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Target-Activated Prodrugs (TAPs) for the Autoregulated Inhibition of MMP12

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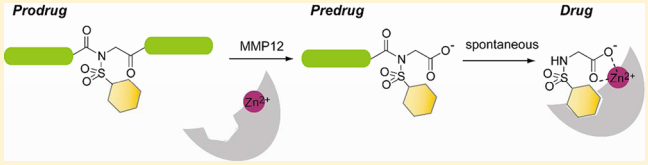
Supporting Information

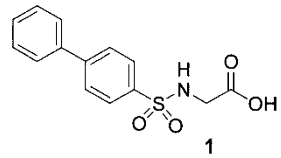
ABSTRACT: We describe a prodrug concept in which the target enzyme MMP12 produces its own inhibitor in a two-step activation procedure. By using an MMP12-specific peptide sequence and a known sulfonamide drug integrated in the backbone, the active inhibitor is released upon enzyme cleavage. In in vitro experiments, we present proof of concept that the activation proceeds with useful kinetics. The approach is highly selective over the closely related MMP8. If applied in vivo in the future, these prodrugs might release the active entity in a highly specific manner only at such sites where enzyme activity resides.



KEYWORDS: *prodrug, MMP, inhibitors, inflammation, proteases*

Proteases are potential drug targets for a broad range of diseases such as cancer or inflammation, and some inhibitors have been successfully used in the clinic.[1] However, developing new drugs in the last years has been challenging, partially due to the difficulty in achieving selectivity and the risk of hitting antitargets, leading to serious side effects. To open doors to new treatments for diseases where proteases have become validated targets, we propose a novel prodrug approach involving target-activated prodrugs (TAPs), to inhibit proteolytic activity selectively and in an autoregulated fashion. Here, we focus on matrix metalloproteinase-12 (MMP12), which is a proteolytic enzyme mainly secreted by macrophages. MMP12 is able to degrade components of the extracellular matrix such as elastin and therefore plays an important role in macrophage migration and extracellular matrix homeostasis.[2] However, under certain pathological conditions, especially when linked to inflammation, its proteolytic activity might result in excessive tissue destruction and lung emphysema. For this reason, MMP12 has been considered a drug target for inflammatory lung diseases for a long time.[3] Consequently, significant effort has been spent on the development of specific MMP inhibitors, and several compound libraries have been created and screened.[4]

Some of the hits obtained were evaluated in mouse models and later in clinical trials against cancer and vascular and inflammatory diseases.[5,6] However, these molecules revealed side effects such as musculoskeletal pain as well as insufficient clinical benefit.[7] The ineffectiveness of synthetic MMP inhibitors was most probably due to the lack of selectivity but also a lack of knowledge about MMP biology. For this reason, it was speculated that an improvement in the selectivity toward a specific MMP and a better understanding of the disease would produce useful treatments in the future.[8] As a result, a large number of low nanomolar MMP inhibitors with improved selectivity were synthesized, but their applicability in the clinic has not yet been proven.[9] Therefore, there is still a need for new strategies to develop MMP inhibitors that avoid side effects and make MMPs druggable targets.[10] A particularly desirable feature would be the exclusive targeting of MMP12 at the site of inflammation rather than the global inhibition of enzyme activity.



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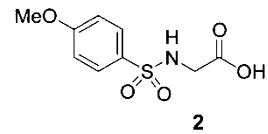

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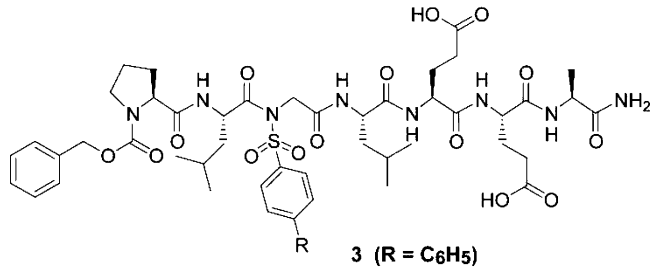

3 (R = C_6H_5)
4 (R = OCH_3)

