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Title: A Peptidomimetic Inhibitor of Matrix Metalloproteinases Containing a Tetherable Linker Group

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ABSTRACT

Successful wound repair and normal turnover of the extracellular matrix relies on a balance between matrix metalloproteinases (MMPs) and their natural inhibitors (the TIMPs). When over-expression of MMPs and abnormally high levels of activation or low expression of TIMPs are encountered, excessive degradation of connective tissue and the formation of chronic ulcers can occur. One strategy to rebalance MMPs and TIMPs is to use inhibitors. We have designed a synthetic pseudopeptide inhibitor with an amine linker group based on a known high-affinity peptidomimetic MMP inhibitor have demonstrated inhibition of MMP-1, -2, -3 and -9 activity in standard solutions. The inhibitor was also tethered to a polyethylene glycol hydrogel using a facile reaction between the linker unit on the inhibitor and the hydrogel precursors. After tethering, we observed inhibition of the MMPs although there was an increase in the IC_{50}s which was attributed to poor diffusion of the MMPs into the hydrogels, reduced activity of the tethered inhibitor or incomplete incorporation of the inhibitor into the hydrogels. When the tethered inhibitors were tested against chronic wound fluid we observed significant inhibition in proteolytic activity suggesting our approach may prove useful in rebalancing MMPs within chronic wounds.

KEYWORDS

matrix metalloproteinase inhibitor, polyethylene glycol, hydrogel, chronic wound fluid, hydroxamic acid

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc ion-containing proteolytic enzymes. During wound repair they play an important role in extracellular matrix (ECM) degradation, growth factor activation and immune system regulation.[1-6] The 20-plus known MMPs share a common domain structure, and are expressed as zymogens, each with an inhibitory pro-domain which must be enzymatically cleaved for activation.[1] In addition to tight control over MMP activation, the duration and location of MMP activity is controlled by the presence of the four members of the tissue inhibitor of metalloproteinases (TIMP) family.[2] The ability of TIMPs to inhibit the MMPs is largely due to the interaction of a wedge-shaped ridge on the N-domain which binds within the active-site cleft of the target MMP.[7]

During wound repair, proper balance between MMPs and TIMPs is crucial for the normal turnover of the ECM.[8] However, the over-expression of several MMPs including MMP-1, 2, 3, 8, 9 and -10, combined with abnormally high levels of activation or low expression of TIMPs, may contribute to excessive degradation of connective tissue and the formation of chronic ulcers.[8,9]. In particular, we have previously shown that MMP-9 activity is abnormally high in chronic wound fluid (CWF), and correlates with the clinical severity of the wound as measured by PUSH (Pressure Ulcer Scale of Healing) scores.[9]

For the treatment of chronic wounds, it has been suggested that the addition of MMP inhibitors prior to topical treatment of the wound with growth factors would reduce the rate of proteolytic degradation of these factors, and reduce the dose required to promote healing.[10-13] We believe, however, that caution is needed when applying synthetic inhibitors to the wound as MMP activity in the wound bed is important to normal tissue remodelling. One strategy to address this problem is to covalently link the inhibitor to a polymer substrate, restricting the inhibition to the wound surface and the exudate. As well as preventing excessive breakdown of ECM at the wound bed surface, this may prevent the breakdown of protein-based treatments

applied to the wound such as the vitronectin:IGF-I:IGFBP complexes pioneered by our laboratory.[14] Furthermore, tethering of the inhibitor prevents it from leaching into the blood circulation, significantly reducing the risk of systemic effects.

A wide range of MMP inhibitors have been studied for treatment of MMP-related diseases, such as cancer, and have shown great success in pre-clinical trials using animal models at the early stages of cancer progression.[15] Four major types of MMP inhibitors for cancer treatment that have been clinically evaluated are hydroxamates, carboxylates, thiols and the tetracycline analogues (doxycyclines).[16] These compounds typically inactivate MMPs by chelating or coordinating to the zinc ion in the active site cleft. Despite the promise in pre-clinical trials, translation of MMP inhibitors to the clinic has been hampered by their link to musculoskeletal syndrome, although the mechanism of the link has not been elucidated.[17] Again, local application of a tethered inhibitor is an appealing strategy to mitigate problems such as musculoskeletal syndrome.

Immobilization of the potent broad-spectrum hydroxamic acid-containing MMP inhibitor, Batimastat, has been previously carried out on a resin then used to concentrate MMPs for cancer diagnostics.[18] This was accomplished by derivatization at the thioproline sulphur group, allowing coupling to an epoxide-functionalised Sepharose resin. A less specific approach using hydroxamic acid functionality to inhibit MMPs has recently been reported by Skarja *et al*.[19] They synthesized beads of poly(methylmethacrylate-*co*-methacrylic acid) and introduced hydroxamic acid groups via reaction of the acid groups and demonstrated inhibition of MMP-2, 3, 8 and -13 in stock solutions as well as MMP-8 in CWF. The disadvantages of these two above approaches are that Batimastat is limited to a small subset of inhibitors, while the hydroxamate-functionalized beads act as non-specific chelators.

There are a number of peptidomimetic inhibitors containing hydroxamate groups which share one of two common backbone structures, mimicking the *N*-terminal (so-called “left hand side” (LHS)) or, more commonly, the *C*-terminal (“right hand side” (RHS)) portion of MMP

substrates,[20] with the hydroxamate group at the *C*- or *N*-terminus. With this in mind, we hypothesized that the structural arrangement of the backbone of these inhibitors would mimic that of native substrates, and that therefore extensions to this backbone should cause minimal disruption to the MMP-binding activity of an inhibitor of this class.

In this study we designed a LHS inhibitor based on the structure of a previously reported inhibitor by the addition of a 6-aminohexanoic acid linker attached to its *N*-terminus, and tested its MMP inhibitory activity. Furthermore, we incorporated this inhibitor into a poly(ethylene glycol) (PEG) hydrogel, and tested its inhibitory effect in active MMP solutions and in a CWF sample with a high PUSH score.

MATERIALS AND METHODS

Materials

The aminohexanoic acid terminated pseudopeptide β-thioproline-L-β-homophenylalanine-L-lysine(4-(4-ethylephenyl)benzoyl)-NHOH (MW 759 Da) was synthesized by Mimotopes Pty Ltd (Melbourne, Australia) and supplied at a purity of 95% by HPLC. Polyethylene glycol (PEG)-dithiol (MW 3.4kDa) was from NOF Corporation (Japan) while PEG-octaacrylate (MW 40kDa) was obtained by functionalization of 8-arm PEG-OH (NOF Corporation, Japan) according to published methodology.[21] The degree of acrylation was approximately 93% as determined by NMR spectroscopy. ω-acryloyl α-NHS Ester PEG MW 3.5kDa was purchased from Jenkem Technology USA Inc. Analytical grade anhydrous dimethyl sulfoxide (DMSO, 99.5+%), 4-aminophenylmercuric acetate (APMA), zinc chloride ($ZnCl_2$), Brij® 35, and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma-Aldrich. Tris(hydroxymethyl) aminomethane (Tris base) was purchased from Roche Diagnostics. Sodium chloride, calcium chloride, pro-enzyme MMP-9 (0.10 mg/mL), -2 (0.373 mg/mL), -1 (0.08 mg/mL) and active form MMP-3 (0.1 mg/mL), as well as MMP fluorogenic substrate III (DABCYL-GABA-Pro-Gln-*Gly-Leu*-Glu(EDANS)-Ala-Lys-NH_2), were purchased from Merck.

