vasculature from their site of origin (primary tumour) to distant sites in the body, where they establish secondary tumours in vital organs (Leber & Efferth 2009, Figure 1.1).

\textbf{Figure 1.1:} Overview of cancer metastasis. Altered expression of cell adhesion molecules and cytoskeletal proteins initiates a shift to a motile mesenchymal phenotype (EMT = epithelial to mesenchymal transition). Cells that have undergone EMT are able to invade through the local stroma and intravasate into blood vessels, allowing transport away from the primary site. Extravasation out of the bloodstream and into secondary tissues can trigger reversion to an epithelial phenotype. This process results in the establishment of metastases in distant organs. Figure adapted from reference 15.

An early event in the metastatic cascade is growth of new vasculature in the tumour mass; a process known as angiogenesis. In healthy tissues, vascularization is tightly regulated by balanced expression of pro- and anti-angiogenic factors (e.g. pro: vascular endothelial growth factor (VEGF), anti: angiostatin). This equilibrium is known as the ‘angiogenic switch’ and is influenced by a wide range of metabolic (e.g. $pO_2$, pH) and mechanical (e.g. compression) stressors and genetic alterations (e.g. oncogene-driven expression of pro-angiogenic signals, Figure 1.2).\textsuperscript{16,17,18}
Figure 1.2: The angiogenic switch. Angiogenesis is regulated by a dynamic balance between pro-angiogenic ‘activators’ and anti-angiogenic ‘inhibitors’. Environmental stimuli like pH and O₂ concentration influence the balance through up/downregulation of inhibitors and activators. VEGF = Vascular Endothelial Growth Factor, FGF = Fibroblast Growth Factor, PDGF-β = Platelet-derived Growth Factor subunit β, EGF = Epidermal Growth Factor, LPA = Lysophosphatidic acid. Figure adapted from reference 17.

Many of these alterations are observed to occur simultaneously in solid tumours (e.g. accumulation of acidic metabolites due to increased glycolytic metabolism, compressive stress due to hyperproliferative and disordered cell growth and overexpression of VEGF).¹⁹,²⁰ This confluence of factors serves to switch the balance toward formation of new vasculature in the tumour mass. The circulatory system and lymphatic network satiates the increasing metabolic needs of the growing tumour mass and provides invasive cells with a pathway for disseminating to distant sites and seeding secondary metastases.²¹

The growth of new blood vessels into tumours often occurs in a disordered and dysregulated manner, creating a structurally and functionally aberrant microvasculature that is unable to
adequately supply O₂ and nutrients to the tissue or remove metabolic waste products. This deficiency produces regions of hypoxia and acidified tumour microenvironments, which drive carcinogenic progression.¹⁸,²²

Genetic mutations and metabolic alterations coinciding with angiogenesis create an invasive phenotype in cancer cells, where they are able to mobilize and spread throughout the body. Invasive cells can remodel their extracellular environment through overexpression of proteolytic enzymes (e.g. uPA, matrix metalloproteinases (MMP) 3, 9, 10, cathepsins B & D), facilitating degradation of ECM/BM components.²³,²⁴,²⁵,²⁶ Accompanying this is a shift from a sedentary epithelial phenotype to a motile fibroblastic phenotype (EMT).²⁷

In normal epithelia the continued production of pro-survival signals is heavily dependent upon maintenance of close contacts between neighbouring cells and the basal lamina. Through altered expression of cell adhesion molecules (e.g. integrins, cadherins, selectins), cells undergoing EMT are able to avoid the programmed cell death that accompanies loss of cell-cell and cell-BM adhesion (anoikis).²²,²⁸-³¹ This shift allows transformed cells to take on a fibroblastic phenotype that is conducive to motile spread of cells away from the primary tumour (refer to Figure 1.1). Together with the enhanced proteolytic activity associated with tumours, these characteristics allow invasive cells to make a path through the primary stroma and intravasate into the circulatory and lymphatic vasculature. Once in the circulation, disseminated tumour cells can extravasate and establish life-threatening secondary tumours; a process that is often accompanied by reversion back to an epithelial phenotype (MET, see Figure 1.1).³²,³³
1.2.1 The urokinase plasminogen activation system (uPAS) and its role in cancer

A central contributor to the process of invasion and metastasis is the trypsin-like serine protease uPA, a component of the uPAS.\textsuperscript{34,35} The fundamental components of the uPAS are uPA, its cognate cell-surface receptor uPAR and the endogenous serine protease inhibitors (serpins) plasminogen activator inhibitor (PAI)-1, -2 and -3 (Figure 1.3).\textsuperscript{7,34,36}

![Diagram of the urokinase plasminogen activation system (uPAS)](image)

**Figure 1.3:** Schematic of the urokinase plasminogen activation system (uPAS). Solid arrows represent activation. ECM = Extracellular matrix, MMP = Matrix metalloproteinase.

The uPAS is an endogenous enzyme cascade that plays a fundamental role in fibrinolysis, tissue remodeling and cell migration.\textsuperscript{5,34} Pro-uPA, the inactive zymogen of uPA, is a single-chain 53 kDa multidomain protein consisting of 411 amino acid residues (Figure 1.4).\textsuperscript{37-40} Upon excretion from cells, cleavage of pro-uPA at residues Lys\textsuperscript{158}-Ile\textsuperscript{159}, primarily by the autocatalytic activity of uPA but also by extracellular proteases (e.g. cathepsin B, kallikrein, trypsin) yields catalytically active, high molecular weight (HMW)-uPA, also referred to as ‘two-chain’ uPA.\textsuperscript{5,41} Cleavage at this position forms the N-terminal (\(\alpha\)) and C-terminal (\(\beta\)) chains of
HMW-uPA, which are joined by a single disulfide bridge between Cys148 and Cys279. The α-chain consists of a kringle domain and an epidermal growth factor-like domain, the amino terminal fragment (ATF) of which mediates interactions with its cell-surface receptor uPAR. The β-chain contains the serine-protease domain, which is formed by the antiparallel arrangement of two subdomains, each composed of six β-strands, with the interface forming the active site of the protease.

**Figure 1.4:** Domain structures of: A) Pro-uPA and B) active-uPA. The single chain zymogen pro-uPA is activated by a variety of extracellular proteases (e.g. cathepsin B, kallikrein, trypsin) through cleavage at Lys^{158}-Ile^{159} to yield active ‘two-chain’ uPA.
uPAR is an atypical multidomain member of the Ly-6 family.\textsuperscript{45} The protein lacks a transmembrane domain, but is able to adhere to the cell surface via a C-terminal glycosyl phosphatidylinositol (GPI) anchor that is attached during endoplasmic reticulum (ER) maturation.\textsuperscript{46} Mature uPAR is 283 amino acids in length and has a molecular weight varying between 55-60 kDa, depending on its glycosylation state. uPAR features three homologous domains (D1-D3) that mediate interactions with uPA and ECM components (e.g. vitronectin).\textsuperscript{47} A highly conserved pattern of intradomain disulphide bonds creates the 3D structure necessary for high affinity binding of the ATF to uPAR.\textsuperscript{5}

uPA displays a relatively narrow substrate scope, selectively cleaving plasminogen at the Arg\textsuperscript{561}-Val\textsuperscript{562} peptide bond to reveal the active, broad spectrum serine protease plasmin.\textsuperscript{42,48} Plasmin is also a feedback activator of Pro-uPA. Plasminogen binds to cell surfaces predominantly via interactions between exposed C-terminal Lys residues and binding sites found within kringle domains 1,4 and 5.\textsuperscript{37,49} A variety of multifunctional cell-surface proteins (e.g. α-enolase, dipeptidyl peptidase-4, tissue factor apoprotein, Plg-R\textsubscript{KT}) bind to plasminogen with high affinity.\textsuperscript{41,50-52} The endogenous inhibitor α\textsubscript{2}-antiplasmin regulates free plasmin activity through interactions with the kringle domain Lys binding sites of plasmin(ogen).\textsuperscript{7,53} These inhibitory sites are obscured upon binding to cell surfaces, preventing inactivation of surface-bound plasmin.\textsuperscript{54} Cell surface binding of plasminogen via Lys residues also initiates conformational changes that increase activation by uPA.\textsuperscript{55}

Binding of Pro-uPA to uPAR co-localizes the complex at the cell surface, initiating a reciprocal activation loop that generates more plasmin and uPA.\textsuperscript{56} Once activated, plasmin degrades a variety of matrix proteins (e.g. fibrin, fibronectin, laminins, aggrecan and assorted proteoglycans) and activates numerous matrix metalloproteinases (e.g. MMPs 1, 2, 3, 9, 10,
13 and 14) and cathepsins that show broad specificity for many ECM and BM components.\textsuperscript{25,26,57,58} uPA-dependent plasmin-mediated activation of this cascade effectively triggers a proteolytic storm in the pericellular environment, leading to degradation of surrounding ECM.\textsuperscript{39}

In the context of cancer, overexpression of uPAS allows cancer cells to initiate proteolysis of the local ECM/BM barriers that confine them within the primary tumour mass, while at the same time liberating latent ECM-associated growth (e.g. TGF-β and Hepatocyte Growth Factor (HGF) and pro-angiogenic factors (e.g. VEGF and FGF-2)).\textsuperscript{59,60} Once activated, these factors act in a paracrine manner on neighbouring cells, promoting proliferation, angiogenesis and further uPA/uPAR overexpression to amplify invasive potential.\textsuperscript{61,62}

uPAS components also regulate cell adhesion and motility via mechanisms distinct from proteolytic activation of plasminogen.\textsuperscript{63} uPA binding to uPAR modulates integrin associations with vitronectin proteins present in the ECM. This occurs through uPAR interactions with the α subunit of α₃β₁, α₅β₁, α₄β₃ and α₄β₅ integrins.\textsuperscript{38,46} uPAR-mediated modulation of integrin binding alters cell-ECM adhesion and promotes migration via the integrin-mediated activation of mitogen-activated protein kinases (MAPK) Erk 1 and 2.\textsuperscript{64-66} In addition, activation of the Erk/MAPK signaling pathway via uPAR-integrin binding causes deactivation of the growth inhibitory p38/MAPK pathway, promoting cell proliferation.\textsuperscript{67,68} uPAR also modulates cell adhesion via direct association of its D1 and D2 domains with the somatomedin-B domain of vitronectin.\textsuperscript{47,69,70}

uPA is efficiently inhibited by three serpins, PAIs 1, 2 and 3. Serpins are highly conserved, irreversible inhibitors of serine and cysteine proteases that circulate in an unstable high-
energy conformation. Reaction of the protease active site with the serpin reactive center loop initiates a rapid conformational change in the complex that distorts the shape of the His57, Asp102, Ser195 catalytic triad, shutting down proteolysis.\textsuperscript{71} Covalent inactivation of uPAR-bound uPA by serpins PAI-1 and PAI-2 leads to internalization of the entire complex via the Low Density Lipoprotein related (LDLR) protein-1 and other endocytosis receptors of the LDLR family.\textsuperscript{72,73} Following lysosomal degradation of the complex, uPAR is recycled back to the cell surface.\textsuperscript{40} In spite of its physiological role as an inhibitor of the uPAS, the paradoxical protumourgenic effect of PAI-1 has been attributed to various interactions with ECM components and endocytosis/signaling co-receptors that promote tumour growth and metastasis.\textsuperscript{40}

1.2.2 uPA as an anticancer target

The prognostic relevance of overexpressed uPAS components in relation to tumour aggressiveness has been established over the past three decades. In 1988, Duffy et al. reported a correlation between uPA activity in primary breast carcinomas and tumour size and the number of auxiliary node metastases.\textsuperscript{74,75} Correlations between uPA, uPAR and PAI-1 overexpression and poor patient prognosis (i.e. shortened disease-free interval and shortened overall survival) have been since observed in breast, ovarian, endometrial, cervical, colorectal, prostate, gastric, head and neck and renal cancers.\textsuperscript{76-84}

The prognostic relevance of uPAS biomarkers is strongest in breast cancer, where high levels of uPA and PAI-1 are better predictors of disease progression than commonly used clinical metrics, including patient age, hormone receptor status, tumour grade and size and HER-2/neu expression.\textsuperscript{85,86} Two retrospective analyses of data pooled from 2780 and 8377 patients, respectively, and a randomized multicenter clinical trial have generated Level 1
evidence (i.e. highest possible) supporting the use of uPA and PAI-1 as prognostic biomarkers in breast cancer.\textsuperscript{76,87-89} Clinically, uPA and PAI-1 can be used to stratify node-negative breast cancer patients who would benefit from adjuvant chemotherapy (intermediate-high expression in primary tissue) from those for whom chemotherapy may be unnecessary (low expression in primary tissue).\textsuperscript{90,91} Recognition of the prognostic utility of uPA/PAI-1 quantitation is reflected in the breast cancer specific guidelines issued by the American Society of Clinical Oncology, the European Society of Clinical Oncology and the German Gynaecological Society.\textsuperscript{86,92} (AGO Website, \url{http://www.ago-online.de/en/guidelines-mamma/march-2016/}, accessed 29\textsuperscript{th} Jan 2017).

Despite the longstanding acceptance of uPA and PAI-1 as valid prognostic biomarkers for various breast cancers, widespread adoption in the clinic has not yet been realized.\textsuperscript{93} This failure has been partly attributed to the need for large amounts of freshly biopsied tissue for analysis using available diagnostic uPA/PAI-1 ELISAs.\textsuperscript{93} Improvements in assay sensitivity that enable use of formalin fixed and paraffin embedded tissue samples would facilitate routine diagnostic use.

uPA and uPAR Knockout (KO) mice show non-lethal developmental phenotypes and no impairment of fertility or altered fibrinolytic activity.\textsuperscript{16,94,95} In comparison, plasminogen KO mice show severely impaired development and other phenotypes, including aberrant fibrin deposition, impaired wound healing and formation of spontaneous thromboses.\textsuperscript{96,97} Redundancy in plasminogen activation in uPA/uPAR KO mice is thought to occur through compensatory activation by tPA.\textsuperscript{98} As such, it is predicted that on-target inhibition of uPA would not cause significant adverse side effects \textit{in vivo}. Importantly, uPA and uPAR-deficient mice show significantly reduced tumour growth and metastasis in xenograft models.\textsuperscript{99-101}
Specific inhibition of uPA proteolytic activity produces antitumour and antimetastatic effects in a variety of murine models (Table 1.1 and Figure 1.5). Xing et al. found that inhibition of uPA in Fisher rats bearing MAT-BIII syngeneic breast xenografts with benzothiophene-based inhibitor 1 decreased primary tumour volume by ~ 40 % and lung, liver and auxiliary lymph node metastases by up to 66% over a 14 day continuous i.p. infusion. An earlier study demonstrated a 60% reduction in lung metastases for C57b mice bearing B16 murine melanoma xenografts that had been previously treated with anti-uPA antibodies.

Figure 1.5: Structures of uPA inhibitors 1-4.

Following on from this work, Ossowski et al. reported a significant decrease in local primary site invasion in BALB/c mice bearing anti-uPA treated HEP3 human squamous cell carcinoma xenografts. More recently (2010), Hennekke et al. observed >40% decrease in lung metastases and more than 60% reduction in primary tumour volume with the peptidomimetic inhibitor CJ-463 2 in C57b/N mice bearing murine Lewis Lung carcinoma xenografts.

Similar results have been reported for compounds that show trypsin-like serine protease (TLSP) inhibitory activity beyond just uPA inhibitory effects. The combination of tranexamic acid 3 with cisplatin completely inhibited ascites formation and prolonged overall survival by
70% in BALB/c mice bearing HRA human cystadenocarcinoma xenografts. Treatment of BALB/c mice receiving SW1990 human pancreatic adenocarcinoma xenografts with the guanidine-containing broad spectrum TLSP inhibitory small molecule gabexate 4 resulted in a 50% decrease in liver metastases. Taken together, these findings support uPA as an attractive target for the development of novel anticancer drugs.

Table 1.1: Representative in vivo data demonstrating the anticancer effects of uPA inhibitors. TLSP = trypsin-like serine protease.

<table>
<thead>
<tr>
<th>Mode of inhibition</th>
<th>Model</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small molecule active site inhibitor – B428</td>
<td>Allograft with Mat B-III Rat breast carcinoma cells or Dunning 3227 Rat prostate carcinoma cells</td>
<td>Significant decrease in tumour volume and number of lung, liver and auxiliary node metastases.</td>
<td>103, 168</td>
</tr>
<tr>
<td>Anti-uPA antibodies</td>
<td>Xenograft with B16 melanoma cells preincubated with antibodies</td>
<td>Up to 60% ↓ in lung metastases</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Xenograft with HEp3 squamous cell carcinoma cells</td>
<td>Significant inhibition of local invasion at primary site</td>
<td>105</td>
</tr>
<tr>
<td>Peptidomimetic inhibitor – CJ-463</td>
<td>Xenograft with murine Lewis Lung Carcinoma cells</td>
<td>↓ number of lung metastases, ↓ primary tumour volume and number of metastatic nodules and clusters</td>
<td>106</td>
</tr>
<tr>
<td>Broad spectrum TLSP inhibitors</td>
<td>Xenoaft with HRA ovarian adenocarcinoma cells</td>
<td>Significant inhibition of ascites formation and prolonged survival</td>
<td>107</td>
</tr>
<tr>
<td>Small molecule – tranexamic acid in combination with cisplatin</td>
<td>Xenograft with SW1990 hepatic carcinoma</td>
<td>50% ↓ in number of mice showing liver metastases</td>
<td>108</td>
</tr>
</tbody>
</table>


1.3 Regulation of cellular pH in cancer and the role of NHE1

Regulation of transmembrane pH gradients is a tightly controlled aspect of cell physiology that involves the concerted action of multiple transmembrane ion transporters and enzymes. In the vast majority of cell types, intracellular pH (pHi) is slightly more acidic (pH 7.0-7.1) than the alkaline extracellular environment (pHe 7.3-7.4). In contrast, a key hallmark of transformed cells is a reversed transmembrane pH gradient, where pHi can exceed pHe by as much as one log unit (tumour pHi 7.1-7.7, tumour pHe 6.2-6.9). Acidification of the tumour microenvironment and the concomitant increase in pHi create a vicious cycle that promotes dysregulation of cell growth and progression towards malignancy. In cancerous cells, an assembly of proteins coordinate their actions to maintain the reversed pH gradient. These include H+-ATPases, monocarboxylate transporters (MCTs), HCO\textsubscript{3}⁻-transporters, carbonic anhydrases and the Na\textsuperscript{+}/H\textsuperscript{+}-exchanger isoform 1 (NHE1). NHE1 is considered an early and primary contributor to the dysregulated pH dynamics that fuel neoplastic progression.

NHE1 is a ubiquitously expressed transmembrane ion transporter that functions primarily to protect cells from intracellular acid. It is a 12-helical protein comprised of 815 amino acids. Residues 1-500 make up the N-terminal transmembrane domain responsible for transporter function. Residues 501-815 constitute the C-terminal cytoplasmic regulatory tail that features numerous serine/threonine phosphoregulatory sites (Figure 1.6). NHE1 is one of 10 characterized isoforms of the SLC9A subfamily of Na\textsuperscript{+}/H\textsuperscript{+}-exchanger proteins. Isoforms 2-5 and 10 show limited tissue and organellar distribution, whereas NHE1 is expressed in the plasma membrane of virtually all mammalian cell types.
Figure 1.6: Schematic of NHE1. Oncogenic modulation of the affinity of the C-terminal tail for intracellular protons leads to constitutive hyperactivation of NHE1 activity. Binding sites on the C-terminal tail for ezrin/radixin/moesin (ERM) family members enable interactions with cytoskeletal proteins, allowing NHE to mediate pseudopodial protrusion and directed cell motility. Figure adapted from reference 6.

NHE1 functions in a number of physiological processes, including regulation of cell volume, Na\(^+\) flux and glycolysis, alteration of functional states and promotion of cell growth, cellular proliferation and differentiation. The transporter's primary role, however, relates to maintenance of pH\(_i\) homeostasis.\(^{116-120}\)

NHE1 mediates an isoelectronic 1:1 exchange of extracellular Na\(^+\) for intracellular H\(^+\) ions, exploiting the inward Na\(^+\) electrochemical gradient to drive extrusion of protons against their
concentration gradient (Figure 1.6). In addition to their role as substrates in the exchange process, intracellular protons act as allosteric modulators of NHE1 activity.\textsuperscript{121}

Under normal conditions NHE1 exists in a quiescent state, becoming activated only when pH\textsubscript{i} drops below a threshold value, referred to as the activation ‘set point’.\textsuperscript{11} In transformed cells, alterations in the cytoplasmic H\textsuperscript{+}-allosteric binding site, believed to occur via phosphoinositide-3-kinase-dependent mechanisms, increase affinity for intracellular protons to lower the set point for exchange activation.\textsuperscript{121,122} These events lead to constitutive hyperactivation of NHE1, driving acidification of the extracellular environment and alkanilization of the cytosol (Figure 1.7).

Expression of functionally active NHE1 has been shown to be necessary for development of transformed phenotypes (e.g. anchorage-independent growth, growth in serum-deprived media, preference for glycolytic metabolism) in E7 oncoprotein-induced NIH3T3 fibroblasts and HPKIA keratinocytes.\textsuperscript{123}

Reversal of transmembrane pH gradients driven by NHE1 hyperactivation promotes progression of transformed cells towards an invasive and malignant phenotype. A well-established hallmark of transformed cells is their preference for glycolytic metabolism over oxidative phosphorylation (OxPhos), even in the presence of sufficient O\textsubscript{2} (aerobic glycolysis), a phenomena known as the “Warburg effect”.\textsuperscript{2,124} Originally thought to be a primary cause of neoplastic transformation, Warburg metabolism is increasingly being recognized as a consequence of pro-oncogenic mutations and alterations that drive dysregulation of energy metabolism.\textsuperscript{85}
Figure 1.7: Summary of NHE1 activity in normal and transformed cells. In normal cells NHE1 is quiescent until pH\textsubscript{i} decreases below the activation set point, after which NHE1 rapidly exchanges extracellular Na\textsuperscript{+} for excess intracellular H\textsuperscript{+}. Oncogene-driven alterations increase the affinity of the NHE1 cytoplasmic tail for H\textsuperscript{+}, lowering the set point and triggering constitutive activation of the exchanger. Hyperactive NHE1 drives acidification of the extracellular space (ECS) while simultaneously alkalinizing pH\textsubscript{i}, resulting in the characteristic reversal of transmembrane pH gradients relative to normal cells. Increased pH\textsubscript{i} promotes aerobic glycolysis, creating acidic metabolites like lactate (Lac\textsuperscript{−}) that activate monocarboxylate transporters (MCT) and maintain the reversed transmembrane pH gradient. Figure adapted from reference 6.

Oncogene-driven activation of NHE1 and subsequent alkalinization of the cytosol cause upregulation of key glycolytic enzymes, including lactate dehydrogenase and the glucose transporter GLUT-4, supporting aerobic glycolytic metabolism.\textsuperscript{117} The preference for glycolysis is reinforced by reliance of glycolytic enzymes (e.g. phosphofructokinase-1) on alkaline pH\textsubscript{i} for optimal activity, compared to the acid-activated OxPhos pathway.\textsuperscript{10} The production of acidic metabolites (e.g. lactate) from increased glycolytic metabolism provides an intracellular H\textsuperscript{+}...
pool that further drives NHE activity, creating a positive feedback loop that delivers steady alkalinization of pH, decreased pH, and increased glycolytic metabolism.\textsuperscript{125}

Increased pH protects transformed cells from acid-dependent apoptosis through a variety of mechanisms. For instance, acidic pH upregulates DNase II activity to promote apoptosis-associated DNA fragmentation and stabilization of the pro-apoptotic BAX protein, triggering the formation of pores in the mitochondrial membrane and release of cytochrome-c (cyt-c).\textsuperscript{112,126,127} Apoptotic effector caspases activated by cytosolic cyt-c show maximal activity at acidic pH ($\text{pH} \sim 6.8$).\textsuperscript{128,129} High pH has also been shown to destabilize acid sensitive mutants of the canonical tumour suppressor gene p53.\textsuperscript{130,131} In this way, NHE1 upregulation is considered an adaptive strategy that protects cancer cells from apoptosis and promotes progression towards malignancy.\textsuperscript{11,132}

NHE1 driven alkalinization of the cytosol drives cell proliferation by facilitating cell-cycle progression past the G\textsubscript{2} checkpoint into mitosis.\textsuperscript{133} It was shown that ras p21 or v-mos-driven oncogenic activation of NHE1 in quiescent NIH 3T3 fibroblasts results in entry into S phase and increased DNA synthesis.\textsuperscript{134,135} Intracellular alkalinization also creates optimal conditions thymidine kinase and thymidylate synthase activities, the rate-limiting enzymes in DNA synthesis.\textsuperscript{136,137}

Dysregulated cell proliferation causes disordered growth of an expanding tumour mass, creating regions of hypoxia as the increasing $O_2$ demands fail to be met by passive diffusion from the surrounding (aberrant) vasculature. This effect supports the shift to glycolytic metabolism and encourages angiogenesis as the tumour attempts to satiate its increasing need for nutrients and $O_2$.\textsuperscript{138} The functionally poor and chaotic blood vessels fail to adequately
remove acidic metabolic byproducts (e.g. carbonic acid, lactate) from the intratumoural space, further acidifying the extracellular environment. The pro-proliferative, pro-angiogenic and anti-apoptotic effects of NHE1 hyperactivation synergize with the increased proteolytic capabilities that accompany acidification of the tumour microenvironment, creating a self-perpetuating cycle that promotes carcinogenic progression and ultimately metastatic spread.

Cancer cell invasiveness requires polarization of cells, a key feature of EMT (Figure 1.1). Rearrangement of the cytoskeleton and altered interactions with ECM components leads to formation of a migratory ‘leading edge’ at the front of moving cells. NHE1 is intrinsically involved in the formation and function of pseudopodia and invadopodia, two pro-invasive structures at the leading edge. Pseudopodia are specialized finger-like structures that project in the direction of the circulatory vasculature, creating a path for cell migration. Invadopodia are discrete acidic, protease-rich microdomains that facilitate focal remodeling of ECM and BM attachments.

NHE1 localizes to invadopodial structures, where its activation leads to acidification of pH_e and increased in pH_i. Acidification of pH_e promotes secretion and activation of co-localized proteases (e.g. MMPs-2, 9, MT1-MMP, cathepsins D & B, uPA etc.), allowing remodeling of cell-matrix attachments and degradation of confining ECM components. Local acidification of pH_e is partially driven by upregulated glycolytic enzymes in the invadopodia. Alkalinization of pH_i facilitates the dynamic reorganization of cytoskeletal components (e.g. coflin association with cortactin) within invadopodial structures. These concerted processes allow successive protrusion of pseudopodia and degradation of ECM at the leading edge of cells, enabling directed invasion of tumour cells through the stromal environment.
hyperactivation is thus considered as a key contributor to cell invasiveness in addition to its central role in the initiation and maintenance of underlying carcinogenic transformation.

Direct interplay between the uPAS and NHE1 at the invasive edge of cells has been studied in non-small cell lung carcinoma (NSCLC). Upon binding of uPA, the uPA/uPAR complex localizes to invadopodial structures, where it facilitates directed uPA-mediated proteolysis. The process is supported by the activity of co-localized NHE1. In addition to its proteolytic activity, uPA binding to uPAR promotes pro-invasive stress-fibre formation, increased cell proliferation and MMP 9 release via activation of the ras/ERK pathway. Stimulation of downstream ERK signaling following uPAR occupation by uPA was shown to be due to NHE1-mediated increases in pH, in HL460 NSCLC cells, with NHE1 knockdown cells showing significantly decreased stress fibre formation and MMP-9 activity.

NHE1 is overexpressed in cervical cancer and hepatocellular carcinoma and its activity is upregulated in breast tumor and glioma cells. In 1989, Rotin et al. demonstrated the importance of NHE activity in tumourigenesis by showing that bladder carcinoma cells lacking functional NHE fail to grow or show greatly reduced tumour growth in murine xenografts. Tumours able to grow in this model consisted of cells that had reverted through gain-of-function mutations to normal NHE1 activity. Taken together, these findings support NHE1 as a promising anticancer target.
1.4 Small molecule inhibitors of uPA

Active site inhibition of uPA proteolytic activity presents unique challenges and opportunities compared to other members of the trypsin-like serine protease (TLSP) family. TLSPs show high homology in the structure of their protein folds and they all feature the canonical His57-Asp102-Ser195 catalytic triad and a deep S1 binding pocket with a highly conserved Asp189 at its base. Asp189 forms an essential salt-bridge interaction with the positively charged side chains of P1 amino acid residues (e.g. Arg, Lys) in their various protein substrates. Substrate specificity in TLSPs is largely dependent on the strength of the P1-S1 interaction.

The TLSP family is divided into subgroups based on the identity of residue 190. In uPA, plasmin, trypsin and factor VIIa this residue is serine, while in tPA, thrombin and factor Xa it is alanine and in kallikrein it is threonine. These differences contribute to the selectivity of small molecule inhibitors as the OH sidechain of Ser190 can form hydrogen bonds to ligands. This H-bond partially compensates for interactions lost through displacement of a water molecule from the S1 site upon inhibitor binding.

The unique insertion of Thr98A and Leu98B in uPA (termed the “99-loop”), greatly reduces the size of its S3 and S4 sites relative to other TLSPs. The S2 site is also reduced in size by the imidazole side-chain of His99 in uPA (Figure 1.8). Reduced access to these regions, which are commonly targeted in TSLP inhibitor design, provides excellent scope for generation of uPA-selective small molecule inhibitors.
Figure 1.8: Schematic representations of the active sites of trypsin, factor Xa, tPA, uPA. The S2, S3 and S4 sites in uPA are reduced in size relative to the other TLSP due to the Thr98A, Leu98B and His99 insertions which collectively form the ‘99-loop’. Figure adapted from reference 158.

Rational design of uPA inhibitors was advanced after publication of the uPA catalytic binding site by Spraggon in 1995 and later through the availability of higher resolution X-ray crystal structures of a re-engineered recombinant uPA (‘micro-urokinase’).160,161 The structures revealed a novel subsite in uPA termed S1β.161 S1β is flanked by the Cys191-Cys220 disulfide
bond, residues Gly216, Gly219, Ser145 and the side chain of Lys143. The site is unique to uPA making it a prime region to target for achieving uPA-selective small molecule inhibitors.

Development of uPA inhibitors began in the 1980’s with early inhibitors based on ε-caproic acid 5, as well as (benz)amidine and phenylguanidine that showed sub-micromolar potencies (Figure 1.9). In 1987 Vassalli and Belin reported that amiloride 6 is a moderately potent ($K_i = 7 \mu M$) and selective inhibitor of soluble uPA. Potent and selective 4-substituted-benzo[b]thiophene-2-carboxamidine inhibitors were reported with IC$_{50}$ values as low at 70 nM (e.g. 7). 4-Iodo-benzo[b]thiophene-2-carboxamidine (B428, 1) inhibited tumour cell growth and invasion in syngeneic mouse breast and rat breast and prostate cancer models. The S1β subsite was identified as a major contributor to the high uPA affinity of 4-substituted-benzo[b]thiophene-2-carboxamidines, where interactions between the substituent at the 4-position on the benzothiophene ring and S1β was critical.
Figure 1.9: Small molecule inhibitors of uPA, including amiloride 6 (red box).

Inhibitors based on a 2-naphthamidine scaffold were designed to specifically target S1β with substituents at the 8-position.4 Additional substitution at the 6-position created potent inhibitors with improved uPA selectivity.169 Optimization of the 8-substituted-2-napthamidines yielded very potent inhibitors (e.g. 8, \( K_i = 4.7 \) nM) that showed further improvements in selectivity for uPA and high oral bioavailability (\( F_{oral} = 55\% \) in rats).170
The high selectivity of 6,8-disubstituted 2-naphthamidine inhibitors was attributed to interactions that complemented those made in the S1 and the S1β subsites, namely H-bonding between the 6-substituents and Asp60A.\textsuperscript{156} Asp60A is a component of the 60-loop region, an area of the uPA binding site that shows large sequence diversity across different TLSPs. Targeting interactions at this site in addition to making the key interactions at the larger sites (i.e. S1, S1β) is thus an attractive strategy for achieving potency and selectivity in uPA inhibitors.

Despite the high potency and selectivity of several reported small molecule inhibitors of uPA, only a single example (to our knowledge) has progressed to clinical evaluation in the cancer setting. WX-UK1\textsuperscript{9}, a peptidomimetic derivative of 3-amidinophenylalanine, (uPA $K_i$ = 410 nM), showed demonstrable antitumor efficacy in phase II trials (Wilex website, http://www.wilex.de/portfolio-english/mesupron/phase-i-ii-with-mesu/, Published 2015, accessed 29\textsuperscript{th} Jan 2017). Phase II evaluation of an orally bioavailable prodrug form (MESUPRON) in combination with gemcitabine led to a 17% increase in 1-year survival in pancreatic cancer patients compared to patients treated with gemcitabine alone.\textsuperscript{171} Similar results were found when the prodrug was trialed in combination with capecitabine in the treatment of HER2-negative breast cancer.\textsuperscript{172}

3-Amidinophenylalanine-based inhibitors (including MESUPRON) show low selectivity, inhibiting both plasmin and trypsin with similar or higher potency than uPA.\textsuperscript{173} Lack of specificity of the compounds confounds interpretation of the clinical data as antitumour and antimetastatic effects may arise from inhibition of multiple cancer-relevant proteases. Since completion of Phase II studies the rights to MESUPRON were acquired by Red Hill Biopharma, who are currently raising funds for further clinical development. (RedHill Website,
http://www.redhillbio.com/Mesupron, accessed 29th Jan 2017). No clinical data with truly selective uPA inhibitors have been reported to date.

Early small molecule inhibitors of uPA were mostly arginine mimetics containing a mono or biaryl core with an appended amidine or guanidine group. These basic functional groups are positively charged at physiological pH (pK_a ~ 11-13) and serve to anchor the inhibitor in the S1 binding site via the salt-bridge with Asp189. Basic groups were considered essential for potent uPA inhibition until recently. High basicity of these groups can restrict efficient intestinal uptake and oral bioavailability and promote rapid clearance. Less basic groups that are able to maintain the Asp189 salt-bridge interaction have long been sought after. The uPA inhibitor amiloride uses the much less basic acylguanidine moiety (pKa = 8.71) to make the key interaction, suggesting it could serve as an attractive scaffold for development of novel inhibitors. Amiloride’s long history as an oral K^+-sparing diuretic provides clear evidence of its excellent drug properties further supporting its potential utility.

Recent efforts have attempted to address the poor pharmacokinetic properties of positively charged arginine mimetic inhibitors by replacing the amidine/guanidine groups with uncharged moieties. Heynekamp et al. developed potent uPA inhibitors based on an isocoumarin scaffold bearing an uncharged bromopropyloxy group after docking simulations predicted that the terminal bromine would interact with Asp189. 3-Bromoalkoxy-4-chloro-7-benzamidoisocoumarin 10 was synthesized and found to inhibit uPA with a K_i of 34 nM. Selectivity of 10 for uPA over other TLSPs was not reported.

Primary amines have also been investigated as replacements for amidine/guanidine groups. Fragment-based screening by X-ray crystallography identified the (R)-enantiomer of the
antiarrhythmic drug mexiletine as a possible uPA inhibitor.\textsuperscript{177} Although mexiletine displayed low affinity for uPA (IC\textsubscript{50} >1mM), the favourable orientation of the interactions it made with the protein coupled with its high bioavailability justified further investigations. Medicinal chemistry efforts led to the development of 11 a potent and moderately selective uPA inhibitor (K\textsubscript{i} = 72 nM).\textsuperscript{177} The favourable pharmacokinetic profile of this compound (F\textsubscript{oral (rat)} = 60%, t\textsubscript{1/2} = 7.5 hours, high volume of distribution) indicates this was an effective strategy for reducing basicity of the S1-occupying moiety. In 2009 West et al. reported a potent and selective uPA inhibitor 12 (IC\textsubscript{50} = 39 nM) based on a linear poly-aryl scaffold that used a primary amine group to make the interaction with Asp189.\textsuperscript{178} Unfortunately this series showed poor bioavailability and high clearance rates \textit{in vivo} (12, F\textsubscript{oral (rat)} = 18%, t\textsubscript{1/2} = 0.26 hours).\textsuperscript{178}

Linear, cyclic and bicyclic peptide inhibitors of uPA have been described, with some showing single digit nM potencies.\textsuperscript{179-181} However, unmodified peptidic inhibitors often suffer from poor bioavailability and short half-lives.\textsuperscript{156} Inclusion of non-natural, chemically modified amino acids (e.g. phenethylsulfonamidino-D-serine) were used to address these issues in some recent peptidomimetics (e.g. 13, K\textsubscript{i} 20 nM).\textsuperscript{182} Compound 13 displayed high antimetastatic activity in mice with HT-1080 fibrosarcoma xenografts, decreasing the number of pulmonary metastatic foci by over 95% and significantly increasing animal lifespan (>3-fold).\textsuperscript{182} Low-nanomolar irreversible peptidic inhibitors of uPA bearing electrophilic diarylphosphonates that react with the active site Ser195 hydroxyl have been reported.\textsuperscript{183} Second generation diarylphosphonates (e.g. 14, IC\textsubscript{50} = 3.1 nM) showed \textit{in vivo} efficacy in rat cancer models, decreasing primary breast tumour weight by up to 27% and lung metastases by up to 70%.\textsuperscript{184}
1.5 Small molecule inhibitors of NHE1

Due to the difficulties in crystalizing membrane proteins, a complete X-ray crystal structure of NHE1 has not yet been described, which has complicated efforts to define the exact target site(s) for small molecule inhibition. Mutagenesis studies and comparisons with prokaryotic homologues identified residues in transmembrane helix IV (TM-IV), TM-IX and TM-X as being involved in inhibitor and/or Na⁺ binding.\textsuperscript{185,186,187-191} Crucially, Phe161 of TM-IV was identified as a key determinant of susceptibility to small molecule inhibition as non-neutral missense mutations cause dramatic increases in inhibitor $K_i$ (see Table 1, Slepkov et al., 2007 for details).\textsuperscript{192} Amiloride and related inhibitors likely bind at or near the Na⁺ binding site via interactions with Glu346 (TM-IX).\textsuperscript{193,194}

The design of small molecule inhibitors of NHE1 effectively began with amiloride in 1982, when it was reported by Benos et al. as an inhibitor of Na⁺ entry into cells that acted through inhibition of the NHE and ENaC families of Na⁺ transporters (Figure 1.10).\textsuperscript{175} Modification of amiloride by double substitution at the 5-NH₂ group yielded NHE inhibitors that have become standard tools for investigating Na⁺ transport. These include HMA \textsuperscript{27} (5-N,N-hexamethyleneamiloride), DMA \textsuperscript{28} (5-N,N-dimethylamiloride), EIPA \textsuperscript{29} (5-(N-ethyl-N-isopropylamiloride) and MIBA \textsuperscript{30} 5-(N-methyl-N-isobutylamiloride) (Figures 1.10 and 1.11).\textsuperscript{195} These inhibitors show greatly improved potency towards NHEs and reduced activity against ENaCs and other Na⁺ transporters (e.g. NCX). Amilorides tend to show only moderate selectivity for NHE1 over other NHE isoforms.\textsuperscript{196,197}
Figure 1.10: Reported small molecule inhibitors of NHE1, including amiloride 6 (red box).

Direct replacement of the pyrazine core of amiloride with an isosteric pyridine produced the more potent 15 (IC$_{50}$ = 1.2 µM). Use of a phenyl acylguanidine led to highly selective NHE1 inhibitors cariporide (16, IC$_{50}$ = 120 nM) and eniporide (17, IC$_{50}$ = 30 nM). These compounds underwent clinical trials, Phase III in the case of cariporide 16 and Phase II in the case of Eniporide 17, for the treatment of post-ischemic cardiac reperfusion injury.$^{198-201}$ Second generation orally-active phenyl and heteroaryl acyl guanidines have since been reported, including 18 (EC$_{50}$ = 31 nM, $F_{oral}$ = 73%, $t_{1/2}$ = 3.2 h) and the eniporide analogue rimeporide (19,
IC50 = 300 nM), which recently received orphan drug designation from the European Medical Administration (EspeRare.org) for the treatment of Duchenne Muscular Dystrophy.202,203

Cyclopropyl acylguanidine BMS-284640, 20 (IC50 9 nM, Foral = 63%, t1/2 = 3 h) and carboximidamide T-162559 21 (IC50 = 0.96 nM) inhibitors have also been reported. Cyclohepta[b]pyridine-3-carbonylguanidine derivative TY-12533 (22, IC50 = 32 nM) showed strong inhibition of NHE1 in vivo along with decreased neural toxicity; a significant issue with earlier NHE1 inhibitors.204,205 Uncharged inhibitors include the substituted benzamide SL59.1227 (23, IC50 = 3.3 nM) and aminopyrimidine compound 9t (24, IC50 = 6.5 nM).206

Clinical studies with NHE1 inhibitors have focused exclusively on treating myocardial infarction-related reperfusion injuries.207-209 Reperfusion injury results from NHE1 hyperactivation that occurs during ischaemia and reperfusion following myocardial infarction.210 Excess accumulation of intracellular Na+ drives the rapid influx of Ca2+ by Na+-gradient-linked transporters (e.g. NCX), leading to apoptosis of cardiac cells.211 NHE1 inhibitors are thought to prevent cardiac tissue damage following ischaemia by preventing the toxic accumulation of Ca2+, provided they are administered within a suitable timeframe following a heart attack.199,212 The potential of these molecules in cancer treatment has only recently attracted interest, with several authors now calling for investigation of NHE1 inhibitors as anticancer agents and, more generally, NHE1 as an anticancer target.13,85,213

1.6 Amiloride analogues

Amilorides as a class show broad utility in the clinic and the research laboratory. More than 1000 amiloride derivatives have been synthesized to date and their pharmacology examined across a wealth of biological platforms. Some analogues have become valuable chemical
biology tools for probing cation transport mechanisms and related processes.\textsuperscript{175,214,215} Potent and selective amiloride based inhibitors have been discovered for epithelial sodium channels (ENaC), sodium hydrogen exchangers (NHEs) and sodium calcium exchangers (NCX). A summary of the structural determinants underpinning activity of amiloride analogues against these exchangers is provided in Figure 1.11.

