Synthesis of Organelle-Specific Inhibitors of Deubiquitinating Enzymes

A thesis submitted to the University of Manchester for the degree of Master of Philosophy in the Faculty of Engineering and Physical Sciences.

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ABSTRACT

In virtually all cellular processes, proteins undergo post-translational modification by the conjugation of the protein ubiquitin, which is a reversible process owing to deubiquitinating enzymes (DUBs). The possibility of conjugating additional ubiquitin moieties to either the protein itself or internal lysine residues on ubiquitin, affords different ubiquitin modifications to be built up. These modifications are recognized as different signals in the cell, hence the ubiquitin signal governs the fate and function of the protein substrate. The signal can also be edited or removed by DUBs, which is a highly understudied class of enzymes, although linked to numerous diseases. Inhibition of DUBs have shown promising anti-cancer abilities, where a prostaglandin pharmacophore (P4F) is well established.

The Whitehead and Swanton groups developed a synthesis for the documented DUB inhibitor Eeyarestatin I (ES₁), and showed that the western domain of the compound targets the ER, but possesses no biological activity in its own right. The aim of the project was to synthesise a new ER targeted DUB inhibitor by coupling the ES₁ western domain with P4F, in addition to synthesising an additional separate DUB inhibitor by coupling the same pharmacophore to a long chain alkyl phosphonium salt, which is known to target mitochondria.

Both inhibitors and the respective targeting domains were subjected to biochemical analysis in order to assess their ability to induce polyubiquitinated material at different concentrations, which resulted in both inhibitors showing some extent of polyubiquitin-accumulative abilities. Cell viability assays with increasing concentrations of the inhibitors and the mitochondrial targeting domain were also carried out, where cell viability was shown to decrease with increasing concentrations of the inhibitors, but to be relatively unaffected by the targeting domain.
DECLARATION

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>α-ubiquitin</td>
<td>anti-ubiquitin</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>anti-tubulin</td>
</tr>
<tr>
<td>Ar</td>
<td>aryl</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cm⁻¹</td>
<td>wavenumber</td>
</tr>
<tr>
<td>¹³C NMR</td>
<td>¹³Carbon nuclear magnetic resonance</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modification of Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DUBs</td>
<td>deubiquitinating enzymes</td>
</tr>
<tr>
<td>E1</td>
<td>ubiquitin activating enzyme</td>
</tr>
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<td>E2</td>
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<tr>
<td>E3</td>
<td>ubiquitin ligase enzyme</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>ES₁</td>
<td>eeyarestatin I</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>¹⁹F NMR</td>
<td>¹⁹fluorine nuclear magnetic resonance</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>¹H COSY</td>
<td>proton Correlation Spectroscopy</td>
</tr>
<tr>
<td>¹H NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HMQC</td>
<td>heteronuclear Multiple Quantum Coherence</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>lit.</td>
<td>literature value</td>
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<tr>
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</tr>
<tr>
<td>Lys29</td>
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</tr>
<tr>
<td>Lys63</td>
<td>lysine 63</td>
</tr>
<tr>
<td>M</td>
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</tr>
<tr>
<td>m</td>
<td>multiplet/medium</td>
</tr>
<tr>
<td>mA</td>
<td>milliamp</td>
</tr>
<tr>
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<td>milligrams</td>
</tr>
<tr>
<td>MHz</td>
<td>megahertz</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MJD</td>
<td>Machado-Joseph disease</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mmol</td>
<td>millimole</td>
</tr>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>OTU</td>
<td>ovarian tumor proteases</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singlet/strong</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UCH</td>
<td>ubiquitin C-terminal hydrolase</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin proteasome system</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin specific proteases</td>
</tr>
<tr>
<td>ν(_{\text{max}})</td>
<td>absorption maxima</td>
</tr>
<tr>
<td>w</td>
<td>weak</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
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Chapter 1

INTRODUCTION
1.1. Ubiquitination

Cells are often referred to as the smallest fundamental units of life, providing structure and activity to organisms. The mammalian eukaryotic cell is built up of membrane enclosed compartments called organelles, a nucleus and the aqueous cytosol. Of the total cell mass, 15% consists of proteins, which are polypeptides of amino acids joined by peptide bonds, and with diverse side-chains fulfil vital functions in basically all chemical processes of life. The activity of proteins can be controlled by many different mechanisms, such as changes in the gene expression, protein synthesis and protein degradation. In addition, post-translational modification such as phosphorylation, acetylation and glycosylation provides a way to rapidly alter the activity and function of proteins. The conjugation of the 76-amino acid, β-grasp folded protein ubiquitin (Figure 1.1) to a protein substrate is referred to as ubiquitination, and is an example of a post-translational modification.

Figure 1.1. Illustration of Ubiquitin. Scheme of the 76-amino acid protein incorporating different lysine residues, in addition to a C-terminus (taken from Groettrup)
Ubiquitin can be removed or edited, after conjugation, by deubiquitinating enzymes (DUBs), hence ubiquitination is a reversible process. Ubiquitin is found as a free monomer or conjugated to a substrate in numerous locations in the cell, such as the cytosol, plasma membrane, nucleus, and ER.\textsuperscript{1,6} It serves as a cellular signalling tag that can alter a protein's fate when conjugated to it, hence protein activity, location and functionality can be tuned.

The conjugation of an ubiquitin moiety to a substrate involves a three-step sequence and is catalysed by a cascade of enzymes, namely ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Figure 1.2). It commences with E1 forming a COOH activated adenylate of the free ubiquitin in an ATP dependent reaction, and then transfers the activated ubiquitin to a cysteine residue on E1 itself \textit{via} thioester bond formation.\textsuperscript{7} The ubiquitin is then transferred over to E2, which functions as a carrier-enzyme for ubiquitin, whilst E3 simultaneously selects suitable protein substrates and catalyses formation of an amide bond between ubiquitin and either the N-terminus or a lysine residue of the protein substrate, in a E2-E3 complex. The ubiquitinated protein is then released.\textsuperscript{8} Specificity of ubiquitination reactions is mainly thought to be due to the 600 different E3s in humans binding to specific catalytic motifs in the protein substrate, and act together with selected E2 to ubiquitinate substrate proteins.\textsuperscript{9}
Figure 1.2. Scheme Showing Ubiquitination of a Protein Substrate. Ubiquitination commences with bond formation between Ub (ubiquitin) and E1 (ubiquitin-activating enzyme), transfer of ubiquitin to E2 (ubiquitin-conjugating enzyme), then ubiquitination of a lysine moiety on the substrate by an E2-E3 (ubiquitin ligase) complex (taken and modified from Liljas).\(^1\)

1.2. The Ubiquitin Signal

Additional ubiquitin moieties can be conjugated to any of the seven internal lysine residues on ubiquitin, which allows a range of different types of polyubiquitin modifications to be built up, linked through different lysine residues and with different chain lengths, which can be linear or branched. Mono-ubiquitination involves formation of an isopeptide linkage between the carboxy-terminal glycine on one ubiquitin molecule and an amino group of a lysine moiety on the protein substrate (Figure 1.3).\(^7\) Also, additional ubiquitin can be added onto other lysine residues of the protein substrate, called multimonoubiquitination.\(^1\)}
Figure 1.3. Scheme Showing Different Ubiquitin Modifications. A) Monoubiquitination, B) Multimonoubiquitination, C) Polyubiquitination with the different modifications (extended conformation, closed conformation and branched conformation). Generated using Adobe Photoshop CS6.

The different nature or identity of ubiquitin modification form different signals that are recognized by ubiquitin binding proteins in the cell, hence different outcomes for the ubiquitinated protein are triggered.\textsuperscript{12,13} The best studied role of ubiquitin is as a signal for protein degradation. Chains of four or more ubiquitin moieties linked by conjugation of the C-terminus of ubiquitin and Lys11 (lysine 11) and Lys48 (lysine 48) of the subsequent ubiquitin serve as signals for degradation of the substrate protein by the 26S proteasome.\textsuperscript{8} In contrast, the Lys63-linkage and a variety of mono-ubiquitinated complexes are mostly involved in lysosomal degradation (Table 1.1).\textsuperscript{11} In addition, the ubiquitin signal plays an important role in many other essential cellular processes, such as protein degradation, DNA damage repair,
endocytosis and regulation in transcription. The ubiquitin signal can however be altered and reversed, making the role of the DUBs highly important.

<table>
<thead>
<tr>
<th>Ubiquitin modification</th>
<th>Outcome/Function</th>
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<tbody>
<tr>
<td>Lys11- and Lys29–linked chains</td>
<td>Proteasomal degradation</td>
</tr>
<tr>
<td>Lys48-linked chains</td>
<td>Proteasomal degradation</td>
</tr>
<tr>
<td></td>
<td>Transcription factor regulation</td>
</tr>
<tr>
<td>Lys63-linked chains</td>
<td>Lysosomal degradation</td>
</tr>
<tr>
<td></td>
<td>Trafficking and recruitment of binding factors</td>
</tr>
<tr>
<td></td>
<td>DNA damage</td>
</tr>
<tr>
<td>Monoubiquitination</td>
<td>Lysosomal degradation</td>
</tr>
<tr>
<td></td>
<td>Endocytosis</td>
</tr>
<tr>
<td></td>
<td>Recruitment of binding factors</td>
</tr>
<tr>
<td>Multimono ubiquitination</td>
<td>Protein localisation</td>
</tr>
</tbody>
</table>

Table 1.1. List of Different Ubiquitin Modifications and Corresponding Outcomes. Different linkages and chain lengths of ubiquitination results in different signalling and are involved in different cellular processes.

1.3. Deubiquitination

DUBs are responsible for the reversibility of ubiquitination, as their action edits ubiquitin chains or cleaves the linked ubiquitin from precursors or protein substrates (Figure 1.4). DUBs are found in various cellular locations, such as the nucleus, endoplasmic reticulum (ER), endosome and mitochondrion (Figure 1.5). The extended cellular processes in which DUBs are involved include degradation pathways, endocytosis, transcription regulation, DNA damage repair and they function as vital regulators in central signalling transduction pathways. Due to the catalysis of deconjugation of ubiquitin, DUBs also maintain free ubiquitin levels in the cell, hence ensuring ubiquitin homeostasis.
DUBs belong to the protease superfamily of enzymes, and of approximately 100 DUBs predicted to be encoded in the human genome, 79 are thought to be functionally active. ⁸
Figure 1.5. Scheme of Eucaryotic Cell Showing Subcellular Location of a Selection of DUBs. DUBs are associated with numerous locations in the cell, such as the plasma membrane (pm), endoplasmic reticulum (er) microtubules (mt), mitochondria (mito), early endosomes (ee) and late endosomes (m vb) (taken from Clague 17)

Through sequence similarity and similar mechanism of action, DUBs have been classified into two different groups, cysteine proteases and metalloproteases.8,18 The cysteine proteases are comprised of four subgroups of DUBs, 58 ubiquitin-specific proteases (USPs), 4 ubiquitin C-terminal hydrolases (UCHs), 14 ovarian tumor proteases (OTUs) and 5 Machado-Josephin domain proteases (MJDs).18 The subgroups are diverse in enzymatic sequence and structure, however share a similar catalytic domain with reactive sites providing a comparable enzymatic mechanism.19

The USPs are the biggest subgroup of cysteine proteases and tend to cleave ubiquitin bound to proteins, whereas the smaller and more conserved UCHs are associated with deubiquitination of smaller peptides and amino acids.20 The ovarian tumor proteases OTUs were first discovered through bioinformatics studies of Drosophila melanogaster ovary development,19 where OTU-like proteases showed
DUB activity. Relatively little information has yet been obtained on the biological functions of many OTUs, however their involvement has been shown in E3 ligase stabilization and involvement in RNA transcription, interaction with ATPase, and negative regulation of NF-κB pathway. The Machado-Josephin domain proteases MJDs have a similar structure and reactivity to UCHs and edit the chains of polyubiquitinated proteins. An example of a MJD is the DUB Ataxin-3, which upon mutation can induce Machado-Joseph disease.

