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Matrix Metalloproteinase Triple-Helical Peptide Inhibitors: Potential Cross-Reactivity with Caspase-11

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Abstract: Triple-helical peptide inhibitors (THPIs) of matrix metalloproteinases (MMPs) have recently been demonstrated to be effective in a variety of animal models of disease, coincidental with knockout studies. However, passenger mutations have been described in MMP knockout mice that impact the activity of other proteins, including caspase-11. Thus, it is possible that the results observed with THPIs may be based on inhibition of caspase-11, not MMPs. The present study evaluated whether THPIs were cross-reactive with caspase-11. Two different THPIs were tested, one that is known to inhibit MMP-1 and MMP-8 (GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI) and one that is selective for MMP-2 and MMP-9 (α1(V)GlyΨ{PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI). No inhibition of caspase-11 was observed with GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI, even at an inhibitor concentration of 5 μM, while 5 μM α1(V)GlyΨ{PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI exhibited 40% inhibition of caspase-11. Further testing of GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI revealed nM inhibition of MMP-2, MMP-9, and MMP-13. Thus, the effectiveness of GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI observed in a sepsis animal model may not be due to caspase-11 inhibition, but may be due to broader MMP inhibition than previously thought.

Keywords: matrix metalloproteinase; caspase; sepsis; phosphinate; protease inhibitor; triple-helical peptide inhibitor

1. Introduction

The matrix metalloproteinase (MMP) family of enzymes has been implicated in a great variety of diseases [1–6], resulting in the pursuit of MMP inhibitors that has spanned decades [1,7–12]. A variety of phosphorus-based peptides have been developed as MMP inhibitors [7,13,14]. Phosphinates/phosphinic peptides contain a hydrolytically stable, tetrahedral phosphinic pseudo-dipeptide moiety which mimics the tetrahedral intermediate formed during Zn²⁺ metalloproteinase-catalyzed amide bond hydrolysis. We have reported that phosphinic triple-helical peptides (THPs) behave as effective transition state analog inhibitors of collagenolytic MMPs [15–19]. Recently, the triple-helical peptide inhibitor (THPI) (Gly-Pro-Hyp)₄-Gly-mep-Flp-Gly-Pro-Gln-{GlyΨ(PO₂H-CH₂)Ile}-His-Lys-Gln-Arg-Gly-Val-Arg-Gly-mep-Flp-(Gly-Pro-Hyp)₄-Tyr-NH₂ (designated GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI) (where mep = (2*S*,4*R*)-4-methylproline, Flp = (2*S*,4*R*)-4-fluoroproline, and Hyp = 4-hydroxy-L-proline) was shown to inhibit MMP-8 in the nanomolar range while being a much weaker inhibitor of MT1-MMP [19]. Gene ablation studies have indicated that MMP-8 facilitates sepsis in animal models, while MT1-MMP is protective [19–21]. GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI was thus applied in an animal model of sepsis [19]. Using the cecal ligation and puncture (CLP) sepsis model, 70% of the wild-type

mice treated with GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI survived after 7 days, while none of the non-treated wild-type mice did [19].

Genetically modified (knockout) mice have been utilized to delineate the roles of MMPs in normal physiological function and pathological states. To examine the specificity of the GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI, *Mmp8* null mice underwent CLP and were then either treated with the THPI or not treated. A survival advantage during sepsis was observed for *Mmp8* null mice compared with wild-type mice [19]. Survival of THPI-treated wild-type mice mirrored that of non-treated *Mmp8* null mice, while survival of *Mmp8* null mice was not augmented by inhibitor treatment [19]. Thus, in consideration of the *Mmp8* null mice data, the GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI was deemed as acting specifically towards MMP-8 in vivo.

The identification of passenger mutations that can accompany MMP knockouts has raised serious concerns as to the interpretation of results from disease models in which MMPs were implicated [22]. For example, *Mmp7*, *Mmp8*, or *Mmp13* null mice were found to be protected from lipopolysaccharide (LPS) lethality (septic shock) [23–25]. However, these knockout mice carried a passenger mutation that inactivated *Casp11* (the mouse ortholog of human *Casp4* and *Casp5*) [22]. Caspase-11 has been demonstrated to participate in LPS-induced endotoxic shock and subsequent lethality [26,27]. Mice possessing a non-functional caspase-11 are more resistant to LPS-induced septic shock [28], and the passenger mutation of *Casp11* resulted in mice resistant to LPS-induced endotoxic shock [22,29].

