Targeting Human Ubiquitin Activating Enzyme UBE1 with Rationally Designed Copper-Based Inhibitors and the Application of a Fluorescent Chemosensor to the Development of an Assay for Adenylationg Enzyme Activity

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Chemistry
University of Toronto

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2015

Abstract

Two projects were explored in this work. In the first, a previously developed, rationally-designed, potent, and selective cupric cyclen-based small molecule library of human ubiquitin activating enzyme 1 (UBE1) inhibitors was explored for its therapeutic potential in hematological malignancies. After significant difficulties reproducing inhibitor validation data, our further investigational findings suggest that the library’s activity observed in western blot-based UBE1-UbcH6 E1-E2 transthiolation assays and a LifeSensors Fluorescence Resonance Energy Transfer (FRET)-based assay was simply the spurious result of excess unbound copper in the compound samples. This work also investigated the application of a dual-emission fluorescent chemosensor, ProxyPhos, to development of a robust high throughput assay for adenylating enzyme activity. With selective detection of pyrophosphate over ATP, ADP, and AMP in aqueous buffer, ProxyPhos was applied in buffer conditions suitable to adenylating enzymes to explore their effects on selectivity and signal.
Acknowledgments

First and foremost, I would like to thank Professor Patrick T. Gunning for providing me the opportunity to complete a M.Sc. in his laboratory. Under his supervision over this past year I have gained invaluable research experience, achieved personal development, and obtained an insight to the field of medicinal chemistry and molecular recognition that I could not have received anywhere else.

I dedicate this thesis to my parents Tahir Iqbal and Fariha Qureshi for everything that they have and continue to give me, and for modelling the incredible importance of both integrity and hard work. I could not have asked for better examples of what a strong will can accomplish.

Next, I thank Jia Nan (Cathy) Xu for her contributions to the work presented in the second half of my thesis. Her scientific curiosity, critical thinking skills, and humorous level of skepticism made her an indispensable asset both in the development of the project and my graduate experience.

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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4HB</td>
<td>4 helix bundle</td>
</tr>
<tr>
<td>AAD</td>
<td>Active adenylation domain</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AE</td>
<td>Adenylating enzyme</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>boc&lt;sub&gt;3&lt;/sub&gt;cyclen</td>
<td>(N,N',N'')-Tri-Boc-cyclen</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CD&lt;sub&gt;3&lt;/sub&gt;OD</td>
<td>Deuterated methanol</td>
</tr>
<tr>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Cyclen</td>
<td>1,4,7,10-tetraazacyclododecane</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DIPEA</td>
<td>(N,N)-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DPA</td>
<td>Dipicolylamine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E1</td>
<td>Activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>Conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ligase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ESI-LC/MS</td>
<td>Electrospray ionization-liquid chromatography-mass spectrometry</td>
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<tr>
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<td>Ethyl acetate</td>
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<tr>
<td>FCCH</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>IAD</td>
<td>Inactive adenylation domain</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MALDI-TOFMS</td>
<td>Matrix-assisted laser desorption/ionization mass spectrometry</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>NAE</td>
<td>NEDD8 E1 activating enzyme</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>Anhydrous sodium sulfate</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium on carbon</td>
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<td>Pyrophosphate</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>SAE</td>
<td>SUMO1 E1 activating enzyme</td>
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<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>TBTU</td>
<td>O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
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<tr>
<td>Triflate</td>
<td>Trifluoromethanesulfonate</td>
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<tr>
<td>U</td>
<td>Enzyme unit</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
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<td>ubiquitin activating enzyme 5</td>
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<td>Ubc12</td>
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<td>Ubiquitin conjugating enzyme</td>
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<tr>
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<td>Ubiquitin-like proteins</td>
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<td>Ubiquitin fold domain</td>
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<tr>
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Chapter 1
Preface

1 Preface

As two projects were explored in this work, this thesis is divided into two parts. In chapter 2, a previously developed rationally-designed cupric 1,4,7,10-tetraazacyclododecane (cyclen)-based small molecule library of human ubiquitin activating enzyme (UBE1) inhibitors was explored for its therapeutic potential in hematological malignancies. This chapter discusses the significant difficulties observed in reproducing library validation data, and the further investigational findings that suggest that the library data derived from western blot-based E1-E2 transthiolation assays and a LifeSensors E1 LITE - UBE1 Activity Assay Kit was the spurious result of excess unbound copper in the compound samples. In chapter 3, the use of a dual-emission fluorescent chemosensor developed by the Gunning Group for development of a robust high throughput assay for adenylating enzyme (AE) activity was explored. The sensor, coined ProxyPhos, demonstrates selective detection of pyrophosphate (PPI) over adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in aqueous buffer. Herein we describe the experimental design of the assay and the experiments used to evaluate ProxyPhos’ suitability for application within AE buffer systems, the challenges that arose, and the problem solving strategies that we employed.

2 Rationally Designed Copper-Based Inhibitors of Ubiquitin Activating Enzyme 1

2.1 Background

2.1.1 Targeting Enzymes for Development of Therapies

2.1.1.1 Enzymes as Targets for Development of Therapies

Without enzymes as biological catalysts, the great array of metabolic processes that occur in organisms would not proceed at a sufficient rate to sustain life. Mechanisms of maintaining homeostasis include the tight control of enzyme expression and activity. When these control measures falter in diseased systems, resultant hyperactivation or hypoactivation of enzyme activity or expression can disturb the delicate balance of biological systems.\(^1\text{-}^3\) Often, small-
molecule inhibitors, rather than agonists of cellular proteins, are employed.\textsuperscript{4-6} These inhibitors act by binding sites on hyperactive and over-expressed enzymes and proteins in such a way to curtail or wholly prevent their functioning in biological pathways. Small-molecule approaches to treatment retain popularity due to their ability to penetrate particular cellular compartments to reach target structures with tunable affinity and selectivity, whilst maintaining the potential to be administered orally and less invasively.\textsuperscript{7-9}

2.1.1.2 Adenylating Enzymes as Druggable Targets

Adenylating Enzymes (AEs) catalyze the two-step process of AMP (5'-adenylic acid) and PPI formation from ATP (Figure 2.1).\textsuperscript{10} First, following ATP and substrate binding to their respective pockets on the AE, PPI is released and a reactive substrate-adenylate is formed via a phosphoester bond with the substrate’s carboxyl group (Figure 2.1).\textsuperscript{10} In the following step, AMP is liberated when a nucleophile attacks the substrate-adenylate’s phosphoester bond; for some AEs, PPI is released in this step.\textsuperscript{10} The formation of the phosphoester bond in the pathway facilitates subsequent nucleophilic attack and substrate-nucleophile conjugate formation by providing a good leaving group.\textsuperscript{10}

![Figure 2.1. Mechanism for adenylating enzyme two step catalysis of ATP conversion to AMP and PPI. Mechanism adapted from Figure 1c of Schmelz and Naismith 2009.\textsuperscript{10}](image-url)
AEs are implicated in many important biological processes, including sub-cellular protein localization and degradation.\textsuperscript{11} As a consequence, abnormal expression and aberrant activity in these enzymes is implicated in diseased states and they are targeted for drug development to investigate if they possess therapeutic potential.\textsuperscript{6,12}

2.1.2 Targeting an Adenylating Enzyme in the Ubiquitin Proteasome System

2.1.2.1 E1 Activating Enzymes: Adenylating Enzymes in the Ubiquitin Proteasome System

AEs are deeply involved in protein labelling processes. In the ubiquitin (Ub) proteasome system, AEs catalyze the first step in the process of ubiquitination. Ubiquitination is the process by which Ub or ubiquitin-like proteins (Ubls) are covalently attached to target proteins. In ubiquitination, Ub protein molecules are linked to target proteins generally via isopeptide bonds between the Ub terminal carboxylates and target protein’s nucleophilic lysine side chain amines (Figure 1.3).\textsuperscript{13} The polyubiquitination of proteins refers to the attachment of multiple Ub molecules in tandem to generate a chain.\textsuperscript{11} The successive Ub molecules are also joined via isopeptide bonds between the Ub terminal carboxylate and nucleophilic Ub lysine side chain amines.\textsuperscript{11} Multiubiquitination on the other hand refers to the attachment of Ub molecules to different lysine residues on a particular target protein.\textsuperscript{11} Polyubiquitination of a target protein with a chain of ubiquitin molecules is usually associated with subsequent protein degradation through the ubiquitin proteasome system, as it serves as a recognition element for the 26S proteasome.\textsuperscript{11} Variation in Ub chain length and in the adjoining linkages between Ubs leads to diversified fates for tagged proteins in cellular processes.\textsuperscript{11,13} When Ubls are conjugated to target proteins, they exert their own control of biological events.\textsuperscript{11} For example, tagging of proteins with some Ubls is associated with nuclear localization, transcriptional regulation, and apoptosis.\textsuperscript{11}
Ub and Ubl proteins are attached to target proteins via a similar cascade of events catalyzed by up to four classes of enzymes that are unique to Ub and each Ubl (Figure 2.2). This process
begins with ATP binding the nucleotide–binding pocket of the E1 activating enzyme (an AE). After, the Ub or Ubl protein binds the Ub/Ubl protein binding pocket of its specific E1, the E1 catalyzes the adenylation of the Ub/Ubl substrate protein, releasing PPi, and forming an AMP-Ub/Ubl adenylate intermediate that remains bound to the nucleotide and Ub/Ubl binding pockets (Figure 2.2). An E1 active site cysteine residue then moves close to the AMP-Ub/Ubl intermediate due to a large induced conformational change in the enzyme. The close proximity of the cysteine to the intermediate facilitates its nucleophilic attack on the intermediate’s phosphoester linkage, to form a high energy thioester E1-S-Ub/Ubl intermediate. A second ATP molecule and Ub/Ubl protein then bind to the E1 which catalyzes the formation of a ternary (Ub/Ubl-AMP)-E1-S-Ub/Ubl intermediate complex. Subsequently, a cross-protein transthiolation reaction between the E1-S-Ub/Ubl thioester and its respective E2 conjugating enzyme active site cysteine occurs. Via the concerted actions of E2 and E3 ligase, the Ub/Ubl is transferred to the target protein to signal variable cellular processes. RING (really interesting new gene) domain-containing E3 ligases, similarly to E2s, become conjugated to their Ub/Ubl E2s via a transthiolation reaction with their catalytic cysteines. After, the Ub is transferred to the target protein in a discreet step. Alternatively, HECT (homologous to the E6-AP carboxyl terminus) domain-containing E3 ligases function as adaptor proteins that bring E2s and target proteins in sufficient proximity to facilitate Ub/Ubl transfer. In some cases, polyubiquitination requires additional activity of E4 ubiquitin-chain elongation factors that work in concert with E1s, E2s and E3s.

In harnessing ATP to facilitate formation of Ub/Ubl-adenylate complexes, E1 activating enzymes’ catalytic cysteines become poised for nucleophilic attack upon the phosphoester bond to drive the pathway forward. Therefore, when hyperactivation or overexpression of these enzymes occur, diseased states can follow.

### 2.1.2.2 Ubiquitin Activating Enzyme 1 (UBE1): an E1 Activating Enzyme Implicated in Disease

One example of an E1 activating enzyme implicated in disease is human UBE1, an AE which catalyzes the first step in ubiquitination. In a 2010 study performed by Xu et al., immunoblotting experiments with leukemia cell lines and primary patient samples revealed higher protein ubiquitination compared to normal hematopoietic cells. When they performed a genetic knockdown of UBE1 in the ubiquitin proteasome system of leukemia and myeloma cells,
decreased protein ubiquitination levels and cell death followed. Interestingly, a small molecule inhibitor of UBE1 1-(3-chloro-4-fluorophenyl)-4-[(5-nitro-2-furyl)methylene]-3,5-pyrazolidinedione (PYZD-4409) (Figure 2.3) was able to induce cell death in malignant cells and prevent clonogenic growth of primary acute myeloid leukemia cells whilst not harming normal hematopoietic cells. PYZD-4409 was also able to reduce tumor weight and volume compared to the inactive control compound PYZDmut (Figure 2.3) in a mouse model of leukemia. They also determined that overexpression of the BI-1 protein, which mitigates cellular endoplasmic reticulum (ER) stress, can prevent cell death when treated with PYZD-4409. Through this work, Xu et al. were able to validate UBE1 as a target for therapeutic potential via chemical inhibition in hematological malignancies.