Cysteine proteases cleave bonds of peptides, amides, esters, thiol esters and thiono esters. The mechanism begins with deprotonation of a cysteine thiol group within the active site by an adjacent histidine, which activates the enzymatic reactive domain and facilitates nucleophilic attack on the ubiquitin carbonyl. The ubiquitin linkage is cleaved by a catalytic triad and proceeds via a stabilised tetrahedral DUB-substrate oxyanion intermediate. Eventual hydrolysis results in formation of a carboxylic acid (Figure 1.6).
Deubiquitinating metalloproteases comprise of a Zn$^{2+}$ dependent subgroup, namely the 14 different JAMMs (JAB1/MON/Mov34 metalloenzyme). The catalytic mechanism of metalloproteases involves non-covalent complexation of three amino acids and a water molecule to one or two bivalent metal ions at the active site, resulting in activation of the peptide bond towards hydrolysis.\textsuperscript{22} Zn$^{2+}$ ions are most common in metalloproteases (as for example in JAMMs), however Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$
and Cu\(^{2+}\) ions can also be present.\(^{22}\) JAMMs have cellular functions related to endocytosis, cleavage of Nedd8 (a ubiquitin-like protein) and as the deubiquitinating Rpn11 subdomain in the proteasome.\(^{23,14}\)

1.4. Functions of DUBs

Their diversity enables some DUBs to act on single or a small number of ubiquitinated substrates, whilst others have a more expanded functionality so the same DUB can contribute in many different cellular processes.\(^{18}\) The catalytic domains of DUBs possess specificity for particular ubiquitinated substrates. This is achieved though ubiquitin chain specificity, determined by several factors such as being co-located to the same sub-cellular localization, or binding to adaptor proteins or to the substrate itself.\(^{17}\) Selectivity towards particular ubiquitin chains is also increased through a sterically favourable rearrangement of the DUB upon binding to ubiquitin, which positions the catalytic residues in an advantageous proximity and protects the active site from other cellular proteins.\(^{12}\)

1.4.1. DUB Functions at the ER

The ER is comprised of a membrane enclosed irregular system of interconnected spaces, and is the main site for synthesis of most secretory and transmembrane proteins. A specific ER-associated degradation (ERAD) pathway removes misfolded, damaged, or otherwise dysfunctional proteins from the ER.\(^{24,25}\) As they could potentially lead to development of diseases, proper detection and degradation of
these dysfunctional proteins is vital. The misfolded protein is transported out of the ER, tagged with degradation-signaling ubiquitin by E3 ligases on the cytosolic face of the ER, and chaperoned to the proteasome. Prior to degradation, DUBs need to remove the ubiquitin tag before the protein substrate can enter the proteasomal core. It has recently been shown that normal proteins that are in the process of being correctly folded in the ER may be mistakenly tagged with degradation-signaling ubiquitin. Hence DUBs play a vital role at the ER as a proofreading mechanism to rescue these substrates, by removing the ubiquitin signal and act in a negative manner with respect to degradation. An example of this is the ER associated transmembrane DUB named USP19 (Figure 1.5).

1.4.2. DUB Functions at Mitochondria

The double-membrane-bound organelles, mitochondria, are involved in many important biological processes. They have diverse functions in the cell, such as ATP generation, cell differentiation, regulation of calcium concentration, fatty acid oxidation, amino acid metabolism, redox signalling and apoptosis. E3 ligases, DUBs and proteins dependent on proteasomal degradation have been located at the mitochondria. Aided by its positioning, the intermembrane bound DUB USP30 (Figure 1.5) has been predicted to have multiple activities in the mitochondria, such as regulation of mitochondrial dynamics and participation in Mitochondrial Associated Degradation (MAD). MAD is a similar process to ERAD, and by cooperating with the cytosolic chaperone p97, USP30 transports protein substrates out of the mitochondria. In addition, the DUB ataxin-3 has been linked to the pathology of Parkinson’s disease as it interacts with the related E3 ligase Parkin.
Damaging reactive oxygen species (ROS) are often generated through the mitochondria electron transport chain\textsuperscript{31} and interestingly, ROS have recently been reported to also have a regulatory functional role in respect to protein degradation through reversible oxidation of cysteine residues, for example in the active site of DUBs.\textsuperscript{32}

\section*{1.5. DUB inhibitors and Disease}

Molecular inhibitors of DUBs are useful for several purposes. Although there is extensive knowledge about ubiquitin conjugation to target proteins, relatively little is known of the reversibility and deubiquitination of these processes. Hence tools to understand cellular function and localization of DUBs are highly sought after, and inhibitors could provide useful tools for such work. In addition, DUB inhibition could be beneficial in disease treatment. Cancer cells possess a characteristic ability to avoid death, escaping the standard cellular program leading to cell death, known as apoptosis, and by distracting the immune system from suppressing tumor growth. Drugs which induce cancer-cell death possess therapeutic potential and may also provide further understanding of cancer-cellular pathways, for example inhibitors that interrupt the degradation of pro-apoptotic proteins (which is often seen in cancer cells) or down-regulate anti-apoptotic proteins.\textsuperscript{33,34}

ERAD and MAD are linked to diseases such as Alzheimer’s disease, Parkinson’s disease, Amyotrophic lateral sclerosis,\textsuperscript{35} cardiomyopathy, diabetes and cancer,\textsuperscript{36} and have evolved as potential targets for inhibition, as disturbance of protein degradation may result in ER or mitochondrial stress.\textsuperscript{37} When such stress is detected, signalling
of a cascade of corrective actions is activated, named the unfolded protein response (UPR), which is linked to, for example, cancer, type II diabetes, Parkinson’s disease and cystic fibrosis.\textsuperscript{37} Benefitting from the resulting upregulated efficiency through corrective actions, cancer cells are often found to operate in tumors under mild UPR conditions.\textsuperscript{38} If UPR prolongs and cannot be reversed, a signalling process is activated so the cell undergoes apoptosis.\textsuperscript{39} An example of an inhibitor which uses this type of mechanism is the dipeptidyl boronic acid compound Bortezomib (Velcade\textsuperscript{®}), approved as an anti-tumor drug in 2003 for treatment of mantle cell lymphoma and relapsed multiple myeloma. Bortezomib inhibits subunits of the 26S proteasome which results in prevention of protein degradation and, as a result, accumulated ubiquitinated proteins activate the UPR, leading to eventual cell death.\textsuperscript{40}

Several types of DUB inhibitors exist, with different specificity towards DUBs. Pan-DUB inhibitors, composed of cyclopentenone prostaglandins, also induce accumulation of polyubiquitinated material resulting in apoptosis. Pan-DUB inhibitors are however unspecific in respect to DUB-group and location, and an example of this is the reversible inhibitor PR-619 which inhibits a broad range of DUB and ubiquitin-like isopeptidases (Table 1.2).\textsuperscript{41} Another class of DUB inhibitors, namely UCH-L1 inhibitors (Table 1.2), incorporate specificity in their inhibition by selectively targeting ubiquitin C-terminal hydrolase L1 DUBs. Similarly, inhibitors that specifically target the DUB USP7/HAUSP have been pursued due to the therapeutic relevance of this DUB in connection with rescuing oncoproteins for degradation and hence causing tumor growth.\textsuperscript{33}
Table 1.2. List of DUB Inhibitors and Corresponding DUB Selectivity. Inhibitors can be general and target a range of DUBs, as for example b-AP15 and pan-DUB inhibitors (PR-619), or target a select few, such as UCH-L1 inhibitors.

Investigations by Mullally and co-workers revealed an electrophilic pharmacophore with anti-cancer properties, incorporating a cross conjugated $\alpha,\beta$-unsaturated dienone bonded to two electrophilic $\beta$-carbons which are both sterically accessible, exemplified by the compound P4F. DUB inhibiting properties were demonstrated by utilisation of the $\beta$-carbons as Michael acceptors, which enables inhibition to occur by covalently binding the cysteine residues of the DUBs (Scheme 1.1).\textsuperscript{44,45}
Scheme 1.1. **P4F inhibition of DUBs.** The cross conjugated α,β-unsaturated dienone P4F inhibits the cysteine residues of DUBs through Michael addition to the β-carbon.

A related pharmacophore is present in the small molecule b-AP15 (Table 1.2), which has been shown to inhibit a range of DUBs, for example UCHL5 and USP14, which are associated with the 19S sub-particle of the 26S proteasome. b-AP15 has recently been shown to also inhibit the selenoprotein thioredoxin reductase TrxR and, although this results in oxidative stress in a similar manner to DUB inhibition, rapid apoptosis is only caused by the latter through enrichment of b-AP15 in cultured cancer cells.46

1.6. Small Molecule Targeting

Selectivity of drugs can be improved by targeting small molecules to different subcellular compartments. Targeted anti-tumor therapy is beneficial compared to untargeted therapy in respect to tumor resistance, as the latter relies on the drug molecule accumulating in the cytoplasm before reaching the nucleus to induce DNA damage. This is a time-consuming process that gives the cancer cell opportunity to regulate DNA repair and other processes for cell survival.28 In addition, a common problem in chemotherapy is insufficient specificity and systemic distribution towards a pathological site. This both increases the toxicity of the drug, and results in a
higher dosage being required for maximum therapeutic efficiency.\textsuperscript{36} Hence, targeted drug delivery has attracted significant interest ever since the 1970s, when immunotoxins and lectin conjugates were introduced, and extended pathogenic research has since been carried out in order to control cellular carcinogenic processes by targeting drugs to specific cellular compartments.\textsuperscript{35} For example, the kinase inhibitor Imatinib is currently used in cancer therapy,\textsuperscript{47} and the inhibitor of mitochondrial hsp90 protein folding chaperone, Gamitrinib, has also shown promise as an anti-tumor agent.\textsuperscript{48}

Mitochondrial targeting essentially requires lipophilicity and delocalized positive charge, the latter in order to drive delivery vector uptake in the membrane, which possesses a more negative potential than the cytosol. Delocalized positive charge increases the ionic radius and reduces solvation activation energy prior to uptake, which is further facilitated using a lipophilic moiety to penetrate the hydrophobic inner membrane of the mitochondria.\textsuperscript{28,49} The triphenylphosphonium ion (TPP) is the most extensively used platform for mitochondrial targeting, owing to its delocalized positive charge and high resonance-stability over three phenyl groups, in addition to a large hydrophobic surface area. Furthermore, other molecules can be conjugated onto a TPP targeting domain, for example inhibitors, enabling a specific delivery of drugs to mitochondria.\textsuperscript{50}

Delivery of small molecules in the cell can often be achieved by the cells own circulation system, by for example endocytic internalization through the cell membrane, and intracellular trafficking can occur through for example the Golgi-ER pathway.\textsuperscript{51} The ER membrane differs from the cytosol by possessing a different
concentration of calcium ions that are bound to proteins within the ER lumen, however it has the same pH value. Trafficking through the ER membrane is regulated by a phospholipid bilayer and imbedded translocons, however is prone to some degree of leakage, making external components able to travel across the membrane. The ER membrane has been shown to be more permeable than other organelles in the cell, for example through isolated ER vehicles in the membrane allowing small molecules and other nonphysiological reagents to pass through.\textsuperscript{52} This and the many ER associated DUBs has led to the ER being an interesting therapeutic target, especially with respect to targeting of inhibitors.