Coomassie Plus™ protein assay reagent and bovine serum albumin (BSA, 2.0 mg/mL) were purchased from Thermo Scientific. Both black and clear 96 well plates were purchased from Nunc. Purified and deionised water with a resistivity of 18.2 MΩ·cm at 25 °C was used throughout this work. A FLUOstar OPTIMA fluorimeter (BMG Labtech GmbH) was used to read the black plates for the MMP fluorescent assay, while a BIO-RAD Benchmark Plus™ microplate spectrophotometer was used to read the clear plates for the Coomassie Plus™ assay.

Pro-enzyme activation

To activate the pro-enzyme, freshly prepared APMA (100 mM in DMSO) was added to MMP-9, -2, and -1 (concentrations as supplied) to give a final APMA concentration of 1 mM, followed by incubation at 37 °C for 3 h. The activated MMPs were then stored in 10 µL aliquots at -80 °C.

MMP Fluorescent Assay

Details of the MMP fluorescent assay using MMP fluorogenic substrate III have been described in detail elsewhere.[22] Briefly, buffer A (50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.01% Brij 35) was used as a dilution buffer for active MMPs and controls. The MMP substrate III was added to a series of active MMPs diluted in buffer A in the presence of 0.1% BSA in a black 96 well plate at 37 °C. The reaction signal was then collected dynamically using a fluorimeter by monitoring fluorescence (λ_{ex}=340 nm, λ_{em}=485 nm) at two-minute time intervals.

PEG-inhibitor Conjugation

The inhibitor was dissolved in 50 µL DMSO to a concentration of 1 mg/mL, then 100 µL of $NaHCO_3$ solution (50 mM; adjusted to pH 8.3 with 1M HCl) was added to the inhibitor solution. A 3x molar excess of acrylate-PEG-NHS (100 µL of a 7.3 mg/mL in $NaHCO_3$ solution) was added and the mixture was stirred in the dark for two hours. A control solution was

prepared using the same methodology with no inhibitor added such that the NHS would hydrolyse to yield acrylate-PEG-COOH.

Hydrogel preparation

Stock solutions of the PEG precursors were prepared by dissolving 162 mg of PEG-dithiol and 401 mg of PEG-octaacrylate in 1.2 and 2.92 mL, respectively, of 50 mM aqueous $NaHCO_3$ buffer adjusted to pH 7.4 with 1M HCl. These stocks were enough to make 96 hydrogel samples and were made up immediately prior to preparing the hydrogels to reduce homopolymer gelation. Stocks of the acrylate-PEG-inhibitor were prepared using a series of 10x dilutions in the acrylate-PEG-COOH blank solution to give solutions ranging from 0 to 10 mM of inhibitor. Hydrogels with approximately 10% dry weight were prepared from three stock solutions using near stoichiometric amounts of thiol and acrylate groups by adding: 30 µL PEG-octaacrylate, 10 µL PEG-dithiol, and 30 µL acrylate-PEG-inhibitor / acrylate-PEG-COOH blank in 2 mL Eppendorf tubes. The ratio of linear PEG to PEG-octaacrylate was 1:3.5. The PEG-dithiol solution was added last and the solutions were immediately agitated using a vortex mixer for 10 s before tapping gently to shift the liquid to the bottom of the tube which acted as the mould. Hydrogels were prepared at room temperature in triplicate for each peptide concentration and typically formed within two minutes, although a further 2 h was allowed for complete curing. Before incubating the hydrogels in active MMP solutions or diluted wound fluid they were washed in 50 mL buffer A on a rotary mixer for 72 h with the washing buffer changed every 24 h.

Hydrogel incubation

Washed hydrogels were placed in 2 mL Eppendorf tubes and incubated with 500 µL of prepared active MMP-9, -3, -2 or -1 solutions (0.1 µg/mL), or in diluted wound fluid for 3 h at room temperature on a table shaker. The incubation solutions were then collected and were

added to a black 96 well plate in the presence of 0.1% BSA for assaying the active MMP content. All experiments were done in triplicate.

Wound fluid

Chronic wound fluid samples were obtained from consenting patients of both Queensland University of Technology and St Luke's nursing services (Brisbane, Australia). All participating patients had chronic venous leg ulcers. Clinical information of the obtained samples is listed in Table 1. As previously described, a standard wound fluid collection technique was performed at the clinical site.[23] Briefly, ulcers were washed with sterile water prior to collecting wound fluid followed by the application of an occlusive dressing over the wound. Exudate accumulated under the dressing after 30 min to 1 h was recovered by washing with 1 mL of saline. The wound fluid samples were centrifuged and the supernatant was filtered. In this study, the protein content for all wound fluid samples was analysed using a Coomassie Plus™ assay. Samples were stored at -80 °C prior to use.

Computational docking

Computational docking of the inhibitor structure was carried out using FlexX version 3.1.4 (BioSolveIT GmbH, Augustin, Germany), which incorporates the methods of modelling metal coordination chemistry developed by Seebeck *et al.*[24] The target for docking was the NMR-determined structure of MMP-2 from an MMP-2-inhibitor complex (PDB ID 1HOV).[25] The search space was confined to the active site cleft and P1' pocket, and constraints were placed on the docking as follows: the P1' pocket was defined as a hydrophobic/aromatic binding site, the active site Zn^{2+} was defined as a metal coordination site, and relevant atoms in the backbone of the β-strand at the base of the cleft were defined as hydrogen bond donors/acceptors. The reported structure is the lowest energy structure which satisfied these constraints.

RESULTS

The design of a "tetherable" MMP inhibitor

An MMP inhibitor was designed based on the LHS hydroxamic acid-containing pseudopeptide inhibitor first synthesised by Brown *et al.*[26] (Figure 1, structure **[1]**) which has reported IC_{50} values of 40, 0.4 and 8 nM for MMP-1, -2 and -3, respectively (Table 2). To enable facile conjugation, the pseudopeptide was synthesized with the benzyl ether adjacent to the thiazolidine substituted with an aminohexanoic acid group, thereby incorporating a spacer and primary amine (Figure 1, structure **[2]**). By maintaining the structure neighbouring the hydroxamic acid group responsible for binding to the catalytic Zn^{2+} of the MMPs, it was anticipated that the inhibition capacity would not be substantially altered. This hypothesis was supported by computational docking of the modified inhibitor to MMP-2 (Figure 2), which indicated that the newly-introduced aminohexanoic acid residue would reside well away from the active cleft, with the *N*-terminal amine freely accessible.

