Title
Esterase and reactive-oxygen species (ROS)-activated prodrug strategies

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Esterase and reactive-oxygen species (ROS)-activated prodrug strategies

A Thesis submitted in partial satisfaction of the requirements for the degree of
Master of Science

in

Chemistry

by

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Committee in charge:

Professor Seth M. Cohen, Chair
Professor Thomas Hermann
Professor Dionicio R. Siegel

2015
The Thesis of Christian Perez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015
DEDICATION

To my mom, family, and friends, thanks for your unconditional support.
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## LIST OF SYMBOLS AND ABBREVIATIONS

<p>| Ac          | Acetyl                                      |
| Ac₂O       | Acetic Anhydride                            |
| AcOH       | Acetic Acid                                 |
| ACE        | Angiotensin Converting Enzyme               |
| ACN        | Acetonitrile                                |
| ADME       | Absorption, Distribution, Metabolism, and Excretion |
| CES        | Carboxylesterase                            |
| COX        | Cycloxygenase                               |
| δ          | Chemical shift, ppm                         |
| d          | Doublet (NMR)                               |
| DCC        | $N,N'$-Dicyclohexylcarbodiimide             |
| DMAP       | 4-Dimethylaminopyridine                     |
| DMF        | $N,N'$-dimethylformamide                    |
| DMSO       | Dimethylsulfoxide                           |
| DNA        | Deoxyribonucleic acid                       |
| EDC        | 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide |
| ESI-MS     | Electrospray Ionization Mass Spectrometry   |
| EtOH       | Ethanol                                     |
| EtOAc      | Ethyl Acetate                               |
| FDA        | Food and Drug Administration                |
| FRET       | Förster Resonance Energy Transfer           |
| GSH        | Glutathione                                 |</p>
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<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HOBT</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HOPO</td>
<td>Hydroxypyridinone</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectrometry</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz (s⁻¹)</td>
</tr>
<tr>
<td>IBU</td>
<td>Ibuprofen</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half Maximal Inhibition Concentration</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>kᵦᵢₙ</td>
<td>Rate Constant</td>
</tr>
<tr>
<td>λ</td>
<td>Wavelength; nm</td>
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<tr>
<td>m</td>
<td>Minute</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet (NMR)</td>
</tr>
<tr>
<td>MBP</td>
<td>Metal-Binding Pharmacophore</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<td>mM</td>
<td>Millimolar</td>
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<td>mmol</td>
<td>Millimoles</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MMPi</td>
<td>Matrix Metalloproteinase Inhibitor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance Spectroscopy</td>
</tr>
<tr>
<td>PLE</td>
<td>Porcine Liver Esterase</td>
</tr>
<tr>
<td>proIBU</td>
<td>Ibuprofen Prodrug</td>
</tr>
<tr>
<td>proMBP</td>
<td>Metal-binding Pharmacophore Prodrugs</td>
</tr>
<tr>
<td>proMMPi</td>
<td>MMP Inhibitor Prodrug</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>s</td>
<td>Singlet (NMR)</td>
</tr>
<tr>
<td>SIL</td>
<td>Self-immolative Linker</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
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Chapter 3, in part, is a reprint of the materials published in the following papers: Christian Perez, Jean-Philippe Monserrat, Yao Chen, and Seth M.
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ABSTRACT OF THE THESIS

Esterase and reactive-oxygen species (ROS)-activated prodrug strategies

by

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Master of Science in Chemistry

University of California, San Diego, 2015
Professor Seth M. Cohen, Chair

Prodrugs are derivatives of bioactive molecules that can become activated in vivo by a chemical or enzymatic stimulus. They serve as tools to help improve physicochemical or pharmacokinetic properties of active agents to overcome
barriers that effect drug formulation. The work in this thesis explores the development of two novel prodrug strategies.

In Chapter 2, several metalloenzyme inhibitor prodrugs that become activated in the presence of esterase were developed. A study that contrasts differences between directly acetylated drugs and ones appended with an acetylated self-immolative linker were investigated. In this study the activation kinetics, aqueous stability and inhibition profile of the prodrugs was evaluated.

Chapter 3 highlights the development of a novel prodrug strategy utilizing thiazolidinones that mask carboxylic acids. The prodrugs developed were reactive in the presence of hydrogen peroxide. Specific activation by hydrogen peroxide may allow for targeted activation of these prodrugs. These strategies were applied to an FDA approved anti-inflammatory drug and a known metalloenzyme inhibitor. Their responsiveness to hydrogen peroxide was investigated, as well as their aqueous stability in the presence of biologically relevant nucleophiles. Lastly, their inhibition profile was evaluated as well as the cytotoxicity of the promoiety.
Chapter 1. Introduction
1.1 Prodrugs

Prodrugs are derivatives of bioactive molecules that can become activated in vivo by a chemical or enzymatic stimulus.\(^1\) They serve as tools to help improve physicochemical or pharmacokinetic properties of active agents to overcome barriers that effect bioavailability and ultimately drug efficacy in vivo.\(^1\) These barriers often involve difficulties associated with absorption, distribution, metabolism, and excretion (ADME).\(^2\)-\(^4\) Prodrugs are typically designed wherein the active drug is appended with a covalent masking/ reactive group, which renders the drug inactive or substantially less active. This masking group differs according to the functional group being masked, and is commonly referred to as a promoiety. The prodrug is designed so that the promoiety is removed in the presence of a specific metabolite or enzyme, which liberates the active drug.\(^3\)

The term “pro-drug” was first introduced by Adrien Albert in 1958 when describing the effects of selective toxicity of certain substances. Albert’s findings described how some substances when administered in this “prodrug” form can be broken down to reveal the true drug.\(^2\) The first examples of prodrugs were not intentionally designed as prodrugs. Like in the case of the antibiotic protonsil, the drug itself was not active, however one of its metabolites was responsible for exerting the desired pharmacological effect. This is regarded as one of the first prodrug examples, although it was not designed to behave this way. However, since the 1960’s the concept of prodrug activation by various chemical and enzymatic stimuli has been largely
explored, and has lead to development of many successful therapeutics (Table 1-1). Presently, the success of the prodrug approach is illustrated by the fact that ~10% of marketed clinical therapeutics are classified as prodrugs.²

**Table 1-1.** Representative list of FDA approved prodrugs.

<table>
<thead>
<tr>
<th>Prodrug Name (Therapeutic Area)</th>
<th>Structure</th>
<th>Strategy</th>
</tr>
</thead>
</table>
| **Enalapril** (angiotensin converting enzyme inhibitor) | ![Structure](https://via.placeholder.com/150) | • Bioconversion by esterases  
• Increased oral bioavailability |
| **Famciclovir** (antiviral) | ![Structure](https://via.placeholder.com/150) | • Bioconversion by esterases and oxidation from purine to guanide |
| **Sulindac** (non-steroidal anti-inflammatory) | ![Structure](https://via.placeholder.com/150) | • Bioprecursor prodrug that is reduced to the active sulfide form after oral absorption  
• ~100 fold increase in aqueous solubility |
<table>
<thead>
<tr>
<th>Drug</th>
<th>Formula</th>
<th>Bioconversion Mechanisms</th>
</tr>
</thead>
</table>
| Miproxifene phosphate         | ![Miproxifene](image) | • Bioconversion by alkaline phosphotases  
• Aqueous solubility increased ~1000 fold |
| Ximelagatran                  | ![Ximelagatran](image) | • Bioconversion by esterases and reductive enzymes  
• Increased oral bioavailability |
| Tenofovir                     | ![Tenofovir](image) | • Bioconversion by esterases and phosphodiesterases  
• Improved oral bioavailability |
| Oseltamivir                   | ![Oseltamivir](image) | • Bioconversion by esterases  
• Increase oral bioavailability |
| Losartan                      | ![Losartan](image) | • Bioconversion by cytochrome P450  
• Alcohol group becomes oxidized to carboxylic acid |
Table 1-1. Representative list of FDA approved prodrugs, continued

|**Fludarabine**  
(anti-viral) | ![Chemical Structure](image) | • Bioconversion by alkaline phosphatases  
• Increase oral bioavailability |

|**Irinotecan**  
(anti-cancer) | ![Chemical Structure](image) | • Bioconversion by carboxylesterases  
• Increased aqueous solubility |

Some of the common functional groups amenable to the prodrug method are hydroxyls, thiols, amines, carboxylic acids, and phosphonates. The high polarity and ionizability of these functional groups can cause undesired effects. Therefore, these functional groups have a tremendous effect on the properties of the drugs, and are commonly masked with promoeities in order to form prodrugs.

### 1.2 Esterase Activated Prodrugs

The most common prodrug approach for delivery of pharmaceuticals is through esterase activation. Bioconversion of the prodrug to the active drug is mediated by carboxylesterases, which are mostly found as two different isoforms, carboxylesterase 1 (CES1) and 2 (CES2). The two isoforms are differentiated by their substrate specificity and tissue distribution. The CES1
isoform is mainly expressed in liver tissue, while CES2 is located mostly in the intestines and kidneys. These differences can be exploited in prodrug design, in order to achieve a specific drug distribution. Prodrugs wherein the promoeity is an acetate or an aryl-acetate group, are predominantly activated in the liver via hydrolysis, mediated by CES1 and butyryl-choline esterases. Esterase activated prodrugs have shown to improve properties of small-molecule drugs including stability, membrane permeability, and oral bioavailability. However, because esterases are ubiquitous and constitutively active, achieving a targeted esterase-activated prodrug strategy can be difficult.

In the case of Irinotecan (Table 1-1), an anticancer topoisomerase I inhibitor, the prodrug is preferentially activated by CES2. Irinotecan is a prodrug of a camptothecin derivative. Camptothecin is cytotoxic compound discovered in a screening of natural products for anticancer activity. It demonstrated effective anticancer activity in the pre-clinical stage, but poor solubility and adverse side effects prevented it from entering clinical trials. However, appending a 4-piperidinopiperidine group through a carbamate linkage, helped increase aqueous solubility 10-fold. Applying a prodrug strategy to a camptothecin derivative allowed it to enter to clinical trials and ultimately achieve FDA approval. This illustrates a success of an esterase prodrug approach.
1.3 Reactive-Oxygen Species Activated Prodrugs

Ideally, a prodrug method could be exploited to achieve targeted release of the active drug. A targeted strategy may be achieved with a promoiety that can be readily cleaved by disease-specific stimuli.\textsuperscript{7,8} Understanding the intrinsic biological differences between normal and cancerous cells and tissues, allows identification of disease specific metabolites that can be exploited for prodrug strategies. Previous work has demonstrated that cancer cells undergo increased oxidative stress, compared to normal cells, caused by oncogenic transformations and changes in metabolism. Consequently, this leads to over production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, hydroxyl radical, and organic peroxides. These ROS contribute to cell proliferation, metastasis, and angiogenesis in addition to DNA alterations and damage, which effectively increase mutation rate within cells directly promoting oncogenic transformations. Therefore ROS are important metabolites found in high concentrations in cancerous cells.

One of the first examples of a ROS-activated prodrug strategy was reported by Jourden and co-workers.\textsuperscript{9,10} This work explored the activation of a matrix metalloproteinase (MMP) inhibitor (MMPi) by hydrogen peroxide ($\text{H}_2\text{O}_2$). MMPs are zinc(II)-dependent proteases capable of degrading all components of the extracellular matrix. They are excreted as zymogens and are activated by proteases, other MMPs, or by ROS. Over activation of MMPs by ROS during an ischemia-induced inflammatory response results in injury.
Due to the correlation between ROS and MMP overactivation, Jourden’s work aimed to develop a strategy to deliver a MMP proinhibitor (proMMPi) that could generate the active inhibitor upon exposure to H$_2$O$_2$.

![Scheme 1](image)

**Scheme 1-1.** The boronic ester promoeity is cleaved by H$_2$O$_2$, initiating a cascade reaction, leading to release of a MMPi.