![Figure 2.3. Chemical structures of human UBE1 inhibitor PYZD-4409 and the inactive PYZDmut molecule reported in Xu et al. 2010.](image)

### 2.1.2.3 Targeting UBE1 in Hematological Malignancies: Why Target Upstream?

UBE1 may be a valid target for inhibition within the ubiquitination pathway, but it may not be the only valid target or the best target. Since Ub/Ubl proteins require many discrete steps to attach to their target proteins, aberrant activity in any of the multiple elements of their pathways could potentially lead to pathologies. Therefore, there are multiple potentially valid targets for inhibition when increased protein ubiquitination exists in hematological malignancies like acute myeloid leukemia. Inhibition of downstream targets is common practice in medicinal chemistry because targeting upstream increases the probability of off-target effects in additional related downstream pathways. Avoiding off-target pathway knockdown is especially important in this system considering that individual E1 enzymes interact with multiple E2 enzymes, and that individual E2 enzymes interact with an even greater number of E3s which have their own unique substrates. However, as inhibition of UBE1 with PYZD-4409 did not lead to cell death of
normal hematopoietic cells, concerns for off-target downstream pathway knockdown are reduced with this strategy. Also, compared to the 8 known E1 enzymes, there are currently 32 E2 enzymes and >600 E3 ligases reported, with considerable structural homology within each class. Therefore, achieving selectivity within an enzyme class is easier for E1s than for E2s and E3s. Moreover, E1s can be targeted at nucleotide binding pockets, Ub/Ubl binding pockets, and at conserved catalytic cysteines, whereas E2 enzymes are characterized by Ub or Ubl conjugating catalytic folds. Thus, with additional structural features, E1s become even more facile targets. Therefore, it appears that targeting human UBE1, the E1 activating enzyme of the ubiquitination pathway, would lead to the greatest potential for successful selective inhibition of a species in this pathway.

2.1.2.4 Current Inhibitors of UBE1

A number of strategies have been employed to inhibit E1 activating enzymes. In a published review of inhibitors targeting E1 activating enzymes by Da Silva et al. (2013) 4 predominant strategies are outlined (Figure 2.4). Since many ultimately unsuccessful UBE1 inhibitors have targeted the catalytic cysteine and the ATP-binding pocket, our lab postulated that the UBE1 Ub-binding pocket, which contains the active adenylation domain be explored as a novel site for inhibition. Targeting the Ub-binding pocket is advantageous for attaining selectivity for UBE1 over other E1s and other enzymes containing catalytic cysteines and ATP-binding pockets. First, inhibition of catalytic cysteines often involves the use of indiscriminate alkylating agents. These inhibitors, despite being effective within simplified in vitro assay systems, will likely find trouble with in cellulo or in vivo target specificity. Also, because ATP-consuming enzymes are both plentiful and ubiquitous in biological systems, achieving selectivity for a single enzyme via targeting the ATP-binding site is a challenging endeavor. For example, there are currently at least 478 reported kinases in the human proteome that utilize ATP as a substrate. In pursuing the Ub-binding pocket, the strategy can both bypass the necessity of applying promiscuous electrophilic molecules required for binding catalytic cysteines and avoid the mountainous challenge of targeting pervasive ATP-binding sites. Finally, as all E1s interact with different Ub/Ubl proteins, selectivity for a particular E1 Ub/Ubl-binding pocket becomes more facile to achieve.
2.1.3 Rationally Designed Cu-based Inhibitors of UBE1

2.1.3.1 Preface

In the following sections, the rationale and steps for generation and validation of a library of human UBE1 inhibitors by Dr. Julie Lukkarila, Sara R. da Silva, Stacey-Lynn Paiva, and Dr. Matt Bancerz of the Gunning Group is described.

2.1.3.2 Rationale for Cu-based Inhibitors of UBE1

To develop a strategy for inhibiting UBE1, crystal structures were consulted. UBA1 (the yeast ortholog of human UBE1) and UBE1 have a sequence identity of 50%, whereas yeast and human Ub share 96% sequence identity. Moreover, the structural domains between UBA1 and UBE1 show considerable conservation. Consequently, the crystal structure for yeast UBA1 was used.
to design a targeting strategy for UBE1, as human UBE1 does not have a published crystal structure.

UBA1 contains six domains: the inactive and active adenylation domains (IAD, AAD), the first and second catalytic cysteine half domains (FCCH, SCCH), a four helix bundle (4HB), and a C-terminal Ub-fold domain (UFD) (Figure 2.5). In UBA1, Ub can be bound at two sites. When Ub binds to free UBA1, Ub C-terminus forms interactions with UBA1’s AAD. Upon formation of the Ub adenylate and subsequent nucleophilic attack by UBA1’s catalytic cysteine, Ub remains covalently bound to the catalytic cysteine domain until an E2 is recruited. Both Ub binding sites are located on the same face of UBA1, but are separated by approximately 31 Å.

Figure 2.5. Structure of the UBA1–Ub₂-AMP complex. Coloured units: active adenylation domain AAD (purple), inactive adenylation domain IAD (cyan), 4 helix bundle 4HB (pale cyan), first catalytic cysteine half domain FCCH (green), second catalytic cysteine half domain SCCH (blue), C-terminal ubiquitin fold domain UFD (red) with the crossover loop connecting the SCCH and AAD domains, Ub-adenylate (Ub(a)) (yellow), Ub-thioesterified (Ub(t)) (orange) linked to catalytic Cys600 (yellow spheres) of UBE1, Cys CAP loop (black). Adapted from Schafer et al. 2014.

For Ub and Ubls to distinguish their Ub/Ubl-binding pockets on E1 activating enzymes, residue 72 in Ub and the prevalent Ubls NEDD8 and SUMO1 appears to be the main determinant for E1 selectivity. Residue 72 is Arg in Ub, an Ala in NEDD8, and a Glu in SUMO1. Therefore, considering that the specificity residues between the prevalent Ub, NEDD8, and SUMO1 proteins are positively charged, lipophilic, and negatively charged respectively, selective
inhibition of UBE1 could be achieved with a species containing positive charge to outcompete the Arg on Ub.

Figure 2.6. Inhibitor library design scheme with variable amino acids appended to zinc or copper cyclen rings via an amide. Library compound naming convention is based upon the metal within the cyclen binding unit and the amino acid appended. For example, Cu-Pro refers to the derivative possessing copper and a proline residue bound to the cyclen ring.

A divalent metal-based small molecule strategy (Figure 2.6) was proposed to competitively and reversibly bind the Ub binding pocket on UBE1, such that the positive charge would selectivity displace Ub by outcompeting the Arg72 (instead of Ala72 and Glu72 within NEDD8 and SUMO1 respectively). Specifically, divalent copper cyclen and zinc cyclen metal complexes bound to various amino acids were generated. Final compounds were named based upon the metal incorporated into the cyclen ring and the amino acid appended to cyclen. For example, the final compound containing copper with a proline residue appended to the cyclen ring is referred to as Cu-Pro. Cyclen was employed as a metal binding unit specifically because its formation constants with divalent copper and zinc are strong \((\log(K_{MC}) = 23.4, 16.2\) respectively, where \(K_{MC}\) refers to the metal (M)-cyclen (C) complex formation constant) and facilitate retention of the metal. Utilizing amino acids allowed for facile synthesis of a preliminary library with functional group diversity.

2.1.3.3 \textit{In vitro} E1-E2 Transthiolation Assays for Inhibitor Screening

2.1.3.3.1 The E1-E2 Transthiolation Assay

To screen the synthesized library for inhibition of ubiquitination, E1-E2 transthiolation assays were developed and performed in collaboration with the Aaron Schimmer group at the
University of Toronto (Figure 2.7). In the normal control for these assays, E1 (50 nM) and E2 enzymes (500 nM), ATP (1 μM), and Ub (500 nM) were incubated in solution for 60 minutes before the reactions were quenched via denaturation using sodium dodecyl sulfate (SDS)-containing 2x laemmli buffer. To quantify the relative proportion of free and Ub-conjugate E1 and E2 formed, the mixture is separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the species were quantified using western blot analysis. In this way, the change in proportion of Ub-enzyme adducts can be used as a metric for reaction progress. Since the E1 and E2 enzymes contained polyhistidine-tags, mouse monoclonal anti-6x his tag® primary antibodies produced by Abcam® were used. To visualize and quantify the antibody-bound enzymes, goat monoclonal anti-mouse secondary antibodies conjugated to horseradish peroxidase (HRP) were employed and treated with luminol and hydrogen peroxide (H₂O₂). Due to the chemiluminescent transformation of luminol and H₂O₂ catalyzed by HRP, the amount of photons released from each band containing HRP-conjugated antibody is proportional to the amount of antibody present, and by extension proportional to the amount of free or Ub-conjugate E1 and E2. Figure 2.7 represents a schematic of a typical blot. For the standard normal reaction, the assay was optimized to yield 1:1 free E2 and E2-Ub conjugate on the blot. Then, varying concentrations of inhibitors can be added to the reaction mixture to assess their effects on E1 or E2 ubiquitin labelling, relative to the normal control.
Figure 2.7. E1-E2 transthiolation assay western blot schematic generated based upon previous unpublished data by the Schimmer Group with example lanes: 1-4, varying inhibitor concentrations with His₆-UBE1, His₆-UbcH6, Ub, and ATP; 5, protein ladder; 6, normal reaction with His₆-UBE1, His₆-UbcH6, Ub, and ATP; 7, normal reaction lacking His₆-UBE1 (no E1); 8, normal reaction lacking UbcH6 (no E2); 9, normal reaction with DTT; 10, normal reaction with MLN4924 or Compound 1.

For negative controls, replicates of the normal trial omitting E1 and E2 enzymes were performed. Dithiothreitol (DTT), MLN4924 (Figure 2.8), and Compound 1 (Figure 2.9), were employed as positive controls for inhibition due to their ability to halt the transthiolation reaction. MLN4924 and Compound 1 were reported by Soucy et al. in 2009 and Chen et al. in 2011 respectively to be irreversible inhibitors of UBE1, whereas DTT disrupts thioester linkages between E1s and E2s with Ub. Compound 1 and MLN4924 operate via a substrate-assisted mechanism by binding to the ATP pocket in the presence of Ub, to become covalently linked to Ub and remain bound to the enzyme. It must be noted that a loading control to ensure that the amount of loaded between each replicate was consistent was not performed. Instead, the amount of E2 within each reaction well was determined via summation of their respective free E2 and E2-Ub conjugate bands. Assuming transfer of free E2 and E2-Ub from the polyacrylamide gel to the nitrocellulose membrane is equally efficient, the total amount of E2 protein collectively amongst both bands should remain constant across trials.
Figure 2.8. MLN4924 developed by Millennium Pharmaceuticals Inc., The Takeda Oncology Company with 1.5 μM IC\textsubscript{50} value against ubiquitin-activating enzyme (UBE1) in E1-E2 transthiolation assays reported by Soucy et al. in 2009.\textsuperscript{37}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC\textsubscript{50}</th>
</tr>
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<tbody>
<tr>
<td>UAE (UBE1)</td>
<td>1.5 ± 0.71 μM</td>
</tr>
</tbody>
</table>

Figure 2.9. 5'-O-sulfamoyl-N(6)-[(1S)-2,3-dihydro-1H-inden-1-yl]-adenosine (Compound 1) developed by Millennium Pharmaceuticals Inc., The Takeda Oncology Company. Compound IC\textsubscript{50} values reported against ubiquitin-activating enzyme (UBE1) in ATP-PPi exchange assays with varying assay concentrations of ATP by Chen et al. 2011.\textsuperscript{38}

<table>
<thead>
<tr>
<th>Compound 1 IC\textsubscript{50} (nM) against UAE (UBE1) with varying [ATP]</th>
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<tbody>
<tr>
<td>10.2 ± 1.7 (0.01 mM ATP)</td>
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2.1.3.4 Screening the Library for Leads

Using an E1-E2 transthiolation assay, the library was screened at 1 μM to determine which complexes possess inhibitory activity (Figure 2.10a-b). In these experiments, the copper-based compounds exhibited greater inhibition relative to zinc-based compounds possessing the same scaffold. Within the copper-based compounds it appears that most compounds halt the transfer of Ub from the E1 to the E2 at the concentration tested, with the exception of the Cu-Lys derivative which exhibits relatively poor inhibition regardless of the metal incorporated. Moreover, it appears that the Cu-Ser, Cu-Trp, Cu-Phe derivatives have reduced potency over Cu-Gly, Cu-Glu, Cu-Gln. However, it must be noted that due to unequal protein loading between tests observable in the blot images, that not all lanes within a particular experiment may be comparable. That said, these copper-based complexes also demonstrated concentration-dependent inhibition of E2-
Ub thioester formation when tested between 250-1000 nM (Figure 2.10c). Although promising, this data does not speak to the selectivity of these compounds.

