Metalloprotein Proinhibitors

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Hydrogen Peroxide Activated Matrix Metalloproteinase Inhibitors: A Prodrug Approach**

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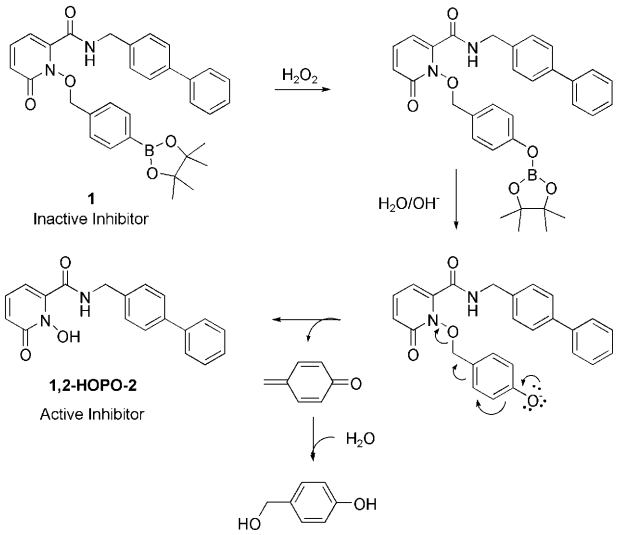
Matrix metalloproteinases (MMPs) are a family of structurally related Zn^{II}-dependent hydrolytic enzymes involved in the breakdown of the extracellular matrix.[1] MMPs are secreted as zymogens, and are activated by cleavage of the propeptide domain by proteases, other MMPs, or by reactive oxygen species (ROS). In particular, the activation of MMPs by ROS during ischemia-induced inflammatory response leads to the breakdown of the blood–brain barrier (BBB) resulting in edema and cell death.[2,3] The strong correlation between ROS-activation of MMPs and the disruption of the BBB has led to several studies on the use of MMP inhibitors (MMPi) as therapeutics for treating reperfusion injury associated with stroke.[4] Inhibition of MMPs after stroke with a variety of broad-spectrum MMPi have shown that MMP inhibition greatly reduced ischemic brain injury.[5,6] While the use of MMPi to reduce the effects of BBB disruption following stroke has been clearly established, the major challenge for MMPi in this area is the need for temporal and spatial control of their inhibitory activity.[7]

A promising strategy in MMPi is through the development of MMP prodrugs or "proinhibitors" that offer the ability to selectively control inhibitory activity. Metalloenzyme inhibitors such as MMPi are particularly suitable to the proinhibitor approach because such compounds generally contain a metal-binding group that can be blocked, which strongly attenuates their inhibitory activity. In the presence of the appropriate stimuli, the protecting group can be removed from the metal-binding group to release the MMPi at the site of activation, and thereby avoiding systemic inhibition of MMPs (which are necessary for normal physiological processes).[8,9] However, metalloenzyme proinhibitors have not been widely investigated, especially in the case of MMP proinhibitors. Recently, MMP proinhibitors that could be activated in the presence of β-glucosidase were reported.[10] In this report, MMP proinhibitors are shown to be activated by H_2O_2 for use as protective therapeutics following ischemia and reperfusion injury during stroke (Scheme 1). As described below, the proinhibitors reported can protect the BBB in two ways, taking advantage of both the triggering mechanism and the resulting MMPi. First, the proinhibitors will consume damaging ROS (e.g. H_2O_2), which would otherwise directly attack the BBB and also activate pathogenic MMPs. Second, the resulting active MMPi serves to inhibit any remaining MMP activity that might damage the BBB. Thus, this unprecedented class of proinhibitors has a dual mode of action: reducing the amount of ROS available to activate MMPs, while also generating an active MMPi.



Scheme 1. Release of the active inhibitor **1,2-HOPO-2** in the presence of H_2O_2 through a self-immolative linker strategy.

Two MMPi, the pyridinone-based molecule **1,2-HOPO-2** and the pyrone-based molecule **PY-2**, were selected for this pilot study. Both compounds are potent, semi-selective MMPi that have been previously described.[11] The hydroxy group of the zinc-binding group (ZBG) of each inhibitor was protected with a self-immolative protecting group containing a boronic ester as the ROS-sensitive trigger (Scheme 2). In the presence of H_2O_2, the boronic ester is cleaved by nucleophilic attack of H_2O_2, facilitating a spontaneous reaction to release the active MMPi through a 1,6-benzyl elimination (Scheme 1). Boronic esters as H_2O_2-reactive protecting groups have been well documented in the literature for H_2O_2-activated fluorophores[12,13] and in the generation of triggered Fe^{III} and Cu^{II} chelates.[14,15] While self-immolative linkers with boronic ester protecting groups have been successfully utilized with H_2O_2 reactive small molecule and dendrimer-based fluorescent probes,[16–19] the present work is the first description of ROS-activated prodrugs.