The promoiety utilized a boronic ester appended benzyl group that is attached to the drug through an ether linkage. Upon exposure to H$_2$O$_2$ the boronic ester becomes hydrolyzed, and the linker undergoes a cascade reaction, which breaks the ether bond, liberating the inhibitor and a quinone methide, which rapidly converts into 4-hydroxybenzyl alcohol in aqueous solution. This work was the first of its kind to show activation of an inhibitor via ROS which can potentially regulate the spatial and temporal activation of an MMP inhibitor.

Another example of a ROS activated prodrug strategy was reported by Mokhir and co-workers.$^{11}$ Mokhir’s work allowed for delivery of an aminoferroocene derivative that became activated in the presence of hydrogen peroxide. The aminoferroocene derivative was linked to a boronic ester of 4-((hydroxymethyl)phenylboronic acid via a carbamate linker. The prodrug was activated in the presence of H$_2$O$_2$ to release two cytotoxic agents.
Scheme 1-2. The boronic ester pro moiety is cleaved by H₂O₂, initiating a cascade reaction, leading to release of two cytotoxic agents and CO₂.

1.4 Focus of the Current Study

The work for this thesis focuses on developing novel prodrug strategies utilizing metalloenzyme inhibitors as model compounds. Two main strategies were developed, an esterase and a ROS-activated strategy. The goal was to expand the options currently available for prodrug strategies.

Chapter 2 explores an esterase-activated strategy for release of alcohols through an acetylated linker. Two MMPis were appended with this strategy, wherein the responsiveness to esterase, aqueous stability, and inhibition profile against MMPs was evaluated.

In Chapter 3, a novel ROS-activated strategy is described which utilizes a thiazolidinone pro moiety to mask carboxylic acids. Here an FDA
approved drug as well as a known MMPi were utilized as model compounds. For this work, the responsiveness to multiple ROS was evaluated, as well as their aqueous stability, and inhibition profile.

1.5 References


Chapter 2. Evaluating Prodrug Strategies for Esterase-Triggered Release of Alcohols
2.1 Introduction

The most common prodrug approach to deliver pharmacologically potent compounds is through esterase bioconversion.\textsuperscript{1,2} The esterases involved in drug metabolism are mainly localized in the liver; among these are carboxyl- and butyrylcholinesterase, which can recognize alkyl and aryl acetate groups as substrates.\textsuperscript{3-5} Ester-based prodrugs have previously been shown to improve the properties of small-molecules.\textsuperscript{3} Esterase-activated prodrugs effectively mask polar moieties with a less polar ester bond, which aids with membrane permeability.\textsuperscript{3} The vast majority of ester prodrugs mask carboxylic acids, with fewer accounts documenting their use to release hydroxyl and phenolic moieties upon hydrolysis. In the latter cases, the hydroxyl moiety (hydroxyl or phenol) is directly esterified and esterase bioconversion leads to release of the drug.

Metalloenzyme inhibitors are a class of compounds that can greatly benefit from a prodrug approach. In fact, the most clinically successful metalloenzyme prodrugs involve alkyl and aryl ester modified carboxylates that target angiotensin-converting enzyme (ACE). In the case of enalapril (marketed as Vasotec, Merck), the ethyl ester prodrug is metabolically converted by esterases to a free carboxylic acid group that can bind to the catalytic Zn(II) ion and attenuate enzyme activity.\textsuperscript{2} Other reports of metalloenzyme prodrug development include matrix metalloproteinase inhibitors (MMPi).\textsuperscript{6-8} MMPs are a family of >20 Zn(II)-dependent endopeptidases that are capable of degrading all components of the extracellular matrix. MMP expression and activity is a highly regulated process under normal physiological conditions.\textsuperscript{9,10} Overexpression and
misregulation of MMPs has implicated these proteases in a number of pathologies including arthritis and tumor cell metastasis.\textsuperscript{11,12} Previous MMP broad-spectrum and selective inhibitors have been developed, but have seen limited clinical success due, in part, to undesired side effects from off-target inhibition and poor bioavailability.\textsuperscript{13} Thus, MMPi stand to benefit from a prodrug strategy. For this reason, MMPs were chosen as our targets of interest for proof-of-concept studies regarding esterase activation of hydroxyl functionalities.

The inspiration for this project came from a several studies that analyzed esterase-activated prodrugs for phosphate functional groups.\textsuperscript{14,15} Our lab has studied prodrug strategies for metalloenzyme inhibitors utilizing glucose and hydrogen peroxide-responsive triggering groups.\textsuperscript{16-18} In an attempt to expand the understanding and chemical tools available for metalloenzyme prodrug design, we hypothesized that a similar esterase strategy (Scheme 2-1, -2) could be appended to metal-binding pharmacophores, (MBPs) that serve as the core scaffold for metalloenzyme inhibitors.

**Scheme 2-1.** Mechanism of activation of an acetylated self-immolative linker that becomes hydrolyzed by carboxylesterases. This then reveals the phosphate functional group of the desired drug. Promoiety highlighted in blue.
Scheme 2-2. Proposed mechanism of activation of an acetylated self-immolative linker that is appended to a hydroxyl functional group of a MBP.

In this study, three different promoieties were coupled to two distinct MBPs. The three different approaches for release of the MBP were studied in the presence of an esterase to identify the best system for the development of potential prodrugs. This then lead to the development of three esterase-responsive strategies that were examined in the context of two MMPi. The aqueous stability, activation kinetics and inhibitory profile were measured for these proMMPi.

2.2 Results and Discussion

2.2.1 Assessment of Ester-Responsive Triggers

The approaches investigated here consist of the following ester-responsive promoieties, all of which are appended to the hydroxyl group of the MBP: (1) direct acetylation, (2) a benzyl ether protecting group containing an acetylated phenol, and (3) a doubly acetylated catechol-based linker. (Scheme 2-3)
Scheme 2-3. Model prodrugs appended with three distinct release strategies.

The two model MBPs used were 3-hydroxy-2-methyl-4H-pyran-4-one (maltol) and 1-hydroxypyridin-2(1H)-one (1,2-HOPO). Approach 1 is comparable to successful strategies widely reported for masking hydroxyl groups, where the direct appendage of the ester moiety inactivates the drug. Approaches 2 and 3 represent self-immolative linker (SIL) strategies wherein the stimulus event (deacetylation) initiates an elimination reaction that leads to release of the inhibitor. Previous studies indicate that the benzyl ether linkage of SILs is superior to the more prevalent carbonate linkage in prodrug design with respect to kinetics of release and stability, thus this linkage was incorporated into our prodrug study here.\textsuperscript{19}
Synthesis the protected MBPs (proMBPs) 1-6 was straightforward and relatively high yielding. For compounds 1 and 4, the MBP was dissolved in acetic anhydride and acetic acid and heated for a period of 18 h.

Scheme 2-4. Synthesis of directly acetylated MBPs. Reagents and conditions: (i) Ac₂O, AcOH, 80°C, 18 h.

Synthesis of compounds 2-3 and 5-6, involved coupling of the MBP to the SIL. The SIL was achieved by using either p-cresol or 4-methylcatechol as the starting material and acetylated with acetic anhydride and scandium triflate. This was followed by benzylic bromination, and consequent coupling to the MBP.

Scheme 2-5. Synthesis of SILs. Reagents and conditions: (i) Ac₂O, Sc(OTf)₃, 25°C, 5 min (ii) CHCl₃, 60°C, 18 h.
Scheme 2-6. Synthesis of MBPs protected with SILs. Reagents and conditions: (i) K$_2$CO$_3$, DMF, 60 °C, 5 h.

The reactivity of compounds 1-6 with an esterase was analyzed via UV-Vis absorption spectroscopy. Upon the addition of porcine liver esterase (PLE), the absorbance of the reaction mixture was monitored. The emergence of a new spectrum with a $\lambda_{\text{max}}$ coinciding with that of the parent MBPs was observed, indicating complete conversion to the respective MBPs, maltol and 1,2-HOPO (Figures 2-1, -2, -3, -4, -5 and -6) was achieved. Similar spectral changes were observed for compounds 1-6, demonstrating that all three approaches were effective for esterase-mediated conversion.
Figure 2-1. Absorbance spectra of 1 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 1 min for 60 min. The dashed line represents the initial spectrum and an authentic sample of maltol is shown in red. The arrows indicate changes in spectra over time.

Figure 2-2. Absorbance spectra of 2 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 30 sec for 30 min. The dashed line represents the initial spectrum and an authentic sample of 1,2-HOPO is shown in red. The arrows indicate changes in spectra over time.
Figure 2-3. Absorbance spectra of 3 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 15 sec for 4.5 min. The dashed line represents the initial spectrum and an authentic sample of maltol is shown in red. The arrows indicate changes in spectra over time.

Figure 2-4. Absorbance spectra of 4 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 30 sec for 9 min. The dashed line represents the initial spectrum and an authentic sample of 1,2-HOPO is shown in red. The arrows indicate changes in spectra over time.
**Figure 2-5.** Absorbance spectra of 5 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 30 sec for 15 min. The dashed line represents the initial spectrum and an authentic sample of maltol is shown in red. The arrows indicate changes in spectra over time.

**Figure 2-6.** Absorbance spectra of 6 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 30 sec for 10 min. The dashed line represents the initial spectrum and an authentic sample of 1,2-HOPO is shown in red. The arrows indicate changes in spectra over time.
It is worth noting that approaches 2 and 3 generate side products that are released upon cleavage. That is, a deacetylation event by esterase at the para position to the ether linkage, leads to a spontaneous cascade reaction releasing a quinone-methide intermediate in both approaches. Quinone-methides are electrophilic Michael acceptors that react rapidly with water to generate 4-hydroxybenzyl alcohol (Scheme 2-3, approach 2) and 3,4-dihydroxybenzyl alcohol (Scheme 2-3, approach 3). This work did not study the effects these side products; however, 4-hydroxybenzyl alcohol is a known neuroprotective agent,\textsuperscript{20} while 3,4-dihydroxybenzyl alcohol is found in virgin olive oil, suggesting an innocuous nature for each.\textsuperscript{21}

### 2.2.2 Applying Strategies to Matrix Metalloproteinase Inhibitors

The successful conversion of the proMBPs to the parent MBPs prompted the exploration of full-length matrix metalloproteinase proinhibitors (proMMPi). Previous studies in our laboratory have led to the discovery of specific inhibitors of MMP-8 and MMP-12 termed **PY-2** and **1,2-HOPO-2** (Scheme 2-7). The biphenyl backbone of these MMPi selects against MMPs possessing shallow S1’ pockets, leading to semi-selective inhibition of deep-pocket MMPs with IC\textsubscript{50} values in the low nanomolar range (Scheme 2-7). Synthesis for these inhibitors as well as inhibition data had been previously reported.\textsuperscript{22} The addition of an esterase-responsive promoiety to the two MMPi was performed in the same manner as **1-6**. (Scheme 2-4, -5, -6)
Scheme 2-7. Full-length proMMPi appended with the three protecting strategies.

Conversion of proMMPi 7-9 was monitored via analytical HPLC, due to complicating spectral overlap between proinhibitors and parent inhibitors observed via UV-Vis spectroscopy. Treating compounds 7-9 with PLE produced HPLC traces corresponded to an authentic sample of PY-2 (Figure 2-7, -8, -9), indicating successful prodrug release. ProMMPi 11-12 were similarly converted by PLE as evidenced by UV-Vis absorption spectroscopy, where the emergence of spectral features matching that of the MMPi 1,2-HOPO-2 were clearly observed (Figure 2-10, -11). Compound 10 was excluded in these studies as it was found to be unstable in aqueous buffer upon preparation. The final absorbance spectrum shown in Figure 1-10 contains both 1,2-HOPO-2 and 4-hydroxybenzyl alcohol in a 1:1 ratio, so that the resulting spectrum possesses features of both compounds. 4-Hydroxybenzyl alcohol was not detected via
HPLC monitoring at 260 nm. Nevertheless, both methods successfully show the responsiveness of the proMMPi to esterase with release of the parent inhibitors observed in every case. A summary of the deprotection mechanisms for each esterase-activated prodrug approach for **PY-2** is summarized in Scheme 2-8.