The parent drug amiloride 6 is a potent inhibitor of ENaC but shows only low/moderate activity against NCX. \textit{N}-substitution of the guanidine in amiloride with alkyl and aryl groups, as in DCB 25, significantly increased potency against both ENaC and NCX.\textsuperscript{175} Alkyl or aryl substitution of the 5-NH\textsubscript{2}, as in HMA 27, selectively removed ENaC activity while greatly enhancing NHE1 inhibition. Aryl/alkyl substitution on both the guanidine nitrogen and 5-NH\textsubscript{2} group yielded potent and selective NCX inhibitors that lack activity against ENaC (e.g. CBDMB 26).\textsuperscript{183} Despite the plethora of amiloride analogues bearing substitutions on the amines in the 3 and 5 positions, or on the acyl guanidine nitrogens, very few 6-substituted amiloride analogues have been described (<20). A single patent describing the synthesis of 6-phenyl HMA, the sole example of a 6-HMA analogue, has been reported.\textsuperscript{216}

In addition to tunable activities against cation transporters, amiloride analogues show a host of side activities against a variety of extracellular and intracellular targets. These include protein kinase-C, serine-threonine kinases, adenylate cyclase, monoamine oxidase, several adrenergic and purinergic receptors and other GPCRs.\textsuperscript{217-223} At a functional level, amiloride analogues have been shown to inhibit DNA, RNA and protein synthesis.\textsuperscript{224}
Inhibition of diverse biological targets by amiloride is due, at least in part, to the cation mimetic properties of its pendant acyl guanidine. Protonation of the guanidine moiety at physiological pH confers analogues with a net positive charge that is functionally similar to a monovalent cation (e.g. Na⁺). This cation mimetic property permits interactions with the monovalent cation binding sites prevalent in many proteins, as evidenced by the competition observed between amiloride analogues and monovalent cations with a variety of targets.\textsuperscript{166,195,225-227}
Amiloride.HCl was first approved for clinical use in 1967.\textsuperscript{228} The drug acts as an inhibitor of ENaCs in the distal nephron of the kidney. As a relatively weak diuretic, amiloride is more commonly used as an antikaliuretic in combination with thiazide or loop diuretics (e.g. frusemide), particularly in patients suffering from hepatic cirrhosis or congestive heart failure.\textsuperscript{229,230} Amiloride is also used in combination with other diuretics to control $K^+$-levels.\textsuperscript{231} The antikaliuretic qualities of amiloride arise as a downstream consequence of ENaC inhibition, which diminishes the $Na^+$ gradient needed to drive $Na^+\cdot K^+$ ATPase-mediated $K^+$ excretion.\textsuperscript{232,233}

Use of amiloride at normal doses (<20 mg/day) produces few side effects and it is generally well tolerated. The drug is contraindicated in patients displaying hyperkalemia, acidosis or impaired renal function. Hyperkalemia and associated cardiac arrhythmias are potentially life threatening side-effects of amiloride overdose due to the increased blood potassium levels, however, when used in combination with thiazide diuretics incidences of these events are rare (<2%).\textsuperscript{234}

Since the early 1980s numerous studies have demonstrated the antitumour properties of amiloride in a variety of animal models. Sparks et al.\textsuperscript{235} first reported on its antitumor effects in 1983 after demonstrating that amiloride produces dose-dependent decreases in tumor growth and tumor cell proliferation in A/J mice bearing H6 hepatocellular carcinoma (HCC) or DMA/J mammary adenocarcinoma xenografts. Later reports showed that amiloride suppresses metastasis and primary tumour growth in allo/xenograft and mutagen-induced murine cancer models. Very high oral doses (200 mg/kg/day) reportedly produced complete remission in a prostate carcinoma xenograft model.\textsuperscript{236} For a comprehensive review on the antitumor effects of amiloide in animals see Matthews et al., 2011.\textsuperscript{13}
In addition to the many observations in animal models, sporadic reports also exist of spontaneous remission of cancers in patients who have received chronic amiloride treatment. Amiloride (5-10 mg three times daily) administered as the sole chemotherapeutic treatment following failure of cyclophosphamide and cisplatin therapy achieved complete remission in a patient with metastatic ovarian carcinoma (i.e. disease free interval >11 years).

1.7 Hypothesis: Anticancer effects of amiloride arise through dual inhibition of uPA and NHE1

There is considerable evidence to support the hypothesis that the in vitro and in vivo anticancer effects of amiloride arise through dual inhibition of both uPA and NHE1.

1.7.1 Amiloride and analogues as uPA inhibitors

As described earlier, amiloride is a moderately potent, reversible and competitive active site inhibitor of uPA. Inhibition of uPA by amiloride has been shown to produce antimetastatic effects in several models. Highly invasive PC-3 prostate adenocarcinoma cells exhibiting amplified expression of PLAU (gene encoding uPA) showed reduced invasiveness in the presence of amiloride compared to cells lacking PLAU amplification (DU145 and LNCaP). Similar results were observed with the small molecule uPA inhibitor B428.

Amiloride significantly inhibited in vitro plasmin formation and invasion by highly invasive (UCT-2) and non-invasive (UCT01) transitional cell carcinomas. UCT-2 cells showed significantly higher uPA mRNA expression, uPA protein levels and uPA activity relative to UCT01 cells. Amiloride also inhibited IGF-1 and HGF-stimulated migration and invasion of L3.6pi pancreatic carcinoma cells. Inhibition of invasiveness was equivalent to treatment
with uPA-specific monoclonal antibodies. Amiloride and B428 1 caused dose-dependent decreases in cell migration and invasion in MDA-MB-231 and MDA-MB-436 breast adenocarcinoma cell lines, with the effects attributed to uPA inhibition.\textsuperscript{243}

A recent report found that amiloride causes dose-dependent decreases in uPA mRNA and protein levels and activity in MKN45 gastric carcinoma cells.\textsuperscript{244} Oral administration of amiloride reduced tumour growth and prolonged survival in BALB/c mice with MKN45 xenografts. Similarly, amiloride suppressed human fibrosarcoma cell invasion \textit{in vitro} (HT-hi/diss, highly intravasating variant of HT-1080 cell line) through inhibition of uPA. \textit{In vivo} experiments using a chick embryo chorioallantoic membrane HT-hi/diss xenograft model showed amiloride treatment decreased tumour weight, intravasation and metastasis.\textsuperscript{245} Amiloride also prevented formation of secondary lymph node metastases and decreased tumour volume in rats bearing with highly metastatic, uPA-overexpressing R3327-AT3 prostatic tumour xenografts.\textsuperscript{246} Finally, oral amiloride administration significantly decreased the number of pulmonary metastatic foci in MATB 13762 rat breast adenocarcinoma xenograft models.\textsuperscript{247-249}

\textbf{1.7.2 Amiloride analogues as NHE1 inhibitors}

Amiloride 6 is a moderately potent inhibitor of NHE1 (IC\textsubscript{50} range = 7-84 \textmu M), although specific values for inhibitory constants vary between experimental techniques and species.\textsuperscript{215} It is known from NHE1 SAR data that amiloride analogues bearing alkyl substitutions at the 5-NH\textsubscript{2} group show dramatically increased potency against NHE1 (Figure 1.12).\textsuperscript{250}
As described in section 1.3, NHE1 overexpression and hyperactivation provides cancer cells with an adaptive advantage over neighbouring healthy cells because the high Na\(^+\)/H\(^+\) exchange activity protects from pro-apoptotic lowering of pH\(_i\).\(^{19,251,252}\) Experimentally, hyperactivation of NHE1 was found to selectively sensitize murine KHT sarcoma and EMT-6 breast adenocarcinoma cells to intracellular acidification following NHE1 inhibition with EIPA 29.\(^{253}\)

To explore this concept further, amiloride analogues have been tested in combination with nigericin, a H\(^+\)-ionophore that equilibrates pH\(_e\) with pH\(_i\). Inhibition of NHE prevents pH\(_i\) recovery (i.e. pH\(_i\)↑) following nigericin treatment in acidified media. Nigericin in combination with amiloride or its analogues DMA 28, EIPA and MIBA 30 caused cell death in MGH-U1 Human or EMT-6 breast adenocarcinoma cells at pH < 7, whereas no cytotoxic effect was observed for each amiloride in isolation. The effect was confirmed as being NHE-1 dependent using NHE-deficient PS120 cells.\(^{254}\) Nigericin in combination with amiloride or EIPA was shown to significantly reduce tumour cell viability following radiotherapy in vivo.\(^{255}\)
NHE1 inhibition by amiloride analogues potentiates thermosensitivity in cancer cells\textsuperscript{256,257} For example, SCK murine mammary carcinoma cell killing is enhanced by treatment with amiloride, HMA \textsuperscript{27} or EIPA in pH 6.6 media at 43 °C, relative to cells in pH 7.5 media.\textsuperscript{258} HMA can delay tumour growth \textit{in vivo} and it significantly increases SCK cell killing post heating of xenografted mice, as determined by clonogenic assay of excised tumours.\textsuperscript{259}

NHE1 overexpression in HCC correlates with increased tumour size, invasion, tumour stage and shortened survival.\textsuperscript{260,261} Amiloride analogues have been shown to inhibit HCC motility and invasion through inhibition of NHE1.\textsuperscript{150} Oral EIPA produced dose-dependent decreases in HCC primary tumour weight in nude mice receiving HepG2 xenografts.\textsuperscript{261} Alkalization of pH\textsubscript{i} in response to tumour promoting xenobiotics (e.g. phorbol esters) or growth factor treatments promoted hepatocyte proliferation through upregulation of DNA synthesis and transcription via an NHE1-driven process.\textsuperscript{262} Amiloride, HMA and EIPA suppressed hepatocyte proliferation in a dose-dependent manner via inhibition of NHE1. Similarly, NHE1-driven hepatocyte proliferation triggered in residual liver tissue following hepatectomy is blocked by HMA.\textsuperscript{263} These results highlight the central role NHE1 in hepatocyte proliferation and suggest the potential of amiloride analogues as possible treatments for HCC.

NHE1-mediated acidification of pH\textsubscript{e} causes selective activation of acid sensing ion channels (ASICs) in adenoid cystic carcinoma cells.\textsuperscript{264} ASICs form part of the ENaC/degenerin superfamily of ion channels and are primarily found on neurons.\textsuperscript{265} The physiological roles of ASICs continue to be elucidated but specific isoforms are thought to be involved in nociception and mechanosensation.\textsuperscript{266} In the context of cancer, it has been proposed that ASIC upregulation may help tumour cells cope with increasingly acidic extracellular environments driven by NHE hyperactivation. Constitutive activation of ASICs regulates cell
volume in glioma cells, allowing alterations in cell size and shape that contribute to high invasive capacity.\textsuperscript{267} Inhibition of ASICs by amiloride disrupts volume regulation in glioma cells, reducing migration \textit{in vitro}.\textsuperscript{267-269} ASIC1 regulation of cell-cycle progression and migration via the ERK1/2 pathway is disrupted by benzamil, leading to potent inhibition of D54-MG glioblastoma cell migration \textit{in vitro}.\textsuperscript{269}

A recent publication demonstrating the role of ASIC1 in cell invasiveness with a panel of breast cancer cell lines showed that amiloride significantly suppresses LM-4142 tumour growth in an orthotopic murine xenograft model.\textsuperscript{270} Aside from the downstream effects of NHE inhibition on ASIC activation, various amiloride analogues inhibit ASICs in their own right.\textsuperscript{266} In 2009 scientists from Merck (the original developers of amiloride) published structure-activity relationship (SAR) study exploring 5-substituted amiloride analogues as ASIC3 inhibitors for the treatment of chronic pain.\textsuperscript{271}

Tumour selective effects of amiloride analogues on NHE inhibition have been noted in leukemic cell lines relative to healthy hematopoietic cells. Constitutive activation of NHE1 in leukemic cells and leukemic patient primary blood samples results in a higher resting pH relative to normal hematopoietic tissues, which sensitizes these cells to the effects of NHE1 inhibition.\textsuperscript{272} HMA treatment was found to trigger apoptosis in $>90\%$ of KG-1a leukaemic cells and primary acute lymphoblastic leukaemia cells, while equivalent doses caused no cell death in healthy hematopoietic cells.\textsuperscript{272} Sequence comparison of cDNA from peripheral blood mononuclear cells (PBMCs) of leukaemic patients and leukaemia cell lines relative to healthy primary PBMCs revealed greater rates of nucleotide polymorphisms, which could contribute to pathogenesis. However leukaemia specific NHE1 sequence mutations were not detected.\textsuperscript{273}
In an earlier study, amiloride was found to block cell cycle progression in murine L 1210 lymphocytic leukaemia cells without effects on cell viability.\(^{274}\)

The NHE1 inhibitor cariporide \(^{16}\) was found to reduce the colony forming ability of primary PBMCs taken from BCR-abl-positive leukaemia patients following relapse after imatinib treatment.\(^{3}\) Cariporide treatment was also able to resensitize K562 BCR-abl-positive cells to imatinib-mediated apoptosis via inhibition of NHE1.\(^{3}\) Another report found that amiloride causes alternate splicing of apoptosis regulators Bcl-x and HIPK3 and oncogenic BCR-abl, leading to potentiation of imatinib-induced apoptosis in K562 and BaF3/Bcr-Ablt315l cells.\(^{275}\)

Alternative splicing was not seen with EIPA, leading the authors to suggest that modulation of apoptosis by amiloride occurs via NHE1-independent mechanisms, although this was not tested experimentally.

Tescalin-mediated NHE1 upregulation was recently shown to play a key role in sorafenib resistance observed in FLT3-ITD acute myeloid leukaemia patients.\(^{276}\) HMA treatment showed increased cell killing of sorafenib-resistant primary AML cells relative to sorafenib-naïve samples, whilst showing no effects on healthy hematopoietic cells. Selectivity for resistant cells was attributed to inhibition of NHE1 by HMA and the associated acidification of pH\(_i\). In combination with sorafenib, HMA potentiated sorafenib-mediated apoptosis in MOLM-13 sorafenib-resistant cells. HMA alone suppressed engraftment of sorafenib-resistant primary acute myeloid leukaemia cells in nude mice and engraftment was completely abolished when HMA was combined with sorafenib.\(^{276}\)

Amiloride, HMA and EIPA decrease pH\(_i\)-dependent DNA synthesis in rat hepatocytes treated with tumour-promoting 12-O-tetradecanoylphorbol-13-acetate, while EIPA decreased
metastasis and invasion of hepatocellular carcinoma cells.\textsuperscript{10,150,262} HMA-induced acidification of pH\textsubscript{i} by NHE blockade promotes maturation of leukocyte elastase inhibitor into its pro-apoptotic form L-DNase II, leading to the apoptotic fragmentation of DNA in corneal endothelial cells.\textsuperscript{277}

### 1.8 Dual uPA/NHE1 inhibitors derived from amiloride

Several lines of evidence support investigation of amiloride \textsuperscript{6} or one of its analogues as an anticancer therapeutic in the clinical setting.\textsuperscript{13} The repeated demonstration of antitumour and antimetastatic effects of amiloride and analogues across a plethora of \textit{in vitro} and \textit{in vivo} cancer models would typically provide a strong motivation for clinical studies. Impressive pre-clinical efficacy coupled with oral bioavailability and a long history of safe usage in humans should make amiloride an attractive clinical candidate. Furthermore, anecdotal reports of amiloride treatments causing remission of neoplastic disease in human patients should bolster interest in clinical anticancer investigations with the drug.\textsuperscript{237} Despite this, only a single report exists of amiloride undergoing clinical evaluation for cancer. In this study, no significant difference in cancer risk was observed in 6614 elderly hypertensive patients receiving a fixed-ratio of 2.5 mg amiloride/25 mg hydrochlorothiazide per day compared to age-matched non-recipients over a 5-year period.\textsuperscript{278}

Assuming that the bulk of amiloride’s anticancer effects arise through dual inhibition of uPA and NHE1, the relatively modest \textit{in vitro} potency of amiloride against these targets presents significant challenges to its successful use clinically as an anticancer drug. Amiloride has a relatively low maximum allowable daily dosage (20 mg/day) due to the risk of hyperkalemia and potentially life-threatening cardiac arrhythmias at higher doses. As a result, cancer
patients receiving amiloride would require costly and inconvenient monitoring of blood K⁺-levels over a prolonged period, making out-patient administration impractical. Indeed, co-administration with a kaliuretic agent (e.g. a thiazide diuretic) might be necessary to mitigate the risk of adverse events. Moreover, administration at the maximum allowable dosage (e.g. 20 mg/day) would be unlikely to afford sufficiently high plasma concentrations to strongly inhibit these two targets and evoke a robust antitumour effect in humans.

A possible solution would be to create an analogue that shows decreased affinity for ENaCs, the target responsible for the drug’s diuretic and antikalliuretic effects. This would allow administration of higher doses without the risk of hyperkalemia. Equally desirable would be to increase the potency of amiloride towards both uPA and NHE1 so that lower plasma concentrations could achieve the desired anticancer effects. Medicinal chemistry campaigns of this type, known as Selective Optimization of Side Activity (SOSA), are a popular modern approach in drug development.279,280

Fortunately, structure activity relationships of amiloride analogues as ENaC inhibitors are well characterized.195,281 As mentioned in section 1.7.2, substitution of the NH₂ group at the 5-position reduced activity against ENaCs, which would be expected to diminish the diuretic and antikalliuretic effects of these analogues in vivo.281 The dramatically reduced inhibition of ENaCs by HMA relative to amiloride is highlighted in Figure 1.13.
Another significant issue facing development of an amiloride analogue into an anticancer drug is the lack of chemical novelty around the scaffold. As the cost of bringing a new drug to market can exceed $1.5 billion dollars (USD), investors are afforded the opportunity to recoup this expenditure and generate profits by securing exclusive rights for 20 years to the manufacture and sale of new drugs through patenting. In order to secure patent rights, an inventor must create a new chemical entity (NCE), a novel chemical structure that is considered non-obvious and for which there exists no prior reference in the scientific or patent literature (prior art). As patent protection for amiloride has long since expired, the incentive to invest in development of amiloride as an anticancer drug is significantly reduced. If, however, novel amiloride-based structures could be discovered that show increased antitumour effects, for example, by improving activity against uPA/NHE1, such compounds would be more attractive in terms of patentability and commercialization.
Previous work from the Kelso and Ranson Labs at UOW saw completion of a comprehensive SAR study of amiloride analogues as inhibitors of uPA. Diverse compounds were synthesized to investigate the effects of various substitution patterns on uPA affinity, including isosteric replacement of the acylguanidine group and pyrazine core, removal of 3 and 5-position exocyclic amines and substitution at the 5 and 6-positions. Key SAR findings from this work are summarized in Figure 1.14.

The authors concluded that amiloride analogues featuring short aryl/alkyl amino substituents at the 5-position and/or bulky substituents at the 6-position show slightly increased affinity for uPA. Specifically, the 5-substituted amiloride analogues HMA and EIPA and the 6-substituted 6-iodoamiloride and 6-phenylacetyleneamiloride showed improved potency against uPA (HMA IC$_{50}$ = 6 µM, EIPA IC$_{50}$ = 6 µM, 6-iodoamiloride IC$_{50}$ = 6 µM and 6-phenylacetylenyl amiloride IC$_{50}$ = 2 µM).

Figure 1.14: Summary of amiloride SAR study completed by Matthews et al. from the Kelso Group. Regions of amiloride altered in each approach are highlighted in red.
This study suggested that further activity gains might be possible by combining favourable substitutions at the 5- and 6-positions (i.e. additive SAR). HMA represents an excellent starting point for exploration of 5,6-disubstituted amilorides due its very high potency against NHE1 \( (K_i = 162 \text{ nM}) \) and greatly reduced activity against ENaCs relative to amiloride.\textsuperscript{197,281} Additionally, HMA has been shown to exhibit a variety of anticancer properties in its own right (see section 1.7). Importantly, the 6-Cl substituent on amiloride is oriented towards the S1β subsite of uPA, forming predominantly hydrophobic interactions with residues Gln192, Gly219 and the Cys220-Cys191 disulfide bond (Figure 1.15).\textsuperscript{238}

![Figure 1.15: X-ray co-crystal structure of amiloride bound to the active site of uPA. S1β subsite is labelled. The 6-Cl atom (Green stick) forms favourable hydrophobic interactions with the S1β residues. Substituents bulkier than Cl may be better able to fill the subsite, improving affinity for uPA. (Image generated from PDB #:1F5L, originally reported in reference 226).](image-url)

Amiloride analogues bearing substituents larger than Cl at the 6-position will likely show more complete occupancy of S1β leading to increased uPA affinity. Provided HMA occupies a similar pose to amiloride upon binding to uPA, it is likely that similar or greater increases in potency might be achieved upon substitution of HMA at the 6-position.
1.9 Thesis Aims

Despite significant advances in targeted anticancer therapeutics over the past 20 years, relatively small gains have been made in chemotherapeutics that could provide general treatments for diverse neoplastic conditions, especially in advanced disease. For this reason, therapeutics able to target phenotypes observed in many different tumour types would provide significant benefits in the clinic. Drugs able to simultaneously inhibit different aspects of carcinogenesis and metastasis processes are particularly well suited to this aim.

Urokinase-type plasminogen activator (uPA) is a key regulator of metastatic potential and is overexpressed in many aggressive cancers. Small molecule inhibitors of uPA show potent antemetastasis activities in vivo, with one compound showing efficacy in Phase II clinical trials. NHE1 activation is an essential contributor to the ‘pro-malignant’ reversal of transmembrane pH gradients observed in virtually all cancers and is upregulated in many cancer cell types.

The amiloride analogue HMA is an ideal starting point for development of novel, dual-targeting uPA/NHE1 inhibitors as anticancer drugs due to its low- µM inhibition of uPA, potent inhibition of NHE1 and demonstrated ability to selectively inhibit multiple cancer-related processes, both in vitro and in vivo. Identification of novel 6-substituted HMA analogues that show increased potency against uPA whilst maintaining high potency against NHE1 and low activity against ENaCs would be attractive candidates for development towards a new class of anticancer drugs with a novel ‘dual’ mechanism of action.

6-Substituted HMA analogues were synthesized in this work with the aim of increasing potency through enhanced binding interactions in the S1β subsite. Targeting S1β to increase ligand affinity was a successful strategy in previous uPA inhibitor programs. Since S1β is unique to uPA, increasing interactions at this site will likely improve selectivity of the
compounds for uPA over other TLSPs. Synthesis of 6-HMA analogues was approached using Pd-catalyzed cross-coupling chemistries (e.g. Suzuki-Miyaura and Sonogashira reactions) due to their wide functional group tolerability, mild reaction conditions and generally high yields. This type of chemistry has not been reported previously in the synthesis of 6-amiloride analogues (6-phenyl HMA). It should be noted that to date only a single example of a 6-HMA analogue has been reported.\textsuperscript{216} As such, generation of a library 6-HMA analogues using this approach represented a significant novel contribution to amiloride/HMA chemistry.

The overall aim of the work was to use the tools of medicinal chemistry to discover novel 6-HMA analogues that show high potency against both uPA and NHE1 and low activity against ENaCs, along with other interesting anticancer properties \textit{in vitro}. Discovery of such compounds could pave the way for further studies of anticancer efficacy in animal models and eventually pre-clinical development of an optimized candidate.
1.9.1 Specific aims

The specific aims of the thesis were to:

- Synthesize a library of 6-HMA analogues through a novel application of Pd-catalyzed cross-coupling chemistry (Chapter 2).
- Evaluate the inhibitory potency of synthesized analogues against uPA using solution-phase enzyme inhibition assays (Chapter 2).
- Obtain X-ray co-crystal structures of lead inhibitors bound to uPA (Chapter 3).
- Measure TLSP selectivity and cytotoxicity of leading compounds (Chapters 2 and 4).
- Characterize the effects of leading compounds on cell growth and behavior (Chapter 4).
- Develop a new medium-throughput assay for quantitating the NHE1 inhibitory potency of 6-HMA analogues (Chapter 5).
CHAPTER 2: Synthesis and in vitro evaluation of 6-HMA analogues

Previous efforts in the Kelso/Ranson laboratories focused on the synthesis and evaluation of amiloride analogues bearing substitutions at the 2, 3, 5 and 6 positions of the pyrazine core, with an emphasis on novel 5-amino substituted compounds. Key insights from the SAR included the requirement for exocyclic amines at the 3 and 5-positions and retention of the pyrazine core. Slight increases in uPA potency were achieved when short, uncharged alkyl or aryl amines occupied the 5-position and groups larger than Cl occupied the 6-position (Figure 1.12). With this in mind, it was hypothesized that analogues bearing substituted amines at the 5-position were suitable scaffolds for structural elaboration at the 6-position, with the goal to increase uPA affinity by establishing new contacts within the S1β subsite.

Of the 5-substituted amiloride analogues examined, HMA 27 was selected as a starting scaffold due to its improved potency against both uPA (~ 2-fold) and NHE1 (~ 520-fold) relative to amiloride. Superposition of the HMA-uPA X-ray co-crystal structure (Jiang et al., unpublished) onto the published AML:uPA structure indicated that the two compounds adopt almost identical binding poses within the uPA active site (Figure 2.1), with the 6-Cl groups of both compounds projecting directly into towards S1β. This provided compelling evidence that 6-HMA analogues would similarly project other 6-substituents into S1β, potentially leading to increased uPA/affinity. In this chapter the synthesis and in vitro evaluation of 6-HMA analogues were explored.
Figure 2.1: X-ray co-crystal structures of AML and HMA bound to the serine protease domain of human uPA. A) AML-uPA complex (PDB:1F5L)\textsuperscript{226}, B) HMA-uPA co-crystal structure (Jiang et al., FJIRSM, Fuzhou, China, unpublished), C): Backbone superposition of the AML:uPA and HMA:uPA structures highlighting the similarity between the binding poses of AML and HMA in the uPA active site. Orientation of the pyrazine core and 2-acylguanidine regions are virtually identical and the 6-Cl substituents project towards S1\(\beta\) in both structures.

2.1 Synthesis of 6-HMA Analogues

The route to 6-HMA analogues commenced with a nucleophilic aromatic substitution at the 5-position of dichloropyrazine methyl ester 31 with hexamethyleneimine to yield the HMA precursor 32 (Scheme 2.1). Pd-catalyzed Suzuki-Miyaura was used to install aryl and heteroaryl substituents at the 6-position to provide 6-HMA methyl esters 33a-55a. The methyl esters were reacted with guanidine.HCl and the products purified by preparatory reverse phase HPLC (rp-HPLC). Anion exchange yielded the 6-substituted acyl guanidines as HCl salts.

The late-stage common intermediate 32 was accessed on multigram scale from the commercially available 3-amino-5,6-dichloropyrazine methyl ester 31 via nucleophilic aromatic substitution with hexamethyleneimine in DMF at 80 °C (Scheme 2.1).
Scheme 2.1: Strategy for the synthesis 6-HMA analogues. Reaction of pyrazine methyl ester 31 with hexamethylenemine afforded the late-stage common intermediate 32. Reaction of 32 with aryl boronic acids or boronic acid pinacol esters via Suzuki-Miyaura-cross coupling afforded the 6-substituted methyl esters 33a-56a. Reaction of 33a-56a with guanidine afforded the 6-substituted acyl guanidines 33-56. Preparative rp-HPLC followed by anion exchange yielded the final products as HCl salts.

A total of 10.42 g of 32 was obtained from 7 reactions in a combined yield of 56% (yield range for individual reactions 46%-74%). The reaction conditions had been previously developed in the Kelso group by Dr. Ghazala Yacoub, who adapted the original method of Cragoe et al. where hexamethylenemine is reacted with 1 in isopropanol (iPrOH) at reflux.281

Selective reaction of hexamethylenimine at the 5-position (as opposed to the 6-position) occurs due to increased resonance stabilization of the nascent anion in the transition state afforded by the electron withdrawing carbonyl group located at the para position (Figure 2.2). Nucleophilic attack of the amine at the 6-Cl position cannot delocalize the anion into the carbonyl group.
**Figure 2.2:** Additional resonance stabilization from the electron withdrawing carbonyl group \textit{para} to C5 favours substitution at the 5-position in 31 rather than the 6-position.\textsuperscript{283}

Intermediate 32 was subjected to Suzuki-Miyaura cross-coupling reactions using a variety of commercial aryl/heteroaryl boronic acids or pinacol boronate esters (Scheme 2.1). The mechanism of the Pd-catalyzed reactions is outlined in Figure 2.3.

**Figure 2.3:** Proposed mechanism for Suzuki-Miyaura cross-coupling reactions with 32.

The initial step involves formation of an organopalladium intermediate via oxidative addition of the Pd(0) catalyst into the carbon-chlorine bond of 32 (Figure 2.3).\textsuperscript{283} Attack of the newly formed Pd(II) centre by the base (CO$_3^{2-}$) displaces the Cl$^-$ ion. Transmetalation between the activated organopalladium(II) intermediate and the boronate species \textit{in situ} yields the aryl-aryl
Pd(II) complex. The cross-coupled product is formed in the final step via reductive elimination, which regenerates the Pd(0) catalyst.

The first attempt at a Suzuki-Miyaura cross-coupling employed the conditions reported by Reyes et al., 2008 (Table 2.1) with phenylboronic acid as the coupling partner. One equivalent of 32 was reacted with 1.5 mole equivalents of phenylboronic acid in the presence of a 10-fold excess of K₂CO₃ and 5 mole % Pd(PPh₃)₄. The reaction was carried out in a 4:1 toluene:EtOH mixture at reflux for 1 h. This initial attempt yielded the desired product along with the transesterified ethyl ester. Substituting EtOH with MeOH solved the problem and the modified conditions were employed in the synthesis of all methyl ester intermediates 33a-55a. Yields for the reactions ranged between 36-98%.

In the majority of cases, reactions were complete after 2 h, as evidenced by the consumption of starting material (SM) by thin layer chromatography (TLC) and electrospray ionization mass spectrometry (ESI-MS). Reaction mixtures were cooled and the catalyst removed by vacuum filtration through celite. After concentration in vacuo the crude products were purified by silica gel column chromatography using gradients of EtOAc in pet. spirit.

Of the two reactions that used aryl pinacol boronates, 4-isoquinoline analogue 37a formed at a similar rate to reactions that used boronic acids (i.e. 2 h total reaction time). In comparison, N-ethylmorpholinopyrazole 39a formed more slowly, requiring 16 h at reflux for complete consumption of the SM.
Table 2.1: Suzuki-Miyaura cross-coupling reactions of 32 with aryl boronic acids to give methyl esters 33a-55a. *Compounds synthesized as described in reference 216. †Reaction conducted using aryl pinacol boronate.

<table>
<thead>
<tr>
<th>Product</th>
<th>R</th>
<th>Yield (%)</th>
<th>Product</th>
<th>R</th>
<th>Yield (%)</th>
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</tr>
<tr>
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<td>77</td>
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<td>44a</td>
<td>S</td>
<td>85</td>
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</table>
The methyl esters were generally not soluble in CH₂Cl₂ or EtOAc:pet.spirit mixtures and required adsorption of the crude products onto silica gel for loading onto silica gel columns. All compounds were obtained in pure form and comprehensively characterized. Spectroscopic data for a representative compound 49a are provided in Figure 2.4.

The ¹H spectrum was referenced to a tetramethylsilane (TMS) internal standard at δ 0.00 (Figure 2.4). The singlet at δ 8.66 integrated to two protons and was assigned to the 2’ and 6’ protons on the pyrimidine ring. A singlet at δ 4.05 with an integration of 3H was assigned to the methyl protons of the para methoxy group. This signal was shifted downfield by the deshielding effects of the pyrimidine ring nitrogens. Further upfield, a second 3H singlet at δ 3.89 was assigned to the methyl ester CH₃. Assignment of the methoxy versus methyl ester signals was provided by the observation that the methyl ester peak appeared at δ 3.89 for all methyl ester products. A triplet with an integration of 4H (J = 5.75 Hz) at δ 3.36 was assigned to the 2” and 7” methylene protons positioned α to the tertiary amino group. Two singlets, each with an integration of 4H at δ 1.67 and δ 1.48, were assigned to the 3”/6” and 4”/5” methylene protons, respectively. Signals observed at δ 1.26 and δ 0.88 were assigned to ‘grease’ impurities from solvents. The methyl ester and methylene signals were observed in the ¹H spectra of all methyl ester products 33a-56a, with variations in chemical shift generally <0.04 ppm.
Figure 2.4: $^1$H and $^{13}$C-APT spectra for 49a.
$^{13}$C-APT spectra were referenced to CDCl$_3$ at δ 77.2 and phased such that CH$_3$ and CH carbon signals are positive and carbonyl groups, CH$_2$ and carbons with no protons attached are negative (Figure 2.4). The carbonyl signal appeared in the expected region at δ 167.0. The 4” pyrimidine carbon was heavily deshielded by the two adjacent nitrogen and one oxygen atoms, appearing at δ 164.5. The single positive peak appearing in the aromatic region (δ 158.1) was assigned to the symmetrical 2’ and 6’ carbons of the pyrimidine ring. These signals were deshielded by inductive electron withdrawal from the neighbouring ring nitrogens. The 5 and 3-position pyrazine carbons were assigned based on their attachment to either a more deshielding cyclic tertiary amine in the case of 5 (δ 155.2), or a less deshielding primary amine in the case of 3 (δ 153.7). The negative signals found at δ 128.7 and δ 125.1 were assigned to the 6 and 1” aromatic carbons respectively. The signal at δ 113.8 was assigned to the carbon at position-2 of the pyrazine ring, this signal being significantly shielded by resonance contributions from the exocyclic amines at the ortho and para positions. The positive peaks observed at δ 55.1 and δ 52.1 were assigned to the methoxy group and methyl ester protons, respectively. The negative peak at δ 51.2 corresponded to the 2’ and 7’ hexamethylene ring methylene units, the signal being significantly deshielded by the neighbouring tertiary amine. The negative peaks at δ 28.0 and δ 27.2 were assigned to the 3’ and 6’ methylene carbons and 4’ and 5’ methylenes carbons, respectively, as the deshielding effects decreased with increasing distance from the electron withdrawing group.

Methyl esters 33a-56a were next subjected to guanidinylation reactions using free base guanidine formed in situ from guanidine.HCl in iPrOH in the presence of Na metal (Scheme 2.1). A proposed mechanism for the guanidinylation reactions is provided in Figure 2.5
Addition of sodium metal to iPrOH forms sodium propoxide and liberates H₂ gas in the process. Next guanidine·HCl is added to obtain the nucleophilic free guanidine by the neutralization of HCl by iPrO⁻. The free guanidine attacks the carbonyl of the methyl ester via nucleophilic acyl substitution, expelling methoxide (MeO⁻) as the leaving group. Loss of a proton on the amide-like NH₂⁺ group affords the desired acyl guanidine (e.g. 49).

Crude reaction products were purified by preparative rp-HPLC and the acylguanidines obtained as trifluoroacetate (TFA) salts after lyophilization. The TFA salts were stirred with the quaternary ammonium Cl⁻ anion exchange resin in MeOH to yield the targets 33-56 as hydrochloride (HCl) salts. Acyl guanidines were fully characterized and their structures confirmed by spectroscopic analysis. Spectroscopic data for a representative acyl guanidine 49 is provided in Figure 2.6. Yields from the guanidinylation reactions and overall yields from 32 over 2-steps for all compounds are provided in Table 2.2.
Figure 2.6: $^1$H and $^{13}$C-APT spectra for 49.
The $^1$H spectrum for $49$ was referenced to a TMS internal standard at $\delta$ 0.00 (Figure 2.6).

Guanidinylation of the methyl ester $49a$ to give $49$ was inferred by the loss of the methyl ester peak at $\delta$ 3.89 and the appearance of three heavily deshielded signals in the aromatic region ($\delta$ 8.00-11.00). The singlet at 10.76 with an integration of 1H was assigned to the amide NH of the acyl guanidine sidechain, this proton being deshielded by the electron withdrawing carbonyl and guandino NH$_2$ groups. The broad singlets at $\delta$ 9.27 and $\delta$ 8.48, each with an integration of 2H, and were assigned to the two guanidino NH$_2$ groups. The singlet at $\delta$ 8.51 showed an integration of protons and was assigned to the 2’- and 6’- aromatic protons. The broad singlet at $\delta$ 6.75 with an integration of 2H and was assigned to the 3-position NH$_2$ group. In concordance with assignments for $49a$, signals for the aromatic OCH$_3$ group ($\delta$ 9.27, 3H) and azepane ring methylene protons appeared in the same chemical shift regions, with each signal integrating to give the expected number of protons ($\delta$ 3.35, 4 H, 2’ and 7’; $\delta$ 1.66, 4 H, 3’ and 6’; $\delta$ 1.47, 4 H, 4’ and 5’).

Analysis of the $^{13}$C-APT spectrum of $49$ supported observations from the $^1$H data (Figure 2.6). As expected, an additional peak at $\delta$ 161.9 was observed for to the guanidine carbon, and the OCH$_3$ signal observed at $\delta$ 52.0 for $49a$ was absent. All other signals varied from the assignments made for methyl ester $49a$ by less than 1.5 ppm. Accordingly, the negative signals at $\delta$ 166.6 and $\delta$ 164.0 were assigned to the carbonyl and 4’’ carbons, respectively. The positive peak at $\delta$ 157.8 corresponded to the 2’ and 6’- carbons of the pyrimidine ring. The 5- and 3- pyrazine carbons were observed at $\delta$ 156.1 and $\delta$ 153.8, respectively. The aromatic peaks at $\delta$ 127.8, $\delta$ 124.7 and $\delta$ 112.5 corresponded to the pyrazine 6- carbon, pyrimidine 1’’-carbon and pyrazine 2- carbons, respectively. The positive peak observed at $\delta$ 55.6 was assigned to the CH$_3$ group and the negative peak at $\delta$ 51.6 corresponded to the 2’ and 7’ methylene carbons. Signals for the 3’ and 6’ and 4’ and 5’ methylene carbons were observed.
at δ 27.8 and δ 27.1, respectively. A small impurity signal at δ 4.9 was also present in the spectrum.

Low to moderate yields were obtained for guanidinylations due to competing formation of side products common in all reactions. $^1$H NMR and MS analysis of samples obtained from preparative rp-HPLC fractions revealed the presence of the isopropyl esters, presumably formed via transesterification with the reaction solvent under the basic reactions conditions. Lower reactivity of the isopropyl esters towards guanidinylation significantly decreased formation of desired products, with transesterification in effect creating a reservoir of unreactive material.

Complete consumption of the methyl ester SM was not observed over the reaction times employed (1-3 h). Closer analysis of the guanidinylation reaction of 42a revealed that longer reaction times were required (up to 18 h) for complete consumption of the methyl ester but this was accompanied by increased formation of isopropyl ester and an unidentified polar side product. Attempts to substitute $^1$PrOH for MeOH as the reaction solvent led formation of complex mixtures, as determined by TLC analysis.
Table 2.2: Guanidinylation of methyl esters 33a-56a to provide target acyl guanidines 33-56. Yields over 2-steps from the starting methyl ester 31 are also shown.

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<th>Overall Yield (%) (2 steps)</th>
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</table>
Previous work from the Kelso and Ranson Labs had shown that 6-phenylacetylenyl amiloride 
(\(IC_{50} = 2 \mu M\)) is ~ 5-fold more potent against uPA than amiloride (\(IC_{50} = 10 \mu M\))\(^6\). Sonogashira cross-coupling was employed to obtain the equivalent 6-phenylacetetyl HMA methyl ester \(56a\) and the target acyl guanidine \(56\) to see whether similar increases in potency were observed with the azepane ring present at C5 (Scheme 2.2).

![Scheme 2.2: Sonogashira cross-coupling of phenylacetylene with 32 yielded 56a (58%), which was converted to acyl guanidine 56 in 14% isolated yield.](image)

Methyl ester 32 was reacted with phenylacetylene using a Pd(PPh\(_3\))Cl\(_2\):CuI catalytic system in DMF with trimethylamine (TEA) present as base. The reaction was stopped by filtering through celite after 24 h and the product isolated by silica gel chromatography. The methyl ester 56a was obtained as a bright yellow solid in 58% yield. Ester 56a was then subjected to the above guanidinylation procedure to give 56 in 14% yield.