The inhibitory effect on active MMPs in solution

To test the above hypothesis, the designed inhibitor (Figure 1, structure **[2]**) was incubated at concentrations of 0.01 – 10000 nM in 0.1 µg/mL solutions of MMP-1, -2, -3 and -9 to test for inhibition using a fluorogenic substrate (DABCYL-GABA-Pro-Gln-*Gly-Leu*-Glu(EDANS)-Ala-Lys-NH_2). When active MMPs cleave the Gly-Leu bond the EDANS and DABCYL fluorophore/quencher combination are liberated enabling quantification of the MMPs present via fluorescence spectroscopy.[22] The activity–concentration curves are shown in Figure 3 and the IC_{50} values in Table 2. Results indicate that inhibitor **[2]** strongly inhibited MMP-1, -2, -3 and -9 at nanomolar concentrations. While the IC_{50} for MMP-2 was comparable (within experimental error) to literature values for the original inhibitor **[1]**, it was significantly lower by

ca. 35- and 3-fold for MMP-1 and -3, respectively, indicating that the addition of the spacer group in **[2]** has increased the broad-spectrum potency of this inhibitor.

The tethered inhibitor removes MMPs from solution

PEG hydrogels incorporating inhibitor **[2]** were prepared by coupling the inhibitor to acrylate-PEG-NHS followed by mixing of PEG-octaacrylate, PEG-dithiol and acrylate-PEG-inhibitor. The thiols and acrylates react via Michael-type addition to form a crosslinked network (Figure 4).[21] The loading of the inhibitor was varied by using a range of acrylate-PEG-COOH and acrylate-PEG-inhibitor feed ratios. The capacity of inhibitor **[2]** tethered in PEG hydrogels to remove MMPs from solution was measured by incubating the hydrogels (*ca.* 200 µL fully swollen) with MMP standard solutions (500 µL, 0.1 µg/mL) for 3 h followed by measurement of MMP activity in the supernatant (Figure 5). Tethering of inhibitor **[2]** to the hydrogels led to an apparent 100 to >1000 fold drop in activity compared with the free inhibitor **[2]**, with the IC_{50} values shifting to the hundreds of nM to low µM range (Table 3).

Protein content and active MMPs in chronic wound fluid samples

To test the inhibitor against would fluid samples we obtained samples from patients with chronic venous ulcers. The size of the ulcers, PUSH scores and protein concentration are summarised in Table 1.

The amount of proteolyic activity in the CWF samples was also measured by firstly diluting the as-received CWF samples 10 fold in buffer, then incubating these solutions with the fluorogenic substrate and measuring the subsequent change in fluorescence. The reason for the 10 fold dilution was to obtain sufficient volume for the assay. The results of the fluorogenic assay are presented in Figure 6.

The tethered inhibitor reduces the proteolytic activity in chronic wound fluid

PEG hydrogels (*ca.* 200 µL fully swollen) incorporating inhibitor **[2]** over a range of concentrations were incubated with a 10-fold dilution of the most active CWF sample, # 584 (volume 500 uL, 3 h incubation), followed by measurement of the residual proteolytic activity in the supernatant using the fluorogenic substrate assay (Figure 7). The PEG hydrogels with the tethered inhibitor showed some reduction in the proteolytic activity, but not to the same degree as observed with the free inhibitor or the tethered inhibitor in MMP standard solutions. At the highest concentration of inhibitor (10000 nM) we did observe approximately 50% reduction in the proteolytic activity (Figure 7).

DISCUSSION

Inhibitory effect comparison

While it is difficult to compare results of Brown *et al.*[26] for their inhibitor without the spacer (inhibitor **[1]**) with our inhibitor **[2]** due to variables such as purity, MMP source and assay conditions, in both cases the lowest IC_{50} value was observed when the inhibitors were incubated with MMP-2 (Figure 3, Table 2). Somewhat unexpectedly, replacement of the benzyl group from inhibitor **[1]** with the less bulky and more flexible aminohexanoic acid residue in inhibitor **[2]** resulted in an inhibitor with significantly higher broad-spectrum potency. This may be an advantage in the proposed application: while MMP-9 activity appears to be a major predictor of chronic wound severity,[9] non-negligible concentrations of a range of other MMPs have been previously noted in chronic wound fluids.[27]

Synthesis of Hydrogels with Inhibitor

One aim of this work was to immobilise an MMP inhibitor into a hydrogel while retaining the ability to inhibit a range of MMPs. PEG was chosen as a substrate hydrogel for its ease of

functionalization, excellent biocompatibility and intrinsic low protein adsorption properties. To prepare PEG hydrogels incorporating inhibitor **[2]** we reacted the *N*-terminal amine with acrylate-PEG-NHS, thereby conjugating a PEG spacer and acrylate group to the inhibitor (Figure 4a). Hydrogel formation was accomplished through a Michael-type conjugate addition between acrylate-PEG-**[2]**, PEG-dithiol and PEG-octaaacrylate (Figure 4b).[21]

An advantage of the conjugate addition reaction between the thiol and acrylate in this case is that the mechanism does not involve radical polymerization. This is important due to the presence of the hydroxamic acid on the pseudopeptide inhibitor which can act as a radical trap analogous to the classical nitroxide traps.[28,29] For this reason, UV-initiated copolymerization of acrylate-PEG-**[2]** with vinyl monomers did not lead to network formation (results not shown). Another advantage of this approach is that by-products such as NHS liberated during the PEGylation of the inhibitor do not need to be removed prior to network formation, thereby allowing the PEGylation and hydrogel formation to proceed as a one-pot reaction.

The hydrogels were washed extensively prior to incubation with MMP solutions using the same buffer composition as for the incubation step to remove any unreacted free inhibitor and any PEGylated but non-crosslinked inhibitor. The washing solutions were also assayed for MMP activity and we observed that in some cases, especially for the hydrogels with high concentration of inhibitor loading, the first washing solutions were able to inhibit MMP activity suggesting the presence of leached inhibitor (not shown). However, after extensive washing the wash buffer no longer contained any detectable inhibitor, implying that the observed inhibitory activity of the hydrogels could be attributed to immobilization of the MMPs by the hydrogel-tethered inhibitor, rather than the release of inhibitor into the MMP solution. It is known that the hydrogels formed from addition of PEG-dithiol to acrylates are hydrolytically unstable and have a half-life of about 11 days at pH 7.4 and 37°C in buffered saline.[21] The timeframe of the MMP incubation step was 3 h – far less than the washing time in which it was established that no inhibitor was being released.