Scheme 2-8. Deprotection mechanisms of proMMPi 7-9 by esterase to generate **PY-2**, a potent inhibitor of MMP-8 and MMP-12.
Figure 2-7. HPLC traces of PY-2 (red), 7 (black), and 7 after the addition of PLE for 1 h. Retention times are 15.4 min for PY-2 and 14.5 min for 7.

Figure 2-8. HPLC trace of PY-2 (black), 8 (red) and 8 after the reaction with PLE (50 U) for 1 h (blue). Retention times are 15.4 min for PY-2, 16.7 min for 8, and 15.4 min for 8 with PLE.
Figure 2-9. HPLC trace of PY-2 (black), 9 (red) and 9 after the reaction with PLE (50 U) for 1 h (blue). Retention times are 15.4 min for PY-2, 16 min for 9, and 15.4 min for 9 with PLE.

Figure 2-10. Absorbance spectra of 11 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 30 sec for 8 min. The dashed line represents the initial spectrum and an authentic sample of 1,2-HOPO-2 is shown in red. The arrows indicate changes in spectra over time.
Figure 2-11. Absorbance spectra of 12 (50 µM) in HEPES (50 mM, pH 7.5) in the presence of PLE (3.6 U) monitored every 30 sec for 6.5 min. The dashed line represents the initial spectrum and an authentic sample of 1,2-HOPO-2 is shown in red. The arrows indicate changes in spectra over time.

2.2.3 Hydrolytic Stability Studies of Full-Length Proinhibitors

ProMMPi were evaluated for aqueous stability under simulated physiological conditions (50 mM HEPES, pH 7.4). An initial HPLC trace was obtained immediately after preparation in aqueous buffer and a second trace was collected after 24 h incubation at 37 °C. After 24 h, ~35% of 7 was hydrolyzed to PY-2, while 10 underwent rapid, complete hydrolysis to 1,2-HOPO-2 (data not shown). However, compounds 8, 9, 11, and 12 were all >90% stable to hydrolysis under these simulated physiological conditions for 24 h. These measurements clearly demonstrate the superior hydrolytic stability of the SIL (approach 2 or 3) over direct acetylation (approach 1) for these inhibitors.
Figure 2-12. HPLC trace of PY-2 (black), 7 in HEPES (50 mM, pH 7.4) at 0 h (red) and after a 24 h incubation in HEPES buffer at 37°C (blue). Retention times are 15.4 min for PY-2, 14.5 min for 7 (0 h), and 14.5 min and 15.4 for 7 (24 h).

Figure 2-13. HPLC trace of PY-2 (black), 8 in HEPES (50 mM, pH 7.4) at 0 h (red) and after a 24 h incubation in HEPES buffer at 37°C (blue). Retention times are 15.4 min for PY-2, 16.7 min for 8 (0 h), and 16.7 min for 8 (24 h).
Figure 2-14. HPLC trace of PY-2 (black), 9 in HEPES (50 mM, pH 7.4) at 0 h (red) and after a 24 h incubation in HEPES buffer at 37°C (blue). Retention times are 15.4 min for PY-2, 16 min for 9 (0 h), and 16 min for 9 (24 h).

Figure 2-15. HPLC trace of 1,2-HOPO-2 (black), 11 in HEPES (50 mM, pH 7.4) at 0 h (red) and after a 24 h incubation in HEPES buffer at 37°C (blue). Retention times are 14.2 min for 1,2-HOPO, 15.9 min for 11 (0 h), and 14.8 min and 15.9 min for 11 (24 h).
Figure 2-16. HPLC trace of 1,2-HOPO-2 (black), 12 in HEPES (50 mM, pH 7.4) at 0 h (red) and after a 24 h incubation in HEPES buffer at 37°C (blue). Retention times are 14.2 min for 1,2-HOPO, 15.4 min for 12 (0 h), and 15.4 min for 12 (24 h).

2.2.4 Activation Kinetics

To determine the sensitivity of these compounds to esterase in a quantitative fashion, pseudo first-order kinetic measurements were performed using UV-Vis absorption spectroscopy (Table 1-1). Pyrone-based proMBPs 1-3 and the full-length proMMPi 7-9 were evaluated to compare the three prodrug approaches. Compounds 2 and 3 displayed similar rate of conversion with $k_{obs}$ of $245 \pm 8$ s$^{-1}$ and $280 \pm 97$ s$^{-1}$, respectively. Surprisingly, these rates were >25× faster than that observed for the directly acetylated proMBP 1 ($k_{obs}$ of $9 \pm 0.4$ s$^{-1}$). A similar trend was observed for the proMMPi, where the directly acetylated compound 7 displayed slower kinetics than the proMMPi containing the
acetylated trigger appended via a SIL (8 and 9). Liberation of 8 and 9 were approximately 4x and 8x faster than that of 7, respectively. These values are consistent with previous reports showing that the rate of deprotection is enhanced with the presence of electron-donating substituents in the aromatic ring. Overall, these findings highlight that the kinetic rates of release can be greatly attenuated by using different promoieties.

**Table 2-1.** Pseudo-first-order rates of conversion in the presence of PLE. Rate constant ($k_{obs}$) values were obtained by averaging three independent trials; data represent the mean ± SD.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{obs}$ [s$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>245 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>280 ± 97</td>
</tr>
<tr>
<td>7</td>
<td>160 ± 12</td>
</tr>
<tr>
<td>8</td>
<td>742 ± 90</td>
</tr>
<tr>
<td>9</td>
<td>1249 ± 60</td>
</tr>
</tbody>
</table>

### 2.2.5 MMP Inhibition Studies

To determine the efficacy of these prodrug approaches, the ability of the proMMPi 7-12 to inhibit MMP-8 and MMP-12 in the absence and presence of esterase was performed. As previously mentioned, compound 10 was excluded in these studies because it was not stable. MMP activity assays utilizing a cleavable fluorescent resonance energy transfer (FRET) substrate were employed (Figure 1-17). Before treatment with PLE, compounds 7, 9, and 11 showed essentially no inhibition against MMP-8 and MMP-12. Compounds 8 and 12 showed minimal inhibition (<10%) of MMP-8 and 12. Upon the addition of
PLE the percent inhibition increased to 40-50% inhibition for all compounds, indicative of activation to PY-2 and 1,2-HOPO-2. These biochemical assays demonstrate that esterase-responsive prodrugs are an effective class of proMMPi.

![Graph showing inhibition assays](image)

**Figure 2-17.** Inhibition assays for compounds 7-9 (300nm for MMP-8, 100nm for MMP-12), 11 and 12 (100nm for MMP-8, 30nm for MMP-12) against MMP-8 (1.82U, grey) and MMP-12 (0.35U, light blue) and in the presence of PLE (dark grey and navy, respectively).

We have demonstrated three different approaches to liberate phenol or hydroxyl moieties upon conversion by esterase, using MMP prodrugs as our proof-of-concept system. The SIL (approach 2 or 3) is superior to the conventional direct linkage of the acetate protecting group (approach 1) with respect to kinetics and
aqueous stability. Testing of these compounds in a biochemical assay shows no inhibition by the proinhibitors against either MMP-8 or MMP-12. Upon treatment with esterase, the promoieties effectively cleave to generate the active MMPI, which inhibits the targets as expected. We hope that the SIL strategies presented here will serve as a platform for esterase-responsive prodrug design.

2.3 Experimental

**General:** All chemicals were purchased from commercial suppliers (Sigma–Aldrich, Acros Organics, TCI America) and were used without further purification. Chromatography was performed using a CombiFlashRf 200 automated system from TeledyneISCO (Lincoln, NE USA). NMR spectra were recorded on a Varian FT 400 NMR instrument. Mass spectrometry was performed at the Molecular Mass Spectrometry Facility (MMSF) in the Department of Chemistry & Biochemistry at the University of California, San Diego.

**Synthesis.**

2-methyl-4-oxo-4H-pyran-3-yl acetate (1). Maltol (0.20 g, 1.6 mmol) was combined reacted with acetic anhydride (15 mL, 158.9 mmol) and glacial acetic acid (3 mL, 52.4 mmol). The reaction mixture was held at 80 °C for 18 h under a nitrogen atmosphere. The solution was concentrated and the resulting residue was dissolved in CH₂Cl₂ (20 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO₄, and filtered. Co-evaporation with MeOH afforded 1 in 84% yield (0.23 g, 1.3 mmol). ¹H NMR (400 MHz, CDCl₃) δ 7.67
(d, J = 6.0 Hz, 1H), 6.41 (d, J = 6.0 Hz, 1H), 2.34 (s, 3H), 2.26 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 172.2, 167.7, 159.3, 154.5, 138.8, 117.0, 20.5, 12.5. ESI-MS(+): m/z 168.95 [M+H]+, 191.02 [M+Na]+.

4-(((2-methyl-4-oxo-4H-pyran-3-yl)oxy)methyl)phenyl acetate (2). Maltol (0.10 g, 0.8 mmol) was dissolved in dry DMF (25 mL). To this solution was added K₂CO₃ (0.33 g, 2.4 mmol) followed by A₂ (0.54 g, 2.4 mmol), and the reaction was held at 60 °C under nitrogen for 5 h. The reaction was cooled to room temperature, and concentrated in vacuo. The resulting residue was brought up in CH₂Cl₂ (30 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO₄ and filtered. The crude product was purified via silica gel chromatography eluting 70% EtOAc in hexanes to afford the desired purified product 2 in 70% yield (0.15 g, 0.6 mmol). ¹H NMR (400 MHz, CDCl₃) δ 7.57 (d, J = 5.6 Hz, 1H), 7.37 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H), 6.31 (d, J = 5.6 Hz, 1H), 5.08 (s, 2H), 2.22 (s, 3H), 2.06 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 169.6, 162.8, 153.7, 150.8, 146.4, 140.4, 134.7, 130.3, 121.8, 117.39, 73.0, 21.4, 15.1. ESI-MS(+): m/z 274.95 [M+H]+, 297.06 [M+Na]+.

4-(((2-methyl-4-oxo-4H-pyran-3-yl)oxy)methyl)-1,2-phenylene diacetate (3). Maltol (0.10 g, 0.8 mmol) was dissolved in dry DMF (10 mL). To this solution was added K₂CO₃ (0.33 g, 2.4 mmol) followed by B₂ (0.68 g, 2.4 mmol), and the reaction was held at 60 °C under nitrogen for 5 h. The mixture was then concentrated, dissolved in CH₂Cl₂ (20 mL) and washed with brine (2×20 mL).
The organic layer was collected, dried over MgSO₄ and filtered. The resulting residue was purified via silica gel chromatography eluting 60% EtOAc in hexanes to afford 3 in 24% yield (0.06 g, 0.2 mmol). ¹H NMR (400 MHz, CDCl₃) δ 7.65 (d, J = 5.6 Hz, 1H), 7.29-7.15 (m, 3H), 6.48 (d, J = 5.6 Hz, 1H), 5.16 (s, 2H), 2.29 (s, 6H), 2.15 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 175.4, 168.4, 160.4, 153.9, 142.2, 135.9, 127.1, 124.1, 123.5, 122.8, 120.8, 117.2, 72.5, 20.8, 15.1. ESI-MS(+) : m/z 333.10 [M+H]⁺, 355.08 [M+Na]⁺.

2-oxopyridin-1(2H)-yl acetate (4). 1-Hydroxypyridin-2(1H)-one (1,2-HOPO) (0.20 g, 1.8 mmol) was combined with acetic anhydride (15 mL, 158.9 mmol) and glacial acetic acid (3 mL, 52.4 mmol). The reaction mixture was held at 80 °C for 18 h under a nitrogen atmosphere. The solution was concentrated and the resulting residue was dissolved in CH₂Cl₂ (20 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO₄, and filtered. Co-evaporation with MeOH afforded 4 in 64% yield (0.18 g, 1.2 mmol). ¹H NMR (400 MHz, CDCl₃) δ 7.45 (d, J = 5.6 Hz, 1H), 7.19 (d, J = 5.6 Hz, 1H), 2.36 (s, 3H), 2.27 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 166.7, 146.4, 139.6, 135.4, 123.1, 105.3, 18.2. ESI-MS(+) : m/z 153.89 [M+H]⁺, 175.94 [M+Na]⁺.

