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Reactive site mutants of N-TIMP-3 that selectively inhibit ADAMTS-4 and ADAMTS-5: Biological and structural implications.

Ngee H. Lim¹, Masahide Kashiwagi¹, Robert Visse¹, Jonathan Jones², Jan J. Enghild³, Keith Brew⁴ and Hideaki Nagase¹ ‡

From the ¹Kennedy Institute of Rheumatology Division, Imperial College London, 65 Aspenlea Rd., London W6 8LH, United Kingdom, ²Orthopaedics and Trauma, Wexham Park Hospital, Slough, Berkshire SL2 4HL, United Kingdom, ³Center for Insoluble Protein Structures (inSPIN) and Interdisciplinary Nanoscience Center (iNANO), Department of Molecular Biology; Science Park, University of Århus, Gustav Wieds Vej 10c, DK-8000 Århus C, Denmark and the ⁴Department of Basic Science, College of Biomedical Science, Florida Atlantic University, Boca Raton, Florida 3341, United States of America.

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‡ To whom correspondence should be addressed. Tel.: 44-20-8383-4488; Fax: 44-20-8356-0399; E-mail address: h.nagase@imperial.ac.uk.

Running Title: Inhibition of ADAMTSs by mutants of N-TIMP-3

Key Words: inhibitor, metalloproteinase, aggrecanase, cartilage, collagenase, osteoarthritis

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; ADAM, a disintegrin and metalloproteinase; GAG, glycosaminoglycan; GST, glutathione-S-transferase; IGD, interglobular domain of aggrecan; IL-1 α , interleukin-1 α ; MMP, matrix metalloproteinase; N-TIMP, N-terminal domain of tissue inhibitor of metalloproteinases; OSM, oncostatin M; TIMP, tissue inhibitor of metalloproteinases;

Abstract

We have previously reported that reactive site mutants of the N-terminal inhibitory domain of TIMP-3 (N-TIMP-3) modified at the N-terminus, selectively inhibited ADAM17 (A Disintegrin and Metalloproteinase) over the MMPs (Matrix Metalloproteinases). The primary aggrecanases, ADAMTS-4 and ADAMTS-5 (ADAM with Thrombospondin motifs) are ADAM17-related metalloproteinases which are similarly inhibited by TIMP-3, but are poorly inhibited by other TIMPs. Using a newly developed recombinant protein substrate based on the interglobular domain (IGD) of aggrecan, *gst-IGD-flag*, these reactive site mutants were found to similarly inhibit ADAMTS-4 and ADAMTS-5. Further mutations of N-TIMP-3 indicated that up to two extra Ala residues can be attached to the N-terminus before the $K_{i(\text{app})}$ for ADAMTS-4 and ADAMTS-5 increased to over 100 nM. No other residues tested at the [-1] position produced inhibitors as potent as the Ala mutant. The mutants N-TIMP-3(T2G), [-1A]N-TIMP-3 and [-2A]N-TIMP-3 were effective inhibitors of aggrecan degradation but not of collagen degradation in both interleukin-1 α (IL-1 α)-stimulated porcine articular cartilage explants and IL-1 α with oncostatin M-stimulated human cartilage explants. Molecular modelling studies indicated that the [-1A]N-TIMP-3 mutant has additional stabilising interactions with the catalytic domains of ADAM17, ADAMTS-4 and ADAMTS-5 that are absent in complexes with MMPs. These observations suggest that further mutation of the residues of N-TIMP-3 which make unique contacts with these metalloproteinases may allow discrimination between them.

Introduction

The TIMPs (Tissue Inhibitor of Metalloproteinases) are a family of four endogenous inhibitors that were originally characterised by their ability to inhibit the MMPs (Matrix Metalloproteinases). TIMPs have now been shown to also inhibit members of two other related metalloproteinase families, the ADAMs (A Disintegrin and Metalloproteinase) and the ADAMTSs (ADAM with Thrombospondin motifs). Specifically, TIMP-1 has been shown to inhibit ADAM10 [1], whereas TIMP-2 has been shown to inhibit ADAM12 [2] and ADAMTS-1 [3]; TIMP-3 has the widest inhibitory activity among the TIMPs, since it has been shown to inhibit ADAM10 [1], ADAM12 [2], ADAM17 [4], ADAM28 [5], ADAM33 [6], ADAMTS-1 [3], ADAMTS-2 [7], ADAMTS-4 [8, 9] and ADAMTS-5 [9]. Not all ADAMs and ADAMTSs are inhibited by the TIMPs; for example none of the four human TIMPs inhibit ADAM8 or ADAM9 [10].

TIMPs comprise two domains, the N-terminal inhibitory domain and a C-terminal domain. The N-terminal inhibitory domain is a wedge like structure that inhibits the MMPs by insertion into the active site of the MMP. The crystal structure of TIMP-1-MMP-3 catalytic domain complex [11] shows that the amino and carbonyl groups of Cys1 of TIMP-1 coordinate the catalytic zinc ion and that the Thr2 and Val4 side chains fit into the S1' and S3' pockets of MMP-3, respectively. The importance of the N-terminus of the TIMP has also been highlighted by several mutagenesis [12-15] and chemical modification studies [16, 17]. These studies indicated that the N-terminal Cys residue is essential for MMP inhibitory activity. Mutation of this residue to serine or blocking the N-terminal α -amino group by the addition of an extra amino acid [15, 17], acetylation [17] or carbamylation [16] severely reduces inhibitory activity against MMPs. The recent crystal structure of the ADAM17 catalytic domain-N-TIMP-3 complex [18] has confirmed that ADAM17 is also inhibited by TIMP-3 in a similar manner as the MMPs, suggesting that the mechanism by which TIMP-3 inhibits ADAMTSs is also similar.

We have previously reported, however, that two N-terminal reactive site mutants of N-TIMP-3, N-TIMP-3(T2G) and [-1A]N-TIMP-3 (N-TIMP-3 with an extra alanine appended to Cys1) which, as predicted from other studies [15-17], are poor MMP inhibitors, still retain inhibitory activity against ADAM17 [19]. This inhibition indicates that there are subtle differences in TIMP inhibition of ADAMs compared to that of the MMPs that are not immediately obvious from the crystal structures. Here we report the testing of these N-terminal reactive site mutants of N-TIMP-3 for their ability to inhibit the two primary aggrecanases, ADAMTS-4 and ADAMTS-5, based on their similar TIMP inhibitory profiles to ADAM17 since they are potently inhibited only by TIMP-3. We have generated additional mutants with varying length of alanine extensions and with other amino acids at the -1 position to determine if these display further selectivity between the two aggrecanases.

Aggrecan degradation in interleukin-1 α (IL-1 α)-stimulated porcine articular cartilage explants, an *ex vivo* model of arthritis, is inhibited by N-TIMP-3, but not by TIMP-1 or TIMP-2 [20], suggesting that the ADAMTSs and not the MMPs are responsible for the degradation of cartilage aggrecan. Furthermore, aggrecan in the cartilage of ADAMTS-5-null mice was protected from degradation when challenged with arthritis induced by surgical joint destabilisation [21], or with antigen-induced arthritis [22], suggesting that in mice, the primary aggrecanase in this disease is ADAMTS-5. However, in cartilage from rheumatoid arthritis and osteoarthritis (OA) patients, there is evidence that aggrecan is degraded by both the ADAMTSs and MMPs; tissue sections stain positive for neo-epitopes produced by both MMP and ADAMTS activity [23]. Aggrecan fragments from both MMP- and ADAMTS-mediated degradation have also been found in OA joint cartilage and synovial fluid [24]. Degradation of the other cartilage structural macromolecule, type II collagen is mediated primarily by the collagenases, which are members of the MMP family [25]. Consequently,

the relative contribution of the ADAMTSs and the MMPs in the progression of human disease remains unclear. We therefore applied our active site mutants of N-TIMP-3 to investigate the role of ADAMTS and MMPs in IL-1 α - induced pig or IL-1 α /Oncostatin M (OSM) - induced human osteoarthritic cartilage degradation.