Ultimately, if these hydrogels are to be used on wounds it is desirable to have a hydrolytically stable network to prevent the inhibitor being cleaved and released. To achieve this other crosslinking chemistry could be employed such as the hydrolytically stable thiol/vinyl sulfone Michael-type addition[30] in place of the thiol/acrylate combination used here.

The effect of tethered inhibitor [2] in MMP solutions

The IC_{50} values for the hydrogel-tethered inhibitors were several orders of magnitude higher than for the free inhibitor (Table 3). As discussed above, the efficiency of the immobilization of the inhibitor into the hydrogels was not 100% as evidenced by the activity found in the early washing solutions. In preparing the solutions for network formation the molar ratio of thiol to total acrylate groups used was close to 1:1, while the molar ratio of linear acrylate-PEG-inhibitor/COOH to PEG-octaacrylate was kept at 1:3.5 so that on average one in 28 acrylate groups would be linked to a linear acrylate-PEG. The low ratio of linear PEG to PEG-dithiol means that the probability of the acrylate-PEG-inhibitor reacting on either side of a PEG-dithiol is small. If the linear polymer containing two inhibitor molecules were to form it would have a molecular weight of 10.5 kDa, would not be incorporated into the network and would be removed during washing. Likewise, during the conjugation step with acrylate-PEG-NHS, a threefold excess of the PEG was used to maximise the reaction between the amine and NHS. Nevertheless, the actual concentration of inhibitor in the hydrogels could not be measured due to the nano-molar sensitivity required so we could not confirm how much inhibitor was present in the hydrogels tested.

Diffusion of the MMPs into the hydrogels to the inhibitor sites will also play a critical role in the inhibitory capacity. The diffusion of proteins within hydrogels has been studied extensively, mainly as a means for drug delivery, and can offer some insight into the diffusion of

MMPs into the MMP inhibitor hydrogels.[31] The PEG hydrogels used here can be described as non-porous but highly swollen gels whereby the diffusion will be mainly controlled by the movement of the proteinases through the spaces between the macromolecular chains, but may also be influenced by complicating factors such as solute aggregation and gel-solute interactions. The mesh size of PEG hydrogels synthesized from multi-arm precursors similar to those used here range from 26.5 Å[21] to 250 Å[32] depending on the number or arms, molecular weight and dry weight used during preparation. The mesh size is, however, only an estimate because of complicating factors such as unreacted chain ends, physical entanglements and intramolecular crosslinking reactions.

The hydrodynamic volume of MMP-2 (72 kDa) has been estimated at 27.5 Å based on its crystal structure.[33] MMP-1 (46 kDa) and MMP-3 (22 kDa) will be smaller, while MMP-9 (92 kDa) will be larger. Accurate volumes are difficult to derive considering the mix of species: some will have the propeptide cleaved while others will the propeptide folded from the active cleft, however, it appears that the pores may be of insufficient size to allow MMP diffusion into the hydrogels.

The diffusion of albumin in PEG hydrogels has been previously studied and can also act as a guide to the expected transport behaviour of MMPs in the hydrogels used here (albumin has a molecular weight of 67 kDa and hydrodynamic volume of 35.6 Å). For example, Elbert *et al.* showed that solid albumin was released from multi-arm PEG hydrogels initially by slow dissolution of the protein followed by hindered diffusion leading to release in the range of 10 days.[21] Conversely, Lutolf *et al.*[34] and Rizzi *et al.*[35] found that when MMP-1 was added to PEG hydrogels with MMP-cleavable crosslinks the gels exhibited bulk degradation suggesting rapid diffusion of the MMP into the network. It is therefore unclear if in our case the hydrogels are partially impermeable to the MMPs or not. If the diffusion is slow it would mean that during the incubation time of 3 hr only a fraction of the bound inhibitor can interact with the MMPs, i.e. it is a surface effect. This theory is the subject of further investigation.

Active MMPs in wound fluid samples

Chronic wound fluid, containing a complex mixture of proteins, peptides and metabolites, is an important modulator of the wound environment.[23] It is clear that the proteolytic activity varies greatly from patient to patient, however, there is a correlation between the severity of the wounds (based on PUSH scores) and the proteolytic activity (Figure 6). Table 1 and Figure 6 show that the three samples with the most potent MMP activity, 311, 276 and 584, all have PUSH scores 13 or above and interestingly, all have high levels of protein content (or protein fragments). Samples 157, 267 and 273, on the other hand, have lower PUSH scores and concomitant lower proteolytic activity. The observed correlation between PUSH scores and proteolytic activity is in agreement with previous reports.[9]

One difficulty encountered when using the fluorescent assay with the wound fluid samples was that a ten-fold dilution was required in order to generate the volume necessary to conduct the assay. This dilution, together with the relatively low proteolytic activity in the CWF samples (compared to the MMP standard solutions), generated a fluorescent signal at the lower limit of detection, hence the large error bars in Figure 6.

Tethered inhibitor 2 on active MMPs in the wound fluid

Figure 7 shows the percentage of proteolytic activity remaining in chronic wound fluid sample 584 after incubation with PEG hydrogels loaded with 0.1 and 10000nM tethered inhibitor. At the highest concentration tested approximately 50% inhibition occurs. As is evident in Figure 7, large error bars are associated with the data. This may be due to the intrinsic technical difficulties in using the fluorescent assay for the detection of proteolytic activity in the wound samples mentioned above, or other contributing factors including the possibility of protein agglomeration or precipitation in the highly proteinaceous CWF samples. We also observed unexplained bleaching of the fluorescence in the CWF samples suggesting a different fluorophore may be needed to accurately assay these samples.

CONCLUSIONS

In this study we demonstrate the modification of a high-affinity hydroxamic acid-based LHS inhibitor by the addition of a 6-aminohexanoic acid linker attached to its *N*-terminus, while maintaining MMP inhibitory activity towards MMP-1, -2, -3 and -9. We further incorporated the inhibitor into poly(ethylene glycol) hydrogels and demonstrated that these hydrogels reduced MMP-1, -2, -3 and -9 activity in test solutions to undetectable levels at inhibitor concentrations in the nanomolar to low micromolar range. Finally we demonstrated that proteolytic activity in a CWF sample as measured using a fluorogenic assay can be decreased by the hydrogel to approximately half.

This approach offers the opportunity to inhibit excess MMPs in CWF while the MMPs within the wound bed required for healing-associated functions remain active. This is likely to be especially relevant in therapies involving the topical application of growth factors as it may prevent the breakdown of the therapy in the wound. Moreover, conceptually since the inhibitor can be chemically tethered, it is not able to leach out into the blood circulation, therefore, safe and locally-effective MMP inhibition is expected. We hypothesize that inhibition of MMPs, including MMP-9, in CWF before the application of active factors to the wound may neutralise the aggressive proteolytic wound environment and may modulate the ulcer towards a healing state.

This study indicates that the utility/flexibility of this approach can be used with other peptide inhibitors.