**Figure 2.10.** UBE1-UbcH6 E1-E2 transthiolation compound screening assays with 1000 nM a) Zn- and Cu-based: Ala, Val, Pro, Lys, Tyr, His, and Arg compounds, b) Zn- and Cu-based Gly, Ser, Trp, Glu, Phe, Gln compounds; c) UBE1-UbcH6 E1-E2 transthiolation assays with 250, 500, 750 and 1000 nM Cu-Gly, Cu-Ser, Cu-Trp, Cu-Glu, Cu-Phe compounds. Inhibitors were incubated with 50 nM His<sub>6</sub>-UBE1, 500 nM His<sub>6</sub>-UbcH6, 1 μM Ub, and 1μM ATP in pH 7.4, 50 mM HEPES, 0.5% BSA, 5 mM MgCl<sub>2</sub> buffer for 1 h. Controls included normal, no E2, no E1, and Compound 1 (1000 nM). An anti-His antibody was employed.

### 2.1.3.4.1 Screening the Library for Selectivity

Next, a subset of the library was tested for their selectivity against the NEDD8 activating enzyme (NAE), the E1 activating enzyme for the NEDD8 Ubl. If the inhibitors perform as desired, inhibition should not be observed at the same concentrations as tested against UBE1 because NEDD8’s key selectivity residue is an alanine. In the analogous E1-E2 transthiolation assay, NAE was incubated with E2 (Ubc12), NEDD8, and ATP for 1 h. However, in this assay an anti-NEDD8 antibody was employed. This means that only E2 labelled with some integer value of NEDD8 will be detected and that unlabelled E2 will not be observed. Therefore, the reduction of the Ubc12-NEDD8<sub>n</sub> conjugate bands intensities is employed to monitor the reaction progress. Excitedly, neither zinc- nor copper-based complexes halted transfer of NEDD8 to Ubc12 at even
1000 nM, compared to compound 1 which prevented Ubc12-NEDD8 conjugation at as low as 10 nM (Figure 2.11).

![Image of gel electrophoresis](image.png)

Figure 2.11. NEDD8-activating enzyme (NAE)–Ubc12 E1-E2 transthiolation compound selectivity assays with 1000, 500, 100, and 10 nM Zn-Val, Cu-Pro, Zn-Arg, and Compound 1. Inhibitors were incubated with 100 nM NEDD8-activating enzyme (NAE, UBA3-APPBP1), 5 μM E2 (Ubc12), 5 μM NEDD8, and 200 μM ATP in pH 7.4, 50 mM HEPES, 0.5% BSA, 5 mM MgCl₂ buffer. An anti-NEDD8 antibody was employed.

With information to support that these copper-based complexes inhibit the ubiquitination pathway selectively, their mode of action remained unclear. Specifically, the complexes could be acting via binding to E1, E2, Ub, or even ATP.

2.1.3.5 *In vitro* Fluorescence-based reversible FRET-binding Assay for Inhibitor Screening

In an attempt to elucidate whether the binding site and mode of inhibition of these compounds is by competitively binding UBE1 for Ub, a reversible FRET-based binding assay by LifeSensors was employed (Figure 2.12). In the assay, a fluorophore was conjugated to Ub and a quencher species was appended to UBE1. In the absence of an inhibitor, the fluorophore becomes quenched because Ub binding to the Ub-binding pocket places the fluorophore in close proximity to the quencher species. Therefore, in the presence of a species that displaces Ub and liberates the fluorophore from the quencher, fluorescence increases. This change in fluorescence can then be monitored as a function of inhibitor concentration.
Consistent with E1-E2 transthiolation assay data, no inhibitory activity was observed for tested zinc-based compounds in the LifeSensors assay (Figure 2.13a). Moreover, the copper-based complexes also observed concentration-dependent displacement of Ub. From this preliminary screen, 5 sub 500 nM compounds were identified: Cu-Gln, Cu-Lys, Cu-His, Cu-Gly, and Cu-Pro derivatives. All copper-based compounds demonstrated similar IC\textsubscript{50} values within the assay, with the exception of Cu-Lys.
Figure 2.13. LifeSensors FRET assay screening data: a) dose-dependent inhibition curves and IC<sub>50</sub> values for identified lead compounds Cu-Gln, Cu-Glu, Cu-Lys, Cu-His, Cu-Gly, Cu-Pro, Zn-Ala, and a control sample of DMSO; b) lead compounds’ chemical structures with Zn-Ala compound.

The results of this assay provide support that these copper-based complexes act by displacing Ub by binding to the UBE1’s AAD or to an allosteric site. Also, the data may support that these complexes act via reversible binding and are not covalent inhibitors. Because ATP was not included in the assay, UBE1 remained inactive. Thus, if these complexes were irreversible inhibitors, they would have to modify UBE1 in its inactive form in such a way to prevent Ub binding. Considering that there is little evidence to support that these complexes can covalently modify inert protein, this mode of action is less likely. Aside from this, the data provides further validation that these compounds exhibit on-target potency for UBE1. With their added selectivity for UBE1 over NAE, these complexes hold potential for development of drugs inhibiting UBE1 in hematological malignancies.
2.2 Aims of This Research

My goal was to develop more potent, selective, reversible Ub-pocket inhibitors of UBE1 from the lead compound library developed in our lab. The following sections of the chapter outline how this task was approached by determining which compounds exhibited the highest potency in E1-E2 transthiolation assays, testing lead compound selectivity against NAE and SAE enzymes in their respective E1-E2 transthiolation assays, and aiming to move toward structure activity relationship (SAR) studies. They also outline the challenges faced in reproducing the data obtained from the original studies, and the troubleshooting that occurred.

2.3 Project Roadblocks and Problem Solving

2.3.1 Reproducibility of Validation Data

To begin, the 6 lead compounds were tested in a dose-dependent manner to determine which possessed greater potency for disrupting E2-Ub formation (Figure 2.14a). After tests were performed, no inhibition was observed for Cu-Lys, Cu-His, Cu-Gly, Cu-Pro, Cu-Glu, Cu-Gln, or Zn-Ala, inconsistent with previous experiments where copper-based compounds held considerable potency.

Since the library was also supported by FRET experiments from Lifesensors, I decided to investigate why I could not reproduce the transthiolation data. This troubleshooting included, but was not limited to, assessing: polyacrylamide gel type, containers for serial dilutions, use of 96 well plates with varying binding, order of component mixing, re-synthesis of inhibitors, verifying reaction conditions, assessing materials purchased for assay, optimization of reaction conditions, and the use of MLN4924 as a positive control (Figure 2.14b-e). However, all attempts led to no marked inhibition in sub μM concentrations.

Though, upon reducing the incubation time of the assay from 1 hour to 20 minutes, inhibition was observed for 10 μM of resynthesized Cu-Gly and Cu-Pro (2.14f). This 2 order of magnitude change in IC50 derived from testing at a shorter incubation time was concerning.
Figure 2.14. Troubleshooting Cu-based inhibitor UBE1-UbcH6 E1-E2 transthiolation assay data reproducibility: a) test of Zn-Ala, Cu-Lys, Cu-His, Cu-Pro, Cu-Glu, Cu-Gln incubated at 250, 500 and 1000 nM; b) test of Cu-His, Cu-Lys incubated at 10, 5 and 1 μM with. In first replicate, inhibitor dilutions were prepared in 96 well tissue culture plates. In second replicate inhibitors dilutions occurred in Eppendorf tubes; c) test of Cu-Pro, Cu-His incubated at 20, 10, and 1. In one replicate, inhibitor was added to His₆-UBE1, Ub, His₆-UbcH6 mix prior to addition of ATP. In second replicate, inhibitor was mixed with His₆-UBE1, after which Ub, His₆-UbcH6, and ATP were added in their respective order; d) UBE1-UbcH6 E1-E2 transthiolation assay optimization with varying incubation time, varying Ub concentration, and varying UbcH6 concentration; e) test of MLN4924 incubated at 250, 10 and 0.25 μM. In one replicate reactions are incubated for 20 min. In second replicate, 500 nM Ub is employed; f) test of Cu-Gly, Cu-Pro at 250, 10, 0.25 μM for with a 20 min incubation period. Unless otherwise stated, inhibitors were incubated with 50 nM His₆-UBE1, 500 nM His₆-UbcH6, 1 μM Ub, and 1 μM ATP in pH 7.4, 50 mM HEPES, 0.5% BSA, 5 mM MgCl₂ buffer for 1 h. Controls included normal, no E2, no E1, MLN4924, and Compound 1. An anti-His antibody was employed for all blots.
2.3.2 Re-Investigation of Lifesensors FRET-Binding Assay:

Considering that the transthiolelation data was not consistent with earlier results, we decided to re-examine the assay developed and performed by LifeSensors. Upon additional evaluation, we found that the company had performed a different assay (Figure 2.15).

![Figure 2.15. Scheme for E1 LITE - UBE1 Activity Assay](image)

Figure 2.15. Scheme for E1 LITE - UBE1 Activity Assay Kit binding assay performed by LifeSensors. A Ub-fluorescein conjugate and UBE1 were incubated with inhibitor for 15 minutes. Then, a Ub-biotin conjugate and ATP were added to incubate for an additional 15 minutes to allow the enzyme’s reaction to proceed forward, leading to transfer of Ub species to UBE1’s catalytic cysteine. Then, terbium conjugated to streptavidin was added. In the absence of an inhibitor displacing Ub, Ub-fluorescein becomes covalently attached to the UBE1 catalytic cysteine. Then, Ub-biotin can occupy empty Ub-binding sites on UBE1, such that when streptavidin-terbium binds biotin, terbium FRET with Ub-fluorescein can occur. The proximity of Ub-fluorescein to streptavidin-terbium facilitates a FRET signal. In the presence of an inhibitor displacing Ub species, FRET signals decrease relative to the control.

In the assay performed by LifeSensors, a Ub-fluorescein conjugate (100 nM) and UBE1 (2.5 nM) were first incubated with inhibitor for 15 minutes. Then, Ub-biotin conjugate (1 nM) and ATP (1 mM) were added to the solution for another 15 minute incubation period. ATP addition allows UBE1 to conjugate Ub species to the enzyme’s catalytic cysteine. Ub-fluorescein becomes covalently attached to the UBE1 catalytic cysteine preferentially to Ub-biotin due to the
order of addition and Ub-fluorescein’s relative abundance in solution. Meanwhile, Ub-biotin is able to occupy remaining empty Ub-binding sites on UBE1. Therefore, addition of streptavidin-terbium conjugates that bind biotin facilitate FRET between terbium (FRET donor) and fluorescein (FRET acceptor). The proximity of streptavidin-terbium to Ub-fluorescein facilitates a FRET signal that can be measured in response to inhibitor. In the presence of an inhibitor displacing Ub species, Ub-fluorescein is not able to conjugate to UBE1’s catalytic cysteine and the FRET signal decreases relative to the control.

This assay is problematic for analysis due to multiple reasons. First, because of the many species in solution (ie. UBE1, Ub-fluorescein, Ub-biotin, Tb-streptavidin, and ATP), the data can become difficult to interpret. In comparison to the original assay, the presence of many species (which includes ATP) means that the copper complexes can act by binding to multiple species and act by any number of mechanisms. Moreover, the presence of ATP specifically means that the enzymatic reaction catalyzed by UBE1 proceeds forward. Consequently, it becomes more difficult to elucidate whether the complex additionally stops the progression of the pathway via binding to the catalytic cysteine, the ATP pocket, or via another method. The inability to distinguish where the complexes bind also means that it is less certain that the inhibitors are reversible binders. Overall, this makes extrapolation from the data problematic beyond stating that the pathway has been halted. Also, because there are 2 different covalently modified Ub species in solution, their varying affinities (due to modification) for the Ub-binding pocket may bias the progression of the assay. This bias is problematic because both species must be bound to UBE1 to elicit a FRET signal. For example, the copper-based complexes may have activity for inhibiting binding of biotinylated Ub, but not fluorescein-labelled Ub, or vice versa. Thus, if the copper complexes can exclusively outcompete one of the two Ub species, a loss of FRET signal will occur and be associated with the absolute ability to outcompete Ub, when this activity may not translate to outcompeting free Ub. Another major challenge to the assay data interpretation comes from the fact that it monitors the loss of signal with inhibition, and not the increase in signal. Monitoring loss of signal is problematic because FRET signal quenching by introduced species (inhibitors) can mimic the loss of signal observed for inhibition. Specifically, Cu^{2+} has been a reported quencher of terbium luminescence, as well as a quencher for various fluorophores.40–43 This knowledge of copper’s quenching ability is interesting considering that almost all copper-based complexes within the library exhibited equivalent inhibition values.
Moreover, with the zinc-based complexes, negative inhibition values were observed. Since zinc has been documented to increase terbium luminescence relative to baseline, it is possible that these complexes exerted their own mechanism on the donor in the assay.\textsuperscript{44} Due to the aforementioned reasons, the UBE1 FRET assay data has been called under question, and is less supportive of the activity of the copper-based inhibitors for UBE1. That said, this information does not invalidate the preliminary E1-E2 transthiolation data which would not be affected by quenching mechanisms.

