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Reactive Oxygen Species (ROS)-Activatable Prodrug for Selective Activation of ATF6 after Ischemia/Reperfusion Injury

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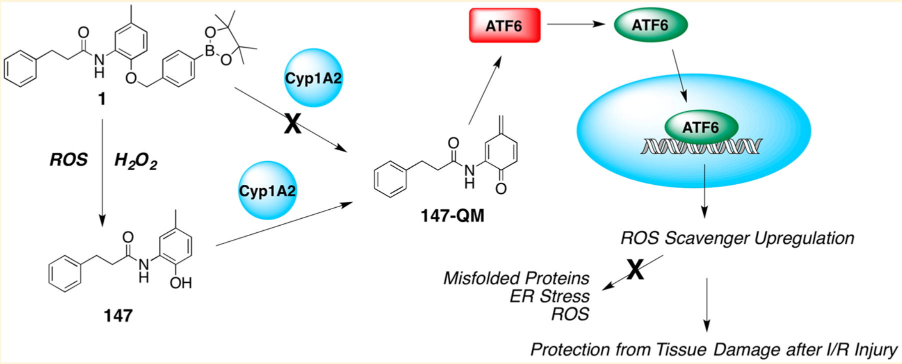
Supporting Information



ABSTRACT: We describe here the design, synthesis, and biological evaluation of a reactive oxygen species (ROS)-activatable prodrug for the selective delivery of **147**, a small molecule ATF6 activator, for ischemia/reperfusion injury. ROS-activatable prodrug **1** and a negative control unable to release free drug were synthesized and examined for peroxide-mediated activation. Prodrug **1** blocks activity of **147** by its inability to undergo metabolic oxidation by ER-resident cytochrome P450 enzymes such as Cyp1A2, probed directly here for the first time. Biological evaluation of ROS-activatable prodrug **1** in primary cardiomyocytes demonstrates protection against peroxide-mediated toxicity and enhances viability following simulated I/R injury. The ability to selectively target ATF6 activation under diseased conditions establishes the potential for localized stress-responsive signaling pathway activation as a therapeutic approach for I/R injury and related protein misfolding maladies.

KEYWORDS: *ROS-activatable prodrug, ATF6 activation, ischemia-reperfusion injury, Cyp1A2*

Modulation of stress-responsive signaling pathways, such as the unfolded protein response (UPR) in the endoplasmic reticulum, has emerged as a promising therapeutic strategy for treatment of a range of protein misfolding diseases.[1−3] In particular, activation of ATF6, a transcription factor involved in UPR signaling, has shown therapeutic potential in models of transthyretin and light chain amyloid diseases,[4−6] diabetes,[7] and protection of tissue damage following ischemia/reperfusion (I/R) injury in pathologies such as myocardial infarction and stroke.[8,9] A selective small molecule ATF6 activator, **147** (Scheme 1), was recently identified by a high-throughput screening effort and has offered the opportunity for pharmacological evaluation of ATF6 transcriptional activation.[6] Compound **147** has advanced to *in vivo* studies in mouse models of I/R, where it was shown to ameliorate cardiac and brain tissue damage and preserve heart and brain function following acute myocardial or cerebral infarction.[10] While these results suggest that ATF6 activation, and **147** in particular, have promising clinical potential, constant and global activation of ATF6 can cause liver disease[11] and apoptosis,[12] motivating the development of prodrug derivatives of **147** that could confer ATF6 activation specifically under I/R conditions, mitigating off-target toxicity and potential side effects.

Reactive oxygen species (ROS) offer a chemical stimulus to incite drug release and target therapy. Significantly elevated and damaging ROS levels are associated with numerous disease states[13] and are particularly damaging during the reperfusion stage of I/R injury.[14−16] We therefore sought to design and synthesize a prodrug of **147** for targeted delivery and ATF6 activation following exposure to ROS during I/R injury. Our approach takes advantage of the phenol functional group on **147** as a chemical handle to attach a ROS-cleavable phenyl boronate ester (Scheme 1). Phenyl boronate linkers of this