1.7. Eeyarestatin I

\begin{center}
\includegraphics[width=0.4\textwidth]{es1.png}
\end{center}

\textbf{Figure 1.7. Structure of Eeyarestatin I.} The established DUB inhibitor ES\textsubscript{1} consists of two domains, namely the western aromatic targeting domain, and the nitrofuran-containing inhibitory eastern domain.

The small molecule Eeyarestatin I (ES\textsubscript{1}) was identified by Fiebiger and co-workers (Figure 1.7) and was shown to inhibit ERAD resulting in ER stress and apoptosis in hematologic cancer cells in a similar manner to Bortezomib.\textsuperscript{38} Recent studies showed that ES\textsubscript{1} can be viewed as being bifunctional, containing two different active
domains, namely the western planar aromatic domain which targets the molecule to parts of the ER membrane, and the eastern nitrofuran domain which has been revealed to be responsible for the compound’s inhibitory and cytotoxic functionality.\textsuperscript{53} The pathogenic value of ES\textsubscript{I} as a drug candidate is however diminished due to the nitro group of the eastern furan domain, which may cause undesired \textit{in vivo} toxicity due to its high reduction potential. In addition, the hydrazone linkage is potentially prone to hydrolysis which would result in the compound losing its efficiency in the cell. The targeting western domain has, however, been shown in several assays not to have any biological functionality nor to have an effect on cell viability. Hence incorporation of the western domain of ES\textsubscript{I} would be feasible with respect to drug potency.\textsuperscript{53} Interest has been shown in ES\textsubscript{I} because of its effect on several ER pathways, including targeting to the ERAD substrate post-translation extraction factor p97/VCP and inhibiting associated deubiquitinating enzymes, such as the downstream DUB ataxin-3,\textsuperscript{54} in addition to the Sec61 ER membrane translocon.\textsuperscript{55,56,57}

\textbf{1.7.1. Synthesis of Eeyarestatins}

A general synthesis of Eeyarestatin 1 and various analogues was developed by the Whitehead group, using a convergent synthetic route where the eastern nitrofuran domain and the western aromatic domain were synthesised independently before coupling them in a final step.\textsuperscript{58} The synthesis of the western domain is illustrated in Scheme 1.2 and involves the initial reaction of 2-methylpropene with isoamyl nitrite and HCl to form 2-chloro-2-methyl-1-nitrosopropane (1). The nitroso compound was then coupled with glycine methyl ester, and the resulting oximino ester (2) was
reacted with 2 equivalents of 4-chlorophenyl isocyanate to give the imidazolidinone (3),\(^{58}\) which was then converted to the corresponding azyl hydrazide (9). The eastern domain, \(E-3-(5\text{-nitrofuran-2-yl})\text{acrylaldehyde}\), was synthesised in a separate step by reacting 5-nitrofuran-2-carbaldehyde and (triphenylphosphanyliden)acetaldehyde in an \(E\)-selective Wittig reaction. The final step involved a condensation between each of the domains to give \(ES_1\).\(^{58}\)

Scheme 1.2. Synthesis of the Western Aromatic Domain of \(ES_1\)
1.8. Project Aims

At the outset, the aim of this project was to synthesise novel DUB inhibitors which selectively target the ER and mitochondria in the cell. The western domain of ES₁ has previously been synthesised and known to target the ER, and the TPP moiety has been shown to localise to the mitochondria. A general DUB inhibitory “warhead” was based on a previously published prostaglandin pharmacophore, namely \((3E,5E)-3,5\text{-bis}(4\text{-fluorobenzylidene})\text{piperidin-4-one}\) (P4F). P4F targets a range of DUBs and possesses favourable thermal stability and increased lipophilicity compared to the ES₁ eastern fragment, in addition to bioavailability and facilitation of hydrophobic interactions at the binding site by the fluorine atoms in the structure.\(^{46}\) Hence, P4F was to be coupled to the respective targeting domains to produce more therapeutically favourable DUB inhibitors, namely ESP4F (6) and MitoP4F (8) (Figure 1.8). The ability of the inhibitors to induce accumulation of polyubiquitinated material in cells, and to effect viable cell numbers was then to be investigated through biochemical assays.

![Figure 1.8. Target Compounds. ESP4F (6) MitoP4F (8)](image)
Chapter 2

RESULTS AND DISCUSSION
2.1 Chemistry

2.1.1 Synthesis of the “Western Domain” of ESP4F

The first steps of the reaction sequence leading to the synthesis of the “western domain” of ES₁, previously developed by the Whitehead group, were used to synthesise the ER targeting domain, and a final step hydrolysis gave the corresponding carboxylic acid. The latter transformation was performed to facilitate peptide bonding to the P4F domain, using different reaction conditions to those used in the group previously, namely employing the peptide coupling reagent 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU).

2.1.1.1. Preparation of 2-chloro-2-methyl-1-nitrosopropane (1)

The first step of the synthesis of the ES₁ “western domain” involved formation of 2-chloro-2-methyl-1-nitrosopropane 1 via reaction of 2-methylpropane and iso-amyl nitrite, and concentrated HCl at -10 °C (Scheme 2.1). This gave 2-chloro-2-methyl-1-nitrosopropane as a white solid in a 35 % yield. Due to the stabilising effect, from induction and hyperconjugation, of tertiary substituted carbocations the halogenated nitroso-compound 1 is reactive towards S_N1 substitution.

Scheme 2.1. Synthesis of 2-chloro-2-methyl-1-nitrosopropane (1)
2.1.1.2. Preparation of Glycine Ester Adduct (2)

The glycine ester adduct was synthesised in the second step of the sequence, via a $S_N1$ reaction, involving nucleophilic attack by the amine lone pair of glycine methyl ester on the carbocation, derived from ionisation of the chloronitrosopropane-compound 1 (Scheme 2.2) Freshly distilled acetonitrile was used as solvent and the reaction was stirred overnight. Triethylamine was used in order to liberate glycine methyl ester from its HCl salt and also to neutralise HCl generated during the reaction. Base-catalysed tautomerism of the nitroso moiety then afforded the oxime-glycine ester product 2 as a white solid in an 82 % yield.

\[
\text{Scheme 2.2. Synthesis of methyl } 2-\{(2-(\text{hydroxyamino})-1,1-\text{dimethylethyl})\text{amino}\}\text{acetate (2)}
\]
2.1.1.3. Preparation of Imidazolidinone (3)

Synthesis of the imidazolidinone during step three proceeded by nucleophilic attack on 4-chlorophenyl isocyanate by the amine of the oximino-glycine ester adduct 2 synthesised in the previous step. This was followed by formation of a C-N bond between the isocyanate nitrogen and the oxime substituted carbon, resulting in formation of a substituted five-membered ring and a hydroxylamine group. Subsequent proton transfer and nucleophilic attack of the hydroxylamine nitrogen lone pair on a second equivalent of the isocyanate, followed by a second proton transfer and tautomerism then afforded the ester-adduct of the “western domain” of ES$_1$ 3 as a white solid in a 66 % yield (Scheme 2.3).
2.1.1.4. Preparation of the Imidazolidinone Carboxylic Acid Adduct (4)

The final step of the “western domain” synthesis involved hydrolysis of the methyl ester moiety of the previously synthesised imidazolidione 3, using lithium hydroxide in THF (Scheme 2.4). This afforded the carboxylic acid variant of the ES₁ “western domain”, and the “western domain” of the ESP4F target compound, 4 as a white solid in a 75 % yield.
A \textsuperscript{1}H NMR spectrum of 4 was acquired in deuterated DMSO (due to poor solubility in deuterated CDCl\textsubscript{3}), and the presence of a characteristic singlet peak at 6.12 ppm confirmed the structure of the western domain of ESP4F. This signal originated from the hydrogen highlighted in Figure 2.1.

**Figure 2.1. Spectroscopic Data Confirming the Identity of the Western Fragment of ESP4F.** Selected region of the \textsuperscript{1}H NMR spectrum of the western fragment of ESP4F, highlighting a diagnostic singlet resonance.
2.1.2. Synthesis of the P4F inhibitory Pharmacophore (5)

Synthesis of the bis-fluorinated benzylidene piperidone domain involved a nucleophilic addition of the 4-piperidone enolate to the carbonyl of 4-fluorobenzaldehyde, in a Claisen-Schmidt crossed aldol reaction (Scheme 2.5). This was followed by dehydration, and the process was repeated with a second equivalent of the benzaldehyde on the opposite α-carbon of the 4-piperidone. Due to the high viscosity of the reaction mixture, addition of 40 mL of water before filtration facilitated the efficiency of the procedure and resulted in an improved yield of the product (28%). Due to the poor solubility of the product in ethyl acetate and petroleum ether, purification of the product was carried out by recrystallization instead of column chromatography.

Scheme 2.5. Synthesis of Bis-Fluorinated Benzylidene piperidone Pharmacophore
Due to poor solubility in deuterated CDCl₃, spectroscopic characterisation was carried out using deuterated DMSO, and the ¹H NMR spectrum confirmed the identity of P4F via the presence of a singlet at 7.66 ppm, representing the vinyl hydrogens (Figure 2.2). The signal had a higher chemical shift than usual alkene signals, which are found in the 5 - 6.5 ppm region, due to the conjugatively electron withdrawing nature of the piperidone carbonyl.

![Figure 2.2: Spectroscopic Data Confirming the Identity of P4F](image)

**Figure 2.2: Spectroscopic Data Confirming the Identity of P4F.** Selected region of the ¹H NMR spectra of P4F, highlighting the signal for the vinyl hydrogen.