In the above example, the results from MMP-8 knockout mice appear to be validated through the use of a THPI which targeted the MMP of interest (MMP-8) in wild-type mice, as the same phenotype was observed for the CLP knockout mice and the CLP wild type mice treated with the GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI. However, if the applied THPI non-specifically inhibited other enzymes, interpretation of the results becomes ambiguous. Given that the *Mmp8* null mice may have had a *Casp11* inactivating mutation [22], the mirroring of survival in the *Mmp8* null mice by the inhibitor treated wild-type mice could have been the result of the THPI inhibiting caspase-11 in the wild-type mice. MMP inhibitors are not anticipated to inhibit caspase-11, due to the different active site chemistries and sequence specificities [30–34]. However, recent research has indicated that caspase-11 recognition of substrates can be strongly influenced by motifs outside of the active site [34], and thus, there is a possibility of non-specific inhibition by MMP inhibitors whose structures may be complimentary to caspase-11 motifs. In addition, MMP inhibitors that are designed to interact with the active site Zn²⁺ can inhibit non-MMP activity by non-selective metal binding [35,36]. The present study has examined the inhibition of (a) caspase-11 by two phosphinate-based THPIs and (b) other collagenolytic MMPs by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI.

2. Results

Caspase-11 hydrolysis of acetyl-Trp-Glu-His-Asp-pNA was examined at several enzyme and substrate concentrations to obtain conditions under which enzyme inhibition could be studied. It was ultimately determined that 3 U/μL (1.08 μM) caspase-11 and 250 μM acetyl-Trp-Glu-His-Asp-pNA provided a reasonably linear rate of hydrolysis over 15 min. Acetyl-Leu-Glu-Val-Asp-CHO was incubated with caspase-11 at a concentration of 5 μM for 2 h prior to the addition of substrate, and was found to completely inhibit enzymatic activity (Figure 1).

The potential inhibition of caspase-11 by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI was examined by adding 5 μM of the inhibitor to the enzyme for 2 h prior to addition of substrate. A 2 h incubation was utilized based on (a) the generally observed behavior of slow on and off rates for tight-binding inhibitors [37], (b) studies demonstrating that high affinity phosphinate inhibitors of Zn²⁺ metalloproteinases are slow binding [38], and (c) our prior studies using THPIs [39]. This concentration was comparable to that used in our prior CLP mouse model studies (13.5 μM) [19]. No inhibition of caspase-11 activity was observed over 15 min (Figure 1).

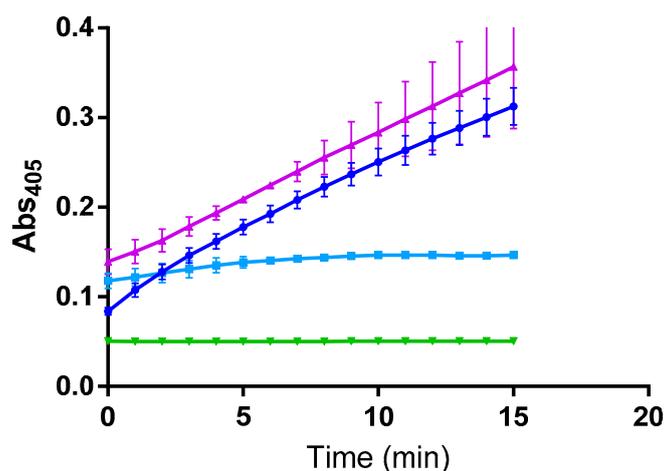


Figure 1. Effect of inhibitors on caspase-11 activity. Hydrolysis of acetyl-Trp-Glu-His-Asp-pNA by caspase-11 (dark blue) and inhibition by 5 μ M acetyl-Leu-Glu-Val-Asp-CHO (light blue) or 5 μ M Gly Ψ {PO₂H-CH₂}Ile-His-Lys-Gln THPI (purple). Acetyl-Trp-Glu-His-Asp-pNA alone (green) was used as a control.