In re-examining the FRET data, we also realized that appropriate controls had not been conducted for these complexes. Specifically, copper ion, zinc ion and the organic scaffolds of the library were not tested in isolation concurrently with the previous tests. Moving forward, we decided to perform these tests, and probe to see if the reported selectivity was reproducible.

\textbf{2.3.3 Re-Investigation of E1-E2 Transthiolation Data:}

When resynthesized inhibitors were tested between 200-1 μM in E1-E2 transthiolation assays, Cu-Gly exhibited a greater potency than Cu-Pro with complete inhibition of E2-Ub (UbcH6-Ub) formation at 100 μM (lanes 3, 10, 17). (Figure 2.16a, b). Also, when non-functionalized copper-cyclen and zinc-cyclen were tested, they demonstrated no significant change from the normal control up to 200 μM (Figure 2.6c, d). When copper triflate (trifluoromethanesulfonate) was assessed at the same concentrations, its potency was slightly greater than the Cu-Gly complex in lanes 3, 10, and 17 (Figure 2.16e). Also, in both the Cu-Gly and copper triflate trials, the appearance of a high molecular weight protein band in lanes of greatest inhibition (lanes 2,3,4, 9,10,11,16,17,18) corresponding to the formation of E2-E2 dimers occurred (Figure 2.16b, e). The appearance of E2-E2 (UbcH6\textsubscript{2}) dimer bands concernedly points to these complexes acting via linking E2 enzymes. Moreover, when Cu-Pro was kept at constant concentration, and the concentration of Ub was increased, the conjugation of Ub to E2 was recovered, pointing to the complex being competitive with Ub (Figure 2.6f). Though, since free copper exhibited strong potency in the assay compared to copper bound in the copper cyclen complex, and we observed the formation of E2-E2 dimers in replicates demonstrating pathway knockdown, we became concerned that Cu-Pro and Cu-Gly potency in the assay could be a result of free copper.
Concernedly, we also determined that Cu-Pro exhibited no selectivity for UBE1 over NAE when tested between 200-1 μM (Figure 2.17). Within this assay inhibition was observed beginning at approximately 50 μM, analogous to its performance in Figure 2.16a.
Moreover, we determined that the final compound preparation was flawed. For each metalation reaction copper triflate was incubated with the organic scaffold in methanol for approximately 2-3 hours, after which the mixture would be filtered through cotton. Initially, it was believed that copper triflate would only dissolve in MeOH upon binding to the organic scaffold’s cyclen nitrogens, and that undissolved (and unbound) copper triflate would remain as solid particulates that could be filtered through the cotton. Unfortunately, all copper triflate dissolved regardless of the presence of ligand as evidenced in Figure 2.18. After measuring the UV-Vis absorbance profiles of our tested species, we found that non-chelated copper triflate and copper chloride samples absorb very strongly about 190 nm, whereas compounds containing cyclen absorb strongly about 264 nm. By normalizing the profiles of compounds containing cyclen to have the same peak height at 264 nm, we found that the compounds with the greatest transthiolation assay inhibitory activity contained the highest relative proportion of 190 nm peak heights. Specifically, Cu-Gly possesses a large 190 nm band relative to the cyclen band, Cu-Pro has a smaller 190 nm band, and Cu-Cyclen (with no appended amino acid) contains the relatively smallest 190 nm absorbance band. Thus, it appears that free (or non-chelated) copper in the copper-based complexes may be the sole determinant of UbcH6-Ub formation disruption in the assay.
Figure 2.18. UV-Vis absorbance spectra for copper chloride, Cu-Gly, Cu-Pro, Cu-Cyclen, and copper triflate dissolved in Milli-Q® water. Samples containing cyclen are normalized to 264 nm, whereas samples lacking cyclen are normalized to 190 nm.

2.4 Discussion

Initially, a rationally designed library of copper-based purportedly potent non-covalent selective inhibitors of UBE1 was developed via E1-E2 transthiolation assay and LifeSensors FRET assay screening. First, the LifeSensors assay data was problematically found to be derived from a monitored loss of fluorescence, instead of an increase in fluorescence. Considering that paramagnetic copper can quench fluorescence and is a known quencher of the terbium donor luminescence, the copper quenching of signal could mimic the signal of inhibition in this assay. The fact that the diamagnetic zinc-based complexes observed no activity also should have raised concern. More interestingly, the negative inhibition values observed for zinc complexes may have even been the result of signal increase by these complexes, as zinc is also known to increase terbium luminescence intensity. In transthiolation assays, the inhibitory data was irreproducible until a third batch of compounds was synthesized. In this batch we have observed higher potency with free copper than the copper-based compounds developed. Moreover, the complexes developed with the highest proportions of free copper signals exhibit the greatest potency in halting UbcH6-Ub formation. In addition, the copper-based complex that exhibited the greatest potency and free copper signal proportions (along with free copper) induced formation of E2-E2
dimers, pointing to inhibition via the same mechanism. That said, inhibition was found to be Ub concentration dependent. Furthermore, tests against NAE demonstrate no selectivity when the assay is performed using the same conditions and concentrations as the UBE1 assay. This lack of selectivity between enzymes, the presence of free copper, and formation of E2-E2 dimers suggest that free copper may be acting by facilitating E1 and E2 catalytic cysteines to create disulphide bridged non-functional dimers when sufficient concentrations in solution. The presence of these catalytic cysteines in both NAE and UBE1 E1-E2 transthiolation assays could explain why both pathways are similarly halted by the same complexes. In summary, investigational transthiolation, absorbance, and LifeSensors assay data support the hypothesis that free copper may be basis of all activity observed for these compounds.

Although this result is disappointing, retrospectively we were able to discern warning signs. For example, it was quite impressive in itself for such a small scaffold (MW≈200-300 g/mol) to exhibit such a high potency for a target and retain selectivity. More concerning was that all compounds, with the exception of Cu-Lys, possessed equivalent potency in both assays, despite being functionalized with amino acids varying greatly in hydrophobicity, bulk, and charged states. Also, upon looking into compounds produced for initial transthiolation assay screening, the compounds with the highest potency exhibited significant yields over 100%; yields over 100% support the hypothesis that excess copper triflate utilized in metalation reactions of the scaffolds occurred and that this copper was unfiltered by cotton, to provide free copper for compound potency. However, these yields could be derived from the hygroscopic nature of the compounds, leading to water absorption increasing the measured mass. This data also supports that my recent “failed” transthiolation assay data attempts where no inhibition was observed were not erroneous, but simply the first occurrence when the compounds were synthesized with insignificant levels of free copper before testing. Much of the issues within this project could have been circumvented with appropriate characterization of the complexes. Typically, copper- and zinc-based cyclen final complexes are characterized by EPR spectroscopy (for copper-based complexes), IR spectroscopy, elemental analysis, and even crystallography prior to use. However, no characterizations were performed for these complexes beyond electrospray ionization-liquid chromatography-mass spectrometry (ESI-LC/MS) experiments where metal was found to dissociate from the ligand. If elemental analysis had been performed with the initially synthesized complexes, it could have revealed whether particular batches of complexes
possessed excess free copper in the samples. For this reason, consistency between batches cannot even be determined. For these reasons, this project, although a medicinal chemistry failure, represents a lesson in diligence for assessing collaborator, peer, and industry data for trends and fallacies in experimental design.

2.5 Future Directions

To assess how to move forward, we considered methods to both achieve affinity and selectivity. We began by re-examining the crystal structure of UBE1 (Figure 2.19) and realized that the majority of its 10 Cys and 16 His residues are solvent exposed. Considering that His and Cys residues can bind to the open coordination sites in the copper with strong affinity, the chances of targeting a specific site on the enzyme using this strategy were low. Therefore, multiple binding sites would have to be considered each time a hit is identified. Also, even if a particular site were successfully targeted, it is unknown if binding could lead to inhibition or even activation. These features may also explain why the concentration of even free copper was three orders of magnitude higher than UBE1 in order to achieve effective pathway knockdown. For these reasons alone this strategy may be ill-fitted. To achieve selectivity for particular sites, more distinctive functionalities could be appended to the cyclen in addition or in exchange for the amino acids to increase surface contacts with the enzyme via hydrogen bonding, dipole-dipole interactions, etc. To combat intermolecular selectivity, it would also be beneficial to test for binding to Ub in addition to NAE and SAE (SUMO1 E1 activating enzyme) since copper-based binders of Ub have already been reported. Though, the use of copper cyclen-based compounds may need to be revisited in itself, considering that they have been reported to catalyze oxidative cleavage of DNA at physiological pH.\(^{45}\) This concern is especially significant if they possess the ability to diffuse across the nuclear membrane.
Figure 2.19. Crystal structure of UBA1 with highlighted features: a) cysteines (yellow), b) histidines (blue), c) adenylation domain (red) (PDB: 4NNJ).

2.6 Contributions

The copper- and zinc-based complex library generated for validation experiments was designed and synthesized in various batches by Dr. Julie Lukkarila, Sara R. da Silva, Stacey-Lynn Paiva, and Dr. Matt Bancerz. E1-E2 transthiolation assay validation experiments were performed in collaboration with Professor Aaron D. Schimmer’s lab at the University of Toronto by Dr. G. Wei Xu, Sara R. da Silva, and Stacey-Lynn Paiva. FRET assay validation data was performed by LifeSensors, Inc. based in Malvern, PA, U.S.A.

Investigational E1-E2 transthiolation assay experiments were planned and performed with Syeda Anika Imam.

2.7 Materials and Methods:

2.7.1 Screening of the Library

2.7.1.1 Reagents

Human recombinant His$_6$-ubiquitin E1 enzyme (UBE1), human recombinant His$_6$-UbcH6, and human recombinant Ub, human recombinant NAE (NEDD8 E1 activating enzyme, APPBP1, UBA3), human recombinant Ubc12 (UBE2M), and human recombinant NEDD8 were purchased from Boston Biochem. Goat monoclonal anti-NEDD8 antibody [Y297] and mouse monoclonal [HIS.H8] to 6X His tag® antibodies were purchased from Abcam. Antibody BLUeye Prestained
Protein Ladder was purchased from FroggaBio, DTT was purchased from Bioshop Canada, MLN4924 was purchased from Active Biochem, and all other reagents including bovine serum albumin (BSA), MgCl₂, and NaCl were purchased from Sigma-Aldrich. 2x laemmli sample buffer, luminol, hydrogen peroxide, goat anti-rabbit-HRP, goat anti-mouse-HRP conjugate and was purchased from Bio-Rad.

2.7.1.2 In vitro E1-E2 Transthioylation Assays

In UBE1-UbcH6 transthioylation assays, compounds were incubated at varying concentrations with 50 nM His₆-UBE1, 500-1000 nM µM Ub, 500 nM His₆-UbcH6, and 1 µM ATP dissolved in fresh pH 7.4, 50 mM HEPES, 0.5% BSA, 5 mM MgCl₂ buffer. Assays were conducted in 96 well plates to a 20 µL final volume, with 20-60 min incubation times. Enzymatic reactions were halted using 10 µL of 2x laemmli sample buffer. Control reactions performed included replicates lacking inhibitor, lacking UBE1, lacking UbcH6, and reactions including DTT and MLN4924.

10 µL fractions from each sample were fractioned on 4-20% gradient SDS-PAGE.

SDS-PAGE was followed by immunoblotting with mouse anti-His antibody and goat anti-mouse-HRP conjugate. Formation of UbcH6-Ub was assessed by Bio-Rad ChemiDoc imaging system after application of 1 mL 1:1 luminol:peroxide solutions.

NAE-Ubc12 transthioylation assays were run analogously to UBE1-UbcH6 transthioylation assays with NAE (APPBP1, UBA3) (500 nM), 1 µM NEDD8, 500 nM Ubc12, and 1 µM ATP. For immunoblotting within this assay rabbit anti-NEDD8 antibody and goat anti-rabbit-HRP conjugate antibodies were employed.

2.7.1.3 Absorbance Experiments

Complexes were dissolved in Milli-Q® H₂O prior to readings in a HP 8452A Diode-Array Spectrometer (Hewlett Packard Corporation, Palo Alto, CA, USA).