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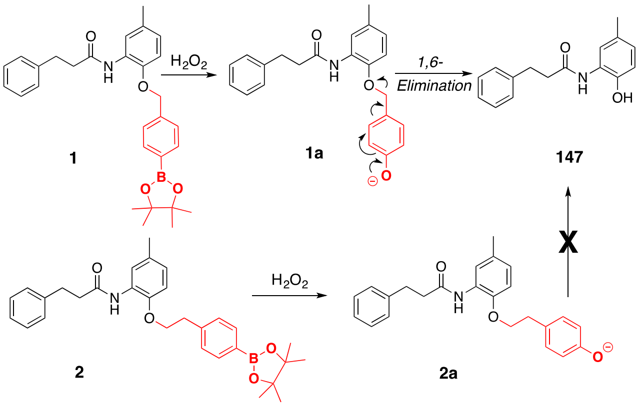
Scheme 1. Structures and Peroxide-Mediated Release Mechanism of Designed Prodrug 1 and Negative Control 2

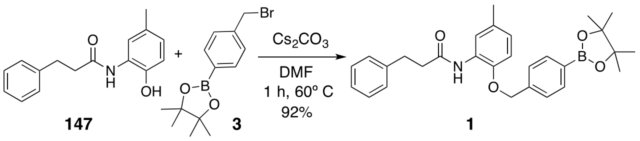


type show selectivity for ROS such as hydrogen peroxide, and have been utilized as peroxide sensors[17−25] and as prodrug activation platforms for the delivery of anticancer[26−33] and anti-inflammatory agents.[34]

ROS-activatable prodrug **1** (Scheme 1) was designed to release **147** and activate ATF6 following reaction with peroxide during I/R, generating intermediate **1a** followed by 1,6-elimination. Esterification of the phenol of **147** should render prodrug **1** biologically inactive, as the intact phenol has been shown to be crucial for activation of ATF6 and **147**'s postulated metabolic oxidation in cells by ER-resident cytochrome P450 enzymes.[35] In order to decouple peroxide scavenging and ATF6 activation effects of prodrug **1**, we further sought to evaluate a negative control compound **2** that retains peroxide reactivity but, due to the extra methylene unit in the linker, is unable to 1,6-eliminate and release **147** (Scheme 1). We set out to synthesize and evaluate these target compounds to explore the therapeutic utility of selective, ROS-mediated ATF6 activation following I/R injury.

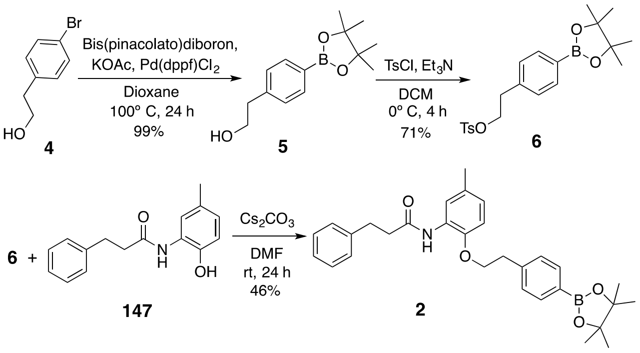
We began by synthesizing in good yield the parent compound **147** from 3-phenylpropanoic acid and 2-amino-4-methylphenol by slight modifications to the literature procedure (see SI).[35] ROS-activatable prodrug **1** was accessed in only one step from **147** by nucleophilic displacement of commercially available phenylboronic ester bromide **3** (Scheme 2).

Scheme 2. Synthesis of ROS-Activatable Prodrug 1



Negative control **2** was accessed in an analogous fashion from **147** and an extended phenylboronic ester tosylate **6** as shown in Scheme 3. The relatively lower yield for the nucleophilic displacement reaction in this case is due to competing elimination chemistry that occurs with the additional carbon atom in tosylate **6** relative to bromo compound **3**. Tosylate **6** was accessed from 4-bromo-phenylethanol by Miyaura borylation followed by tosylation in good yield (Scheme 3).

Scheme 3. Synthesis of Negative Control 2



With requisite compounds in hand, we evaluated the stability and peroxide-mediated release of the designed prodrug by analytical HPLC. Incubation of prodrug **1** under physiologically relevant conditions (Hepes Buffered Saline (HBS), pH 7.3, 37 °C) demonstrated that the prodrug is relatively stable in the absence of peroxide, with a degradation half-life ($t_{1/2}$) of 25.8 days. In contrast, incubation of prodrug **1** with 20 mol equiv of H_2O_2 gives smooth conversion to free **147** within 2 h at 37 °C (Figure 1). Notably, the conversion at 2 h is dependent on the peroxide concentration, where substoichiometric peroxide concentrations (such as the lowest tested 0.1 mol equiv peroxide/prodrug) do not release significant amounts of **147** (Figure 1). This implies that background peroxide levels, such as observed in normal tissues, would not be sufficient to activate the prodrug in a meaningful therapeutic time frame and that locally high concentrations of peroxide, as observed following I/R events, could be sufficient to trigger prodrug activation and **147** release selectively *in vivo*.