With the exception of 33a and 33, all methyl ester and acyl guanidine products were novel compounds that had not reported in either the patent or scientific literature (SciFinder search, 1\(^{st}\) December, 2016). For 33a and 33, specific methods had been described in a single patent concerned with the use of aroylguanidines as HIV and HCV ion channel inhibitors.\(^{216}\)
2.2 Solution phase uPA inhibition assays

The synthesized acyl guanidines **33-56** were subjected to a fluorometric solution phase uPA activity assay to measure inhibitory potencies. Assays were conducted in black 96-well plates using a commercially available fluorogenic substrate (Calbiochem 672159, Z-Gly-Gly-Arg-AMC, Merck Millipore, Massachusetts, USA), Urokinase from human kidney cells (Sigma-Aldrich, Cat # U4010) and a POLARStar fluorescence plate reader (BMG-Labtech, Ortenberg, Germany) (Figure 2.7). Compounds were dissolved in anhydrous DMSO to create 10-50 mM stock solutions. Stocks were serially diluted in assay buffer in the 96-well plate then added to a separate black assay plate containing fluorescent substrate (in assay buffer) via multichannel pipette. Assays were conducted such that each well contained a final concentration of 1% DMSO (v/v). Detailed experimental methods for the assays are provided in Chapter 7.3. Two compounds were assayed in each plate with each dilution was carried out in triplicate. Drug blanks were included to correct for intrinsic fluorescence in the compounds. An inhibitor-free vehicle control was included in each assay to determine maximal enzymatic activity, allowing for correction of day to day and enzyme batch variations. Assay plates were prepared and kept on ice until final addition of enzyme immediately prior to first reading. Assays were conducted at 37 °C with double orbital shaking before each read. Wells were excited at 355 nm and the emitted fluorescence at 460 nm was measured. Data points from the linear portion of reaction progress curves were averaged and used to determine initial velocities, which were then blank corrected and plotted to calculated IC$_{50}$ values for each compound. IC$_{50}$ is defined as the concentration of compound needed to inhibit one half of enzyme activity under the conditions used. Representative concentration-response curves for compounds **42** and **50** are provided in Figure 2.8. Modification of the assay allowed determination of $K_i$ values for selected inhibitors (Table 2.3, as described in detail in Chapter 7.3), where $K_i$ is the
concentration of compound at which the reaction rate is one half of the maximal reaction rate (Vmax) under saturating substrate conditions.

Figure 2.7: Schematic of uPA activity assay. uPA cleaves the pro-fluorogenic tripeptide substrate to liberate the highly fluorescent 7-amino-4-methylcoumarin (AMC), providing a quantifiable measure of enzymatic activity. When 6-HMA analogues are added, reversible competition with the substrate for binding to the uPA active site inhibits proteolytic activity, reducing fluorescence. Decreased fluorescence in the presence of 6-HMA analogues was used to calculate IC₅₀ values, and in some cases Kᵢ values.
Figure 2.8: Curves from solution-phase uPA inhibition assays with representative compounds 42 and 50. Data points represent the mean ± SEM (n = 3) from a representative experiment.

The aryl-boronic acid coupling partners for the Suzuki-Miyaura cross-coupling reactions were selected based on commercial availability and structural diversity. To probe the SAR of the 6-position of HMA, boronic acids were initially chosen to cover a breadth of structural diversity that appeared to have the correct size for occupancy of the S1β subsite. Later compounds were chosen as the SAR trends emerged and as X-ray co-crystal structures came to hand (Chapter 3).
Table 2.3: Inhibitory potencies of 6-HMA analogues 33-56 against Human uPA. For compounds where $n > 1$, values represent the mean ± SEM from identical repeat assays conducted on different days.

<table>
<thead>
<tr>
<th>Product</th>
<th>R</th>
<th>uPA IC$_{50}$ (nM) [$n$]</th>
<th>uPA $K_i$ (nM) [$n$]</th>
<th>Product</th>
<th>R</th>
<th>uPA IC$_{50}$ (nM) [$n$]</th>
<th>uPA $K_i$ (nM) [$n$]</th>
</tr>
</thead>
</table>
2.2.1 Structure-activity analysis

The 6-Phenyl derivative 33 (IC$_{50}$ = 8,158 nM) showed a ~ 3.7-fold decrease in potency relative to HMA (IC$_{50}$ = 2,206 nM). Addition of a methylthioether group at the para position 34 resulted in a ~ 12.1-fold decrease in potency (IC$_{50}$ = 26,717 nM). Substitution of the methylthioether of 34 with a trifluoromethyl group 35 produced a ~ 3.1-fold decrease in potency (IC$_{50}$ = 6,914 nM). Methyleneedioxy derivative 36 showed a ~ 1.5-fold increase in activity over HMA (IC$_{50}$ = 1,518 nM). The 4-isoquinoline compound 37 showed ~ 2.6-fold lower activity than HMA (IC$_{50}$ = 5,751 nM) while the N-methylpyrazole 38 showed increased activity, ~ 2.7-fold higher than HMA (IC$_{50}$ = 825 nM). Substitution of the $N$-methyl group of 38 with $N$-ethylmorpholine 39 (IC$_{50}$ = 1,070 nM) did not improve activity.

The 2-Indole derivative 40 (IC$_{50}$ = 9,214 nM) showed ~ 4.2-fold lower activity than HMA. Similarly, substitution of the indole N with S to yield benzothiophene 41 did not afford potent inhibition, with 41 showing ~ 2.4-fold decreased activity relative to HMA (IC$_{50}$ = 5,375 nM). However, substitution of the ring heteroatom with O to yield benzofuran 42 provided a ~ 7.4-fold boost in potency (IC$_{50}$ = 297 nM). Potency was further increased by fluorination of 42 at the benzofuran 4-position to give compound 43, which showed ~ 15.4-fold greater potency than HMA (IC$_{50}$ = 143 nM).

The 2-thiophene derivative 44 was slightly better than its benzothiophene analogue 41 (IC$_{50}$ = 3,927 nM). The 3-thiophenyl isomer 45 slightly more potent (IC$_{50}$ = 1,669 nM). Removal of the phenyl ring of 42 to yield the 2-furanyl derivative 46 had little effect on activity, with both compounds showing similar potency (IC$_{50}$ = 254 nM). Similarly, 3-furanyl isomer 47 (IC$_{50}$ = 270 nM) showed activity comparable to 42 and 46.
The 5-pyrimidine derivative 48 (IC$_{50}$ = 175 nM) showed ~ 12.6-fold higher activity than HMA. Methoxy substitution at the *para* position of 48 to yield 49 increased potency into the <100 nM range, affording a ~ 25.6-fold increase relative to HMA (IC$_{50}$ = 175 nM). Dimethoxy substitution of the 5-pyrimidine to give 50 afforded the most potent inhibitor arising from this work, some 32-fold more potent than the parent HMA (IC$_{50}$ = 69 nM). The importance of the 5-pyrimidinyl scaffold for high potency was highlighted with the 2,3-dimethoxy-3-pyridinyl analogue 51. Here, removal of the second ring N resulting in a dramatic loss in potency (IC$_{50}$ = 1,437 nM). The 2-fluoro-3-pyridinyl derivative 52 was ~ 1.3-fold less potent than HMA. Removal of ring nitrogens altogether from 50 carbocyclic analogue 53 reduced activity ~ 1.6-fold relative to HMA (IC$_{50}$ = 3,571 nM). The 3,5-dimethoxyphenyl isomer 54 (IC$_{50}$ = 2,143 nM) was approximately equipotent with HMA. The 3,6-Dimethoxypyridazine derivative 55 showed ~ 4.3-fold greater potency than HMA (IC$_{50}$ = 512 nM). The phenylacetylene derivative 56 proved to be the least potent inhibitor studied (IC$_{50}$ = 29,816 nM). In comparison, the analogous 6-phenylacetylamiloride reported by Matthews et al., showed a 5-fold increase in uPA inhibitory activity relative to amiloride (6-phenylacetylamiloride IC$_{50}$ = 2 µM; Amiloride IC$_{50}$ = 10 µM, using a chromogenic assay). It is possible that the steric bulk of the azepane ring at the 5-position of 56 prevents the 6-phenylacetylene substituent from orienting favourably in the S1β subsite.

Inhibitory constants ($K_i$) were determined for HMA and 11 inhibitors using a modified fluorogenic assay (Table 2.3). Double reciprocal plots of initial reaction velocities (1/RFU.min$^{-1}$) versus substrate concentration (1/S) for representative inhibitors 43 and 48 confirmed competitive inhibition of uPA, since $K_m$ increased with increasing [inhibitor] while $V_{max}$ remained unchanged (Figure 2.9).
The rank order of potencies according to $K_i$ agreed well with the IC$_{50}$ values obtained earlier (Table 2.3). Compounds 43 and 48 were exceptions, however, occupying slightly different positions in their two rank orders. With the exception of HMA, all values indicated affinities that were higher than the IC$_{50}$ concentrations determined for each compound, as expected for competitive inhibitors.  

2.3 Selectivity for uPA over related trypsin-like serine proteases

A shortcoming of previous small molecule inhibitors of uPA has been their lack of selectivity for uPA over related trypsin-like serine proteases, such as thrombin, plasmin and tPA. Off-target inhibition of these proteases raises the risk of unwanted side effects being observed if the compounds were to be used as cancer drugs in humans.

To examine the uPA selectivity of the 6-HMA analogues, three examples were screened against a panel of human TLSPs using chromogenic enzyme inhibition assays (detailed experimental methods in Chapter 7.3). Representative inhibition curves from these experiments are presented in Figure 2.10. In experiments using the chromogenic uPA substrate S-2288
(Chromogenix, Massachusetts, USA) benzofuran analogue 42 showed IC$_{50}$ = 529 nM, or approximately 2-fold higher than the value observed in the fluorogenic assay (IC$_{50}$ = 297 nM). This general trend was observed across all inhibitors, with the chromogenic assay IC$_{50}$ values only 2 to 4-fold higher than those obtained from assays from fluorescence-based assays. The exception was compound 49, which showed IC$_{50}$ = 39 nM (n = 1) in the chromogenic assay and IC$_{50}$ = 86 nM in the fluorescence assay. Nevertheless, the rank order of inhibitor potencies against uPA was preserved across both assays (e.g. 49 < 43 < 48 << HMA). The variation between assays likely arises from difference in the affinity of the two substrates for the uPA active site. Differences in assay buffer composition may have also contributed to the variations. For all other TLSPs tested, half-maximal inhibitory concentrations were not reached at the highest concentrations tested (10 µM). Compound 42 showed slight inhibition of trypsin, but this was only 24% inhibition at 10 µM.

Similar results were observed for compounds 43 and 49 (Table 2.4), where half-maximal inhibition of tPA, plasmin or thrombin was not reached. Of the enzymes tested, plasmin activity was inhibited to the greatest extent by 43 (16%) and 49 (48%) at 20 µM. These results clearly demonstrated that 6-HMA analogues with high uPA inhibitory potency are 10-100-fold less potent against closely related TLSP off-targets.
Figure 2.10: Activity of 42 against uPA and related trypsin-like serine proteases determined using chromogenic enzyme activity assays. Data represent the mean ± SEM (n = 3).
Table 2.4: Inhibitory potencies of 6-HMA analogues 42, 43 and 49 against uPA and related trypsin-like serine proteases. N/C = Experiment not conducted.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
<th>uPA</th>
<th>tPA</th>
<th>Trypsin</th>
<th>Thrombin</th>
<th>Plasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="42" /></td>
<td>529 nM</td>
<td>&gt;10 mM (&gt;19)</td>
<td>&gt;10 mM (&gt;19)</td>
<td>&gt;10 mM (&gt;19)</td>
<td>&gt;10 mM (&gt;19)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="43" /></td>
<td>429 nM</td>
<td>&gt;20 mM (&gt;47)</td>
<td>N/A</td>
<td>&gt;20 mM (&gt;47)</td>
<td>&gt;20 mM (&gt;47)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="49" /></td>
<td>39 nM</td>
<td>&gt;20 mM (&gt;19)</td>
<td>N/A</td>
<td>&gt;20 mM (&gt;19)</td>
<td>&gt;20 mM (&gt;19)</td>
<td></td>
</tr>
</tbody>
</table>

A wider selection of 6-HMA analogues were also screened against a panel of TSLPs involved in the blood coagulation cascade, including human plasma kallikrein, Factor Xa, Factor Xla and Activated Protein C (Table 2.5). Experiments used the chromogenic substrates S-2288, S-2366 or S-2444 (Chromogenix, Massachusetts, USA), depending on their suitability for each enzyme based on manufacturer Vₘₐₓ values. Due to time constraints (experiments were performed during a three week international research visit to the laboratory of the Prof. Mingdong Huang, Fujian Institute for Research into the Structure of Matter (FJIRSM), Fuzhou (China), compounds were not tested simultaneously against wildtype human uPA, thus a determination of fold-change in activity relative to uPA was not possible. Nevertheless, a similar profile to that observed with 43, 48 and 49 against tPA, plasmin and thrombin was seen with most of the compounds, where half-maximal inhibition was not observed at the highest concentration tested (50 μM).
Half-maximal inhibition of Factor Xa activity was not observed for any of the compounds in the range of concentration range tested (50 nM-50 μM). For Factor XIa, half-maximal inhibition was achieved at concentrations lower than 50 μM for 5 of the 12 compounds screened. The 3,6-dimethoxy-4-pyridazine 55 showed the greatest inhibition, returning an IC$_{50}$ of 8.7 μM. The 4-fluoro-2-benzofuran analogue 43 (IC$_{50}$ = 23.3 μM) showed slightly higher inhibition of Factor XIa than the unadorned 2-benzofuran 42 (IC$_{50}$ = 31.0 μM). The 2,4-dimethoxypyrimidine analogue 50 showed intermediate potency (IC$_{50}$ = 25.8 μM) and IC$_{50}$ was not reached for any of the other compounds against Factor XIa. The 5-pyrimidine analogue 48 was the only compound for which an IC$_{50}$ could be calculated against Activated Protein C (IC$_{50}$ = 26.9 μM).

While slight activity was observed for some of the compounds against Factor XIa, Plasma kallikrein and Activated protein C, a >10-fold difference in potency was generally seen between uPA potency and potency against the off-targets.
Table 2.5: Inhibitory potencies of selected 6-HMA analogues against TLSPs from the blood coagulation cascade.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Human PKα</th>
<th>Factor Xaβ</th>
<th>Factor Xlaα</th>
<th>Activated Protein C¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMA</td>
<td></td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>40.7</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>&gt;50</td>
<td>N/A</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>41</td>
<td></td>
<td>&gt;50</td>
<td>N/A</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>11.3</td>
<td>&gt;50</td>
<td>31.0</td>
<td>&gt;50</td>
</tr>
<tr>
<td>43</td>
<td></td>
<td>16.7</td>
<td>&gt;50</td>
<td>23.3</td>
<td>&gt;50</td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>26.9</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>24.5</td>
<td>&gt;50</td>
<td>25.8</td>
<td>&gt;50</td>
</tr>
<tr>
<td>51</td>
<td></td>
<td>&gt;50</td>
<td>N/A</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>&gt;50</td>
<td>N/A</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>55</td>
<td></td>
<td>16.7</td>
<td>&gt;50</td>
<td>8.7</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>
2.4 Selectivity of inhibitors for human versus murine uPA

Species specificity and potency differences of active site inhibitors against human and murine uPA have been noted in the literature.\textsuperscript{22,287,288} Smaller inhibitors that interact primarily with the S1 binding pocket tend to show similar potencies against the two enzymes, (selectivity ratios = 0.5-5), whereas Inhibitors that make contacts in other sites (e.g. S1β, S2) show a pronounced preference (10-100 fold or more) for the human enzyme.\textsuperscript{22} These trends arise because the four active site amino acid substitutions between the species are located in sites outside S1. HuPA:MuPA D60Q is located near the catalytic triad, H99Y is on the edge of S2/S4, S146E is in S1β and Q192K is on the boundary of S1/ S1β.\textsuperscript{22}

The preference of inhibitors for human over murine uPA presents challenges for conducting studies with compounds in mouse xenograft tumour and metastasis models. This is because enzymes derived from both the xenografted human tissue and the murine host may contribute to plasminogen activation, invasion and metastasis. The issue is further complicated by the variable propensity of uPA/uPAR-upregulating tumour cells from different tissues to recruit uPA from the surrounding stromal environment. For example, breast carcinomas recruit the majority of their uPA complement from surrounding stromal cells.\textsuperscript{289,290} The differing affinities of human uPAR for human and murine uPA and the higher rate of activation of both human and murine plasminogen by the human enzyme add even more complexity.\textsuperscript{291}

If an inhibitor being studied \textit{in vivo} showed a marked preference for HuPA there is a possibility that a xenograft model may underestimate its efficacy, making it difficult to demonstrate \textit{in vivo} proof-of-concept. The activity of two of the more potent analogues \textbf{42} and \textbf{50} analogues against wild-type murine uPA was therefore assessed using a fluorogenic assay. Comparative
data for the two compounds, amiloride and HMA against the human and murine enzymes is presented in Table 2.6.

As expected amiloride, which binds to uPA primarily through contacts within the S1 pocket, showed virtually no difference in activity against the two enzymes. HMA was found to lose the most potency against MuPA (IC$_{50}$ = 13.3 µM), experiencing a ~ 6-fold drop in activity. As amiloride and HMA differ only in the identity of the 5-amino substituent, the drop in potency can be attributed to unfavourable interactions between the 5-azepane ring of HMA and the active site of MuPA. Assuming that HMA adopts a similar binding pose when bound to both enzymes, the 5-substituent would occupy space corresponding to the junction of S1 and S2 and the oxyanion hole (Figure 2.11).

Table 2.6: Inhibitory potencies of amiloride, HMA and 6-substituted analogues 42 and 50 against human and murine uPA.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human uPA IC$_{50}$ (nM)</th>
<th>Murine uPA IC$_{50}$ (nM)</th>
<th>Human/Mouse selectivity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>3,954</td>
<td>3,310</td>
<td>0.8</td>
</tr>
<tr>
<td>HMA</td>
<td>2,206</td>
<td>13,321</td>
<td>6.0</td>
</tr>
<tr>
<td>42</td>
<td>297</td>
<td>2,579</td>
<td>8.7</td>
</tr>
<tr>
<td>50</td>
<td>69</td>
<td>2,305</td>
<td>33.4</td>
</tr>
</tbody>
</table>
In murine uPA, the S2 residue His\textsuperscript{99}_{HuPA} is substituted by a bulkier Tyr (His van der Vaal’s volume = 118 Å\textsuperscript{3}; Tyr = 141 Å\textsuperscript{3}), with the three other active site substitutions lying outside of this area.\textsuperscript{292,293} Assuming that these distant substitutions do not cause major structural differences in the S1/S2 and oxyanion hole regions, it is possible that the drop in activity observed for HMA and 6-HMA analogues relative to amiloride is due to unfavourable interactions between the 5-hexamethyelene ring and the side chain of Tyr\textsuperscript{99} in murine uPA.

**Figure 2.11:** X-ray co-crystal structure of HMA bound to WT human uPA highlighting the position of His\textsuperscript{99}, presented in coral, and its close proximity to the azepane ring. In Murine uPA, residue 99 is substituted with a larger Tyr. Unfavourable interactions between the Tyr\textsuperscript{99} sidechain and the azepane ring of HMA and analogue(s) may be responsible for reducing activity against MuPA. Gln\textsuperscript{192} and Ser\textsuperscript{146}, two other active site residues that differ between the human and murine enzymes, are presented in purple and orange respectively.

Benzofuran analogue 42 showed improved potency relative to HMA against both human (IC\textsubscript{50} = 297 nM, c.f. 2,206 nM \textasciitilde 7.4-fold increase) and murine uPA (IC\textsubscript{50} = 2,579 nM, c.f. 13.3 \mu M for HMA, \textasciitilde 5.2-fold increase). This compound showed \textasciitilde 8.7-fold selectivity for Human uPA.
Species differences were most pronounced for the dimethoxypyrimidine analogue 50, where 33-fold greater potency was observed against (IC$_{50}$ = 69 nM vs. MuPA IC$_{50}$ = 2,305 nM). The compound showed 5.8-fold higher potency than HMA against MuPA. Taken together, these results indicate that substitution of HMA at the 6-position is beneficial for activity against MuPA. The presence of the 2-benzofuranyl and 2,4-dimethoxypyrimidinyl groups in 42 and 50 restored some of the activity lost due to the presence of the 5-azepane ring. Restoration of potency is likely due to the new favourable interactions established between the 6-substituents and the S1β subsites in both enzymes.

The similarity in inhibitory potencies of 42 and 50 (42 IC$_{50}$ = 2,579 nM, 50 IC$_{50}$ = 2,305 nM) against MuPA suggests that the S1β subsite of this enzyme is less able to make favourable contacts with 6-substituents relative to HuPA, where increases in inhibitory potency approaching two orders of magnitude were observed upon varying the 6-substituent against the human enzyme (see Table 2.4).

Decreased tolerability of these substituents in MuPA is likely due to the Ser$^{146}$Glu and Gln$^{192}$Lys substitutions in S1β, as these residues define part of the pocket boundary in HuPA (Figure 2.11). These data corroborate previously described selectivity trends for S1β-targeting small molecule inhibitors, where the subsite was generally more accepting of ligand substituents in the human enzyme.$^{22}$

To further explore the effect of H99Y substitution on inhibitory potency, selected, analogues were screened against a recombinant human uPA mutant carrying the H99Y mutation (HuPA$^{H99Y}$, Table 2.7).$^{294}$ In line with predictions, the “murinized” H99Y uPA substitution decreased the activity of all 6-HMA analogues relative to WT HuPA (e.g. HMA IC$_{50}^{H99Y}$ > 50 μM,
HMA IC\textsubscript{50}^{WT} 2206 nM). While amiloride was not tested against HuPA\textsuperscript{H99Y}, the results suggest that the ~ 4-fold lower potency of HMA relative to amiloride against MuPA is, at least in part, a result of unfavourable interactions with Tyr\textsuperscript{99}. The losses in activity observed for HMA and 6-HMA analogues against HuPA\textsuperscript{H99Y} generally aligned with those observed between WT HuPA and MuPA.

The observation that 6-substitution was generally beneficial to interactions in both WT HuPA and MuPA was recapitulated with compounds 38, 42, 43, 48, 50 and 55, which returned IC\textsubscript{50} values in the range 3.0-9.7 μM. Supporting X-ray co-crystal structures of some 6-HMA analogues bound to H99Y are presented in Chapter 3.2.
Table 2.7: Inhibitory activities of 6-HMA compounds against a site-specific mutant of human uPA featuring a His to Tyr substitution at position 99 (HuPA$^{H99Y}$).

<table>
<thead>
<tr>
<th>R</th>
<th>HuPA$^{H99Y}$ IC$_{50}$ (μM)</th>
<th>Selectivity Factor (WT/HuPA$^{H99Y}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMA</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>37</td>
<td>40.7</td>
<td>7.1</td>
</tr>
<tr>
<td>38</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>40</td>
<td>&gt;50</td>
<td>N/A</td>
</tr>
<tr>
<td>41</td>
<td>&gt;50</td>
<td>N/A</td>
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<tr>
<td>42</td>
<td>11.3</td>
<td>38.0</td>
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<tr>
<td>48</td>
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<tr>
<td>50</td>
<td>9.7</td>
<td>140.6</td>
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<tr>
<td>51</td>
<td>&gt;50</td>
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<td>53</td>
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</tr>
<tr>
<td>55</td>
<td>16.7</td>
<td>32.7</td>
</tr>
</tbody>
</table>
2.5 Physicochemical properties of 6-HMA analogues

In order to understand how the intrinsic properties of 6-HMA analogues affect uPA potency, relationships between inhibitory activity \( pIC_{50} \) and a variety of physicochemical parameters were calculated for each using Biovia Insight for Excel (Dassault Systemes, Velizy-Villacoublay, France, Figure 2.12).

A weak positive correlation \( (r^2 = 0.2025) \) between polar surface area (PSA) and \( pIC_{50} \) (Figure 2.12A) was observed for compounds 33-56.\(^{29}\) A stronger correlation \( (r^2 = 0.3141) \) was found between the number of hydrogen bond acceptors, which ranged between 8 and 12, and inhibitory potency (Figure 2.12C). Sub-micromolar \( IC_{50} \) values \( (pIC_{50} > 6) \) were achieved only for compounds bearing more acceptors than HMA (i.e. > 8). All compounds contained the same number of hydrogen bond donors (i.e. 6) except indole analogue 40 (7 H-bond donors), making correlation analysis for this variable uninformative. No correlation was found between inhibitory potency and the number of rotatable bonds present in each structure \( (r^2 = 0.001) \), with clustering of data around 4 and 6 rotatable bonds (4 rotatable bonds: 15 of 25 compounds; 6 rotatable bonds: 5 of 25 compounds). Likewise, no relationship was observed between molecular weight and \( pIC_{50} \) \( (r^2 = 0.0005, \) Figure 2.12B).

Due to the basic nature of the acyl guanidine moiety (amiloride \( pK_a = 8.71 \)) present in all analogues a significant proportion of each compound would exist in a charged state in solution at physiological pH.\(^{295}\) For this reason, LogD values were calculated at \( \text{pH} \) 7.4 to account for the contributions of both the charged and uncharged species to the partitioning coefficient. Comparison of CLogD to CLogP values (which considers partitioning of uncharged species only) for each compound revealed that both metrics were virtually equivalent, with CLogD values < 0.04 units higher for all analogues. The exception was 4-isoquinoline analogue
37, whose CLogD was affected by the basic isoquinoline ring nitrogen (pKa ~ 5.4-5.5). This comparison suggests that the identity of the distal 6-substituent does not strongly influence the pKa of the acyl guanidine. 6-substituents bearing ionizable functional groups featuring pKa values closer to 7.4 would experience greater shifts in LogD relative to LogP due to the functional group contribution to the total molecular pKa, as observed with 37 (ClogP 2.37 vs. ClogD 2.60).

An inverse correlation was found between uPA inhibitory potency and ClogD (Figure 2.12D, r² = 0.2772). This correlation was strengthened by exclusion of the hydrophobic 2-benzofuranyl analogues 42 and 43 (r² = 0.5158). The presence of 2-benzofuranyl 42 or 4-fluoro-2-benzofuranyl 43 substituents at the 6-position caused large increases in ClogD relative to HMA (HMA ClogD 1.52; 42 3.19; 43 3.39). The finding that two structural classes of 6-HMA analogues that differ significantly in hydrophobicity (5-pyrimidinyl 48 ClogD 0.82, IC₅₀ = 175 nM; 4-fluoro-2-benzofuranyl 42 ClogD 3.39, IC₅₀ = 143 nM) are nearly equipotent suggests that uPA inhibition with this class is not a function of ClogD and that affinity is instead driven by specific intermolecular interactions between the inhibitors and uPA.

The physicochemical differences between 2-benzofuranyl 42 and 5-pyrimidinyl 48 were further highlighted by their lipophilic ligand efficiencies (LLE) (Figure 2.12D, diagonal lines). LLE is an estimation of the preference of a compound for a target over partitioning into 1-octanol, and is used to evaluate the benefit of increasing hydrophobicity relative to effects on potency.32 For compounds showing properties typical of orally active drugs (ClogD ~ 2.5-3, target potency ~ 1-10 nM) LLE is typically > 5, which is considered an indicator of a well optimized drug candidates.34 A compound with LLE 6 will have a 10⁶-fold greater affinity for
interaction with a given target than 1-octanol.\textsuperscript{34} The diagonal lines in Figure 2.12D indicate LLE units.

Only the pyrimidine-based analogues displayed LLEs >5, with the unadorned 5-pyrimidinyl 48 showing the highest LLE (LLE: 48 5.94, 49 5.74, 50 5.32). The 2-benzofuranyl analogues 42 and 43 showed LLEs below 4 (42 3.33, 43 3.45), or lower than the parent HMA (LLE 4.13). Increases in potency associated with inclusion of the benzofuranyl moieties was thus not sufficient to offset the concomitant increases in hydrophobicity. Inclusion of the 2-benzofuranyl substituent would require a 46-fold increase in potency relative to HMA (required IC\textsubscript{50} = 48 nM, observed IC\textsubscript{50} = 297 nM, observed fold-increase 7.4-fold) to maintain an LLE equivalent to the parent structure (HMA LLE 4.13). Additionally, to match the ‘well optimized’ LLE of the 5-pyrimidinyl 48 (LLE 5.94) the potency of 48 would have to increase 2,997-fold, giving an IC\textsubscript{50} = 741 pM.

Despite the increases in potency afforded by 6-substitution, none of the 6-HMA analogues were able to match the high LLE of amiloride (LLE 6.16). This finding illustrates the effect of polarity relative to potency on LLE calculations. In the case of amiloride, high LLE arising from its negative ClogP (ClogP -0.76) dramatically offsets its moderate potency (IC\textsubscript{50} = 3,954 nM).
Figure 2.12: Relationship between uPA inhibitory activity ($pIC_{50}$) and calculated physicochemical parameters for a library of $6$-HMA analogues. A) $pIC_{50}$ vs polar surface area. B) $pIC_{50}$ vs molecular weight. C) $pIC_{50}$ vs number of H-bond acceptors. D) LLE plot, solid lines represent linear regression fits for each data set and dotted curves describe 95% confidence intervals. Analogues lying above the blue line (LLE >5) are considered well optimized in terms of their activity against uPA relative to their intrinsic hydrophobicity.
The changes in CLogD and inhibitory potency relative to HMA further highlights the intrinsic differences between the 2-benzofuranyl and 5-pyrimidinyl-based lead classes (Figure 2.13, red and green boxes, respectively). During the optimization process it is desirable for leads to progress towards the top left corner of a $\Delta pIC_{50}/\Delta CLogD$ plot. The 2-benzofuranyl class clearly deviates from this trajectory due to accompanying increases in CLogD, while the 5-pyrimidinyl analogues clearly localize in the correct region. Relative to HMA, 5-pyrimidinyl analogue 48 and the $p$-methoxy-5-pyrimidinyl 49 showed improvements in both CLogD and potency.

**Figure 2.13:** Changes in ClogD and $pIC_{50}$ for 6-HMA analogues relative to HMA.

Based on calculated physicochemical properties (Table 2.8), the 6-HMA analogues were evaluated for ADMET liabilities using a set of predictive computational tools within the Biovia Insight for Excel software suite (Dassault Systemes, Velizy-Villacoublay, France). The majority of 6-HMA analogues (56%, 14/25 compounds) were predicted to cause dose-dependent hepatotoxic injuries (e.g. hepatitis, zonal necrosis, cholestasis etc.) or trigger elevated blood levels of aminotransferase enzymes in over 10% of the human population.
In contrast, the 5-pyrimidinyl analogues 48, 49 and 50 were predicted to be non-hepatotoxic. Compounds with CLogP values exceeding 3, including benzofuran 42 and 43, were predicted to have very low aqueous solubility (at the lower limit of 95% of orally active drugs). All other compounds, including the 5-pyrimidinyl leads 48, 49 and 50 and HMA, were predicted to have low solubility (towards the lower end of 95% of orally active drugs), with the exception of N-1-methyl-1H-4-pyrazole 38, which was predicted to have good solubility.41

The 5-pyrimidinyl leads 48, 49 and 50 and structures featuring methoxy (51, 53, 54 and 55), morpholino (39) or methylenedioxy (36) substituents, were predicted to show very low passive intestinal absorption following oral administration (outside 99% confidence ellipse for orally active compounds). Good-moderate (within 95% confidence ellipse) absorption was predicted for the 2 and 3-thiophenyl analogues 44 and 45, HMA and phenyl analogue 33. All other compounds, including the 2-benzofuran leads 42 and 43, were predicted to exhibit low passive intestinal absorption (between 95% and 99% confidence ellipses).

Predictions regarding favourable solubility and absorption characteristics for small molecules are often made using the well-known Lipinski’s “rule of five” (MW <500 g.mol⁻¹, CLogP < 5, ≤ 10 H-bond acceptors and ≤ 5 H-bond donors).42 HMA and the 6-HMA analogues all exceed the H-bond donor criteria as the guanidine and 3-NH₂ groups provide 6 potential donors. Compounds 36, 38, 39, 51, 54 and 55 and the 5-pyrimidines 48, 49 and 50 violate both the H-bond donor and acceptor criteria.

Reliable predictions of blood-brain barrier permeability were not possible as the compounds were predicted to lie outside of the 99% confidence ellipse in the model used by Biovia Insight for Excel. All compounds were considered unlikely to be substrates for cytochrome P450.
Isozyme CYP2D6 and no compound was predicted to bind strongly to human plasma proteins.

(strong binder = > 90% bound).
Table 2.8: Summary of uPA inhibitory potencies, predicted physiochemical properties and calculated ADMET metrics for 6-HMA analogues. All physicochemical parameters and ADMET predictions were calculated using Biovia Insight for Excel v2.6. tPSA = topographical polar surface area. HAC = Heavy atom count. LE = ligand efficiency \(((1.37/\text{heavy atoms}) \times pIC_{50})\). Rotatable bonds = number of bonds within structure that possess meaningful rotational freedom for molecular mechanics, excluding terminal hydrogens. Liver toxicity = Predicted dose-dependent hepatotoxicity. Predicted aqueous solubility (see reference 41). Predicted intestinal absorption (see reference 53).

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<th>ClogD (Δ rel. HMA)</th>
<th>ClogP (Δ rel. HMA)</th>
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2.6 Summary and Conclusions

The new synthetic methods described herein provided divergent access to 6-substituted HMA analogues using common, commercially available reagents. Based on published X-ray co-crystal structures of amiloride bound to uPA, we correctly hypothesized that 6-substitution of HMA 27 may afford increases in inhibitory potency by establishing new interactions within the S1β subsite of uPA. Screening the synthesized library of analogues against Human uPA revealed that, in general, the S1β subsite is able to tolerate a variety aryl and heteroaryl substituents. The greatest increase in potency was observed with the dimethoxypyrimidine analogue 50 (IC$_{50}$ = 69 nM). The 2-benzofuranyl and 5-pyrimidinyl classes emerged as two structurally and physicochemically distinct lead classes.

Screening of selected inhibitors against a variety of potential TLSP off-targets revealed good uPA selectivity across all analogues. As half-maximal inhibition was not achieved for trypsin, plasmin and tPA, retesting against these enzymes at higher concentrations would be needed to determine selectivity factors. We conclude that off-target TLSP inhibition should not be a major issue with this class of compounds if used as drugs.

Experiments with murine uPA revealed that the 6-HMA analogues show substantially lower potency against the murine enzyme relative to human uPA. Experiments with a ‘murinized’ variant of human uPA carrying a H99Y substitution suggested that the decreased potency was at least partially caused by unfavourable interactions between the larger, Tyr99 side chain in mouse uPA and the 5-azepane ring present in all HMA analogues. These findings have significant implications for in vivo xenotransplantation studies in mice as the decreased activity may lead to underestimation of the antimetastatic efficacy of the inhibitors since both human and endogenous
murine uPA will play roles in this process. Ongoing studies in the Kelso and Ranson Labs are seeking to identify compounds that potently inhibit both mouse and human uPA to address this.

Analysis of the calculated physicochemical properties of the 6-HMA analogues suggested that high potency is not reliant on intrinsic hydrophobicity, given that nM inhibitors were generated from structures varying widely in ClogD. Indeed, strong correlations were not found between potency and a range of physicochemical and structural parameters, suggesting that uPA affinity likely arises from a combination of specific and non-specific interactions, in addition to hydrophobicity. LLE analysis revealed that the large increases in hydrophobicity associated with inclusion of 2-benzofuranyl moieties cancelled out the moderate increases in potency in terms of LLE. Conversely, all members of the 5-pyrimidine class were calculated to have LLE values in the ‘well optimized’ range (LLE > 5), supporting further studies with these compounds in particular.
CHAPTER 3: X-ray co-crystal structures of HMA and 6-HMA analogues bound to uPA

X-ray crystallographic analysis of ligand-protein complexes occupies a central position in modern drug discovery.\cite{297,298} Three dimensional visualization of drug-target interactions allows the rationalization of SAR trends arising from biological assays and can be used to guide the direction of chemical synthesis towards analogues with improved potency and selectivity. This process is known as structure-based drug design.\cite{299} Collaborative efforts between the Kelso/Ranson laboratories at UOW and scientists at the Fujian Institute for Research into the Structure of Matter (FJIRSM), Fuzhou (China), allowed for integration of X-ray structural data into the selection of synthetic targets. Novel co-crystal structures were obtained for HMA \textbf{27} (Figures 2.1 and 3.1) and the 6-HMA analogues \textbf{38}, \textbf{42}, \textbf{43} and \textbf{46-50} at resolutions ranging from 1.6-3.0 Å. Complexes were obtained by soaking each inhibitor into pre-formed Human uPA crystals over a period of 14 days prior to X-ray diffraction experiments. All co-crystals were obtained and their structures solved by Dr. Longguang Jiang at the FJIRSM. A summary of the data collection and refinement statistics is provided in Appendices 9.1 and 9.2.

3.1 HMA:uPA co-crystal structure

The expected salt bridge was observed between the positively charged acylguanidine of HMA and the Asp189 side chain at the base of the S1 binding pocket (Figure 3.1). H-bonds were present between the backbone carbonyl of Gly219 and the terminal NH\textsubscript{2} and amide NH groups of the acyl guanidine. The other guanidino NH\textsubscript{2} formed a H-bond to the Ser190 side chain OH and the exocyclic NH\textsubscript{2} group at C3 was H-bonded to the side chain OH of Ser195. A bridged H-bonding network was formed between a surface-bound water molecule, the acyl guanidine carbonyl, the Ser190 side chain hydroxyl, and possibly the guanidino NH\textsubscript{2}.
Figure 3.1: A) X-ray co-crystal structure of HMA bound to the serine protease domain of uPA (1.6 Å). The electron density map \((2mF_o-DF_o)\) is contoured at 1.5 \(\sigma\). Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. A bridging water molecule is shown as a red ball. All non-bridging water molecules have been omitted for clarity. A sulfate from the crystallization buffer is bound to the oxyanion hole and is presented in stick representation. B) Equivalent structure to A) with surfaces coloured according to electrostatic potential (uPA) or cyan (HMA). C) Summary of the binding interactions between HMA and uPA. Residues comprising the S1\(\beta\) subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.

The hydrophobic nature and compact size of the S1\(\beta\) subsite is highlighted by the electrostatic potential surface map in Figure 3.1A. The accessibility to S1\(\beta\) from the 6-position of the HMA scaffold was clear. The 6-Cl group of HMA interacted primarily at the mouth of S1\(\beta\), participating
in van der Waals interactions with the Cys191-Cys220 disulfide bond, Gly219 and Gln192Cβ (Figure 3.1C).

3.2 Co-crystal structures of 6-HMA analogues

Evaluation of the 6-HMA analogue:uPA X-ray co-crystal structures revealed that the hydrogen bonding and ionic interactions observed between the acyl guanidine of HMA and Asp189, Gly219, Ser190 and the H-bond from the exocyclic NH₂ group at C3 and Ser195 in the HMA-uPA structure were preserved, showing only slight variations in bond distances (measured as the distance between the two heteroatoms in all cases). Participation of the terminal guanidino NH₂ in the bridging network comprised of the surface H₂O, Ser190 and the acyl guanidine carbonyl showed more variation as prospective H-bonds distances for these interactions exceeded accepted limits in some cases (i.e. >3.3 Å). The SAR trends observed for the 6-HMA analogues can largely be rationalized in terms of formation of new favourable (or unfavourable) interactions between the 6-substitutent and the S1β subsite.

3.2.1 Furanyl analogues 46 and 47

2-Furanyl analogue 46 (IC₅₀ = 254 nM) showed 8.7-fold higher potency than HMA (IC₅₀ = 2,206 nM). The increase in affinity appeared to arise, at least in part, by formation of new H-bonds between the furanyl O, a bridging water molecule and the amide NH of Gly219 (Figure 3.2C). Interactions were also observed between the furan ring and Gly219, Cys191-Cys220, Ser146 backbone carbonyl and the side chain Cβ and Cγ of Gln192. Figure 3.2B highlights the increased occupation of the S1β subsite by the 2-furanyl substituent relative to the Cl atom in HMA.
Figure 3.2: A) X-ray co-crystal structure of 46 bound to the serine protease domain of human uPA (1.7 Å). The electron density map ($2mF_o-DF_C$) is contoured at 1.5 σ. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. Bridging water molecules are represented as red balls. All non-bridging water molecules have been omitted for clarity. A sulfate from the crystallization buffer is bound to the oxyanion hole and is presented in stick representation. B) Equivalent structure to A) with surfaces coloured according to electrostatic potential (uPA) or cyan (46). C) Summary of the binding interactions between 46 and uPA. Residues comprising the $S_1\beta$ subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.
Altering the position connecting the furan ring to produce the 3-furanyl analogue 47 had little effect on the activity (IC₅₀ = 270 nM). Movement of the furanyl oxygen abolished the H-bond interaction with the bridging water molecule bound to Gly219, as seen in the 2-furanyl 46:uPA structure (Figure 3.3C). Flexibility of the Arg217 side chain was apparent in this structure as it had moved significantly to participate in the Gly219-bridging water H-bonding network (Figure 3.3A). Relative to 46, the plane of the furan ring in 47 had tilted 11°, adopting a pose that appears to facilitate van der Waals interactions with Cys220. Interactions between S1β residues Cys191-Cys220, Ser146 and the Gln192 side chain were preserved and there appeared to be a polar interaction between the side chain carbonyl of Gln192 and the δ+ CH at the 2-position of the furanyl ring. The new combination of interactions appeared to compensate for the lost H-bonding interaction to the Gly219-bridging water-Arg217 bonding network seen for 46.
Figure 3.3: A) X-ray co-crystal structure of 47 bound to the serine protease domain of uPA (1.7 Å). The electron density map ($2mF_o-DF_C$) is contoured at 1.5 $\sigma$. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. Bridging water molecules are represented as red balls. All non-bridging water molecules have been omitted for clarity. B) Equivalent structure to A) with surfaces coloured according to electrostatic potential (uPA) or cyan (47). C) Summary of the binding interactions between 47 and uPA. Residues comprising the S1$\beta$ subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.
3.2.2 1-Methyl-pyrazolyl analogue 38

In contrast to furanyl analogues 46 and 47, 1-methyl-pyrazolyl analogue 38 positioned its ring coplanar with the pyrazine core, reducing its penetration into S1β (Figure 3.4). This orientation limited potential interactions between the N-methyl group and the Lys143-Gln192-Ser146 hydrophobic dimple, instead projecting it out towards solvent. Interactions appeared to exist between the pyrazole C3 and C5 CH groups and the Gly219 backbone and Gln192 side chain, respectively. The surface water bound to Gly219 in the furanyl structures was observed, however it did not interact with the 6-substituent of 38. The decreased occupation of S1β due to the coplanar orientation of the pyrazole with the pyrazine core probably contributed to its lower activity against uPA (IC$_{50}$ = 825 nM) relative to the furanyl analogues.
**Figure 3.4:** A) X-ray co-crystal structure of 38 bound to the serine protease domain of human uPA (1.9 Å). The electron density map (2mFo-DFc) is contoured at 1.5 σ. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. Bridging water molecules are represented as red balls. All non-bridging water molecules are omitted for clarity. B) Equivalent structure to A) with interacting surfaces coloured according to electrostatic potential (uPA) or cyan (38). C) Summary of the binding interactions between 38 and uPA. Residues comprising the S1β subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.