4-(((2-oxopyridin-1(2H)-yl)oxy)methyl)phenyl acetate (5). 1,2-HOPO (0.10 g, 0.9 mmol) was dissolved in dry DMF (10 mL). To this was added K₂CO₃ (0.37 g, 2.7 mmol) followed by A2 (0.21 g, 0.9 mmol), and the reaction was held at 60°C under nitrogen for 5 h. The mixture was cooled to room temperature, and
concentrated in vacuo. The resulting residue was dissolved in CH$_2$Cl$_2$ (20 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO$_4$ and filtered. The crude product was purified via silica gel chromatography eluting 70% EtOAc in hexanes to afford the desired product 5 in 34% yield (0.08 g, 0.3 mmol). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.40 (d, J = 8.6 Hz, 2H), 7.23 (td, J$_1$ = 6.8 Hz, J$_2$ = 2.0 Hz, 1H), 7.12 (dd, J$_1$ = 7.6 Hz, J$_2$ = 2.0 Hz, 1H), 7.09 (d, J = 8.6 Hz, 2H), 6.68 (dd, J$_1$ = 7.6 Hz, J$_2$ = 1.0 Hz, 1H), 5.96 (td, J$_1$ = 6.8 Hz, J$_2$ = 2.0 Hz, 1H), 5.25 (s, 2H), 2.28 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 169.4, 159.1, 151.6, 146.4, 138.9, 136.8, 131.4, 122.9, 122.1, 104.8, 77.8, 21.3. ESI-MS(+) m/z 260.0 [M+H]$^+$, 282.0 [M+Na]$^+$.

4-(((2-oxopyridin-1(2H)-yl)oxy)methyl)-1,2-phenylene diacetate (6). 1,2-HOPO (0.10 g, 0.9 mmol) was dissolved in dry DMF (10 mL). To this was added K$_2$CO$_3$ (0.37 g, 2.7 mmol) followed by B2 (0.77 g, 2.7 mmol), and the reaction was held at 60°C under nitrogen for 5 h. The mixture was then concentrated in vacuo, dissolved in CH$_2$Cl$_2$ (20 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO$_4$ and filtered. The resulting residue was purified via silica gel chromatography eluting 60% EtOAc in hexanes to afford 6 in 37% yield (0.11 g, 0.3 mmol). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.31 (J = 1.9 Hz, 1H), 7.28-7.16 (m, 4H), 6.65 (dd, J$_1$ = 8.0 Hz, J$_2$ = 1.2 Hz, 1H), 5.95 (td, J$_1$ = 6.7 Hz, J$_2$ = 1.7 Hz, 1H), 5.25 (s, 2H), 2.28 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 168.3, 168.2, 159.0, 143.1, 142.4, 139.1, 136.7, 132.7, 128.2, 125.2,
123.9, 122.9, 105.0, 77.3, 20.9, 20.8. ESI-MS(+) : m/z 318.12 [M+H]^+, 335.02 [M+NH₄]^+, 340.15 [M+Na]^+.

2-(((1,1'-biphenyl)-4-ylmethyl)carbamoyl)-4-oxo-4H-pyran-3-yl acetate (7). PY-2 (0.05 g, 0.2 mmol) was combined with acetic anhydride (15 mL, 158.9 mmol) and glacial acetic acid (3 mL, 52.4 mmol), and the reaction was held at 80°C for 18 h under nitrogen. The solution was concentrated and the resulting residue was dissolved in CH₂Cl₂ (20 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO₄, and filtered. The resulting residue was purified via silica gel chromatography eluting 70% EtOAc in hexanes to afford 7 in 37% yield (0.02 g, 0.06 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ 9.48 (s, 1H), 8.29 (d, J = 5.6 Hz, 1H), 7.65-7.63 (m, 4H), 7.45-7.35 (m, 5H), 6.62 (d, J = 5.6 Hz, 1H), 4.68 (d, J = 6.0 Hz, 2H), 2.23 (s, 3H). ¹³C NMR (100 MHz, DMSO-d₆) δ 173.0, 167.9, 158.4, 156.6, 149.7, 140.5, 139.7, 138.2, 129.6, 128.6, 128.0, 127.4, 127.2, 117.5, 42.7, 20.7. HRMS calcd for C₂₁H₁₇NO₅Na: 386.0999; Found: 386.1000.

4-(((2-(((1,1'-biphenyl)-4-ylmethyl)carbamoyl)-4-oxo-4H-pyran-3yl)oxy)methyl)phenyl acetate (8). PY-2 (0.10 g, 0.3 mmol) was dissolved in dry DMF (10 mL). To this was added K₂CO₃ (0.13 g, 0.9 mmol) followed by A1 (0.21 g, 0.9 mmol), and the reaction was held at 60°C under nitrogen for 5 h. The mixture was then cooled to room temperature and concentrated in vacuo. MeOH was added to crude which caused the formation of a white precipitate. The
precipitate was filtered afford the desired product 8 in 56% yield (0.08 g, 0.2 mmol). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.18 (t, \(J = 5.6\) Hz, 1H), 8.22 (d, \(J = 5.6\) Hz, 1H), 7.63 (d, \(J = 7.2\) Hz, 2H), 7.58 (d, \(J = 8.4\) Hz, 2H), 7.47 (t, \(J = 7.2\) Hz, 2H), 7.38-7.33 (m, 4H), 7.05 (d, \(J = 8.4\) Hz, 2H), 6.54 (d, \(J = 5.6\) Hz, 1H), 5.14 (s, 2H), 4.45 (d, \(J = 6\) Hz, 2H), 2.22 (s, 3H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 175.7, 169.7, 159.5, 156.2, 150.9, 150.8, 145.3, 140.5, 139.5, 138.2, 134.5, 130.3, 129.6, 128.7, 128.0, 127.3, 127.2, 122.3, 117.7, 73.7, 42.9, 21.5. HRMS calcd for \(C_{28}H_{23}NO_6Na\): 492.1418; Found: 492.1419.

4-(((2-(([1,1'-biphenyl]-4-ylmethyl)carbamoyl)-4-oxo-4H-pyran-3-yl)oxy)methyl)-1,2-phenylene diacetate (9). PY-2 (0.10 g, 0.3 mmol) was dissolved in dry DMF (10 mL). To this was added K\(_2\)CO\(_3\) (0.129 g, 0.9 mmol) followed by B2 (0.27 g, 0.9 mmol), and the reaction was held at 60°C under nitrogen for 5 h. The mixture was then cooled to room temperature, concentrated in vacuo, brought up in CH\(_2\)Cl\(_2\) (20 mL), and washed with brine (2×50 mL). The organic layer was collected, dried over MgSO\(_4\) and filtered. The resulting residue was purified via silica gel chromatography eluting 70% EtOAc in hexanes to afford the desired product 9 in 46% yield (0.08 g, 0.2 mmol). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.21 (br, s, 1H), 8.24 (d, \(J = 5.6\) Hz, 1H), 7.64-7.56 (m, 4H), 7.47 (t, \(J = 7.2\) Hz, 2H), 7.36-7.30 (m, 5H), 7.22 (d, \(J = 8.0\) Hz, 2H), 6.56 (d, \(J = 5.6\) Hz, 1H), 5.13 (s, 2H), 4.46 (\(J = 6.0\) Hz, 2H), 2.25 (s, 3H), 2.23 (s, 3H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 175.7, 168.8, 159.5, 156.3, 151.0, 145.2, 142.4, 140.5, 139.6, 138.2, 136.0, 129.5, 128.6, 128.0, 127.3, 127.2, 127.0,
124.1, 123.9, 117.8, 73.3, 42.8, 21.0, 20.9. HRMS calcd. for C$_{30}$H$_{25}$NO$_8$Na: 550.1472; Found: 550.1471.

6-(((1,1'-biphenyl]-4-ylmethyl)carbamoyl)-2-oxopyridin-1(2H)-yl acetate (10). 1,2-HOPO-2 (0.10 g, 0.3 mmol) was dissolved with acetic anhydride (15 mL, 158.9 mmol) and glacial acetic acid (3 mL, 52.4 mmol), and the reaction was held at 80°C for 18 h under nitrogen. The solution was concentrated and the resulting residue was dissolved in CH$_2$Cl$_2$ (20 mL) and washed with brine (2×20 mL). The organic layer was collected, dried over MgSO$_4$, and filtered. The resulting residue was purified via silica gel chromatography eluting 70% EtOAc in hexanes to afford 10 in 76% yield (0.09 g, 0.2 mmol). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 7.61-7.30 (m, 10H), 6.70 (d, $J = 9.2$ Hz, 1H), 6.56 (d, $J = 6.5$ Hz, 1H), 4.50 (s, 2H), 2.17 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ = 166.2, 160.4, 158.0, 141.3, 140.7, 140.5, 140.0, 137.1, 128.7, 128.1, 127.2, 127.0, 122.9, 106.2, 42.9, 16.3. ESI-MS (+): m/z 384.98 [M+Na]$^+$. 

4-(((6-((1,1'-biphenyl]-4-ylmethyl)carbamoyl)-2-oxopyridin-1(2H)-yl)oxy)methyl)phenyl acetate (11). 1,2-HOPO-2 (0.15 g, 0.5 mmol) was dissolved in dry DMF (10 mL). To this was added K$_2$CO$_3$ (0.19 g, 1.4 mmol) followed by A2 (0.32 g, 1.4 mmol), and the reaction was held at 60°C under nitrogen for 5 h. The mixture was then cooled to room temperature and concentrated in vacuo. MeOH was added to crude which caused the formation of a white precipitate. The precipitate was filtered to afford the desired product
11 in 38% yield (0.08 g, 0.2 mmol). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.46 (br, s, 1H) 7.62 (d, $J = 8.0$ Hz, 2H), 7.54 (d, $J = 7.6$ Hz, 2H), 7.50-7.43 (m, 3H), 7.41 (d, $J = 8.4$ Hz, 2H), 7.38-7.36 (m, 3H), 7.08 (d, $J = 7.2$ Hz, 2H), 6.68 (d, $J = 7.2$ Hz, 1H), 6.38 (d, $J = 6.8$ Hz, 1H), 5.24 (s, 2H), 4.48 (d, $J = 6.0$ Hz, 2H), 2.23 (s, 3H). 

$^13$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 161.2, 160.9, 158.1, 155.3, 151.5, 144.6, 139.7, 139.5, 138.2, 132.0, 131.5, 129.6, 128.0, 127.3, 127.2, 123.1, 122.5, 104.6, 78.3, 42.9, 21.5. HRMS calcd for C$_{28}$H$_{24}$N$_2$O$_5$Na: 491.1577; Found: 491.1578.

4-(((6-(((1,1'-biphenyl)-4-ylmethyl)carbamoyl)-2-oxopyridin-1(2H)-yl)oxy)methyl)-1,2-phenylenediacetate (12). 1,2-HOPO-2 (0.10 g, 0.3 mmol) was dissolved in dry DMF (10 mL). To this was added K$_2$CO$_3$ (0.129 g, 0.9 mmol) followed by B2 (0.267 g, 0.933 mmol), and the reaction was held at 60°C under nitrogen for 5 h. The mixture was then cooled to room temperature, concentrated, brought up in CH$_2$Cl$_2$ (40 mL) and washed with brine (2×50 mL). The organic layer was collected, dried over MgSO$_4$ and filtered. The resulting residue was purified via silica gel chromatography eluting 70% EtOAc in hexanes to afford the desired product 12 was obtained in 16% yield (0.03 g, 0.05 mmol). 

$^1$H NMR (400Hz, DMSO-$d_6$) $\delta$ 7.58-7.52 (m, 5H), 7.45 (t, $J = 7.2$ Hz, 2H), 7.37-7.33 (m, 3H), 7.23 (td, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz, 2H), 7.16 (s, 1H), 7.07 (d, $J = 8.0$ Hz, 1H), 6.48 (d, $J = 8.4$ Hz, 1H), 6.35 (d, $J = 5.6$ Hz, 1H), 5.15 (s, 2H), 4.53 (d, $J = 6.0$ Hz, 2H), 2.26 (s, 3H), 2.20 (s, 3H). 