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Figure captions

Figure 1 – Structures of pseudopeptidic MMP inhibitors **[1]** from Brown *et al*,[26] and **[2]** the inhibitor modified with a aminohexanoic acid spacer group.

Figure 2. Predicted structure of the complex between **[2]** and MMP-2: Left: inhibitor **2** (sticks, coloured according to CPK standard) was docked to MMP-2 (ribbon and solvent-accessible surface) using BioSolveIt FlexX. The active site zinc ion and *N*-terminal nitrogen are shown as spheres and marked by an arrowhead and arrow respectively. The bulky, hydrophobic (ethylphenylbenzoyl)lysine side chain occupies the P1' site hydrophobic tunnel, contributing the bulk of the stabilizing interactions. As expected given the peptidomimetic structure of this inhibitor, the amine group (analogous to the N-terminal end of an MMP substrate) is very accessible (blue sphere indicated by arrow).

Figure 3. The inhibitory effect of inhibitor **[2]** on active MMPs in solution.

Figure 4. Synthesis of polyethylene glycol hydrogels tethered with inhibitor **[2].** (a) Inhibitor **[2]** is PEGylated at the amine using acrylate-PEG-NHS as a means to add a PEG spacer and acrylate group. (b) The PEGylated inhibitor was mixed with PEG-octaacrylate and crosslinked with PEG-dithiol.

Figure 5. The inhibitory effect of tethered inhibitor **[2]** in PEG hydrogels on active MMPs in solution.

Figure 6. Proteolytic activity of chronic wound fluid (WF) samples as expressed as fluorescence following incubation with the fluorogenic substrate. PUSH scores are labelled above each respective bar.

Figure 7. The inhibitory effect of tethered inhibitor **[2]** in PEG hydrogels on chronic wound fluid sample # 584 at 0.1 nM and 10000 nM loading.

Table Captions

Table 1. Clinical information and protein content of obtained chronic wound fluid samples.

Table 2. A comparison of IC_{50} value (unit in nM) of inhibitors **[1]**[26] and **[2]** in free form.

Table 3. A comparison of IC_{50} value (unit in nM) of the inhibitor **[2]** incorporated into PEG hydrogels.

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TABLES

Table 1

Sample Code	Ulcer Size, cm^2	Pressure Ulcer Healing Scale (PUSH) score	Protein content (mg/mL)
157	6.8	12	0.35
267	6.5	10	0.15
273	5.5	12	0.25
276	5.5	13	1.18
311	8.3	13	1.35
584	47.4	15	1.85

Table 2

	Inhibitor [1]	Inhibitor [2] in Free Form*
MMP-1	40	1.13 ± 0.61
MMP-2	0.4	0.532 ± 0.014
MMP-3	8	2.55 ± 0.30
MMP-9	-	0.382 ± 0.054

***(Error represents 95% confidence intervals)**

Table 3

	Inhibitor [2] in PEG Hydrogel*
MMP-1	134 ± 100
MMP-2	180 ± 98
MMP-3	>1000
MMP-9	392 ± 162