### 2.1.3. Synthesis of ESP4F (6)

The coupling agent 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), which operates with an entropic mechanistic driving force, was used in the peptide coupling of the secondary amine group of P4F 5 and the carboxylic acid of the western ER-targeting domain 4. Due to its good basicity, but poor nucleophilicity, N,N-diisopropylethylamine (DIEA) was used to deprotonate the carboxylic acid, leaving the negatively charged oxygen to attack HATU and form an O-acyl(tetramethyl)isouronium salt (Scheme 2.6). The
negative charge on the oxygen of HATU then attacks the carbonyl and tetramethylurea is released as a side product of the reaction; the carbonyl is now activated towards nucleophilic attack by the amine of P4F. The resulting tetrahedral intermediate then collapses with formation of a π-bond, and release of 3H-[1,2,3]triazolo[4,5-b]pyridin-3-ol as a side product. The coupled product ESP4F (6) was isolated as a yellow solid in a 62 % yield.

Scheme 2.6. Synthesis of ESP4F. R=“Western Domain” of ESP4F and R’=P4F
Figure 2.3. Spectroscopic Data Confirming the Identity of ESP4F. Selected region of the $^1$H NMR spectra of ESP4F, showing the signal from the different aromatic rings and alkene hydrogens.

The $^1$H NMR spectrum of ESP4F was carried out in deuterated DMSO, due to poor solubility in CDCl$_3$. The spectrum showed different aromatic domains (Figure 2.3), originating from the aromatic and alkene hydrogens. Together with supplementary NMR data and characterisation methods this confirmed the structure of the target compound.

2.1.4. Synthesis of the Mitochondrial Targeting Domain (7)

Synthesis of the mitochondrial targeting domain involved an S$_2$N$_2$ reaction between 10-bromodecanoic acid and triphenylphosphine in toluene (Scheme 2.7), which was carried out under reflux for two days. Extraction with toluene and subsequent solvent removal afforded the phosphonium salt as a brown oil in an 82 % yield.
Scheme 2.7. Synthesis of Phosphonium Salt (7)

### 2.1.5. Synthesis of MitoP4F (8)

For the coupling of P4F 5 with the phosphonium salt 7, the same reagents and conditions were used as for the synthesis of ESP4F 6, i.e the HATU procedure described in section 2.1.3. (Scheme 2.6) was employed. This resulted in the formation of a yellow solid, which was isolated in a 76% yield. The $^1$H NMR spectrum confirmed the structure, however the triplet-signal from the protons in the ortho-positions to fluorine on the fluorophenyl rings (which was previously seen in the NMR data of P4F 5) overlapped with the aromatic signal from the triphenyl groups, hence was not seen.

Scheme 2.8. Synthesis of MitoP4F (8)
2.2 Biochemistry

The compounds used for biochemical analysis included the target compounds (ESP4F and MitoP4F), the DUB inhibitory pharmacophore P4F, the mitochondrial targeting domain (phosphonium salt), and the azyl hydrazide adduct of the ER targeting western domain of ESP4F (ES) (Figure 2.4). The latter was previously synthesised in the Whitehead group.

![Compounds Diagram]

**Figure 2.4. Structures of the Compounds Used in Biochemical Assays.** 6) Target compound ESP4F; 9) ER-targeting domain ES; 8) target compound MitoP4F; 7) mitochondrial-targeting domain phosphonium salt; 5) DUB inhibitory domain P4F

The different compounds in Figure 2.4 were biochemically analysed upon their ability to induce an accumulation of polyubiquitinated proteins, and subsequently
suggesting inhibitory effects on DUBs. This was investigated using SDS-PAGE electrophoresis and Western blotting, by ubiquitin specific antibodies reflecting quantities of polyubiquitinated proteins. In addition, the viable cell number relative to DMSO after treatment of these compounds was investigated by MTT assays.

2.2.1. Determination of Levels of Polyubiquitin Conjugates

D’Arcy and co-workers have shown that an accumulation of polyubiquitinated proteins will result from inhibition of proteasomal DUBs, which therefore provides a useful readout of the ability of the target compounds to inhibit DUBs.\textsuperscript{59} To investigate this inhibition, HeLa cells were cultured and treated with increasing concentrations of each compound, then harvested after 7 hours. The cells were then lysed in sodium dodecyl sulfate (SDS) sample buffer, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate high-molecular weight ubiquitinated proteins. Following transfer onto nitrocellulose membrane, Western Blotting with anti-ubiquitin antibodies then enabled ubiquitinated proteins to be visualised and quantified.

The anionic nature of the SDS detergent causes binding to proteins when exposed to cell samples, and hence produce an equal distribution of mass to charge ratio. When samples are loaded onto a polyacrylamide gel with an applied current, the proteins will migrate towards the anode. Due to the tunable pore size of the gels, the proteins will migrate separate distances depending on their size. The larger proteins will experience more resistance and less freedom to move through the pores than smaller proteins, hence will travel shorter distances. In contrast, smaller proteins will travel
longer distances. Due to the possibility of conjugating numerous ubiquitin moieties onto cellular proteins, ubiquitinated proteins often have relatively high molecular weights and hence migrate shorter distances, and will be found in the top section of the gel further away from the anode.

In order to detect the ubiquitinated proteins, Western blotting with anti-ubiquitin antibodies was carried out. Firstly, transfer of the proteins on the gel to a nitrocellulose membrane was necessary, and accomplished by using an electric current. The membrane was then incubated overnight with a solution containing primary anti-ubiquitin antibodies, hence the protein of interest (ubiquitin) was distinguished by exclusive bonding to ubiquitin moieties. In order to visualise the ubiquitin antibodies, the membrane was then incubated with an infrared dye-labelled secondary antibody, which consequently detects and binds to the primary ubiquitin antibody. Hence the ubiquitin proteins were indirectly visualised using an infrared scanner. Due to possibility of ubiquitinated proteins with a range of ubiquitin moieties and chains, in addition to ubiquitination of differently sized proteins taking place, the molecular weight of ubiquitinated proteins can vary. Polyubiquitination in the cell generally result in relatively high molecular weight proteins however, hence the ubiquitin signals were found in the top section of the membrane and seen as a dark/intensely-coloured smear or region of bands. In order to normalise the signal relative to the amount of cell lysate that was loaded on to the gel, a loading control was used. This was done by incubating the membrane with an additional primary antibody, namely anti-tubulin, which recognises the cellular abundant protein tubulin, followed by a separate secondary infrared dye labelled antibody to visualise the tubulin signal. The tubulin signal was seen as a distinct band at 55 kDa.
An accurate quantitative measurement of the intensity of these signals was carried out using pixel counting in Licor Odyssey software. During quantification, each ubiquitin signal was normalised against the corresponding loading control (tubulin signal) to obtain an accurate polyubiquitin signal in cells treated under different concentrations. The normalised signals were then expressed as a percentage of DMSO for comparative reasons. In cases where three separate experiments were carried out, a mean of the signals and standard errors of the mean was calculated using GraphPad Prism 6.04 software.

2.2.1.1. The ER and Mitochondrial Targeting Domains Do Not Induce Accumulation of Polyubiquitin Conjugates in Human Cells.

Investigation of the organelle targeting domains of the target compounds was crucial in order to determine any potential contribution of these domains to the overall effect of the target compound on inducing accumulated polyubiquitinated material.

HeLa cells were treated for seven hours with the ER targeting domain (ES (9)) and the mitochondrial targeting domain (phosphonium salt (7)), at concentrations of 2.5, 5, 7.5, 10 and 20 µM and DMSO as solvent control. SDS-page and Western blotting was then carried out. Three separate sets of experiments using phosphonium salt were carried out, however as the effect of ES have already been investigated, only one experiment was carried out treating with ES. The ubiquitin signal was visualised using anti-ubiquitin antibodies and a secondary antibody conjugated to infrared dye, and detected using Licor Odyssey Software.
The scanned image of the separated proteins (Figure 2.5.A and B) revealed no apparent effect by DMSO on inducing polyubiquitinated material, suggesting that ubiquitinated protein levels are relatively low under normal cell conditions. There appeared to be similar ubiquitin signal intensity in lysates of cells treated with all concentrations of each compound, comparable to that of the solvent control, i.e. there seemed to be no increase in the ubiquitin signal intensity upon treatment (Figure 2.5.A and B).

The ubiquitin signal from phosphonium salt at each concentration was quantified using Odyssey Software and normalised relative to the tubulin signal (loading control) (Figure 2.5.C). In order to provide a measurement of the effect of each compound on the ubiquitin signal, the normalised ubiquitin signal from lysates of cells treated with each compound was expressed as a percentage of the ubiquitin signal from DMSO treated cells, hence the DMSO signal was set to 100% (Figure 2.5.C). The ubiquitin level appeared to confirm the Western blot observations as no increase or decrease in ubiquitin signal intensity was observed following treatment with increased concentrations of each targeting domain. Hence, together with the Western blot observations, this suggests that the targeting domains ES and phosphonium salt do not induce an accumulation of ubiquitinated material in HeLa cells.
Figure 2.5. Effect of the Targeting Domains ES (9) and Phosphonium Salt (7) on Level of Polyubiquitin Conjugates in Human Cells.
HeLa cells were treated for 7 hours with the indicated concentrations. Lysates were analysed by Western blotting using anti-ubiquitin and anti-tubulin antibodies, and visualised using a secondary antibody, conjugated to an infrared dye, and detected using Odyssey software. A) Image of one of the scanned ES Western blot membranes, showing the effect of increasing concentrations. B) Image of one of the scanned phosphonium salt Western blot membranes, showing the effect of increasing concentrations. C) Dose response curve of accumulation of ubiquitinated material upon treatment with phosphonium salt. The intensity of ubiquitin signal was quantified and normalised with reference to the loading control, and expressed relative to the solvent control. Data represents mean ±SEM of three experiments (n=3).
2.2.1.2. The Inhibitory Domain, P4F, Induces Accumulation of Polyubiquitin Conjugates in Human Cells.

The ability of the prostaglandin pharmacophore, P4F (5), to induce accumulation of polyubiquitin conjugates on its own, was determined. HeLa cells were treated with P4F at concentrations of 2.5, 5, 10 and 20 µM, with DMSO as solvent control, in three separate experiments. After 7 hours SDS-PAGE and Western blotting was carried out. Cells were lysed, and lysates analysed as previously described.

In comparison with DMSO treated cells, lysates of cells treated with P4F gave a stronger signal in the Western blot membranes (Figure 2.6.A), seen as intense staining near the top of the membrane, consistent with high molecular weight polyubiquitin proteins being present in the lysates. In addition, this would be consistent with P4F being a known DUB inhibitor, hence this signal was assumed to originate from polyubiquitinated proteins.

Quantification of three independent experiments revealed that levels of high molecular weight polyubiquitin conjugates increased steadily when cells were treated with 2.5 µM – 10 µM (Figure 2.6.B). The polyubiquitin level at 20 µM seemed to be similar to that of 10 µM, with an approximate fourfold increase compared to that of the initial concentration of 2.5 µM.

Hence, P4F does indeed inhibit DUBs in HeLa cells, leading to accumulation of polyubiquitinated proteins.
Figure 2.6. Effect of the DUB Inhibitory Domain P4F (5) on Level of Polyubiquitin Conjugates in Human Cells.
HeLa cells were treated for 7 hours with the indicated concentrations. Lysates were analysed by Western blotting using anti-ubiquitin and anti-tubulin antibodies, and visualised using a secondary antibody, conjugated to an infrared dye, and detected using Odyssey software. A) Image of one of the scanned P4F Western blot membranes, showing the effect of increasing concentrations. B) Dose response curve of accumulation of ubiquitinated material upon treatment with P4F. The intensity of ubiquitin signal was quantified and normalised with reference to the loading control, and expressed relative to the solvent control. Data represents mean ±SEM of three experiments (n=3).
2.2.1.3. The Target Compounds ESP4F and MitoP4F Induce Accumulation of Polyubiquitin Conjugates in Human Cells.

