ADAMTS13 and 15 are not regulated by the full length and N-terminal domain forms of TIMP-1, -2, -3 and -4

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Received June 29, 2015; Accepted July 15, 2015

DOI: 10.3892/br.2015.535

Abstract. A disintegrin and metalloproteinase with thombospondin motifs (ADAMTS) 13 and 15 are secreted zinc proteinases involved in the turnover of von Willebrand factor and cancer suppression. In the present study, ADAMTS13 and 15 were subjected to inhibition studies with the full-length and N-terminal domain forms of tissue inhibitor of metalloproteinases (TIMPs)-1 to -4. TIMPs have no ability to inhibit the ADAMTS proteinases in the full-length or N-terminal domain form. While ADAMTS13 is also not sensitive to the hydroxamate inhibitors, batimastat and ilomastat, ADAMTS15 can be effectively inhibited by batimastat (K_i^{app} 299 nM). In conclusion, the present results indicate that TIMPs are not the regulators of these two ADAMTS proteinases.

Introduction

A disintegrin and metalloproteinase with thombospondin motifs (ADAMTSs) are secreted members of the zinc-dependent metalloproteinases that contain one or more thrombospondin type 1 repeats (TSP1) as the ancillary domains. There are ≥26 ADAMTS proteinases identified thus far; however, not all of them are known to possess enzymatic activity. The ADAMTS proteinases are important regulators of cellular events, as the enzymes have been shown to exhibit a vast array of activities, including cleavage of pro-collagen and von Willebrand factor (VWF), tumor suppression and proteolysis activities associated with arthritis, morphogenesis, angiogenesis and even ovulation [as reviewed previously (1,2)].

Also known as the VWF-cleaving protease, ADAMTS13 is noted for its ability in cleaving and reducing the size of the ultra-large (UL) form of the VWF. Reduction in ADAMTS13 activity from either hereditary or acquired deficiency causes accumulation of UL-VWF multimers, platelet aggregation and arterial thrombosis that leads to fatal thrombotic thrombocytopenic purpura [as reviewed previously (1,3)]. By contrast, ADAMTS15 is a potential tumor suppressor. Only a limited number of in-depth investigations have been carried out on the enzyme; however, expression and profiling studies have shown that the *ADAMTS15* gene is genetically inactivated in colon cancer and breast carcinoma, although the exact role of the enzyme remains to be delineated (4,5).

Structure-wise, ADAMTS13 and 15 share a common domain organization with the other members of the ADAMTS proteinases. At the N-terminal of their polypeptides is a pro-peptide and a metalloproteinase domain in which the catalytic activity of the proteinases resides. Succeeding the metalloproteinase domain is a disintegrin-like domain followed by the first TSP1, a cysteine-rich domain, a spacer domain and 7 more TSP1 repeats and 2 CUB domains. As with the members of the matrix metalloproteinase (MMP) and ADAM proteinases, the metalloproteinase domain of ADAMTS13 bears the typical hallmarks of the reprolysin or adamalysin proteinases, namely a zinc-binding motif 'HExxHxxGxxH' that is essential for substrate turnover. Notably, human ADAMTS15 has a slightly modified zinc-binding motif, 'HExxNxxGxxH,' instead of the usual conserved sequence (2,6,7).

Tissue inhibitor of metalloproteinases (TIMPs) are the endogenous inhibitors of the MMPs, ADAMs and certain members of the ADAMTS family, including ADAMTS1, 4 and 5 (8-11). TIMPs are small proteins of 22-28 kDa in molecular mass. There are 4 human TIMPs and each has its own distinct profile of metalloproteinase inhibition. While the activity of TIMPs against the MMPs and ADAMs has been studied rather extensively, the inhibitory profiles of TIMPs against the ADAMTS proteinases remain largely unexplored.

To expand the current understanding of the role of TIMPs in ADAMTS13 and 15 regulation, the enzymes were subjected to direct inhibition with the full-length and the N-terminal domain forms of TIMP (N-TIMP)-1, -2, -3 and -4. The responses of the ADAMTS proteinases to broad spectrum hydroxamate inhibitors, batimastat (also known as BB-94)

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Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thombospondin type 1 motifs; E:I, enzyme:inhibitor; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; N-TIMP, N-terminal domain of TIMP

Key words: a disintegrin and metalloproteinase, a disintegrin and metalloproteinase with thombospondin type 1 motifs, matrix metalloproteinase, tissue inhibitors of metalloproteinases, enzyme inhibition

and ilomastat (GM6001, galardin), were also examined. The data show that ADAMTS13 and 15 are not inhibited by the full-length or the N-TIMP-1 to -4. Notably, while ADAMTS13 shows little or no inhibition by batimastat and ilomastat, ADAMTS15 can be effectively inhibited by batimastat with a K_i^{app} value of <300 nM.

Materials and methods

Materials. Human ADAMTS5, 13 and 15, full-length TIMP-3 and the recombinant human aggrecan substrate G1-IGD-G2 were all products of R&D Systems (Minneapolis, MN, USA). Full-length TIMP-1, -2 and -4, as well as N-TIMP-1 to -4 were produced in house either from Sf9 insect cells or *Escherichia coli* inclusion bodies, as described previously (12-15). The fluorescence-quenching peptide for the ADAMTS13 assay, FRETS-VWF73 [DRE-Dap(Nma)-APN LVY MVTG-Dpa- PAS DEI KRL PGD IQV VPI GVG PNA NVQ ELE RIG WPN API LIQ DFE TLP REA PDL VLQR], was purchased from AnaSpec, Inc. (Fremont, CA, USA). The hydroxamate inhibitors batimastat (BB-94) and ilomastat (GM6001, galardin) were purchased from Selleck Chemicals Inc. (Houston, TX, USA). All the chemicals and reagents were products of Thermo Scientific (Rockford, IL, USA) unless stated otherwise.