***(Error represents 95% confidence intervals)**

FIGURES

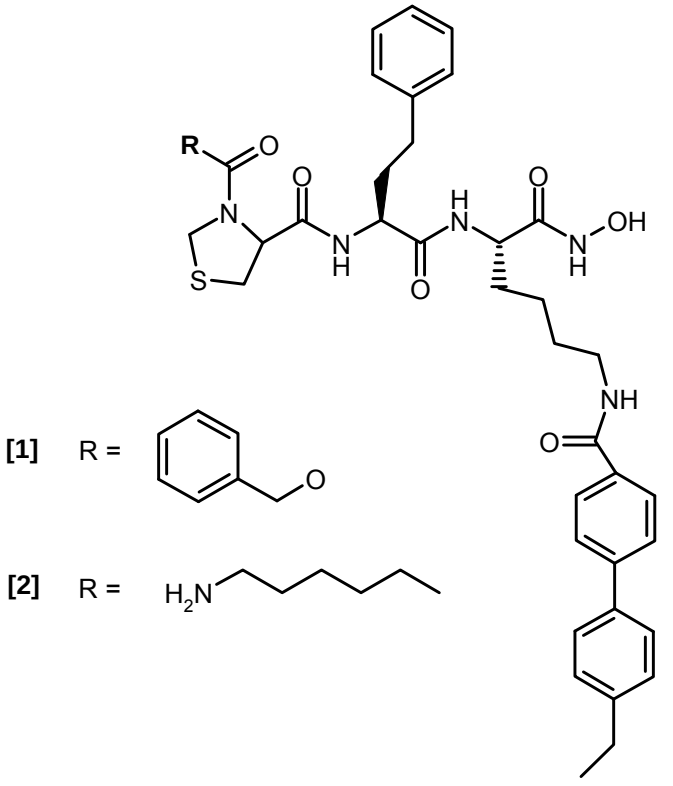


Figure 1

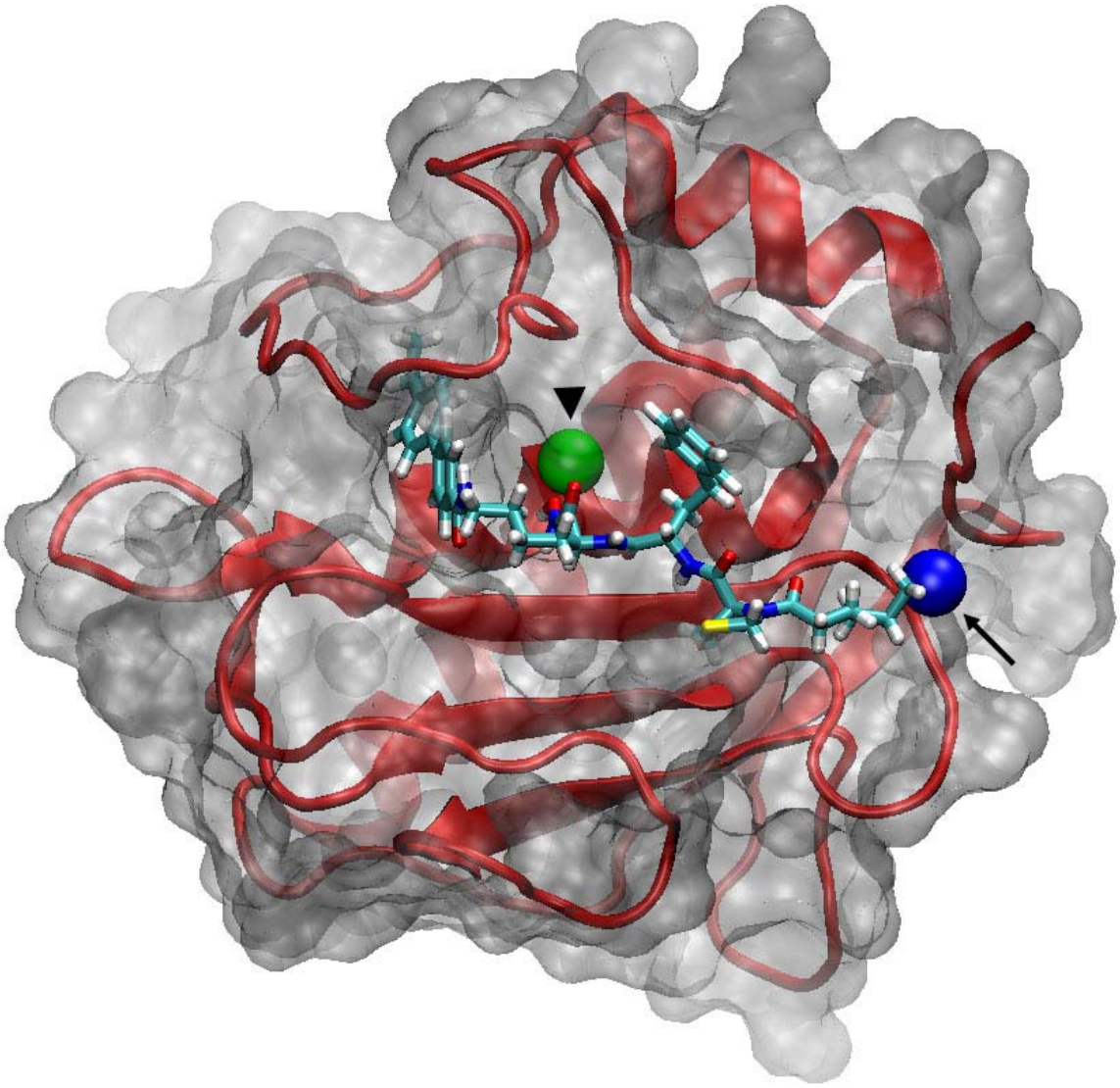


Figure 2

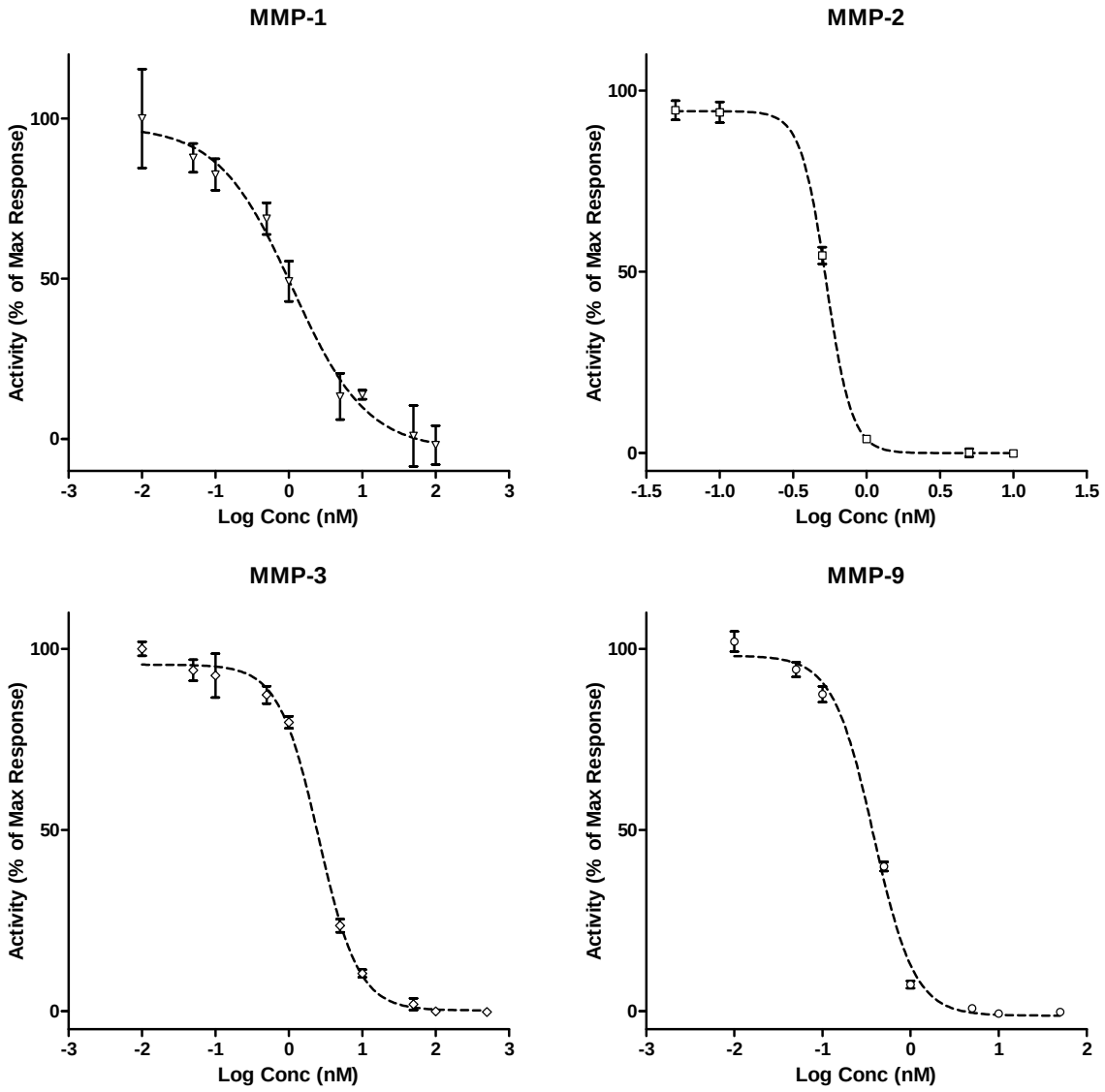
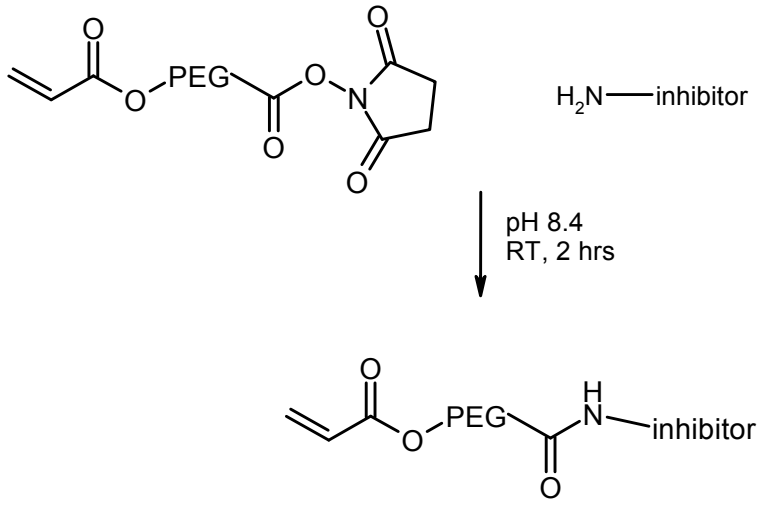
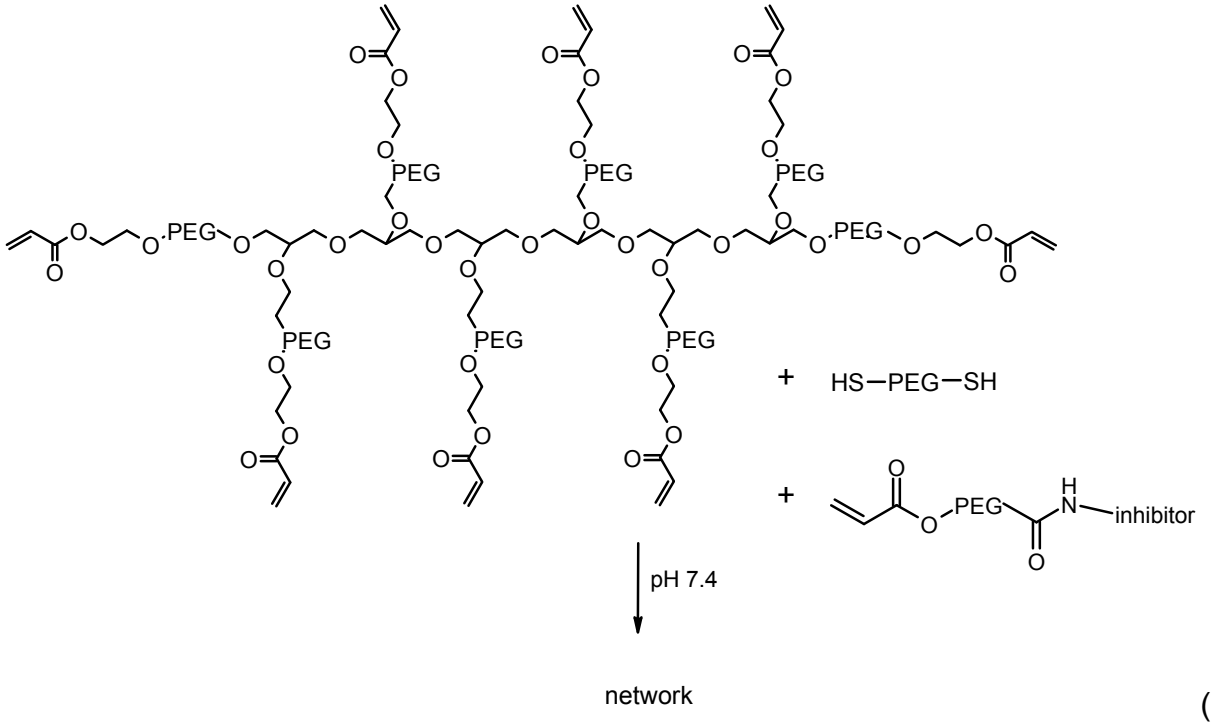