2.7.2 General Synthetic Methods and Characterization of Molecules

2.7.2.1 Chemical Methods

Dichloromethane (DCM), methanol (MeOH), ethyl acetate (EtOAc), hexanes, dimethylformamide (DMF), and deuterated solvents (chloroform (CDCl₃) and methanol
(CD_{3}OD)) were purchased from Sigma-Aldrich and used as received. With the exception of boc anhydride and cyclen which were purchased from Alfa Aesar and Toronto Research Chemicals respectively, all reagents were purchased from Sigma-Aldrich. Reactions were monitored for completeness by thin-layer chromatography (TLC) using silica gel with UV light visualization and/or development by treatment with KMnO_{4} stain. NMR spectra were measured using a Bruker 400 MHz spectrometer in deuterated solvents such as CDCl_{3} and CD_{3}OD. Chemical shifts (\delta) were reported in parts per million, and coupling constants (J) are reported in hertz (Hz). LCMS measurements were performed using a Waters Micromass ZQ 2000 Liquid Chromatography/Mass Spectrometer (LC/MS), with samples dissolved in either dH_{2}O or MeOH. All compounds were analyzed using the positive electrospray mode, with some including negative electrospray modes. Ligand purity was evaluated via analytical HPLC using a Phenomenex Luna 5u C18(2) 100 Å 150 x 4.60 mm 5 micron column with eluent flow set at 1 mL/min. Retention times for compounds in water with 0.1% trifluoroacetic acid (TFA) for 30 minutes followed by a gradient to 100% acetonitrile by 35 minutes with UV detection at 214 nm are reported with purities following.

2.7.3 Synthesis of Inhibitor Library

Generation of the proposed inhibitor library began with the synthesis of N,N',N''-Tri-Boc-cyclen (boc_{3}cyclen) from commercially available 1,4,7,10-tetraazacyclododecane (cyclen) and di-tert-butyl dicarbonate (Boc_{2}O, boc anhydride) (Scheme 1). After, N-boc protected amino acids were coupled to the free nitrogen on boc_{3}cyclen using O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU). This step was followed by removal of amino acid protecting groups where appropriate and global deprotection by via incubation with trifluoroacetic acid to remove the Boc groups. The final products were prepared via incubation of the deprotected molecules with either copper or zinc trifluoromethanesulfonate salts in methanol (MeOH) and subsequent evaporation of the solvent to yield crystalline product.

For investigational data, I was tasked with resynthesizing Cu-Gly and Cu-Pro. All other synthesis was performed by Dr. Julie Lukkarila, Sara R. da Silva, Stacey-Lynn Paiva, and Dr. Matt Bancerz.
Specific Procedure 1: tri-N-Boc Protection

Di-tert-butyl dicarbonate (2.6 eq) dissolved in chloroform (CHCl₃) was added to cyclen in CHCl₃ (1.0 eq.) dropwise over 3 hours with stirring at 0°C. The reaction continued to stir at room temperature overnight under nitrogen gas. After the reaction was judged complete by TLC analysis, the mixture was concentrated, azeotroped with DCM. The crude was dissolved in...
EtOAc:Hexanes (4:1), and then subjected to flash silica gel column chromatography using the same solvent to yield 2.1 (N,N',N''-Tri-Boc-cyclen).

**General Procedure 2: Amide Coupling**

2.1 (1.0 eq.), TBTU (2.2 eq.), and N,N-diisopropylethylamine (DIPEA) (2.3 eq.) were dissolved in DMF and the reaction mixture was stirred at room temperature for 5 minutes. Depending on the compound synthesized, appropriate N-boc amino acids (eg. Boc-Gly-OH) (1.0 eq.) were added to the reaction mixture, and left to stir at room temperature overnight under nitrogen gas. After the reaction was judged complete by TLC analysis, a dH2O/EtOAc work-up was performed. The organic fractions were combined, washed with brine, dried over anhydrous sodium sulfate (Na2SO4), concentrated once more, and azeotroped 3 times with DCM to yield crude. The crude was then subjected to flash silica gel column chromatography to yield product.

**General Procedure 3: Hydrogenation**

Palladium on carbon (Pd/C) (10% w/w) (0.2 eq.) was added to ligand (general procedure 2 product) (1.0 eq.) in MeOH. The reaction mixture was stirred at room temperature overnight under hydrogen gas. After the reaction was judged complete by TLC analysis, the reaction was filtered through Celite, and concentrated. The crude was then subjected to flash silica gel column chromatography to obtain pure product.

**General Procedure 4: Fmoc Deprotection**

(20 % v/v) piperidine in DMF was added to solid ligand (general procedure 2 product). The reaction mixture was stirred at room temperature for 15 minutes. After the reaction was judged complete by TLC analysis, a dH2O/EtOAc work-up was performed. The organic fractions were combined, washed with brine, dried over anhydrous Na2SO4, concentrated once more, and azeotroped 3 times with DCM to yield crude. The crude was then subjected to flash silica gel column chromatography to yield product.

**General Procedure 5: Global Deprotection**

Ligand (products from general procedures 2, 3, and 4) (1.0 eq.) was dissolved in TFA:H2O (3:1). The reaction mixture was stirred at room temperature for 90 min. After the reaction was judged
complete by TLC analysis, the mixture was concentrated, then azeotroped with MeOH, and then DCM. The crude was dissolved in Milli-Q® H₂O, and subjected to reverse-phase preparative HPLC. The solvent system transitioned between a pure 0.1% TFA (by volume) in Milli-Q® solution to 0.1% TFA and 10% Milli-Q® H₂O in ACN (% by volume) over 40 minutes. Using low-resolution mass spectrometry, the fractions containing product were identified, combined, and concentrated. The sample was dissolved in MeOH and subjected to a flash amberlite ion-exchange resin column in MeOH. The eluent was then concentrated, and azeotroped with chloroform to obtain product.

**General Procedure 6: Copper Metallation**

Ligand (products from general procedures 5) (1.0 eq.) and copper (II) trifluoromethanesulfonate (1.0 eq.) were dissolved in MeOH. The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then filtered through cotton, concentrated, and azeotroped with DCM to yield crystalline product.

**General Procedure 7: Zinc Metallation**

Ligand (products from general procedures 5) (1.0 eq.) and zinc (II) trifluoromethanesulfonate (1.0 eq.) were dissolved in MeOH. The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then filtered through cotton, concentrated, and azeotroped with DCM to yield crystalline product.
2.8 Characterizations:

**Tri-tert-butyl 1,4,7,10-tetraazacyclododecane-1,4,6-tricarboxylate (2.1)** Compound 2.1 was synthesized according to specific procedure 1, yielding 49.0% colourless solid; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.63 (br, 4H, 2 x CH$_2$), 3.34 (br, 8H, 4 x CH$_2$), 2.84 (brs, 4H, 2 x CH$_2$), 1.53 (s, 1H, NH), 1.480 (s, 27H, 9 x CH$_3$); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 25.46, 27.35, 61.88, 81.64, 83.53, 86.63, 89.95, 113.41, 119.35, 140.04, 149.10, 152.96, 156.40; LRMS [ESI$^+$] $m/z$ calc'd for C$_{23}$H$_{44}$N$_4$O$_6$ [M]: 473.33, found 473.14.

**tri-tert-butyl 10-((tert-butoxycarbonyl)prolyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2e)** Compound 2.2e was synthesized according to general procedure 2 with Boc-Pro-OH, yielding 38.8% colourless solid; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.20-1.34 (m, 2H, CH$_2$), 1.36-1.50 (m, 36H, 12 x CH$_3$), 1.74-1.99 (m, 2H, CH$_2$), 2.02-2.25 (m, 2H, CH$_2$), 2.80-3.98 (m, 15H, 7½ x CH$_2$), 4.13 (brs, 1H, CH), 4.48-4.63 (m, 1H, ½ x CH$_2$); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 20.87, 23.51, 24.11, 28.32, 32.08, 46.714, 50.80, 51.41, 55.36, 60.22, 78.936, 79.389, 79.995, 80.331, 80.386, 153.4, 171; LRMS [ESI$^+$] $m/z$ calculated for C$_{33}$H$_{59}$N$_5$O$_9$ [M+Na$^+$]: 692.42, found 692.26.
1-prolyl-1,4,7,10-tetraazacyclododecane (2.5e). Compound 2.5e was synthesized according to general procedure 5 with 2.2e, yielding 35.0% colourless solid; $^1$H NMR (400 MHz, MeOD): $\delta$ 1.51-1.79 (m, 3H, CH), 1.99-2.14 (m, 1H, CH$_2$), 2.45-2.91 (m, 13H, 5½ x CH$_2$), 2.99-3.09 (m, 1H, ½ x CH$_2$), 3.18-3.36 (m, 2H, CH$_2$), 3.59-3.78 (m, 2H, CH$_2$), 3.88 (t, $J = 7.58$ Hz, 1H, CH); $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 25.8, 29.9, 43.95, 45.22, 48.58, 51.77, 57.05, 57.87, 106.8, 117.2, 175.47; LRMS [ESI$^+$] $m/z$ calculated for C$_{13}$H$_{27}$N$_5$O [M]: 269.39, found 270.20; HPLC $t_R = 30$ min (92.0%).

Cu-Pro. Cu-Pro was synthesized according to general procedure 6 with 2.5e, yielding 100% blue solid.
tri-tert-butyl 10-((tert-butoxycarbonyl)glycyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2f). Compound 2.2f was synthesized according to general procedure 2 with Boc-Gly-OH, yielding 70.0% colourless solid; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.39-1.53 (m, 36H, 12 x CH$_3$), 3.25-3.60 (m, 16H, 8 x CH$_2$), 3.94 (s, 2H, CH$_2$); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 28.0262, 28.1243, 28.1720, 29.3231, 41.9614, 49.2545, 49.5408, 49.8252, 50.9046, 76.7186, 77.0383, 78.9688, 80.0056, 80.1083, 155.2520, 155.3412, 156.5237, 169.0391; LRMS [ESI$^+$] m/z calculated for C$_{30}$H$_{55}$N$_5$O$_9$ [M]: 630.41, found 630.43.

2-amino-1-(1,4,7,10-tetraazacyclodecan-1-yl)ethan-1-one (2.5f). Compound 2.5f was synthesized according to general procedure 5 with 2.2f, yielding 33.5% colourless solid. $^1$H NMR (400 MHz, MeOD): $\delta$ 2.45-2.91 (m, 13H, 5½ CH$_2$), 2.99-3.09, 3.18-3.36 (m, 2H, CH$_2$), 3.59-3.78 (m, 2H, CH$_2$), 3.88 (t, $J$ = 7.58, 1H, CH); $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 42.1196, 43.9711, 45.2135, 53.2768, 174.3188; LRMS [ESI$^+$] m/z calculated for C$_{10}$H$_{23}$N$_5$O [M]: 230.20, found 230.27; HPLC $t_R$ = 30 min (90.0%).

Cu-Gly. Cu-Gly was synthesized according to general procedure 6 with 2.5f, yielding 100% blue solid.
tri-tert-butyl 10-((tert-butoxycarbonyl)alanyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2g).

2-amino-1-(1,4,7,10-tetraazacyclododecan-1-yl)propan-1-one (2.5g).

Zn-Ala.
tri-tert-butyl 10-(N6-((benzyloxy)carbonyl)-N2-(tert-butoxycarbonyl)lysyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2b).

tri-tert-butyl 10-((tert-butoxycarbonyl)lysyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.3b).

2,6-diamino-1-(1,4,7,10-tetraazacyclodecan-1-yl)hexan-1-one (2.5c).
Cu-Lys.

tri-tert-butyl 10-((Na-(((9H-fluoren-9-yl)methoxy)carbonyl)-Nt-(tert-butoxycarbonyl)histidyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2d).

tri-tert-butyl 10-(Nt-(tert-butoxycarbonyl)histidyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.4d).
2-amino-1-(1,4,7,10-tetraazacyclododecan-1-yl)-3-(1H-imidazol-5-yl)propan-1-one (2.5d).

Cu-His.

tri-tert-butyl 10-(5-(benzyloxy)-2-((tert-butoxycarbonyl)amino)-5-oxopentanoyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2a).
4-((tert-butoxycarbonyl)amino)-5-oxo-5-(4,7,10-tris(tert-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid (2.3a).

4-amino-5-(1,4,7,10-tetraazacyclododecan-1-yl)-5-oxopentanoic acid (2.5a).

Cu-Glu.
tri-tert-butyl 10-((N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N5-tritylglutaminy)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.2c).

tri-tert-butyl 10-(N5-tritylglutaminy)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (2.4c).

4-amino-5-(1,4,7,10-tetraazacyclodecan-1-yl)-5-oxopentanamide (2.5c).
3 Application of ProxyPhos, a Selective Chemosensor for Pyrophosphate to Development of an Adenylating Enzyme Activity Assay

3.1 Background

3.1.1 Studying Adenylation Enzyme Activity for Drug Discovery

3.1.1.1 Studying Enzyme Activity for Drug Discovery

An enzyme unit (U) is the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per minute. Enzyme activity refers to the number of activity units per mL. Enzyme activity can therefore be quantified by measuring enzyme substrate consumption or product accumulation. Measuring enzyme activity is important for characterizing substrate turnover rates, and for understanding how turnover rates change in relation to other species in biological systems, diseased states, and small molecules developed for drug discovery. For example, incubation of enzyme and substrate with an additional protein or small molecule can reveal pathway regulatory features through their effects on substrate consumption rates. Specifically, the types of interactions between enzymes and added ligands can be elucidated based upon effects on substrate consumption and product formation rates. In this way, methods measuring enzyme activity are important tools for assessing small molecules for drug discovery.