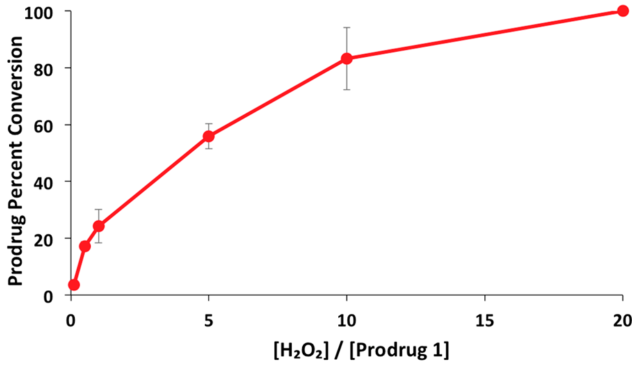


Figure 1. Conversion of prodrug **1** to **147** upon incubation with various molar equivalents of H_2O_2 for 2 h at 37 °C in HBS pH 7.4 by analytical HPLC (254 nm). Compound **147** integrations were normalized by full conversion and compared to an internal standard, napthalene methanol. Error bars represent standard error of $n = 3$ trials.

Further analysis of the peroxide-mediated release profile of prodrug **1** supports the designed release mechanism outlined in Scheme 1. Intermediate **1a** is detectable by LC−MS after 10 min of excess peroxide incubation (Figure S1a). Comparing the relative integrations of **1**, **147**, and **1a** over time suggests that prodrug **1** initially coverts to intermediate **1a**, which then releases **147** by a rate-limiting 1,6-elimination step (Figure S1b). Additionally, negative control **2** does not release any detectable **147** upon incubation with excess H_2O_2 (20 mol equiv), even with incubation times in excess of 24 h, although conversion of **2** to intermediate **2a** is clearly detected under these conditions (Figure S2).

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Following confirmation that prodrug **1** releases **147** upon exposure to ROS, we next probed the ability of intact prodrug **1** to block the purported metabolic oxidation of **147**. A previous report investigating the mechanism of **147**'s ATF6 activation bioactivity has suggested that **147** undergoes metabolic oxidation by previously unidentified cytochrome P450 enzymes to a quinone methide (**147-QM**), which serves as an electrophile for ER protein disulfide isomerase enzymes involved in ATF6 activation (Figure 2a).[35] Luminescent ATF6 reporter experiments supported the requirement for the free phenol and methyl group as required for bioactivity, and ATF6 activation was reduced in the presence of exogenous sulfur nucleophiles and by a suggested cytochrome P450 inhibitor, resveratrol.[35,36] Here, we provide direct support for the quinone methide bioactivation of **147** and identify a particular ER-membrane resident P450 enzyme (Cyp1A2) capable of this transformation. Incubation of **147** for 24 h at 37 °C with recombinant Cyp1A2 shows full consumption of **147** by LC–MS analysis, whereas **147** incubated under identical conditions without Cyp1A2 remains intact (Figure 2b, Figure S3b). Further, the Cyp1A2-generated quinone methide of **147** can be trapped by incubation in the presence of an exogenous nucleophile, glutathione (GSH), and formation of a **147-GSH** adduct was directly observed by MS analysis (Figure S4). These experiments provide the first direct evidence of **147** metabolic activation by a cytochrome P450 enzyme and implicate a particular Cyp, Cyp1A2, as capable of this transformation. Importantly, similar experiments with intact prodrug **1** demonstrate that **1** is not a substrate of Cyp1A2 (Figure 2c), which suggests that prodrug **1** remains biologically inactive until its ROS-mediated activation to free **147**.