MMP-1
Activity (% of Max Response)
100
50
0
-3
-2
-1
0
1
2
3
Log Conc (nM)
MMP-2
Activity (% of Max Response)
100
50
0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Log Conc (nM)
MMP-3
Activity (% of Max Response)
100
50
0
-3
-2
-1
0
1
2
3
Log Conc (nM)
MMP-9
Activity (% of Max Response)
100
50
0
-3
-2
-1
0
1
2
Log Conc (nM)

Figure 3


PEG
H_2N—inhibitor
pH 8.4
RT, 2 hrs
PEG
inhibitor

(a)


PEG
HS—PEG—SH
PEG
inhibitor
pH 7.4
network

(

b)

Figure 4

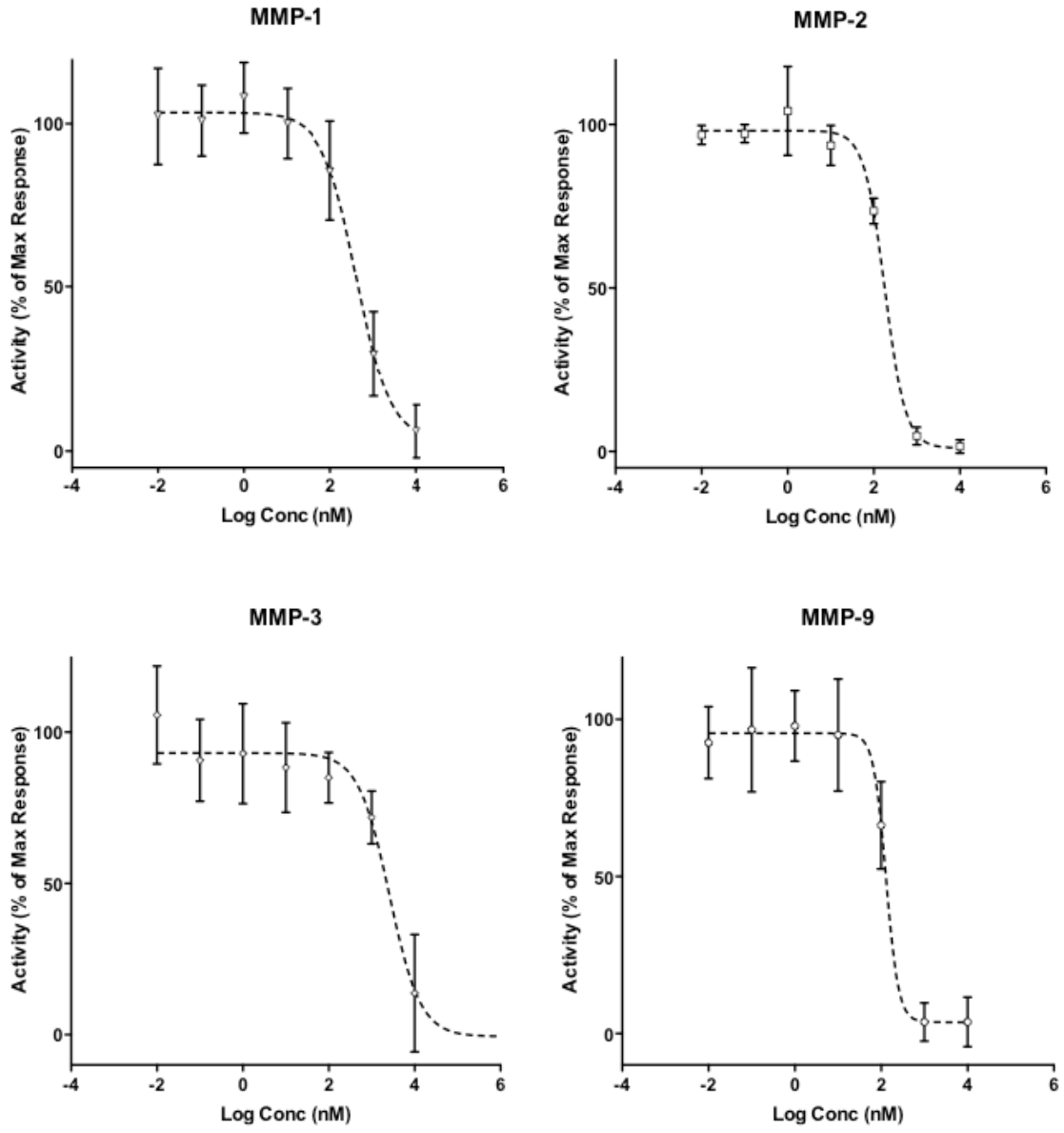

MMP-1
MMP-2
MMP-3
MMP-9
Activity (% of Max Response)
Log Conc (nM)
100
50
0
-4
-2
0
2
4
6