Experimental Procedures

Materials

Restriction enzymes were from New England Biolabs (Hitchin, UK). The plasmid pGEX-4T1, Pfu DNA polymerase, GFX Gel Band PCR Purification Kit, GFX Microplasmid Purification Kit and glutathione-Sepharose 4B column material were from Amersham Biosciences UK Limited (Bucks, UK). Precision Protein Standards for SDS-PAGE were from BioRad (Hemel Hempstead, UK). Proteinase inhibitor cocktail set II was purchased from Calbiochem (Merck Chemicals Ltd, Nottingham, UK). Chloramine T, dimethylaminobenzaldehyde (DMBA), dimethylmethylene blue (DMMB) and OSM were obtained from Sigma-Aldrich Company (Dorset, UK). ADAM17 and the Mca-PLAQAV-Dpa-RSSSR fluorogenic substrate were from R&D systems (Abingdon, UK). The Mca-PLGL-Dap(Dnp)-AR fluorogenic substrate was supplied by Bachem (Merseyside, UK). TIMP-1, TIMP-2, N-TIMP-3, MMP-1 Δ C, MMP-2, MMP-3 Δ C, ADAMTS4-2 and ADAMTS5-4 were prepared as described previously [9, 17, 26-30]. Purified IL-1 α was a gift from Professor J. Saklatvala (Imperial College London, UK). Anti-ARGSV antibody was a gift from Professor B. Caterson (Cardiff, UK). Porcine metacarpophalangeal joints were supplied by Fresh Tissue Supplies (London, UK) and dissected on-site within 24 hours of slaughter. Human cartilage was obtained from consenting osteoarthritic patients undergoing hip or knee joint replacement at Heatherwood and Wexham Park Hospital (Slough, Berkshire).

Construction of N-TIMP-3 mutants

The plasmid pET-42b-N-TIMP-3-His was used as the template for mutagenesis by PCR as described [19]. The common reverse primer (NotI restriction site in italics) 5'-AAAAGCGGCCGCGTTACAACCCAGGTGATA-3') was used in all mutagenesis procedures. The forward primers used were (codons for the mutated amino acids underlined and the NdeI restriction site in italics): [-2A]N-TIMP-3, 5'-AAAACATATGGCAGCATGCACATGCTCGCCCAGC-3'; [-3A]N-TIMP-3, 5'-AAAACATATGGCAGCAGCATGCACATGCTCGCCCAGC-3'; [-5A]N-TIMP-3, 5'-AAAACATATGGCAGCAGCAGCAGCATGCACATGCTCGCCCAGC-3'; [-8A]N-TIMP-3, 5'-AAAACATATGGCAGCAGCAGCAGCAGCAGCAGCATGCACATGCTCGCCCAGC-3'; [-1G]N-TIMP-3, 5'-AAAACATATGGGTTGCACATGCTCGCCCAGC-3'; [-1M]N-TIMP-3, 5'-AAAACATATGATGTGCACATGCTCGCCCAGC-3'; [-1E]N-TIMP-3, 5'-AAAACATATGGAGTGCACATGCTCGCCCAGC-3' and [-1K]N-TIMP-3, 5'-AAAACATATGAAGTGCACATGCTCGCCCAGC-3'.

Expression and purification of N-TIMP-3 and mutants.

N-TIMP-3 and its mutants were expressed in *E. coli* BL-21(DE3), and purified by Ni²⁺ chelate chromatography as previously described [9]. Additionally, endotoxin was removed by including a wash step with 60% isopropanol, 6M guanidine hydrochloride, during column chromatography [31]. The amount of endotoxin after purification was determined using the limulus amoebocyte lysate assay (Cambrex) to be less than 1pg/ pmol of N-TIMP-3. The level of endotoxin that stimulated cartilage matrix degradation in porcine articular cartilage explants was > 1ng/ml.

MMP and ADAM17 inhibition kinetics

The ability of N-TIMP-3 mutants to inhibit the MMPs and ADAM17 was tested using the fluorogenic substrate Mca-PLG~L-Dap(Dnp)-AR (~ indicates the scissile bond) [32] for the MMPs and Mca-PLA~QAV-Dpa-RSSSR for ADAM17. The N-TIMP-3 mutants were preincubated with MMP (1 nM catalytic domain of MMP-1 (MMP-1ΔC), 125 pM MMP-2, 500 pM catalytic domain of MMP-3 (MMP-3ΔC)) or 250 pM of ADAM17 ectodomain for 1 h at 37 °C before residual activity was measured by fluorescence increase (excitation 323nm, emission 395 nm) using 2 μM substrate. The data were fitted to the tight binding inhibitor equation: $v = [(E-I-k+[(E-I-k)^2+4Ek]^{1/2})/(2E)]$, where v is the velocity of the reaction, E the enzyme concentration, I the initial inhibitor concentration and k the apparent inhibition constant, using the software Prism (Graphpad, La Jolla, USA).

Construction, expression and purification of the *gst-IGD-flag*

The expression plasmid pGEX-4T1 containing the glutathione S-transferase (GST) sequence fused with the interglobular domain (IGD) of aggrecan (Tyr330 to Gly457), with a C-terminal FLAG (DYKDDDDK) sequence, cloned into the EcoRI and XhoI cloning sites (Figure 1 A). The plasmid was transformed into *E. coli* BL-21 (DE3). Recombinant protein expression was induced with IPTG (100 μM) overnight at 25 °C. Bacteria were harvested, washed and mechanically disrupted with a French Press (5x 1500 Psi). After centrifugation, the supernatant containing the *gst-IGD-flag* was applied to a glutathione-Sepharose 4B column equilibrated with 50 mM Tris-HCl, pH 8.0. The column was washed with 0.5 M NaCl, 50 mM Tris-HCl pH 8.0 and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. The eluted protein substrate was dialysed three times against 10 volumes of 50 mM Tris-HCl pH 8.0, 150 mM NaCl for 4 hours at 4 °C and concentrated to $A_{280} > 2.5$ using polyethyl sulphate membrane spin concentrators. The concentration of intact substrate was determined to be approximately 34 μM. The yield of substrate (about 20 mg of partially purified *gst-IGD-flag* per litre of bacterial culture) was sufficient for over 2000 assays.

Gst-IGD-flag aggrecanase assays

Equal volumes (5 μl) of 17 μM *gst-IGD-flag* and 1 nM ADAMTS-4 which lacks the C-terminal spacer domain (ADAMTS 4-2, [29]) or 0.5 nM ADAMTS-5 which lacks the C-terminal cysteine rich, spacer and thrombospondin domains (ADAMTS5-4, [30]) were incubated at 37 °C for the indicated period of time. Inhibitor, if used, was preincubated with the ADAMTS enzyme (total volume 5 μl) at 37 °C for one hour before addition of 5 μl of the substrate for 1 h. The reactions were stopped by addition of an equal volume (10 μl) of 2x SDS-PAGE sample buffer containing 10 mM EDTA. SDS-PAGE (10% acrylamide) was run under reducing conditions using a modification of the ammediol/glycine/HCl buffer system of Wyckoff *et al.* [33]. Following staining by Coomassie Brilliant Blue R-250 (CBB), the amount of product was determined by densitometric quantitation of the 17 kDa band using the GS-710 scanning densitometer (Biorad, Hertfordshire) and analysed using the 1D Phoretix Software (Non-linear dynamics, Newcastle upon Tyne). Under conditions where substrate cleavage was linear, the amount of substrate cleaved is directly related to the velocity of the reaction. For inhibition kinetics, the data were fitted to the same tight binding equation as in the MMP inhibition kinetics.

Cartilage explant cultures

Porcine articular cartilage explants were prepared as previously described [20]. Explanted human cartilage pieces (approx. 5 mm x 5 mm x 2.5 mm) from either the femoral head or the knee were rested overnight in DMEM with 20% of foetal calf serum before being washed twice with DMEM without serum. Each cartilage piece was placed in one well of a round bottomed 96-well plate with 200 μl of serum-free DMEM with or without IL-1α (10 ng/ml) and OSM (50 ng/ml), together with various concentrations of each TIMP. Each

treatment was prepared in triplicate. After 3 days, the conditioned media were harvested and stored at -20°C until use. For longer term culture of porcine articular cartilage, the media were replaced every 4 days and the harvested media were stored as above.

Analysis of cartilage degradation

Glycosaminoglycan (GAG) release into the culture medium was determined as previously described [20]. Hydroxyproline (hypro) released into the culture medium was determined using a modification of the assay as described by Bergman and Loxley [34].