$^13$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 168.5, 168.2, 160.5, 158.8, 143.2, 142.8, 142.1, 140.8, 140.7, 138.5, 136.7,
132.2, 129.1, 129.0, 128.7, 127.8, 127.7, 127.3, 125.7, 124.1, 123.9, 106.5, 64.3, 43.7, 20.8, 20.7. HRMS calcd. for C$_{30}$H$_{26}$N$_2$O$_7$Na: 549.1632; Found: 549.1631.

**p-Tolyl acetate (A1).** $p$-Cresol (1.50 g, 13.9 mmol) was dissolved in CH$_2$Cl$_2$ (140 mL) and acetic anhydride (4 mL, 41.6 mmol). To this was added scandium(III) triflate (0.13 g, 0.3 mmol) at room temperature and stirred for 5 min. The mixture was washed with a saturated NaHCO$_3$ solution (2×50 mL). The organic layer was collected, dried over MgSO$_4$, filtered and co-evaporated with MeOH to afford A1 in 88% yield (1.82 g, 12.2 mmol). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.19 (d, $J$ = 8.4 Hz, 2H), 6.98 (d, $J$ = 8.4 Hz, 2H), 2.35 (s, 3H), 2.29 (s, 3H). ESI-MS(+): m/z 167.95 [M+NH$_4$]$^+$, 172.96 [M+Na]$^+$.

**4-(bromomethyl)phenyl acetate (A2).** A1 (1.82 g, 12.1 mmol) was dissolved in CHCl$_3$ (50 mL) and combined with N-bromosuccinimide (2.91 g, 16.4 mmol) and benzoyl peroxide (0.59 g, 2.4 mmol) in a flame dried vessel. The reaction was held at reflux under nitrogen gas for 18 h. The mixture was cooled to room temperature, concentrated, brought up in CH$_2$Cl$_2$ (50 mL) and washed with brine (2×50 mL). The organic layer was collected, dried over MgSO$_4$ and filtered. The resulting residue was purified via silica gel chromatography eluting 5% EtOAc in hexanes to afford A2 in 43% yield (1.20 g, 5.2 mmol). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.40 (d, $J$ = 7.2 Hz, 2H), 7.07 (d, $J$ = 7.2 Hz, 2H), 4.46 (s, 2H), 2.28 (s, 3H). ESI-MS(+): m/z 245.93 [M+NH$_4$]$^+$, 250.91 [M+Na]$^+$.
4-methyl-1,2-phenylene diacetate (B1). 4-methylcatechol (0.50 g, 4.0 mmol) was dissolved in CH₂Cl₂ (40 mL). To this was added acetic anhydride (1.5 mL, 16.1 mmol) and scandium (III) triflate (0.04 g, 0.08 mmol) at room temperature. After 5 min, the reaction was complete and the resulting solution was washed with saturated NaHCO₃ (2×50 mL). The organic layer was collected, dried over MgSO₄, filtered and co-evaporated with MeOH to afford B1 in a 98% yield (0.82 g, 3.9 mmol). ¹H NMR (400 MHz, CDCl₃) δ 7.07-7.02 (m, 2H), 6.99 (s, 1H), 2.33 (s, 3H), 2.26 (s, 3H). ESI-MS(+) : m/z 226.0 [M+NH₄]⁺, 231.0 [M+Na]⁺, 247.0 [M+K]⁺.

4-(bromomethyl)-1,2-phenylene diacetate (B2). B1 (0.82 g, 3.9 mmol) was dissolved in CHCl₃ (50 mL) and combined with N-bromosuccinimide (0.95 g, 5.3 mmol) and benzoyl peroxide (0.19 g, 0.80 mmol) in a flame-dried vessel. The reaction was held at reflux for 18 h. The mixture was cooled to room temperature and concentrated. The resulting residue was brought up in CH₂Cl₂ (20 mL) and washed with brine (2×50 mL). The organic layer was collected, dried over MgSO₄ and filtered. The crude product was purified via silica gel chromatography eluting 15% EtOAc in hexanes to afford B2 in 72% yield (0.82 g, 2.8 mmol). ¹H NMR (400 MHz, CDCl₃) δ 7.23-7.13 (m, 3H), 4.43 (s, 2H), 2.26 (s, 6H). ESI-MS(+) : m/z 306.01 [M+NH₄]⁺.

UV-Vis Spectroscopy. Absorption spectra of compounds 1-6, 11, and 12 were collected on a Perkin-Elmer Lambda 25 UV-visible spectrophotometer. To a 1.0
mL solution at 0.05 mM concentration in HEPES buffer (50 mM, pH 7.5) was added PLE (3.57 U). Spectra were monitored over time at room temperature (Figures S1-S7).

**Calculation of Kinetic Rate Constant.** Pseudo-first order rate constants were calculated by monitoring the absorption spectra over time in the presence of PLE. To a 1.0 mL solution of 50 µM of each compound in HEPES buffer (50 mM, pH 7.5) was added PLE so that each sample contained 0.178 U of protein. Spectra were monitored over 10-20 min at room temperature with at least 100 spectra recorded for each sample. The change in absorption was monitored at 274 nm for the maltol series (1-3) and at 338 nm for the PY-2 series (7-9) we term $A_{max}$. The rate constant ($k_{obs}$) was determined by monitoring the appearance of the absorption peak by plotting the linear slope of $\ln[(A_{max} - A)/(A_{max})]$.

**HPLC Analysis.** Analytical HPLC was performed on a HP Series 1050 system equipped with a Vydac® C18 reverse phase column (218TP, 250×4.6 mm, 5 µm). Separation was achieved with a flow rate of 1 mL/min and the following solvents: solvent A is 5% MeOH and 0.1% formic acid in H2O and solvent B is 0.1% formic acid in MeOH. Starting with 95% A and 5% B, an isocratic gradient was run for 15 min to a final solvent mixture of 5% A and 95% B, which was held for 5 min before ramping back down to 95% A and 5% B in 2 min and holding for an additional 4 min. Compounds were prepared in HEPES buffer (50 mM, pH
7.5) at a concentration of 1 mM. Retention times of compounds PY-2, and 1,2-HOPO-2 were determined under identical HPLC conditions prior to evaluation of esterase cleavage of the protected compounds.

To determine the efficiency of esterase cleavage for the proMMPi, 1 mL samples of each compound were prepared at a concentration of 1 mM in HEPES buffer (50 mM, pH 7.5). To each sample was added 50 U of PLE and incubated at 25 °C for 1 h prior to analysis (Figures S8-S9).

To evaluate the hydrolytic stability of the proMMPi, a 1.0 mL sample of each compound was prepared at a concentration of 1 mM in HEPES buffer (50 mM, pH 7.4) and a trace was obtained immediately. This sample was incubated in the buffer solution for 24 h at 37° C before a second trace was obtained. The stability of each sample was determined based on the area under the curve.

**MMP Inhibition Assays.** Inhibition values of 7-9, and 11-12 were determined using a previously described commercially available fluroscent-based assay kit. MMP activity was measured in 96-well plates using a Bio-Tek Flx800 fluorescent plate reader. The protected MMPi were dissolved in DMSO to a concentration of 1 mM and diluted in HEPES buffer (50 mM, pH 7.5) to a concentration of 50 µM. To each sample was added PLE such that 50 U of protein was present. This mixture was incubated for 1 h at room temperature. The esterase was removed via micro centrifugation using 10 kDa molecular weight cut-off filters. The filtered esterase-treated compounds were then added to appropriate wells at their respective IC50 values. Each well contained 20 µL of MMP-8 or MMP-12 (1.82
U/mL or 0.35U/mL, respectively), 60 µL MMP assay buffer (50 mM HEPES, 10mM CaCl2, 0.10% Brij-35, pH 7.5), and the esterase-treated MMPi (10 µL). After a 30 min incubation at 37 °C, a reaction was initiated with the addition of 10 µL (40 µM) of the fluorescent substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2) where Mca = (7-methoxycoumarin-4-yl)-acetyl and Dpa = N-3-(2,4-dinitrophenyl)-L-α-β-diaminopropionyl)) and kinetic activity was monitored every 40 sec for 30 min with excitation and emission wavelengths at 335 nm and 405 nm, respectively. Enzymatic activity and thus inhibition was calculated with respect to the control experiment (no inhibitor present). Measurements were performed in duplicate in two independent experiments.

2.4 Acknowledgements


The thesis author was the primary researcher for the data presented and was the primary author on the publication. The permission to reproduce this paper was granted by John Wiley and Sons, Inc. Copyright 2013, John Wiley and Sons, Inc.
2.5 References.


Chapter 3. Exploring Hydrogen Peroxide Responsive Thiazolidinone Based Prodrugs
3.1 Introduction

Prodrugs are inactive derivatives of bioactive molecules that are designed to become activated in vivo by a variety of stimuli. As discussed in Chapter 1, prodrug strategies often serve to improve the drug-like properties of bioactive molecules including bioavailability, cell permeability, and pharmacokinetics.\(^1\)\(^-\)\(^4\)

Most often prodrug strategies involve the covalent appendage of a chemical group, termed the promoiety, to the active drug. The basis for promoiety selection depends on the specific functional group being masked and on the specific attribute being improved. Carboxylic acids are a common functional group amenable to the prodrug method, since they are highly polar and ionizable, which often leads to a decrease in membrane permeability.\(^2\) A common approach to overcome this drawback is through an esterase-activated prodrug strategy. This approach involves esterification of the acid moiety, which can be hydrolyzed in vivo by ubiquitous esterases.\(^6\) This strategy is the most popular method used to overcome issues associated with carboxylic acid containing drugs such as Oseltamivir an antiviral used for prevention of influenza, Pivampicillin an antibiotic useful for treatment of both gram-positive and gram-negative bacteria, and Ximelagatran a direct thrombin inhibitor that acts as an anticoagulant. (Figure 3-1).\(^2\)
Since esterases are ubiquitous and constitutively active, ester-based prodrugs do not target disease-specific environments, which limits the full potential of this approach. Ideally, a prodrug method could be exploited to achieve targeted release of the active drug. A targeted strategy may be achieved with a promoiety that can readily be cleaved by disease-specific stimuli.

Reactive oxygen species (ROS) are naturally occurring species that result from cellular metabolism of molecular oxygen.7-9 The reduction of molecular oxygen results in the production of intermediates such as superoxide anion, hydrogen peroxide, hydroxyl radical, and organic peroxides.10 Cells maintain tightly controlled and rather complex systems of enzymatic and non-enzymatic antioxidants that balance and minimize levels of ROS.11 However,
overproduction of these ROS can result in impaired cellular functions and formation of toxic metabolites. Several pathologies are associated with increased levels of ROS including inflammation, cancer, cardiovascular, and neurodegenerative diseases. Cancer cells are an ideal target for ROS-prodrug activation, because many tumors exhibit elevated levels of H$_2$O$_2$ (5 µM to 1.0 mM). Therefore, ROS-activated prodrugs may serve as a platform for targeted release of potent therapeutics to these specific microenvironments, while limiting off-target interactions. Such ROS-mediated activation could result in spatially and temporally controlled release of therapeutics. Successful efforts to release therapeutics through ROS activation have been achieved through incorporation of sulfonate esters and boronic acid/esters promoieties, for the masking of alcohols and amine groups in bioactive molecules. However, none of these strategies have been proven to be amendable for the release of carboxylic acids.