ESP4F (6) and MitoP4F (8) were synthesised with the intention to show accumulation of polyubiquitinated material in cells upon treatment, suggesting inhibition of DUBs taking place. Having shown that the targeting domains had little, if any, effect on levels of polyubiquitin conjugates, and that P4F does appear to inhibit DUBs, the next goal was therefore to examine the effect of ESP4F and MitoP4F. Hence, HeLa cells were treated with ESP4F and MitoP4F at concentrations of 2.5, 5, 10 and 20 µM, in addition to DMSO as solvent control, in three separate experiments. After 7 hours SDS-PAGE and Western blotting was carried out and cells were lysed, and lysates analysed as previously described.

Scanned Western blot ubiquitin signals revealed that lower concentrations (2.5-10 µM) of ESP4F did not induce accumulation of polyubiquitin conjugates in HeLa cells (Figure 2.7.A), and seemed similar to the signal from cells treated with DMSO. When cells were treated with 20 µM of ESP4F however, a stronger intensity was observed in the top area of the membrane. This signal appeared similar to the signal produced from P4F, hence it was assumed to originate from polyubiquitinated material. Quantification of the ubiquitin signal intensity confirmed these observations, and a gradual increase in polyubiquitin level was observed as cells were treated with increasing concentrations of ESP4F (Figure 2.7.B). The highest peak of intensity was observed at 20 µM. Hence, ESP4F appeared to induce accumulation of polyubiquitinated material at 20 µM.
The Western blot ubiquitin signal from cells treated with MitoP4F appeared to increase with the initial increasing concentrations (2.5-5 µM) (Figure 2.7.C), however the signal appeared to decrease in cells treated with higher concentrations (7.5, 10 and 20 µM). Quantification of the ubiquitin signals confirmed these observations (Figure 2.7.D). When looking at the tubulin signal, a similar decrease was observed at the higher concentrations. Visual inspection of the cells using a light microscope during treatment revealed that a large amount of cells had adopted a round shape and detached from the plate surface. These observations suggested that MitoP4F is toxic to human cells at the higher concentrations, hence ubiquitin and tubulin signal was decreased as cells had died.

Hence, ESP4F and MitoP4F appeared to have an accumulative effect of polyubiquitinated material in cells, suggesting that DUBs are being inhibited, with the highest ubiquitin signal intensity being observed in cells treated at 20 µM and 5 µM respectively.
Heidi Korsberg

RESULTS AND DISCUSSION

2.5 µM DMSO

5 µM

10 µM

20 µM

7.5 µM

Figure 2.7. Legend on following page
**Figure 2.7. Effect of the Target Compounds ESP4F (6) and MitoP4F (8) on Level of Polyubiquitin Conjugates in Human Cells.**

HeLa cells were treated for 7 hours with the indicated concentrations. Lysates were analysed by Western blotting using anti-ubiquitin and anti-tubulin antibodies, and visualised using a secondary antibody, conjugated to an infrared dye, and detected using Odyssey software. **A)** Image of one of the scanned ESP4F Western blot membranes, showing the effect of increasing concentrations after 7 hours of treatment. **B)** Dose response curve of accumulation of ubiquitinated material upon treatment with ESP4F. The intensity of ubiquitin signal was quantified and normalised with reference to the loading control, and expressed relative to the solvent control. Data represents mean ±SEM of three experiments (n=3). **C)** Image of one of the scanned MitoP4F Western blot membranes, showing the effect of increasing concentrations after 7 hours of treatment. **D)** Dose response curve of accumulation of ubiquitinated material upon treatment with MitoP4F. The intensity of ubiquitin signal was quantified and normalised with reference to the loading control, and expressed relative to the solvent control. Data represents mean ±SEM of three experiments (n=3), **p <0.01 as compared to 10μM and 20 μM (one way ANOVA followed by Turkey’s multiple comparison test).
2.2.1.4 Comparison of Compounds

In order to compare the effect of the different compounds in their ability to accumulate polyubiquitinated proteins, HeLa cells were treated with fixed concentrations of each compound, in addition to a positive control (b-AP15). Cell lysates were analysed in parallel in the same gel, using the same method as previously described.

An image of the Western blot membrane revealed an intense ubiquitin signal from cells treated with both concentrations of P4F, in addition to ESP4F and MitoP4F (Figure 2.8.A). The intensities compared to that produced by the known proteasomal DUB inhibitor b-AP15, which supports the assumption of the signal originating from polyubiquitin conjugates. Quantification of the signal confirmed these observations (Figure 2.8.B), and revealed the greatest levels of ubiquitin conjugates were found in cells treated with P4F at 10 µM. These were similar to that in cells treated with b-AP15 at 5 µM. Conjugated P4F showed to be most effective in MitoP4F at 5 µM, which compared to that of P4F at 7.5 µM, whereas signal intensity from ESP4F at 10 µM compared to that of b-AP15 at 1 µM. Both of the organelle targeting domains, ES and phosphonium salt, showed similar levels of polyubiquitin conjugates to cells treated with the solvent alone, supporting the assumption that their contribution to the overall effect of accumulated polyubiquitinated material was negligible.

Hence, comparison of the ubiquitin signal intensities showed that P4F at 10 µM induced the greatest accumulation of polyubiquitin conjugates, whereas MitoP4F (5 µM) appeared to be most effective out of the two target compounds.
Figure 2.8. Legend on following page
Figure 2.8. Overview of Effect of Previously Mentioned Compounds and Positive Control (b-AP15) on Level of Polyubiquitin Conjugates in Human Cells.

HeLa cells were treated for 7 hours with the indicated concentrations. Lysates were analysed by Western blotting using anti-ubiquitin and anti-tubulin antibodies, and visualised using a secondary antibody, conjugated to an infrared dye, and detected using Odyssey software. **A)** Image of one of the scanned Western blot membranes, showing the effect of phosphonium salt and ES (targeting domains), P4F (inhibitory domain), ESP4F and MitoP4F (target compounds) and positive control b-AP15. **B)** Chart showing the effect of accumulation of ubiquitinated material upon treatment with the indicated compounds. The intensity of ubiquitin signal was quantified and normalised with reference to the loading control, and expressed relative to the solvent control.
2.2.2. Cell Viability

DUBs are found throughout the cell and act in various essential cellular processes, which are linked to numerous human diseases. Hence, it is likely that prolonged exposure to inhibitors of DUBs would have an effect on cells and potentially cause cell death. In order to evaluate cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out. Mitochondrial succinate dehydrogenase enzymes in the cell reduce MTT solution to formazan, which results in a colour change. The amount of colour/dye present is directly proportionate to the amount of living cells, and can be read using a spectrophotometer. Results from treated cells can be compared to results from cells treated with the solvent alone, to obtain a measure of the extent of cell death being caused by the compound.

2x10^4 HeLa cells/mL were seeded and grown for 24 hours, and then treated with increasing concentrations of each compound, and DMSO as solvent control, for 48 hours. MTT assays were performed and the resulting absorbance was measured at each concentration at 570 nm. As solvents can alter the results, the absorbance was normalised with reference to the blank (solvents without cells). In order to compare the results from each compound, the normalised absorbance was expressed as a percentage of the solvent control (DMSO) so that the absorbance from DMSO corresponded to 100 %. Three sets of experiments were carried out for each compound in order to calculate a mean and standard errors of the mean (SEM).
2.2.2.1. P4F and ESP4F Have a Decreasing Effect on Viable Cell Number Relative to DMSO in Human Cells

Results from the assays of the inhibitory domain P4F (5) showed a rapid decrease in viable cell number (relative to DMSO) at the initial concentration (0.01 µM), then a rise and a relatively gradual decrease over the subsequent concentrations (Figure 2.9). At the highest concentration (0.5 µM) the viable cell number relative to DMSO was at 9%.

![Graph showing the effect of P4F on viable cell number relative to DMSO in human cells.](image)

**Figure 2.9. Effect of P4F on Viable Cell Number Relative to DMSO in Human Cells.** HeLa cells (2x10^4 cells/mL) were treated with increasing concentrations of P4F for 48 hours. MTT assays were performed and the resulting absorbance was measured and normalised relative to the blank. The normalised absorbance was expressed as a percentage of the solvent control. Data represents mean ±SEM of three experiments (n=3).

Results from the assays of the target compound ESP4F (6) showed a similar curve to that of P4F, with rapid decrease in viable cell number (relative to DMSO) at the initial concentration (0.1 µM), then a rise and a relatively gradual decrease over the
subsequent concentrations (Figure 2.10). The concentrations were however, at 10 fold higher in this case, and at the highest concentration (5 µM) the viable cell number was at 16 %. In order to confirm that the targeting domain ES did not drastically contribute to this effect, cells were treated with ES at 5 µM and showed a viable cell number relative to DMSO of 82 %.

**Figure 2.10. Effect of ESP4F and ES on Viable Cell Number Relative to DMSO in Human Cells.** HeLa cells (2x10⁴ cells/mL) were treated with increasing concentrations of ESP4F and 5 µM of ES for 48 hours. MTT assays were performed and the resulting absorbance was measured and normalised relative to the blank. The normalised absorbance was expressed as a percentage of the solvent control. Data represents Mean ±SEM of three experiments (N=3).

Hence, the lowest viable cell number resulted from cells treated with the inhibitory domain P4F. The target compound ESP4F also seemed to also have a decreasing effect on viable cell number, although this was seen at approximately 10 fold higher concentrations to those used for P4F.
2.2.2.2. MitoP4F Has a Decreasing Effect on Viable Cell Number Relative to DMSO in Human Cells

As in the previous cases, absorbance results from MitoP4F (8) showed a rapid decrease in the first concentration (0.1 µM) then a rapid increase at the following concentrations to more than 100 %, indicating that cell growth was stimulated at 0.125 – 0.25 µM (Figure 2.11). At the three final concentrations however, viable cell number decreased gradually, and at 2.5 µM corresponded to 5%.

Evaluation of effect on cell viability number and any contribution from the targeting domain was also crucial to get an accurate result. The same procedure as with MitoP4F was therefore performed, treating cells with phosphonium salt (7) with the same concentrations. After a rapid decrease and increase at the two initial concentrations, an even greater cell growth simulation effect was seen as viable cell number increased to 134%. A decrease was then seen over the subsequent concentrations, however as the final concentration showed a viable cell number of 84%, the overall effect of Phosphonium salt on viable cell number was negligible.