Molecular Modelling with AutoDock 4.2

Molecular docking of N-TIMP-3 and the mutants onto the three-dimensional X-ray structure of the catalytic domain of MMP-1 and ADAMTS-5 was carried out using the AutoDock software package (version 4.2) as implemented through the graphical user interface AutoDockTools (ADT 1.5.4) [35]. The structures used were from the N-TIMP-1-MMP-1 complex (pdb 2J0T [36]), the N-TIMP-3-ADAM17 complex (pdb 3CKI [18]), ADAM17 (pdb 3G42 [37]), ADAMTS-4 (pdb 2RJP [38]) and ADAMTS-5 (pdb 2RJQ [38]). To create the initial coordinates for docking studies, the water molecules were removed from the crystal structures and excluded from the calculations. The non-catalytic zinc chloride found in the ADAMTS-5 structure was also removed. Hydrogen atoms were added and Gasteiger charges computed. For the catalytic zinc atom, the formal charge of +2 and van der Waals radius of 0.87 \AA were used, as suggested by Hu *et al.* [39]. To minimise computation time, the only bonds allowed to rotate were in mutated residues of the TIMP. The docking area was defined by a box, centred on the TIMP binding position above the metalloprotease. Grids points of $100 \times 100 \times 100$ with 0.5 \AA spacing were calculated around the docking area for all the ligand atom types using AutoGrid4. Two hundred separate docking calculations were performed for each N-TIMP. Each docking calculation consisted of 2,500,000 energy evaluations using the Lamarckian genetic algorithm local search over 27,000 generations. A mutation rate of 0.02 and a crossover rate of 0.8 were used to generate new docking trials for subsequent generations. The results of the docking experiments were visualised using pymol (<http://www.pymol.org/>).

Results

Aggrecanase assay using the *gst-IGD-flag* substrate

The *gst-IGD-flag* substrate purified from the soluble extract of the *E. coli* contained a considerable amount of lower molecular weight products after purification (Figure 1 B lane 1). Either the GST-tag or the FLAG-tag was associated with these bands, indicating degradation (data not shown). The degradation was not prevented by the addition of protease inhibitors (data not shown). Further purification using ion-exchange and size exclusion chromatography did not separate the degradation products from the intact substrate under native conditions (data not shown). However, the intact substrate accounted for over 70% of the CBB-stainable protein bands (Figure 1 B lane 2), and the expected product after ADAMTS cleavage (Figure 1 B, arrow) was distinguishable from the degradation products. The apparent molecular sizes of the intact substrate and cleavage products were 52 kDa, 35 kDa and 17 kDa, respectively. These were higher than the calculated molecular masses (Figure 1 A). The 17 kDa band reacted with the neo-epitope antibody which recognises the newly generated N-terminal ARGS(374-377) of the aggrecan core protein, indicating that it is an authentic C-terminal fragment generated by aggrecanase activity (Figure 1 B).

The linear range of *gst-IGD-flag* cleavage was determined by incubating $17 \mu\text{M}$ *gst-IGD-flag* with 0.5 nM ADAMTS5-4 for up to 32 h at 37°C (Figure 1 B). Densitometric analysis of the products after SDS-PAGE separation and CBB-staining indicated that cleavage was linear up to 400,000 pixel volumes, equivalent to incubation with 0.5 nM ADAMTS5-4 for 20 h (Figure 1 C). The enzyme concentration-dependence of substrate

cleavage was determined by the incubating *gst-IGD-flag* with increasing amounts of ADAMTS5-4 (0-2.5 nM) for 20 hours at 37 °C before visualisation by CBB staining (Figure 1 D). Cleavage was linear up to 0.5 nM enzyme (Figure 1 E).

Inhibition of aggrecanases by reactive site mutants of N-TIMP-3

Using the *gst-IGD-flag* assay, we tested the ability of N-TIMP-3(T2G) and [-1A]N-TIMP-3 to inhibit the aggrecanases. The remaining activity after inhibition was plotted as % activity against inhibitor concentration in Figure 2A and 2B for ADAMTS4-2 and ADAMTS5-2 respectively, and the data fitted to the tight binding inhibition equation, to obtain the $K_{i(\text{app})}$ values shown in Table 1, with the standard errors representing goodness of fit. The mutants retained inhibitory activity for ADAMTS, with $K_{i(\text{app})}$ values between 18 and 30 nM for ADAMTS4-2 and about 1.5 nM for ADAMTS5-4. N-TIMP-3 shows cooperativity in its inhibition of ADAM17 with a Hill coefficient, h , for N-TIMP-3 inhibition of ADAM-17 of 3.59 [19], but the inhibition curves reported here fitted the equation for tight binding non-cooperative inhibition, reflecting an h value of 1. All the N-terminal reactive site mutants tested show selective inhibition for the ADAMTS enzymes over the MMPs.

Effect of extending the number of alanines appended to the N-terminus on the inhibition of ADAM17 and the aggrecanases

The alanine extension mutants [-2A]N-TIMP-3, [-3A]N-TIMP-3, [-5A]N-TIMP-3 and [-8A]N-TIMP-3 with 2, 3, 5 and 8 additional Ala residues at the N-terminus respectively, were tested for their ability to inhibit ADAMTS-4 and ADAMTS-5 (Figure 2C and 2D respectively). These mutants were also tested against MMP-1 Δ C, MMP-2, MMP-3 Δ C and ADAM17. The summary of the results is shown in Table 2. All the alanine extension mutants were poor MMP inhibitors, with less than 50% inhibition observed at the highest concentration tested (1 μ M). The [-2A] N-TIMP-3 remained a potent inhibitor for ADAMTS-5 ($K_{i(\text{app})}$ = 1.8 nM), but was weaker for ADAMTS-4 ($K_{i(\text{app})}$ = 35.9 nM) and ADAM17 ($K_{i(\text{app})}$ = 65.3 nM). With 3 additional alanines, the apparent inhibition constants for ADAM17, ADAMTS-4 and ADAMTS-5 were about 100-times worse compared to that of the wild type inhibitor. The $K_{i(\text{app})}$ for ADAM17 was 239 nM, similar to that for ADAMTS-4. The inhibitory ability of the [-5A]N-TIMP-3 and the [-8A]N-TIMP-3 mutants decreased further, where the [-8A]N-TIMP-3 showed no significant inhibition at the highest concentration tested (1 μ M). N-terminal sequence analysis indicated all these mutants start with the expected alanine.

Effect of changing the amino acid at position [-1] on the inhibition of ADAMTS-4 and ADAMTS-5

To investigate the properties of the enzyme pocket that accommodates the N-terminus, the mutants [-1G]N-TIMP-3, [-1E]N-TIMP-3, [-1M]N-TIMP-3 and [-1K]N-TIMP-3 were generated and their ability to inhibit MMPs, ADAM17, ADAMTS-4 and ADAMTS-5 tested. N-terminal sequencing indicated that the N-termini of these mutants were differently processed by *E. coli*. The N-terminal residues of [-1E]N-TIMP-3, [-1M]N-TIMP-3 and [-1K]N-TIMP-3 were Met, making them [-ME]N-TIMP-3, [-MM]N-TIMP-3 and [-MK]N-TIMP-3. The mutant [-1G]N-TIMP-3 did not return a sequence, suggesting that the N-terminus was chemically modified, probably by N-acetylation (henceforth [-xG]N-TIMP-3). As the [-2A]N-TIMP-3 mutant was still active against the ADAMTSs, the $K_{i(\text{app})}$ values of these mutants for the various metalloproteinases were determined.

As summarised in Table 3, all the mutants were poor MMP inhibitors, with less than 50% inhibition at the highest concentration tested (1 μ M), apart from [-MK]N-TIMP-3, which inhibited MMP-1 and MMP-2 with $K_{i(\text{app})}$ of 0.5 and 0.75 μ M, respectively. On the other hand, the [-MM]N-TIMP-3 inhibited ADAMTS-4, ADAMTS-5 and ADAM17 with $K_{i(\text{app})}$ values of 75 nM, 69 nM and 153 nM, respectively. The other [-1] mutants were weaker inhibitors of these enzymes.

Inhibition of porcine cartilage degradation by reactive site mutants of N-TIMP-3

The aggrecanase-selective mutants, N-TIMP-3(T2G), [-1A]N-TIMP-3 and [-2A]N-TIMP-3 were tested in the IL-1 α -stimulated porcine articular cartilage explant model of cartilage degradation. As previously reported [20], upon stimulation with IL-1 α , there was a 5-fold increase in GAG release from explanted porcine articular cartilage, which was not inhibited by TIMP-1 or TIMP-2, but was dose-dependently inhibited by N-TIMP-3. Additionally, all three of the N-TIMP-3 mutants tested were able to inhibit this release dose-dependently, although they were slightly weaker than N-TIMP-3 (Figure 3).