There are several classes of inhibitors that stand to benefit from a ROS prodrug strategy. Of particular interest are inhibitors targeting cyclooxygenases (COX), matrix metalloprotenaises (MMP), and angiotensin converting enzyme (ACE). Prodrug strategies for these inhibitors have been thoroughly explored. Ibuprofen is a non-selective cyclooxygenase (COX) inhibitor, and its chronic use as an analgesic and anti-inflammatory has been associated with formation of ulcers and gastrointestinal bleeding. A ROS-responsive prodrug strategy could aid in diminishing adverse side effects, since release of the active drug would be limited in healthy tissues where normal levels of COX-1 activity are required for
proper cell functioning, yet activated in inflamed tissues bearing higher concentrations of ROS in concordance with aberrant COX-2 expression. MMP inhibitors also stand to benefit from a ROS-targeted prodrug strategy, as MMP and ROS hyperactivity have been associated with cancer and ischemic reperfusion injury, which leads to accelerated and undesirable matrix degradation. Lastly, ACE inhibitors are major class of therapeutics with several FDA approved compounds in the clinic. ACE inhibitors, such as the tripeptide dicarboxylate enalapril, utilize an esterase prodrug approach in which one of the carboxylic acid pharmacophores is esterified.\textsuperscript{27} We hypothesized that by appending a ROS-sensitive functionality to the carboxylic acid group of these inhibitors, we could attenuate the inhibition by these compounds until the active drug is selectively liberated in the presence of H\textsubscript{2}O\textsubscript{2}.

In this study, we explored a novel approach for ROS-activated prodrugs based on a thiazolidinone promoiety. This strategy was inspired by the use of H\textsubscript{2}O\textsubscript{2} to cleave oxazolidinone protecting groups that are used as auxiliaries for asymmetric reactions, and are commonly referred to as Evan's auxiliaries.\textsuperscript{28,29} Similar protecting groups have also been used as auxiliaries.\textsuperscript{30,31} By examining modified oxazolidinones, we report a prodrug strategy that can effectively mask the carboxylic acid groups of several pharmacologically potent agents. In the presence of elevated levels of H\textsubscript{2}O\textsubscript{2}, the promoiety is hydrolyzed to generate the bioactive compound with a free carboxylic acid (Scheme 3-1).
3.2 Results and Discussion

3.2.1 Development of Model Compounds

In order to develop carboxylic acid prodrugs, the responsiveness of several oxazolidinone derivatives to H$_2$O$_2$ and their stability in simulated physiological conditions were examined. Four distinct, but closely related, promoieties were studied: oxazolidinone (A), thiazolidinone (B), oxazolidinethione (C), and thiazolidinethione (D). Our approach evaluated each of these derivatives with respect to stability and rate of activation in order to identify a suitable promoiety. The promoiety showing the best stability balanced with the fastest rate of H$_2$O$_2$-activation was selected for further investigations. Each of these four groups was appended to two test compounds: benzoic acid and phenyl acetic acid. These model compounds were chosen for their structural simplicity and to highlight differences between the reactivity and stability of aryl and alkyl (benzylic) carboxylic acids. Compounds A and D are commercially available and the synthesis for B and C have been previously reported.$^{32,33}$ Synthesis of several of the model compounds has also been described.$^{34-37}$

![Scheme 3-1. Proposed mechanism for activation of a thiazolidinone prodrug.](image-url)
Amide bond formation between the carboxylic acid compounds and the promoieties (A-D) was performed via one of two different methods: addition of DCC and DMAP in CH₂Cl₂ (Method i) or through the Schotten-Baumann reaction (Method ii), with the corresponding acid-chloride. All compounds were rigorously characterized via NMR and mass spectrometry. In addition, the single-crystal X-ray structure of compounds 2 and 3 were determined, unambiguously confirming their composition and connectivity.

\[ \text{Scheme 3-2. Synthesis of model compounds 1-8. Reagents and conditions: (i) } N,N'-\text{Dicyclohexylcarbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), CH}_2\text{Cl}_2, 25^\circ\text{C (ii) NaOH, H}_2\text{O, Acetone, 25^\circC.} \]
Figure 3-2. Crystal structure (asymmetric unit) of compound 2. Thermal ellipsoids are shown at 50% probability. Hydrogen atoms are omitted for clarity. Color scheme: carbon (grey), nitrogen (blue), oxygen (red), and sulfur (yellow).

Figure 3-3. Crystal structure (asymmetric unit) of compound 3. Thermal ellipsoids are shown at 50% probability. Hydrogen atoms are omitted for clarity. Color scheme: carbon (grey), nitrogen (blue), oxygen (red), and sulfur (yellow).

3.2.2 Evaluation of Hydrogen Peroxide Responsiveness

Compounds 1-8 were treated with H₂O₂ (20 equiv, 100 mM Tris-Cl, pH 7.4) in a 40% DMSO/60% buffer (100 mM Tris-Cl, pH 7.4) solution, and cleavage was monitored via analytical HPLC after 1 h and 4 h incubation. The high DMSO content was used in order to ensure that all of the compounds were completely
soluble under the reaction conditions. After 4 h of incubation with H₂O₂, compounds 1, 2, 5, and 6 displayed a wide range of cleavage rates to the corresponding acids (i.e., benzoic acid or phenyl acetic acid, Table 3-1), with compounds 2 and 6 (both which utilize protecting group B) showing the fastest rates of conversion.

**Table 3-1.** Activation of compounds upon treatment with H₂O₂. All values represent percent conversion to the carboxylic acid product and are an average of two independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time (1 h)</th>
<th>Time (4 h)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>37%</td>
<td>70%</td>
</tr>
<tr>
<td>2</td>
<td>&gt;95%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>3</td>
<td>N.D.*</td>
<td>N.D.*</td>
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<tr>
<td>4</td>
<td>N.D.*</td>
<td>N.D.*</td>
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<tr>
<td>5</td>
<td>&lt;5%</td>
<td>10%</td>
</tr>
<tr>
<td>6</td>
<td>37%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>7</td>
<td>N.D.*</td>
<td>N.D.*</td>
</tr>
<tr>
<td>8</td>
<td>N.D.*</td>
<td>N.D.*</td>
</tr>
</tbody>
</table>

*Conversion to multiple products was observed.

The activation of compounds 2 and 6 were tested at a lower DMSO concentration 5% DMSO/95% buffer (100 mM Tris-Cl, pH 7.4) in which conversion to the corresponding product was also observed (Figures 3-4, -5). The conversion of compounds 3, 4, 7, and 8, all of which contain a thione-based protecting group (e.g. exocyclic sulfur in C and D), were not determined under these reduced DMSO conditions, because these reactions were not clean, showing conversion to multiple products (data not shown).
Figure 3-4. HPLC trace of an authentic sample of 2 (Ret. Time = 8.1 min, black), 2 (1 mM, 5% DMSO/95% 100 mM Tris-Cl, pH 7.4 buffer) in the presence of \( \text{H}_2\text{O}_2 \) (20 mM, 37°C) at 1 h (blue) and at 4 h (green). The expected product, benzoic acid, is also shown for comparison (Ret. Time = 6.2 min, brown).

Figure 3-5. HPLC trace of an authentic sample of 6 (Ret. Time = 9.6 min, black), 6 (1 mM, 5% DMSO/95% 100 mM Tris-Cl, pH 7.4 buffer) in the presence of \( \text{H}_2\text{O}_2 \) (20 mM, 37°C) at 1 h (blue) and at 4 h (green). The expected product, phenylacetic acid is also shown for comparison (Ret. Time = 6.4 min, brown).
3.2.3. Aqueous Stability of Model Compounds

In addition to stimulus-mediated activation, prodrugs must have good aqueous stability, which in this study can be tested by incubation in buffer in the absence of H₂O₂. The stability of each compound was examined in a 40% DMSO/60% buffer solution (100 mM Tris-Cl, pH 7.4) at physiological pH, as well as in the presence of glutathione (GSH) (20 equiv, 100 mM Tris-Cl, pH 7.4), with incubation at 37 °C for 24 h. Compounds 1, 2, 5, and 6 displayed good stability in the presence and absence of GSH, all showing <35% degradation (Table 3-2). In contrast, compounds 3, 4, 7, and 8, all showed poor stability, with rapid degradation under these incubation conditions (Table 3-2). This trend parallels the complex reactivity observed with these compounds (all of which contain protecting group C or D with an exocyclic sulfur) in the presence of H₂O₂ (vide supra). Overall, the model compounds that displayed the optimal combination of rate of cleavage and good stability were 2 and 6.

Table 3-2. Stability of model compounds. All values represent percent conversion to the carboxylic acid product or unidentified side products and are an average of two independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Buffer*</th>
<th>Buffer* + GSH (20 equiv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;95%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>2</td>
<td>&gt;95%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>3</td>
<td>40%</td>
<td>10%</td>
</tr>
<tr>
<td>4</td>
<td>50%</td>
<td>78%</td>
</tr>
<tr>
<td>5</td>
<td>&gt;95%</td>
<td>65%</td>
</tr>
<tr>
<td>6</td>
<td>74%</td>
<td>67%</td>
</tr>
<tr>
<td>7</td>
<td>10%</td>
<td>11%</td>
</tr>
<tr>
<td>8</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

*Buffer = 100 mM Tris-Cl, pH 7.4 (37 °C)
Figure 3-6. HPLC trace of an authentic sample of 2 (Ret. Time = 8.1 min, black), 2 (1 mM, 40% DMSO/ 60% 100 mM Tris-Cl, pH 7.4 buffer) after 24 h (37 °C) incubation (blue), and 2 under the same conditions, with the addition of GSH (20 mM, green). The expected product, benzoic acid, is also shown for comparison (Ret. Time = 6.2 min, brown).

Figure 3-7. HPLC trace of an authentic sample of 6 (Ret. Time = 9.6 min, black), 6 (1 mM, 40% DMSO/ 60% 100 mM Tris-Cl, pH 7.4 buffer) after 24 h (37 °C) incubation (blue), and 6 under the same conditions, with the addition of GSH (20
mM, green). The expected product, phenylacetic acid is also shown for comparison (Ret. Time = 6.4 min, brown).

### 3.2.4 Applying Thiazolidinone Promoiety to Inhibitors

The success of model compounds 2 and 6, both of which utilize promoiety B, prompted the investigation of the scope of this prodrug approach with an FDA approved drug, ibuprofen, and a well-studied matrix metalloproteinase inhibitor. Both prodrugs (proIBU and proMMPi) were readily synthesized (Schemes 3-3 and 3-4) and evaluated for ROS activation (Figures 3-8 and 3-9) and hydrolytic stability via analytical HPLC under simulated physiological conditions (Figures 3-11-12).

![Scheme 3-3. Synthesis of proIBU](image-url)

Scheme 3-3. Synthesis of proIBU
Scheme 3-4. Synthesis of proMMPi

ProMMPi activation by two different ROS was evaluated. Experiments were carried out in which a proMMPi solution (100 µM) was added to either 20 equiv of H₂O₂ or 20 equiv of NaClO (Figures 3-9, -10). Activation by H₂O₂ to the corresponding MMPI was observed. However, exposure of proMMPi to NaClO lead to the degradation to an unidentified product.
Figure 3-8. HPLC trace of an authentic sample of proIBU (Ret. Time = 14.1 min, black), proIBU in the presence of H_2O_2 (20 equiv, 37°C) in buffer (100 mM Tris-Cl) at several time points. The expected IBU product is also shown for comparison (Ret. Time = 11.6 min, brown).

Figure 3-9. HPLC trace of an authentic sample of proMMPi (Ret. Time = 12.6 min, black), proMMPi (100 µM, 40% DMSO/60% 100 mM Tris-Cl, pH 7.4 buffer) in the presence of H_2O_2 (2 mM, 37°C) at several time points. The expected MMPi product is also shown for comparison (Ret. Time = 11.0 min, brown).
**Figure 3-10.** HPLC trace of an authentic sample of proMMPi (Ret. Time = 12.6 min, black), proMMPi (100 µM, 40% DMSO/ 60% 100 mM Tris-Cl, pH 7.4 buffer) in the presence of NaClO (2 mM, 37°C) at several time points. The expected MMPi product is also shown for comparison (Ret. Time = 11.0 min, brown).

In order to test the aqueous stability, the compounds were incubated in DMSO and buffer (100 mM Tris-Cl, pH 7.4) for 24 h at 37 °C. Additionally, each prodrug was incubated under the same conditions with the addition of 20 equiv of lysine, serine, or GSH, in order to demonstrate that the promoiety is not readily cleaved in the presence of biologically relevant nucleophiles. Both proIBU and proMMPi displayed remarkable stability of >95% under all of the conditions tested.
Figure 3-11. HPLC trace of an authentic sample of proIBU (Ret. Time = 14.1 min, black), proIBU after 24 h (37 °C) incubation in buffer (100 mM Tris-Cl, blue), proIBU in buffer with serine (20 equiv, teal), proIBU in buffer with lysine (20 equiv, yellow), proIBU in buffer with GSH (20 equiv, purple). The expected IBU product is also shown for comparison (Ret. Time = 11.6 min, brown).