### 3.2.3 Benzofuran analogue 42

Fusion of a phenyl ring to the 2-furanyl ring of 46 to create benzofuran 42 resulted in a slight decrease in potency (42 IC₅₀ = 297 nM). Figure 3.5B shows that the benzofuran ring system extends well into S1β, almost completely filling the subsite. The plane of the benzofuran ring was almost perpendicular to the pyrazine core, but the ring oxygen appeared to have moved to the opposite side of the pyrazine relative to the furanyl oxygen of 46. This orientation allowed the benzofuran 7-position CH to fill the Lys143-Gln192-Ser146 hydrophobic dimple. Additional
hydrophobic contacts were observed between the benzofuran 3- and 4-position CHs and Gly219. As seen with the HMA:uPA and 46:uPA structures, the Arg217 side chain adopted a conformation well away from S1β. The surface water bound to the Gly219 carbonyl O, as present in other structures, was not observed.
**Figure 3.5:** A) X-ray co-crystal structure of 42 bound to the serine protease domain of human uPA (1.6 Å). The electron density map ($2mF_o-DF_c$) is contoured at 1.5 σ. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. Bridging water molecules are represented as red balls. All non-bridging water molecules have been omitted for clarity. B) Equivalent structure to A) with interacting surfaces coloured according to electrostatic potential (uPA) or cyan (42). C) Summary of the binding interactions between 42 and uPA. Residues comprising the S1β subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.
3.2.4 4-F Benzofuran analogue 43

A 2-fold improvement in uPA-inhibitory potency was achieved by adding fluorine to the 4-position of the benzofuranyl ring of 42 to yield 43 (IC$_{50} = 143$ nM versus IC$_{50} = 297$ nM for 42).

The X-ray co-crystal structure with 43 revealed that the increased potency possibly arises from favourable interactions between the 4-fluoro group and the side chain guanidine of Arg217 and the backbone amide NH of Gly219 (Figure 3.6A). These interactions were not observed in the 42:uPA structure as the sidechain of Arg217 adopted a position away from S1β, as observed with 38, 46 and HMA. The interaction between the 4-fluoro group and Arg217 caused a slight rotation about the benzofuran-pyrazine axis, where the dihedral angles were 78° for 42 and 68° for 43 (Figure 3.7). Similar to the 42:uPA structure, the surface water interacting with the Gly219 backbone carbonyl was not observed.
Figure 3.6: A) X-ray co-crystal structure of 43 bound the serine protease domain of human uPA (1.9 Å). The electron density map \(2mF_o-DF_c\) is contoured at 1.5 σ. Electrostatic potential surfaces
of uPA are shown with blue = negative and red = positive. A bridging water molecule is represented as a red ball. All non-bridging water molecules have been omitted for clarity. B) Equivalent structure to A) with interacting surfaces coloured according to electrostatic potential (uPA) or cyan (43). C) Summary of the binding interactions between 43 and uPA. Residues comprising the S1β subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.

Figure 3.7: Superposition of 42 (gold) and its 4-fluorinated analogue 43 (green). The plane of the benzofuran moiety relative to the pyrazine ring is rotated slightly in 43 (dihedral angle = 78°) compared to 42 (dihedral angle = 68°), allowing formation of a favourable interaction between the polarized fluorine and the positively charged sidechain of Arg217.

3.2.5 Pyrimidine analogues 48, 49 and 50

X-ray diffraction data sets of varying quality (1.7-3.0 Å) were obtained for the three 5-pyrimidine-based compounds 48, 49 and 50. Analysis of the unadorned 5-pyrimidine 48:uPA structure at 2.0 Å showed that this substituent partially fills S1β (Figure 3.8). Interactions with the Cys191-Cys220 disulfide, Ser146 and Gln192 residues were preserved with the addition of an apparent dipole interaction between the δ+ CH at the 4-position of the pyrimidinyl and the Gln192 side chain carbonyl (Figure 3.8A). A H-bond was observed between the backbone amide NH of Gly219 and the lone pair of electrons on the pyrimidine nitrogen. The pyrimidine ring was oriented favourably
to form a polar interaction between the $6^+\text{CH}$ at 6-position and the Gly219 backbone carbonyl. The sidechain of Arg217 was positioned close to the pyrimidine 6-substituent, potentially forming a favourable polar interaction with the ring. The bridging water molecule contributing to the H-bonding network between the acylguanidine carbonyl, $\text{NH}_2$ groups and the side chain hydroxyl of Ser190, which was consistently observed in other structures, was not present. This may have been due to the lower resolution of the 48:uPA structure.
Figure 3.8: A) X-ray co-crystal structure of 48 bound to the serine protease domain of human uPA (2.0 Å). The electron density map ($2mF_o-DF_C$) is contoured at 1.5 $\sigma$. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. Water molecules have been omitted for clarity. B) Equivalent structure to A) with interacting surfaces coloured according to electrostatic potential (uPA) or cyan (48). C) Summary of the binding interactions between 48 and uPA. Residues comprising the S1β subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts. The backbone NH of the Gly219 was observed to donate a H-bond to the pyrimidine nitrogen lone pair.
Methoxy derivatization at the pyrimidine 2-position of 48 to create 49 resulted in a 2-fold increase in potency (49 $IC_{50} = 86$ nm, 48 $IC_{50} = 175$ nM). Superposition of the 48:uPA and 49:uPA co-crystal structures revealed that the inhibitors adopt very similar poses, with rotation about the plane of pyrimidinyl ring relative to the pyrazine core varying by only 22° (Figure 3.9).

Figure 3.9: Superposition of the uPA:48 (green, uPA in orange) and uPA:49 (magenta, uPA in mauve) structures. In both structures the sidechain of Arg217 is positioned close to the pyrimidine substituents to form dipolar interactions.

The methoxy CH$_3$ group of 49 was oriented towards bulk solvent (Figure 3.10A). Compared to 48, the Gly219 NH-pyrimidine nitrogen H-bond was lengthened considerably, weakening the interaction (2.9 Å in 48 versus 3.4 Å in 49, Figure 3.10C). The sidechain of Arg217 had again flipped into close proximity with the substituent to make polar interactions with the pyrimidine nitrogen. The bridging water molecules observed in other structures were not present, most likely due to the low resolution of this data set (3.0 Å).
Figure 3.10: A) X-ray co-crystal structure of 49 bound to the serine protease domain of human uPA (3.0 Å). Electron density map (2mFo-DFc) is contoured at 1.5 σ. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. Water molecules have been omitted for clarity. B) Equivalent structure to A) with interacting surfaces coloured according to electrostatic potential (uPA) or cyan (49). C) Summary of the binding interactions between 49 and uPA. Residues comprising the S1β subsite are labeled in blue. Dashed lines represent H-bonds and ionic interactions. Curved dashed lines represent van der Waals contacts.
Introduction of a second methoxy substituent onto the 5-pyrimidinyl ring (48) resulted in a small increase in potency relative to the mono-methoxy 49 (IC$_{50}$ = 69 nM, 49 IC$_{50}$ = 86 nM), affording the most potent 6-HMA analogue identified in this study. Compared to pyrimidine analogues 48 and 49, compound 50 displayed greater rotation about the pyrimidine-pyrazine axis, adopting a dihedral angle of 80° (c.f. 66° for 49 and 44° for 48). This led to increased occupancy of S1β and allowed the o-methoxy CH$_3$ to fill a small hydrophobic dimple formed by the side chains of Lys143, Gln192 and the Ser146 backbone (Figure 3.11). The p-methoxy substituent was projected towards bulk solvent, with its CH$_3$ group (δ+) appearing to interact with the Gly219 backbone NH lone pair (3.0 Å). The pyrimidine ring appeared to make an additional hydrophobic contact with the Gly219 Cα. In contrast to 48 and 49, the Arg217 side chain was positioned away from S1β, apparently to
avoid a steric clash with the $p$-methoxy substituent. It would appear that loss of favourable interactions with the Arg217 sidechain, as observed in the uPA:48 and uPA:49 structures, was compensated for by the new interaction between the $o$-methoxy CH$_3$ group and the hydrophobic dimple in S1$\beta$, as indicated by the higher potency of 50.
Figure 3.11: A) X-ray co-crystal structure of 50 bound to the serine protease domain of Human uPA (1.7 Å). The electron density map ($2mF_o-DF_c$) is contoured at 1.5 $\sigma$. Electrostatic potential surfaces of uPA are shown with blue = negative and red = positive. A bridging water molecule is represented as a red ball. All non-bridging water molecules have been omitted for clarity. A sulfate from the crystallization buffer is bound to the oxyanion hole and is presented in stick representation. B) Equivalent structure to A) with interacting surfaces coloured according to electrostatic potential (uPA) or cyan (50). C) Summary of the binding interactions between 50 and uPA. Residues comprising the S1$\beta$ subsite are labeled in blue. Straight dashed lines represent H-bonds and ionic interactions. Dashed lines represent van der Waals contacts. Favourable interactions were observed between the $\alpha$-methoxy CH$_3$ and the small hydrophobic dimple of S1$\beta$ formed by the side chains of Lys143, Gln192 and the Ser146 backbone. The sidechain of Arg217 was positioned away from S1$\beta$, preventing interaction with 50.
3.3 X-ray co-crystal structures of HMA, 42 and 50 in complex with H99Y ‘murinized’ uPA

A key active site difference between murine and human uPA is the substitution at residue 99 (occupying S2 and S4), where His in the human enzyme is replaced by the bulkier Tyr in the mouse equivalent. X-ray co-crystal structures of HMA, 42 and 50 in complex with HuPA H99Y were solved to aid explanation of the SAR trends for HuPA\textsuperscript{H99Y} (and by extension MuPA) presented in Chapter 2.4.

The Tyr\textsuperscript{99} sidechain OH was found to be located close to the proximal methylene group of HMAs 7-membered ring (3.2 Å). In the equivalent huPA structure, the closest distance from the same CH\textsubscript{2} group to His99 was 4.1 Å (Figure 3.12). Similar observations were made for 42 and 50, where the Tyr\textsuperscript{99} OH resided closer to the proximal CH\textsubscript{2} group (3.5 Å versus 5.3 Å for 42, 3.4 Å versus 5.1 Å for 50).
Figure 3.12: Superpositions of X-ray co-crystal structures of H99Y uPA with WT HuPA; A) HMA, B) 42 and C) 50. H99Y X-ray co-crystal structures HuPA structures. The H99Y is presented in orange and the WT backbone in mauve. Residue 99 in each variant (His$^{99}$ in WT, Tyr$^{99}$ in H99Y mutant) is highlighted by a black box. Positioning of backbone residues in each uPA variant was preserved across all structures and inhibitor binding poses were very similar across matched complexes. In the H99Y structures, the Tyr sidechain is positioned closer to the proximal CH$_2$ of the azepane ring than in the HuPA structures, potentially creating unfavourable interactions. Structures were obtained at the following resolutions: HMA 2.3 Å, 42 2.9 Å and 50 2.4 Å.

This close orientation suggests that an unfavorable interactions between the polar Tyr$^{99}$ sidechain and the hydrophobic azepane ring may be occurring, which could explain the greatly decreased potency of these analogues against H99Y relative to HuPA. The topology of the S1β subsite was preserved across both co-complexes for all three inhibitors. The binding poses of HMA and 50
were very similar across the WT and mutant structures and the two methoxy groups of 50 occupied virtually identical positions. Interestingly, the pyrazine core and azepane ring of 42 were shifted towards the S2 pocket in the H99Y structure, apparently to present the 6-benzofuranyl substituent in identical configurations in both structures. The positioning of the sidechain of Arg217 was similar in both the HMA and 42 structures. However, the Arg217 sidechain in the 50 H99Y structure was positioned close to the pyrimidine methoxy group of 50. In the HuPA structure it was positioned well away from S1β. Despite the potential for H-bonds allowed by this positioning, potency was actually decreased against the murinzed enzyme (H99Y IC₅₀ = 9.7 μM).

3.4 Summary and conclusions

The X-ray co-crystal data presented herein allowed rationalization of some of the potency trends observed for the 6-HMA series. In general, appending substituents to the 6-position did not alter the binding pose or interactions formed between the parent structure and the HuPA active site. Favourable interactions between the 6-substituents and S1β were observed in all examples, confirming that this subsite is accessible from the 6-position of HMA and that increased S1β occupancy can drive increases in potency.

The observation that significant modifications (e.g. 46 vs 43 and 48 vs 50) resulted in modest changes in potency may reflect contributions from bulk effects, such as differences in desolvation energies for the respective substituents as the formation of specific and distance-dependent interactions appeared not to fully explain the observed trends. Experiments with the H99Y ‘murinized’ human uPA suggested that unfavourable interactions between the Tyr99 sidechain and the 5-azepane ring of 6-HMA analogues may be contributing to the decreased activity relative to HuPA. By extension, analogous (unfavourable) interactions between the 6-HMA analogues and
MuPA may be responsible for the generally lower activity observed with these compounds against murine uPA.
CHAPTER 4: *In vitro* and *ex vivo* characterization of 6-HMA analogues

Solution phase enzyme inhibition assays and X-ray crystallographic analysis allowed identification of promising lead structures from the synthesized library of 6-HMA analogues. The effects of selected leads on various aspects of cancer cell biology were characterized using multiple *in vitro* and *ex vivo* platforms. As is common practice during evaluating novel drug leads, cytotoxicity was also evaluated against human established cell lines.

4.1 Effects on cell proliferation and cytotoxicity

The cytotoxic effects of 2-benzofuranyl and 5-pyrimidinyl leads were investigated using the Promega CellTitre 96° Aqueous One Solution Cell Proliferation Assay. The assay measures the extent to which intracellular dehydrogenase enzymes convert a tetrazolium reagent (MTS) into a chromogenic formazan dye that can be quantified using a spectrophotometer. As the assay relies on the activity of enzymes upregulated during growth, the technique is unable to differentiate between cells that have died or entered senescence in response to drug treatment. As such, the assay is not strictly a measure of treatment-induced cytotoxicity, but rather is a measure of cell viability. Microscopic examination of cell cultures immediately prior to addition of MTS reagent provides a way of confirming cytotoxic effects, as indicated either by loss of cells or induction of a granular appearance. Despite their shortcomings, MTS and equivalent assays are commonly used as surrogate measures of compound cytotoxicity.

Compounds from the 2-benzofuranyl (42) and 5-pyrimidinyl (50) lead classes differed markedly in their effects on U-937 human histiocytic lymphoma cell viability (Figure 4.1). Inhibition of U-937 cell viability by 42 first appeared at concentrations between 1 and 10 μM (28% inhibition at 10 μM), with complete inhibition observed at 100 μM. In contrast, no inhibition was observed for
2,4-dimethoxypyrimidine analogue 50 in the range 1 nM-100 μM. Microscopic analysis of cultures post-treatment revealed a rapid onset for the cytotoxic effects of 42 at 100 μM, with cells exhibiting phenotypes characteristic of cytotoxic shock (e.g. decreased cell number, granular appearance, decreased surface area). The cells did not recover over the course of the experiment (50 h).

Figure 4.1: Effects of 42 and 50 on viability of U-937 Human histiocytic lymphoma cells. Cells were harvested 48 h post-passage, plated at a density of 5000 cells/well and incubated at 37 °C, 5% CO₂ for 18 h prior to drug treatment. Vehicle (DMSO) was present in all solutions at 1% (v/v). Cells were cultured for 48 h post-drug treatment, incubated for 2 h with MTS reagent and absorbance readings obtained at 490 nm. Bars represent the mean ± SEM (n = 4).

To determine whether similar results were observed in adherent cells, compound 42 was tested for cytotoxicity against MDA-MB-231 human epithelial breast adenocarcinoma cells. As seen with U-937 cells, 42 exhibited a pronounced inhibitory effect at 100 μM (Figure 4.2). HMA was similarly cytotoxic at this concentration, while no significant effects were observed for amiloride or the selective NHE1 inhibitor cariporide.
Figure 4.2: Effects of 42, HMA, amiloride and cariporide on viability of MDA-MB-231 human breast adenocarcinoma cells. Assays were conducted as described in Figure 4.1. Data represent the mean ± SEM (n = 4).

Microscopic analysis of the cells confirmed the cytotoxicity of 42 and HMA at 100 μM, as indicated by the greatly decreased cell numbers and (dead) appearance of cells (Figure 4.3). It appeared that the compounds elicited an immediate cytotoxic effect as very low cell numbers were present at the time of imaging, indicating that mitotic duplication had not occurred (i.e. the cells had died prior to initial doubling).
Figure 4.3: Phase-contrast photomicrographs of MDA-MB-231 cells 48 h after treatment with compound 42 or HMA. A pronounced decrease in cell number was observed with 42 and HMA at 100 µM relative to vehicle control (1% DMSO v/v).
To examine whether the anti-proliferative/cytotoxic effects were consistent within the 2-benzofuranyl and 5-pyrimidinyl lead classes, other analogues from each class were next investigated using MDA-MB-231 cells. 4-Fluoro-2-benzofuranyl analogue 43 was found to be more toxic than the unsubstituted analogue 42 in the same cell line, exhibiting almost complete loss of viability at 10 µM (Figure 4.4). In agreement with the low cytotoxicity of 50 observed in U-937 cells, pyrimidine-based analogue 48 exhibited only a slight anti-proliferative effect on MDA-MB-231 cells (30% inhibition) at 100 µM.

![Graph showing effects of compounds 43 and 48 on viability of MDA-MB-231 human breast adenocarcinoma cells. Assays were conducted as described in Figure 4.1. Data represent the mean ± SEM (n = 4).](image)

**Figure 4.4:** Effects of compounds 43 and 48 on viability of MDA-MB-231 human breast adenocarcinoma cells. Assays were conducted as described in Figure 4.1. Data represent the mean ± SEM (n = 4).

To examine whether the cytotoxic effects of the compounds were species-specific, compounds 42 and 50 were tested in MTS assays using murine macrophage RAW 264.7 cells. The murine myeloid cells displayed greater sensitivity to both lead classes relative to human U-937 cells, with complete loss of cell viability observed at 100 µM for both 42 and 50 (Figure 4.5).
Figure 4.5: Effects of 42 and 50 on viability of RAW 264.7 murine macrophage cells. Assays were conducted as described in Figure 4.1. Data represent the mean ± SEM (n = 4).

To complement the data obtained using the MTS assay, analogues 42 and 50 were investigated using the Roche Cytotoxicity Detection kit PLUS LDH release assay. Compromised plasma membrane integrity and resulting loss of cytoplasmic contents is a well characterized marker of cytotoxic cell death. The assay functions by detecting activity of lactate dehydrogenase (LDH), an enzyme released from the cytoplasm of necrotic cells in response to drug treatment. Catalytic turnover of a chromogenic substrate provides a quantifiable response. To obtain a representative maximal signal (control) for cytoplasmic LDH activity, vehicle-treated cells were lysed at the assay endpoint using a detergent solution (9% v/v Triton-X100), referred to as lysis control. A matched negative control was also included to confirm that LDH is not secreted into the cell culture supernatant by healthy cells (vehicle control).

The amount of LDH activity in U-937 cells treated with 42 and 50 (1 nM-100 µM) was measured 48 h after initial compound exposure (Figure 4.6). In line with MTS assay results, only the 2-benzofuranyl analogue 42 caused a significant release in LDH activity at 100 µM, indicative of cell
death. Evidence of slight LDH release at 10 µM was apparent. No significant LDH release was observed with 50 at any concentration.

**Figure 4.6:** Effects of 42 and 50 on LDH release from U-937 cells. $\text{Abs}_{490}$ corresponds to the degree of cytotoxic cell death in response to drug treatment. Cells were harvested, cultured and treated as described in Figure 4.1. Lysis control cells were subsequently treated for 15 min with 5 µl lysis Buffer, incubated for 10 min with reagent mixture and readings obtained at 490 nm. Data represent the mean ± SEM (n = 4).

Microscopy immediately prior to LDH assay revealed that 100 µM 42 had caused a substantial loss of cells relative to the lower doses or vehicle-treated controls, further confirming that 42 is cytotoxic at this concentration (Figure 4.7).
**Figure 4.7:** Phase contrast photomicrographs of U-937 cells 48 h after treatment with 42 or 50. A pronounced decrease in cell number was observed for 42 at 100 µM relative to the vehicle control (1% DMSO v/v).

Taken together, these results indicate a marked difference in the cytotoxic effects of the two lead series, with the 2-benzofuranyl analogues being clearly more cytotoxic than the pyrimidines. It is unclear whether the cytotoxic effects of the benzofuran compounds arose from specific interactions with pro-cytotoxic target(s) or whether they were simply a function of their higher lipophilicity relative to 5-pyrimidinyl class (benzofurans 42 and 43 ClogD 3.19-3.40; pyrimidines 48, 49 and 50 ClogD 0.82-1.85), or a combination of these factors.

HMA is known to trigger dose-dependent apoptosis via depletion of endoplasmic Ca$^{2+}$ stores in human umbilical vein endothelial cells (HUVEC). In some recent reports, HMA (40 µM) was used
as a specific inducer of apoptosis.\textsuperscript{304,305} To examine whether the cytotoxic effects of the benzofuran analogues are caused by induction of apoptotic cell death, compound 42 was tested using the Promega APO-One Homogenous Caspase 3/7 Assay.

The assay probes the release of pro-apoptotic cysteine aspartic acid proteases (Caspase) 3 and 7 into the cytoplasm by measuring turnover of a rhodamine-conjugated fluorogenic substrate of these enzymes. Cariporide is a potent and selective inhibitor of NHE1 and was included to examine whether NHE1 inhibition was also able to induce apoptosis (Cariporide uPA IC\textsubscript{50} >> 100 \(\mu\)M, see Appendix 9.3). Of the compounds 42, HMA and cariporide, only 42 at the highest concentration tested (30 \(\mu\)M) produced a significant increase in caspase 3/7 activity in HEK-293 cells after 5 h of treatment exposure (Figure 4.8). This result supports the cytotoxic effects of 42 being linked to induction of apoptosis.

**Figure 4.8:** Caspase 3/7 activation in HEK-293 cells following treatment with 42, HMA and cariporide. Cells were plated at 2.0x10\(^4\) cells/well in black 96-well cell culture plates and incubated for 18 h at 37° C, 5% CO\(_2\). Cells were treated with compounds or vehicle (1% v/v DMSO) for 5 h, shaken for 1 h at 500 rpm using a motorized plate shaker and incubated for 2 h at RT. Caspase 3/7 fluorogenic substrate in lysis buffer was added and after incubation at RT for 3 h the plates were read (ex. 485 nm, em 520 nm). Data represent the mean ± SEM (n = 3).
4.2 Effects on cell proliferation in real time via xCELLigence assay

To further investigate the cellular effects of the benzofuranyl class, cells were examined in response to treatment with 42 using the xCELLigence Real Time Cell Analyzer (Roche Diagnostics Australia Pty. Ltd., NSW, Australia). The xCELLigence system measures changes in electrical impedance inside a specialized multiwell plate and can be used to monitor various aspects of cell behavior, including viability and proliferation rate. As cells proliferate they impede the flow of electrons between electrodes fused to the bottom of each well. The impedance change is measured and used to calculate a cell index (CI); a dimensionless value that allows relative comparison of different compound treatments and conditions on cell behavior (e.g. growth rate).

A highly metastatic variant of the MDA-MB-231 breast adenocarcinoma cell line (MDA-MB-231HM) was used as it is known to overexpress both uPA and uPAR (M. Ranson, personal communication). Cells were plated into proprietary E-plate culture plates and allowed to grow for 24 h prior to drug addition. Figure 4.9 shows that cells in all treatment groups showed similar proliferation rates prior to drug addition at 24 h. Compound 42 at 10 μM caused an immediate toxic shock to cells with no recovery over time, indicative of cell death. This confirmed the rapid onset of cytotoxicity induced by the benzofuranyl analogues, as observed earlier with MTS/LDH assays and microscopic analyses.

HMA at 10 μM slowed the proliferative rate of MDA-MB-231HM cells, but the cells were able to recover and achieve a CI comparable to the vehicle control by the 72 h endpoint. Recovery of HMA treated cells suggests that it is not cytotoxic at this dose, corroborating results from the MTS, and Caspase 3/7 activation assays and microscopic analysis. Amiloride and cariporide showed minimal differences to vehicle control cells. These results highlight intrinsic differences between the
cytotoxic effects of HMA, the parent compound amiloride and 6-benzofuran analogue 42. The negligible effect of cariporide showed that inhibition of NHE1 does not affect proliferation in MDA-MB-231HM cells.

Figure 4.9: xCELLigence real-time analysis of the antiproliferative effects of compounds on MDA-MB-231HM human breast adenocarcinoma cells over 72 h. Cell index (CI) is a dimensionless measure of changes in electrical impedance caused by proliferation of cells. Cells were plated at 5x10^3 cells/well. Data points represent the average of two intra-assay replicates. The sharp decrease observed at the time of drug addition (24 h) is caused by requilibration of the system after mechanical perturbation.

To examine whether the antiproliferative effects of HMA and 42 in MDA-MB-231HM cells were uPA-mediated, the xCELLigence assay was repeated using uPA/uPAR-null HEK-293 human embryonic kidney cells. As seen in MDA-MB-231HM cells, treatment with 10 μM 42 caused immediate cell death with no recovery over 72 h (Figure 4.10). Likewise, HMA treatment slowed proliferation of HEK-293 cells without evidence of cytotoxicity, with CI recovery approaching the vehicle control by the end of the assay. Amiloride or cariporide treatment had no effect on proliferative rate or final CI. Recapitulation of the MDA-MB-231HM results in uPA/uPAR-null HEK-
293 cells indicates that the cytotoxic effects of 42 (and modest cytostatic effects of HMA) operate via uPA/uPAR-independent mechanisms.

Figure 4.10: xCELLigence real-time analysis of the antiproliferative effects of compounds on the uPA/uPAR-null HEK-293 human embryonic kidney cells over 72 h. Assays were conducted as described in Figure 4.9. The plateau in cell index after ~ 60 h indicates cells reaching confluence.

Dose-dependency of the effects of 42 and HMA was probed by repeating the assay with uPA/uPAR-null HEK-293 cells at lower concentrations. Compound 42 at 1 µM did not show the dramatic cytotoxic effects observed at 10 µM, although CI was reduced by 22% relative to vehicle control cells after 70 h (Figure 4.11), suggesting a mild-antiproliferative effect. HMA at 10 µM was again found to inhibit the rate of cell proliferation, with CI recovering to approximately half that of control cells by the assay endpoint. Amiloride treatment at 1 µM and 10 µM had no effect on CI. Compound 42 showed no difference to control cells when present at 100 nM. These findings complemented results from the cytotoxicity and apoptosis assays showing that compound 42 has no effect at concentrations below 10 µM.
4.3 Effects of 6-HMA analogues in a 3D organotypic cell invasion model

Despite their prevalence, two-dimensional models for studying cancer cell migration and invasion do not accurately reflect the dynamic three-dimensional (3D) environment experienced by cancer cells in vivo. This shortcoming has fueled much research into the development of 3D cell culture models that capture the spatiotemporal milieu of invasive cancers. To this end, and to examine the extent to which 6-HMA analogues are able to inhibit invasion in an ex vivo setting, a recently described collagen I organotypic cell invasion model was employed.

In this model, a preparation of collagen I isolated from rat tail tendons is seeded with fibroblasts and grown in culture. Due to their ECM-component secreting and ordering behaviors, the seeded fibroblasts contract the collagen I into a solid, dermal-mimicking plug. Once formed, the contracted plugs are overlaid with invasive epithelial cells that form a contiguous monolayer along the upper surface. The epithelial cells are then induced to invade into the collagen I matrix by transferring the plug to a steel grid and raising the cell culture medium to contact the bottom of the matrix, establishing an air-liquid interface. Growth factors and nutrients present in the culture.
media diffuse into the matrix, providing a chemotactic gradient and trophic incentive that attracts epithelial cell invasion downwards into the plug. After an incubation period (3-21 days), cell plugs are formalin fixed and paraffin embedded, sectioned and stained, providing a measure of cell invasion. This system is an excellent model for examining the effectiveness of uPA inhibitors against epithelial cells that rely on the plasminogen activation pathway for invasiveness as inhibition of uPA and plasminogen activation should decrease migration of cells into the collagen I matrix.

Overexpression of uPAS components is a key determinant of metastatic potential in malignant ovarian disease, where uPA levels correlate with FIGO (International Federation of Gynaecology and Obstetrics) grade and poor progression-free survival and overall survival.\textsuperscript{308,309} Differences in uPA expression between primary tumour and peritoneal metastasis have been validated as a prognostic biomarker for surgical outcome and overall survival.\textsuperscript{67} These findings suggested invasive SKOV-3 ovarian carcinoma cells as being suitable for use in the 3D organotypic model.

SKOV-3 cells seeded at a density of $1.875 \times 10^5$ cells per plug were allowed to invade for 21 days. Compound or vehicle containing media was replaced every 72 h, with all compounds present at 2 \textmu M. Cell invasion was quantified by calculating the invasion index (II) for each image, where II = number of epithelial cells invaded into collagen plug/number epithelial cells forming a contiguous monolayer along top of the plug x 100\% (Figures 4.12 & 4.13).

A small but significant decrease in invasion index was observed for the 2-benzofuranyl analogue 42 relative to the DMSO control group (Figure 4.12, mean II DMSO control = 63.65, 42 = 57.83, \(p \leq 0.0287\)). The difference between HMA and 42 did not reach significance (mean II HMA = 62.41, \(p \leq 0.1099\)).
Figure 4.12: Inhibition of SKOV-3 ovarian adenocarcinoma cell invasion by compounds. Collagen I plugs contracted by human skin-derived fibroblasts were seeded with SKOV-3 cells at a density of 1.875x10⁵/plug and allowed to invade for 21 days prior to fixing. Stained plug sections were imaged and the images quantified for invasion index (II). Compounds were present at a concentration of 2 μM. Data points represent invasive indices from 10 individual images across identically treated plugs. Central lines represent the mean ± SEM.

No significant differences in invasion index were detected for amiloride, HMA, cariporide or the 2,4-dimethoxy-5-pyrimidinyl compound 50 versus the DMSO control. The lack of effect of both amiloride and HMA on SKOV-3 invasion is likely due to too a low a concentration being used given the moderate potency of each compound against uPA (amiloride IC₅₀ = 3954 nM, HMA IC₅₀ = 2206). The finding that both HMA and cariporide did not inhibit cell invasion suggests that NHE1 does not play a role in invasion in this model. The lack of inhibition of cell invasion by compound 50 is surprising as this analogue is an ~ 4.3-fold more potent uPA inhibitor than 42 (50 IC₅₀ = 68 nM, 42 IC₅₀ = 297 nM). Analytical HPLC analysis of the DMSO stock from which compound 50 treatment was prepared revealed the appearance of 3 peaks in addition to the peak for the pure compound, suggesting degradation of 50 had occurred. As such, the concentration of 50 present
during the experiment would have been lower than 2 μM. Higher doses of 50 (and 42) may be needed in future experiments to demonstrate inhibition of uPA-mediated cell invasion in this model.

It is possible that the small decrease in II caused by 42 arose not from inhibition of uPA-mediated cell invasion but instead from cytotoxic effects of the compound on SKOV-3 and/or TIF cells. MTS assays showed that 42 is cytotoxic to both human and murine cells at concentrations > 7.5 μM. While plausible, this explanation is unlikely given that the concentration used (2 μM) was well below the threshold for cytotoxicity observed in MDA-MB-231 and HEK-293 cells (10 μM). Experiments measuring the effects of 42 on SKOV-3 and TIF cell viability would be necessary to disprove this hypothesis.

Variations in collagen plug density and integrity between experiments and batches of collagen I have been identified by colleagues in our laboratory (Nathanial Harris, personal correspondence). Degree of contraction, plug width and matrix density is heavily dependent on the functional state of fibroblasts, where contraction times can vary significantly between cultures (6-14 days). As shown in Figure 4.13, matrices were imperfect and contained small gaps. These variables may have contributed to the variability of responses observed between experiments.
Figure 4.13: Representative images showing SKOV-3 human ovarian carcinoma cell invasion matrices contracted by human skin-derived fibroblasts. Cells were allowed to invade for 21 days prior formalin fixing. Cytokeratin stained plugs were imaged using bright-field microscopy with 10 images collected across three plugs for each treatment group. All compounds were present at 2 μM, with compound-containing media refreshed every 72 h.
SKOV-3 cells are known to overexpress large amounts of uPAR.\textsuperscript{310} However, relative to other ovarian cancer cell lines, SKOV-3 cells secrete only moderate amounts of HMW-uPA (full-length, active uPA that’s able to bind uPAR).\textsuperscript{69} In addition, telomerase-immortalized fibroblasts do not secrete large amounts of HMW-uPA, making endogenous uPA expression by SKOV-3 cells the sole source of the enzyme in this system (Harris et al., manuscript submitted). It is therefore possible that uPAR occupancy and uPA-mediated cell invasion was low, decreasing the dynamic range for a measurable effect. Addition of exogenous HMW-uPA in future experiments would ensure complete occupation of uPAR has been used previously in our laboratory with success (N. Harris, personal communication).

SKOV-3 cells are considered to be moderate to highly invasive but they showed only mild invasiveness in this model, requiring a long time (21 days) for sufficient invasion.\textsuperscript{74} Selection of a more aggressive cell line that relies on uPA/plasminogen for invasion (e.g. MDA-MB-231 breast adenocarcinoma cells, 10 day invasion) may provide a more sensitive platform for measuring the anti-invasive effects of 6-HMA analogues and other uPA inhibitors in general.
4.4 Effects of 6-HMA analogues in combination with chemotherapy drugs on neoplastic cell phenotypes

The acidic tumour microenvironment is increasingly being linked to the responsiveness of cancerous cells to a variety of conventional chemotherapy drugs.\textsuperscript{311,312} Drugs with pK\textsubscript{a} values near the pH range observed in tumour microenvironments will exist as charged or neutral species, depending on their acidic/basic groups.\textsuperscript{313} Maximizing the proportion of drug present in an uncharged state is crucial for compounds that act on intracellular targets as the neutral species is able to passively diffuse across the plasma membrane.\textsuperscript{314}

NHE1 blockade has been suggested as a possible approach for sensitizing tumour cells to weakly basic drugs acting on intracellular targets. The theory is that preventing NHE1-driven acidification of the extracellular space will increase the amount of drug present in the membrane-permeable neutral form.\textsuperscript{85} A commonly used, weakly basic anticancer drug is the vinca alkaloid vinblastine (pK\textsubscript{a} ~ 8.8).\textsuperscript{295} Vinblastine is an antimitotic agent that binds to β-tubulin subunits, preventing functional microtubule assembly and mitotic spindle formation that leads to cell cycle arrest during mitosis, and ultimately apoptosis.\textsuperscript{87} Clinically, vinblastine is often used in combination with cisplatin or mitomycin C for ovarian, lung and brain tumours and Hodgkin’s lymphoma. It is also used as a salvage therapy in metastatic breast cancers non-responsive to first-line therapies.\textsuperscript{315,316}

To test this hypothesis, the effects of combinations of 6-HMA analogues \texttt{42}, \texttt{43} and \texttt{49} and vinblastine on MDA-MB-231 cell growth and viability were explored using MTS assays. NHE1 activity in cells was stimulated by serum starvation (0.2% FCS).\textsuperscript{122} The use of low serum media increased the cytotoxic effects of benzofuran compounds \texttt{42} and \texttt{43}, with complete cell death observed at 5 μM for both compounds (c.f. 10 μM using 10% FCS media, section 4.3). Vinblastine
alone at 5 μM decreased cell viability by ~ 25% relative to vehicle treated cells and 1 nM vinblastine had no effect (Figure 4.14 A). The cytotoxicity of Vinblastine at 5 nM was increased by 36% and 30% when combined with 1 μM and 0.25 μM 42, respectively (p 0.0039 and 0.0002). Slightly less potentiation was observed with 1 nM vinblastine and 42 at 1 μM or 0.25 μM, respectively (19% and 29% decreases in cell viability, p 0.0196 and p 0.001). In contrast, no potentiation of the effect of 5 nM vinblastine was observed with any combination of 43. However, small but significant increases were observed with 1 nM vinblastine and both 1 μM and 0.25 μM 43 (18% and 11% decreases in cell viability, p 0.0004 and p 0.0039). The differences between the benzofuran and 4-fluorobenzofuran analogues suggest that potentiation of vinblastine cytotoxicity is probably not general for the 2-benzofuranyl class.

No significant potentiation was observed for any combination of vinblastine with the pyrimidine analogue 49 compared to vinblastine alone. Compared to the benzofuran analogues 42 and 43, the highest concentration of 49 (5 μM) showed no effects on cell viability, again highlighting the differences in the intrinsic cytotoxicity of the 5-pyrimidine versus 2-benzofuranyl lead classes.

Contrary to expectation, potent NHE1 inhibitor cariporide appeared to protect MDA-MB-231 cells from the effects of vinblastine, increasing viability by 69% at 5 μM cariporide compared to when vinblastine was present at 5 nM alone (Figure 4.14). In cells treated with cariporide alone viability was decreased in the range 19-35%.
Figure 4.14: Effects of vinblastine/6-HMA analogue combinations on MDA-MB-231 cell viability using MTS assays. Data represent the mean ± SEM (n = 4). Statistical significance between groups was tested using the unpaired parametric t-tests in GraphPad PRISM v6.0. Level of significance was denoted as: * p < 0.05, ** p < 0.02, *** p < 0.005.

When co-administered with cariporide, 1 nM vinblastine showed negligible effects at all cariporide doses. At 5 nM vinblastine alone cell viability was decreased by ~60%. The incomplete killing of cells at this high dose of vinblastine suggested the existence of a resistant subpopulation.

A concentration-response experiment conducted to determine the effects of vinblastine on MDA-MB-231 cell viability gave an IC$_{50}$ value of 2.33 μM and showed the presence of a resistant subpopulation of ~45% the total cell population (Figure 4.15).
MDA-MB-231 cell growth was also monitored in real time using the Essen Biosciences Incucyte ZOOM Live-Cell Analysis System, which allowed real time evaluation of the effects of the compound combinations on cell confluency (Figure 4.16). An immediate and potent cytotoxic response was observed with 5 μM 42, both alone and in combination with vinblastine (Figure 4.14 A & B, 4.16 A). Vinblastine alone showed dose-dependent inhibition of cell growth, with 1 nM and 5 nM concentrations decreasing normalized confluency by 13% and 33%, respectively, after 52 h. The initial slow growth of cells treated with 5 nM vinblastine suggests the presence of a resistant population of cells, as an appreciable increase in normalized confluency was not seen until 18 h post-treatment.

No cytotoxic effect relative to control was seen for 42 at 1 μM. The combination of 1 μM 42 with 1 nM or 5 nM vinblastine resulted in 20% and 10% decreases, respectively, in normalized confluency at 52 h relative to cells treated with vinblastine alone (Figure 4.16A). No cytotoxic effect was observed for the pyrimidine-based compound 49 at the highest concentration tested (5 μM, Figure 4.16 B).
Figure 4.15: Real time analysis of the effects of vinblastine/6-HMA analogue combinations on MDA-MB-231 cell growth. Representative images from each treatment group were acquired every 2 h under a 10X objective using the Incucyte ZOOM Live-Cell Analysis System. All data are normalized relative to their respective confluence at time point zero (~ 30 min after initial drug treatment).
No significant effects of 49 in combination with vinblastine were observed, with confluency tracking closely relative to cells treated with vinblastine alone. Data from combinations of vinblastine with 0.25 μM 42 and 49 were omitted due to their similarity to that obtained with the compounds present at 1 μM.