3.1.1.2 Current Adenylating Enzyme Activity Assays

Currently, there are 4 widely used methods for measuring the activity of AEs for the purpose of drug discovery. These methods include ATP/PPi exchange assays, SDS-PAGE- and western blotting-based assays, chemosensor-based assays, and luciferase assays. These assays measure AE activity via quantification of ATP consumption, PPi production, or formation of additional...
AE products. In this chapter, luciferase assays will not be discussed due to their limited use in drug discovery owed to both sensitivity to changes in reaction conditions and the possession of an ATP binding site on luciferase that is problematically often targeted for inhibition on AEs.

3.1.1.2.1 ATP/PPi Exchange AE Activity Assays

ATP-PPi exchange assays track reaction progress by monitoring incorporation or loss of labelled isotopes in ATP and P Pi. In radioactive ATP-PPi exchange assays, enzyme, ATP, and substrate are incubated for a particular time period (Figure 3.1a). Then, excess radioactive phosphorous-labelled pyrophosphate \([^{32}\text{P}]\text{PPi}\) is incorporated to drive the reverse reaction and concomitant incorporation of \(^{32}\text{P}\) into ATP. From here, activated charcoal can be added to adsorb the newly generated radioactive ATP, which can be separated from the solution via centrifugation and decanting the supernatant. The charcoal is then re-suspended for quantification in a scintillation counter.\(^{47-49}\) The amount of radioactive ATP formed is then assessed as proportional to the reaction progress. In heavy atom ATP-PPi exchange assays, enzyme, \(\gamma-18\text{O}_4\)-ATP, and substrate are incubated, followed by addition of excess unlabeled P Pi.\(^{50}\) Through the forward reaction ATP loses heavy oxygen and the back reaction generates unlabeled ATP. From here, the mixture can be assessed by ESI-LC/MS or MALDI-TOFMS. As reaction progresses, an 8 Da shifted peak will be generated. The activity of the enzyme can be determined by finding the integral ratio of the \(\gamma-16\text{O}_4\)-ATP peak to other ATP species.
Figure 3.1. ATP-PPI exchange assay type I: measures $^{32}$P-PPI back incorporation into unlabeled ATP  a) assay scheme and b) mechanism of labelling; Assay type II: measures consumption of $\gamma$-18O<sub>2</sub>-ATP c) assay scheme and d) mechanism of labelling. Figure generated with information from Otten et al. 2007 and Phelan et al. 2009.
ATP-PPI exchange assays benefit from their sensitivity, precision, and little intervention on equilibria under investigation. Specifically, by monitoring reaction progress via detection of ATP formation or consumption, additional species like sensors do not have to be added to the solution that may influence the equilibria under investigation. That said, these assays are not short of drawbacks. Since these assays involve labelled species, they are costly, can be time-consuming, and require significant training. The safety and radioactive waste disposal concerns with \[^{32}\text{P}]\text{PPi}\) in themselves are reason enough to make this method inaccessible to some scientists. Overall, ATP-PPI exchange assays are robust and sensitive methods for measuring AE activity, but are not accessible to all scientists due to limiting set-up requirements.

3.1.1.2.2 SDS-PAGE- and Western Blotting-based AE Activity Assays

SDS-PAGE and western blot-based assays for measuring AE activity are applied in systems that have ATP-dependent covalent modification-mediated molecular weight changes in species. In these assays, reaction progress is monitored via detection and quantification of newly formed protein species. After incubation of reaction protein species for a desired time period, the reaction mixture is separated on an SDS-PAGE where the disappearance of an initial species and the concomitant formation of product species can be observed with protein stains. This reaction progress can also be evaluated in response to increasing inhibitor or activator species (Figure 3.2). For additional information, western blots can be performed by transferring the protein species from the gel to a membrane and probing for species identification and quantification. The use of antibodies in western blots can be advantageous for identification of key species when general protein visualization in SDS-PAGE presents too many species for reliable distinction.
These SDS-PAGE- and western blot-based assays are widely used because they are robust and largely insensitive to changes in the reaction conditions of the system studied. The added benefit of western blot analysis of enzymatic reactions is that antibody detection systems eliminate off-target signals. Problematically though, these assays are time consuming, with many steps. A typical western-blot coupled assay will involve a 1-2 hour separation of 10-26 enzymatic reaction samples via SDS-PAGE, a 15 minute transfer of proteins to a nitrocellulose or Polyvinylidene fluoride (PVDF) membrane, a 1-2 hour blocking of the membrane, an overnight incubation of the membrane with a primary antibody, three 15 minute washes of the membrane, a 1-2 hour incubation of the membrane with a secondary antibody, three 15 minute washes of the membrane, and then a visualization step. Due to the number of steps involved in these assays, troubleshooting when assays fail to work can also become time-consuming as each step requires multiple components. Regrettably, the use of antibody detection methods also means that these assays can be costly to complete in high amounts. Finally, these assays can only be applied to monitor systems with covalent modifications large enough to be teased apart using SDS-PAGE. For these reasons, SDS-PAGE- and western blot-based assays for measuring AE activity are useful but can be costly and troublesome methods for systems that involve covalent modification of species coupled with detectably large changes in molecular weight.
3.1.1.2.3 **Chemosensor-Based AE Activity Assays**

Chemosensor-based enzyme activity assays employ organic molecules that produce signals upon binding target analyte species. AE activity assays require the application of ATP, AMP, or PPI sensing species to enzymatic reactions, and studying changes to signal over time and in response to inhibitors, activators, and other species under investigation. For example, reaction progress can be monitored by measuring sensor absorbance (for colorimetric sensors) or fluorescence (for fluorescent chemosensors) in the presence of analyte.

![Figure 3.3. Chemosensor-based AE activity assay example scheme.](image)

Chemosensor-based assays benefit from being relatively quick and inexpensive. Since chemosensors can be incubated with a reaction mixture for as little as 10-20 minutes before measurement, they permit rapid study of enzymatic systems.\(^{51}\) Moreover, the sensors can be relatively inexpensive to produce and sell with respect to other methods because they are small molecules. That being said, there are a number of concerns that must be addressed when utilizing chemosensors. First, sensors must demonstrate a strong affinity and selectivity for binding an AE substrate or product to monitor AE systems, such as ATP, AMP, or PPI. However, most sensors cannot be universally selective, and depending on the system, will demonstrate off-target binding. Sensors that demonstrate significant non-specific binding can create difficulties in monitoring enzymatic breakdown of substrates or generation of products due to confounding signal generation. This can be even more difficult because many sensors are sensitive to changes in sample buffer conditions, leading to altered selectivity, affinity, or signal transduction. For these reasons, chemosensor-based assays for enzyme activity are a more accessible and a less time-consuming option. Though, these assays are very sensitive to changes in conditions and require significant optimization.
3.1.1.3 Necessity for High Throughput Adenylating Enzyme Activity Assays

Of the aforementioned assays, chemosensor-based approaches are the most ideal for application to high throughput systems. The time and cost constraints of ATP-Pi exchange assays and SDS-PAGE- and western blot-based assays limit the feasible amount of data that scientists can obtain within a period of time whilst consuming a sizeable amount of research funding. With completion times in as low as 10 minutes and relatively inexpensive pricing, chemosensor-based assays can be applied in large scale testing of enzyme activity.

Thus, the need for developing a high throughput assay for AE activity is clear. Having inexpensive high throughput methods for assaying AE activity increases accessibility of tools to a greater number of scientists to advance research. In addition, such assays can increase drug development for AEs and spur additional fundamental studies of the mechanisms behind these enzymes. For these reasons, there is a utility for robust AE activity assays.

3.2 Aims of This Research

This chapter outlines how we proposed to adapt ProxyPhos, a fluorescence-based PPi chemosensor for the development of high throughput screening assays for AE activity, with the goal of utilizing these screens as a drug development platform. In the following sections the sensor and its utility for AE activity assays, the assay experimental design, the buffer condition optimizations performed, and the troubleshooting that took place are described.

3.3 ProxyPhos: Structure and Spectroscopic Properties

ProxyPhos (Figure 3.4) is an excimer-based fluorescent chemosensor developed by the Gunning Group. The sensor is composed of a zinc cyclen unit for binding negatively charged analytes, a methylene linker, and a pyrene reporter unit which characteristically fluoresces between 365-430 nm (Figure 3.5b) when excited at its most red-shifted absorbance band (350 nm) (Figure 3.5a). However, when two sensor molecules’ pyrene units come into close proximity, red-shifted excited state dimer (excimer) fluorescence is observable at between 440 and 600 nm (Figure 3.5b). Thus, when two sensor molecules are able to bind species in close proximity, their pyrene units are able to come in sufficient proximity to exhibit increased excimer fluorescence. For example, with increasing concentrations of PPi, excimer fluorescence increases because...
ProxyPhos binding to multiple charges on PPi facilitates more than one sensor molecule coming into close proximity (Figure 3.5b).

Figure 3.4. Chemical Structure of ProxyPhos reported by Kraskouskaya et al.\textsuperscript{52}
Figure 3.5. a) UV-Vis absorbance spectrum of ProxyPhos dissolved in 10% ethanol in MilliQ water; b) fluorescence intensity scan of ProxyPhos in pH=7.5 50 mM HEPES 20% DMSO 75 mM NaCl buffer with increasing concentrations of pyrophosphate: 0, 5, 10, 20, 40, 80 μM. Figure highlights changes in fluorescence signals for monomer and excimer regions in response to PPI concentration with a schematic of hypothesized underlying process.

Because ProxyPhos exhibits two characteristic fluorescence emission maxima, there are a number of different data transformations that can be employed to monitor changes in the proportion of excimer and monomer populations. First, there is the fluorescence intensity change (ΔFI) parameter (Equation 1). This parameter is determined by dividing the fluorescence emission of the sensor at a particular wavelength when incubated with an analyte, by the emission of the sensor without analyte at the same wavelength. For example, $\Delta F_{I,\lambda=476\,\text{nm}}$
refers to the ratio of fluorescence intensity of ProxyPhos emission ($\lambda=476$ nm) with analyte divided by its intensity ($\lambda=476$ nm) without analyte. For most experiments, $\Delta FI_{\lambda=476\text{nm}}$ and $\Delta FI_{\lambda=376\text{nm}}$ are determined because they allow for monitoring changes in excimer and monomer proportions in response to analyte respectively. The larger the proportion of target analyte, the greater the changes in $\Delta FI_{\lambda=476\text{nm}}$ and $\Delta FI_{\lambda=376\text{nm}}$. Additionally, fluorescence enhancement ($Fe$) (Equation 2) is calculated by dividing $\Delta FI_{\lambda=476\text{nm}}$ by $\Delta FI_{\lambda=376\text{nm}}$, and combines information from both wavelengths. Fe is important because it reflects changes in both excimer and monomer proportions.

ProxyPhos is applied to in vitro assays involving incubation of the sensor with analyte in a buffered solution for 20 minutes prior to fluorescence measurements.

**Equation 1.** Fluorescence intensity change formula for a particular wavelength, $\lambda$.

$$\Delta FI_{\lambda=\text{n}} = \frac{FI_{\lambda=\text{n},\text{analyte}}}{FI_{\lambda=\text{n},\text{noanalyte}}}$$

**Equation 2.** Fluorescence enhancement formula: fluorescence intensity of a solution at $\lambda=476$ nm divided by the fluorescence intensity of the same solution at $\lambda=376$ nm.

$$Fe = \frac{\Delta FI_{\lambda=476\text{nm}}}{\Delta FI_{\lambda=376\text{nm}}}$$

### 3.3.1 ProxyPhos: a Selective Binder of Pyrophosphate in vitro

In order to determine ProxyPhos’ suitability for the development of a high throughput assay for AE activity, we assessed its ability to detect a number of biologically relevant phosphoanions. ProxyPhos’ ability to undergo excimer formation in the presence of these species was assessed by incubating 40 $\mu$M sensor with the following analytes between 80 $\mu$M to 40 nM: ATP, ADP, AMP, UTP, UDP, UMP, TTP, GTP, GDP, GMP, CTP, CMP, PPi, Pi, and $\beta$-casein in 50 mM HEPES, pH 7.5, 75 mM NaCl in 20% dimethyl sulfoxide (DMSO) (Figure 3.6a).
Figure 3.6. a) Fe signal of ProxyPhos incubated with varying concentrations of phosphoanions: Analytes (80, 40, 20, 10, 5, 2.5, 1.25, .625, .3125, .15625, .078125, .039063 μM were combined with 40 μM ProxyPhos sensor in 50 mM HEPES pH 7.5, 75 mM NaCl, 20% DMSO; b) Fe signal of ProxyPhos incubated with 80 μM phosphoanions from 3.6a: Analytes were combined with 40 μM ProxyPhos sensor in 50 mM HEPES pH 7.5, 75 mM NaCl, 20% DMSO. Error bars reported are 1 standard deviation calculated between 3 replicate trials.