Collagen degradation of IL-1 α -stimulated cartilage was not inhibited by aggrecanase selective TIMP-3 mutants

Upon stimulation with IL-1, collagen degradation in porcine cartilage mediated by MMPs occurs after 21 days [40]. The cartilage stimulation experiments were therefore extended for a longer time period at a single dose of 100 nM of the various TIMPs to determine the effects of the mutants on aggrecan and collagen catabolism.

As in the previous experiment, TIMP-1 and TIMP-2 did not affect the amount of GAG release from the cartilage upon IL-1 α stimulation (Figure 4A). There was no significant difference in GAG release between stimulated cartilage explants in the presence of N-TIMP-3 and the unstimulated cartilage explants, indicating that TIMP-3 is an effective inhibitor of aggrecanases. However, there was a gradual loss of aggrecan from the tissue even without IL-1 α stimulation, and by the end of 38 days, this equated to the loss of approximately 45% of the total GAG content. The N-TIMP-3 mutants significantly reduced the release of GAG at the early time points, compared to TIMP-1 and TIMP-2. However, there was a slow gradual loss of aggrecan above that observed for N-TIMP-3. This equated to roughly 85% of the total GAG content lost after 38 days.

Collagen degradation as measured by Hypro release into the medium started at day 21 and rapidly progressed thereafter: At days 35-38, the cartilage tissue was almost completely dissolved. TIMP-1, TIMP-2 and N-TIMP-3 inhibited collagen degradation, whereas the aggrecanase-selective N-TIMP-3 mutants were unable to inhibit collagen degradation (Figure 4B).

Inhibition of aggrecan degradation but not collagen degradation in human cartilage explants stimulated by IL-1 α and OSM by N-TIMP-3 mutants

Stimulation of cartilage from human OA patients by IL-1 α alone was insufficient to cause aggrecan degradation in 3 days (data not shown), but in combination with OSM there was a 2-3-fold increase in GAG release (Figure 5A), which represented roughly 3% of total GAG in the tissue. Cartilage from one knee joint was used to test the effects of the different doses of inhibitor. TIMP-1 unexpectedly showed dose-dependent inhibition of GAG release in this tissue, in contrast to porcine cartilage. TIMP-2, however, showed no inhibition of GAG release, making it unlikely that an MMP was involved. N-TIMP-3 was the most potent inhibitor of stimulated GAG release, with 0.1 μ M completely inhibiting it. The three reactive site mutants, N-TIMP-3(T2G), [-1A]N-TIMP-3 and [-2A]N-TIMP-3 inhibited GAG release dose-dependently, although they were not as potent as N-TIMP-3. Within the 3 days of culture, there was no stimulatory increase in collagen degradation even when the tissue was treated with IL-1 α and OSM (Figure 5B). The collagen degraded in 3 days of culture represented about 1.5% of the total collagen in the tissue. This suggests that the collagenolytic MMPs are already activated in OA cartilage. The Hypro release from the OA cartilage was dose-dependently inhibited by TIMP-1, TIMP-2 and N-TIMP-3, but the aggrecanase-selective N-TIMP-3 mutants had no effect on the collagen degradation (Figure 5B).

The experiments were repeated on cartilage obtained from the hips of 4 other patients using a single concentration of 0.1 μM of the respective TIMPs. The GAG and Hypro released upon stimulation without TIMP was normalised to a value of 100 in order to compare the different patients (Figure 5C and 5D). At a concentration of 0.1 μM , TIMP-1 showed a 50% inhibition of GAG release, whereas N-TIMP-3 inhibited GAG release to below that of control levels. TIMP-2 had no effect on GAG release. The three N-TIMP-3 mutants inhibited GAG release to control levels, but did not have any effect on collagen degradation, whereas the three wild-type TIMPs inhibited collagen degradation about 50% at a concentration of 0.1 μM .

Docking of N-TIMP-3 and modelled mutants to MMP-1, ADAM17, ADAMTS-4 and ADAMTS-5

In the absence of a crystal structure, we carried out molecular docking experiments to attempt to explain the inhibitory capabilities of the various mutants of TIMP-3. Docking of N-TIMP-3 with the ADAM17 component from the N-TIMP-3-ADAM17 complex (pdb 3CKI) was first performed to validate the docking method. All 200 docking runs returned structures that fitted in the active site cleft of the enzyme. The same N-TIMP-3 structure docked with a different ADAM17 structure (pdb 3G42) returned 52 active site fits. The positions of residues Lys315 and Met345, which are located on the active site cleft of ADAM17, differ between the two ADAM17 structures. In the ADAM17 (pdb 3G42) structure, they create a more restricted active site cleft, which may explain the fewer active site fits in this docking experiment. The same N-TIMP-3 structure docked into MMP-1, ADAMTS-4 and ADAMTS-5 returned 69, 2 and 27 active site fits, respectively. The modelled complex of N-TIMP-3 with MMP-1 model was similar to the experimentally determined structure of N-TIMP-1-MMP-1 complex [36]. The fit of N-TIMP-3 into the active site of ADAMTS-4 and ADAMTS-5, however, differed from those with MMP-1 and ADAM17: The catalytic zinc was still chelated by the N-terminal Cys residue of the TIMP, but the TIMP was tilted towards the prime side of the active site so that Phe34 of the TIMP that normally interacts with YV(351-352) of ADAM17 was not in contact with either ADAMTS, creating a water-accessible gap between the TIMP-3 and the ADAMTS components. The interaction of the bulky Arg84 residue of TIMP-3 with the loop GVST(323-326) in ADAMTS-4 or GHHS(372-375) of ADAMTS-5 probably prevented TIMP-3 binding in the same way as in previously characterised TIMP/metalloproteinase complexes. Mutation of all these residues on both molecules to alanine *in silico* generated complexes that are more similar to the canonical TIMP-metalloproteinase interaction, supporting the above possibility.

The model of [-1A]N-TIMP-3 was created by the addition of an alanine at the N-terminus of the N-TIMP-3 crystal structure, with the alanine being allowed to rotate in the docking experiments. When docked with MMP-1, 29 structures in the active site were obtained containing TIMP-3 docked in the MMP-1 active site. However, to accommodate the extra alanine, the TIMP was tilted in such a manner that Phe34 was not in contact with the enzyme, and the TIMP leant to one side of the active site cleft (Figure 6A). When [-1A]N-TIMP-3 was docked with ADAM17 (pdb 3CKI), 108 structures were returned containing TIMP-3 in the active site cleft. The TIMP molecule was tilted towards the prime side of the active site, such that Phe34 of the TIMP was no longer in contact with the enzyme, as in the models of N-TIMP-3 docked into ADAMTS-4 and ADAMTS-5 (Figure 6B). In this model however, the access of the catalytic zinc to water is blocked by the side chain of the alanine residue. Docking [-1A]N-TIMP-3 with ADAMTS-4 and ADAMTS-5 produced 4 and 28 models in which TIMP-3 is in contact with the active site, respectively. These were very similar to the corresponding complexes generated with N-TIMP-3, except that access of the catalytic zinc to water was blocked by the NH_2 -group of the alanine (Figure 6C and 6D). The

models of [-1A]N-TIMP-3 had additional interactions with the deeper active site clefts of ADAM17, ADAMTS-4 and ADAMTS-5 that were not present in the MMP-1 complexes. These were with Met345 in ADAM17, the loop GVST(323-326) in ADAMTS-4 and GHHS(372-375) in ADAMTS-5 (highlighted in Figure 6).

Discussion:

Using the newly developed *gst-IGD-flag* substrate, we demonstrate that the N-terminal mutants of N-TIMP-3 that have essentially lost their MMP-inhibitory activities, N-TIMP-3(T2G) and [-1A]N-TIMP-3, retain their ability to inhibit ADAMTS-4 and ADAMTS-5. Furthermore, the [-2A]N-TIMP-3 mutant also retained strong affinity with ADAMTS-5 but has a lower affinity for ADAMTS-4 and ADAM17.