A second THPI was examined for inhibitory potential towards caspase-11. (Gly-Pro-Hyp)₄-Gly-mep-Flp-Gly-Pro-Pro-Gly Ψ {PO₂H-CH₂}Val-Val-Gly-Glu-Gln-Gly-Glu-Gln-Gly-Pro-Pro-Gly-mep-Flp-(Gly-Pro-Hyp)₄-NH₂ (designated α 1(V)Gly Ψ {PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI) is an effective, and selective, inhibitor of MMP-2 and MMP-9, with K_i values of 2.24 and 0.98 nM at 37 °C for MMP-2 and MMP-9, respectively, and exhibiting no or weak (K_i = 10–50 μ M) inhibition of MMP-1, MMP-3, MMP-8, MMP-13, and MT1-MMP [15,19]. α 1(V)Gly Ψ {PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI was recently shown to be efficacious in an animal model of multiple sclerosis [19]. 5 μ M of α 1(V)Gly Ψ {PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI was added to the enzyme for 2 h prior to addition of substrate. Approximately 40% inhibition of caspase-11 activity was observed after 15 min (Figure 2). The extent of this inhibition did not increase after 1 h (data not shown). We examined the inhibition of caspase-11 by the α 1(V)Gly Ψ {PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI using no inhibitor pre-incubation time, and found no inhibition to occur at early time points (data not shown), in similar fashion to what was observed for a 2 h inhibitor pre-incubation (Figure 2).

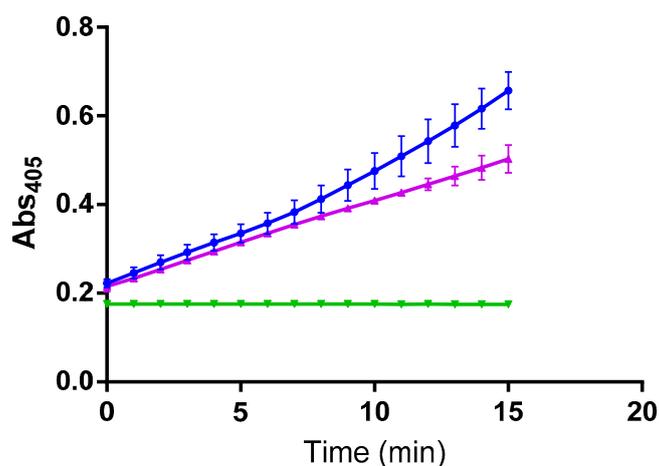


Figure 2. Effect of α 1(V)Gly Ψ {PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI on caspase-11 activity. Hydrolysis of acetyl-Trp-Glu-His-Asp-pNA by caspase-11 (dark blue) and inhibition by 5 μ M α 1(V)Gly Ψ {PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI (purple). Acetyl-Trp-Glu-His-Asp-pNA alone (green) was used as a control.

The inhibitory potency of GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI was initially examined for MMP-1, MMP-3, MMP-8, and MT1-MMP (Table 1) [19]. Selectivity within the collagenolytic MMPs was further examined by treating MMP-2, MMP-9, and MMP-13 with GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI. All three enzymes were inhibited by the THPI (Table 1). The K_i value for MMP-13 was better than that observed for MMP-8 (Table 1). MMP-2 and MMP-9 were very efficiently inhibited by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI, with K_i values below 10 nM (Table 1).

Table 1. Inhibition of matrix metalloproteinases (MMPs) by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI at 37 °C.

Enzyme	K _i (nM)
MT1-MMP	4704 ± 708.4 ¹
MMP-8	124.6 ± 6.9 ¹
MMP-8	378.1 ± 40.6
MMP-1	169.2 ± 28.4 ¹
MMP-3	NI ^{1,2}
MMP-2	2.2 ± 0.20
MMP-9	3.7 ± 0.40
MMP-13	31.7 ± 7.6

¹ Previously reported [19]. ² NI = No inhibition at a triple-helical peptide inhibitor (THPI) concentration of 1000 nM.

3. Discussion

The *Mmp8* null mice utilized in our CLP study were on a C57BL/6J background [19], which may still harbor the *Casp11* mutation depending upon the extent of backcrossing [22]. The inhibition of caspase-11 by a THPI could occur by two non-exclusive mechanisms: (a) chelation of a caspase-11 metal ion by the THPI phosphinate; and (b) binding of the THPI sequence to a complementary motif on the surface of caspase-11. To consider possibility (a), the mechanism by which caspase-11 contributes to CLP/LPS-induced lethality needs to be taken into account. The CLP sepsis model induces polymicrobial peritonitis and results in translocation of bacteria into the bloodstream (bacteremia) [40,41]. Both Gram negative and Gram positive bacteria enter the bloodstream and tissues, with a shift to Gram negative bacteria with disease progression [40]. Gram negative, but not Gram positive, bacteria activate the caspase-11 non-canonical inflammasome pathway [28]. Caspase-11 directly binds the lipid A tail of LPS via the caspase recruitment domain motif [28]. LPS binding first results in the formation of caspase-11 dimers, followed by auto-hydrolysis at the interdomain linker residue Asp285 [42]. Caspase-11 then undergoes oligomerization, ultimately inducing pyroptosis and secretion of the pro-inflammatory cytokines interleukin-1β and interleukin-18 [28].