In this screen we observed that nucleotide triphosphates generated greater signals than nucleotide diphosphates, and that nucleotide monophosphates possess the smallest signals. This trend is enhanced specifically in Figure 3.6b, where Fe is reported for 40 μM ProxyPhos incubated with these analytes at 80 μM concentrations in 50 mM HEPES, pH 7.5, 75 mM NaCl in 20% DMSO. Interestingly though, PPi generates a significantly larger signal than any of the adenosine phosphates. For both the ability to selectively detect PPi over adenosine phosphates and the ability to observe concentration-dependent changes in fluorescence of ProxyPhos with these ions, we became interested in applying ProxyPhos to assays for adenylating enzyme activity. Specifically, because the sensor generates strong selective signals with PPi, it could be applied to sense production of PPi as a measure of ATP consumption and AE activity.
3.4 Experimental Design

3.4.1 Method

Figure 3.7 describes the experimental design for a ProxyPhos-based AE activity assay. The method begins with incubation of the enzyme UBA5 (an E1 activating enzyme for the UFM1 Ubl), UFM1 (ubiquitin-fold modifier 1), and ATP for a set period of time in a 384 black well plate. The reaction can then be quenched with addition of stopping buffer, after which ProxyPhos can be added for 20 minute incubation. During this incubation time, the sensor can bind to target analytes (ideally) in 2:1 ratios to facilitate excimer fluorescence upon excitation at 350 nm. It would not be ideal to add the sensor to the mixture prior to reaction incubation, because the sensor could influence the progression of the reaction by binding to a site on an enzyme, ATP, cofactor, or buffer species.

Figure 3.7. Experimental design for application of ProxyPhos to adenylating enzyme assay. First, E1 activating enzyme, E2 conjugating enzyme, ATP, Ub/Ubl will be incubated for a set period follow by addition of ProxyPhos. Half of each reaction well will be used for fluorescence measurements, with the other half applied to SDS-PAGE and or western blotting as a validation tool. Fluorescence data and SDS-PAGE/western blot data can then be compared to a study by Bacik et al. 2010 where the activity of UBA5 was studied.24
An E1 activating enzyme (UBA5) was picked because it is an AE that incorporates ATP-dependent molecular weight changes in protein species that can be monitored via robust western blot-based E1-E2 transthiolation assays. Therefore, product formation in transthiolation assays can be utilized as a validation strategy by correlating the data with ProxyPhos’ fluorescence measurements. UBA5 was chosen over UBE1 because of a recently published paper supporting that UBA5, unlike UBE1, does not require formation of a ternary UBA5-UFM12-AMP (UFM1-AMP)-UBA5-S-UFM1 (where -S- refers to a thioester linkage) complex prior to transfer of UFM1 to the E2 enzyme (Ufc1). Instead, the UBA5-S-UFM1 complex can directly transfer UFM1 to the E2. Since the pathway requires less steps prior to transfer of UFM1, the simpler system allow for relatively more facile experimental design and data analysis.

To validate ProxyPhos’ ability to monitor the breakdown of ATP to AMP and PPi, each trial well (60 μL) will be split between E1-E2 transthiolation assays (30 μL) and fluorescence measurements with ProxyPhos (30 μL). Because of the molecular weight changes in UBA5 upon UFM1 conjugation, the transthiolation assays allow for tracking UBA5-UFM1 formation and concomitant ATP consumption. Therefore, the transthiolation assay data ideally should correlate with ProxyPhos fluorescence data if it functions as desired. For transthiolation and fluorometric methods, buffer conditions such as composition, component concentrations, reaction volumes, and incubation times must extensively optimized to obtain the highest signal to noise ratio in fluorescence measurements for PPi whilst retaining enzyme activity.

3.4.2 Measurement Types

Kinetic measurement and end-point measurement experimental designs will be explored. In kinetic measurement experiments, individual replicate reactions will be stopped at various time points with a stopping buffer prior to addition of ProxyPhos to monitor PPi formation over time. In end-point measurements, samples are incubated for a fixed period of time prior to termination and addition of stopping buffer.

3.5 Validation Studies and Buffer Optimization

3.5.1 Validation Studies: Control Experiments

In order for ProxyPhos to be applied in enzymatic systems, a series of experiments were conducted. First, we probed the role of zinc in ProxyPhos’ binding to target analytes by
incubating 40 μM sensor lacking metal with PPI, ATP, AMP, and BSA between 80 μM and 40 nM in 50 mM HEPES, pH 7.5, 75 mM NaCl, and 20% DMSO buffer (Figure 3.8). As the profile of each analyte with ProxyPhos became flat without zinc in the sensor (relative to Figure 3.6a), it appears that the metal is necessary for detection of these analyte species and facilitating excimer formation.

![Figure 3.8](image)

Figure 3.8. Fe signal of unmetallated ProxyPhos (40 μM) incubated with varying concentrations of ATP, AMP, PPI, and BSA (80, 40, 20, 10, 5, 2.5, 1.25, .625, .3125, .15625, .078125, .039063 μM) in HEPES pH 7.5, 75 mM NaCl, 20% DMSO. Error bars reported are 1 standard deviation calculated between 3 replicate trials.

3.5.2 Validation Studies: Buffer Optimization

Next, in recognizing that the original buffer optimized for ProxyPhos, is not optimized for AEs (Table 1) (eg. UBE1, UBA5), we decided to explore the effects of buffer changes upon the sensor selectivity profile (Figure 3.9). The optimization of the UBA5 transthiolation reaction buffer was not performed concurrently with the buffer for ProxyPhos because UBA5, UFM1 and Ufc1 were not available at the time. To assess ProxyPhos’ selectivity, buffers with varying pH, salt concentrations, DTT, and DMSO were tested. Early on we recognized that it would be neither cost effective nor time efficient to generate and test all possible buffers. Instead, a hierarchical approach was decided in which buffer components hypothesized to have the greatest impact on sensor selectivity were tackled first. The order of components in the hierarchy is listed as follows: pH>MgCl₂>DTT>[NaCl],[DMSO]. pH was tackled first because of its ability to alter the protonation states of both sensor and analyte species and the potentially dramatic effect of this protonation on sensor analyte interactions. At pH 6.5, the selectivity of ProxyPhos (40 μM) for PPI over ATP, AMP, and BSA decreased along with signal enhancement for PPI which fell approximately by half (Figures 3.9a, b). This loss of selectivity and signal enhancement for PPI between Figure 3.9a and 3.9b could be explained by protonation of PPI negative charges in the
pH 6.5 solution, masking the charges with which zinc could form electrostatic interactions. Though, regardless of the buffer pH, MgCl₂ almost entirely eliminated both any signal and any selectivity for substrates (Figure 3.9c, d).

Table 1. Buffer Composition for ProxyPhos, UBE1, and UBA5

<table>
<thead>
<tr>
<th>ProxyPhos Buffer</th>
<th>UBE1</th>
<th>UBA5</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM HEPES</td>
<td>50 mM HEPES</td>
<td>50 mM Bis-Tris</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>pH 7.5</td>
<td>pH 6.5</td>
</tr>
<tr>
<td>75 mM NaCl</td>
<td>50 mM MgCl₂</td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td>20 % DMSO</td>
<td>50 mM BSA</td>
<td>10 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 mM DTT</td>
</tr>
</tbody>
</table>
Figure 3.9. Fe signal of ProxyPhos (40 μM) incubated with varying concentrations of ATP, AMP, PPI, and BSA (80, 40, 20, 10, 5, 2.5, .125, .625, .3125, .15625, .078125, .039063 μM) in varying buffers: a) 50 mM HEPES pH 7.5, 75 mM NaCl, 20% DMSO, b) 50 mM Bis-Tris, pH 6.5, 75 mM NaCl, 20% DMSO, c) 50 mM HEPES, pH 7.5, 75 mM NaCl, 20% DMSO, 10 mM MgCl₂, d) 50 mM Bis-Tris, pH 6.5, 75 mM NaCl, 20% DMSO, 10 mM MgCl₂. Error bars reported are 1 standard deviation calculated between 3 replicate trials.

Next, buffer optimization with MgCl₂ was performed because magnesium’s coordination to PPI could lead to outcompeting the sensor. When a fluorescence intensity scan was conducted with ProxyPhos incubated with PPI at various concentrations in a buffer containing 10 mM MgCl₂ (in 50 mM HEPES, pH 7.5, 75 mM NaCl, 20% DMSO), all excimer-based fluorescence about 470 nm was abolished (Figure 3.10b) relative to solution lacking MgCl₂ (Figure 3.10a). Although
incredibly problematic, this magnesium-accompanied loss of signal is not entirely surprising as magnesium is known to coordinate to ATP, and PPI and likely is in such excess (10 mM) that it strongly outcompetes the sensor in binding (40 μM). However, this interpretation does not explain how magnesium decreased background excimer formation of ProxyPhos without analyte in solution (Figure 3.10b, 0 μM PPI) relative to buffer conditions without MgCl₂ (Figure 3.10a, 0 μM PPI). This loss of background excimer formation may be attributed to Cl⁻ (20 mM) coordination to the open coordination sites of the ProxyPhos Zn²⁺ ions. Since chloride is in excess, binding to sensor may lead to dispersion of ProxyPhos in solution, a reduced probability of ProxyPhos pyrene reporters moving into close proximity, and reduction of basal excimer formation.

Figure 3.10. Fluorescence intensity scan of ProxyPhos (40 μM) with varying concentrations of PPI (80, 10, 0 μM) in a) pH 7.5, 50 mM HEPES, 20% DMSO, 75 mM NaCl, and b) pH 7.5, 50 mM HEPES, 20% DMSO, 75 mM NaCl, 10 mM MgCl₂.

The drastic reduction of ProxyPhos excimer signal for PPI in the presence of MgCl₂ presented a significant impetus for mitigating its effects because of the importance of magnesium for AE activity meant that it could not be removed. As a result, we explored the chelation of excess magnesium prior to incubation of ProxyPhos and the application of an unmetallated sensor.

First, we attempted to mitigate the effects of magnesium on ProxyPhos by masking the ions with the chelating agents ethylenediaminetetraacetic acid (EDTA), dipicolylamine (DPA), and 18-crown-6 (Figure 3.11a-c). These molecules were chosen due to their known ability to coordinate to magnesium and, because they were available in our lab. To assess the chelating agents, we
increased their concentrations in the presence of sole ProxyPhos (40 μM) and ProxyPhos (40 μM) with PPI (80 μM) to determine their effects on the sensor in isolation and on sensor detection of PPI. To determine the chelating agents their abilities to recover ProxyPhos signal, these experiments were performed again in solutions containing and omitting 10 mM MgCl₂. An optimal chelating agent for application would recover of ProxyPhos signal with PPI (80 μM) in the presence of 10 mM MgCl₂ when employed with increasing concentration, without adversely affecting analyte detection.
Figure 3.11. Fe and $\Delta F \lambda=476\text{nm}$ of ProxyPhos (40 μM) with increasing concentrations of a) EDTA, b) DPA, and c) 18-crown-6 in varying mixtures of 10 mM MgCl$_2$ and 80 μM PPI in 50 mM HEPES, pH 7.5, 75 mM NaCl, 20% DMSO. Error bars reported are 1 standard deviation calculated between 3 replicate trials.

Increasing EDTA concentration, when magnesium was not present, led to loss of signal with both ProxyPhos in isolation and ProxyPhos with 80 μM PPI (Figure 3.11a). This is not surprising because the higher concentrations of EDTA permit it to potentially remove the zinc from the cyclen binding unit, eliminating all selectivity of ProxyPhos for its analytes and its ability to induce excimer formation. In the presence of magnesium, recovery of ProxyPhos signal with PPI was observed at equimolar EDTA:MgCl$_2$ (10 mM:10 mM). With DPA, recovery of signal was
observed above 1:1 DPA:MgCl₂ (20 mM DPA). However, this signal increase of ProxyPhos with DPA in the presence of MgCl₂ also occurred when PPI was absent. Further, the curve has the same profile, suggesting that DPA itself can increase the signal of the sensor in isolation. The signal increase could be derived from the two pyridine-type nitrogens on a DPA molecule coordinating to the zins within two separate sensor molecules, facilitating excimer formation and signal increase. Regardless, it appears that DPA is ill-fitted for use in our assay. 18-crown-6 on the other hand exhibited no effects within any of the conditions tested. This is not entirely surprising as most crown ethers preferentially bind alkali metals over divalent metal ions. 18-crown-6 was tested because an analogous crown ether with two appended cyclohexyl groups was found to bind magnesium. That said, the relatively high concentration of NaCl (75 mM) could have easily outcompeted the magnesium for chelation. In retrospect, 18- and 21-membered diazacrown ethers would have been more appropriate for application due to both their larger ring sizes and stronger affinity for Mg²⁺.