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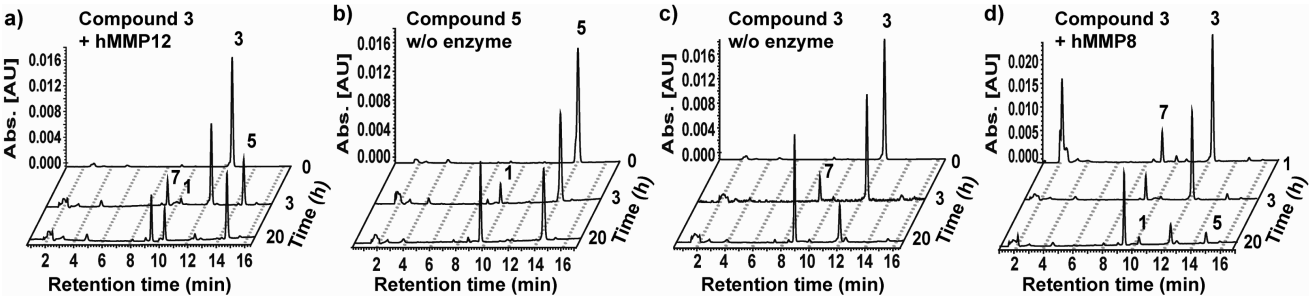
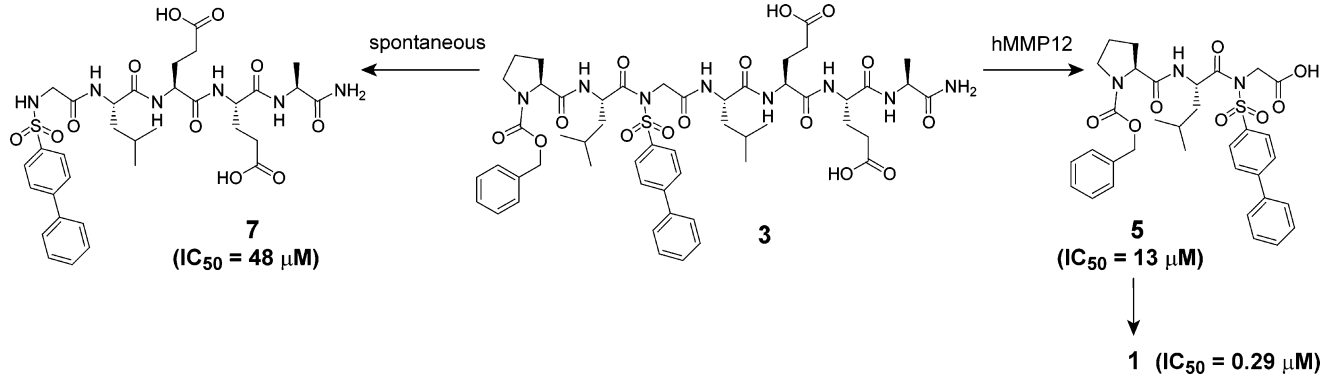


Figure 1. HPLC traces showing hMMP12-mediated drug release from TAP. (a) Prodrug **3** (10 μM) was incubated with hMMP12 (12 nM) in TCN buffer at 37 °C for different times. After 3 h, the prodrug had been significantly cleaved by hMMP12 to generate predrug **5**. Over time, **5** hydrolyzed spontaneously to the potent inhibitor **1**. Uncleaved prodrug **3** hydrolyzed to compound **7**. (b) Compound **5** (10 μM) was incubated without enzyme to study the spontaneous hydrolysis of the molecule. After 20 h, a substantial amount of **5** had converted to the free inhibitor (**1**). (c) In the absence of hMMP12, prodrug **3** degraded slowly to the noninhibitory compound **7**. (d) When prodrug **3** (10 μM) was incubated with hMMP8 (20 nM), the enzyme did not cleave the prodrug substantially, and thus, inhibitor release was poor. Instead, most of the prodrug degraded to compound **7** over time.

Scheme 1. Mechanism of TAP Activation by hMMP12 and Inhibitor Release[a]



[a]An MMP12 inhibitor is inserted into a peptidic sequence (**3**), which is specifically cleaved by hMMP12. Upon proteolytic cleavage, the released molecule (**5**) shows a moderate IC_{50} towards hMMP12 but degrades spontaneously, generating the more potent hMMP12 inhibitor **1**. In the absence of enzyme, the prodrug eventually degrades to the poor hMMP12 inhibitor (**7**).

Our aim was to develop a prodrug that is selectively activated by its target, MMP12, to release its own inhibitor. This approach of TAPs will ensure that the release of the inhibitor will be closely linked to the localization and activity of the target enzyme. Unlike other protease-activated prodrugs,[11] TAPs are unique in that the released inhibitor is not designed to act on a different target than the activating protein. Additionally, the prodrug requires proteolytic activation by the target to become an inhibitor, quite contrary to approaches where the prodrug itself is already a good inhibitor of the target.[12] Areas of enzyme activity will therefore produce local inhibitor release at the site of inflammation.