The ability of these three mutants to dose-dependently inhibit aggrecan degradation in the IL-1 α -stimulated model of porcine cartilage degradation reinforce our previous observations that the enzymes involved in the degradation of aggrecan in this system are from the ADAMTS family and are not MMPs [20]. There was no inhibition of collagen degradation when the N-TIMP-3 mutants were tested in the long-term IL-1-stimulated porcine articular cartilage. As the TIMP-3 mutants used in our study lack the ability to inhibit the MMPs, they were not expected to exhibit a direct effect on MMP mediated collagen degradation, confirming that the selectivity of these N-TIMP-3 mutants was maintained *ex vivo*. Pratta *et al.* [41] reported that, in IL-1-induced bovine nasal cartilage degradation, the blocking of aggrecan degradation by an ADAMTS-selective chemical inhibitor resulted in inhibition of collagen degradation. The discrepancy between the two studies may be due to different levels of aggrecan protection: In this study a slower persistent loss of aggrecan was observed in the explant cultures when the mutants were used at a concentration of 0.1 μ M above the amount observed in the control and N-TIMP-3 treated explants, whereas aggrecan degradation was almost completely blocked in the study by Pratta *et al.* [41]. The amount of intact aggrecan left in the explants therefore may not have been sufficient to influence collagen degradation. Interestingly, mice deficient in MMP-13 are also protected in an animal model of osteoarthritis [42], indicating that inhibition of MMP-13 is also an effective cartilage protectant. The inhibition of both aggrecanases and collagenases, as shown with 0.1 μ M wild-type TIMP-3, may be an effective way to protect cartilage from degradation during the progression of OA.

The N-TIMP-3 reactive site mutants are able to inhibit IL-1 α with OSM stimulated GAG release from human cartilage explants in culture. This inhibition, however, had no effect on the amount of collagen degradation detected, indicating that by the stage where joint replacement surgery was required, protection of aggrecan degradation had little effect on the outcome of collagen degradation. Furthermore, hydroxyproline release was fully activated in human OA cartilage without IL-1 α and OSM treatment and it was dose-dependently inhibited by TIMP-1, TIMP-2 and TIMP-3, suggesting that the collagenolytic MMPs were fully active at this stage. Further studies using our ADAMTS-selective mutants in combination with TIMP-2 and TIMP-3, will enable us to dissect the relative contributions of the ADAMTSs and the MMPs during the progression of joint cartilage degradation and the benefits of targeting either protease at different stages of the disease.

Based on the crystal structure of ADAM17-N-TIMP-3 complex it was considered that there would not be sufficient space in the active site of the enzyme to accommodate TIMPs with one or two additional amino acids at the N-terminus. However, our enzyme inhibition studies presented here have shown that up to two alanine residues can be accommodated. The *in silico* docking experiments performed indicate that the [-1A]N-TIMP-3 mutant is most likely tilted so that Phe34 of the TIMP is no longer in contact with the enzyme, while still blocking the access of the catalytic zinc ion to the water molecule required for peptide

hydrolysis. Our *in vitro* inhibition data suggests that with MMP-1, this leads to a large loss of binding energy which renders the TIMP inactive but this is not the case in ADAM17, ADAMTS-4 and ADAMTS-5. Additional interactions with Met345 in ADAM17, GVST(322-325) in ADAMTS-4 and GHHS(372-375) in ADAMTS-5 may compensate for the loss of binding energy associated with the loss of interactions involving Phe34. The amino acids within the TIMP molecule that are likely to be in contact with these extended loops include the residues around Leu94 and Arg 84, which were identified by Lee *et al.* [43, 44] as being important in ADAM17 inhibition. In addition, comparison of the crystal structures of ADAMTS-4 in the presence and absence of a synthetic inhibitor, and those of ADAMTS-5, showed that the loops GVST(322-325) and GHHS(372-375), designated the S2' loop by Mosyak *et al.*, are able to adopt at least two different conformations [38]. Similarly, ADAM17 has been shown to have a more flexible active site than MMP-2 upon binding to a non-competitive inhibitor [45]. The flexible nature of these active sites may allow TIMP-3 with one or two extra alanines at the N-terminus to still form tight enzyme-inhibitor complexes. Further structural studies of [-1A]N-TIMP-3 and the [-2A]N-TIMP-3 in complex with these enzymes will be required to validate these docking predictions, whereas the Leu94 and Arg84 residues may provide a further avenue in TIMP engineering to obtain TIMPs capable of differentiating between the two aggrecanases.

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TABLE 1. $K_{i(\text{app})}$ (nM) of N-terminal reactive site mutants for MMP-1 Δ C, MMP-2, MMP-3 Δ C, ADAMTS4-2 and ADAMTS5-4.

Inhibitor	$K_{i(\text{app})}$ nM					
	MMP-1 Δ C	MMP-2	MMP-3 Δ C	ADAM17	ADAMTS4-2	ADAMTS5-4
N-TIMP-3	1.7 \pm 0.2	2.7 \pm 0.3	53.6 \pm 5.4	13.7 \pm 0.2	1.8 \pm 0.2	0.5 \pm 0.1
N-TIMP-3 (T2G)	>1000	>1000	>1000	35.6 \pm 1.9	18.3 \pm 0.1	1.5 \pm 0.1
[-1A] N-TIMP-3	800	970	>1000	33.9 \pm 2.8	22.2 \pm 0.1	1.7 \pm 0.1

TABLE 2. $K_{i(\text{app})}$ (nM) of N-terminal alanine extension mutants for MMP-1 Δ C, MMP-2, MMP-3 Δ C, ADAMTS4-2 and ADAMTS5-4.

Inhibitor	$K_{i(\text{app})}$, nM					
	MMP-1 Δ C	MMP-2	MMP-3 Δ C	ADAM17	ADAMTS 4-2	ADAMTS 5-4
[-1A] N-TIMP-3	800	970	>1000	33.9 \pm 2.8	22.2 \pm 0.1	1.7 \pm 0.1
[-2A] N-TIMP-3	>1000	790	>1000	65.3 \pm 5.2	35.9 \pm 8.4	1.8 \pm 0.9
[-3A] N-TIMP-3	>1000	>1000	>1000	239	173.7	87.6
[-5A] N-TIMP-3	>1000	>1000	>1000	843	385	593
[-8A] N-TIMP-3	>1000	>1000	>1000	>1000	>1000	>1000

TABLE 3. $K_{i(\text{app})}$ (nM) of position [-1] mutants for MMP-1 Δ C, MMP-2, MMP-3 Δ C, ADAMTS4-2 and ADAMTS5-4.

Inhibitor	$K_{i(\text{app})}$, nM					
	MMP-1 Δ C	MMP-2	MMP-3 Δ C	ADAM17	ADAMTS 4-2	ADAMTS 5-4
[-1A] N-TIMP-3	800	970	>1000	33.9 \pm 2.8	22.2 \pm 0.1	1.7 \pm 0.1
[-xG] N-TIMP-3	>1000	>1000	>1000	465	107	118
[-ME] N-TIMP-3	>1000	>1000	>1000	529	326	327
[-MM] N-TIMP-3	>1000	>1000	>1000	153	75.7	69.4
[-MK] N-TIMP-3	500	750	>1000	325	280	217

Legends

Figure 1. Characterisation of the *gst-IGD-flag* substrate.

(A) Schematic representation of the *gst-IGD-flag* substrate and its products upon aggrecanase activity. The observed molecular masses are bracketed. (B) Time course of cleavage of *gst-IGD-flag* by ADAMTS5-4. *Gst-IGD-flag* (17 μ M) was incubated with recombinant ADAMTS5-4 (500 pM) at 37 °C. At different time points (0-32 hours), reactions were stopped and analysed by SDS-PAGE (10% acrylamide). The 17 kDa product band (arrow) also stained positive for the aggrecanase generated neo-epitope ARGSV. (C) The product was analysed by densitometry to calculate the extent of cleavage, expressed as pixel volumes. (D,E) Cleavage of *gst-IGD-flag* by different amounts of enzyme. *Gst-IGD-flag* (16 μ M) substrate was incubated for 16 hours at 37 °C with increasing amounts of ADAMTS5-4 (0-2 nM).

Figure 2. The $K_{i(\text{app})}$ of N-TIMP-3 mutants for ADAMTS4-2 and ADAMTS5-4.

N-TIMP-3 or the mutants (0-1 μ M) were incubated with ADAMTS4-2 (1 nM, (A,C)) or ADAMTS5-4 (500 pM, (B,D)) for 1 hour at 37 °C, before the addition of *gst-IGD-flag* (17 μ M). After 16 hours at 37 °C, SDS-PAGE and densitometric analysis of the 17 kDa product band was used to determine the percentage of residual activity. Data were fitted to the tight binding equation to obtain the $K_{i(\text{app})}$.