Phase contrast photomicrographs taken 52 h post-treatment illustrate the effects of 42/vinblastine combinations on MDA-MB-231 morphology (Figure 4.17). Cytotoxic effects of 42 at 5 μM are evident in panels A-C, where the remaining cells show greatly reduced cell area, rounded appearance and high granularity. Lower doses of 42 did not alter morphology relative to vehicle treated controls. Cells treated with 1 μM or 0.25 μM 42 in combination with 5 nM vinblastine displayed a smaller, more rounded morphology and decreased cell number relative to cells treated with 5 nM vinblastine alone. Cells treated with 1 nM vinblastine in the presence 1 μM or 0.25 μM 42 showed a similar appearance to matched combinations at 5 nM vinblastine, albeit with slightly increased cell area. Vinblastine alone at 5 nM and 1 nM induced cells towards a tightly packed ‘cobblestone-like’ appearance compared to control cells, which displayed a more elongated and polarized morphology.

In general, the vinblastine/6-HMA analogue combinations did not show notable potentiation relative to vinblastine-treated MDA-MB-231 cells. The modest potentiation of 5 nM vinblastine by 42 may be anomalous as the readout for cells treated with this dose of vinblastine alone appears to be high relative to similar treatments in matched experiments (Figure 4.14A rightmost black bar c.f. 4.14 B-D).
Figure 4.16: Phase contrast photomicrographs of MDA-MB-231 human breast adenocarcinoma cells treated with combinations of 6-HMA analogue 42 and vinblastine. Representative images from each treatment group were acquired 52 h post-treatment under a 10X objective using the Incucyte ZOOM Live-Cell Analysis. Yellow bars represent scale (100 μm).
Paclitaxel is an atypical antimitotic agent used in the treatment of aggressive cancers, including ovarian and breast. In contrast to microtubule destabilizing drugs like vinblastine, paclitaxel promotes formation of αβ-tubulin heterodimers upon binding to β-tubulin, causing the stabilization of microtubules and consequent cell cycle arrest during late G2 or M phase. Paclitaxel in combination with cisplatin is a standard therapy for patients with triple-negative and HER2+ metastatic breast carcinomas who have relapsed following first line chemotherapy. NHE1 downregulation by paclitaxel via the PKA/p38 MAPK signaling pathway is thought to be a mediator of proapoptotic effects of paclitaxel. NHE1 inhibition by the 5-substituted amiloride analogue DMA 28 has been shown to increase paclitaxel-induced cell death in MDA-MB-435 melanoma-like cells.

More recently, low dose HMA (10 nM) was shown to potentiate the cytotoxic and anti-migratory effects of paclitaxel in aggressive, hormone receptor triple-negative MDA-MB-231 breast adenocarcinoma cells. Similar results were reported using the NHE1 specific inhibitor EMD 87580 (Rimeporide), supporting NHE1 inhibition as the mechanism underlying potentiation. Given the relevance to this thesis work, combinations of 6-substituted HMA analogues, HMA and the NHE1-specific inhibitor cariporide with paclitaxel were investigated for their effects on MDA-MB-231 cells.

As found previously, 5 μM concentrations of 2-benzofuranyl analogues 42 and 43 caused immediate and complete cell death, when administered alone and in combination with 0.5 nM or 5 nM paclitaxel (Figure 4.18). No cytotoxicity was observed in cells treated with the 5-pyrimidinyl analogues 48 or 49 (5 μM), HMA (5 μM) or cariporide (1 μM).
Disappointingly, no potentiation of paclitaxel cytotoxicity was observed in combination with HMA, 6-HMA analogues or cariporide at any of the concentrations tested (Figure 4.18).

**Figure 4.17**: Effects of paclitaxel/6-HMA analogue combinations on MDA-MB-231 cell viability. MTS assays were conducted as described in Figure 4.14.

Cell viability assays using real-time analysis of cell growth following compound/combination treatments showed that 43 and 49 produced no synergistic effects when used with paclitaxel (Figure 4.19). Similar results seen with the 6-HMA analogues 43 and 49, co-administration of cariporide did not potentiate paclitaxel induced cytotoxicity.
Combinations of HMA with paclitaxel were found to show the greatest effects on cell confluence (Figure 4.20). Normalized confluency decreased ~ 20% in cells receiving 5 nM paclitaxel + 5 μM or 1 μM HMA relative to cells treated with 5 nM paclitaxel alone. Greater effects on confluence were observed for 0.5 nM paclitaxel combinations with 1 μM HMA or 5 μM HMA, where 23% and 30% reductions in confluency were seen, respectively. These effects on confluency did not coincide with decreases in cell viability, as shown for HMA in Figure 4.18F. Data from 43, 49 and HMA at 0.25 μM +/- paclitaxel were omitted due to similarity with to data obtained at 1 μM.
Figure 4.18: Real time analysis of the effects of Paclitaxel/HMA analogue combinations on MDA-MB-231 cell growth. Images were acquired as described in Figure 4.15.

It is challenging to understand why we were not able to reproduce results similar to those reported by Amith et al. with HMA and MDA-MB-231 cells. Misidentification of the cell line used for the assays was ruled out using short tandem repeat analysis of cell DNA (92.8% match to ATCC standard c.f.; 95% match for cells used in reference 112, L. Fliegel personal communication).
One difference between experimental protocols, however, was the use of far greater compound concentrations in our experiments relative to those reported for HMA (10 nM). Intuitively, it would be expected that synergistic effects of HMA or 6-HMA analogues with paclitaxel should be larger at higher concentrations. To rule out the possibility that synergy is only observed at low concentrations, HMA 42, 48 and 49 were examined at 25 nM in combination with paclitaxel at 0.5 nM (Figure 4.21).
Figure 4.219: Effects of low-dose combinations of HMA and analogues 42, 48 and 49 with paclitaxel on cell viability in MDA-MB-231 cells. Assays were conducted as described in Figure 4.14.

No significant effect was observed for any compound, either alone or in combination with paclitaxel, relative to vehicle treated control cells.

4.5 Inhibitory effects of 6-HMA analogues on ENaCs

The diuretic and antikaliuretic properties of amiloride arise through inhibition of epithelial sodium channels (ENaCs) in the distal nephron of the kidney. Loss of ENaC inhibitory effects upon substitution at the amiloride 5-position have been documented with alkyl substitution of the 5-NH$_2$ group resulting in large decreases in potency. As diuretic and antikaliuretic activities would be undesirable in an anticancer drug, a key hope in this project was to establish that substitution of HMA at the 6-position would produce compounds that lack activity against ENaCs. Amiloride, HMA and a representative subset of 6-HMA analogues were screened for inhibitory activity against HEK-293 cells that transiently express the $\alpha$, $\beta$ and $\gamma$ subunits of Human ENaC (Figure 4.22). The assays used a fluorescent membrane potential dye kit and an automated fluorescence plate
reader (FLIPRTETRA, Molecular Devices, Sunnyvale, California, USA). The experiments were performed under contract by Charles River Laboratories, Cleveland, Ohio, USA. My role was to liaise with the company, advising on compound solubility and other experimental parameters.

![Graph](image)

**Figure 4.20**: Inhibition of ENaC activity in ENaC-overexpressing recombinant HEK-293 human embryonic kidney cells. All cells were stimulated with ENaC agonist S3969. All compounds were present at 10 μM. Data were normalized to uninhibited (0%) and benzamil treated (ENaC antagonist, 100%) controls. Data represent the mean ± SD (n = 4).

Substitution of the 6-position of HMA had no effect on ENaC activity, with HMA and all 6-HMA analogues showing similar effects (12-21% inhibition). In comparison, amiloride showed 68% inhibition at the same concentration. The results clearly demonstrate that 6-HMA analogues retain
the low ENaC potency previously noted for HMA. As such, it is unlikely that 6-HMA analogues 
would display the antikaliuretic and diuretic properties of amiloride in vivo. This is a key finding in 
this work as it implies that if anticancer therapeutics were eventually developed from this class 
they would be unlikely to carry the potentially life-threatening cardiac effects associated with K⁺ 
retention, as observed with amiloride overdose.
4.6 Summary and conclusions

Cell viability and proliferation assays conducted with a variety of human and murine cell lines revealed a large difference in the intrinsic cytotoxicity of compounds from the two lead classes of 6-HMA amiloride analogues. In general, members of the 2-benzofuranyl class (42 and 43) showed greater *in vitro* cytotoxic effects than the 5-pyrimidine analogues (48, 49 and 50) with complete cell death observed for compounds 42 and 43 at concentrations as low as 5 µM. In contrast the 5-pyrimidines showed no significant effects on cell viability up to 100 µM against the majority of human cell lines. In the case of 42, caspase 3/7 activity assays revealed that the cytotoxicity observed with this class was mediated through induction of apoptosis.

2-Benzofuranyl analogue 42 showed modest inhibition of SKOV-3 cell invasion in a 3D organotypic cell invasion model. Potentiation of the cytotoxic effects of the standard chemotherpay drugs vinblastine and paclitaxel was not observed with analogues from either lead class. A selection of 6-substituted analogues, including representatives from both lead classes, were found (like HMA) to not inhibit ENaC, providing evidence that the diuretic effects of the parent amiloride had been removed.

Taken together, these findings show that compounds from both lead classes do not possess potent cytotoxic effects and are unlikely to show diuretic activity *in vivo*. As such, they are well suited for further investigation as potential non-cytotoxic antimetastatic candidates.
CHAPTER 5: Development of a 96-well plate-based assay for measuring NHE1 inhibition

5.1 Introduction

Na\textsuperscript{+}/H\textsuperscript{+} exchange activity has been measured using a variety of techniques, including \textsuperscript{22}Na\textsuperscript{+}-flux, patch clamping, atomic absorption spectroscopy (AAS) and fluorescence-based methods.\textsuperscript{321-323} The major disadvantages of radiometric, electrophysiological and AAS approaches are the requirement for expensive, specialist equipment and expertise and low throughput. Confocal microscopic and flow cytometry-based fluorescence methods suffer from similar limitations.\textsuperscript{324,325} Fluorescence plate readers, on the other hand, are designed for high sample throughput (up to 1536-well capability in some cases) and are common instruments in academic and industrial cell biology laboratories. The development of a new plate-based method for medium throughput screening of compounds for NHE1 activity would constitute a significant contribution to the field. This chapter describes the first reported 96-well plate-based fluorescence assay for the quantitation of NHE1 activity and inhibition in the presence of antagonists.

5.2 Fluorescence plate assay and comparison to gold standard

Fluorescence-based methods for measuring NHE1 activity have typically used the ratiometric dye (2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). The poly acetoxyethyl ester form (BCECF-AM) is able to diffuse across plasma membranes and enter the cytosol, where it is cleaved by non-specific esterases to an active, charged form that becomes trapped within cells. The pH sensitivity of the fluorescence properties of the
‘trapped’ BCECF allows dynamic measurement of changes in $pH_i$. $pH_i$ can be calibrated by exposing dye-loaded cells to buffer solutions at set pH values containing the membrane permeable $H^+$ ionophore nigericin. Nigericin acts to shuttle protons across the cell membrane, causing rapid equilibration of $pH_i$ with $pH_e$, and allowing the creation of a standard curve. BCECF features pH-sensitive and insensitive (isobestic point) fluorescence excitation maxima at 500 nm and 439 nm, respectively, and an emission maximum at 535 nm (Figure 5.1).

![Fluorescence excitation](image)

**Figure 5.1:** Excitation spectra of BCECF showing the pH sensitivity across the physiological range of its maximum at ~ 500 nm. Excitation at 500 nm increases with increasing pH. pH-insensitive isobestic point is present at 439 nm (shown at 10x magnification). Adapted from Molecular Probes BCECF Technical Manual.

Using dual-excitation with single emission measurements, ratiometric calculations (e.g. pH sensitive signal/isobestic signal) are able to be employed to correct for sample variability; e.g. degree of dye loading, activity of esterases, photobleaching etc.\(^{326}\)
Fluorescence plate reader assays utilizing the pH-sensitive ratiometric dye BCECF to measure intracellular pH have been reported with a variety of cell types, including *S. pneumoniae* (bacteria), *S. cerevisiae* (yeast), *Trypanosoma* (parasitic protist) and primary and established mammalian epithelial cells (Obexer et al., 1997). Of these reports, Obexer et al. 1997 is the only example that exploited the high throughput capacity of a multiwell plate reader. This report used BCECF-AM to screen a library of anti-trypanosomal compounds and determine IC$_{50}$ values.

In addition to assays using standard fluorescence plate readers, there have been reports using a proprietary automated fluorescence plate reader (FLIPR) to measure NHE activity. The FLIPR High-throughput Cellular Screening System (Molecular Devices, Sunnyvale, California, USA) is a robotically controlled fluorescence plate reader specifically designed for screening compound libraries against transmembrane ion channels and GPCR targets. Despite the FLIPR system being well suited to the aims of this work, its prohibitive cost (+$800K AUD) limits broad uptake (G. Monteith, personal communication). To our knowledge, a straightforward assay for quantitating NHE1 inhibitory potency of compounds in adherent mammalian cells using commonly available fluorescence plate readers has not been described.

Validation of the novel plate-based assay required comparison of the data produced to data derived from currently accepted standard methods. To obtain such validation, a collaboration was established with Prof. Larry Fliegel at the University of Alberta, Canada, an internationally renowned expert in the molecular and cellular biology of NHE1. The Fliegel lab uses a traditional cuvette-based fluorometric method for determination of
NHE1 activity in adherent cell monolayers. This method is considered ‘gold standard’ for the evaluation of NHE1 inhibitor potency and is used by Prof. Fliegel in a number of active collaborations with groups developing NHE1-targeting compounds within academia and industry. Figure 5.2 shows a pH<sub>i</sub> calibration trace as it varies in response to different treatments throughout an experiment conducted using the Fliegel fluorimeter protocol.

**Figure 5.2:** pH<sub>i</sub> calibration trace for MDA-MB-231 breast adenocarcinoma cells using a fluorimeter-based NHE activity assay. Figure adapted from reference 112. See text for details.

Cells are initially exposed to Na<sup>+</sup>-containing buffer, allowing them to equilibrate pH<sub>i</sub> to a resting state value (1). Introduction of NH<sub>4</sub>Cl to the buffer causes immediate alkalinization of pH<sub>i</sub> (2), as NH<sub>3</sub> rapidly enters the cell and establishes a new NH<sub>3</sub> ⇌ NH<sub>4</sub><sup>+</sup> equilibrium as NH<sub>3</sub> molecules sequester H<sup>+</sup> in the cytosol. This transient alkalinization diminishes over time extracellular NH<sub>4</sub><sup>+</sup> crosses the plasma membrane. This process is orders of magnitude slower than NH<sub>3</sub> passage due the species positive charge. Switching the buffer quickly to a Na<sup>+</sup>-free/NH<sub>4</sub>Cl-free solution causes rapid acidification of pH<sub>i</sub> (3) as NH<sub>3</sub> rushes out of the cell.
down its concentration gradient. The absence of Na\(^+\) in the system deprives NHE1 of the Na\(^+\) gradient necessary to drive NHE1-mediated translocation of intracellular H\(^+\), thus preventing recovery of pH\(_i\). Reintroduction of Na\(^+\) (4) to the buffer switches on NHE1 activity, restoring pH\(_i\) to a new steady state within minutes.\(^{128}\)

The fluorometric method involves culturing adherent epithelial cells on cell culture treated coverslips in complete media. Once 80% confluence is achieved, cells are serum starved (cultured in 0.2% FCS-containing media) for approximately 24 h to stimulate NHE1 activity. Cells are then incubated for 20 min with the cell membrane-permeable fluorescent dye BCECF-AM. After dye loading, the coverslips are transferred to a custom-made glass cuvette designed to position the cell monolayer in the light path of a fluorimeter (PTI Deltascan Illumination System, Photon Technology International, New Jersey, USA). The cuvette is filled with Na\(^+\)-containing assay buffer for 3 minutes, allowing the cells to equilibrate pH\(_i\) through the action of NHE1. Cells are then subjected to a transient acid load with a pulse of NH\(_4\)Cl (50 mM) before being transferred to a second cuvette containing Na\(^+\)-free media. This results in a rapid decrease in pH\(_i\) as NH\(_3\) diffuses out of the cells. pH\(_i\) remains acidic until Na\(^+\) is reintroduced, causing NHE1 function to be restored and returning pH\(_i\) to resting values. Following NHE1-mediated pH recovery, intracellular pH signal is calibrated by sequentially equilibrating the cells in nigericin-containing buffers at set pH values. By measuring initial recovery of pH\(_i\) in the presence of different concentrations of inhibitors, dose-response curves can be plotted and IC\(_{50}\) values for inhibition of NHE obtained.

A plate reader assay could follow the same general principles with key modifications to adapt the assay to a 96-well plate format. All liquid addition steps were performed using a
multichannel air-displacement pipette. Liquids were removed by vigourously shaking out the plate into a receptacle followed by visual inspection of wells to ensure total removal of liquids. A schematic overview of the assay is presented in Figure 5.3.

**Figure 5.3:** Summary of fluorescence plate assay for determining NHE activity/inhibition.

Cells were plated at a density of $2 \times 10^4$ cells/well in black cell culture-treated 96-well plates and cultured in RPMI 1640/10%FCS for ~ 42 h to ensure formation of a confluent monolayer. On the day of experiments, cells were starved for 3-4 h in low serum (0.2% FCS) media. Cells were then loaded with 5 μM BCECF-AM in serum-free media and incubated for 30 min. The dye solution was then removed and cells washed once with 100 μL Na\(^+\)-containing buffer and equilibrated for 3 min with additional Na\(^+\)-containing buffer (Step 1). The buffer was
then replaced with Na\(^+\)-containing buffer containing 50 mM NH\(_4\)Cl and equilibrated for 3 min (Step 2). NH\(_4\)Cl-containing buffer was replaced by 100 µL Na\(^+\)-free buffer, decreasing pH, as NH\(_3\) diffuses out of the cell (Step 3). Compounds diluted in Na\(^+\) containing buffer in a separate 96-well plate were then added allowing measurement of NHE1-driven recovery in the presence or absence of inhibitors. (Step 4, n = 3 wells for each compound concentration). Intrinsic fluorescence of compounds was corrected for by including identically treated dye-free control cells at each compound concentration. Finally, control cells were successively equilibrated using nigericin-containing buffers at set pH values (Steps 5-7), allowing initial rates of recovery (ΔpH/s) to be calculated for each compound concentration with normalization against untreated control cells. Throughout Steps 1-7 readings were obtained using a POLARstar Omega Fluorescence plate reader with the settings outlined in Table 5.1

<table>
<thead>
<tr>
<th>Setting</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement type</td>
<td>Fluorescence Intensity, top optic</td>
</tr>
<tr>
<td>Read Mode</td>
<td>Plate, multichromatic</td>
</tr>
<tr>
<td>No. of cycles</td>
<td>1-3 dependent on step</td>
</tr>
<tr>
<td>Cycle time</td>
<td>107 seconds</td>
</tr>
<tr>
<td>Scan mode</td>
<td>Orbital averaging</td>
</tr>
<tr>
<td>Scan diameter/No. flashes</td>
<td>1 mm/ 8 flashes per well</td>
</tr>
<tr>
<td>Filter settings</td>
<td>Excitation</td>
</tr>
<tr>
<td>1. pH-sensitive couple</td>
<td>1. 485 ± 12, 520 nm, 1450</td>
</tr>
<tr>
<td>2. Isobestic couple</td>
<td>2. 440 nm, 520 nm, 1450</td>
</tr>
<tr>
<td>Assay Temperature</td>
<td>37 °C</td>
</tr>
</tbody>
</table>

In addition to Na\(^+\)/H\(^+\)-exchange, HCO\(_3\)\(-\) driven processes (e.g. Cl\(-\)/HCO\(_3\)\(-\) exchanger and Na\(^+\)/HCO\(_3\)\(-\) co-transporters) are also contributors to transmembrane pH homeostasis.\(^{130}\)
Dependence on HCO$_3^-$ or Na$^+$-driven processes for regulating transmembrane pH has been shown to vary between established neoplastic cell lines, with some lines displaying a preference for one pathway over the other. MDA-MB-231 breast adenocarcinoma cells were chosen for this work due to their reliance on Na$^+$/H$^+$-exchange driven regulation of pH$_i$. MDA-MB-231 cells also express NHE1 exclusively over other plasma membrane NHE isoforms (L. Fliegel, personal communication). HCO$_3^-$-driven contributions to pH recovery were negated by using HCO$_3^-$-free solutions in all experiments. Identical buffer compositions were used in all experiments across both NHE1 measurement techniques (Table 5.2).

**Table 5.2:** Composition of buffers used in NHE1 activity assays. The membrane-impermeant N-Methyl-D-glucamine was used in place of Na$^+$ to maintain isotonicity in Na$^+$-free buffers.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$-containing buffer</td>
<td>135 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 5.5 mM glucose, 10 mM HEPES.</td>
</tr>
<tr>
<td>Na$^+$/NH$_4$Cl-containing buffer</td>
<td>135 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 5.5 mM glucose, 10 mM HEPES, 50 mM NH$_4$Cl.</td>
</tr>
<tr>
<td>Na$^+$-free buffer</td>
<td>135 mM N-methyl-D-glucamine, 5 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 5.5 mM glucose, 10 mM HEPES.</td>
</tr>
<tr>
<td>pH calibration buffer</td>
<td>5 mM N-methyl-D-glucamine, 135 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 5.5 mM glucose, 10 mM HEPES, 2 μM nigericin.</td>
</tr>
</tbody>
</table>
5.3 Results

A representative pH calibration curve obtained from the successive equilibration of vehicle control cells in nigericin-containing buffer at pH 6-8 is presented in Figure 5.4. By performing linear regression analysis on the respective fluorescence ratios (pH sensitive signal/isobestic signal) at each pH, an equation relating $pH_i$ to the observed fluorescence ratio was derived. Using the equation, observed fluorescence ratios were converted to discrete pH values, allowing determination of $\Delta pH/s$ for each sample. Calibration was performed each time in each Individual assay to account for interassay variations.

![Fluorescence ratio vs pH](image)

**Figure 5.4:** Representative pH calibration curve from fluorescence plate reader assay. Fluorescence ratios from pH sensitive/isobestic signals were determined for control cells equilibrated in nigericin-containing buffers at different pHs. Linear regression analysis was performed and the resulting linear equation used to calibrate fluorescence ratios relative to pH$_i$. Data represent mean ± SEM (n = 6).

Data were obtained from both the plate-based and traditional cuvette assays and dose-response curves were calculated using GraphPad PRISM 6.0v. Representative dose-response curves from the two assay types are presented in Figure 5.5.
**Figure 5.5:** Representative dose response curves for standard NHE1 inhibitors from A) cuvette and B) fluorescence plate reader assays. Data represent: A) mean ± SEM (n = 3-4); B) mean ± SEM (n = 3). MDA-MB-231 cells were used in all assays. Fluorescence measurements were obtained using: A) PTI Deltascan Illumination System (Photon Technology International, New Jersey, USA) B) POLARstar OMEGA Fluorescence Plate reader (BMG-Labtech). Data were analyzed using Graphpad PRISM v.6.0 and fitted using log inhibitor vs normalized response – variable slope algorithm to calculate IC₅₀ values. Data in A) was provided by J. Wilkinson (Fliegel Lab), University of Alberta, Canada.

IC₅₀ values obtained for selected 6-HMA analogues and known NHE1 inhibitors using each technique are provided in Table 5.3. Goodness of fit analysis of curves generated from the two assay types revealed that the fluorescence plate technique generally yielded more sigmoidal dose-response relationships (fluorimeter R² range: 0.5745-0.8326, mean: 0.7009 n = 6; plate R² range: 0.7553-0.9580, mean: 0.8877, n = 5). Standard errors of the mean for each data point were found to be significantly lower in the plate-based assay compared to the fluorimeter assay (fluorimeter SEM range: 1.71%-18.46%, Plate SEM range: 0.81%-11.10%; mean Fluorimeter SEM = 7.866 ± 0.6912, n = 37, mean plate SEM = 4.070 ± 0.4663, n = 28, p <0.0001).
Table 5.3: Comparison of inhibitory potencies of amiloride, HMA, selected 6-HMA analogues and known NHE1 inhibitors (EMD87580 and cariporide) as determined using traditional cuvette and plate-based fluorometric assays in MDA-MB-231 breast adenocarcinoma cells. N/D = not determined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cuvette NHE1 IC50 (nM)</th>
<th>Plate Reader NHE1 IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>6,849</td>
<td>-</td>
</tr>
<tr>
<td>HMA</td>
<td>523</td>
<td>240</td>
</tr>
<tr>
<td>42</td>
<td>553</td>
<td>932</td>
</tr>
<tr>
<td>43</td>
<td>782</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>397</td>
<td>2,046</td>
</tr>
<tr>
<td>49</td>
<td>21,512</td>
<td>19,650</td>
</tr>
<tr>
<td>EMD87580</td>
<td>44</td>
<td>-</td>
</tr>
<tr>
<td>Cariporide</td>
<td></td>
<td>628</td>
</tr>
</tbody>
</table>
Concordance between IC$_{50}$ values and order of NHE1 inhibitory potencies among the 6-HMA analogues obtained from the two assays was not evident. HMA and 2-benzofuranyl analogue 42 were found to be nearly equipotent in the cuvette-based protocol (IC$_{50}$ = 523 nM vs. 553 nM), whereas HMA was 3.9-fold more potent (IC$_{50}$ = 240 nM) then 42 (IC$_{50}$ = 932 nM) in the plate-based method. Poor agreement was observed between assays for 5-pyrimidinyl analogue 48, which appeared as the most potent 6-HMA analogue via the cuvette assay (IC$_{50}$ = 397 nM) but showed 5-fold lower activity in the plate assay (IC$_{50}$ = 2,046 nM). Good agreement between both techniques was observed, however, for the p-methoxy-5-pyrimidinyl analogue 49, the least potent NHE1 inhibitor of the series (IC$_{50}$cuvette = 21,512 nM vs IC$_{50}$plate = 19,650 nM).

A wider selection of 6-HMA analogues were subsequently screened for NHE1 inhibition at a single concentration (10 μM) as a way of quickly identifying active compounds (Figure 5.6).
Figure 5.6: Inhibition of NHE1 activity in MDA-MB-231 cells using the plate-based assay. All compounds were present at a single concentration (10 μM). Data represent the mean ± SEM (n = 3-6). Normalized data were pooled across multiple assays.
Results from the single concentration assays revealed similar trends to those observed in the fluorimetric and plate-based dose-response assays. In agreement with the plate-based experiments, HMA was the most active inhibitor. 2-Benzofuranyl analogue 42 showed ~2-fold lower activity than HMA. Fluorination at the 4-position of the benzofuran to give 43 decreased activity further 2-fold. Substitution of the furanyl oxygen with S to give benzothiophene 41 or NH indole 40 decreased inhibition 2- to 3-fold relative to 42. 2-Furanyl and 3-furanyl isomers 46 and 47 showed only slightly decreased activity relative to HMA (~2 to 3-fold). In contrast, pyrazole compound 38 was ~10-fold less active than HMA.

As noted in the fluorimetric dose-response experiments, 5-pyrimidinyl analogue 48 showed strong activity and was, being equipotent with HMA. p-Methoxy substitution of the pyrimidine to give 49 decreased activity considerably (~7-fold). Trifluoromethylphenyl 35, 3,5-dimethoxyphenyl 54, 2,4-dimethoxypyridinyl 51 and 2,5-dimethoxypyridazinyl 55 derivatives all showed some activity at 10 μM. Isoquinolinyl analogue 37 was a poor inhibitor, as was p-methoxy pyrimidinyl analogue 49. The 6-phenylethynyl analogue 56 showed the lowest activity, being ~15-fold less potent than HMA.

These results provide the first insights into SAR at the 6-position of HMA against NHE1. Generally speaking, substitution with 2-benzofuranyl (42 and 43) or 5-pyrimidinyl substituents (48) was well tolerated, with potency varying within 2-fold of HMA in the cuvette-based assay. Substitution of 48 with a single methoxy group between the two pyrimidine nitrogens to give 49 resulted in a remarkable decrease in NHE1 inhibitory potency (IC50cuvette = 21,512 nM, ~55-fold loss relative to 48). Compounds 42 and 43 both feature bulky benzofuran moieties at the 6-position, which would project beyond the region...
of space occupied by the p-methoxy group of 43. As such, it is unlikely that the deleterious interactions formed by the p-methoxy group are purely steric. This supposition, of course, assumes that the two compounds adopt similar poses in the NHE1 binding site.

5.4 Discussion

Increased sample throughput enabled by the 96-well plate assay format for NHE1 inhibitor screening is a major advantage relative to the labour and time-intensive cuvette assay, where maximum throughput for a skilled worker generates IC\textsubscript{50} determinations for 2 compounds per day only (n = 3-4 replicates at each concentration, L. Fliegel personal correspondence). In contrast, 7-point dose-response curves (n = 3 at each concentration) can be obtained by a single person for 8+ compounds per day using the plate-based technique. Throughput could be further increased to 14 compounds in triplicate per plate (~112 per day) at a single concentration (for screening purposes) without alterations to the protocol.

NHE1-mediated recovery of pH\textsubscript{i} begins almost instantaneously upon reintroduction of extracellular Na\textsuperscript{+} following NH\textsubscript{4}Cl acid challenge. Ideally, rates of pH\textsubscript{i}-recovery should be determined from the linear portion of plots comparing pH\textsubscript{i} versus time. The manual addition of solutions via a standard 8-channel pipette creates a delay in time between each preceding row of cells, reaching a total of ~45-60 sec between rows A and H. This delay is accounted for by configuring the reader to reach each row in the order A-H in a left to right fashion. Nevertheless, NHE1 will have been active for between 45-60 sec before initial readings are obtained for any well. A second read is required to calculate the rate of pH recovery, which
occurs 107s after the initial read. It is possible that pH$_i$ recovery may have started to plateau by this point and, as such, rates calculated may not be truly representative of the initial (linear) rate period.

This potential shortcoming might be mitigated by conducting experiments on unstimulated cells under standard serum concentrations (5-10% FCS), as serum starvation increases NHE1 activity and hence the rate of pH$_i$ recovery. The rate of pH$_i$ recovery is likely to be cell line dependent and vary with the conditions used. Efforts should thus be made to measure baseline rates of NHE activity when optimizing the assay for new cell types. The traditional fluorimeter protocol does not suffer from this issue as pH$_i$ recovery is measured immediately and continuously after reintroduction of Na$^+$, allowing a real-time analysis of recovery kinetics.

A confluent cell monolayer was found to be essential for cell adherence throughout the assay. Seeding density was optimized to ensure confluence on the day of experimentation (~42 h post seeding). An appropriate shakeout technique was also crucial as residual liquid in wells were found to increase variability in the signals. Liquid removal via multichannel pipette was trialed but found to be too slow. Pipetting must be performed such that all replicates of a particular sample receive solutions at the same time to prevent staggering of equilibration/pH$_i$ across a treatment group. The time taken to add Na$^+$-containing buffer/drug dilutions to wells must also be kept to an absolute minimum to prevent large differences in the start time of pH$_i$ recovery.
When using the ‘plate multichromatic’ read mode, the plate is first read using the pH-sensitive filter couple, which is followed by a second read cycle using the isobestic filter couple. The time lag between reading a particular well using each filter couple (~ 30-50s depending on the exact protocol used) is inconsequential given the static nature of the isobestic signal across the relevant pH range. This was verified using the alternative ‘well multichromatic’ read mode, in which each individual well is read rapidly using both filter couples prior to moving to the next well. While this approach decreases the potential for changes in the sample that may accrue over time it was not used because of the large increase in time per read cycle relative to the ‘plate multichromatic read mode’.

The limitations due to reliance of this assay on manual handling for the addition and removal of buffers were apparent. As such, automation of these steps using liquid handling robotics would likely improve the accuracy and reproducibility of NHE1 activity measurements. The Illawarra Health and Medical Research Institute (Ranson Lab) has recently acquired a Molecular Devices FlexStation 3 Multimode plate reader. The FlexStation 3 is a multimode fluorescence/luminescence plate reader with an integrated liquid handling microfluidics system capable of automating all liquid addition steps in the assay. Use of the FlexStation 3 should allow for a large increase in data density as the minimum time needed to read plates at both wavelengths is ~ 30s (c.f. 107s for the POLARstar assay). These advantages may support the POLARstar system as the preferred platform for performing this assay. Efforts to optimize the assay for use with the FlexStation 3 are targeted for the near future.
In conclusion, the protocols described in this chapter constitute a novel, straightforward and affordable means for medium-throughout determination of NHE1 inhibition by small molecule antagonists.
CHAPTER 6: Thesis conclusions and Future Directions

The potassium-sparing diuretic drug amiloride shows robust anticancer activities in animal models. We proposed that this anticancer side activity arises from amiloride’s moderate inhibition (low μM) of two unrelated biological targets; the pro-metastatic serine protease uPA and the Na⁺/H⁺ exchanger isoform 1 (NHE1), a key driver of oncogenic transformation.

The primary aim of this work was to develop novel (and patentable) uPA/NHE1 dual-targeting amiloride analogues suitable for advancement towards anticancer drugs. A library of 24 6-HMA analogues was successfully synthesized and evaluated, with compounds showing sub-μM potency against uPA identified. As it was unknown whether substitution at the 6-position would affect the high potency of HMA against the pro-carcinogenic ion channel NHE1, a secondary aim was to evaluate whether 6-HMA analogues retained activity against this target.

Development of novel synthetic methodology allowed the expeditious generation of structurally diverse 6-heteroaryl-HMA analogues. Recent efforts in the Kelso laboratory have led to the development of a superior acyl guanidinylation method utilizing a 2M solution of free guanidine in MeOH with DMF as solvent. Final products were easily obtained in good yields as the free base form without the need for further purification, avoiding costly and labourious preparative HPLC.
Evaluation of the uPA inhibitory potency of these compounds via enzyme activity assays identified 15 analogues with improved potency over the parent HMA, with gains ranging from 1.03 to 32-fold. From these, active analogues, two physicochemically diverse lead classes; the 2-benzofuranyl and 5-pyrimidinyl analogues (exemplified by compounds 42 and 49 respectively, Figure 6.1) were further examined in enzyme selectivity and cell assays.

Figure 6.1: Properties of lead compounds 42 and 49.

Favourable cytotoxicity profiles for both lead classes were seen across a variety of human and murine cell lines. Preliminary organotypic cell invasion assays demonstrated the ability of 42 to inhibit epithelial cell invasion in a physiologically relevant context. X-ray co-crystallographic analysis of uPA:6-HMA analogue complexes enabled the rationalization of SAR trends and shed light on the structural requirements for favourable occupancy of the S1β subsite by 6-substituents on analogues.

Development of a novel fluorescence-based high throughput assay allowed elucidation of SAR around the 6-position of the HMA scaffold. Excitingly, the high potency of HMA against NHE1 was maintained in several 6-substituted analogues, with the exception of 49, which represents a selective uPA inhibitor (uPA IC$_{50}$ = 86 nM; NHE1 IC$_{50}$ = 21 µM). This new
technique will soon be submitted for publication, providing the NHE1 drug discovery community with a rapid and straightforward method for the screening of compounds against this important target.

The work has satisfied the aims described in Chapter 1 and provides a wealth of exciting avenues for future research. Analogues from both lead classes met activity, selectivity and toxicity endpoints necessary for entry into in vivo efficacy models. In addition to the discovery of leads with dual uPA/NHE1 potency, identification of p-methoxypyrimidine analogue 49, which shows activity only for uPA (not NHE1), provides scope for the examination of truly uPA selective pharmacological probes. This separation of uPA/NHE1 activity for members of the amiloride class is a significant advance given the abundance of reports using amiloride and its 5-substituted analogues to study diverse physiological phenomena and the potential of these activities, namely inhibition of uPA and NHE1, to simultaneously effect the processes studied. We hope to make these improved probes available to the broader research community.

It is worthwhile noting that while this work satisfied aims, the medicinal chemistry described herein is by no means exhaustive. Further work could involve synthesis of an expanded library of 6-HMA analogues using the Suzuki-Miyaura and other transition metal-catalyzed cross-coupling chemistries (e.g. Sonogashira chemistry, as well as ‘click’ chemistry), with the goal of expanding the set of active analogues against both targets with varying ADMET properties.
This project formed part of a larger campaign focused on the development of novel dual-targeting anticancer and anti-tuberculosis compounds from amiloride. Parallel efforts from other group members have resulted in the generation of analogous 6-substituted and 5,6-disubstituted amiloride analogues that possess similar activity, selectivity and toxicity profiles to those described herein. Competitive grant funding from the Australian National Health and Medical Research Council and the Health Research Council of New Zealand has provided the resources necessary to advance development of promising 6-AML and 6-HMA analogues. Recent in vivo ADMET experiments revealed that compounds 42 and 49 possess desirable pharmacokinetic parameters (e.g. plasma $t_{1/2}$ 3-7 h, favourable $V_d$s, no indication of acute toxicities etc.), with some compounds showing similar profiles to amiloride. Pharmacokinetic parameters will be used to inform dose scheduling for in vivo efficacy experiments planned over the next 6-18 months.

In vitro studies confirmed that the 6-HMA and 6-AML analogues are not active against ENaC, the diuretic and antikaliuretic target of amiloride, suggesting success in our efforts to selectively optimize amiloride’s side activity with loss of the original clinically-relevant interaction. Studies to confirm the lack of effect of these leads on urinary Na$^+$/K$^+$ levels in animals will commence shortly.

In vivo anticancer activity of 42 and 49 will be evaluated using orthotopic xenograft models of pancreatic and breast (highly uPA/uPAR overexpressing) metastasis in mice, as well as in a spontaneous model of metastatic breast carcinoma. The finding that 6-HMA analogues show pronounced species specificity, where inhibitory activity is substantially decreased against the murine enzyme, is a concern for these studies as the lower inhibition of MuPA may lead
to an underestimation of the anticarcinogenic/antimetastatic effects. Ultimately, the species selectivity issue may be redundant as the robust anticancer effects of high-dose amiloride across various murine cancer models has been repeatedly observed despite amiloride showing only moderate inhibitory potency against both human and murine uPA (e.g. low µM, similar to the potency of 6-HMA analogues against MuPA). Future work will involve the design of analogues where interspecies specificity has been addressed, namely 6-HMA analogues that replace the 5-azepane ring with smaller groups.

It is hoped that a demonstration of in vivo efficacy will enable selection of genuine pre-clinical candidates for further study and entry into first-in-man studies within the next 24 months. In addition, it is hoped that the broader research community will relish the opportunity to screen novel members of a long-studied class of compounds against the known and undiscovered targets of amilorides. Further elucidation of the SAR around 6-substituted amilorides against such targets, may result in the discovery of leads for the treatment of other disease indications.
CHAPTER 7: Experimental

7.1 Synthesis – General

Reagents and solvents were used as obtained from suppliers without further purification, unless otherwise stated. Toluene and \(N,N\)-dimethylformamide (DMF) were decanted from a PureSolv Solvent purification system (InertTechnology Inc., Amesbury, Massachusetts, USA) immediately prior to use. \(^1\)PrOH was distilled from BaO powder and stored over 4 Å molecular sieves under Ar. Boronic acids/pinacol esters were obtained from various suppliers and used without further purification. All other reagents were obtained from Sigma Aldrich (St. Louis, MI, USA).

Compounds were weighed using a Sartorius Extend 220g electronic balance (Sartorius AG, Göttingen, Germany) or an OHAUS Adventurer Analytical electronic balance (Cat. #AX224, OHAUS Corporation, Parsippany, New Jersey, USA). Solvents were removed \textit{in vacuo} at RT-60 °C with a Büchi RotavaporR-200 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) attached to a Vacuubrand CVC2 vacuum pump (Vacuubrand GmbH, Wertheim, Germany). Solvent residues were removed \textit{in vacuo} using a Javac Vector RD-90 double stage high vacuum pump (JAVAC Pty. Ltd., Homebush, NSW, Australia). Fractions collected from preparative rp-HPLC purifications were concentrated by lyophilization using a freeze-drying system consisting of a Christ Alpha 1-4 LOC-1M (MartinChrist GmbH, Osterode am Harz, Germany) condenser attached to an Alcatel Pascal 2015 SD high vacuum pump (Pfeiffer Vacuum GmbH, Annecy, France). Analytical thin layer chromatography was performed on Merck 0.2 mm silica gel 60 \(F_{254}\) coated aluminium plates (Merck Millipore, Massachusetts,
USA). Compounds on silica plates were visualized under UV light (λ = 254 nm). Flash column chromatography was performed on Merck silica gel 60 (230-400 mesh). All compounds were purified to >95% purity for biological evaluation using a Waters PLC/DSC LC150 system (Waters Corporation, Milford, Massachusetts, USA) with dual detection at 254 nm and 210 nm. Purifications were performed using gradient elutions with solvents A (100% H₂O, 0.1% TFA) and B (100% ACN, 0.1% TFA) on a Waters Sunfire PREP C18 OBD 5 µm (dimensions) column at a flow rate of 20 mL.min⁻¹. Purity analysis of fractions collected was performed using a Shimadzu CLASS-VP LC10 Analytical HPLC system (Shimadzu Corporation, Kyoto, Japan) with detection at 254 nm, gradient elutions of solvents A and B on a Waters VisionHT 3 µm C18 column (150 mm x 4.6 mm) and at a flow rate of 0.5 mL.min⁻¹.