As a starting point for the development of a TAP for hMMP12, we chose as a starting point two MMP inhibitors containing an aryl-sulfonamide scaffold (**1** and **2**). This class of compounds has been extensively investigated[13] and presents various positions that are easily functionalized.[14] The molecules are able to block MMP12 activity by chelating the catalytic zinc ion in the active site of the enzyme via their carboxylic acid group.[15] Although hydroxamic acid derivatives would have a higher affinity,[16] the carboxylic acid provides higher stability and bioavailability[17,18] and is synthetically more accessible.

Our first aim was to mask the inhibitory potency of compounds **1** and **2** and at the same time generate a specific substrate for MMP12. We therefore incorporated the inhibitors into a peptidic sequence cleavable by the target protease. We chose the sequence PLGLEEA, previously shown to be specific for hMMP12 over other hMMPs, where the cleavage site is located between glycine and leucine and the specificity relies on two glutamates located at the P′ site.[19] The inhibitor was incorporated between the two leucines as an N-substituted glycine generating compounds **3** and **4**. The P′ site should destroy the inhibitory activity from these compounds as it masks the zinc binding group (ZBG). On the other hand, the ZPL sequence located on the P site is known to be crucial for enzyme recognition.[20,21] The combination should provide specificity toward hMMP12 over other hMMPs and make the inhibitory effect sensitive to hMMP12. All peptides were prepared by solid-phase peptide synthesis (see the Supporting Information).

To test the modified peptide **3** as a substrate for recombinant hMMP12, we incubated the prodrug with the enzyme and monitored its integrity by HPLC. Only in the presence of hMMP12 (Figure 1a), we observed the conversion of the starting material into compound **5** (Scheme 1), which is the product resulting from the predicted proteolytic cleavage at the N terminus of leucine. This demonstrated the unaltered substrate behavior of the prodrugs against hMMP12 whose catalytic efficiency [K_{cat}/K_m = (3.7 ± 0.1) 10^3 M^{-1} s] was determined by HPLC (see Figure S1a in the Supporting Information). Prodrug **3** showed a substrate behavior toward five other MMPs (see Figure S1b in the Supporting

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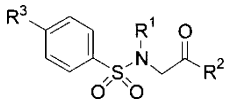
Information) very similar to what was previously published for the unmodified peptide.[19] Only MMP13 showed significant cleavage of **3**, albeit much slower than MMP12.

Furthermore, **5** hydrolyzed over time to the more hydrophilic free MMP12 inhibitor **1**. Hence, the successful TAP design produced inhibitor **1** in a completely MMP12-dependent fashion through a two-step process: the initial enzymatic cleavage of prodrug **3** to release predrug **5** followed by its spontaneous conversion into the final drug (**1**) (Scheme 1). However, there was a significant delay between enzyme activity and production of its own inhibitor. This is desirable to produce a burst of predrug **5** that upon hydrolysis to **1** effectively inhibits MMP12. Together with the MMP12 inhibitor (**1**), we found an additional peak corresponding to compound **7** (Scheme 1), a result of the sulfonamide hydrolysis of **3**. N-acylated sulfonamides hydrolyze spontaneously to secondary sulfonamides as we showed by incubating predrug **5** and prodrug **3** in TCN buffer pH 7.5 at 37 °C in the absence of enzyme (Figure 1b,c). In both cases, the starting materials converted over time into compounds **1** and **7**, respectively, at a different rate (Table 1). A similar degree of stability was

Table 1. Compounds Containing the Arylsulfonamide Scaffold Tested as Substrates of hMMP12 and Half Lifetimes in TCN Buffer pH 7.5[a]



compd	R^1	R^2	R^3	t_{50} pH 7.5 (h)
3	Z-Pro-Leu(CO)−	−HN-Leu-Glu-Glu-Ala(NH_2)	−Ph	5.1 ± 0.5
4	Z-Pro-Leu(CO)−	−HN-Leu-Glu-Glu-Ala(NH_2)	−OMe	28 ± 4
5	Z-Pro-Leu(CO)−	−OH	−Ph	27 ± 0.9
6	Z-Pro-Leu(CO)−	−OH	−OMe	77 ± 9
7	−H	−HN-Leu-Glu-Glu-Ala(NH_2)	−Ph	NH
8	−H	−HN-Leu-Glu-Glu-Ala(NH_2)	−OMe	NH
9	Z-Pro-Leu-Gly(CO)−	−OH	−OMe	27 ± 1
10	Z-Pro-Gly(CO)−	−HN-Leu-Glu-Glu-Ala(NH_2)	−OMe	6.4 ± 0.6

[a]NH, no hydrolysis.