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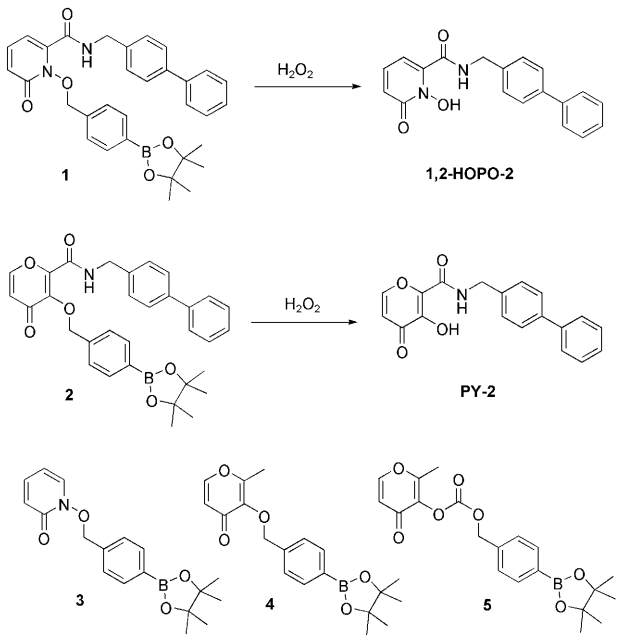

Scheme 2. Structures of proinhibitors **1** and **2** and their active inhibitors **1,2-HOPO-2** and **PY-2**, respectively, and the protected ZBGs **3**–**5**.

The ROS-triggered self-immolative protecting group can be attached to the MMPi by using either an ether (**3**, **4**) or carbonate ester (**5**) linkage at the hydroxy group of the ZBG (Scheme 2). To determine which linker strategy provided the best overall approach, both the cleavage kinetics and solution stability of protected ZBGs **3**–**5** were examined (see Supporting Information). The ability of these compounds to be activated by H_2O_2 was evaluated by using electronic spectroscopy. A sample of each compound in HEPES buffer (50 mM, pH 7.5) was activated with an excess (18 equiv)[12–15] of H_2O_2 and the change in absorbance was monitored over time. In all cases, the spectra of the protected ZBG compounds decreased over time while the spectra of the free ZBG appeared, demonstrating the expected cleavage reaction (Supporting Information, Figure S1–S3). To confirm that the boronic ester moiety was necessary for H_2O_2 cleavage, the ZBGs were prepared with benzyl protecting groups without the boronic ester. For these compounds, no change in absorbance was observed over time in the presence of H_2O_2 (Figure S4). Additionally, the selectivity of the boronic ester towards H_2O_2 was confirmed by examining cleavage in the presence of KO_2 and catalase (Figure S5). As expected,[12,20] the superoxide anion was unable to activate the protected ZBGs.

The rates of conversion of compounds **3**–**5** to their respective activated ZBGs were then determined by monitoring the change in absorption using pseudo-first order reaction conditions with an excess of H_2O_2. The calculated rate constants indicated that the carbonate ester linkage in compound **5** provided the fastest conversion with a rate constant of $6.7\,M^{-1}s^{-1}$, while rate constants of $4.0\,M^{-1}s^{-1}$ and $2.9\,M^{-1}s^{-1}$ were found for compounds **3** and **4**, respectively (see Supporting Information). Upon examination of the solution stability of these compounds, **3** and **4** were stable in buffer over a 24 h time period, while **5** showed >50% hydrolysis (Figure S6). Although the use of carbonate and carbamate ester linkages in self-immolative systems are more common (due to the additional thermodynamic driving force from the release of CO_2 in the cascade reaction),[21,22] our findings suggest that the carbonate ester linkage was not optimal because of the low aqueous stability observed for **5**. In addition, we found that incorporation of the carbonate ester linkage was synthetically more challenging and less reliable (i.e. when comparing the synthesis of **4** versus **5**), which further discouraged its use in a metalloprotein proinhibitor approach. Indeed, despite numerous attempts, we were unable to achieve a satisfactory synthesis for the carbonate ester analog of compound **3**.