Figure 3. Dose dependent inhibition of GAG release upon IL-1 α stimulation in porcine articular cartilage by N-TIMP-3 reactive site mutants.

Porcine articular cartilage explants were stimulated with IL-1 α (10 ng/ml) for 3 days with the various TIMPs at the concentrations indicated (0.01, 0.1, 1 μ M). GAG released in the medium was measured by DMMB. $n = 3$ for each treatment.

Figure 4. The effects of the N-TIMP-3 mutants on aggrecan and collagen degradation in porcine articular cartilage under long term stimulation with IL-1 α .

Porcine articular cartilage explants were stimulated with IL-1 α (10 ng/ml) with the various TIMPs at 0.1 μ M for 38 days. The amount of GAG and Hypro released into the medium was measured using the DMMB and DMBA assay respectively. Graphs of the cumulative amount of GAG (A) and Hypro (B) loss against time were plotted. $n = 3$ for each treatment.

Figure 5. Inhibition of GAG release but not hydroxyproline release of explanted human articular cartilage stimulated with IL-1 α .

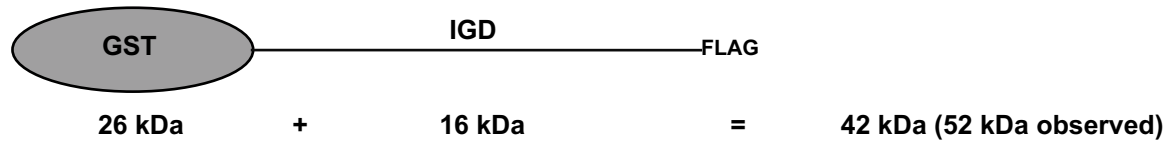
Cartilage from the knee of an OA patient was stimulated with IL-1 α (10 ng/ml) + OSM (50 ng/ml) and the various TIMPs (0.01, 0.1 and 1 μ M concentrations) for 3 days. GAG (A) and hydroxyproline (B) released into the medium were taken as measures of cartilage degradation. $n = 3$ for each treatment. Cartilage samples from the hips of 5 OA patients were treated with IL-1 α (10 ng/ml) and the various TIMPs at a concentration of 100 nM. Data from the different patients were normalised by taking the amount of (C) GAG released upon stimulation as 100 and (D) hydroxyproline released without stimulation as 100, shown as mean \pm standard deviation, $n = 3$ for each patient, * = $p < 0.03$

Figure 6. *In silico* models of N-TIMP-3 and [-1A]N-TIMP-3 with ADAM17, MMP-1, ADAMTS-4 and ADAMTS-5.

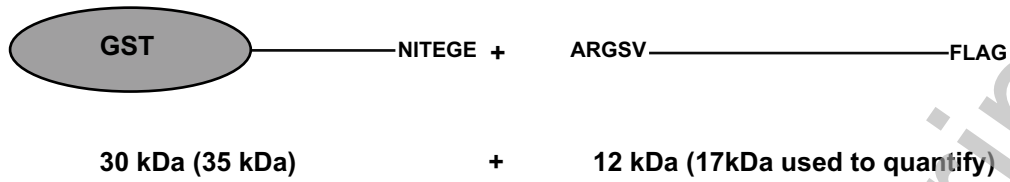
Computer simulated lowest free energy ranked models of complexes of N-TIMP-3 (salmon ribbon) or [-1A]N-TIMP-3 (blue ribbon) with MMP-1 (A), ADAM17 (B), ADAMTS-4 (C) or ADAMTS-5 (D) are shown. The catalytic zinc is shown in magenta, water accessible

enzyme surfaces are grey and regions which interact only with N-TIMP-3, [-1A]N-TIMP-3 or with both are in salmon, blue and green respectively. The Phe34 and N-terminal extra Ala are shown with sticks. In the [-1A]N-TIMP-3 complexes with MMP-1 and ADAM17, the TIMP molecule is tilted and the interaction of Phe34 of the inhibitor with the enzymes are lost. Both N-TIMP-3 and [-1A]N-TIMP-3 are bound to ADAMTS-4 and ADAMTS-5 in a similar tilted manner. Residues which make the deeper active site clefts of ADAM17 (Met345), ADAMTS-4 (GVST(322-325)) and ADAMTS-5 (GHHS(372-375)) that interact with the TIMP and are not present in MMP-1 are highlighted in red.

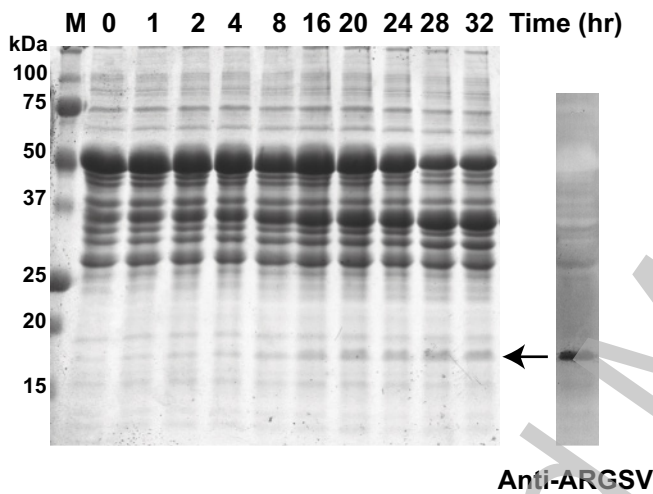
(A)



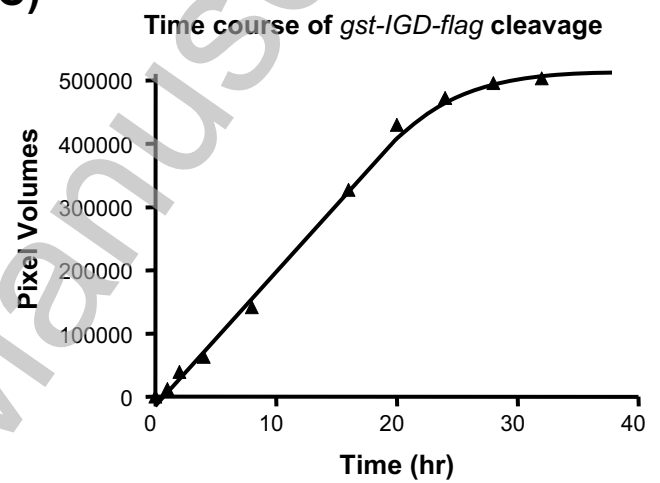
Aggrecanase-cleavage



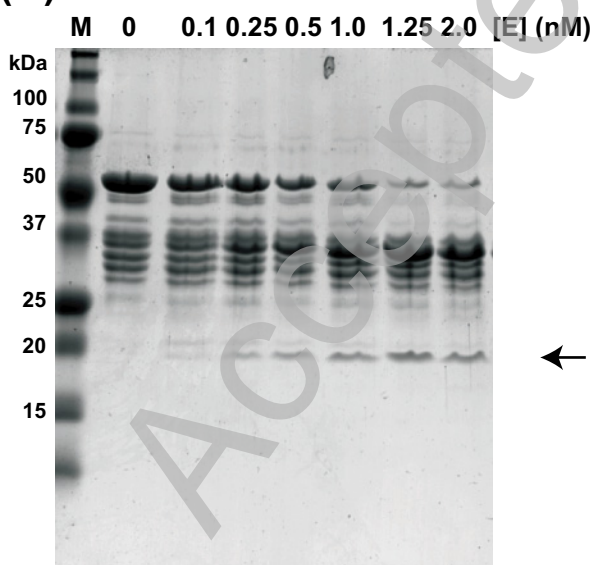
(B)



(C)



(D)



(E)