Figure 5

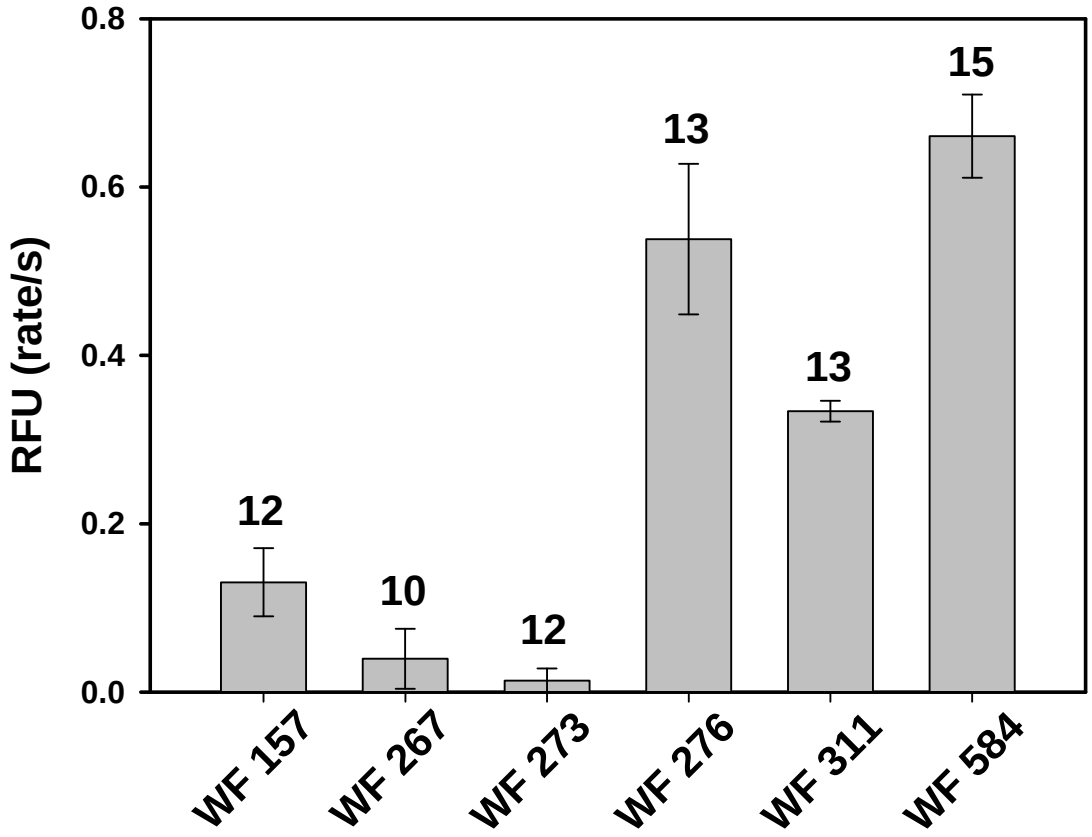

RFU (rate/s)
0.8
0.6
0.4
0.2
0.0
12
10
12
13
13
15
WF 157
WF 267
WF 273
WF 276
WF 311
WF 584

Figure 6

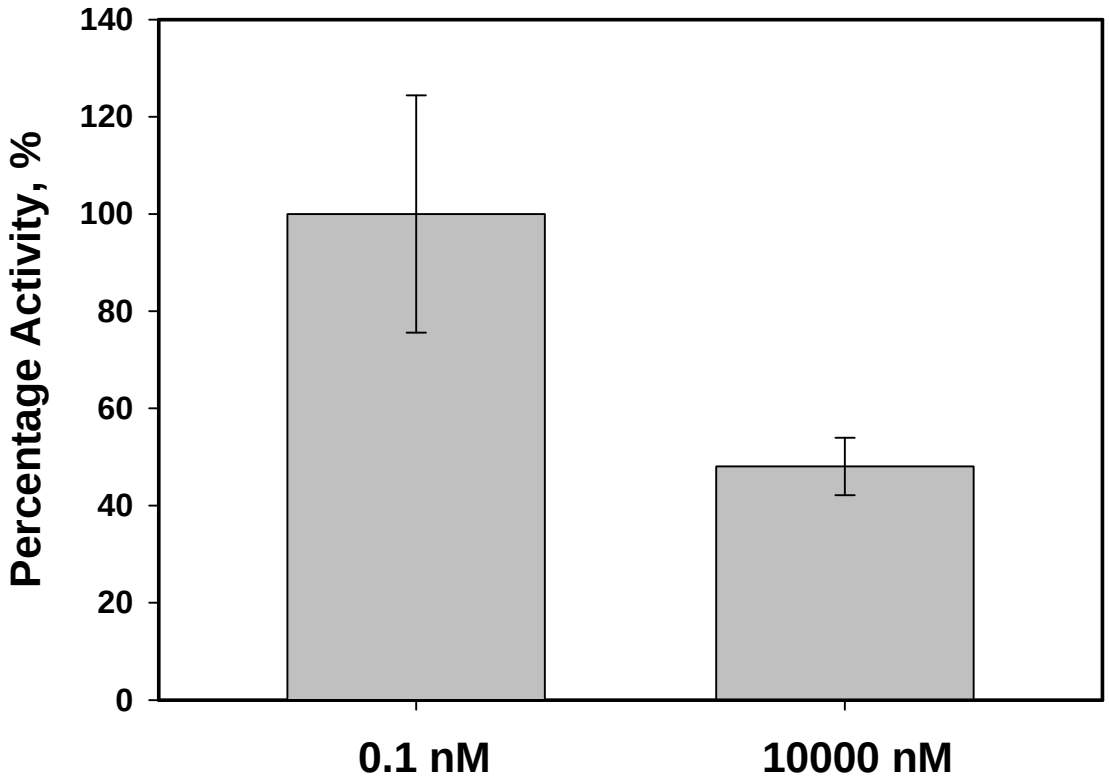


Figure 7