Figure 3-12. HPLC trace of an authentic sample of proMMPi (Ret. Time = 12.6 min, black), proMMPi (1mM, 40% DMSO/ 60% 100 mM Tris-Cl, pH 7.4 buffer) after 24 h (37 °C) incubation (blue), proMMPi under the same conditions, with the addition serine (20 mM, teal), lysine (20 mM, yellow), and GSH (20 mM,
purple). The expected MMPi product is also shown for comparison (Ret. Time = 11.0 min, brown).

### 3.2.5 Inhibition Assays

To determine the efficacy of the prodrug approach, inhibition assays were performed for each metalloenzyme target. Inhibition by activated proIBU (proIBU + H₂O₂) using a commercially available fluorescent assay for COX-1 could not be determined because H₂O₂ interfered with the assay. However, no inhibition by proIBU (<5%) of COX-1 was observed, indicating the promoiety did suppress the activity of proIBU against the target (Figure 3-13).

**Figure 3-13.** Inhibition assay results for proIBU against COX-1 in the absence and presence of H₂O₂. Inhibition for IBU was measured 37±2% and proIBU 2±2%.
The efficacy of the proMMPi to inhibit its target was also evaluated. The MMP assay was carried out as previously described against MMP-2, by utilizing a cleavable Förster resonance energy transfer (FRET) substrate. The proMMPi demonstrated essentially no inhibition against MMP-2 (<5%, Figure 3-14). In contrast, inhibition of MMP-2 after treatment of proMMPi with 20 equiv of H₂O₂ increased to ~37%, close to the expected value of 50% and demonstrating that the inhibitory prolife was readily restored. The data confirm that the proMMPi is inactive against its target (MMP-2), but regains near full activity after being treated with H₂O₂.

![Graph showing inhibition assay results for proMMPi against MMP-2 in the absence and presence of H₂O₂. Inhibition for MMPi (1.16U) was measured 47±6%, proMMPi (350nM) 3±2%, and proMMPi + H₂O₂ (350nM + 50 equiv.) at 37±6%.]

**Figure 3-14.** Inhibition assay results for proMMPi against MMP-2 in the absence and presence of H₂O₂. Inhibition for MMPi (1.16U) was measured 47±6%, proMMPi (350nM) 3±2%, and proMMPi + H₂O₂ (350nM + 50 equiv.) at 37±6%.
3.2.6 Evaluation of Thiazolidinone Cytotoxicity

Based on our proposed scheme (Scheme 3-1), a thiazolidinone molecule is liberated as the prodrug becomes activated by H$_2$O$_2$. Therefore, a cell viability assay was conducted to determine if the thiazolidinone moiety is cytotoxic. An MTS assay utilizing NIH/3T3 cells was performed in which the cytotoxicity of promoiety B was determined. Compound B demonstrated no significant cytotoxicity up to a concentration of 100 µM (Figure 3-15).

![Cytotoxicity assay for thiazolidinone promoiety B](image)

**Figure 3-15.** Cytotoxicity assay for thiazolidinone promoiety B. Each data point is the result of three independent experiments (error bars shown).

In summary, our findings demonstrate the development of a prodrug strategy for the H$_2$O$_2$-dependent release of carboxylic acids. A study using
model compounds revealed the thiazolidinone promoiety B to possess high stability in a mixed DMSO/aqueous solvent and was not prone to attack by common biological nucleophiles. Prodrugs based on the thiazolidinone B displayed essentially no activity against their targets, but in the presence of \( \text{H}_2\text{O}_2 \) activity was restored for an MMP inhibitor. Overall, these findings indicate that a thiazolidinone (B) prodrug strategy is viable for derivatizing carboxylic acid-based therapeutics for ROS-targeted release. Considering the widespread use of ester-based prodrugs, such as ACE inhibitors, it is expected that the strategy here will have applicability to produce ROS-activated, disease-targeted analogs of various valuable bioactive compounds.

3.3 Experimental

**General Experimental Details:** All chemicals were purchased from commercial suppliers (Sigma–Aldrich, Acros Organics, TCI America) and were used without further purification. Chromatography was performed using a CombiFlashRf 200 automated system from TeledyneISCO (Lincoln, NE USA). NMR spectra were recorded on a Varian FT 400 NMR instrument. Mass spectrometry was performed at the Molecular Mass Spectrometry Facility (MMSF) in the Department of Chemistry & Biochemistry at the University of California, San Diego.
**Synthesis.** Synthesis of compounds B, D, 1 and 4-8 had been previously reported. Oxazolidin-2-one (A) and thiazolidine-2-thione (D) are commercially available.

**Thiazolidin-2-one (B).** To a solution of anhydrous EtOH (10 mL) in a dry vessel was added sodium (0.25 g, 11 mmol). The mixture was kept under a nitrogen atmosphere and stirred at room temperature for ~30 min. To this was added thiazolidine-2-thione (D) (1.2 g, 10 mmol) and 2-bromoethanol (1.3 g, 10 mmol). The mixture was heated to reflux for 4 h, then allowed to cool to room temperature, and finally filtered to remove insoluble white solids, which were rinsed with anhydrous EtOH (3x10 mL). The filtrate was concentrated, then purified via silica gel chromatography eluting with hexanes and EtOAc to afford B in 56% yield (0.64 g, 6.2 mmol). $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$ 6.91 (br s, 1H), 3.62 (t, $J = 7.2$ Hz, 2H), 3.44 (t, $J = 7.2$ Hz, 2H). ESI-MS(+): $m/z$ 104.0 [M+H]$^+$, 126.0 [M+Na]$^+$.

**Oxazolidine-2-thione (C).** To a solution of 2-aminoethanol (6.1 g, 100 mmol) in EtOH (250 mL) was added K$_2$CO$_3$ (6.9 g, 50 mmol) and carbon disulfide (15.2 g, 200 mmol). The mixture was heated to 40 °C, and H$_2$O$_2$ (30% w/w) (15.3 mL, 150 mmol) was added over 1 h. The reaction was cooled to room temperature and stirred for an additional 4 h, then followed by the addition of sat. NH$_4$Cl (aq, 10 mL). The resulting mixture was extracted with EtOAc (3x150 mL). The organic phases were combined, dried with MgSO$_4$, concentrated, and purified via
silica gel chromatography, eluting with hexanes and (0-50%) EtOAc to afford C in 78% yield (4.0 g, 39 mmol). $^1$H NMR (400 MHz, Acetone-\textit{d$_6$}) $\delta$ 8.63 (br s, 1H), 4.66 (t, $J = 8.7$ Hz, 2H), 3.81 (t, $J = 8.7$ Hz, 2H). ESI-MS(+): $m/z$ 104.0 [M+H]$^+$, 126.0 [M+Na]$^+$.

General Procedure for Synthesis of Model Compounds 1-8:

Protocol for the amide coupling (Method i):

To a solution of carboxylic acid (1 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), was added DCC (1.1 mmol) and DMAP (1.1 mmol). The mixture was stirred at room temperature for 20 min followed by the addition of the corresponding amine (A-D) (1 mmol) and stirring for an additional 4 h. The resulting solution was concentrated, then purified via silica gel chromatography eluting with a gradient of 0-30% EtOAc in hexanes.

Protocol for Schotten-Baumann reaction (Method ii)
To a solution of A-D (12 mmol) in H₂O (5 mL) was added NaOH (15 mmol), followed by acetone (45 mL), and then the corresponding acyl chloride (15 mmol). The mixture was stirred for 30 min at room temperature. Acetone was removed from the solution under reduced pressure and the remaining aqueous solution was further diluted with H₂O (20 mL), followed by extraction with EtOAc (3x20 mL). The organic phases were combined and dried with MgSO₄, filtered, concentrated, and purified via silica gel chromatography eluting with a gradient of 0-30% EtOAc in hexanes.

3-Benzoyloxazolidin-2-one (1). Yield via Method i: 72% (0.14 g, 0.72 mmol). 

$^1$H NMR (400 MHz, Acetone-$_d$₆) $\delta$ 7.64-7.66 (m, 2H), 7.52-7.56 (m, 1H) 7.40-7.45 (m, 2H) 4.55 (t, $J$ = 7.7 Hz, 2H) 4.18 (t, $J$ = 7.7 Hz, 2H). ESI-MS(+): m/z 192.2 [M+H]$^+$, 214.1 [M+Na]$^+$.

3-Benzoylthiazolidin-2-one (2). Yield via Method i: 50% (0.10 g, 0.50 mmol. $^1$H NMR (400 MHz, Acetone-$_d$₆) $\delta$ 7.62-7.65 (m, 2H), 7.52-7.56 (m, 1H), 7.41-7.45 (m, 2H), 4.25 (t, $J$ = 7.0 Hz, 2H), 3.55 (t, $J$ = 7.0 Hz, 2H). $^{13}$C NMR (100 MHz, Acetone-$_d$₆) $\delta$ 172.4, 169.5, 134.8, 132.0, 129.1, 128.0, 48.7, 25.9. ESI-MS(+): m/z 230.0 [M+Na]$^+$.

Phenyl(2-thioxooxazolidin-3-yl)methanone (3). Yield via Method i: 72% (0.15 g, 0.72 mmol). $^1$H NMR (400 MHz, Acetone-$_d$₆) $\delta$ 7.71-7.73 (m, 2H), 7.54-7.58 (m, 1H), 7.42-7.46 (m, 2H), 4.77 (t, $J$ = 7.0 Hz, 2H), 4.36 (t, $J$ = 7.0 Hz, 2H). $^{13}$C
NMR (100 MHz, Acetone-$d_6$) $\delta$ 187.7, 171.1, 134.5, 132.3, 129.5, 128.1, 68.2, 48.4. ESI-MS(+) $m/z$ 230.0 [M+Na]$^+$.  

**Phenyl(2-thioxothiazolidin-3-yl)methanone (4).** Yield via Method i: 86% (0.19 g, 0.86 mmol). $^1$H-NMR (400 MHz, Acetone-$d_6$) $\delta$ 7.73-7.76 (m, 2H), 7.55-7.59 (m, 1H), 7.43-7.47 (m, 2H), 4.58 (t, $J$ = 7.2 Hz, 2H), 3.68 (t, $J$ = 7.2 Hz, 2H). ESI-MS(+) $m/z$ 224.1 [M+H]$^+$, 246.0 [M+Na]$^+$.  

**3-(2-Phenylacetyl)oxazolidin-2-one (5).** Yield via Method ii: 82% (2.5 g, 12.3 mmol). $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$ 7.24-7.31 (m, 5H), 4.47 (t, $J$ = 7.8 Hz, 2H) 4.34 (s, 2H), 4.03 (t, $J$ = 7.8 Hz, 2H). ESI-MS(+) $m/z$ 206.0 [M+H]$^+$, 226.0 [M+Na]$^+$.  

**3-(2-Phenylacetyl)thiazolidin-2-one (6).** Yield via Method i: 41% (0.09 g, 0.41 mmol). $^1$H-NMR (400 MHz, Acetone-$d_6$) $\delta$ 7.24-7.32 (m, 5H), 4.17-4.20 (m, 4H), 3.42 (t, $J$ = 7.4 Hz, 2H). ESI-MS(+) $m/z$ 222.1 [M+H]$^+$, 244.0 [M+Na]$^+$.  