observed in fetal calf serum (FCS) and heat inactivated FCS, suggesting a lack of enzymatic activity in these media that could accelerate hydrolysis or promote undesirable degradation pathways (see Figure S2 in the Supporting Information). The hydrolysis of N-acylated sulfonamides leading to secondary sulfonamides has been observed in vivo.[22] On the other hand, the lability of prodrug **3** indicates that the present TAP design leads to byproduct that might affect the target enzyme. Therefore, we investigated the inhibitory potency of all molecules generated upon incubation of compound **3** with and without hMMP12. Inhibition constants were determined using the MMP12 FRET reporter LaRee5, previously developed in our lab[23] (see Figure S3 in the Supporting Information). Compound **1** was by far the most potent inhibitor with an IC_{50} = 0.29 μM. Compound **5**, which also has a free carboxylic acid group, inhibited hMMP12 with an IC_{50} = 13 μM. Byproduct **7** was only able to inhibit hMMP12 with a weak IC_{50} of 48 μM, most probably due to its inability to chelate zinc. This indicates that in the absence of hMMP12, nonproductive degradation will occur, whereas in the presence of hMMP12, a weak inhibitor will be formed followed by productive degradation to the potent hMMP12 inhibitor **1** over time (Scheme 1).

Following this mechanism, the limiting step for the conversion to the inhibitor is the spontaneous degradation of acylated sulfonamides; therefore, the concentration of inhibitor released will be proportional to the concentration of active hMMP12 since predrug **5** does not strongly inhibit MMP12 (see Figure S4a,b in the Supporting Information). To expand this concept to similar inhibitors, the same set of experiments was performed with prodrug **4**. Again, the generation of the inhibitor, in this case compound **2**, happened exclusively in the presence of hMMP12 (see Figure S5 in the Supporting Information). Next, we studied the molecular factors affecting the spontaneous hydrolysis of the prodrugs and intermediates. It was previously demonstrated that the hydrolysis of N-acylated sulfonamides highly depends on the pH.[24] We examined how different substitutions affect the stability of these model compounds. When leucine at the P1 site in **4** was substituted by glycine (**10**, Table 1), the stability in buffer pH 7.5 decreased. However, the terminal free carboxylic acid as in **5** or **6** increased the stability of the molecule as compared to the amide (**3** or **4**), suggesting that the conversion occurs via a typical nucleophilic attack by water on the sulfonamide. The biphenyl derivatives degraded generally faster than those containing the methoxyphenyl group (Table 1). These data indicate that by modifying either the peptidic sequence or the aryl-sulfonamide scaffold, it is possible to control the timing of drug release and therefore the window between the enzymatic activation and the end of drug delivery. In this example, the spontaneous conversion of **3** into **7** is slow as compared to its proteolytic cleavage in the presence of hMMP12 (Figure 1a). Therefore, enzymatic cleavage will dominate. Once converted into predrug **5**, irreversible release of the potent drug **1** will occur (see Figure S4c in the Supporting Information). Additionally, we used an activity-based assay to demonstrate the TAP concept and confirm the HPLC results (Figure 2). The longer the incubation time of prodrug **3** with hMMP12, the more inhibitor was released, and the lower was the enzymatic activity (Figure 2a). The same result was obtained when compound **5** was incubated without hMMP12, since **5** spontaneously degraded to generate the potent MMP12 inhibitor **1**, as observed by HPLC (Figure 2b). On the other hand, the incubation of **3** without hMMP12 did not lead to any reduced enzymatic activity, showing once more that the spontaneous degradation of the prodrug will not have an effect on the target enzyme (Figure 2c). As a control, the stability of hMMP12 activity over time was monitored (see Figure S6a in the Supporting Information). One of the key features of the TAP approach is that a broad-spectrum inhibitor can be converted to a specific inhibitor. To prove this, we tested the prodrug with hMMP8, another protease that is inhibited by **1** (see Figure S7 in the Supporting Information). Unlike hMMP12, hMMP8 fails to significantly cleave the prodrug due to the specificity of the peptidic sequence toward hMMP12; thus, inhibitor **1** is only sparsely released as compared to hMMP12. Instead, the prodrug mainly degrades over time to compound **7**, which has no effect on the enzymatic activity of MMPs (Figures 1d and 2d). To support the importance of the peptidic sequence as supplier of drug