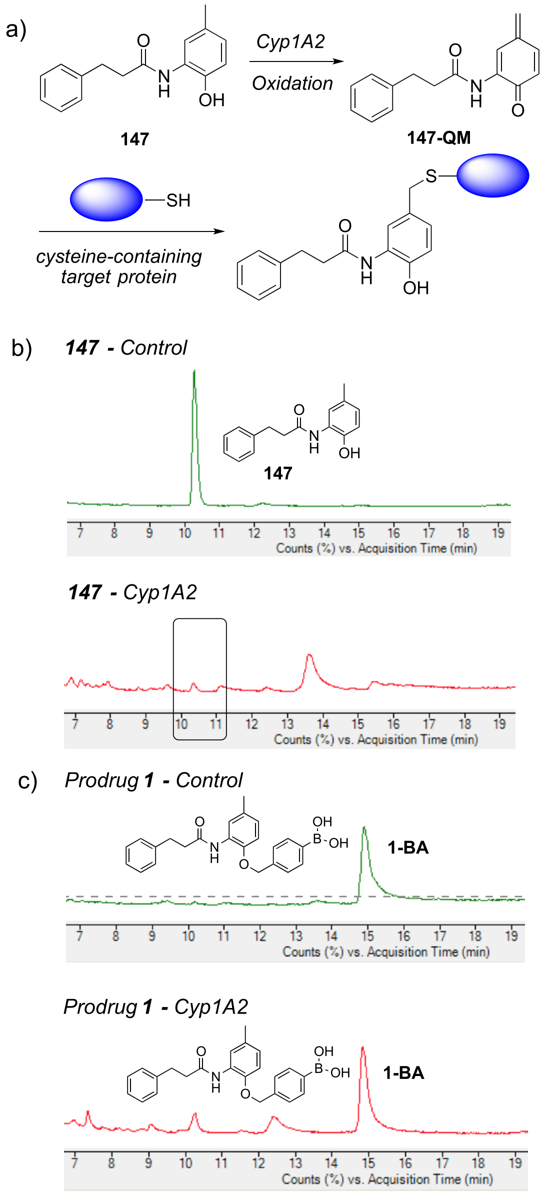

Figure 2. (a) Schematic of proposed **147** metabolic oxidation by Cyp1A2. (b) Mass spectrometry chromatogram of **147** incubated with control or Cyp1A2 baculosomes for 24 h at 37 °C by LC–MS. (c) Mass spectrometry chromatogram of prodrug **1** incubated with control or Cyp1A2 baculosomes for 24 h at 37 °C. Compound **1-BA** indicates the boronic acid of prodrug **1**.

The biological activity of ROS-activatable prodrug **1** was next examined in living cells. Cultured neonatal rat ventricular myocytes (NRVM) were treated with **147**, ROS-activatable prodrug **1**, negative control **2**, or preactivated **1** and **2** by peroxide incubation prior to NRVM administration. The induction of ATF6 target genes GRP78 and Catalase were then analyzed by qRT-PCR (Figure 3). ATF6 target gene induction was observed by **147** treatment as expected, and by prodrug **1**, only after preactivation with peroxide, indicating that prodrug **1** is biologically inactive until **147** is released following peroxide activation. Notably, negative control **2** was unable to induce ATF6 target genes, even following peroxide pretreatment.

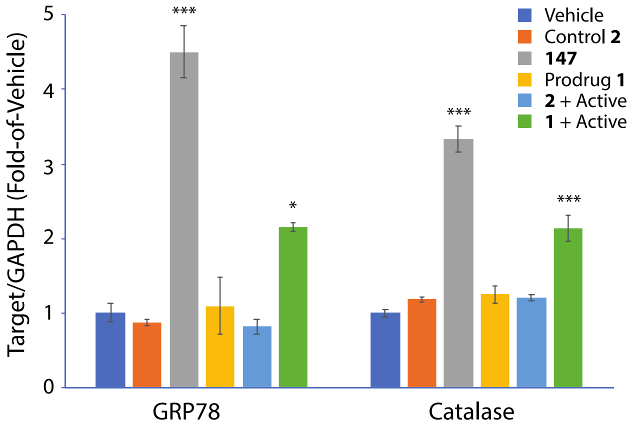

Figure 3. ATF6 target gene induction in NRVM incubated with the indicated compounds (10 μM) for 16 h followed by RNA extraction and qRT-PCR. Control **2** and prodrug **1** (+ Active) indicates compound preactivation in media containing 50 μM H_2O_2 in the absence of cells for 8 h prior to NRVM administration. Error bars represent SEM for $n = 3$ trials. *$p < 0.05$, ***$p < 0.001$ relative to vehicle as determined by ANOVA and Tukey's posthoc analysis.