For the sake of trying, we opted to test unmetallated sensor to see if it could recover signal for PPI over ATP, AMP, and BSA in the presence of magnesium, which ordinarily binds tightly to the target species (Figure 3.12). Expectedly, the selectivity profile between analytes was not improved, and recovery of signal did not occur.

![Figure 3.12. Fe signal of metallated and unmetallated ProxyPhos (40 μM) incubated with varying concentrations of ATP, AMP, PPI, and BSA (80, 40, 20, 10, 5, 2.5, 1.25, .625, .3125, .15625, .078125, .039063 μM) in HEPES pH 7.5, 75 mM NaCl, 20% DMSO, 10 mM MgCl₂. Error bars reported are 1 standard deviation calculated between 3 replicate trials.](image-url)
In summary, it appears that EDTA holds promise for recovery of magnesium-mediated signal and selectivity loss when employed at equimolar concentrations to magnesium.

Figure 3.13. Fe, $\Delta F_{\lambda=476\text{nm}}$ and $\Delta F_{\lambda=376\text{nm}}$ of ProxyPhos (40 μM) with increasing concentrations of DTT in varying mixtures of 10 mM MgCl$_2$ and 80 μM PPI in 50 mM HEPES, pH 7.5, 75 mM NaCl, 20% DMSO. Error bars reported are 1 standard deviation calculated between 3 replicate trials.

After, DTT was explored as a buffer component. DTT was tested similarly to EDTA in the presence and absence of magnesium and PPI (Figure 3.13). All four profiles were extremely similar despite the presence or absence of 10 mM MgCl$_2$ or 80 μM PPI. Moreover, all profiles increased in Fe in response to increasing DTT before later decreasing. This increase may be explained by DTT acting in a similar fashion to the hypothesized mechanism for DPA, in that DTT could be facilitating excimer formation by binding to two zinc ions in two ProxyPhos molecules via its sulfhydryl groups. The soft sulfhydryl donor and soft zinc ions could be generating complexes that are stable in the presence and absence of PPI and or magnesium. However, in interpreting the data it must be recognized that DTT can oxidize in solution, to varying degrees depending on the species in solution. Because of this variable oxidation, the proportion of DTT populations may vary even between different data points with different DTT concentrations. Regardless, considering that DTT is used up to 100 μM in the UBA5 buffer, it may still be employable in the buffer at concentrations that reduce its purported adverse effects.
Finally, in an effort to increase the observed signal of ProxyPhos in response to PPI, we attempted to determine the approximate binding stoichiometry by increasing ProxyPhos concentration in the presence of 1 and 80 μM PPI (Figure 3.14). Interestingly, Fe increased with increasing ProxyPhos concentration until roughly a 1:1 ratio was observed, after which Fe decreased. This result is surprising considering that the premise for this sensor’s application is that signal increase is coupled to two sensors becoming proximal upon binding PPI to facilitate excimer formation. Also, where $\Delta F_{\lambda=476\text{nm}}$ similarly increases until 80 μM, $\Delta F_{\lambda=376\text{nm}}$ decreases till just after 1 μM, after which a slight increase is observed until 80 μM. These latter two profiles dictate that excimer formation increased and that monomer signal decreased in concert until 1 μM, after which excimer formation increased while monomer signal slightly increased. This unexpected binding stoichiometry could be facilitated by a higher order complex beyond 2:1 ProxyPhos:analyte (Figure 3.15). Specifically, the 1:1 binding stoichiometry could be evidence of a 2:2 ProxyPhos:analyte ratio. The negatively charged oxygen atoms on both ends of a PPI could bind to two sensor molecules’ zinc atoms, and another PPI’s oxygen atoms could bind to the other axial sites on the zincax of the same sensor molecules to generate two zinc octahedral complexes with 4 donor nitrogen atoms (cyclen) and 2 donor oxygens (PPI). This binding mode is supported by the increase in excimer signal despite approaching 1:1 ProxyPhos:analyte. Moreover, this binding mode could explain the slight increase in monomer signal by rationalizing the resultant higher order complex as slightly less stable, allowing for greater spacing between pyrene reporter units. Irrespective, this experiment demonstrates that the
studies previous performed with 40 μM sensor were understating the optimal signal that could be observed with optimized sensor concentrations.

Figure 3.15. Schematic of hypothesized binding interactions between ProxyPhos and PPI within solutions of increasing PPI concentration.

3.6 Discussion

In this chapter we explored the application of ProxyPhos, a fluorescence-based chemosensor to an enzyme activity assay for AEs. In our studies, we were able to determine that ProxyPhos is a selective binder of PPI over ATP, ADP, AMP, Pi, and BSA in pH 7.5, 50 mM HEPES, 75 mM NaCl, and DMSO. Interestingly, ProxyPhos appears to exhibit an unusual 1:1 (or possible 2:2) sensor: analyte binding stoichiometry despite the sensor having an excimer-based reporter group. Studies without Zn$^{2+}$ also suggest that the divalent zinc of ProxyPhos plays a role in binding as the absence leads to loss of sensor selectivity and signal enhancement.

In optimization of the sensor conditions for the application to an enzymatic system, ProxyPhos (40 μM) and analyte (80-80.039063 μM) concentrations remained constant as reaction conditions were varied. Lower pH was found to decrease signal for PPI, and resultantly also decrease selectivity for PPI. This result was likely caused by protonation of PPI’s negative charges, reducing the affinity of the zinc cyclen binding group on the sensor for analyte. The addition of MgCl$_2$ problematically removed any selectivity of ProxyPhos for analytes, and abolished all foreseeable excimer fluorescence in fluorescence intensity scans. To mitigate this problematic finding, EDTA appears to hold promise for its ability to recover signal of ProxyPhos with PPI in the presence of 10 mM MgCl$_2$ (when 1:1 EDTA: MgCl$_2$). In contrast to MgCl$_2$, DTT presents the opposite problem by inducing excimer formation with increasing concentrations possibly by bridging pairs of sensors with its sulfhydryl groups. Although not ideal, the concentration of
DTT can be optimized and assessed, as a maximum concentration of 100 μM is required for in vitro UBA5 enzymatic assays.

We also determined that the sensor concentration employed for most of the studies presented in this chapter understated the true signal of ProxyPhos in the presence of PPI. The knowledge of a possible 2:2 ProxyPhos:PPI binding ratio could lead to greater enhancement for PPI in future assays, excitedly reducing the effects of magnesium and DTT-induced signal changes. Though, the exact application of ProxyPhos in this 2:2 ratio is difficult because final PPI concentrations produced by enzymatic reactions are difficult to predict prior to sensor incubation. For this reason, optimization may have to occur for each application of ProxyPhos within enzymatic systems to maximize signal enhancement with PPI, with previous studies of these enzymes used as a starting point. That said, limitations to this approach will likely occur in detection of small quantities of PPI by employing concentrations of ProxyPhos lower than the tested 40 μM. With decreasing concentrations of sensor, which as evidenced in Figure 3.14 an appreciably low change in signal is observed. Until the final buffer conditions are established, this lower limit of sensor application cannot be sufficiently hypothesized.

Due to ProxyPhos’ ability to selectively bind PPI over other phosphoanions, our application of the sensor to conditions typical to testing the activity of AEs, our recovery of lost signal with magnesium, and the ability to increase the signal ratio via sensor concentration optimization, we are optimistic as to the potential for future application of ProxyPhos to an AE activity assay for drug discovery.

### 3.7 Future Directions

In order to move forward with application of ProxyPhos in an enzymatic system, a number of experiments need to be performed. First, alternative EDTA concentrations for MgCl₂ must be explored. Currently, the concentrations of EDTA tested closest to signal recovery were 5, 10, and 20 mM. Considering that 10 mM was the only concentration to observe recovery of signal, the experiment must be repeated with greater data points about 10 mM to determine the optimal ratio. From here, another selectivity screen of ProxyPhos for PPI, ATP, AMP, and BSA can be conducted in pH 7.5, 50 mM HEPES, 75 mM NaCl, 20% DMSO, and 10 mM MgCl₂, in the presence of an optimal EDTA concentration for maximal magnesium chelation. This study would allow for assessment of whether EDTA can both lead to recovery of signal, and selectivity.
for PPi over the other anions and BSA. This study would be followed with a trial including 0.1 mM DTT to ascertain the acceptable levels of this species in solution. Application of the sensor to UBA5’s system also requires assessment of sensor compatibility with competition studies. Specifically, when probing the effects of various proteins’ and ligands’ effects on reaction equilibria, their concentrations are varied between trials. Since ProxyPhos’ positively charged zinc-based binding group does not have universal selectivity for binding negative charges and that the pyrene reporter group’s hydrophobicity can facilitate indiscriminate interactions with lipophilic molecules, hydrophobic and negatively charged species must be screened for their off-target affinities with ProxyPhos to better understand the limitations of applicability for the assay.

3.8 Contributions

The experiments within this chapter were designed and performed by Harris I. Qureshi and Jia Nan (Cathy) Xu. Dr. Claire V. Sauvée and Dr. David Rosa contributed to the synthesis of ProxyPhos for its application within these experiments.

3.9 Materials and Methods:

3.9.1 Reagents

384 well black flat bottom polystyrene plates were purchased from Corning. TCEP was purchased from Alfa Aesar, DTT was purchased from Bioshop Canada, and all other reagents including nucleotides, PPi, Pi, BSA, MgCl₂, DMSO, and NaCl were purchased from Sigma-Aldrich.

3.9.2 Fluorescence Assays

The following buffers were prepared: a) 50 mM HEPES, pH 7.5, 75 mM NaCl, 20% DMSO, b) 50 mM Bis-Tris, pH 6.5, 75 mM NaCl, 20% DMSO, c) 50 mM HEPES, pH 7.5, 75 mM NaCl, 20% DMSO, 10 mM MgCl₂, d) 50 mM Bis-Tris, pH 6.5, 75 mM NaCl, 20% DMSO, 10 mM MgCl₂.

ProxyPhos (40-100 μM) was incubated with varying concentrations of protein and nucleotides dissolved in fresh buffers. Samples were incubated in 384 well black flat bottom polystyrene plates for 20 minutes prior to taking readings on Tecan Infinite® m1000, or Cytation™ 3
machines. For each experiment, a separate sample was prepared with ProxyPhos dissolved in buffer without analyte.

For all experiments, 350 nm (5 nm bandwidth) excitation, 376 and 476 nm (20 nm bandwidth) emission wavelengths were selected.

3.9.3 Absorbance Experiments

Complexes were dissolved in Milli-Q® H₂O prior to readings in a HP 8452A Diode-Array Spectrometer (Hewlett Packard Corporation, Palo Alto, CA, USA).

4 Concluding Remarks

This thesis’ focus has been concerned with targeting AEs implicated in diseased states using small molecules to assess their therapeutic potential. In chapter 2, the assessment of a previously developed rationally designed copper-cyclen based library of potent, reversible, and selective UBE1 inhibitors was conducted. Through extensive investigation, it is supported that inhibitor activity was derived from excess copper impurity from improper sample preparation. Due to copper’s ability to provide false positives in enzymatic assays by binding Ub and its ability to quench fluorescence and mimic an inhibitory signal in a collaborator assay, these compounds provided the optimal conditions for error. Through examination of the crystal structure, it was apparent that the use of copper may have been inappropriate given its ability to indiscriminately bind the high number of cysteine and histidine residues on the surface of the enzyme. The report of copper-based inhibitors of the pathway that act via Ub also raised concern that the method employed could have inappropriately even led to inhibition via substrate binding. Despite the failure of the compounds, UBE1 still remains a validated target worthy of focus for drug development, albeit with alternative small molecule inhibitors. In chapter 3, the development of a high throughput screening assay for AE activity was explored. Due to the low cost and incubation times necessary, a fluorescent chemosensor-based approach was targeted as it possesses the greatest potential to increase access to drug discovery and pathway elucidation research tools. The ProxyPhos sensor developed by the Gunning group demonstrated selectivity for PPi over other AE substrates and products and can monitor concentration dependent changes in these species. Optimization of the sensor buffer conditions provided challenges with pH and MgCl₂ that have potential to be overcome with the application of EDTA to chelate the
magnesium and increasing sensor concentration to achieve ideal ProxyPhos:analyte ratios. Recognizing that chemosensors cannot be universally selective, some investigation as to the limits of ProxyPhos’ selectivity must occur in addition to salt and DMSO concentration optimization. However, given the results, there is optimism that this sensor could allow for the generation of a new chemosensor-based high throughput screening assay for adenylating enzymes.
References


(7) *J. Clin. Oncol.*