Hence, the target compound MitoP4F seemed to also have a decreasing effect on viable cell number, although at approximately 10 fold higher concentrations to those used for P4F. The organelle targeting domain phosphonium salt seemed to however stimulate cell growth at lower concentrations, and did not appear to have a decreasing effect on viable cell number overall.
Figure 2.11. Effect of MitoP4F and Phosphonium Salt on Viable Cell Number (Relative to DMSO) in Human Cells. HeLa cells (2x10⁴ cells/mL) were treated with increasing concentrations of P4F for 48 hours. MTT assays were performed and the resulting absorbance was measured and normalised relative to the blank. The normalised absorbance was expressed as a percentage of the solvent control. Data represents mean ±SEM of three experiments (n=3).
2.3 Conclusion

At the outset, the aim of this project was to synthesise two organelle-targeted compounds, which through biochemical assays would show accumulation of polyubiquitin conjugates in cells, suggesting DUB inhibition taking place. Both target compounds MitoP4F (8) and ESP4F (6) were synthesised successfully in relatively good yields. The ER-targeting domain ES (9) was obtained from the Swanton group, and the inhibitory domain P4F (5) was synthesised as previously reported by the Whitehead group, but they were coupled together using a different coupling agent, namely HATU. Using the same reagent, P4F was also coupled to phosphonium salt (7), incorporating a 10-carbon lipophilic chain and a triphenylphosphine moiety, which are known to target mitochondria. The phosphonium salt was synthesised in good yield from 10-bromodecanoic acid and triphenylphosphine.

When deubiquitinating enzymes (DUBs) in the cell are inhibited, a resulting accumulation of ubiquitinated material occurs. As P4F is a well-established DUB inhibitor, due to covalent inhibition of cysteine residues in the DUB active site, this effect was predicted to be seen in cells after treatment with the target compounds. By resolving treated cells lysates using SDS-page electrophoresis and estimating the amount of ubiquitin conjugates by Western blotting, P4F treated cells appeared to inhibit DUBs as an accumulation of polyubiquitinated proteins was observed. Though not to the same extent as P4F, the effect was also observed with the target compounds, hence MitoP4F and ESP4F caused accumulation of ubiquitinated material in human cells, suggesting DUB inhibition to occur. The decrease in ubiquitin signal intensity compared to P4F might be due to the target compounds.
incorporating the organelle-targeting domains, which intends them to only inhibit DUBs at the specific targeted cellular locations.

As DUBs are associated with numerous pathways that have been linked to human diseases, in addition to anti-tumour abilities of many DUB inhibitors being demonstrated, evaluation of the synthesised compounds ability to kill cultured cancer cells was carried out by subjecting the compounds to MTT assays. All compounds incorporating the P4F inhibitory domain appeared more toxic to cells, though non-conjugated P4F seemed more toxic at lower concentrations. This is consistent with its ability to induce great accumulation of high molecular mass ubiquitin conjugates. The organelle targeting domains appeared to not have a significant effect on cell viability when investigated on their own.

It was concluded that accumulation of ubiquitinated material was induced by the target compounds. Compared to previously published work using other known inhibitors such as WP1130$^{60}$ and b-AP15$^{61}$ (Table 1.2), the effect of MitoP4F on cells appears to be similar at similar concentrations, though similar effect is only seen in cells treated with ESP4F at higher concentrations (20 µM). Additional experiments are however necessary to establish that this was indeed due to DUBs being inhibited, such as assays using purified DUBs. In addition, although the organelle targeting domains have been shown to target the ER and mitochondria individually, this has not been proved when conjugated to the P4F. Hence, the exact location of the target compounds in the cell needs establishing to support proof of inhibition of the associated DUBs occurring at these locations. In addition, it is unknown whether subsets of DUBs, or a select few, are inhibited. A suitable
technique which could be performed to explore the location of the compounds in the cell, and the link to their ability to accumulate ubiquitin conjugates, is immunofluorescence. The technique involves using antibodies to target fluorescent dyes to specific cellular targets, which can be visualised, and corresponds to the location of the compound in the cell.
Chapter 3

EXPERIMENTAL
3.1 Chemistry

3.1.1. General Procedures

Infra-Red Spectroscopy
Infra-Red spectra of compounds in the solid state were recorded using either a Bruker Alpha-P FT-IR or a Perkin Elmer FT-IR spectrometer, where the absorption maxima ($v_{\text{max}}$) were measured in wavenumbers (cm$^{-1}$). The peak intensities are described using abbreviations; w, weak; m, medium; s, strong.

Mass Spectrometry
All mass spectrometry data were recorded by staff in the Mass Spectrometry Laboratory at the University of Manchester, School of Chemistry, using Micromass Platform II for electrospray and Waters Q2OF for High Resolution Accurate Mass. Accurate mass values are reported within ±10 ppm, and electrospray within ±5 ppm. Molecular ions, fraction from molecular ions or other major peaks are reported in a mass to charge ratio (m/z).

Nuclear Magnetic resonance
A Bruker avance 400 MHz spectrometer was used to record $^1$H, $^{13}$C and $^{19}$F NMR spectra, in addition to COSY and HMQC spectra for assignment-supporting purposes. Chemical shifts are represented as parts per million (ppm) to the nearest 0.01 ppm. Proton chemical shifts ($\delta_H$) were referenced to TMS, and $^1$H NMR data are reported in the format chemical shift, integration, multiplicity (s, singlet; d,
doublet; t, triplet; q, quartet; m, multiplet), coupling constants ($J$), and assignment. $^{13}$C ($\delta_C$) and $^{19}$F ($\delta_F$) NMR data are reported in the format chemical shift, multiplicity, coupling constant and assignment, and referenced to the solvent peak.

**Melting Points**

A Sanyo Gallenkamp MPD350 heater was used to record melting points.

**Solvents**

Dichloromethane (DCM), triethylamine (TEA) and acetonitrile (MeCN) were distilled over calcium hydride and THF was distilled from sodium benzophenone ketyl, all under an atmosphere of nitrogen. Solvents were evaporated using an IKA rotary evaporator, equipped with an IKA water bath and deionized water was obtained from Milipore Elix.

**Reagents**

Reagents were purchased from Alpha, Fischer and Sigma-Aldrich and WERE handled according to the recommended hazard and safety procedures. Room temperature corresponds to 20-25 °C, the reactions that required temperatures above room temperature was carried out in an oil bath and the reactions that required 0 °C were carried out in an ice bath.

**Structures**

All chemical structures and names were generated using the software ChemBioDraw Ultra 13.02.
3.1.2. Experimental Procedures

2-Chloro-2-methyl-1-nitrosopropane (1)

2-methylpropene (16 mL, 178 mmol) was condensed into a three-necked round bottomed flask, cooled to -35 °C and equipped with a cold finger at -78 °C. The temperature was allowed to rise to -15 °C, and iso-amyl nitrite (24 mL, 178 mmol) was added. Cold conc. HCl (15 mL, 178 mmol) was added dropwise, and the reaction was stirred for two hours at -10 °C. Without stirring, the solution was then cooled to -20 °C and the resulting precipitate was collected by filtration and washed with cold MeOH (30 mL) and dried under vacuum. The title compound was thereby isolated as a white solid (7.60 g, 35 %). Mp 101-108 °C [lit.62 104-105 °C]; ν<sub>max</sub>/cm<sup>-1</sup> 2980m (C-H), 1463m (N=O), 1368s (N=O); δ<sub>H</sub> (400 MHz; CDCl<sub>3</sub>) 4.71 (2H, s, C(1)<sub>H</sub>), 1.78 (6H, s, C(3)<sub>H</sub> and C(4)<sub>H</sub>); δ<sub>C</sub> (100.6 MHz; CDCl<sub>3</sub>) 66.4 (C(1)<sub>H</sub>), 65.9 (C(2)Cl), 31.3 (C(3)<sub>H</sub> and C(4)<sub>H</sub>); m/z (+ve ion electrospray) 243 ([M+H]<sup>+</sup>, 81 %), 144 ([M+Na]<sup>+</sup>, 27 %).
Methyl 2-[(2-(hydroxyamino)-1,1-dimethylethyl)amino]acetate (2)

Glycine methyl ester hydrochloride (1.04 g, 8.26 mmol) was added to a solution of 2-chloro-2-methyl-1-nitrosopropane (1.00 g, 8.26 mmol) in freshly distilled acetonitrile (17 mL). Triethylamine (2.3 mL, 16.52 mmol) was then added dropwise and the reaction was left stirring under an atmosphere of nitrogen for 24 hours at room temperature. Saturated aqueous NaHCO₃ (8 mL) was added in order to quench the reaction, and the solution was extracted with DCM (3 x 10 mL). The combined organic layers were then washed with brine (15 mL), dried over MgSO₄ and concentrated under vacuum. This yielded the title compound as a white solid (1.18 g, 82%). Mp 79.5-84 °C [lit. 58 86-86.9 °C]; ν_{max}/cm⁻¹ 3292m (OH), 3165w (NH), 3059w (NH), 2976m (C-H), 2954m (C-H), 2853m (C-H), 2748m (C-H), 1743s (C=O); δ_1H (400 MHz; CDCl₃) 7.24 (1H, s, C(1)H), 3.68 (3H, s, C(5)H₃), 3.38 (2H, s, C(3)H₂), 1.25 (6H, s, C(6)H₃ and C(7)H₃); δ_13C (100.6 MHz; CDCl₃) 172.9 (C(1)H), 155.4 (C(4)), 54.1 (C(2)), 52.1(C(5)H₃), 44.9 (C(3)H₂), 25.3 (C(6)H₃ and C(7)H₃); m/z-(+ve ion electrospray) 175 ([M+H]⁺ 60 %).
Methyl 2-(3-(4-chlorophenyl)-4-(3-(4-chlorophenyl)-1-hydroxyureido)-5,5-dimethyl-2-oxoimidazolidin-1-yl)acetate (3)