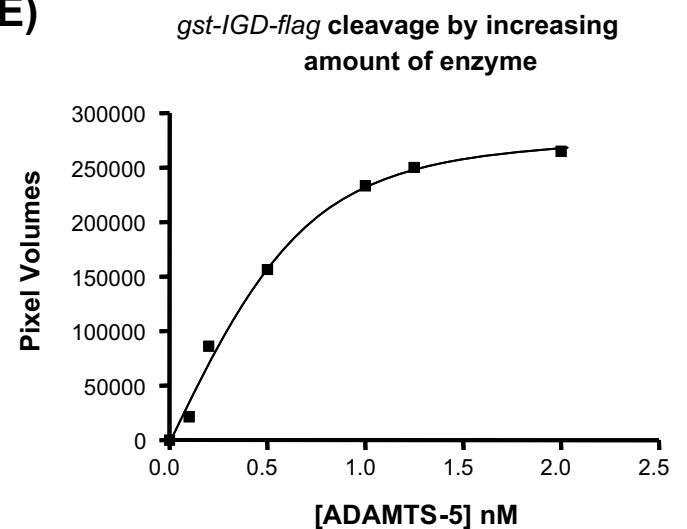
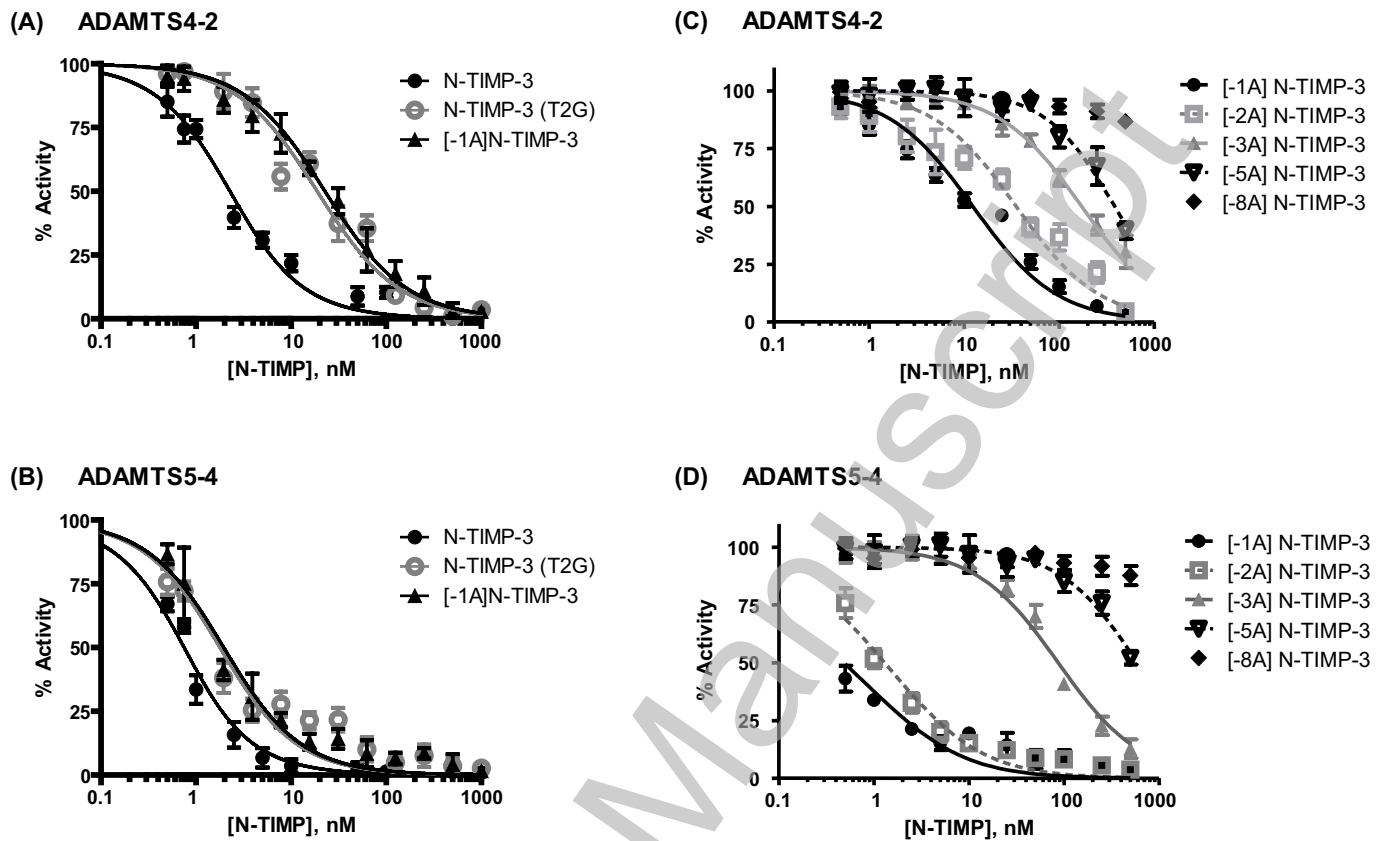


Figure 1 Lim *et al.*

Figure 2 Lim *et al.*

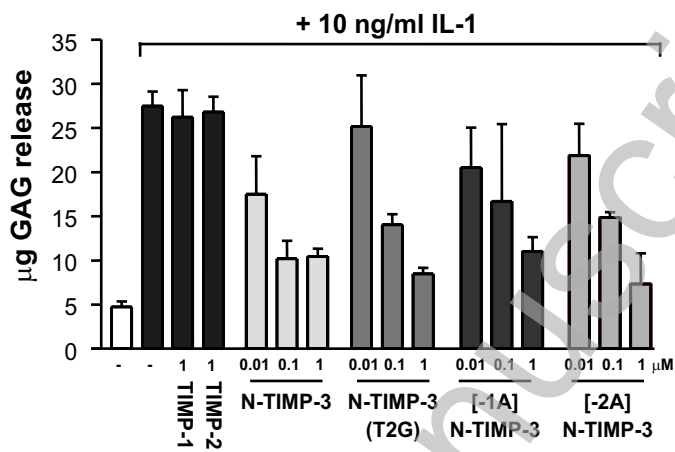


Figure 3 Lim *et al.*

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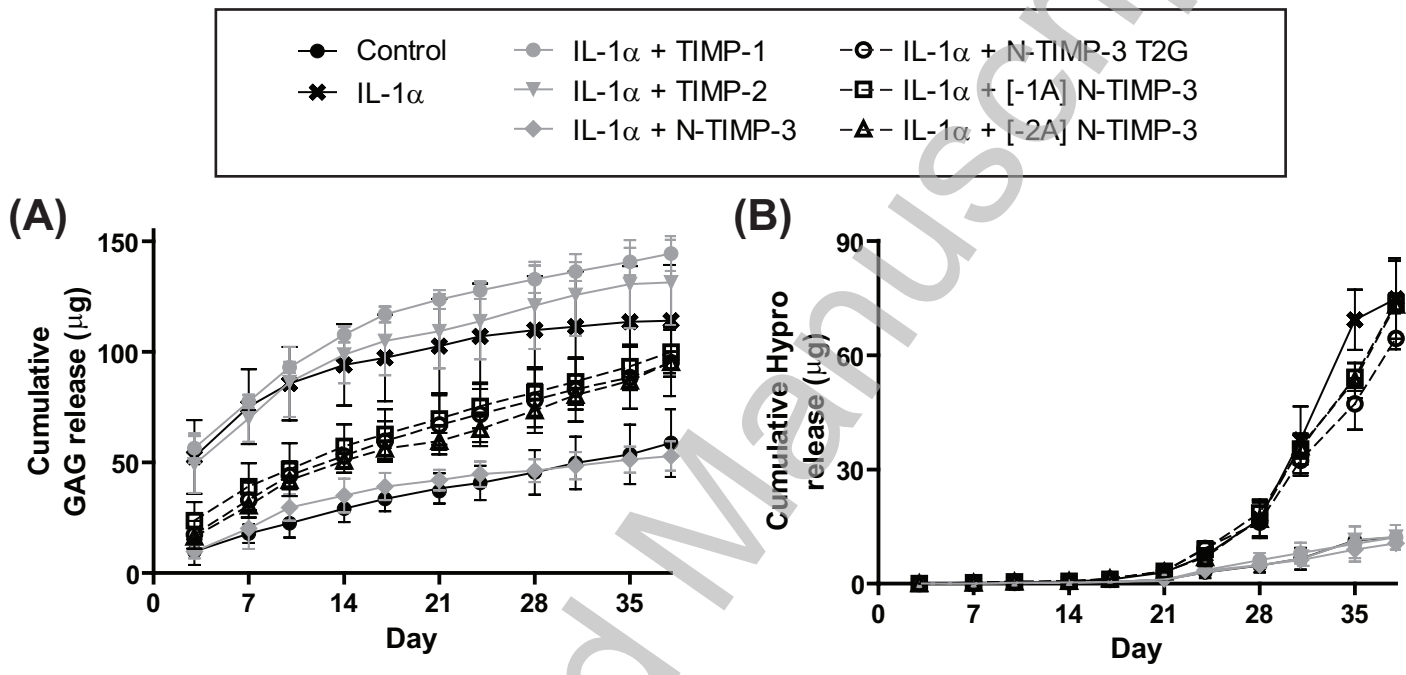