MMP inhibitors have an unfortunate history of non-selectivity. Of specific concern is the ability of zinc binding groups to behave in a non-selective fashion. For example, hydroxamic acids have been commonly used in MMP inhibitors, but the ability of hydroxamic acids to non-specifically chelate metal ions, in some cases with greater affinity than zinc, has been noted [35,36]. Caspase-11 is a Cys protease, and it is not anticipated that non-selective metal chelation would be a concern as caspase family members caspase-1, caspase-2, caspase-3, caspase-7, caspase-8, and caspase-9 do not possess structural metals [43,44]. However, the human orthologs of caspase-11, caspase-4 and caspase-5, have been reported to each have one Mg²⁺ ion present (<https://swissmodel.expasy.org/repository/uniprot/P49662> and <https://swissmodel.expasy.org/repository/uniprot/P51878>). Homology modeling indicated that in caspase-4 the Mg²⁺ binds to His338 of the A chain, His338 of the B chain, and Tyr370 of the B chain, while in caspase-5, the Mg²⁺ binds to His395 of the A chain and Glu398 of the B chain. Thus, the Mg²⁺ ion could stabilize caspase-11 dimers, and dimerization is an important step in the caspase-11 pathway contributing to endotoxic shock (see above). As indicated by studies with nucleic acids, phosphates will readily bind Mg²⁺ [45,46]. The GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI did not show inhibition of caspase-11, and thus, Mg²⁺ ions most likely do not undergo non-specific chelation by THPIs (see above).

In consideration of mechanism (b), THPIs have extended sequences that interact with regions well outside of the active site in MMPs [16,17]. These regions of interaction are referred to as secondary binding sites (exosites). The THPI extended sequences could interact with caspase-11 exosites. It has been proposed that caspase-11 substrate specificity is governed by exosite interactions, as the substrate preferences of caspase-11 cannot be explained by the sequences at the active site [34]. One possibility is that negatively charged residues in the P₇-P₁₀ region may be unfavorable for substrate interaction with caspase-11 [34]. GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI possesses no negative charges, and thus could interact with caspase-11. However, as this THPI did not inhibit caspase-11, we can assume that there is no exosite interaction.

It is interesting to note that α1(V)GlyΨ{PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI exhibited some inhibition of caspase-11 activity, although it occurred at a relatively high inhibitor concentration (5 μM). It is not likely that this inhibition was due to Mg²⁺ chelation (based on the lack of caspase-11 inhibition by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI). This suggests a possible interaction between α1(V)GlyΨ{PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI and a caspase-11 allosteric site, although α1(V)GlyΨ{PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI possesses 6 negatively charged residues, which are not favored in the P₇-P₁₀ region by caspase-11 (see above).

There are two caveats to the interpretation of results from the present study. First, the caspase-11 substrate used here is small (4 amino acids), whereas in the CLP/LPS animal models caspase-11 is processing proteins. An exosite-binding inhibitor might not impact caspase-11 hydrolysis of a small substrate but could impact processing of a protein, unless binding to the exosite had an allosteric effect on the enzyme. Second, chelation of Mg²⁺ ions might not impact caspase-11 activity towards a small substrate, but rather inhibit dimerization of the enzyme. Dimerization might be required to facilitate specific proteolytic activities in CLP/LPS models (see above).

We presently observed that GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI did not inhibit caspase-11 activity. This result supports our prior report on the modulation of sepsis by MMP-8 inhibition, not caspase-11 inhibition [19]. However, our prior studies did not examine the full range of collagenolytic MMPs that might have been inhibited by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI. We found that MMP-2, MMP-9, and MMP-13 are effectively inhibited by the THPI, along with MMP-8. Thus, the observed protection from sepsis in the CLP mouse model by GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI may have been due to inhibition of multiple MMPs. The use of more selective inhibitors will elucidate the role of each MMP in sepsis.