4-chlorophenyl isocyanate (0.85 g, 5.57 mmol) was dissolved in freshly distilled THF (2 mL) and added to a solution of methyl 2-[(2-(hydroxyamino)-1,1-dimethylethyl)amino]acetate 1 (0.50 g, 2.87 mmol) in freshly distilled THF (12 mL). The reaction was left stirring under an atmosphere of nitrogen at room temperature for 17 hours. The reaction was then concentrated under reduced pressure and the resulting solid was recrystallized from DCM. The crystals were collected by filtration and washed with diethyl ether. The title compound was thereby obtained as a white solid (0.91 g, 66 %). Mp 216-225 °C [lit.58 217.5-218.8 °C]; ν_max/cm⁻¹ 3331m (O-H), 3284m (N-H), 2958w (C-H), 1734s (C=O), 1695s (C=O), 1671s (C=O), 1589s (C=C), 1526s (C=C); δ_H (400 MHz; CDCl₃) 8.17 (1H, s, N_H or O_H), 7.92 (1H, s, N_H or O_H), 7.67 (2H, d, J 8 Hz, C(8)H and C(12)H or C(14)H and C(18)H), 7.55 (2H, d, J 8 Hz, C(8)H and C(12)H or C(14)H and C(18)H), 7.30-7.26 (4H, m, C(9)H and C(11)H and C(15)H and C(17)H), 6.19 (1H, s, C(1)H), 4.43 (1H, d, J 16 Hz, C(19)HₐHₕ), 3.61 (1H, d, J 16 Hz, C(19)HₐHₕ), 3.59 (3H, s, C(21)H₆), 1.37 (3H, s, C(4)H₃ or C(5)H₃), 1.31 (3H, s, C(4)H₃ or C(5)H₃); δ_C (100.6 MHz; CDCl₃, some signals are coincident), 170.0 (C(20)), 156.5 (C(3)), 155.5 (C(6)), 138.0 (Ar-Cl), 128.3 (Ar-C), 128.2 (Ar-CH), 121.3 (Ar-CH), 120.1 (Ar-CH), 74.3
(C(1)H), 58.6 (C(2)), 51.7 (C(21)H₃), 30.7 (C(19)H₂), 26.4 (C(4)H₃ or C(5)H₃), 19.0 (C(4)H₃ or C(5)H₃); m/z-(ve ion electrospray) 481 ([M]\(^{35}\text{Cl}+{35}\text{Cl})^−,[M]\(^{37}\text{Cl}+{37}\text{Cl})^−, 100 \%), 483 ([M]\(^{35}\text{Cl}+{37}\text{Cl})^−, 64 \%), 485 ([M]\(^{37}\text{Cl}+{37}\text{Cl})^−, 9\%); (Found 481.1034 C_{21}H_{22}O_{5}N_{4}^{35}\text{Cl}_{2} ([M]), requires 481.1040).
3-(4-chlorophenyl)-4-(3-(4-chlorophenyl)-1-hydroxyureido)-5,5-dimethyl-2-oxoimidazolidin-1-yl)acetic acid (4)

LiOH·H₂O (0.25 g, 59.27 mmol) was added to a solution of methyl 2-(3-(4-chlorophenyl)-4-(3-(4-chlorophenyl)-1-hydroxyureido)-5,5-dimethyl-2-oxoimidazolidin-1-yl)acetate 3 in THF (10 mL). The reaction was left stirring under an atmosphere of nitrogen at room temperature for 24 hours. The reaction was then concentrated under reduced pressure and the residue was partitioned between water (10 mL) and DCM (10 mL). The aqueous layer was separated and HCl (10 mL) was added to it, and the resulting precipitate was collected by filtration and dried, yielding the title compound as a white solid (0.33 g, 75%). Mp 201.1- 203.7 °C [lit. 207.8-208.5 °C]; ν<sub>max</sub>/cm<sup>-1</sup> 3314w (O-H, N-H), 17.53m (C=O), 1696m (C=O), 1619m (C=O), 1596m (C=C), 1536m (C=C); δ<sub>H</sub> (400 MHz; DMSO-d<sub>6</sub>) 7.66 (2H, d, J 8 Hz, Ar-CH), 7.43 (2H, d, J 8 Hz, Ar-CH), 7.33-7.27 (4H, m, Ar-CH), 6.12 (1H, s, C(1)H), 4.05 (1H, d, J 20 Hz C(19)H<sub>a</sub>H<sub>b</sub>), 4.00 (1H, d, J 20 Hz C(19)H<sub>a</sub>H<sub>b</sub>), 1.44 (3H, s, C(4)H<sub>3</sub> or C(5)H<sub>3</sub>), 1.41 (3H, s, C(4)H<sub>3</sub> or C(5)H<sub>3</sub>); δ<sub>C</sub> (100.6 MHz; DMSO-d<sub>6</sub>, some signals are coincident) 173.2 (C(20)), 159.3 (C(3)) or (C(6)), 158.5 (C(3) or (C(6)), 138.7 (Ar-C), 129.7 (Ar-C), 129.6 (Ar-CH), 123.2 (Ar-CH), 77.4 (C(1)H), 60.7 (C(2)), 41.7 (C(19)H<sub>2</sub>), 26.7 (C(4)H<sub>3</sub> or (C(5)H<sub>3</sub>), 19.8 (C(4)H<sub>3</sub> or (C(5)H<sub>3</sub>); m/z (+ve ion electrospray) 489 ([M<sup>35</sup>Cl<sup>35</sup>Cl]+Na<sup>+</sup> 100 %), 491
A mixture of NaOH (4.00 g, 100 mmol) in ethanol (40 mL) was stirred until all of the NaOH had dissolved, at room temperature under a nitrogen atmosphere. 4-fluorobenzaldehyde (1.38 mL, 13 mmol) and 4-piperidone hydrochloride (1.00 g, 6.50 mmol) was slowly added, and the reaction was further stirred for 17 hours. Water (20 mL) was added and the precipitate was collected by filtration and recrystallised from ethanol to yield the title compound as a yellow solid (0.56 g, 28%). Mp 217.0-219.0 °C [lit. 218-220 °C]; $\nu_{\max}$/cm$^{-1}$ 3305 (N-H), 2938 (C-H), 1657 (C=O), 1598 (C=C); $\delta_H$ (400 MHz; DMSO-$d_6$) 7.77 (2H, s, C(6)H and C(6’)H), 7.54-7.50 (4H, m, C(8)H, C(8’)H, C(12)H, and C(12’)H), 7.22 (4H, t, J 8.7 Hz, C(9)H, C(9’)H, C(11)H and C(11’)H), 4.12 (4H, s, C(1)H$_2$ and C(2)H$_2$); $\delta_C$ (100.6 MHz; DMSO-$d_6$) 186.1 (C(4)), 162.5 (d, J 161 Hz, C(10) and C(10’)), 146.2 (C(6)H and C(6’)H), 142.3 (C(3) and C(5)), 132.6 (d, J 9.1 Hz, C(8)H, C(12)H, C(8’)H and C(12’)H), 130.9 (C(7) and C(7’)), 115.6 (d, J 21.1 Hz, C(9)H, C(11)H and C(9’)H and C(11’)H), 47.4 (C(1)H$_2$ and C(2)H$_2$); $\delta_F$ (376.5 MHz; DMSO-$d_6$) -111.41 (s, C(10)F and C(10’)F); m/z (+ve ion electrospray) 312 ([M+H]$^+$ 100 %); (Found 312.1198, C$_{19}$H$_{16}$O$_3$N$_4$F$_2$ ([M+H]$^+$), requires 312.1194).
1-(1-(2-(3,5-bis((E)-4-fluorobenzylidene)-4-oxopiperidin-1-yl)-2-oxoethyl)-3-(4-chlorophenyl)-5,5-dimethyl-2-oximidazolidin-4-yl)-3-(4-chlorophenyl)-1-hydroxyurea (6)

3-(4-Chlorophenyl)-4-(3-(4-chlorophenyl)-1-hydroxyureido)-5,5-dimethyl-2-oximidazolidin-1-yl) acetic acid 4 (0.15 g, 0.32 mmol) was dissolved in DMF (7 mL) and (3E,5E)-3,5-bis(4-fluorobenzylidene)piperidin-4-one 5 (0.12 g, 0.38 mmol) was added. DIEA (0.11 mL, 0.64 mmol) and HATU (0.14 g, 0.38 mmol) were added and the reaction was left stirring for 24 hours at room temperature under an atmosphere of nitrogen. The solution was diluted with ethyl acetate (10 mL) and saturated NaHCO$_3$ (aq) (15 mL) was added. The resulting precipitate was dispersed in water and then collected by filtration and dried, yielding the title compound as a yellow solid (0.15 g, 62%). 