**2-Phenyl-1-(2-thioxooxazolidin-3-yl)ethan-1-one (7).** Yield via Method i: 63% (0.14 g, 0.63 mmol). $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$ 7.25-7.32 (m, 5H), 4.70 (s, 2H), 4.64 (t, $J$ = 7.6 Hz, 2H), 4.29 (t, $J$ = 7.6 Hz, 2H). ESI-MS(+) $m/z$ 244.0 [M+Na]$^+$. 
2-Phenyl-1-(2-thioxothiazolidin-3-yl)ethan-1-one (8). Yield via Method i: 73% 
(0.17 g, 0.73 mmol). $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$ 7.25-7.33 (m, 5H), 4.63 
(s, 2H), 4.61 (t, $J = 7.6$ Hz, 2H), 3.44 (t, $J = 7.6$ Hz, 2H). ESI-MS(+): $m/z$ 260.0 
[M+Na]$^+$. 

3-(2-(4-Isobutylphenyl)propanoyl)thiazolidin-2-one (proIBU). Yield via Method i: 70% 
(0.2 g, 0.7 mmol). $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$ 7.19 (d, $J = 8.1$ Hz, 2H), 7.10 (d, $J = 8.1$ Hz, 2H), 4.93 (q, $J = 7.0$ Hz, 1H), 4.15-4.20 (m, 2H), 
3.28-3.37 (m, 2H), 2.44 (d, $J = 7.2$ Hz, 2H), 1.84 (sep $J = 6.7$ Hz, 1H), 1.30 (d, $J 
= 7.0$ Hz, 3H), 0.88 (d, $J = 6.7$ Hz, 6H). $^{13}$C NMR (100 MHz, Acetone-$d_6$) $\delta$ 174.0, 
172.2, 140.1, 138.5, 129.1, 127.8, 47.8, 44.7, 43.7, 30.1, 24.6, 21.8, 
19.21. ESI-MS(+): $m/z$ 292.2 [M+H]$^+$, 314.2 [M+Na]$^+$. HRMS calcd for C$_{16}$H$_{21}$N 
O$_2$ S Na: 314.1185; Found: 314.1192. 

Methyl([1,1'-biphenyl]-4-ylsulfonyl)-D-phenylalaninate (9). A solution of D- 
phenylalanine methyl ester hydrochloride (0.5 g, 2.3 mmol) and [1,1'-Biphenyl]-4- 
sulfonyl chloride (1.76 g, 6.9 mmol) in pyridine (11 g, 139 mmol) was irradiated in 
a microwave reactor (CEM Discover) for 12 min (130 °C, 250 max psi). The 
resulting solution was concentrated in vacuo. The crude oil was purified via silica 
gel chromatography eluting a gradient of 0-50% EtOAc in hexanes. Yield = 0.85 
g (93%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.79 (d, $J = 8$ Hz, 2H), 7.63 (d, $J = 8$ Hz, 
2H), 7.59 (d, $J = 8.0$ Hz, 2H), 7.49-7.09 (m, 8H), 5.45 (d, $J = 9.2$ Hz, 1H), 4.25 
(dd, $J = 8$ Hz, 6 Hz, 1H), 3.49 (s, 3H), 3.06-3.02 (m, 2H).
([1,1'-biphenyl]-4-ylsulfonyl)-D-phenylalanine (10). To a solution of 9 (0.85 g, 2.16 mmol) in THF (30 mL) was added 2M NaOH (25 mL), and the mixture heated to 55 °C for 4 h. THF was removed in vacuo. The aqueous layer was acidified to pH 2-3 using 6M HCl, which caused the formation of an off-white precipitate. The precipitate was filtered to afford the carboxylic acid product. Yield = 0.81 g (99%). \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)) \(\delta\) 7.71 (d, \(J = 8.4\) Hz, 2H), 7.58-7.40 (m, 7H), 7.21-7.08 (m, 5H), 5.28 (d, \(J = 9.2\) Hz, 1H), 4.24 (m, 1H), 3.13 (dd, \(J = 13.6\) Hz, 4.8 Hz, 1H), 2.97 (dd, \(J = 14\) Hz, 7.2 Hz, 1H). ESI-MS(-): \(m/z\) 380.26 [M-H]^-.

(R)-N-(1-oxo-1-(2-oxothiazolidin-3-yl)-3-phenylpropan-2-yl)-[1,1'-biphenyl]-4-sulfonamide (proMMPi). To a solution of 10 (0.25 g, 0.65 mmol) in DMF (10 mL) was added hydroxybenzotriazole (HOBT) (0.15 g, 0.98 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (0.18 g, 0.98 mmol), and the reaction was stirred under nitrogen atmosphere at room temperature for 15 min. To this was then added B (0.34 g, 3.3 mmol) and the mixture was allowed to stir overnight. The solvent was removed in vacuo, and the resulting crude oil was purified via silica gel chromatography eluting 0-80% EtOAc in hexanes. Yield = 0.06 g (19%). \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)) \(\delta\) 7.69 (d, \(J = 8.4\) Hz, 2H), 7.59-7.42 (m, 7H), 7.18-7.12 (m, 5H), 5.50 (d, \(J = 10.4\) Hz, 1H), 5.37 (m, 1H), 3.95 (m, 1H), 3.15 (t, \(J = 7.2\) Hz ,2H), 3.07 (dd, \(J = 13.6\) Hz, 4.8 Hz, 1H), 2.77 (dd, \(J = 8.8\) Hz,
4.8 Hz, 1H). ESI-MS(+): m/z 467.09 [M+H]^+, 489.19 [M+Na]^+. HRMS calcd for C_{24}H_{22}N_{2}O_{4}S_{2}Na: 489.0913; Found: 489.0906.

**Activation of Compounds by ROS.** Activation was determined via analytical HPLC on a HP Series 1050 System equipped with an Agilent Poroshell 120 reverse-phase column (EC-C18, 4.6x100mm, 2.7μm). Separation was achieved with a flow rate of 1 mL min\(^{-1}\) and the following mobile phase: 2.5% ACN + 0.1% formic acid in H\(_2\)O (A) and 0.1% formic acid in ACN (B). Starting with 95% A and 5% B, a linear gradient was run for 15 min to a final solvent mixture of 5% A and 95% B, which was held for 5 min before ramping back down to 95% A and 5% B over the course of 2 min, with constant holding at this level for 4 additional min. Injections consisted of 100 μL. Activation of compounds 1, 3-5, 7-8 and proIBU was determined in a 1 mM solution in 40% DMSO/60% Buffer (100 mM Tris-Cl, pH 7.4) by adding H\(_2\)O\(_2\) (20 equiv, 20 mM) and incubating at 37 °C. For compounds 2 and 6, activation was determined in a 1 mM solution in 5% DMSO/95% Buffer (100 mM Tris-Cl, pH 7.4) by adding H\(_2\)O\(_2\) (20 equiv, 20 mM) and incubating at 37 °C. ProMMPi activation was determined in a 100 μM solution in 40% DMSO/60% Buffer (100 mM Tris-Cl, pH 7.4) by adding H\(_2\)O\(_2\) (20 equiv, 2 mM) or NaClO (20 equiv, 2 mM) and incubating at 37 °C. HPLC traces were obtained at different time points. Conversion to corresponding acid was determined by integration of area under the curve.
**Stability of Model Compounds.** The stability was determined via the analytical HPLC method previously described. Aqueous stability of compounds was determined by making a 1 mM in 40% DMSO/60% Buffer (100 mM Tris-Cl, pH 7.4) and incubating at 37 °C for 24 h. Stability in the presence of nucleophiles was determined by making a 1 mM in 40% DMSO/60% Buffer (100 mM Tris-Cl, pH 7.4) and adding the corresponding nucleophile (20 equiv, 20 mM) and incubating at 37 °C for 24 h.

**MMP Inhibition Assays.** Inhibition values of proMMPi were determined using a commercially available fluorescent-based assay kit. MMP-2 and OmniMMP fluorogenic subsbtrate were purchased from Enzo Life Sciences (Farmingdale, NY). MMP activity was measured in 96-well plates using a Bio-Tek Synergy HT fluorescent plate reader. ProMMPi was dissolved in DMSO to a concentration of 100 mM and further diluted with Tris-Cl buffer (100 mM, pH 7.4) to a concentration of 1 mM (40% DMSO/ 60% Buffer). To the sample was added H$_2$O$_2$ (50 equiv, 50 mM), followed by incubation at 37 °C until full deprotection was observed via analytical HPLC. The treated compound was then added to appropriate wells near its IC$_{50}$ value (350 nM). Each well contained 20 µL of MMP-2 (1.16 U), 60 µL MMP assay buffer (50 mM HEPES, 10mM CaCl$_2$, 0.10% Brij-35, pH 7.5), and the H$_2$O$_2$-treated MMPi (10 µL). After a 30 min incubation at 37 °C, a reaction was initiated with the addition of 10 µL (40 µM) of the fluorescent substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$) where Mca = (7-methoxycoumarin-4-yl)-acetyl and Dpa = N-3-(2,4-dinitrophenyl)-L-α-β-
diaminopropionyl)) and activity was monitored every 30 sec for 30 min with excitation and emission wavelengths at 320 nm and 400 nm, respectively. Enzymatic activity and thus inhibition was calculated with respect to the control experiment (no inhibitor present). Measurements were performed in triplicate with three independent experiments.

**COX-1 Inhibition Assays.** Inhibition values of proIBU was determined using a commercially available fluorescent-based assay kit *Cayman Chemical – Cox Fluorescent Inhibitor Screening Assay Kit Item No. 700100*. COX-1 activity was measured in 96-well plates using a Bio-Tek Synergy HT fluorescent plate reader. ProIBU was dissolved in DMSO to a concentration of 100 mM and further diluted in Tris-Cl buffer (100 mM, pH 8.0) to a concentration of 1 mM (40% DMSO/60% Buffer). To the sample was added H\textsubscript{2}O\textsubscript{2} (50 equiv), and incubation at 37 °C was allowed until full deprotection was observed via analytical HPLC. The treated compound was then added to appropriate wells near its IC\textsubscript{50} value (2µM). Protocol was carried out as instructed by supplier.

**Crystallographic Data.** A concentrated solution of either compound 2 or 3 was in prepared in a 1:1 (by volume) mixture of CH\textsubscript{2}Cl\textsubscript{2} and EtOAc. The solution was transferred to a vial, layered with hexanes, and sealed. The solution was allowed to stand at room temperature for several days, yielding X-ray quality crystals of the desired compound.

A single crystal of 2 or 3 taken from a mixture of CH\textsubscript{2}Cl\textsubscript{2}:EtOAc:hexanes
was mounted on nylon loops with paratone oil and placed under a nitrogen cold stream. Data was collected on a Bruker Apex diffractometer using Mo Kα (λ= 0.71073 Å) radiation controlled using the APEX 2010 software package. The data was collected up to 0.83 Å. A multi-scan method utilizing equivalents was employed to correct for absorption. All data collections were solved and refined using the SHELXTL software suite. Details for these structures can be obtained from the Cambridge Crystallographic Data Centre (CCDC) under deposition numbers 1038268-1038269.

**Cytotoxicity Assay.** NIH/3T3 cell line was kindly donated by Dr. Richard Klemke and grown on recommended medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C in an incubator with 5% CO₂. The CellTiter 96 AQueous One Solution Cell Proliferation assay (MTS) kit was purchased from Promega (Madison, WI, USA). The NIH/3T3 cell line was maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). The cytotoxicity of thiazolidinone was measured using the MTS assay according to the manufacturer protocol. To start the assay, NIH/3T3 cells were counted with a hemocytometer and diluted with fresh medium to the proper concentration, such that 5000 cells per well were seeded in a 96 well plate. The NIH/3T3 cells were then incubated at 37 °C with 5% CO₂ for 16 h prior to the drug treatment for cell attachment. The cells were then treated with various concentrations of thiazolidinone (ranging from 0.25 µM to 170 µM) for 60 h. At this point, 20 µL of CellTiter 96 AQueous One Solution was added per well, and the plate was
incubated at 37 °C for 2 h. The absorbance at 490 nm was read using a BioTek Synergy HT microplate reader. Each concentration was conducted in triplicate for one trial and repeated for 2-3 trials.

3.4 Acknowledgements


The thesis author was the primary researcher for the data presented and was the primary author on the publication. The permission to reproduce this paper was granted by The Royal Society of Chemistry. Copyright 2015, The Royal Society of Chemistry.

3.5 References