Melting points were obtained using a Griffin Analogue Melting Point Apparatus (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). Electrospray ionization mass spectra were obtained using a Shimadzu LCMS-2010 (Shimadzu Corporation, Kyoto, Japan). High resolution electrospray ionization mass spectra were obtained using a Waters XEVO QToF mass spectrometer (Waters Corporation, Milford, Massachusetts, USA) with leucine encephalin as the internal standard.

7.2 Compound Characterization

¹H and ¹³C NMR spectra were obtained using Varian Inova 500 MHz or Varian Premium Shielded 500 MHz spectrometers at 25 °C. Chemical shift values are reported in δ (ppm) relative to TMS (δ 0.00 ppm), internal standards (CDCl₃, δ = 7.26 ppm) or other residual solvent peaks. The following abbreviations are used throughout: Ar = aryl, bs = broad singlet,
s = singlet, d = doublet, t = triplet and m = multiplet, SM = starting material, RM = reaction mixture and RT = room temperature. All compounds in this work bear several exchangeable protons attached to nitrogen atoms. The $^1$H chemical shifts for these protons were temperature and concentration dependent. Compounds marked with * denote structures previously described in the literature for which full characterization data was not reported.

**Methyl 3-amino-5-(azepan-1-yl)-6-phenylpyrazine-2-carboxylate (33a)**

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (101 mg, 3.54x10$^{-4}$ mol) was combined with K$_2$CO$_3$ (488 mg, 3.53x10$^{-3}$ mol), phenylboronic acid (65 mg, 5.32x10$^{-4}$ mol) and Pd(Ph$_3$)$_4$ (21 mg, 18.0x10$^{-5}$ mol) and the reaction vessel purged with Ar. Anhydrous toluene (12 mL), followed by MeOH (3 mL) were added via syringe and the mixture heated at reflux for 1 h. TLC analysis indicated absence of SM and the presence of a UV active spot ($R_f = 0.95$, 1:4 EtOAc:Pet. spirit) after 1 h. The RM was allowed to cool to RT, vacuum filtered through celite and washed through with CH$_3$CN (3x20 mL) to remove catalyst. Solvent was removed under reduced pressure. The crude product was re-dissolved in EtOAc and adsorbed onto silica gel under reduced pressure. The product was isolated via silica gel flash chromatography (5:95 EtOAc:Hexane) to yield 33a as a light yellow solid (106 mg, 91%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.50 (d, $J = 7.5$ Hz, 2H, ArC$_2$ and ArC$_6$), 7.38 (t, $J = 7.5$ Hz, 2H, ArC$_3$ and ArC$_5$), 7.29 (t, $J = 7.5$ Hz, 1H, ArC$_4$), 3.89 (s, 3H, Ester C$_3$H$_3$), 3.33 (t, $J = 5.75$ Hz, 4H, C$_2'$H$_2$ and C$_7'$H$_2$), 1.61 (s, 4H, C$_3'$H$_2$ and C$_6'$H$_2$), 1.47 (s, 4H, C$_4'$H$_2$ and C$_5'$H$_2$). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 167.67 (Ester C=O), 154.99 (ArC$_5$), 153.66 (ArC$_3$), 140.99 (ArC$_1$), 132.03 (ArC$_5$), 128.63 (ArC$_3$ and ArC$_5$), 128.09 (ArC$_2$ and ArC$_6$), 127.52 (ArC$_4$), 112.45 (ArC$_2$), 52.13 (Ester C$_3$H$_3$), 51.12 (C$_2'$ and C$_7'$), 28.11 (C$_3'$ and C$_6'$), 27.20 (C$_4'$ and C$_5'$). LR ESI-MS (M + H)$^+$ 327.
Methyl 3-amino-5-(azepan-1-yl)-6-(4-(methylthio)phenyl)pyrazine-2-carboxylate (34a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (102 mg, 3.60x10^{-4} moles), (4-(methylthio)phenyl)boronic acid (93 mg, 5.45x10^{-4} moles), K₂CO₃ (508 mg, 3.87x10^{-3} moles) and Pd(PPh₃)₄ (9.9 mg, 1.05x10^{-5} moles) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. After 15 min a UV active spot (R_f = 0.5, 1:4 EtOAc:Pet spirit) was detected via TLC, with disappearance of SM after 1 h. After 30 min the RM was allowed to cool to RT, stirred for 1 h, filtered through celite and washed with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was dissolved in EtOAc and adsorbed to silica and purified by silica gel flash column chromatography (gradient elution:2.5:97.5 EtOAc:Pet. Spirit → 5:95 EtOAc:Pet. Spirit) to give 34a as a yellow solid (104 mg, 77%): MP: 132-134° C; ¹H NMR (500 MHz, CDCl₃) δ 7.42 (d, J = 8.1 Hz, 2H, ArH3” and ArH5”), 7.25 (d, J = 8.1 Hz, 2H, ArH2” and ArH6”), 6.20 (bs, 2H, Ar3NH₂), 3.88 (s, 3H, Ester CH₃), 3.36 (t, J = 5.5 Hz, 4H, C2’ H₂ and C7’ H₂), 2.48 (s, 3H, thioetherCH₃), 1.62 (s, 4H, C3’ H₂ and C6’ H₂), 1.43 (s, 4H, C4’ H₂ and C5’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.42 (Ester C=O), 154.77 (ArC5), 153.43 (ArC3), 137.77 (ArC4”), 137.47 (ArC1”), 131.19 (ArC6), 128.29 (ArC2” and ArC6”), 126.77 (ArC3” and ArC5”), 112.45 (ArC2), 51.90 (Ester CH₃), 51.01 (C2’ and C7’), 27.96 (C3’ and C6’), 27.08 (C4’ and C5’) 16.05 (thioetherCH₃); HRESI-MS m/z (M + H)^+: Calc 373.1698, Obs 373.1688. Anal-HPLC (70:30 A:B/100% B over 30 min, t_r = 36.1 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(4-(trifluoromethyl)phenyl)pyrazine-2-carboxylate (35a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate **32** (100 mg, 3.53x10⁻⁴ mol) was combined with K₂CO₃ (501 mg, 3.62x10⁻³ mol), 4-trifluoromethylphenylboronic acid (100 mg, 5.26x10⁻⁴ mol) and Pd(Ph₃)₄ (21 mg, 18.0x10⁻⁵ mol) and the reaction vessel purged with Ar. Anhydrous toluene (12 mL), followed by MeOH (3 mL) were added via syringe and the mixture heated to reflux for 10 min. TLC analysis indicated absence of SM and the presence of a UV active spot (R_f = 0.90, 1:4 EtOAc:Pet. spirit) after 10 min. The RM was allowed to cool to RT, vacuum filtered through celite and washed through with CH₃CN (3x20 mL) to remove catalyst. Solvent was removed under reduced pressure. The crude product was re-dissolved in EtOAc and adsorbed onto silica gel under pressure. The product was isolated via silica gel flash column chromatography (5:95 EtOAc:Hexane) to yield **35a** as a light brown solid (101 mg, 72%): mp 154-156˚C; ¹H NMR (500 MHz, CDCl₃) δ 7.63 (s, 4H, Ar"Hs) 3.89 (s, 3H, Ester CH₃), 3.34 (s, 4H, C2’H₂ and C7’H₂) 1.65 (s, 4H, C3’H₂ and C6’H₂), 1.44 (s, 4H, C4’H₂ and C5’H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.40 (Ester C=O), 154.89 (ArC5), 153.72 (ArC3), 144.58 (ArC1”), 129.42 (ArC2” and ArC6”), 129.16 (ArC3” and ArC5”), 128.23 (ArC4”), 125.58 (Ar-CF₃), 123.35 (ArC6), 113.11 (ArC2), 52.20 (Ester CH₃), 51.26 (C2’ and C7’), 28.04 (C3’ and C6’), 27.30 (C4’ and C5’); HRESI-MS m/z (M + Na)⁺: Calc. 417.1526, Obs 417.1514. Anal-HPLC (100 A:B → 100% B over 30 min, t_r = 29.3 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(benzo[d][1,3]dioxol-5-yl)pyrazine-2-carboxylate (36a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (99 mg, 3.49x10^{-4} mol), benzo[d][1,3]dioxol-5-ylboronic acid (85 mg, 5.15 x10^{-4} mol), K$_2$CO$_3$ (486 mg, 3.70 x10^{-3} mol) and Pd(PPh$_3$)$_4$ (20 mg, 1.70 x10^{-5} mol) were added to a stoppered two-neck round bottom flask and the flask was charged with Ar. The flask was fitted to a reflux condenser followed by addition of anhydrous toluene (12 mL) and MeOH (3 mL) via syringe. The flask was heated to reflux for 1 h. A UV active spot (R$_f$ = 0.75, 1:4 EtOAc:Pet. Spirit) was detected via TLC. After 1 h the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (2.5 EtOAc:97.5 Pet spirit – 10:90 EtOAc:Pet. spirit) to give 36a as a yellow solid (66 mg, 51% yield); MP: 156-158° C; $^1$H NMR (500 MHz, CDCl$_3$) δ 7.02 (s, 1H, ArH$_2$”), 6.93 (d, J = 8.0 Hz, 1H, ArH$_9$”), 6.80 (d, J = 8.0 Hz, 1H, ArH$_8$”), 5.96 (s, 2H, C5”H$_2$), 3.88 (s, 3H, Ester C$_3$H$_3$), 3.38 (s, 4H, C2’H$_2$ and C7’H$_2$) 1.63 (s, 4H, C3’ H$_2$ and C6’ H$_2$), 1.44 (s, 4H, C4’ H$_2$ and C5’ H$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 167.55 (Ester C=O), 154.89 (ArC5), 153.46 (ArC3), 147.86 (ArC3”), 147.05 (ArC7”), 134.93 (ArC1”), 131.51 (ArC6), 121.69 (ArC9”), 112.20 (ArC2), 108.73 (ArC2”), 108.34 (ArC8”), 101.12 (C5”), 51.94 (Ester C$_3$H$_3$), 50.99 (C2’ and C7”), 28.08 (C3’ and C6’), 27.10 (C4’ and C5’); HRESI-MS m/z (M + H)$^+$: Calc. Mass 371.1719. Obs 371.1734; Anal-HPLC (100% A: → 100% B over 30 min, t$_r$ = 31.8 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(isoquinolin-4-yl)pyrazine-2-carboxylate (37a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate (203 mg, 7.12x10⁻⁴ mol), 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoquinoline (271 mg, 1.06x10⁻³ mol), K₂CO₃ (970 mg, 7.01x10⁻³ mol) and Pd(PPh₃)₄ (40 mg, 3.44x10⁻⁵ mol) were added to a round bottom flask. Anhydrous toluene (24 mL) followed by MeOH (6 mL) were added via syringe and heated to reflux. After 2 h a UV active spot (Rᵢ = 0.25, 1:4 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 2.5 h. After 1 h the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (5:95 EtOAc:Pet. Spirit → 10:90 EtOAc:Pet. Spirit) to give 37a as a brown solid (156 mg, 58%); MP 140-142°C; ¹H NMR (500 MHz, CDCl₃) δ 9.23 (s, 1H, ArH₂”), 8.58 (s, 1H, ArH₄”), 8.00 (d, J = 8.0 Hz, 1H ArH₆”), 7.81 (d, J = 8.0 Hz, 1H. ArH₉”), 7.68 (t, J = 7.5 Hz, 1H, ArH₇”), 7.62 (t, J = 7.5 Hz, 1H, ArH₈”), 3.86 (s, 3H, EsterCH₃), 3.21 (t, J = 5.5 Hz, 4H, C₂’ H₂ and C₇’ H₂), 1.47(s, 4H, C₃’ H₂ and C₆’ H₂), 1.38 (s, 4H, C₄’ H₂ and C₅’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.29 (Ester C=O), 155.77 (ArC₅), 154.02 (ArC₃), 152.06 (ArC₄”), 143.01 (ArC₂”), 134.26 (ArC₁₀”), 131.78 (ArC₁”), 130.82 (ArC₆”), 128.42 (ArC₆), 127.97 (ArC₈”), 127.37 (ArC₇”), 126.66 (ArC₅”), 124.50 (ArC₉”), 112.90 (ArC₂), 52.02 (Ester CH₃), 50.38 (C₂’ and C₇’), 27.87 (C₃’ and C₆’), 26.83 (C₄’ and C₅’); HRESI-MS m/z (M + Na)⁺: Calc. 400.1749, Obs. 400.1747. Anal-HPLC (100% A → 100% B over 30 min, tᵣ = 22.3 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrazine-2-carboxylate (38a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate (32) (101 mg, 3.53x10^{-4} mol), (1-methyl-1H-pyrazol-4-yl)boronic acid (110 mg, 5.26 x10^{-3} mol), K$_2$CO$_3$ (526 mg, 3.70 x10^{-3} mol) and Pd(PPh$_3$)$_4$ (21 mg, 1.76 x10^{-5} mol) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. A UV active spot ($R_f$ = 0.5, EtOAc) was detected via TLC along with disappearance of SM. After 1 h the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in acetone and adsorbed to silica prior to purification by silica gel flash chromatography (5:95 EtOAc:Pet spirit – 10:90 EtOAc:Pet. spirit) to give 38a as a yellow solid (89 mg; 77%); MP: 134-136°; $^1$H NMR (500 MHz, CDCl$_3$) δ 7.64 (s, 1H, ArH$_{2”}$), 7.57 (s, 1H, ArH$_{6”}$), 6.17 (bs, 2H, 3NH$_3$), 3.91 (s, 3H, 4″CH$_3$), 3.89 (s, 3H, Ester C$_3$H$_3$), 3.47 (t, $J$ = 6 Hz, 4H, C2″H$_2$ and C7″H$_2$), 1.66 (s, 4H, C3″H$_2$ and C6″H$_2$), 1.47 (s, 4H, C4″H$_2$ and C5″H$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 167.34 (Ester C=O), 155.54 (ArC5), 153.40 (ArC3), 138.73 (ArC6”), 129.11 (ArC2”), 125.25 (ArC1”), 122.56 (ArC6), 112.42 (ArC2'), 52.08 (Ester CH$_3$), 51.16 (C2’ and C7’), 39.00 (C4’ CH$_3$), 28.00 (C3’ and C6’), 27.17 (C4’ and C5’); HRESI-MS m/z (M + H)$^+$: Calc 331.1882, Obs. 331.1894. Anal-HPLC (70:30 A:B $\rightarrow$100% B over 30 min, $t_r$ = 25.2 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(1-(2-morpholinoethyl)-1H-pyrazol-4-yl)pyrazine-2-carboxylate (39a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (99 mg, 3.48x10^{-4} mol), 4-(2-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazol-1-yl)ethyl)morpholine (165 mg, 5.36x10^{-4} mol), K_{2}CO_{3} (522 mg, 3.78 x10^{-3} mol) and Pd(PPh_{3})_{4} (21 mg, 1.77 x10^{-5} mol) were added to a stoppered two-neck round bottom flask and the flask was charged with Ar. The flask was fitted to a reflux condenser followed by addition of anhydrous toluene (12 mL) and MeOH (3 mL) via syringe. The flask was heated to reflux for 16 h. A UV active spot (R_f = 0.44, 85:10:5 EtOAc:MeOH:NH_{3}) was detected via TLC, along with the disappearance of SM (R_f = 0.95) after 16 h. After 17 h the RM was allowed to cool to RT, filtered through celite and washed with CH_{3}CN (4x20 mL). Solvent was removed under reduced pressure. The crude material was dissolved in CH_{3}CN and adsorbed to silica prior to purification by silica gel flash column chromatography (EtOAc → 6% MeOH:EtOAc) to give 39a as a yellow solid (119 mg, 80%): MP: 132-134° C; 1H NMR (500 MHz, CDCl_{3}) δ 7.70 (s, 1H, ArH5”), 7.61 (s, 1H, ArH2”), 6.24 (bs, 2H, ArNH_{2}3), 4.25 (s, 2H, C7H_{2}), 3.89 (s, 3H, Ester C_{H}3), 3.69 (s, 4H, C11H_{2} and C13H_{2}), 3.46 (s, 4H, C2’H_{2} and C7’H_{2}), 2.84 (s, 2H, C8H_{2}) 2.50 (s, 4H, C10H_{2} and C14H_{2}), 1.65 (s, 4H, C3’ H_{2} and C6’ H_{2}), 1.47 (s, 4H, C4’ H_{2} and C5’ H_{2}); 13C NMR (126 MHz, CDCl_{3}) δ 167.20 (Ester C=O), 155.50 (ArC5), 153.33 (ArC3), 138.62 (ArC5’), 128.67 (ArC2’), 125.13 (ArC1’), 122.10 (ArC6), 112.41 (ArC2), 66.94 (C11CH_{2} and C13CH_{2}), 58.19 (C8CH_{2}), 53.68 (C10CH_{2} and C14CH_{2}), 51.91 (Ester C_{H}3), 51.05 (C2’ and C7’), 49.55 (C7CH_{2}), 27.94 (C3’ and C6’), 27.02 (C4’ and C5’); HRESI-MS m/z (M + H)^{+}: Calc. 430.2567, Obs. 430.2553. Anal-HPLC (70:30 A:B → 100% B over 30 min, t_r = 21.6 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(1H-indol-2-yl)pyrazine-2-carboxylate (40a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (201 mg, 7.06x10^{-4} moles), (1H-indol-2-yl)boronic acid (278 mg, 1.73x10^{-3} moles), K_{2}CO_{3} and Pd(PPh_{3})_{4} (41 mg, 3.52x10^{-5} moles) were added to a round bottom flask. Anhydrous toluene (24 mL) followed by MeOH (6 mL) were added via syringe and heated to reflux. After 30 min a UV active spot (R_{f} = 0.85, 2:3 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 1 h. After 1 h the RM was allowed to cool to RT, filtered through celite and washed with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give 40a of a orange solid (88 mg, 34%); MP 152-154°C; ¹H NMR (500 MHz, CDCl₃) δ 9.14 (s, 1H, ArNH₂”), 7.57 (d, 1H, J = 7.7 Hz, ArH7”), 7.37 (d, 1H, J = 7.7 Hz, ArH4”), 7.16 (t, 1H, J = 7.0 Hz, ArH5”), 7.08 (t, 1H, J = 7.0 Hz, ArH6”), 6.52 (s, 1H, ArH9”), 3.93 (s, 3H, Ester CH₃), 3.58 (t, J = 5.75 Hz, 4H, C₂’ H₂ and C₇’ H₂), 1.70 (s, 4H, C₃’ H₂ and C₆’ H₂), 1.50 (s, 4H, C₄’ H₂ and C₅’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.18 (Ester C=O), 155.09 (ArC₅), 153.15 (ArC₃), 135.96 (ArC₃”), 135.68 (ArC₁”), 128.95 (ArC₈”), 124.51 (ArC₆), 122.36 (ArC₅”), 120.67 (ArC₇”), 119.92 (ArC₆”), 112.71 (ArC₂), 111.06 (ArC₄”), 101.95 (ArC₉”), 52.05 (Ester CH₃), 51.65 (C₂’ and C₇’), 28.12 (C₃’ and C₆’), 27.53 (C₄’ and C₅’); HRESI-MS m/z (M + H)^+: Calc. 388.1730, Obs. 388.1731; Anal-HPLC (100%A → 100% B over 30 min, tᵣ = 29.8 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(benzo[b]thiophen-2-yl)pyrazine-2-carboxylate (41a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (203 mg, 7.12x10^{-4} mol), benzo[b]thiophen-2-ylboronic acid (193 mg, 1.08x10^{-3} mol), K$_2$CO$_3$ (1.02 g, 7.39x10^{-3} mol) and Pd(PPh$_3$)$_4$ (41 mg, 3.57x10^{-5} mol) were added to a round bottom flask. Anhydrous toluene (24 mL) followed by MeOH (6 mL) were added via syringe and heated to reflux. After 10 min a UV active spot ($R_f = 0.85$, 2:3 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 30 min. After 30 min the RM was allowed to cool to RT, filtered through celite and washed with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (2.5:97.5 EtOAc:Pet. Spirit $\rightarrow$ 10:90 EtOAc:Pet. Spirit) to give 41a of a bright yellow solid (245 mg, 90% yield); MP 146-148 °C; $^1$H NMR (500 MHz, CDCl$_3$) δ 7.80 (d, $J = 7.5$ Hz, 1H, ArH4”), 7.71 (d, $J = 7.5$ Hz, 1H, ArH7”), 7.22 (m, 2H, ArH5” and ArH6”), 7.21 (t, $J = 7.0$ Hz , 1H, ArH9”), 3.91 (s, 3H, Ester C$_3$H$_3$), 3.51 (t, $J = 5.75$ Hz, 4H, C2’ H$_2$ and C7’ H$_2$), 1.69 (s, 4H, C3’ H$_2$ and C6’ H$_2$), 1.48 (s, 4H, C4’ H$_2$ and C5’ H$_2$); $^{13}$C NMR (CDCl$_3$): δ 167.10 (Ester C=O), 155.01 (ArC5), 153.40 (ArC3), 143.09 (ArC8”), 140.02 (ArC5”), 139.90 (ArC6”), 125.22 (ArC8”), 124.23 (ArC5”), 123.63 (ArC6”), 123.46 (ArC6), 122.15 (ArC7”), 122.83 (ArC4”), 112.98 (ArC2”), 52.18 (Ester C$_3$H$_3$), 51.10 (C2’ and C7’), 27.98 (C3’ and C6’), 27.23 (C4’ and C5’). HRESI-MS $m/z$ (M + Na)$^+$: Calc. 405.1394. Obs. 405.1361; Anal-HPLC (100% A $\rightarrow$100% B over 30 min, $t_r = 31.9$ min).
Methyl 3-amino-5-(azepan-1-yl)-6-(benzofuran-2-yl)pyrazine-2-carboxylate (42a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate (32) (250 mg, 8.78x10^{-4} mol), benzofuran-2-ylboronic acid (490 mg, 3.02x10^{-3} mol), K₂CO₃ (485 mg, 3.70x10^{-3} mol) and Pd(PPh₃)₄ (51 mg, 4.39x10^{-5} mol) were added to a round bottom flask. Anydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. A UV active spot (Rᵥ = 0.45, 1:4 EtOAc:Pet. spirit) was detected via TLC after 1 h. Methyl ester SM was not detectable by TLC after 21 h. After 24 h the RM was allowed to cool to RT and filtered through celite, washed through with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (1 EtOAc:99 Pet spirit – 15:85 EtOAc:Pet. spirit) to give 42a as a bright yellow solid (116 mg, 36%); MP: 118-120 °C; ¹H NMR (500 MHz, CDCl₃) δ 7.57 (d, J = 7.5 Hz, 1H, ArH₄”), 7.47 (d, J = 7.5 Hz, 1H, ArH₇”), 7.25 (m, 2H, ArH₅” and ArH₆”), 6.92 (s, 1H, ArH₉”), 3.90 (s, 3H, Ester C₃H₃), 3.46 (t, J = 5.8 Hz, 4H, C₂’ H₂ and C₇’ H₂), 1.68 (s, 4H, C₃’ H₂ and C₆’ H₂), 1.48 (s, 4H, C₄’ H₂ and C₅’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.13 (Ester C=O), 154.79 (ArC₅), 154.64 (ArC₃”), 154.47 (ArC₁”), 154.11 (ArC₃), 129.16 (ArC₈”), 124.30 (ArC₅”), 123.07 (ArC₆”), 121.38 (ArC₆), 121.18 (ArC₇”), 112.84 (ArC₂), 111.46 (ArC₄”), 105.07 (ArC₉”), 52.24 (Ester C₂H₃), 50.16 (C₂’ and C₇’), 28.11 (C₃’ and C₆’), 27.24 (C₄’ and C₅’); HRESI-MS m/z (M + H): Calc. 367.1831, Obs. 367.1770; Anal-HPLC (70:30 A:B → 100% B over 30 min, tᵣ = 29.5 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(4-fluorobenzofuran-2-yl)pyrazine-2-carboxylate (43a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (101 mg, 3.55x10^{-4} mol), (4-fluorobenzofuran-2-yl)boronic acid (95 mg, 5.31x10^{-4} mol), K_{2}CO_{3} (485.0 mg, 3.51x10^{-3} mol) and Pd(PPh_{3})_{4} (22 mg, 1.89x10^{-5} mol) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. After 1 h a UV active spot (R_{f} = 0.25, 20:80 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 2 h. After 2 h the RM was allowed to cool to RT and filtered through celite, washed through with EtOAc (4x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give 43a as a yellow solid (80 mg, 58%); MP: 108-110° C; ^1H NMR (500 MHz, CDCl_{3}) δ 7.27 (d, 1H, ArH5”), 7.20 (m, 1H, ArH7”), 6.99 (s, 1H, H3”), 6.93 (t, 1H, J = 8.5 Hz, ArH6”), 3.91 (s, 3H, EsterCH_{3}), 3.46 (s, 4H, C2’ H_{2} and C7’ H_{2}), 1.69 (s, 4H, C3’ H_{2} and C6’ H_{2}), 1.49 (s, 4H, C3’ H_{2} and C6’ H_{2}); ^13C NMR (126 MHz, CDCl_{3}) δ 167.0 (Ester C=O), 156.9 (ArC5), 156.2 (ArC3), 154.7 (ArC8”), 154.0 (ArC4”), 138.3 (ArC1”), 124.6 (ArC6”), 120.5 (ArC6), 118.0 (ArC2), 115.9 (ArC3”), 110.4 (ArC5”), 108.6 (ArC7”), 107.6 (ArC2”), 52.2 (Ester CH_{3}), 50.0 (C2’ and C7’), 28.0 (C3’ and C6’), 27.1 (C4’ and C5’); HRESI-MS m/z (M + Na)^+: Calc. 407.1495, Obs. 407.1504 Anal-HPLC (70:30 A/B → 100% B 30 min, t_{r} = 21.2 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(thiophen-2-yl)pyrazine-2-carboxamide (44a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate (32) (100 mg, 3.52 x 10^{-4} mol) was combined with K$_2$CO$_3$ (512 mg, 3.70 x 10^{-3} mol), thiophene-2-ylboronic acid (70 mg, 5.49 x 10^{-4} mol) and Pd(Ph$_3$)$_4$ (20 mg, 17.5 x 10^{-5} mol) and the reaction vessel purged with Ar. Anhydrous toluene (12 mL), followed by MeOH (3 mL) were added via syringe and the mixture heated towards reflux for 3 h. TLC analysis indicated absence of SM and the presence of a UV active spot (R$_f$ = 0.80, 1:4 EtOAc:Pet. spirit) after 3 h. The RM was allowed to cool to RT. The RM was vacuum filtered through celite and washed through with CH$_3$CN (3x20 mL) to remove catalyst. Solvent was removed under reduced pressure. The crude product was re-solubilized in MeOH and adsorbed onto silica gel under reduced pressure. The product was isolated via silica gel flash column chromatography (gradient elution, 5:95 to 10:90 EtOAc:Hexane) to yield 44a as a light yellow solid (99 mg, 85%): MP: 118-120 °C; $^1$H NMR (500 MHz, CDCl$_3$) δ 7.27 (d, J = 5 Hz, 1H, ArH$_3$”), 7.03 (d, J = 3 Hz, 1H, ArH$_5$”) 6.98 (m, 1H, ArH$_4$”) 3.89 (s, 3H, Ester C$_3$H$_3$), 3.43 (t, J = 5.7 Hz, 4H, C2’H$_2$ and C7’H$_2$), 1.67 (s, 4H, C3’H$_2$ and C6’H$_2$), 1.47 (s, 4H, C4’H$_2$ and C5’H$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 167.28 (Ester C=O), 155.24 (ArC5), 153.52 (ArC3), 142.83 (ArC1”), 126.89 (ArC3”), 125.96 (ArC4”), 125.78 (ArC5”), 125.54 (ArC6), 112.70 (ArC2), 52.13 (Ester CH$_3$), 51.11 (C2’ and C7’), 28.15 (C3’ and C6’), 27.26 (C4’ and C5’); HRESI-MS m/z (M + Na)$^+$: Calc. 355.1205, Obs. 355.1193. Anal-HPLC (100% A: $\rightarrow$ 100% B over 30 min, t, = 28.8 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(thiophen-3-yl)pyrazine-2-carboxylate (45a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (100 mg, 3.55x10⁻⁴ mol) was combined with K₂CO₃ (492 mg, 3.56x10⁻³ mol), thiophen-3-ylboronic acid (75 mg, 5.84x10⁻⁴ mol) and Pd(Ph₃)₄ (20 mg, 1.73x10⁻⁵ mol) and the reaction vessel purged with Ar. Anhydrous toluene (12 mL), followed by MeOH (3 mL) were added via syringe and the mixture heated to reflux for 2 h. TLC analysis indicated absence of SM and the presence of a UV active spot (Rᶠ = 0.75, 1:4 EtOAc:Pet. Spirit) after 2 h. The RM was allowed to cool to RT, vacuum filtered through celite and washed through with CH₃CN (3x20 mL) to remove catalyst. Solvent was removed under reduced pressure. The crude product was re-solubilized in MeOH and adsorbed onto silica gel under reduced pressure. The product was isolated via silica gel flash column chromatography (gradient elution, 5:95 to 10:90 EtOAc:Hexane) to yield 45a as an off white solid (100 mg, 85%). MP: 118-120°C. ¢H NMR (500 MHz, CDCl₃) δ 7.38 (s, 1H, ArH2”), 7.29 (m, 1H ArH4”), 7.25 (d, J = 5 Hz, 1H, ArH5”), 3.88 (s, 3H, Ester C=O), 3.38 (t, J = 5.8 Hz, 4H, C2’H2 and C7’H2), 1.63 (s, 4H, C3’H2 and C6’H2), 1.44 (s, 4H, C4’H2 and C5’H2); 13C NMR (126 MHz, CDCl₃) δ 167.35 (Ester C=O), 155.30 (ArC5), 153.53 (ArC3), 141.34 (ArC1”), 128.06 (ArC5”), 127.86 (ArC6), 125.39 (ArC4”), 122.83 (ArC2”), 112.16 (ArC2), 51.97 (Ester CH₃), 50.90 (C2’ and C7’), 28.01 (C3’ and C6’), 27.05 (C4’ and C5’); HRESI-MS m/z (M + H)⁺: Calc. 355.1205, Obs. 355.1197; Anal-HPLC (100%A → 100% B over 30 min, tᵣ = 28.3 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(furan-2-yl)pyrazine-2-carboxylate (46a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate (102 mg, 3.59x10^{-4} mol) was combined with K$_2$CO$_3$ (519 mg, 3.76x10^{-3} mol), furan-2-ylboronic acid (59 mg, 5.26x10^{-4} mol) and Pd(Ph$_3$)$_4$ (20 mg, 1.75x10^{-5} mol) and the reaction vessel purged with Ar. Anhydrous toluene (12 mL), followed by MeOH (3 mL) were added via syringe and the mixture heated to reflux for 1.5 h. TLC analysis indicated absence of SM and the presence of a UV active spot (R$_f$ = 0.90, 1:4 EtOAc:Pet. spirit) after 2 h. The RM was allowed to cool to RT, vacuum filtered through celite and washed through with CH$_3$CN (3x20 mL) to remove catalyst. Solvent was removed under reduced pressure. The crude product was solubilized in MeOH and adsorbed onto silica gel under reduced pressure. The product was isolated via silica gel flash column chromatography (gradient elution, 5:95 to 15:85 EtOAc:Hexane) to yield 46a as an off white solid (111 mg, 98%): MP: 114-116 °C; $^1$H NMR (500 MHz, CDCl$_3$) δ 7.41 (s, 1H, ArH$_3$”), 6.54 (d, J = 2.75 Hz, 1H, ArH5”), 6.45 (s, 1H, ArH4”), 3.89 (s, 3H, Ester CH$_3$), 3.37 (t, J = 5.8 Hz ,4H, C2’H$_2$ and C7’H$_2$), 1.66 (s, 4H, C3’H$_2$ and C6’H$_2$), 1.47 (s, 4H, C4’H$_2$ and C5’H$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 167.16 (Ester C=O), 154.76 (ArC5), 154.00 (ArC3), 152.24 (ArC1”), 141.49 (ArC3”), 122.04 (ArC6), 112.15 (ArC2), 111.68m (ArC4”), 108.73 (ArC5”), 52.11 (Ester CH$_3$), 50.06 (C2’ and C7’), 28.11 (C3’ and C6’), 27.03 (C4’ and C5’); HRESI-MS m/z (M + Na)$^+$: Calc. 339.1418, Obs. 339.1433. Anal-HPLC (100% A →100% B over 30 min, t$_r$ = 27.1 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(furan-3-yl)pyrazine-2-carboxylate (47a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (107 mg, 3.89x10^{-4} mol), furan-3-yl boronic acid (81 mg, 4.16 x10^{-4} mol), K₂CO₃ (483 mg, 3.68 x10^{-3} mol) and Pd(PPh₃)₄ (20 mg, 1.69 x10^{-5} mol) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. A UV active spot (Rf = 0.55, 1:4 EtOAc: Pet spirit) was detected via TLC along with disappearance of SM. After 1 h the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (5:95 EtOAc:Pet spirit – 10:90 EtOAc:Pet. spirit) to give 47a of a yellow solid (114 mg , 93%); MP: 122-124° C; ¹H NMR (500 MHz, CDCl₃) δ 7.64 (s, 1H, ArH₂”), 7.41 (s, 1H, ArH₄”), 6.61 (s, 1H, ArH₅”), 6.20 (Ar3NH₂), 3.89 (s, 3H, Ester C₃H₃), 3.48 (t, J = 5 Hz, 4H, C2’ H₂ and C7’ H₂), 1.66 (s, 4H, C3’ H₂ and C6’ H₂), 1.47 (s, 4H, C4’ H₂ and C5’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.30 (Ester C=O), 155.56 (ArC₅), 153.60 (ArC₃), 142.62 (ArC₄”), 140.63 (ArC₂”), 125.82 (ArC1”), 124.77 (ArC6), 112.55 (ArC₂), 111.15 (ArC₅”), 51.97 (Ester C₃H₃), 50.99 (C2’ and C7’), 28.02 (C3’ and C6’), 27.10 (C4’ and C5’); HRES-MS m/z (M + H)⁺: Calc. 316.1614, Obs. 316.1604; Anal-HPLC (70:30 A:B →B over 30 min, tᵣ = 30.9 min).

Methyl 3-amino-5-(azepan-1-yl)-6-(pyrimidin-5-yl)pyrazine-2-carboxylate (48a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (100 mg, 3.50x10^{-4} mol), pyrimidin-5-ylboronic acid (69 mg, 5.58x10^{-4} mol), K₂CO₃ (508 mg, 3.67x10^{-3} mol) and Pd(PPh₃)₄ (25 mg, 2.12x10^{-5} mol) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3...
mL) were added via syringe and heated to reflux. After 15 min a UV active spot ($R_f = 0.25, 2:3$ EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 40 min. After 45 min the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit $\rightarrow$ 40:60 EtOAc:Pet. Spirit) to give 48a as an off white solid (95 mg, 83%); MP 178-180° C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.13 (s, 1H, ArH$_4$"), 8.89 (s, 2H, ArH$_2$" and ArH$_6$"), 3.91 (s, 3H, EsterCH$_3$), 3.35 (t, $J = 5.0$ Hz, 4H, C$_2'$ H$_2$ and C$_7'$ H$_2$), 1.68 (s, 4H, C$_3'$ H$_2$ and C$_6'$ H$_2$), 1.48 (s, 4H, C$_4'$ H$_2$ and C$_5'$ H$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 167.06 (Ester C=O), 157.05 (ArC$_4$"), 155.63 (ArC$_2$" and ArC$_6$"), 155.22 (ArC$_5$), 153.82 (ArC$_3$), 134.85 (ArC$_1$"), 124.47 (ArC$_6$), 114.38 (ArC$_2$), 51.45 (C$_2$" and C$_7$"), 27.95 (C$_3$" and C$_6$"), 27.35 (C$_4$" and C$_5$"). HRESI-MS $m/z$ (M + Na)$^+$: Calc. 351.1545, Obs. 351.1535; Anal-HPLC (70:30 A:B $\rightarrow$ B over 30 min, $t_r = 23.4$ min).

**Methyl 3-amino-5-(azepan-1-yl)-6-(2-methoxypyrimidin-5-yl)pyrazine-2-carboxylate (49a)**

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (100 mg, 3.50x10$^{-4}$ mol), 2-methoxypyrimidin-5-ylboronic acid (79 mg, 5.13 x10$^{-4}$ mol), K$_2$CO$_3$ (479 mg, 3.60 x10$^{-3}$ mol) and Pd(PPh$_3$)$_4$ (20 mg, 1.74 x10$^{-6}$ mol) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. After 20 min a UV active spot ($R_f = 0.13, 1:4$ EtOAc: Pet spirit) was detected via TLC along with disappearance of SM. After 20 min the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel
flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give 49a as a light yellow solid (111 mg, 88%); MP: 141-143° C; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.66 (s, 2H, ArH\(_2\)"and ArH\(_6\)"), 4.05 (s, 3H, p-methoxyCH\(_3\)), 3.90 (s, 3H, Ester CH\(_3\)), 3.38 (t, \(J = 6\) Hz, 4H, C2' H\(_2\) and C7' H\(_2\)), 1.67 (s, 4H, C3' H\(_2\) and C6' H\(_2\)), 1.48 (s, 4H, C4' H\(_2\) and C5' H\(_2\)); \(^13\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 167.03 (Ester C=O), 164.54 (ArC4"), 158.09 (ArC2" and ArC6"), 155.14 (ArC5), 153.67 (ArC3), 128.66 (ArC6), 125.05 (ArC1"), 113.75 (ArC2), 55.06 (p-methoxyCH\(_3\)), 52.03 (Ester CH\(_3\)), 51.21 (C2' and C7'), 27.85 (C3' and C6'), 27.16 (C4' and C5'); HRESI-MS m/z (M + H): Calc. 359.1832, Obs. 359.1848; Anal-HPLC (70:30 A:B → 100% B over 30 min, \(t_r = 28.0\) min).

**Methyl 3-amino-5-(azepan-1-yl)-6-(2,4-dimethoxypyrimidin-5-y)pyrazine-2-carboxylate (50a)**

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate \(32\) (101 mg, 3.53x10\(^{-4}\) mol), 2,4-dimethoxypyrimidin-5-ylboronic acid (100 mg 5.13x10\(^{-4}\) mol), K\(_2\)CO\(_3\) (479 mg, 3.65x10\(^{-3}\) mol) and Pd(PPh\(_3\))\(_4\) (12 mg, 1.02x10\(^{-5}\) mol) were added to a round bottom flask. Anhydrous toluene (12 mL) followed by MeOH (3 mL) were added via syringe and heated to reflux. After 30 min a UV active spot (\(R_f = 0.8\), EtOAc) was detected via TLC along with disappearance of SM. After 30 min the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give \(50a\) of a light yellow solid (124 mg, 91%); MP: 150-152° C; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.36 (s, 1H, ArH\(_6\)"), 4.03 (s, 3H, p-methoxyCH\(_3\)), 3.94 (s, 3H, o-methoxyCH\(_3\)), 3.87 (s, 3H, Ester CH\(_3\)), 3.39 (s, 4H, C2' H\(_2\) and C7' H\(_2\)), 1.60 (s,
4H, C3’ H₂ and C6’ H₂, 1.46 (s, 4H, C4’ H₂ and C5’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 168.30 (ArC₂”), 167.18 (Ester C=O), 164.76 (ArC₄”), 158.09 (ArC₆”), 155.34 (ArC₅), 154.05 (ArC₃), 123.75 (ArC₆), 117.06 (ArC₁”), 112.64 (ArC₂), 54.96 (p-methoxyC₆H₃), 54.10 (m-methoxyC₆H₃), 51.95 (Ester C₆H₃), 50.06 (C₂’ and C7’), 27.98 (C₃’ and C₆’), 26.95 (C₄’ and C₅’); HRESI-MS m/z (M + H)⁺: Calc. 389.1937, Obs. 389.1929; Anal-HPLC (70:30 A:B → B over 30 min, tᵣ = 27.1 min).

Methyl 3-amino-5-(azepan-1-yl)-6-(2,6-dimethoxypyridin-3-yl)pyrazine-2-carboxylate (51a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate (32) (200 mg, 7.03x10⁻⁴ moles), 2,6-dimethoxypyridin-3-ylboronic acid (200 mg, 1.09x10⁻³ moles), K₂CO₃ (1.04 g, 7.53x10⁻³ moles) and Pd(PPh₃)₄ (35 mg, 3.30x10⁻⁵ moles) were added to a 10 mL glass sample vial charged with Ar. To the flask was added anhydrous toluene (4 ml) followed by MeOH (1 ml) and the mixture stirred at RT for 18 h. After 18 h a UV active spot (Rᵣ = 0.3 40:60 EtOAc: Pet spirit) was detected via TLC along with SM (Rᵣ = 0.5, 1:4 EtOAc: Pet. Spirit). After 18 h the RM was filtered through celite and washed through with EtOAc (3x20 mL). Solvent removed under reduced pressure. The crude material was dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give 51a as a yellow solid (115 mg, 42%); MP: 134-136° C; ¹H NMR (500 MHz, CDCl₃) δ 7.66 (d, J = 8.0 Hz, 1H, ArH₅”), 6.38 (d, J = 8.0 Hz, 1H, ArH₄”), 3.92 (s, 3H, p-methoxyCH₃), 3.87 (s, 3H, Ester CH₃), 3.86 (s, 3H, m-methoxyCH₃), 3.45 (bs, 2H, C₂’ and C7’), 3.31 (bs, 2H, C₂’ and C7’), 1.57 (s, 4H, C₃’ H₂ and C₆’ H₂), 1.44 (s, 4H, C₄’ H₂ and C₅’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.52 (Ester C=O), 162.79 (ArC₆”), 159.68 (ArC₄”), 153.92 (ArC₃), 141.52 (ArC₄”), 127.61 (ArC₆), 116.24 (ArC₃”), 112.21 (ArC₂), 101.33 (ArC₅”),
53.72 (p-methoxyCH₃), 53.48 (o-methoxyCH₃), 51.90 (Ester CH₃), 50.14 (C2’ and C7’), 28.12 (C3’ and C6’), 27.07 (C4’ and C5’); HRESI-MS m/z (M + H)^+ : Calc. 388.1985, Obs. 388.1983; Anal-HPLC (70:30 A:B → 100% B over 30 min, tᵣ = 31.6 min).