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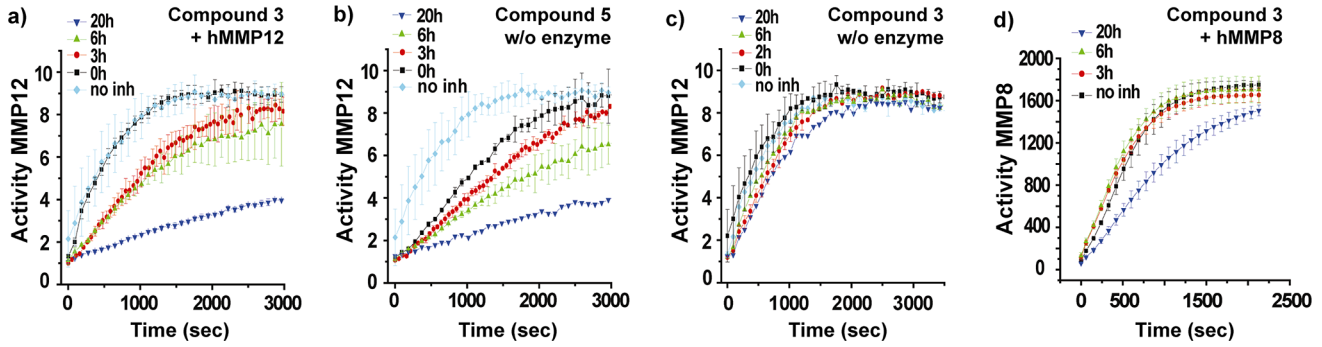

Figure 2. Activity-based assay showing the selective inhibition of hMMP12 by TAP. (a) Prodrug **3** (10 μM) was preincubated with hMMP12 (12 nM) in TCN buffer at 37 °C for different times. After that, the activity of the enzyme was measured with the fluorogenic substrate LaRee5. With longer preincubation times, the enzyme was significantly inhibited due to the presence of compound **1**. (b) Compound **5** (10 μM) was preincubated in buffer without hMMP12. After that, we added hMMP12 (12 nM) and measured its activity. Strong inhibition occurred after long preincubation times due to the spontaneous conversion of **5** into **1**. (c) Incubation of prodrug **3** in the absence of hMMP12 did not generate any inhibitory molecule over time; thus, hMMP12 activity remained high. (d) When prodrug **3** was incubated with hMMP8 (20 nM), the enzyme activity also remained high over time, as measured with a commercial fluorogenic substrate, since compound **1**, which also inhibits hMMP8, is not significantly formed.

specificity, we synthesized a modified substrate (**10**) where leucine at the P1 site of **4** was substituted by glycine. hMMP12 failed to cleave this substrate and release the inhibitor (see Figure S8 in the Supporting Information).

In conclusion, we have described a novel concept to inhibit proteolytic activity using a prodrug approach that relies on the local catalytic activity of the target enzyme itself. Only in the presence of the target enzyme, in this case hMMP12, the prodrug gets activated and releases its inhibitor (Scheme 1). This mechanism of drug release has the following advantages over the direct application of an inhibitor or other known prodrug concepts: (i) Only the active target protease is able to promote the release of the inhibitor. (ii) An unspecific inhibitor (here **1**) is employed to produce a specific inhibitory effect. (iii) For a given reaction and inhibitor, the release of the drug will be proportional to the concentration of enzyme and substrate in the system. If there is little enzyme to be inhibited, only a small portion of the prodrug will be converted. (iv) The window between enzymatic activation and inhibitor release is tunable by hydrolytic stability rather than drug−enzyme interaction. (v) The release of the drug will occur locally at the sites of enzymatic hydrolysis and a gradient of inhibitor concentration will be generated locally only where (damaging) proteolysis occurs. It needs to be stressed that so far only the release of the predrug is local and that the lack of its spontaneous hydrolysis still hampers spatially restricted activity. One solution would be much faster hydrolysis. Alternatively, the flexibility of the TAP design would allow the insertion of chemical moieties, that is, lipidation, to modulate its residence time. If applied in vivo, the combined advantages would likely avoid that off-targets are hit in areas unaffected by the inflammation. This might largely reduce side effects and opens the opportunity to use known potent inhibitors with already studied pharmacological properties. Of course, pharmacokinetic aspects of the prodrugs need to be considered to go beyond the proof of concept presented in this study. Expanding the TAP concept to other proteases by using different small-molecule inhibitors and other peptidic sequences will open new doors to personalized medicine, where the individual equipment of enzyme activities will take care of releasing as much inhibitor as needed. This will avoid excessive and unspecific inhibition and, thus, the main cause of side effects. Especially for the cancer antitarget MMP12 and its dramatic role in lung emphysema formation, the TAP concept may lead to successful MMP inhibitors in the future.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures, experimental protocols for synthesis, and biochemical assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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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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