After establishing a strategy for the addition of H_2O_2 activated protecting groups to the appropriate ZBGs, the full-length inhibitors **1,2-HOPO-2** and **PY-2** were protected with 4-bromomethylphenyl boronic acid pinacol ester in the presence of K_2CO_3 in DMF to yield compounds **1** and **2**, respectively. Activation of **1** and **2** by H_2O_2 to release **1,2-HOPO-2** and **PY-2** was confirmed by absorption spectroscopy (Figure S7 and S8). The spectral changes observed for **1** and **2** (obtained under the same reaction conditions as those used for compounds **3** and **4**) suggest that the cleavage kinetics for the proinhibitors are comparable to the ZBGs. The IC_{50} values of the proinhibitors **1** and **2** against MMP-9 were found to be greater than 1 mM, representing a >100 fold-increase than the active inhibitor (Table 1). When **1** and **2** were tested against MMP-12, their IC_{50} values were found to be in the micromolar range (Table 1), which was again >100-fold less effective than their activated counterparts. Both sets of experiments show that when the ZBG of the inhibitor is protected, the ability of the compounds to inhibit MMPs is severely attenuated.

Table 1: IC_{50} values of proinhibitors and inhibitors against MMP-9 and MMP-12 as measured using a fluorescence based assay. Data are the average of two experiments.

Proinhibitor	IC_{50}	Inhibitor	IC_{50}	Enzyme
1	>1 mM[a]	**1,2-HOPO-2**	6.1(±0.2) μM	MMP-9
1	17.8(±1.1) μM	**1,2-HOPO-2**	0.053(±0.01) μM	MMP-12
2	>1 mM[b]	**PY-2**	9.8(±0.7) μM	MMP-9
2	12.9(±0.03) μM	**PY-2**	0.035(±0.003) μM	MMP-12

[a] 46% inhibition at 1 mM. [b] 27% inhibition at 1 mM.

Having established that proinhibitors **1** and **2** could be effectively protected and activated in the presence of H_2O_2, the ability of these compounds to inhibit MMPs after activation was evaluated. Using a fluorescence-based assay, compounds **1** and **2** were tested with MMP-9 and MMP-12 in the presence of H_2O_2 at concentrations close to their reported IC_{50} values.[11] MMP-9 is considered a high-value MMP target in the context of ischemia-reperfusion injury associated with stroke.[7] The percent inhibition of proinhibitors **1** and **2** were evaluated after one hour of activation with and without H_2O_2. As expected, when there is no hydrogen peroxide present,

there is little inhibition observed for the proinhibitors (Figure 1). However, after activation with 100 μM H_2O_2, the percent inhibition observed for **1** was similar to that observed for the active inhibitor **1,2-HOPO-2**. Assuming that the rate

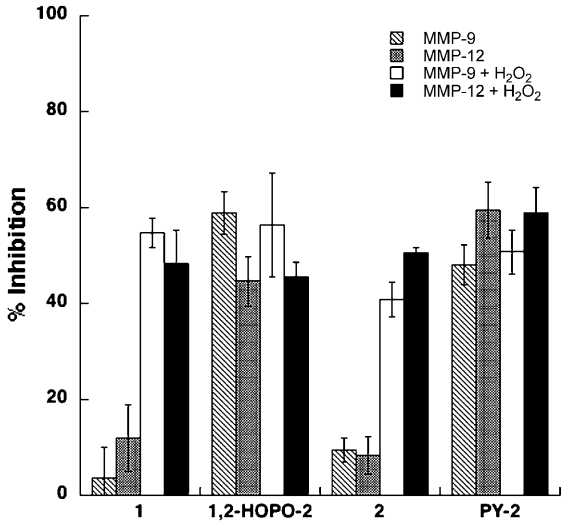

Figure 1. Percent inhibition of MMP-9 and MMP-12 with proinhibitors **1** and **2** tested at 10 μM for MMP-9 and 50 nM for MMP-12 in the absence and presence of 100 μM H_2O_2 after one hour of activation. Data are the average of four experiments.

constants found for the cleavage of **3** and **4** are essentially the same when incorporated into **1** and **2**, then the 50% inhibition observed is consistent with the calculated amount of active inhibitor present when exposed to 100 μM H_2O_2 for 1 h (see Supporting Information).

The proinhibitors introduced in this work demonstrate an effective means to passivate MMPi and activate them in the presence of H_2O_2. Through addition of a boronic ester protecting group to the metal-binding moiety of MMPi via a self-immolative linker, proinhibitors based on two different ZBGs were developed. These compounds were found to be sufficiently stable in buffer and were found to have high rates of cleavage allowing for efficient activation with H_2O_2. These compounds should display a dual mode of action in the prevention of reperfusion injury, by neutralizing ROS and generating an active MMPi. To the best of our knowledge, this is the first example of a H_2O_2 activated prodrug which offers a novel way to provide both spatial and temporal control over MMP inhibition for use in reperfusion injury. The potential of these drugs is currently being investigated to reduce the effects of the BBB disruption following stroke.

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