**Methyl 3-amino-5-(azepan-1-yl)-6-(2-fluoropyridin-3-yl)pyrazine-2-carboxylate (52a)**

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (100 mg, 3.52x10⁻⁴ mol), 2-fluoropyridin-3-ylboronic acid (75 mg, 5.29x10⁻⁴ mol), K₂CO₃ (519 mg, 3.75x10⁻³ mol) and Pd(PPh₃)₄ (21 mg, 1.77x10⁻⁵ mol) were added to a stoppered two-neck round bottom flask and the flask was charged with Ar. The flask was fitted to a reflux condenser followed by addition of anhydrous toluene (12 mL) and MeOH (3 mL) via syringe. The flask was heated to reflux for 45 min. A UV active spot (Rᵣ = 0.55, 2:3 EtOAc:Pet. spirit) was detected via TLC after 3 min. TLC at 45 min revealed the disappearance of methyl ester SM (Rᵣ = 0.8, 2:3 EtOAc:Pet. spirit). The RM was allowed to cool to RT, filtered through celite and washed through with CH₃CN (3x20 mL). Solvent removed under reduced pressure. The crude product was re-solubilized in MeOH and adsorbed onto silica gel under reduced pressure. The product was isolated via silica gel flash column chromatography (gradient elution, 5:95 to 15:85 EtOAc:Hexane) to give 52a as a light brown solid (92 mg, 76%): MP: 156-158°C; ¹H NMR (500 MHz, CDCl₃) δ 8.17 (d, J = 4.3 Hz, 1H, ArH₆”), 8.06 (m, 1H, ArH₅”), 7.29 (t, J = 5.5 Hz, 1H, ArH₄”), 3.89 (s, 3H, Ester CH₃), 3.36 (s, J = 5.7 Hz, 4H, C2’H₂ and C7’H₂), 1.63 (s, 4H, C3’H₂ and C6’H₂), 1.46 (s, 4H, C4’H₂ and C5’H₂). ¹³C NMR (126 MHz, CDCl₃) δ 167.21 (Ester C=O), 161.32 (ArC2”), 155.23 (ArC5), 154.10 (ArC3), 146.80 (ArC6”), 141.07 (ArC4”), 124.54 (ArC5”), 123.84 (ArC6), 122.15 (ArC3”), 113.34 (ArC2), 52.27 (Ester CH₃), 50.34 (C2’ and C7’), 28.13 (C3’ and C6’), 27.29 (C4’
and C5’); HRESI-MS m/z (M + Na)^+: Calc. 368.1499, Obs. 368.1489. Anal-HPLC (100% A → 100% B 30 min, t_r = 26.3 min).

**Methyl 3-amino-5-(azepan-1-yl)-6-(2,4-dimethoxyphenyl)pyrazine-2-carboxylate (53a)**

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (201 mg, 7.07x10^-4 mol), 2,4-dimethoxyphenylboronic acid (128 mg, 7.02x10^-4 mol), K_2CO_3 (1.02 g, 7.41x10^-3 mol) and Pd(PPh_3)_4 (40 mg, 3.48x10^-5 mol) were added to a round bottom flask. Anhydrous toluene (24 mL) followed by MeOH (6 mL) were added via syringe and heated to reflux. After 1 h a UV active spot (R_f = 0.4, 1:4 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 2 h. After 2.75 h the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give 53a as a light yellow solid (143 mg, 53%): MP: 106-108 °C; ^1H NMR (500 MHz, CDCl_3): ^1H NMR (500 MHz, CDCl_3) δ 7.33 (d, J = 8.3 Hz, 1H, ArH_6”), 6.54 (dd, J = 8.3 Hz, J = 2.1 Hz 1H, ArH5”), 6.42 (d, J = 2.1 Hz, 1H, ArH3”) 3.85 (s, 3H, p-methoxyC_6H_3), 3.82 (s, 3H, o-methoxyC_6H_3), 3.70 (s, 3H, Ester CH_3), 3.44 (s, 2H, C2’ H_2 and C7’ H_2), 3.26 (s, 2H, C2’ H_2 and C7’ H_2), 1.58 (s, 2H, C3’ H_2 and C6’ H_2), 1.53 (s, 2H, C3’ H_2 and C6’ H_2), 1.46 (s, 2H, C4’ H_2 and C5’ H_2), 1.39 (s, 2H, C4’ H_2 and C5’ H_2).

**^13C NMR (126 MHz, CDCl_3)** δ 165.95 (Ester C=O), 160.93 (ArC4”), 158.21 (ArC2”), 155.42 (ArC5), 153.99 (ArC3), 131.10 (ArC6”), 129.22 (ArC1”), 123.71 (ArC6), 111.77 (ArC2), 105.07 (ArC5”), 98.67 (ArC3”), 55.74 (d, J = 2.1 Hz, p-methoxyCH_3), 55.66 (d, J = 2.1 Hz, m-methoxyCH_3), 51.99 (EsterCH_3), 50.62 (C2’ and C7’), 27.81 (C3’ and C6’), 26.93 (C4’ and C5’).
Methyl 3-amino-5-(azepan-1-yl)-6-(3,5-dimethoxyphenyl)pyrazine-2-carboxylate (54a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (200 mg, 7.02x10⁻⁴ mol), 3,5-dimethoxyphenylboronic acid (128 mg, 7.02x10⁻⁴ mol), K₂CO₃ (1.02 g, 7.41x10⁻³ mol) and Pd(PPh₃)₄ (40 mg, 3.48x10⁻⁵ mol) were added to a round bottom flask. Anhydrous toluene (24 mL) followed by MeOH (6 mL) were added via syringe and heated to reflux. After 1 h a UV active spot (Rf = 0.4, 1:4 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 3 h. After 3 h min the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (2.5:97.5 EtOAc:Pet. Spirit → 10:90 EtOAc:Pet. Spirit) to give 54a of a light yellow solid (166 mg, 61%); MP: 96-98° C; ¹H NMR (500 MHz, CDCl₃) δ 6.64 (s, 2H, ArH₂" and ArH₆"), 6.40 (s, 1H, ArH₄"), 3.88 (s, 3H, Ester 𝐶H₃), 3.81 (s, 6H, 𝑚-methoxy𝐶H₃), 3.38 (t, J = 6.0 Hz, 4H, C₄’ H₂ and C₅’ H₂), 1.64 (s, 4H, C₃’ H₂ and C₆’ H₂), 1.44 (s, 4H, C₄’ H₂ and C₅’ H₂); ¹³C NMR (126 MHz, CDCl₃) δ 167.50 (Ester 𝐶=O), 160.98 (ArC₃” and ArC₅”), 154.73 (ArC5), 153.67 (ArC3), 142.75 (ArC1”), 131.59 (ArC6), 111.97 (ArC2), 106.32 (ArC2” and ArC6”), 99.95 (ArC4”), 55.61 (m-methoxy𝐶H₃), 51.98 (Ester𝐶H₃), 50.89 (C₂’ and C7’), 28.09 (C₃’ and C6’), 27.14 (C₄’ and C₅’). HRESI-MS m/z (M + Na)⁺: Calc. 409.1852, Obs. 409.1867. Anal-HPLC (70:30 A:B → 100% B over 30 min, tᵣ = 30.0 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(3,6-dimethoxypyridazin-4-yl)pyrazine-2-carboxylate (55a)

Methyl 3-amino-5-(azepan-1-yl)-6-chloropyrazine-2-carboxylate 32 (102 mg, 3.59x10^{-4} mol), 3,6-dimethoxypyridazin-4-ylboronic acid (97 mg, 5.26x10^{-4} mol), K$_2$CO$_3$ (485 mg, 3.51x10^{-3} mol) and Pd(PPh$_3$)$_4$ (22 mg, 1.89x10^{-5} mol) were added to a round bottom flask. Anhydrous toluene (24 mL) followed by MeOH (6 mL) were added via syringe and heated to reflux. After 1 h a UV active spot ($R_f$ = 0.5, 2:3 EtOAc:Pet. Spirit) was detected via TLC along with disappearance of SM after 2 h. After 2 h the RM was allowed to cool to RT, filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The crude material was re-dissolved in EtOAc and adsorbed to silica prior to purification by silica gel flash column chromatography (10:90 EtOAc:Pet. Spirit → 20:80 EtOAc:Pet. Spirit) to give 55a as a yellow solid (94 mg, 68%): MP: 152-154°C; $^1$H NMR (500 MHz, CDCl$_3$) δ 7.17 (s, 1H, ArH$_2^\prime$), 4.08 (s, 3H, o-methoxyCH$_3$), 4.00 (s, 3H, m-methoxyCH$_3$), 3.88 (s, 3H, EsterCH$_3$), 3.43 (s, 2H, C2’ H$_2$ and C7’ H$_2$), 3.29 (s, 2H, C2’ H$_2$ and C7’ H$_2$), 1.63 (s, 4H, C3’ H$_2$ and C6’ H$_2$), 1.44 (s, 4H, C3’ H$_2$ and C6’ H$_2$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ 166.97 (Ester C=O), 162.75 (ArC3’), 159.61 (ArC6’), 154.70 (ArC5), 154.14 (ArC3), 135.05 (ArC1’), 122.88 (ArC6), 119.39 (ArC2’), 113.08 (ArC2), 54.82 (o-methoxyCH$_3$), 54.62 (m-methoxyCH$_3$), 52.07 (Ester C=O), 49.74 (C2’ and C7’), 27.98 (C3’ and C6’), 27.20 (C4’ and C5’); HRESI-MS m/z (M + Na)$^+$: Calc. 411.1757, Obs. 411.1762. Anal-HPLC (70:30 A:B → 100% B over 30 min, $t_r$ = 21.2 min).
Methyl 3-amino-5-(azepan-1-yl)-6-(phenylethynyl)pyrazine-2-carboxylate (56a)

Methyl 3-amino-5(azepan-1-yl)-6-pyrazine-2-carboxylate 32 (101 mg, 3.53x10^{-4} mol), Pd(PPh_{3})Cl_2 (12 mg, 1.65x10^{-5} mol) and Cul (9.9 mg, 5.2 x10^{-5} mol) were added dry to a 10 mL round bottom flask and dissolved in anhydrous THF (3.5 ml). To this solution Et_{3}N (147 µL, 1.05 x10^{-3} mol) and phenylacletylene (58 µL, 5.27 x10^{-4} mol) were added via pipette. The solution was sparged with N_{2} and heated to reflux with vigorous stirring. After 24 h the reaction was allowed to cool to RT. The crude RM was filtered through celite and washed through with EtOAc (3x20 mL). Solvent was removed under reduced pressure. The product was isolated via silica gel flash column chromatography (gradient elution 1-7.5% EtoAc:petroleum spirit) to yield 56a as a brown solid (73 mg, 59%): MP: 126-128 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.50 (m, 2H, ArH_2” and ArH_6”), 7.32 (m, 3H, ArH_3”, ArH_4” and ArH_5”), 4.00 (t, J = 5.5 Hz, 4H, C2’H_2 and C7’H_2), 3.91 (s, 3H, Ester CH_3), 1.87 (s, 4H, C3’H_2 and C6’ H_2), 1.58 (s, 4H, C4’H_2 and C5’ H_2); ^13C NMR (126 MHz, CDCl_3) δ 166.65 (Ester C=O), 155.26 (ArC5), 153.79 (ArC3), 131.45 (ArC4”), 128.41 (ArC3” and ArC5”), 128.34 (ArC2” and ArC6”), 123.43 (ArC1”), 113.71 (ArC2), 113.47 (ArC6), 90.44 (C7), 89.04 (C8), 52.15 (Ester CH_3), 50.44 (C2’ and C7’), 28.60 (C3’ and C6’), 26.84 (C4’ and C5’); HRESI-MS m/z (M + H)^+: Calc. 351.1829, Obs. 351.1821; Anal-HPLC (70:30 A:B → 100% B over 30 min, t_r = 36.8 min).
**General Method for acyl guanidinylation:**

*General Method*

Free guanidine was generated via neutralization of guanidine.HCl with Na$^+$ iPrO$^-$ in iPrOH to create a 2-fold molar excess relative to the 6-substituted pyrazinoyl methyl ester. A stock solution of iPrO$^-$ was prepared by adding Na(s) (typically 30-70 mg) in anhydrous iPrOH under N$_2$ and heated at 70 °C for 30 min. From this stock solution an amount of iPrO$^-$ was removed via syringe and made up to 10 mL in anhydrous iPrOH. To this solution was added a stoichiometric amount of guanidine hydrochloride and the mixture stirred at 70 °C for 30 min. This solution was gravity filtered onto the neat 6-substituted pyrazinoyl methyl ester. Once filtration was complete, the mixture was heated to reflux and allowed to react for between 1-6 h. The reactions were monitored by MS and TLC and terminated by removal of solvent under reduced pressure. Final products were isolated by rp-HPLC. All compounds were isolated as TFA salts and exchanged to their respective HCl salts by stirring with quaternary ammonium Cl$^-$ anion exchange resin in MeOH prior to biological evaluation.
3-amino-5-(azepan-1-yl)-6-phenylpyrazine-2-carboxamide (33)

The compound was prepared using the General Method (2.5 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-phenylpyrazine-2-carboxylate 33a (70 mg, 2.14x10⁻⁴ mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 33 as a bright yellow solid (TFA salt, 7.3 mg, 7%) after HPLC; MP: 126-130 °C; ¹H NMR (500 MHz, CD₃OD) δ 8.98 (s, 1H, Amide NH), 8.26 (s, 2H Guanidine NH), 7.59 (bs, 2H Guanidine NH₂), 7.45 (d, J = 7.5 Hz, 2H, ArH₂” and ArH₆”), 7.40 (d, J = 7.5 Hz, 2H, ArH₃” and ArH₅”), 7.33 (d, J = 7.5 Hz, 1H, ArH₄”), 3.38 (t, J = 5.0 Hz 4H, C₂’H₂ and C₇’H₂), 1.63 (s, 4H, C₃’H₂ and C₆’H₂), 1.45 (s, 4H, C₃’H₂ and C₆’H₂). ¹³C-APT δ 167.93 (Amide C=O), 160.25 (Guanidine C), 155.77 (ArC₅), 155.04 (ArC₃), 145.55 (ArC₁”), 130.13 (ArC₆), 130.01 (ArC₂” and ArC₆”), 129.88 (ArC₃” and ArC₅”), 125.23 (ArC₆), 124.78 (Ar-CF₃), 112.89 (ArC₂), 51.61 (C₂’ and C₇’), 28.63 (C₃’ and C₆’), 27.58 (C₄’ and C₅’). LRESI-MS m/z (M + H)⁺ 353; Anal-HPLC (70:30 A:B → 100% B over 30 min, tᵣ = 25.4 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(4-(methylthio)phenyl)pyrazine-2-carboxamide (34)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(4-(methylthio)phenyl)pyrazine-2-carboxylate 34a (81 mg, 2.16x10⁻⁴ mol). The residue was taken up in A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 34 as a yellow solid (TFA salt, 5.5 mg, 5% after HPLC).
MP 152-154°C; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 10.31 (s, 1H, Amide NH), 9.44 (s, 2H, Guanidine NH\(_2\)), 8.47 (s, 2H, Guanidine NH\(_2\)), 7.44 (d, \(J = 8.1\) Hz, 2H, ArH3” and ArH5”), 7.28 (d, \(J = 8.1\) Hz, 2H, ArH2” and ArH6”), 3.40 (t, \(J = 5.5\) Hz, 4H, C2’H\(_2\) and C7’H\(_2\)), 1.60 (s, 4H, C3’H\(_2\) and C6’ H\(_2\)), 1.46 (s, 4H, C4’H\(_2\) and C5’ H\(_2\)). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 166.04 (Amide C=O), 156.63 (Guanidine C), 155.71 (ArC5), 153.56 (ArC3), 138.99 (ArC4”), 136.40 (ArC1”), 131.19 (ArC6), 128.14 (ArC2” and ArC6”), 126.61 (ArC3” and ArC5”), 110.96 (ArC2), 51.47 (C2’ and C7’), 27.89 (C3’ and C6’), 27.11 (C4’ and C5’), 15.45 (thioether C\(_3\)). HR-ESI-MS \(m/z\) (M + H)+ 400, Anal. for C\(_{19}\)H\(_{26}\)N\(_7\)OS: Calc. 400.1920, Obs. 400.1935. Anal-HPLC (70:30 A:B → 100% B over 30 min, \(t_r\) = 28.4 min).

\(3\)-amino-5-(azepan-1-yl)-N-carbamidoyl-6-(4-(trifluoromethyl)phenyl)pyrazine-2-carboxamide (35)

The compound was prepared using the General Method (2.5 h reaction time) with methyl \(3\)-amino-5-(azepan-1-yl)-6-(4-(trifluoromethyl)phenyl)pyrazine-2-carboxylate \(35a\) (101 mg, 2.56x10^{-4} mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B → 100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 35 as a bright yellow solid (TFA salt, 16 mg, 12% after HPLC); MP: 126-130 °C; \(^1\)H NMR (500 MHz, CD\(_3\)OD) :7.68 (d, \(J = 8.0\) Hz, 2H, ArH3” and ArH5”), 7.65 (d, \(J = 8.0\) Hz, 2H, ArH2” and ArH6”), 3.38 (t, \(J = 5.0\) Hz 4H, C2’H\(_2\) and C7’H\(_2\)), 1.65 (s, 4H, C3’H\(_2\) and C6’H\(_2\)), 1.46 (s, 4H, C3’H\(_2\) and C6’H\(_2\)). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 167.93 (Amide C=O), 160.25 (Guanidine C), 155.77 (ArC5), 155.04 (ArC3), 145.55 (ArC1”), 130.13 (ArC6), 130.01 (ArC2” and ArC6”), 129.88 (ArC3” and ArC5”), 125.23 (ArC6), 124.78 (Ar-CF\(_3\)), 112.89 (ArC2), 51.61 (C2’ and C7’), 28.63 (C3’ and C6’), 27.58 (C4’ and C5’). HR-ESI-
MS m/z (M + H)^+: Calc. 422.1911, Obs. 422.1912; Anal-HPLC (70:30 A:B → 100% B over 30 min, t_r =27.9 min).

3-amino-5-(azepan-1-yl)-6-(benzo[d][1,3]dioxol-5-yl)-N-carbamimidoylpyrazine-2-carboxamide (36)

The compound was prepared using the General Method (3 h reaction time) using methyl 3-amino-5-(azepan-1-yl)-6-(benzo[d][1,3]dioxol-5-yl)pyrazine-2-carboxylate 36a (54 mg, 1.46x10^-4 mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 36 as a yellow solid (HCl salt, 5.7 mg, 9% after IEX) MP 154-156°C; ^1H NMR (500 MHz, DMSO-d_6) δ 10.39 (s, 1H, Amide NH), 8.71 (s, 2H, Guanidine NH_2), 8.27 (s, 2H, Guanidine NH_2), 7.31 (s, 1H, ArC2”), 6.97 (d, J = 8.0 Hz, 1H, ArH9”), 6.93 (d, J = 8.0 Hz, 1H, ArH8”), 6.08 (s, 2H, C5”)H_2), 3.40 (t, J= 5.75 Hz, 4H, C2’ H_2 and C7’ H_2), 1.60 (s, 2H, C3’ H_2 and C6’ H_2), 1.39 (s, 2H,C4’ H_2 and C5’ H_2). ^13C NMR (126 MHz, DMSO-d_6) δ 166.23 (Amide C=O), 155.15 (Guanidine C), 154.75 (ArC5), 153.61 (ArC3), 147.26 (ArC3”), 146.71 (ArC7”), 133.39 (ArC1”), 130.28 (ArC6), 121.43 (ArC9”), 112.73 (ArC2), 108.47 (ArC2”), 107.96 (ArC8”), 101.15 (C5”), 50.44 (C2’ and C7’), 27.21 (C3’ and C6’), 26.07 (C4’ and C5’). HRESI-MS m/z (M + H)^+ 398: Calc. 398.1941, Obs. 398.1929. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r =26.4 min).
3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(isoquinolin-4-yl)pyrazine-2-carboxamide (37)

The compound was prepared using the General Method (2.5 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(isoquinolin-4-yl)pyrazine-2-carboxylate 37a (156 mg, 4.13x10^-4 mol). The residue was taken up in anhydrous DMSO (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 37 as an orange solid (TFA salt, 31 mg, 15% after HPLC) MP 158-160°C; ^1H NMR (500 MHz, CD3OD) δ 10.47 (s, exchange with solvent, Amide NH), 9.57 (bs, 1H, ArH2”), 9.19 (s, exchange with solvent, Guanidine NH2), 9.09 (s, exchange with solvent, Guanidine NH2), 8.69 (bs, 1H, ArH4”), exchange with solvent, Guanidine NH2), 8.40 (d, J = 8.2 Hz, 1H ArH6”), 8.01 (t, J = 7.5 Hz, 1H ArH7”), 7.91 (t, J = 7.5 Hz, 1H ArH8”), 7.81 (d, J = 8.2 Hz, 1H ArH9”), 3.37 (s, 2H, C2‘ H2 and C7’ H2), 3.23 (s, 2H, C2’ H2 and C7’ H2), 1.53 (s, 4H, C3’ H2 and C6’ H2), 1.44 (s, 4H, C4’ H2 and C5’ H2). ^13C NMR (126 MHz, CD3OD) δ 167.51 (Amide C=O), 157.74 (Guanidine C), 156.87 (ArC5), 156.36 (ArC3), 149.67 (ArC4”), 137.60 (ArC1”), 136.92 (ArC2”), 136.87 (ArC10”), 136.82 (ArC6”), 131.61 (ArC8”), 131.42 (ArC7”), 129.044 (ArC6), 125.72 (ArC9”), 124.73 (ArC5”), 112.97 (ArC2), 51.58 (C2’ and C7’), 28.94 (C3’ and C6’), 27.75 (C4’ and C5’). HRESI-MS m/z (M + H)+: Calc. 405.2164, Obs. 405.2151. Anal-HPLC (70:30 A:B→100% B over 30 min, t, 22.7 min).
3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(1-methyl-1H-pyrazol-4-yl)pyrazine-2-carboxamide (38)

The compound was prepared using the General Method (2 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrazine-2-carboxylate 38a (69 mg, 2.07x10^-4 mol). The residue was taken up in solvent A:B (2 mL) plus 3 drops CH3CN and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 38 as a yellow solid (HCl salt, 31 mg, 38% after IEX) MP 150-152°C; ¹H NMR (500 MHz, CDCl₃) δ 10.49 (s, 1H, Amide NH), 8.83 (s, 2H, Guanidine NH₂), 8.52 (s, 2H, Guanidine NH₂), 8.00 (s, 1H, ArH₂”), 7.57 (s, 1H, ArH6”), 5.49 (bs, 2H, Ar3N NH₂), 3.93 (s, 3H, ArN₃”CH₃), 3.50 (t, J = 5.75 Hz, 4H, C2’H₂ and C7’H₂), 1.69 (s, 4H, C3’H₂ and C6’H₂), 1.49 (s, 4H, C4’H₂ and C5’H₂). ¹³C NMR (126 MHz, CDCl₃) δ 166.57 (Amide C=O), 156.35 (Guanidine C), 156.07 (ArC5), 153.40 (ArC3), 137.39 (ArC6”), 130.73 (ArC2”), 125.04 (ArC1”), 121.70 (ArC6), 111.08 (ArC2), 51.54 (C2’ and C7’), 38.67 (ArN₃”CH₃), 27.85 (C3’ and C6’), 27.27 (C4’ and C5’). HRESI-MS m/z (M + H)+: Calc. 358.2104, Obs. 358.2118. Anal-HPLC (70:30 A:B→100% B over 30 min, tᵣ =21.0 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(1-(2-morpholinoethyl)-1H-pyrazol-4-yl)pyrazine-2-carboxamide (39)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(1-(2-morpholinoethyl)-1H-pyrazol-4-yl)pyrazine-2-carboxylate 39a (99 mg, 2.29x10^-4 mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions
containing purified product were pooled concentrated via freeze drying to yield 39 as an orange solid (TFA salt, 38 mg, 30% after HPLC) MP 136-138°C; 1H NMR (500 MHz, CDCl3) δ 10.48 (s, 1H, Amide NH), 8.86 (s, 2H, Guanidine NH$_2$), 8.65 (s, 2H, Guanidine NH$_2$), 7.89 (s, 1H, ArH5”), 7.56 (s, 1H, ArH2”), 4.67 (s, 2H, C7H2), 3.93 (s, 4H, C11H2 and C13H2), 3.69 (s, 2H, C8H2), 3.47 (s, 4H, C10H2 and C14H2), 3.42 (bs, 2H, C2’ and C7’), 3.05 (bs, 2H, C2’ and C7’), 1.67 (s, 4H, C3’ H2 and C6’ H2), 1.49 (s, 4H, C4’ H2 and C5’ H2). 13C NMR (126 MHz, CDCl3) δ 166.63 (Amide C=O), 156.35 (Guanidine C), 156.06 (ArC5), 153.63 (ArC3), 139.96 (ArC5”), 130.58 (ArC2”), 124.10 (ArC1”), 122.90 (ArC6), 111.31 (ArC2), 63.78 (C11CH2 and C13CH2), 57.19 (C8CH2), 53.29 (C10CH2 and C14CH2), 51.40 (C2’ and C7’), 46.38 (C7CH2), 27.89 (C3’ and C6’), 27.17 (C4’ and C5’). HRESI-MS m/z (M + H)$^+$: Calc. 457.2788, Obs. 457.2796. Anal-HPLC (70:30 A:B →100% B over 30 min, t, =19.0 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(1H-indol-2-yl)pyrazine-2-carboxamide (40)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(1H-indol-2-yl)pyrazine-2-carboxylate 40a (76 mg, 2.09x10⁻⁴ mol). The residue was taken up in solvent A:B (4 mL) and purified by preparative HPLC (70:30 A:B →100% B over 30 min, 2 separate 2 mL injections). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 40 as an orange solid (TFA salt, 29 mg, 31% yield after HPLC) MP 152-154°C; 1H NMR (500 MHz, CDCl3) δ 11.09 (s, 1H, ArNH2”), 10.82 (s, 1H, Amide NH), 8.82 (s, 2H, Guanidine NH$_2$), 7.92 (s, 2H, Guanidine NH$_2$), 7.55 (d, 1H, J = 7.7 Hz, ArH7”) 7.41 (d, 1H, J = 7.7 Hz, ArH7”), 7.12 (t, 1H, J = 7 Hz, ArH5”), 7.03 (t, 1H, J = 7 Hz, ArH6”), 6.44 (s, 1H, ArH9”), 3.64 (t, J = 5.0 Hz, 4H, C2’ H2 and C7’ H2), 1.73 (s, 4H, C3’ H2 and C6’ H2), 1.50(s, 4H, C4’ H2 and C5’ H2). 13C NMR (126 MHz,
\[ \text{CDCl}_3 \delta 166.66 \text{ (Amide C=O)}, 156.22 \text{ (Guanidine C)}, 155.69 \text{ (ArC5)}, 152.98 \text{ (ArC3)}, 136.62 \text{ (ArC3")}, 134.89 \text{ (ArC1")}, 128.32 \text{ (ArC8")}, 125.72 \text{ (ArC6}), 122.71 \text{ (ArC5")}, 120.49 \text{ (ArC7")}, 119.81 \text{ (ArC6")}, 117.74 \text{ (ArC2)}, 110.83 \text{ (ArC4")}, 102.31 \text{ (C2' and C7')}, 27.88 \text{ (C3' and C6')}, 27.55 \text{ (C4' and C5')}; \]

HRESI-MS \( m/z \) (M + H)<sup>+</sup>: Calc. 393.2151, Obs. 393.2159. Anal-HPLC (70:30 A:B→100% B over 30 min, \( t_r =24.4 \) min).

**3-amino-5-(azepan-1-yl)-6-(benzo[b]thiophen-2-yl)-N-carbamimidoylpyrazine-2-carboxamide (41)**

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(benzo[b]thiophen-2-yl)pyrazine-2-carboxylate 41a (225 mg, 5.88x10<sup>-4</sup> mol). The residue was taken up in solvent A:B (2 mL, partially soluble) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 41 as a yellow solid (HCl salt, 10 mg, 4% after IEX) MP: 220-224°C (dec); \(^1\)H NMR (500 MHz, CD<sub>3</sub>OD) \( \delta \), 7.78 (d, \( J = 7.5 \) Hz, 1H, ArH4’’), 7.71 (d, \( J = 7.5 \) Hz, 1H, ArH7’’), 7.31-7.26 (m, 2H, ArH5’’ & 6’’), 7.24 (s, 1H, ArH9’’), 3.48 (t, \( J = 6.0 \) Hz, 4H, C2’ H<sub>2</sub> and C7’ H<sub>2</sub>), 1.66 (s, 4H, C3’ H<sub>2</sub> and C6’ H<sub>2</sub>), 1.47 (s, 4H, C4’ H<sub>2</sub> and C5’ H<sub>2</sub>). \(^{13}\)C NMR (126 MHz, CD<sub>3</sub>OD) \( \delta \) 168.23 (Amide C=O), 164.88 (ArC9’’), 160.75 (Guanidine C), 156.54 (ArC5), 155.41 (ArC3), 144.08 (ArC1’’), 141.34 (ArC8’’), 141.16 (ArC3’’), 125.75 (ArC5’’), 125.51 (ArC6’’), 124.66 (ArC6), 123.45 (ArC7’’), 123.07 (ArC2), 113.47 (ArC2’ and C7’), 52.01 (C2’ and C7’), 29.22 (C3’ and C6’), 28.07 (C4’ and C5’). HRESI-MS \( m/z \) (M + H)<sup>+</sup>: Calc. 410.1763, Obs. 410.1780. Anal-HPLC (70:30 A:B→100% B over 30 min, \( t_r =27.5 \) min).
3-amino-5-(azepan-1-yl)-6-(benzofuran-2-yl)-N-carbamimidoylpyrazine-2-carboxamide (42)

The compound was prepared using the General Method (6 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(benzofuran-2-yl)pyrazine-2-carboxylate 42a (116 mg, 3.17x10^-4 mol). The residue was taken up in anhydrous DMSO (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 42 as a yellow solid (TFA salt, 52 mg, 34% yield after HPLC) MP: 126-132°C; ¹H NMR (CD₃OD) δ 7.64 (d, J = 7.5 Hz, 1H, ArH4”), 7.50 (d, J = 8.0 Hz, 1H, ArH7”), 7.31 (t, J = 8.0 Hz, 1H, ArH6”), 7.26 (t, J = 7.5 Hz, 1H, ArH5”), 7.05 (s, 1H, ArH7”), 3.51 (t, J = 6.0 Hz, C2' H₂ and C7' H₂), 1.71 (s, 4H, C3' H₂ and C6' H₂), 1.54 (s, 4H, C4' H₂ and C5' H₂). ¹³C NMR (CD₃OD) δ 167.44 (Amide C=O), 164.32 (Guanidine C), 154.61 (ArC5), 154.47 (ArC3”), 154.37 (ArC1”), 154.09 (ArC3), 129.67 (ArC8”), 124.98 (ArC5”), 123.66 (ArC6”), 121.64 (ArC6), 121.08 (ArC7”), 112.44 (ArC2), 111.52 (ArC4”), 105.63 (ArC9”), 50.31 (C2’ and C7’), 28.62 (C3’ and C6’), 27.39(C4’ and C5’). HRESI-MS m/z (M + H)⁺: Calc. 394.1991, Obs. 394.1975. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r =26.4 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(4-fluorobenzofuran-2-yl)pyrazine-2-carboxamide (43)

The compound was prepared using the General Method (3 h reaction time) using moles methyl 3-amino-5-(azepan-1-yl)-6-(4-fluorobenzofuran-2-yl)pyrazine-2-carboxylate 43a (54 mg, 1.39x10^-4). The residue was taken up in anhydrous DMSO (2 mL) and purified by preparative HPLC (100% A→100% B over 30 min). Collected fractions
containing purified product were pooled concentrated via freeze drying to yield 43 as an orange solid (TFA salt, 15 mg, 26% yield after HPLC), MP 132-136 °C. \(^1\)H NMR (500 MHz, CD\(_3\)OD) δ 10.47 (s, exchange with solvent, Amide NH), 8.98 (s, exchange with solvent, Guanidine NH\(_2\)), 8.33 (s, exchange with solvent, Guanidine NH\(_2\)), 7.36 (t, J = 8.7Hz, 1H, ArH7"), 7.32 (q, J = 5.5Hz, 1H, ArH6"), 7.22 (s, 1H, ArH2"), 7.02 (t, J = 8.7Hz, 1H, ArH5"), 3.44 (t, J = 5.8 Hz, 4H, C2′ H\(_2\) and C7′ H\(_2\)) 1.72 (s, 4H, C3′ H\(_2\) and C6′ H\(_2\)) 1.51 (s, 4H, C4′ H\(_2\) and C5′ H\(_2\)). \(^{13}\)C NMR (126 MHz, CD\(_3\)OD) δ 167.32 (Amide C=O), 158.20 (ArC8"), 157.38 (Guanidine C), 156.89 (ArC5), 156.23 (ArC4"), 156.18 (ArC1"), 155.73 (ArC3), 126.57 (ArC6"), 118.90 (ArC6), 112.16 (ArC2), 109.75 (ArC5"), 108.65 (ArC7"), 51.08 (C2′ and C7′), 29.02 (C3′ and C6′), 27.88 (C4′ and C5′). HRESI-MS m/z (M + H): Calc. 412.1897, Obs. 412.1909. Anal-HPLC (100% A→100% B over 30 min, t, =25.6 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(thiophen-2-yl)pyrazine-2-carboxamide (44)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(thiophen-2-yl)pyrazine-2-carboxylate 44a (99 mg, 2.98x10\(^{-4}\) mol). The residue was taken up in 70:30 solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 44 as a bright yellow solid (TFA salt, 1.9 mg, 2% yield after HPLC); MP \(^1\)H NMR (500 MHz, CDCl\(_3\)) δ 10.10 (s, 1H, Amide NH), 10.04 (bs, 2H Guanidine NH\(_2\)), 8.08 (bs, 2H Guanidine NH\(_2\)), 7.26 (d, J = 5 Hz, 1H, ArH3"), 7.03 (d, J = 3 Hz, 1H, ArH5"), 3.47 (t, J = 5.75 Hz, 4H, C2′H\(_2\) and C7′H\(_2\)), 1.68 (m, 4H, C3′H\(_2\) and C6′H\(_2\)), 1.49 (m, 4H, C4′H\(_2\) and C5′H\(_2\)). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) δ 166.97 (Amide C=O), 156.30 (Guanidine C), 156.05 (ArC5), 153.39 (ArC3), 139.86 (ArC1"), 128.44 (ArC5"), 128.18 (ArC6), 125.24 (ArC4"), 124.54 (ArC2"), 51.08 (C2′ and C7′), 29.02 (C3′ and C6′), 27.88 (C4′ and C5′)
3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(thiophen-3-yl)pyrazine-2-carboxamide (45)

The compound was prepared using the General Method (2 reaction time) using (103 mg, 3.09x10^-4 mol) moles methyl 3-amino-5-(azepan-1-yl)-6-(thiophen-3-yl)pyrazine-2-carboxylate 45a. The residue was taken up in 50:50 solvent A:B (2 mL) and purified by preparative HPLC (100% A→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 45 as a bright yellow solid (TFA salt, 24 mg, 17% yield after HPLC); MP: 138-140°C; 1H NMR (500 MHz, CDCl3) δ 10.44 (s, 1H, Amide NH), 8.71 (bs, 4H, 2x Guanidine NH), 7.29 (m, 1H ArH4" and ArH5"), 7.29 (m, 1H ArH4" and ArH5"), 3.43 (t, J = 5 Hz, 4H, C2'H2 and C7'H2), 1.65 (s, 4H, C3'H2 and C6'H2), 1.46 (s, 4H, C4'H2 and C5'H2); 13C NMR (126 MHz, CDCl3) δ 166.66 (Amide C=O), 156.24 (Guanidine C), 156.02 (ArC5), 153.58 (ArC3), 140.12 (ArC1"), 128.40 (ArC5"), 127.79 (ArC6), 125.88 (ArC4"), 123.57 (ArC2'"), 110.74 (ArC2), 51.27 (C2' and C7'), 27.92 (C3' and C6'), 27.14 (C4' and C5'). HRESI-MS m/z (M + H)^+: Calc. 360.1607, Obs. 360.1611. Anal-HPLC (100% A→100% B over 30 min, t_r = 24.8 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(furan-2-yl)pyrazine-2-carboxamide (46)

The compound was prepared using General Method (2 h reaction time) using methyl 3-amino-5-(azepan-1-yl)-6-(furan-2-yl)pyrazine-2-carboxylate 46a (111 mg, 3.52x10^-4 mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min).
Collected fractions containing purified product were pooled concentrated via freeze drying to yield 46 as a bright yellow solid (TFA salt, 25 mg, 16% yield after HPLC); MP: 146-150 °C. 

$^1$H NMR (500 MHz, CD$_3$OD), 7.50 (s, 1H, ArH3”), 6.55 (d, J = 3.0 Hz, 1H, ArH5”), 6.50 (s, 1H, ArH4”), 3.37 (t, J = 6.0 Hz, 4H, C2’H$_2$ and C7’H$_2$), 1.64 (s, 4H, C3’H$_2$ and C6’H$_2$), 1.47 (s, 4H, C4’H$_2$ and C5’H$_2$). $^{13}$C NMR (126 MHz, CD$_3$OD) δ 168.1 (Amide C=O), 156.3 (ArC5), 153.4 (ArC3), 142.8 (ArC1”), 141.49 (ArC3”), 122.4 (ArC6), 112.6 (ArC2), 112.4 (ArC4”), 109.9 (ArC5”), 50.9 (C2’ and C7’), 29.2 (C3’ and C6’), 27.8 (C4’ and C5’). HRESI-MS m/z (M + H)$^+$ 344: Calc. 344.1835, Obs. 344.1830. Anal-HPLC (70:30 A:B→100% B over 30 min, $t_r$ = 24.3 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(furan-3-yl)pyrazine-2-carboxamide (47)

The compound was prepared using the General Method (2 h reaction time) with moles methyl 3-amino-5-(azepan-1-yl)-6-(furan-3-yl)pyrazine-2-carboxylate 47a (90 mg, 2.85x10$^{-4}$). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 47 as a bright yellow solid (HCl salt, 41 mg, 35% yield after HPLC); MP 146-148°C; $^1$H NMR (500 MHz, CDCl$_3$) δ 10.42 (s, 1H, Amide NH), 8.54 (s, 4H, 2X Guanidine NH$_2$), 7.63 (s, 1H, ArH2”), 7.42 (s, 1H, ArH4”), 6.63 (s, 1H, ArH5”), 5.35 (bs, 2H, Ar3NH$_2$), 3.52 (t, J = 5.75 Hz, 4H, C2’H$_2$ and C7’H$_2$), 1.68 (s, 4H, C3’H$_2$ and C6’H$_2$), 1.49 (s, 4H, C4’H$_2$ and C5’H$_2$). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 166.44 (Amide C=O), 156.29 (Guanidine C), 155.84 (ArC5), 153.61 (ArC3), 143.19 (ArC4”), 141.10 (ArC2”), 125.32 (ArC1”), 124.87 (ArC6), 110.82 (ArC2), 110.72 (ArC5”), 51.37 (C2’ and C7’), 27.86 (C3’ and C6’), 27.14 (C4’ and C5’).
The compound was prepared using the General Method (3 h reaction time) using methyl 3-amino-5-(azepan-1-yl)-6-(pyrimidin-5-yl)pyrazine-2-carboxylate 48a (86 mg, 2.63x10^-4 mol). The residue was taken up in anhydrous DMSO (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 48 as an orange solid (TFA salt, 81 mg, 26% yield after HPLC); MP: 186-190 °C; 1H NMR (500 MHz, CD3OD) δ 10.47 (Amide NH), 9.14 (s, 1H, ArH4”), 8.98 (s, 2H, ArH2” and ArH6”), 8.83 (s, 2H, Guanidine NH2), 8.33 (s, 2H, Guanidine NH2), 3.44 (t, J = 5.75 Hz, 4H, C2’ H2 and C7’ H2) 1.72 (s, 4H, C3’ H2 and C6’ H2), 1.51 (s, 4H, C4’ H2 and C5’ H2). 13C NMR (126 MHz, CD3OD) δ 167.44 (Amide C=O), 157.71 (Guanidine C), 157.33 (ArC4”), 156.91 (ArC5), 156.87 (ArC2” and ArC6”), 155.84 (ArC3), 135.87 (ArC1”), 125.37 (ArC6), 113.35 (ArC2), 52.43 (C2” and C7”), 28.85 (C3” and C6”), 28.10 (C4” and C5”). HRESI-MS m/z (M + H)+: Calc. 356.1947, Obs. 356.1944. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r = 20.1 min).