Figure 4 Lim *et al.*

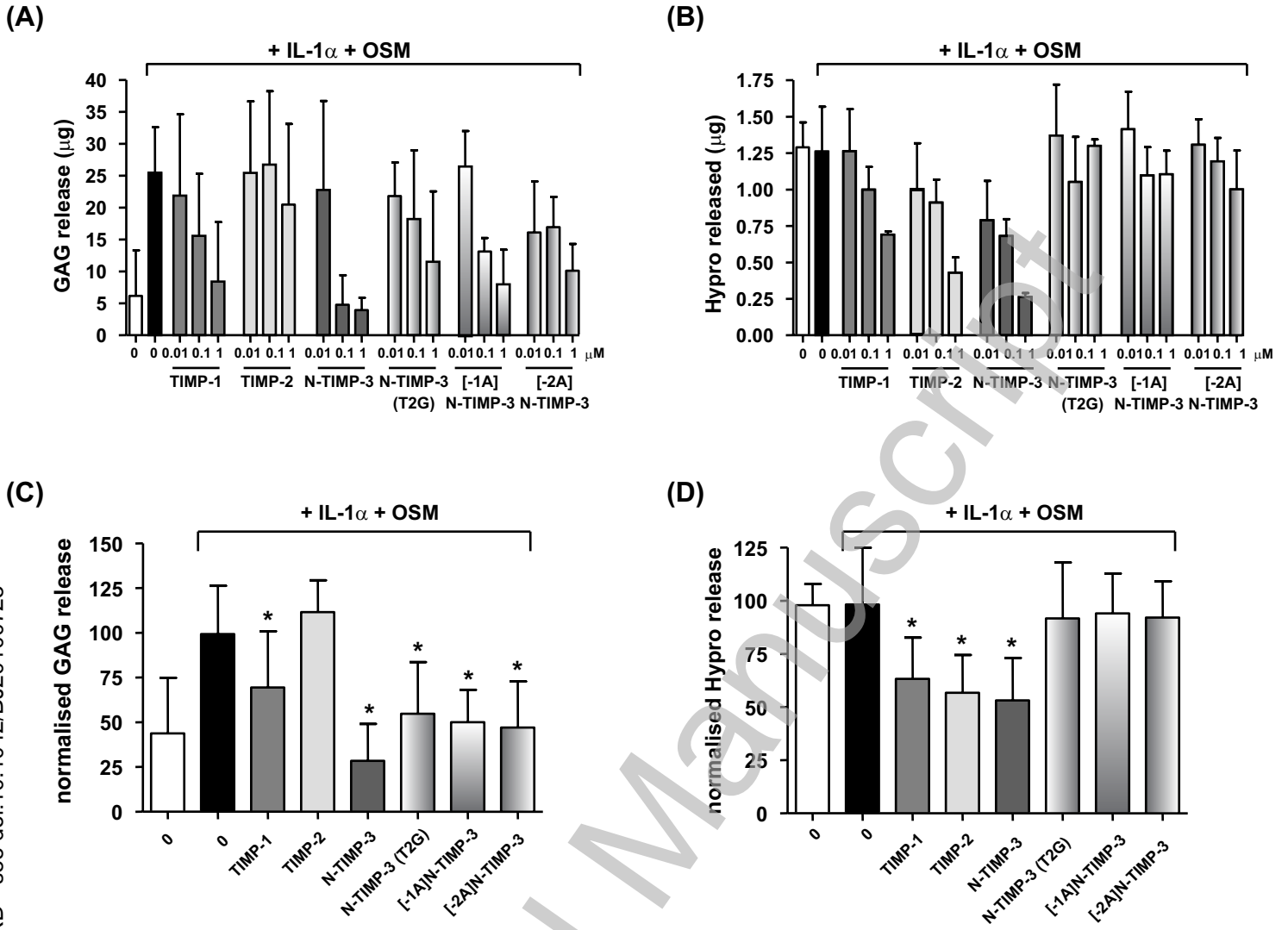


Figure 5 Lim *et al.*

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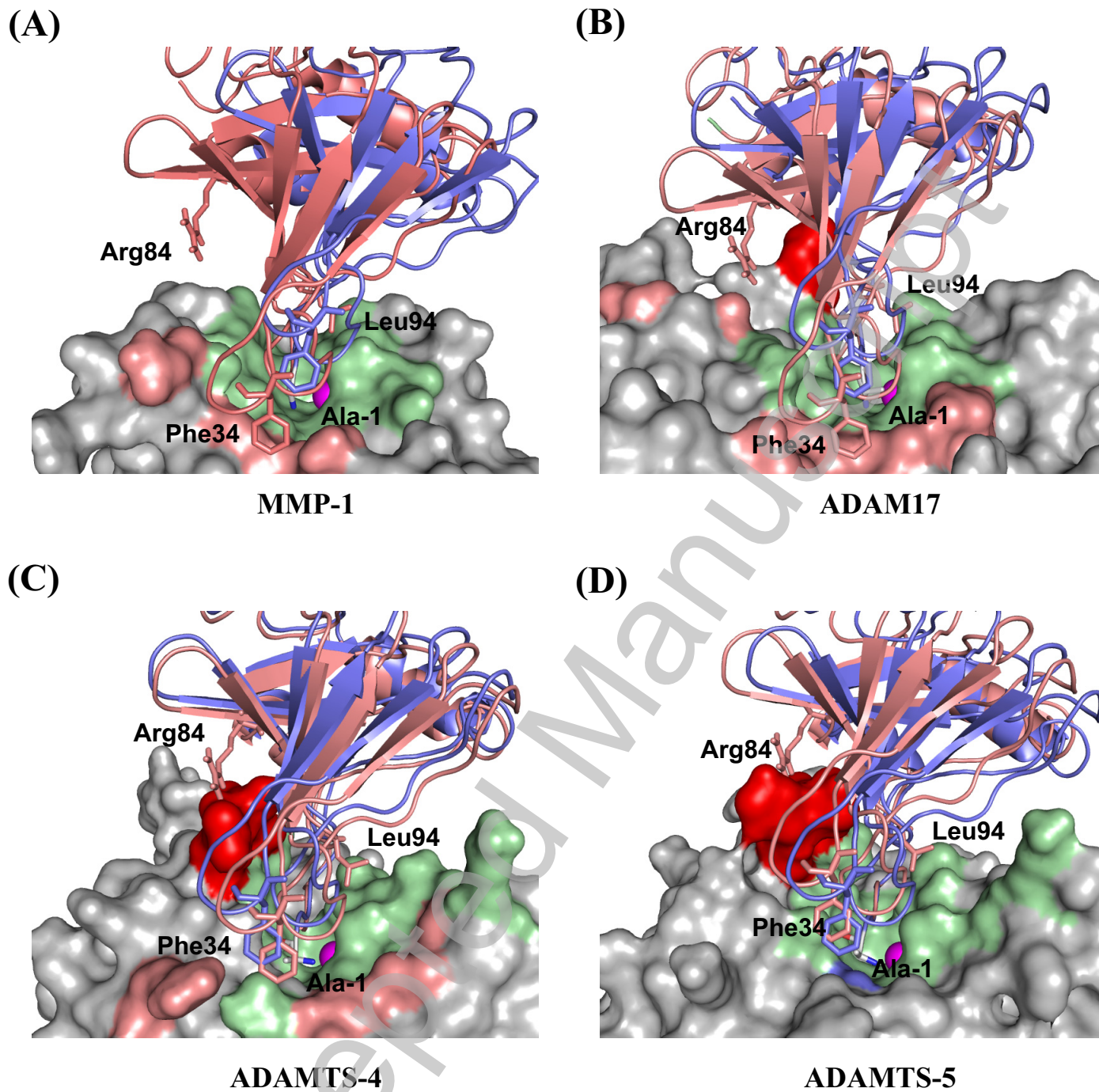


Figure 6 Lim *et al.*

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