Mp 218.7-223.3 °C [lit.$^6$ 226.2-230.6 °C]; $\nu_{\text{max}}$/cm$^{-1}$ 3306w (N-H or O-H), 3184w (N-H or O-H), 2973w (C-H), 2844w (C-H) 1700s (C=O), 1669s (C=O), 1628s (C=O), 1597s (C=O); $\delta_H$ (400 MHz; DMSO) 9.24 (1H, s N-H or O-H), 7.57-7.72 (10H, m, 8 x Ar-CH and C(26)H and C(26')H), 7.29-7.36 (8H, m, Ar-CH), 6.01 (1H, s, C(1)H), 4.90-4.98 (4H, m, C(21)H$_2$ and C(25)H$_2$), 4.11 (2H, d, $J$ 20 Hz C(19)H$_2$H$_6$), 4.02 (2H, d, $J$ 20 Hz C(19)H$_2$H$_6$), 1.22 (3H, s, C(4)H$_3$)
or C(5)H₃), 1.15 (3H, s, C(4)H₃ or C(5)H₃); δC (100.6 MHz; DMSO, some signals are coincident) 186.0 (C(23)), 167.6 (C(20)), 163.8 (d, J 112 Hz, C(30 and 30’)), 162.3 (C(3)), 155.8 (C(6)), 138.0 (Ar-Cl), 133.4 (Ar-CH or C(26)H and C(26’)H), 132.5 (d, J 93 Hz, C(32)H, C(32’)H, C(28)H and C(28’)H), 130.8 (C(22) and C(24)), 128.2 (Ar-CH or C(26)H and C(26’)H), 126.3 (Ar-Cl), 121.2 (Ar-CH), 120.0 (Ar-CH), 115.8 (d, J 21 Hz, C(29)H, C(29’)H, C(31)H and C(31’)H ), 74.1 (C(1)H, 58.6 (C(2)), 46.0 (C(21)H₂ or C(25)H₂), 42.4 (C(19)H₂), 26.3 (C(4)H₃ or C(5)H₃), 18.9 (C(4)H₃ or C(5)H₃); δF (376.5 MHz; DMSO) -113.3 (s, C(30)F and C(30’)F); m/z- (+ve ion electrospray) 761 ([M+H(³⁵Cl+³⁵Cl)]⁺ 100 %), 763 ([M+Na(³⁵Cl+³⁷Cl)]⁺ 64%); (Found 761.1930, C₃₉H₄₀O₅N₅³⁵Cl₂F₂ ([M+H]⁺), requires 761.1978).
10-Bromodecanoic acid (2.00 g, 7.96 mmol) and triphenylphosphine (2.09 g, 7.96 mmol) were dissolved in toluene (20 mL) and the reaction mixture was heated under reflux for two days under an atmosphere of nitrogen. The reaction was then cooled and concentrated under reduced pressure and triturated with toluene (5 x 20 mL) to yield the title compound as a brown oil (4.75 g, 82%). $\nu_{\text{max}}/\text{cm}^{-1}$ 3391w (O-H), 3054w (Ar-H), 2952w (C-H), 1721s (C=O), 1437s (C=C); $\delta_H$ (400 MHz; CDCl$_3$) 7.78-7.71 (9H, m, Ar-C$_H$), 7.70-7.61 (6H, m, Ar-C$_H$), 3.5-3.65 (2H, m, C(10)H$_2$), 2.28 (2H, t, $J$ 6 Hz, C(2)H$_2$), 1.60-1.44 (6H, m, C(3)H$_2$, C(8)H$_2$ C(9)H$_2$), 1.24-1.09 (8H, m, C(4)H$_2$, C(5)H$_2$, C(6)H$_2$ and C(7)H$_2$); $\delta_C$ (100.6 MHz; CDCl$_3$, some signals are coincident) 177.4 (C(1)), 135.2 (d, $J$ 3 Hz, C(14)H), C(20)H, C(26)H), 133.7 (d, $J$ 20 Hz, C(12)H, C(16)H, C(18)H, C(22)H), C(24)H, C(28)H) 130.7 (d, $J$ 10 Hz, C(13)H, C(15)H, C(19)H, C(21)H), C(25)H, C(27)H), 118.6 (d, $J$ 87 Hz, C(11), C(17) and C(23)), 34.5 (C(2)H$_2$), 30.3 (C(9)H$_2$), 30.1 (C(8)H$_2$), 28.7 (C(7)H$_2$ - C(4)H$_2$), 24.6 (C(3)H$_2$), 22.5 (C(10)H$_2$); m/z (+ve ion electrospray) 433 ([M]$^+$ 100 %); (Found 433.2289, C$_{28}$H$_{34}$O$_2$P ([M+H]$^+$), requires 433.2291).
9-(Carboxynonyl)triphenylphosphonium bromide 7 (0.35 g, 0.68 mmol) was dissolved in DMF (8 mL) and (3E,5E)-3,5-bis(4-fluorobenzylidene)piperidin-4-one 5 (0.25 g, 0.80 mmol) was added. DIEA (0.23 mL, 1.34 mmol) and HATU (0.31 g, 0.80 mmol) was added and the reaction was stirred for 21 hours under an atmosphere of nitrogen at room temperature. The solution was diluted with ethyl acetate (10 mL) and washed with water and saturated Na$_2$CO$_3$ (aq) (15 mL), then extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with water (10 mL) and brine (10 mL) and dried over MgSO$_4$, to give the title compound as a yellow solid (0.4 g, 76 %). Mp 75.5-81.6 °C; $\nu_{\text{max}}$/cm$^{-1}$ 2926w (C-H), 2856w (C-H), 1639m (C=O), 1599m (C=O); $\delta_{\text{H}}$ (400 MHz; CDCl$_3$) 7.55-7.74 (17H, m, Ar-C and C(6)H and C(6'))H, 7.32-7.39 (4H, m, C(8)H and C(8')H and C(12)H and C(12')H), 6.99-7.10 (4H, m, C(9)H and C(9')H and C(11)H and C(11')H), 4.79 (2H, s, C(2)H$_2$ or C(1)H$_2$), 4.63 (2H, s, C(2)H$_2$ or C(1)H$_2$), 3.02-3.09 (2H, m, C(22)H), 2.05 (2H, t, J 7.7 Hz, C(14)H$_2$), 1.49-1.54 (4H, m, C(21)H$_2$ and C(20)H$_2$), 1.38-1.45 (2H, m,
C(18)H₂ or C(19)H₂, 1.27-1.34 (2H, m, C(15)H₂), 1.10-1.20 (2H, m, C(18)H₂ or C(19)H₂), 0.97-1.03 (4H, m, C(16)H₂ and C(17)H₂); δC (100.6 MHz; CDCl₃, some signals are coincident) 187.7 (C(4)), 172.2 (C(13)), 163.8 (d, J 126 Hz, C(10) and C(10')), 136.9 (C(6)H and C(6')H), 136.1 (C(5) and C(3)), 135.3 (d, J 3 Hz, (C(26)H, C(32)H and C(38)H)), 133.4 (d, J 10 Hz, (C(25)H, C(27)H, C(31)H, C(33)H, C(37)H, C(39)H)), 132.6 (d, 28 Hz, C(8)H, C(8')H, C(12)H and C(12')H), 131.7 (C(7) and C(7')), 130 (d, J 13 Hz, (C(24)H, C(28)H, C(30)H, C(34)H, C(36)H, and C(40)H)), 118.3 (d, J 85 Hz, (C(23), C(29), C(35)), 116.2 (d, J 21, C(9)H, C(9')H, C(11)H and C(11')H), 46.2 (C(1)H₂ or C(2)H₂), 43.3 (C(1)H₂ or C(2)H₂), 32.9 (C(14)H₂), 30.2 (C(18)H₂), 28.8 ((C(16)H₂, C(17)H₂ and C(19)H₂), 24.9 (C(15)H₂), 22.4 (C(20)H₂ and C(21)H₂), 21.8 (C(22)H₂); δF (376.5 MHz; CDCl₃) -109 (s, C(10)F or C(10')F), -110 (s, C(10)F or C(10')F); m/z-(+ve ion electrospray) 726 ([M]+ 100 %).
3.2 Biochemistry

3.2.1. General Procedures

Cell Cultures

HeLa cells were cultured in DMEM (Dubecco’s modified Eagle’s Medium), which was supplemented with 10 % FBS (Fetal Bovine Serum), non-essential amino acids and 2 nM L-glutamine. They were kept in sterile cell culture dishes in a 37 °C incubator at 5 % CO₂ atmosphere.

Splitting of Cells

The DMEM in the cell culture dishes was aspirated and replaced with warm phosphate buffered saline (PBS), and aspirated again. Warm Trypsin (2 mL) was added and the dish was returned to 37 °C incubation for 5 minutes. In order to ensure all cells were detached from the surface, the dish was tapped gently on the side. Warm DMEM (8 mL) was then added to the dish and the trypsinated cells were resuspended by pipetting up and down on the side of the dish, three times. For maintenance purposes, cells were split in a 1:10 dilution every 2-3 days. For western blot purposes, cells were split in a 1:5 dilution and grown in 12-well dishes until approximately 70 % confluent. For MTT assay purposes, 20 000 cells were seeded in each well of a 24-well dish.
**Counting of Cells**

In order to seed 20,000 cells for MTT experiments, a 1:1 solution of cells and Tryphan blue was made up and added to a haemocytometer, where the average number of cells in the sample was calculated. A stock solution was then made up and was pipetted out into a 24-well dish so that each well contained 20,000 cells.

**Treatment of Cells with Compounds**

Target compounds were synthesised as described above, ES was previously synthesised in the Swanton and Whitehead groups. All compounds were solubilised in DMSO with a 20 mM concentration, and then serial diluted to obtain stock solutions of an appropriate range of concentrations. In addition, a 10 mM solution of b-AP15 was obtained from the Swanton group and used as a positive control. The stock solutions were stored at -80 °C.

**Western blotting**: Cells grown in 12-well dishes with 1 mL of DMEM were treated by adding 1 µL of the stock solutions, rocking the plate to ensure an even distribution, and incubated at 37 °C for seven hours. For comparison, wells were also treated with DMSO (solvent control). The DMEM was then aspirated and washed twice with 1 x PBS (1 mL).

**MTT assays**: Cells grown in 24-well dishes with 0.5 mL of DMEM were treated by adding 0.5 µL of the stock solutions, rocking the plate to ensure an even distribution, and incubated at 37 °C for 48 hours. For comparison, wells were also treated with DMSO (solvent control), and one well was treated with DMSO but not used for MTT assay to obtain a (blank) control.
SDS-Page

The cells were solubilised in 250 µL SDS sample buffer (150 µL; 62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2% SDS, 0.0025 % Bromophenol blue). In order to prevent the tendency of 3D protein conformation through forming stabilizing inter and/or intramolecular sulfide bonds, and hence potentially alter the migration distance through the gel (and indirectly Western blot results), dithiothreitol (DTT) (500 µL) was added. In addition, the cell samples were heated to 96 ºC to completely denature the proteins, sonicated for five minutes and centrifuged for three minutes in order to avoid any aggregated material.

SDS-page gels were then ran using the Laemmli method$^{64}$ with 20 µL of each sample. Electrophoresis was carried out using 5 % stacking gels and 7.5 % separating gels (tris-glycine acrylamide gels) at 30 mA in running buffer.

Western Blotting

The separated proteins on the acrylamide gels were transferred onto a nitrocellulose membrane (Licor biosciences) in transfer buffer at 300 mA for one hour. The membranes were then blotted with 2 % milk (2 g in 100 mL TBS) for 20 minutes, in order to block unspecific active sites. The membranes were incubated with primary antibodies overnight at 4 ºC, then washed with TBS (3 x 5 mL) for 5 minutes each, then blotted with the corresponding secondary fluorescent antibody for one hour at room temperature. Lastly, the TBS (3 x 5 mL) wash was repeated and the proteins were visualized, using an Odyssey-SA (Licor biosciences) scanner.
MTT Assays

The DMEM was aspirated from each well and replaced with 0.5 mL of a 1.5 M solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a sterile tetrazolium dye, in DMEM solution, and left at 37 °C incubation for two hours. The MTT solution was then aspirated and replaced with 0.5 mL of DMSO, then left rocking for 30 minutes in order to dissolve any resulting formazan crystals. 150 µL triplicates of the solution in each well were then pipetted into a 96-well dish, and the absorbance was recorded using a BioTek Synergy H1 Hybrid Multi-Mode Microplate reader at 570 nm.

Analysis of Data

Western blotting: Using Licor Odyssey software, fluorescence-signals from the desired proteins were quantified using pixel counting, then normalised relative to the loading control. The data was then expressed as a percentage of the normalised solvent control signal (DMSO). In cases where three sets of experiments were carried out, mean intensities and standard errors of the mean (SEM) was calculated from the quantified data using GraphPad Prism 6.04, and ANOVA analysis was also carried out.

MTT assays: The software Gen5 1.11 was used to obtain absorbance signals from each well, and were normalised relative to the control (blank) and then expressed as a percentage of the well treated with the solvent control (DMSO). Three sets of assays were carried out, hence three sets of data were obtained, in order to calculate mean intensities and standard errors of the mean (SEM), using GraphPad Prism 6.04.
3.2.2 Accumulation of Polyubiquitinated Material

Cells were prepared as described above and treated with P4F (5), ESP4F (6), ES (9), MitoP4F (8) and phosphonium salt (7) using concentrations of 2.5, 5, 7.5, 10 and 20 µM. Cells were also treated with an equal volume of DMSO. SDS-page electrophoresis was carried out, followed by Western blotting. In order to detect polyubiquitinated material, the membranes were blotted with a mouse anti-ubiquitin antibody (P4D1), followed by a green donkey anti-mouse secondary antibody, both in 2 % milk solution. For a quantitative comparison, blotting with rabbit anti-tubulin (TAT1), along with a red goat anti-rabbit secondary antibody, was used as a loading control.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ubiquitin (P4D1)</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>α-tubulin (TAT1)</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

Table 3: Antibodies used in Western blotting

3.2.3. Cell Viability

In order to assess cell viability, 20 000 cells were prepared and treated for 48 hours, using P4F at concentrations 0.01, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2, 0.25 and 0.5 µM. ESP4F was used at concentrations of 0.1, 0.25, 0.3, 0.6, 0.75, 1, 1.5, 2.5 and 5 µM, and cells were also treated with ES at 5 µM. The same treatment was performed using MitoP4F and phosphonium salt at concentrations of 0.1, 0.125, 0.15, 0.2, 0.25, 0.5, 1 and 2.5 µM. Cells were also treated with DMSO as a solvent control,
and a well from each set was not treated with MTT, but still measured, as a blank control. Cell viability was assessed by measuring the corresponding absorbance at each concentration at 570 nm, where decreased absorbance corresponded to increased cell viability.
Chapter 4

REFERENCES
4. References


REFERENCES