3-amino-5-(azepan-1-yl)-N-carbamidoyl-6-(2-methoxypyrimidin-5-yl)pyrazine-2-carboxamide (49)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(2-
methoxypyrimidin-5-yl)pyrazine-2-carboxylate 49a (111 mg, 3.09x10^{-4} mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B → 100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 49 as a yellow solid (HCl salt, 64 mg, 49% yield after IEX) MP 148-150°C; \(^{1}\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 10.76 (Guanidine NH), 9.27 (s, 2H, Guanidine NH\(_2\)), 8.51 (s, 2H, Ar\(_{2}H^2\) and Ar\(_{6}H^6\)), 8.48 (s, 2H, Guanidine NH\(_2\)), 4.08 (s, 3H, p-methoxyCH\(_3\)), 3.35 (t, \(J = 5.75\) Hz, 4H, C\(_2\)H\(_2\) and C\(_7\)H\(_2\)), 1.66 (s, 4H, C\(_{3}\)H\(_2\) and C\(_6\)H\(_2\)), 1.46 (s, 4H, C\(_{4}\)H\(_2\) and C\(_5\)H\(_2\)) \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 166.62 (Amide C=O), 163.99 (ArC\(_4\)"), 161.93 (Guanidine C), 157.84 (ArC\(_2\)" and ArC\(_6\)"), 156.07 (ArC\(_5\)), 153.87 (ArC\(_3\)), 127.83 (ArC\(_6\)), 124.71 (ArC\(_1\)"), 112.51 (ArC\(_2\)), 55.59 (p-methoxyCH\(_3\)), 51.59 (C\(_{2}\) and C\(_7\)), 27.76 (C\(_3\) and C\(_6\)), 27.13 (C\(_4\) and C\(_5\)).HRESI-MS \(m/z\) (M + H): Calc. 386.2076, Obs. 386.2080. Anal-HPLC (70:30 A:B → 100% B over 30 min, \(t_r = 20.7\) min).

3-amino-5-(azepan-1-yl)-N-carbamimoyl-6-(2,4-dimethoxypyrimidin-5-yl)pyrazine-2-carboxamide (50)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(2,4-dimethoxypyrimidin-5-yl)pyrazine-2-carboxylate 50a (104 mg, 2.67x10^{-4} mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B → 100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 50 as a yellow solid (HCl salt, 65 mg, 54% yield after IEX) MP 184-186°C; \(^{1}\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 10.46 (s, exchange with solvent, Amide NH), 8.79 (s, exchange with solvent, Guanidine NH\(_2\)), 8.45 (s, 1H, Ar\(_{6}H^6\)), 8.22 (s, exchange with solvent, Guanidine NH\(_2\)), 4.06 (s, 3H, p-methoxyCH\(_3\)), 4.00 (s, 3H, o-
methoxyCH₃), 3.60 (bs, 2H, C2’ H₂ and C7’ H₂), 3.41 (s, 2H, C2’ H₂ and C7’ H₂), 1.64 (s, 2H, C3’ H₂ and C6’ H₂), 1.51 (s, 2H, C4’ H₂ and C5’ H₂) ¹³C NMR (126 MHz, CDCl₃) δ 168.25 (Amide C=O), 166.45 (ArC₄”), 164.14 (ArC₂”), 156.81 (ArC₆”), 155.89 (ArC₅), 153.92 (ArC₃), 123.28 (ArC₆), 111.40 (ArC₂), 108.06 (ArC₁”), 55.36 (p-methoxyCH₃), 54.56 (o-methoxyCH₃), 50.22 (C2’ and C7’), 27.84 (C3’ and C6’), 26.93 (C4’ and C5’)

HRESI-MS m/z (M + H)+: Calc. 416.2159, Obs. 416.2158. Anal-HPLC (70:30 A:B→100% B min over 30 min, tᵣ =21.2 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(2,6-dimethoxypyridin-3-yl)pyrazine-2-carboxamide (51)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(2,6-dimethoxypyridin-3-yl)pyrazine-2-carboxylate 51a (98 mg, 2.53x10⁻⁴ mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled and concentrated via freeze drying to yield 51 as a yellow solid (HCl salt, 15 mg, 13% yield after IEX); MP: 152-154° C; ¹H NMR (500 MHz, CDCl₃) δ 10.28 (s, 1H, Guanidine NH), 8.77 (s, 2H, Guanidine NH₂), 8.14 (s, 2H, Guanidine NH₂), 7.61 (d, J = 8.0 Hz, 1H, ArH₅”), 6.39 (d, J = 8.0 Hz, 1H, ArH₄”), 3.94 (s, 3H, p-methoxyCH₃), 3.87 (s, 3H, o-methoxyCH₃), 3.52 (s, 2H, C2’ CH₂ and C7’ CH₂), 3.35 (s, 2H, C2’ CH₂ and C7’ CH₂), 1.60 (s, 4H, C3’ CH₂ and C6’ CH₂), 1.51 (s, 2H, C4’ CH₂ and C5’ CH₂), 1.44 (s, 2H, C4’ CH₂ and C5’ CH₂). ¹³C NMR (126 MHz, CDCl₃) δ 166.00 (Amide C=O), 163.16 (ArC₆”), 160.10 (Guanidine C), 159.19 (ArC₂”), 155.44 (ArC₅), 153.54 (ArC₃), 141.10 (ArC₄”), 127.98 (ArC₆), 114.64 (ArC₃”), 110.19 (ArC₂), 101.28 (ArC₅”), 53.82 (o-methoxyCH₃), 53.54 (p-methoxyCH₃), 50.39 (C2’ and C7’), 27.65 (C3’ and
C6’), 26.81 (C4’ and C5’). HRESI-MS (M + H): Calc 415.2206, Obs. 415.2191. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r = 26.1 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(2-fluoropyridin-3-yl)pyrazine-2-carboxamide (52)

The compound was prepared using the General Method (2.5 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(2-fluoropyridin-3-yl)pyrazine-2-carboxylate 52a (100 mg, 2.90x10^-4 mol). The residue was taken up in 70:30 solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 52 as a bright yellow solid (TFA salt, 18 mg, 13% yield after HPLC); MP: ^1H NMR (500 MHz, CDCl_3) δ 10.42 (s, 1H, Amide NH), 8.59 (bs, 4H, 2x Guanidine NH), 8.17 (m, 2H, ArH4” and ArH6”); 7.29 (t, J = 5.4 Hz, 1H, ArH5”) 5.13 (bs, 2H, Ar3NH), 3.38 (s, 4H, C2’H_2 and C7’H_2), 1.64 (s, 4H, C3’H_2 and C6’H_2), 1.47 (s, 4H, C4’H_2 and C5’H_2). ^13C NMR (126 MHz, CDCl_3) δ 166.45 (Amide C=O), 160.67 (ArC2”), 155.98 (ArC5), 155.90 (Guanidine C), 154.01 (ArC3), 146.91 (ArC6”), 141.41 (ArC4”), 124.19 (ArC5”), 123.41 (ArC6), 122.45 (ArC3”), 111.79 (ArC2), 50.40 (C2’ and C7’), 27.86 (C3’ and C6’), 27.16 (C4’ and C5’). HRESI-MS m/z (M + H)^+ 355: Calc. 373.1901, Obs. 373.1916. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r = 23.2 min).
3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(2,4-dimethoxyphenyl)pyrazine-2-carboxamide (53)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(2,4-dimethoxyphenyl)pyrazine-2-carboxylate 53a (123 mg, 3.17x10^{-4} mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 53 as a yellow solid (HCl salt, 12 mg, 8.1% yield after IEX) MP 156-158°C; {^1}H NMR (500 MHz, CDCl₃) δ 10.19 (s, 1H, Amide NH), 9.10 (bs, 2H, Guanidine NH₂), 7.79 (bs, 2H, Guanidine NH₂), 7.23 (d, J = 8.3 Hz, 1H, ArH2”), 6.58 (dd, J = 8.3 Hz, J = 2.1 Hz 1H, ArH3”), 6.48 (d, J = 2.1 Hz, 1H, ArH5”), 3.86 (s, 3H, p-methoxyC₆H₃), 3.74 (s, 3H, o-methoxyC₆H₃), 3.51 (s, 2H, C₂’ H₂ and C₇’ H₂), 3.36 (s, 2H, C₂’ H₂ and C₇’ H₂), 1.59 (s, 4H, C₃’ H₂ and C₆’ H₂), 1.50 (s, 2H, C₄’ H₂ and C₅’ H₂), 1.45 (s, 2H, C₄’ H₂ and C₅’ H₂) {^{13}C} NMR (126 MHz, CDCl₃) {^{13}C} NMR (126 MHz, CDCl₃) δ 165.95 (Amide C=O), 161.54 (ArC₄’’), 157.83 (ArC₂”), 155.71 (Guanidine C), 155.40 (ArC₅), 153.75 (ArC₃), 130.62 (ArC₆”), 129.59 (ArC₁”), 122.11 (ArC₆), 109.78 (ArC₂), 104.99 (ArC₅”), 98.72 (ArC₃”), 55.67 (p-methoxyC₆H₃), 55.64 (m-methoxyC₆H₃), 50.62 (C₂’ and C₇’), 27.81 (C₃’ and C₆’), 26.93 (C₄’ and C₅’). HRESI-MS m/z (M + H)^+ 414: Calc. 414.2254, Obs. 414.2269. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r = 26.7 min).
3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(3,5-dimethoxyphenyl)pyrazine-2-carboxamide (54)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(3,5-dimethoxyphenyl)pyrazine-2-carboxylate 54a (147 mg, 3.81x10^{-4} mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 54 as a yellow solid (HCl salt, 44 mg, 26% yield after IEX) MP 138-140°C; ^1H NMR (500 MHz, CDCl$_3$) δ 10.75 (s, 1H, Amide NH), 8.81 (bs, 2H, Guanidine NH$_2$), 8.60 (bs, 2H, Guanidine NH$_2$), 6.73 (d, J = 2.2 Hz, 2H, ArH" and ArH6"), 6.58 (t, J = 2.2 Hz, 1H, ArH4"), 3.84 (s, 6H, m-methoxyC$_3$H$_3$), 3.38 (s, 2H, C2' H$_2$ and C7' H$_2$), 1.64 (s, 4H, C3' H$_2$ and C6' H$_2$), 1.45 (s, 2H, C4' H$_2$ and C5' H$_2$). ^13C NMR (126 MHz, CDCl$_3$) δ 167.04 (Amide C=O), 160.96 (ArC3" and 5"), 156.35 (Guanidine C), 155.60 (ArC5), 153.77 (ArC3), 141.64 (ArC1"), 132.92 (ArC6), 110.93 (ArC2), 106.46 (ArC2" and C6"), 100.28 (ArC4"), 55.92 (m-methoxyC$_3$H$_3$), 50.99 (C2' and C7'), 27.95 (C3' and C6'), 27.12 (C4' and C5'). HRESI-MS m/z (M + H)$^+$: Calc. 414.2254, Obs. 414.2244. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r =25.8 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(3,6-dimethoxypyridazin-4-yl)pyrazine-2-carboxamide (55)

The compound was prepared using the General Method (3 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(3,6-dimethoxypyridazin-4-yl)pyrazine-2-carboxylate 55a (94 mg, 2.43x10^{-4} mol). The residue was taken up in anhydrous DMSO (2 mL) and purified by preparative HPLC (100% A→100% solvent B over 30 min). Collected fractions containing
purified product were pooled concentrated via freeze drying to yield 55 as a bright yellow solid (TFA salt, 21 mg, 17% yield after HPLC; δ 202-204°C; 1H NMR (500 MHz, CD3OD) δ 10.38 (s, exchange with solvent, Amide NH), 8.77 (s, exchange with solvent, Guanidine NH2), 8.21 (s, exchange with solvent, Guanidine NH2), 7.35 (s, 1H, ArH2), 4.06 (s, 3H, o-methoxyCH3), 3.99 (s, 3H, m-methoxyCH3), 3.35 (s, 4H, C2′ H2 and C7′ H2) 1.66 (s, 4H, C3′ H2 and C6′ H2), 1.55 (s, 2H, C4′ H2 and C5′ H2), 1.45 (s, 2H, C4′ H2 and C5′ H2). 13C NMR (126 MHz, CD3OD) δ 167.31 (Amide C=O), 164.10 (ArC3′), 160.89 (ArC6′), 156.90 (Guanidine C), 156.75 (ArC5), 156.10 (ArC3), 136.33 (ArC1′), 123.95 (ArC6), 121.38 (ArC2′), 112.19 (ArC2), 55.44 (o-methoxyCH3), 55.26 (m-methoxyCH3), 50.98 (C2′ and C7′), 28.89 (C3′ and C6′), 27.92 (C4′ and C5′). HRESI-MS m/z (M + H)+: Calc. 416.2159, Obs. 416.2150. Anal-HPLC (100% A→100% solvent B over 30 min, t =19.0 min).

3-amino-5-(azepan-1-yl)-N-carbamimidoyl-6-(phenylethynyl)pyrazine-2-carboxamide (56)

The compound was prepared using the General Method (1 h reaction time) with methyl 3-amino-5-(azepan-1-yl)-6-(phenylethynyl)pyrazine-2-carboxylate 56a (54 mg, 1.42x10⁻⁴ mol). The residue was taken up in solvent A:B (2 mL) and purified by preparative HPLC (70:30 A:B→100% B over 30 min). Collected fractions containing purified product were pooled concentrated via freeze drying to yield 56 as a brown solid (TFA salt, 9.1 mg, 14% yield after HPLC) MP -°C; 1H NMR (500 MHz, CD3OD) δ 7.51 (m, 2H, ArH2” and ArH6”), 7.39 (m, 3H, ArH3”, ArH4” and ArH5”), 4.07 (t, J = 5.75 Hz, 4H, C2’H2 and C7’H2), 1.88 (s, 4H, C3’H2 and C6’H2), 1.60 (s, 4H, C4’H2 and C5’H2). 13C NMR (126 MHz, CD3OD) δ 166.82 (Amide C=O), 156.97 (Guanidine C) 156.88 (ArC5), 155.57 (ArC3), 132.20 (ArC4”), 129.96 (ArC3” and ArC5”), 129.76 (ArC2” and ArC6”), 123.92 (ArC1”), 114.13
(ArC2), 112.93 (ArC6), 91.45 (C7), 89.29 (C8), 51.66 (C2' and C7'), 29.55 (C3' and C6'), 27.62 (C4' and C5'); HRESI-MS m/z (M + H)+: Calc. 378.2037, Obs. 378.2042. Anal-HPLC (70:30 A:B→100% B over 30 min, t_r =28.6 min).

7.3 Biology Experimental

Cell culture conditions

Human skin-derived telomerase-immortalized fibroblasts (TIFs) were sourced from Dr. Paul Timpson and the Kinghorne Cancer Centre, Sydney, NSW, Australia. All other cells used were subcultured from ATCC certified cell lines. SKOV-3 human ovarian adenocarcinoma, MDA-MB-231 human breast adenocarcinoma, HEK-293 human embryonic kidney and RAW 264.7 murine Abelson murine leukemia virus induced macrophage and U-937 human histiocytic lymphoma cells cell lines were cultured at 37° C, 95% humidity and 5% (v/v) CO_2 in a ThermoScientific Heracell 150i CO_2 incubator (ThermoScientific). ATCC cell lines were maintained in RPMI 1640 media supplemented with 5% (v/v) heat-inactivated FBS unless otherwise stated (Sigma-Aldrich, St. Louis, MI, USA). TIFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS. Adherent cells were passaged via the addition of trypsin/0.05% EDTA solution (GIBCO), followed by centrifugation and resuspension in fresh media using a 5 mL Luerlock syringe fitted with an 18-gauge drawing-up needle (Terumo, Tokyo, Japan). U-937 cells were detached from cell culture flasks via mechanical agitation in the absence of trypsin/EDTA detachment solution prior to harvest. Haemocytometry with Trypan Blue staining was used to determine the concentration of live suspended cells and to ensure a single cell suspension had been achieved. Cells were passaged weekly and media changed every 2-3 days. Testing for the
presence of mycoplasma was routinely performed using a MycoAlert™ Mycoplasma Test Kit (Lonza Group, Basel, Switzerland). No mycoplasma contaminations were detected. All experiments were conducted on cells with a passage number lower than 20.

**Statistical analysis**

Significance testing was performed using the unpaired parametric t-test function in GraphPad PRISM v6.0 (GraphPad Software, San Diego, California, USA). Level of significance was denoted as follows: * = $p < 0.05$, ** = $p < 0.02$, *** = $p < 0.005$. Correlation analysis was performed using the linear regression function in GraphPad PRISM v6.0.

**CellTitre 96® Aqueous One Solution Cell Proliferation Assay (MTS) assay**

Sub-confluent cells (~70-80% confluent) were harvested and counted and dissociated into a single cell suspension as described above. Cells were seeded at a density of 5000 cells/well (final volume 90 µL) into a Greiner CELLSTAR® 96-well plate (Greiner Bio-One GmBH, Kremsmünster, Austria) via multichannel pipette and incubated for 18h prior to the addition of compounds. Compounds were serially diluted from 20-50 mM DMSO stocks in RPMI 1640 media containing 5% v/v heat inactivated FBS in a separate 96-well plate under sterile conditions to give 10x stocks (10 nM-1 mM) on the day of compound addition. 10 µL of the 10x compound/media stocks or matched vehicle/media solutions (for vehicle controls) were then transferred via multichannel pipette into the assay plate containing 90 µL cell suspension via multichannel pipette to give the treated assay plate at the final 1X drug concentrations (1 nM-100 µM, $n = 4$ wells at each concentration). Relevant drug blanks or vehicle media blanks were included at each concentration to correct for the intrinsic colour of the compounds and of the Phenol Red containing media. 200 µL media was added to
wells A1-12 and H1-12 to decrease evaporation and were not used in the assay itself. Compound-treated plates were incubated for 48h prior to development. After 48h treatment time, plates were removed from the incubator and 20 µL CellTitre 96® Aqueous One Solution Cell Proliferation Assay (Cat # G3581, Promega Corporation, Fitchburg, Wisconsin, USA) was added to each well. Plates were incubated for a further 2h prior to reading at 490 nm using a SpectraMax Plus 384-well plate reader (Molecular Devices LLC, Sunnyvale, California, USA). Blank subtracted data was analyzed and graphed using GraphPad PRISM v6.0 (GraphPad Software, San Diego, California, USA).

**Cytotoxicity Detection Kit**(PLUS) (LDH) Assay

Assay and compound dilution plates were prepared as described in Section 7.3. After 48h treatment time assay plates were removed from the incubator and 5 µL of Lysis solution (Bottle 3) of the Roche Cytotoxicity Detection Kit**(PLUS) (LDH) (Roche Diagnostics Australia Pty. Ltd., NSW, Australia), was added to cell lysis controls. The plate was gently mixed by hand in a circular motion atop a bench (similar to the manner one might wax a car) for 30 s and incubated for a further 15 min. During this period 2.5 µL Catalyst solution (Bottle 1) was added to 112.5 µL Dye solution (Bottle 2) to form Reaction mixture for each well to be assayed. The plate was removed from the incubator and 100 µL Reaction mixture added to each well. The plate was then wrapped in aluminium foil and incubated for 30 min. After 30 min 50 µL Stop solution was added to each well and read at 490 nm using a SpectraMax Plus 384-well plate reader (Molecular Devices LLC, Sunnyvale, California, USA). Blank subtracted data was analyzed and graphed using GraphPad PRISM v6.0 (GraphPad Software, San Diego, California, USA).
**Apo-ONE® Homogeneous Caspase-3/7 Assay**

HEK-293 cells were harvested as described in Section x.xx and seeded into a black Greiner CELLSTAR® 96-well plate (Cat # 655079, Greiner Bio-One GmBH, Kremsmünster, Austria) at a density of 5000 cells/well in a volume of 40 µL RPMI 1640 media with incubation for 18h. On day of treatment 5x compound stocks in 5%DMSO/media were prepared in a separate 96-well plate under sterile conditions. 10 µL of each 5x compound stock was added to 40 µL of cell suspension on assay plate to give 1X compound dilutions in 1% v/v DMSO (1-30 µM) and the plate incubated for 5 h (time sufficient to trigger detectable induction of apoptosis according to manufacturer’s instructions). Using the Apo-ONE® Homogeneous Caspase-3/7 Assay Kit (Cat # G7790, Promega Corporation, Fitchburg, Wisconsin, USA) 30 µL of Z-DEVD-R110 Caspase substrate was diluted into 2970 µL Assay buffer and 50 µL of the mixture added to each assay well and the plate shaken using a plate shaker at 300 rpm for 2 h followed by reading using a POLARstar OMEGA Fluorescence Plate reader (BMG-Labtech GmBH, Ortenberg, Germany) ex. 480 nm, em 520 nm. Blank subtracted data was analyzed and graphed using GraphPad PRISM v6.0 (GraphPad Software, San Diego, California, USA).

**Solution-phase uPA inhibition assay**

Urokinase from human kidney cells (Cat # U4010, Sigma-Aldrich, St. Louis, MI, USA) was diluted to 900 nM, aliquoted and stored at -80° C until thawed for use in a series of experiments (typically 4-5 plates assayed on the same day). Thawed enzyme stocks were maintained at -20° C between each respective assay using a Nalgene Labtop Cooler Jr (Cat# 5115-0012, Thermo-Fisher Scientific, Waltham, Massachusetts, USA). A 5 mM stock solution of Urokinase fluorescent substrate III (Z-Gly-Gly-Arg-AMC, Calbiochem Cat # 672159, Merck Millipore, Massachusetts, USA) was prepared in dH₂O/10% DMSO and aliquoted with
storage at -20°C. Assay buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 0.5 mM EDTA, 0.01% v/v Tween-20) was prepared and used within 7 days of preparation with storage at 4°C.

On the day of experimentation compound stocks (20-50 mM in anhydrous DMSO) were serially diluted in assay buffer in a clear Greiner CELLSTAR® 96-well plate on ice (Greiner Bio-One GmbH, Kremsmünster, Austria) to give each dilution at 2X the final assayed concentration. In a separate black Greiner CELLSTAR® 96-well plate (Greiner Bio-One GmbH, Kremsmünster, Austria) residual volumes of assay buffer were added via multichannel pipette to compound fluorescence blanks and inhibited enzyme control wells as appropriate. Next 90 μL of dilute fluor (250 μM final) was added to all wells, followed by 100 μL of 2x compound dilution to appropriate wells. Immediately prior to assay 900 nM uPA stock was diluted to 15 nM and 10 μL of this dilution was added to all relevant wells, initiating reaction. Reaction progress was monitored using a POLARStar OMEGA fluorescence plate reader (Fluorescence intensity mode, top-optic, ex. 355 nM em. 450 nM, 20 flashes per well, double orbital shaking for 3s prior to every read, cycle time 60 s, 45-60 cycles, incubation at 37°C, BMG-Labtech, Ortenberg, Germany). Blank subtracted data was analyzed and graphed using GraphPad PRISM v6.0 (GraphPad Software, San Diego, California, USA).

**Chromogenic Trypsin-like serine protease activity experiments**

For experiments using uPA (Cat # U4010, Sigma-Aldrich, St. Louis, MO, USA), trypsin, tPA, thrombin and plasmin (all from Marker Gene Technologies Inc., Eugene, OR, USA) concentrated stocks were prepared in dH2O and stored at -80°C until thawed for use. Thawed enzyme stocks were maintained at -20°C between each respective assay using a Nalgene Labtop Cooler Jr (Cat# 5115-0012, Thermo-Fisher Scientific, Waltham, Massachusetts, USA). A 312.5 μM stock solution of S-2288 chromogenic substrate 2288 (D-
Ile-L-Pro-L-Arg-p-nitoraniline, Chromogenix, Massachusettes, USA) was prepared in dH$_2$O and aliquoted with storage at -20° C. Assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% v/v Tween-20, pH 7.4) was prepared and used within 7 days of preparation with storage at 4° C.

On the day of experimentation compound stocks (20-50 mM in anhydrous DMSO) were serially diluted in assay buffer in a clear Greiner CELLSTAR® 96-well plate on ice (Greiner Bio-One GmbH, Kremsmünster, Austria) to give each dilution at 10X the final assayed concentration. In a separate Greiner CELLSTAR® 96-well plate (Greiner Bio-One GmbH, Kremsmünster, Austria) residual volumes of assay buffer were added via multichannel pipette to compound fluorescence blanks and inhibited enzyme control wells as appropriate. Next 80 μL of dilute fluor (250 μM final) was added to all wells, followed by 10 μL of 10x compound dilution to appropriate wells. Immediately prior to assay concentrated enzyme stocks were diluted to 100 nM and 10 μL of this dilution was added to all relevant wells, initiating reaction. DMSO was present at a final concentration of 1% v/v. Change in absorbance overtime at 405 nm was measured at 37°C using a Molecular Devices SpectraMax Plus 384-well plate reader (Molecular Devices LLC, Sunnyvale, California, USA). IC$_{50}$ values were determined by plotting percentage of residual activity (V0) versus log drug concentration and fitted to a sigmoidal dose response curve using GraphPad Prism v.6.0 (GraphPad Software, La Jolla, CA, USA).

For experiments using Human Plasma Kallikrein, Factor Xa, Factor XIa, Activated Protein C or uPA H99Y the chromogenic substrates S-2366 (pyroGlu-Pro-Arg-pNA) Human Plasma Kallikrein, Factor XIa and Activated Protein C), S-2288 (H-D-Ile-Pro-Arg-pNA, Factor X) or S-2444 (pyroGlu-Gly-Arg-pNA, uPA H99Y) were used (all substrates sourced from Chromogenix, Massachusetts, USA). All enzymes were sourced from Marker Gene
Technologies, Inc., Eugene, OR, USA. Enzymes were used at the following final concentrations: Human Plasma Kallikrein 23.5 nM, Factor Xa 10 nM, Activated Protein C 10 nM, Factor Xia 2 nM and uPA H99Y 2 nM. Concentrated enzyme stocks were prepared in dH₂O and stored at -80°C until thawed for use. Experiments were conducted as described above for experiments using other trypsin-like serine proteases. DMSO was present at a final concentration of 1% v/v. Change in absorbance overtime at 405 nm was measured at 37°C using a BioTek Synergy 4 384-well plate reader (BioTek Instruments Inc., Winooski, Vermont, USA). IC₅₀ values were determined by plotting percentage of residual activity (V₀) versus log drug concentration and fitted to a sigmoidal dose response curve using GraphPad Prism v.6.0 (GraphPad Software, La Jolla, CA, USA).

**Determination of inhibitory constant values (Kᵢ)**

Urokinase substrate III (Fluorogenic) (Cat #672159 - Merck Millipore, Merck KgaA, Darmstadt, Germany) was serially diluted in HEPES assay buffer (20 mM HEPES, 100 mM NaCl, 0.5 mM EDTA, 0.01% (v/v) Tween-20, pH 7.6) to give final concentrations between 2.0 μM and 250 μM. 200x inhibitor stocks in DMSO were diluted 1 in 100 into HEPES assay buffer to yield a 2x working stock in 1% DMSO. The diluted substrate was added to a Greiner CELLSTAR black 96-well microtitre plate (Cat. # M9936, Greiner Bio-One GmbH, Kremsmünster, Austria), followed by addition of 2x working stock drug dilutions or equivalent volumes of assay buffer for controls such that all wells contained 200 μL solution, 0.5% DMSO v/v. The assay plate was allowed cool on a bed of ice and Urokinase from human kidney cells (Cat # U4010, Sigma-Aldrich, St. Louis, MI, USA) from a 900 nM stock was diluted in cold assay buffer to give a concentration of 15 nM. 10 μL of 15 nM enzyme stock was added to appropriate wells (final enzyme concentration = 0.75 nM) and the plate
immediately read at ex. 355 nm, em. 460 nm using a BMG Labtech POLARStar Omega Fluorescence Plate Reader (BMG Labtech, Ortenberg, Germany), cycle time 60 s, 45-60 cycles, 3s orbital shaking before each read). Blank subtracted data from the linear portion of the kinetic curve was used to determine initial velocity and data plotted using GraphPad PRISM v6.0 (GraphPad Software, La Jolla, California, USA). Data was fitted using the ‘competitive inhibition’ function to determine $K_i$ for each inhibitor.

**xCiLLigence Cell Index Assay**

Sub-confluent cells (~ 70-80% confluent) were harvested and counted and dissociated into a single cell suspension as described above. Cells were seeded at a density of 5000 cells/well (final volume 180 μL, RPMI 1640 + 10% FCS) into a ACEA Biosciences E-16 plate (Roche Diagnostics Australia Pty. Ltd., NSW, Australia) and incubated for 18 h prior to experimentation. On the day of experimentation the xCELLigence RTCA DP was allowed to equilibrate at 37° C, 95% humidity and 5% (v/v) CO$_2$ in a ThermoScientific Heracell 150i CO$_2$ incubator (ThermoScientific). Using the ‘plate scan’ program within the xCELLigence software package, the impedance of the cells was monitored for 24 h. The next day 10x drug dilutions were prepared in a Greiner CELLSTAR* 96-well plate (Greiner Bio-One GmBH, Kremsmünster, Austria). After 24 h the cell plate was removed from the xCelligence system and 10x drug dilutions added to give the desired final concentrations in a total volume of 200 μL (1% DMSO v/v final). Impedence was measured for 48 h following addition of drugs. Data was analyzed in the RTCA 2.0 v3.4 software package and graphed using GraphPad Prism v.6.0 (GraphPad Software, La Jolla, CA, USA).
**IncuCyte experiments**

IncuCyte ZOOM Live-Cell Analysis System (Essen Bio Science, Inc., Ann Arbor, MI, USA) was maintained in at 37° C, 95% humidity and 5% (v/v) CO₂ in a ThermoScientific Heracell 150i CO₂ incubator (ThermoScientific) throughout the course of all experiments. Sub-confluent cells (~ 70-80% confluent) were harvested and counted and dissociated into a single cell suspension as described above. Cells were seeded at a density of 5000 cells/well (final volume 90 μL) into a Greiner CELLSTAR® 96-well plate (Greiner Bio-One GmbH, Kremsmünster, Austria) via multichannel pipette and incubated for 18 h prior to the addition of compounds. On the day of drug addition compound dilutions were prepared and added to plates as described above for MTS assays. Images were acquired every 2 h for ~ 52 h following the addition of compounds using a 10x objective. Cell confluency for each image was calculated using the cell confluence processing definition inside the IncuCyte ZOOM software package v.2016A. Cell confluence masks were optimized using representative images to exclude cell debris and accurately measure cell area. Data from all images was normalized to an initial image taken of the same well immediately prior to drug addition. Data was exported from IncuCyte ZOOM and graphed using GraphPad Prism v.6.0 (GraphPad Software, La Jolla, CA, USA). All treatments were conducted in triplicate with a single image taken from each well at each time point.

**Organotypic cell invasion assay**

Tendons from excess deceased laboratory rats were surgically removed after washing in 70% v/v ethanol/water solution. Isolated tendons were frozen at -80°C and mashed using a glass rod prior to dissolution of collagen I in 1500 mL 0.5 M Acetic acid at 4°C for 48 h with magnetic stirring. Extracted collagen was filtered through wet household paper towel to
remove any remaining sheath. The extract was centrifuged at 10,000 rpm at 4°C for 1 h and the pellet discarded. Sufficient NaCl was added to the supernatant to form a 10% w/v NaCl solution in a 2 L conical flask with excess NaCl added until the solution exhibited a persisting white colour and no longer separated upon standing. The solution was centrifuged at 10,000 rpm at 4°C for 30 min and supernatant discarded. The precipitate was dissolved in 0.25 M acetic acid at a 1:1 ratio for 24 h at 4°C with magnetic stirring. The resulting collagen solution was dialyzed against 6-8 changes of 5 L 0.174 mM acetic acid with changes occurring twice daily. Dialyzed collagen was centrifuged at 20,000 rpm at 4°C for 1.5 h. Isolated collagen I was adjusted to a concentration of 2.0 mg/mL for use. Purity of extracted collagen I was verified by SDS-PAGE. Human skin-derived telomerase-immortalized fibroblasts were maintained in culture for 4-14 days post-confluency without changing of media prior to harvesting to ensure quiescence. On day of collagen I seeding a collagen I matrix solution was prepared by adding 3 mL 10x MEM solution to 25 mL collagen I solution along with 3 mL 0.22 M NaOH and subsequent dropwise addition of NaOH until a pH of ~7.4 was achieved as determined by colour change of internal Phenol Red indicator. Harvested Fibroblasts were resuspended in 3 mL FBS and seeded at a density of 1.0x10^6 cells/12 matrices into the neutralized collagen I matrix solution and 2.5 mL aliquoted into separate sterile 35 mm sterile cell culture dishes, followed by. After ~ 30 min ~ 2 mL fibroblast growth medium (DMEM/10% FBS/1xPenstrep) was added to and nascent collagen I/fibroblast matrices were manually detached from the sides of each dish using a sterile pipette tip with rotation of each dish. Matrices were allowed to contract for 8-12 days until they had reached a diameter of ~ 20 mm. Following contraction matrices were transferred using tweezers to a sterile 24-well cell culture plate. Harvested SKOV-3 ovarian adenocarcinoma cells were seeded with a volume of 100 µL of cell suspension at a density of 1x10^6 cells/mL.
Seeded plugs were transferred to sterile steel mesh grids in 60 mm cell culture dishes (3 plugs per grid, 1 grid/treatment group per 60 mm dish) and ~10 mL media added such that an air liquid interface was established with the bottom of the plug (as growth media diffuses from the bottom of the plug towards the top a chemotactic gradient is established, inducing epithelial cells seeded atop to invade downwards through the collagen matrix towards the bulk growth media). On day of initial drug treatment, culture media was removed from each dish and ~10 mL of the appropriate drug treatment/vehicle control was added to establish an air-liquid interface as described above. After 21 days invasion time plugs were fixed in 4% neutral buffered formalin for 24-48h. Fixed samples were processed using a Peloris Dual Retort tissue processor (Leica, Germany). Histological staining was conducted on 4 μm sections deparfinized in xylene and rehydrated via graded washing in ethanol (70% v/v ethanol/water-100% ethanol). Cytokeratin staining was performed using an Autostainer XL (Leica, Germany). Ten images per treatment group were acquired using a DM4000 bright field microscope (Leica, Germany) and analyzed for epithelial cell invasive index where invasive index is defined as: (# epithelial cells invaded into collagen plug / # epithelial cells forming contiguous monolayer along top of the plug) x100. Scores for each image were collated and statistical analysis performed using Microsoft Excel for Mac 2011 v.14.1.0. Graphs were generated using GraphPad PRISM v6.0 (GraphPad Software, San Diego, California, USA).
Whole cell fluorescent membrane potential dye assay for the determination of ENaC inhibition

HEK-293 Cells were cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and appropriate selection antibiotics. Semiconfluent cells were transfected with cDNA for ENaC α, β and γ subunits (genes SCNN1A, SCNN1B and SCNN1G) prior to experimentation. Cells were plated at a density of 20,000 cells/well in a 384-well black wall, flat clear bottom microtiter plates (Type: BD Biocoat Poly-D-Lysine Multiwell Cell Culture Plate) and incubated at 37 °C overnight. Following manufacturer instructions, cells were loaded with 20 µl of membrane potential dye solution for 30 min at 37 °C from the FLIPR Membrane Potential Red Dye Fluorescence Assay Kit (Molecular Devices, Sunnyvale, California, USA). This assay allows the fluorometric detection of changes in membrane potential following modulation of transmembrane ion channel/exchanger activity, namely antagonism of stimulated ENaC activity in this case. Drug, vehicle and control solutions were prepared from frozen stocks in DMSO on the day of experimentation through dilution into HEPES-buffered physiological salt solution (NaCl 137 mM, KCl 4 mM, CaCl₂ 1.8 mM, MgCl₂ 1 mM, HEPES 10 mM, Glucose 10 mM, DMSO 0.3% v/v, pH 7.4) in a 384-well microtiter plate ready for addition to the assay plate by the FLIPR TETRA instrument. All amiloride analogues were tested at 8 concentrations (n = 4 wells at each concentration). Positive control cells were stimulated with 1 µM S-3969 (ENaC agonist, n = 4 wells). Negative control cells were first stimulated with 1 µM S-3969 and then treated with 30 µM benzamil (potent ENaC antagonist, n = 4 wells). Following dye loading cells were preincubated with 5 µl drug, vehicle or control solutions for 5 min. Next 5 µl of S-3939 solution (final [S-3969] = 1
µM) was added to stimulate ENaC activity. All data was normalized to agonist-treated positive and antagonist-treated negative controls such that agonist-treated signal equaled 100% activity and antagonist-treated signal equaled 0% activity. Normalized data was used to determine IC₅₀ values for each amiloride analogue using the log[inhibitor]-normalized response variable slope algorithm in GraphPad PRISM v6.0 (GraphPad Software, La Jolla, California, USA). This experiment was performed under contract by Charles River Laboratories, Cleveland, Ohio, USA.
Chapter 8 References


Ploug, M. & Ellis, V. Structure-function relationships in the receptor for urokinase-type plasminogen activator. Comparison to other members of the Ly-6 family and snake venom a-neurotoxins. FEBS letters 349, 163-168 (1994).


Plesner, T., Behrendt, N. & Ploug, M. Structure, function and expression on blood and bone marrow cells of the urokinase-type plasminogen activator receptor, uPAR. Stem Cells 15, 398-408 (1997).


Yuan, C. & Huang, M. Does the urokinase receptor exist in a latent form? Cell and Molecular Life Sciences. 64, 1033-1037 (2007).


Han, J. & Burgess, K. Fluorescent Indicators for Intracellular pH. *Chemical Reviews* 110, 2709-2728 (2010).


## Chapter 9: Appendix

### Appendix 9.1: Data collection and refinement statistics of Human uPA in complex with 5-(N,N-hexamethylene)amiloride (HMA) and novel 6-HMA analogues. Statistics for the highest resolution shell are shown in parentheses.

<table>
<thead>
<tr>
<th>Data set</th>
<th>HMA 38</th>
<th>42</th>
<th>47</th>
<th>46</th>
<th>48</th>
<th>43</th>
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<th>50</th>
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<td><strong>Space group</strong></td>
<td>R 3</td>
<td>R 3</td>
<td>R 3</td>
<td>R 3</td>
<td>R 3</td>
<td>R 3</td>
<td>R 3</td>
<td>R 3</td>
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<tr>
<td><strong>Cell dimensions</strong></td>
<td>120.462</td>
<td>120.768</td>
<td>120.51</td>
<td>120.851</td>
<td>120.33</td>
<td>120.66</td>
<td>20.698</td>
<td>120.434</td>
</tr>
<tr>
<td>(a,b,c)</td>
<td>120.462</td>
<td>120.768</td>
<td>120.51</td>
<td>120.851</td>
<td>120.33</td>
<td>120.66</td>
<td>20.698</td>
<td>120.434</td>
</tr>
<tr>
<td><strong>Resolution range (Å)</strong></td>
<td>34.8 - 1.9</td>
<td>60.4 - 1.9</td>
<td>60.26 - 1.9</td>
<td>60.43 - 1.9</td>
<td>23.92 - 1.9</td>
<td>60.35 - 1.9</td>
<td>60.35 - 1.9</td>
<td>28.82 -</td>
</tr>
<tr>
<td></td>
<td>1.7 (1.76)</td>
<td>(1.97) - 1.62</td>
<td>1.65</td>
<td>1.73 (1.79</td>
<td>1.95</td>
<td>1.9</td>
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<td>1.65</td>
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<td>3.6 (3.6)</td>
<td>5.1 (5.0)</td>
<td>3.6 (3.8)</td>
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<td>(2487)</td>
<td>(1828)</td>
<td>(2839)</td>
<td>(2816)</td>
<td>(2419)</td>
<td>(1679)</td>
<td>(1839)</td>
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<td>99.83</td>
<td>98.82</td>
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<td>0.068</td>
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<td>0.121</td>
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<td>(0.553)</td>
<td>(0.973)</td>
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<td>0.022</td>
<td>0.010</td>
<td>0.017</td>
<td>0.018</td>
<td>0.027</td>
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Appendix 9.2: Data collection and refinement statistics of Human uPA H99Y in complex with 5-(N,N-hexamethylene)amiloride (HMA) and novel 6-HMA analogues. Statistics for the highest resolution shell are shown in parentheses.

<table>
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<th>X-ray data and model statistics</th>
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<td>Total reflections</td>
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<td>Unique reflections</td>
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<tr>
<td>Completeness (%)</td>
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<td>Overall (final shell)</td>
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<td>91.54</td>
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<td>(99.07)</td>
<td>(94.48)</td>
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Appendix 9.3: Concentration-response curves from solution-phase uPA inhibition assays with HMA and the NHE1 inhibitor Cariporide. Cariporide did not inhibit uPA activity at concentrations up to 100 μM. Data points represent the mean ± SEM (n = 3).