Following confirmation that prodrug **1** releases **147** capable of ATF6 activation in living cells, we tested the ability of prodrug **1** to enhance cardiac cell viability under peroxide stress. Incubation of NRVM cells with prodrug **1** followed by peroxide challenge demonstrated that the ROS-activatable prodrug offers significant protection from peroxide-induced toxicity, albeit to a lesser extent than the protection observed for **147** alone (Figure S5). This effect reflects the ability of **147** to activate ATF6 target genes prior to peroxide exposure, whereas prodrug **1** remains inactive until peroxide administration.

Finally, we assessed the ability of prodrug **1** to enhance viability and reduce cardiac tissue damage following simulated I/R injury. NRVM cells were treated with 10 μM of control **2**, **147**, or prodrug **1** and subjected to 8 h of simulated ischemia followed by 24 h of reperfusion, then viability was determined by lactate dehydrogenase (LDH) leakage and calcein-AM labeling relative to cells under normoxia conditions (Figure 4, Figure S6).[8] Cardiomyocyte treatment with prodrug **1** significantly reduced the media levels of LDH, a marker for necrosis,[37] relative to treatment with vehicle or control **2** following simulated I/R injury (Figure 4). The ability of prodrug **1** to protect cells from I/R injury damage was further observed whether **1** was administered during the duration of the experiment or only during the reperfusion step (Prodrug **1** R, Figure 4). This result indicates that the high ROS levels during reperfusion are sufficient to cleave prodrug **1** to free **147** and activate ATF6 in living cells. Similar results are observed for NRVM viability as measured by calcein-AM labeling (Figure S6b). In all cases, the protective ability of prodrug **1** is moderately reduced relative to **147** alone, which is expected due to the requirement for prodrug **1** to be activated by ROS prior to onset of activity, giving **147** a “head start” to the beneficial effects of ATF6 activation. Speeding up the kinetics of prodrug release upon peroxide exposure could potentially mitigate the differences observed in activity and represent an opportunity for further development.

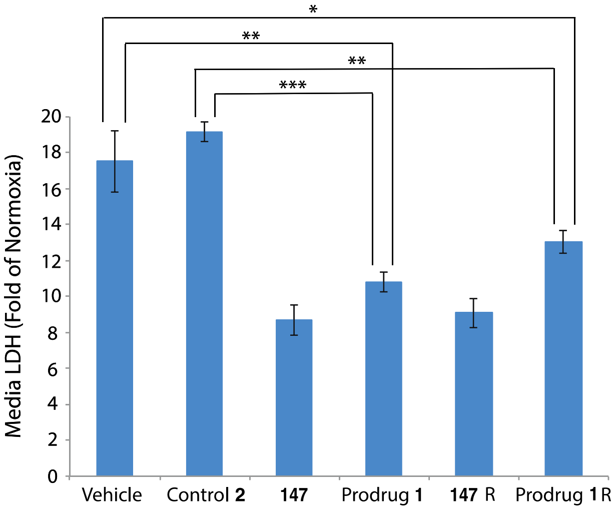

Figure 4. Viability of NVRM determined by LDH activity in media following 8 h of simulated ischemia then 24 h of reperfusion. Vehicle, negative control **2**, **147**, or prodrug **1** (10 μM) was added to cells during the entirety of the experiment or at the reperfusion step only (**147** R, Prodrug **1** R). Error bars represent SEM for $n = 3$ trials. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by ANOVA and Tukey’s posthoc analysis.

In summary, a ROS-activatable prodrug was designed and synthesized for targeted activation of ATF6 during I/R injury. The prodrug does not undergo metabolic oxidation by Cyp1A2 as revealed for the free drug until prodrug conversion is facilitated by peroxide exposure. Further, prodrug administration offers protection from I/R injury in living cells. This study offers proof-of-concept that activation of stress-responsive signaling pathways under disease conditions could offer a targeted approach for the treatment of a myriad of protein misfolding disorders. We hope to further assess the opportunities of targeted stress-responsive signaling pathway activation by translation of this study into animal models for I/R injury and expand this approach to the targeted treatment of other protein misfolding diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00299.

> Experimental details for chemical synthesis, spectroscopic analysis, stability and peroxide release assays, biological evaluation in primary cardiomyocytes for ATF6 targets, peroxide toxicity, and simulated ischemia/reperfusion injury assays, and Figures S1−S6 (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ROS, reactive oxygen species; ER, endoplasmic reticulum; ATF6, activating transcription factor 6

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