4. Materials and Methods

GlyΨ{PO₂H-CH₂}Ile-His-Lys-Gln THPI and α1(V)GlyΨ{PO₂H-CH₂}Val [mep_{14,32},Flp_{15,33}] THPI were synthesized and characterized as described previously [19]. Recombinant caspase-11 (residues 81-373), caspase substrate acetyl-Trp-Glu-His-Asp-pNA (where pNA = *p*-nitroaniline) (MW 747.8 Da), and caspase-4 and caspase-5 inhibitor acetyl-Leu-Glu-Val-Asp-CHO (MW 500.6 Da) were obtained from Enzo Life Sciences, Inc.

4.1. Caspase Enzyme Assay

Caspase-11 (9 U/μL in 10 μL caspase activity buffer (100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)), pH 6.5, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10% poly(ethylene-glycol) (PEG)-8000, 10 mM dithiothreitol (DTT)) was added to 10 μL of caspase-11 inhibitor (5 μM), THPI (5 μM), or no inhibitor in caspase activity buffer. For testing THPIs as inhibitors, enzyme was incubated with THPIs for 2 h at 37 °C. Residual enzyme activity was monitored by adding 10 μL of caspase substrate in caspase activity buffer to produce a final concentration of <0.1K_M. pNA cleavage from the peptide was monitored at 37 °C over 1 h using λ = 405 nm on a Bio-Tek Synergy H1 Reader or H4 Hybrid Reader.

4.2. Matrix Metalloproteinases (MMPs)

Human recombinant, full-length proMMP-8 and proMMP-13 were purchased from R&D Systems (Minneapolis, MN, USA) and activated according to the manufacturer's instructions prior to use. *p*-Aminophenylmercuric acetate was purchased from EMD Biosciences (San Diego, CA, USA), and trypsin (TPCK-treated) was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Recombinant MMP-2 and MMP-9 catalytic domain were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used directly without further activation in the desired concentration.

ProMMP-8 and proMMP-13 were activated by mixing an equal volume of stock enzyme solution and activator (*p*-aminomercuric acetate to a final concentration of 1 mM) followed by 2–3 h incubation in a 37 °C water bath. Activated MMPs were diluted to 200–400 nM in ice cold TSB*Zn (50 mM Tris, 100 mM NaCl, 10 mM CaCl₂, 0.05% Brij-35, 0.02% NaN₃, 1 μM ZnCl₂, pH 7.5) to prevent autoproteolysis. Enzyme aliquots were either kept on wet ice and used the same day or immediately frozen at −80 °C. MMP activity was initially evaluated by using the Knight substrate (Mca-Lys-Pro-Leu-Gly-Leu-Lys-(Dnp)-Ala-Arg-NH₂, where Mca = 7-methoxycoumarin-4-acetyl and Dnp = 2,4-dinitrophenyl) and compared with prior data [16,17]. In this way, activity towards the substrate was used as an indicator of enzyme integrity, rather than TIMP titration, as performed previously [47].

4.3. Inhibition Kinetic Studies

Peptide substrate and THPI solutions were prepared using TSB*Zn. 1–10 nM enzyme was incubated with varying concentration of inhibitor for 2 h at 37 °C. Residual enzyme activity was monitored by adding Knight substrate solution in TSB*Zn to produce a final concentration of <0.1K_M. Initial velocity rates were determined from the first 10 min of hydrolysis when product release is linear with time. Fluorescence was measured on a Bio-Tek Synergy H1 Reader or H4 Hybrid Reader using λ_{excitation} = 324 nm and λ_{emission} = 393 nm. Apparent K_i values were calculated from the following formulas [15]:

$$v_i/v_o = \{E_t - I_t - K_i^{(app)} + ((E_t - I_t - K_i^{(app)})^2 + 4E_tK_i^{(app)})^{0.5}\}/2E_t \quad (1)$$

$$K_i^{(app)} = K_i \{(A_t + K_M)/K_M\} \quad (2)$$

where I_t is the total inhibitor concentration, E_t is the total enzyme concentration, A_t is the total substrate concentration, v_o is the activity in the absence of inhibitor, and K_M is the Michaelis constant. In our assays the value of $E_t/K_i^{(app)}$ does not exceed 100 so that the inhibitor is distributed in both free and bound forms, and $K_i^{(app)}$ can be calculated by fitting inhibition data to equation 1. Because the substrate concentration is less than $K_M/10$, $K_i^{(app)}$ values are insignificantly different from true K_i values. In cases where weak inhibition occurred, $K_i^{(app)}$ values were calculated using Prism 7.0 (GraphPad, San Diego, CA, USA) by fitting data to the equation $v_i = v_o/(1 + I_t/K_i^{(app)})$.

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Conflicts of Interest: The authors declare no conflict of interest.

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