Inhibition studies of ADAMTS5 and 15 with the full-length form and N-TIMPs using recombinant human aggrecan G1-IGD-G2 domains. The procedure for the assay was modified from the one designed for ADAMTS4 by Hashimoto *et al* (16). In brief, ADAMTS5 and 15 (≤20 nM) were pre-incubated with TIMPs (various concentrations, ≤1 μM for certain N-TIMPs) or hydroxamate inhibitors in FAB buffer [10 mmol/l $CaCl_2$, 50 mmol/l Tris-HCl (pH 7.5), 0.05% Brij-35, 1% dimethyl sulfoxide and 0.02% NaN_3] in a total volume of 20 μl for 1 h at room temperature before 2 μg of recombinant human aggrecan domains G1-IGD-G2 was added to initiate digestion. Digestion occurred for 4 h at 37˚C before the samples were analyzed on an 8% reducing SDS-PAGE followed by staining in 0.2% Coomassie Brilliant Blue. The K_i^{app} value of the inhibitors was calculated based on densitometric analysis of the 130 kDa undigested G1-IGD-G2 bands using ImageJ software developed at the National Institutes of Health (Bethesda, MD, USA).

Inhibition studies of ADAMTS13 with the full-length form and N-TIMPs using VWF73 fluorescence-quenching peptide. The assay for ADAMTS13 was carried out in 2 ml cuvettes in Na acetate buffer [25 mmol/l Na acetate, 50 mmol/l NaCl, 20 mmol/l $CaCl_2$ and 0.05% Brij (pH 6.0)] in a manner similar to the protocol developed for evaluating the K_i^{app} of ADAM17 and MMPs (12,17). To measure the inhibitory activity of TIMPs on ADAMTS13, ≤2 nM of ADAMTS was pre-incubated with increasing concentrations of TIMPs (≤300 nM) in a total volume of 2 ml for 1 h at room temperature. Reactions were initiated by adding 2.5 μM of VWF73 fluorescence-quenching peptide and the activity of the enzyme was followed for ≥45 min using a LS55 spectrofluorometer (Perkin-Elmer, Waltham, MA, USA) pre-warmed to 30˚C.

The K_i^{app} values of TIMPs were calculated by plotting the steady-state rates against TIMP concentrations with GraphPad Prism software using the Morrison equation: $V_s = (V_0/2E_t) \times [(E_t - I_t - K_i^{app}) + \{(K_i^{app} + I_t - E_t)^2 + 4E_tKi^{app}]^{1/2}\}$, where V_0 denotes the rate of reaction in the absence of inhibitor, E_t is the total enzyme concentration and I_t is the total inhibitor concentration.

Results

ADAMTS13 is not inhibited by the full-length form or N-TIMP-1, -2, -3 or -4. Fig. 1A summarizes the inhibition patterns of full-length and N-TIMP-1, -2, -3 and -4 against ADAMTS13. As shown, only full-length TIMP-2, full-length TIMP-3 and N-TIMP-4 exhibited a notable degree of inhibitory activity against the enzyme. The effects of other TIMPs were either minimal or negligible. Overall, the K_i^{app} values of the majority of the TIMPs were >200 nM. Fig. 1A also shows that the K_i^{app} value of the most potent TIMP for ADAMTS13, namely full-length TIMP-2, was 186 ± 8 nM. The value is well above the normal range of affinity for a tight-binding inhibitor, which is typically in the low nM to pM range, as in the cases of TIMP-3 and ADAMTS4 and 5 (9,16).

To illustrate the contrasting effect of the TIMPs, in particular TIMP-3, on other ADAMTS proteinases, another set of inhibition studies was performed using ADAMTS5 (also known as aggrecanase-2) as the subject. As shown in Fig. 1B, full-length TIMP-3 was a potent inhibitor against the proteinase. At 25 nM [enzyme:inhibitor (E:I) ratio, 1:1], full-length TIMP-3 almost completely inhibited the ability of ADAMTS5 to digest the aggrecan substrate G1-IGD-G2 (K_i^{app} <2 nM), similar to the results of a previous study (9).

ADAMTS15 is not inhibited by the full-length form or N-TIMP-1, -2, -3 or -4. Due to the lack of a suitable fluorescence-quenching peptide substrate, the catalytic activity of ADAMTS15 was evaluated by its ability to cleave the 130 kDa recombinant human aggrecan G1-IGD-G2 domains into 70- and 55-kDa fragments. Fig. 2 shows that despite pre-incubation with an excessive dose of the full-length or N-TIMPs (E:I, ~1:50), none of the TIMPs exhibited any sign of inhibition against the enzyme. Switching the incubation condition to a neutral one (pH 7.4) with FAB buffer yielded the same outcome. Therefore, the enzymatic activity of ADAMTS15 was not inhibited by any of the TIMPs.

Inhibition of ADAMTS13 and 15 by broad spectrum MMP inhibitors batimastat and ilomastat. To understand whether ADAMTS13 and 15 were sensitive to hydroxamate-based small molecule inhibitors, the proteinases were subjected to two doses (0.3 and 3 μM) of batimastat and ilomastat. The results are summarized in Fig. 3A. As shown, batimastat was able to partially inhibit ADAMTS15 at 300 nM. As the concentration of batimastat was increased to 3 μM, the effect of the inhibitor became even more pronounced. By contrast, ilomastat has no noticeable activity even at the high dose of 3 μM. To quantify the affinity of batimastat for ADAMTS15, further investigation was carried out to examine the concentration effect. Fig. 3B illustrates how the K_i^{app} value of batimastat was derived by densitometric analysis of the undigested G1-IGD-G2 bands.

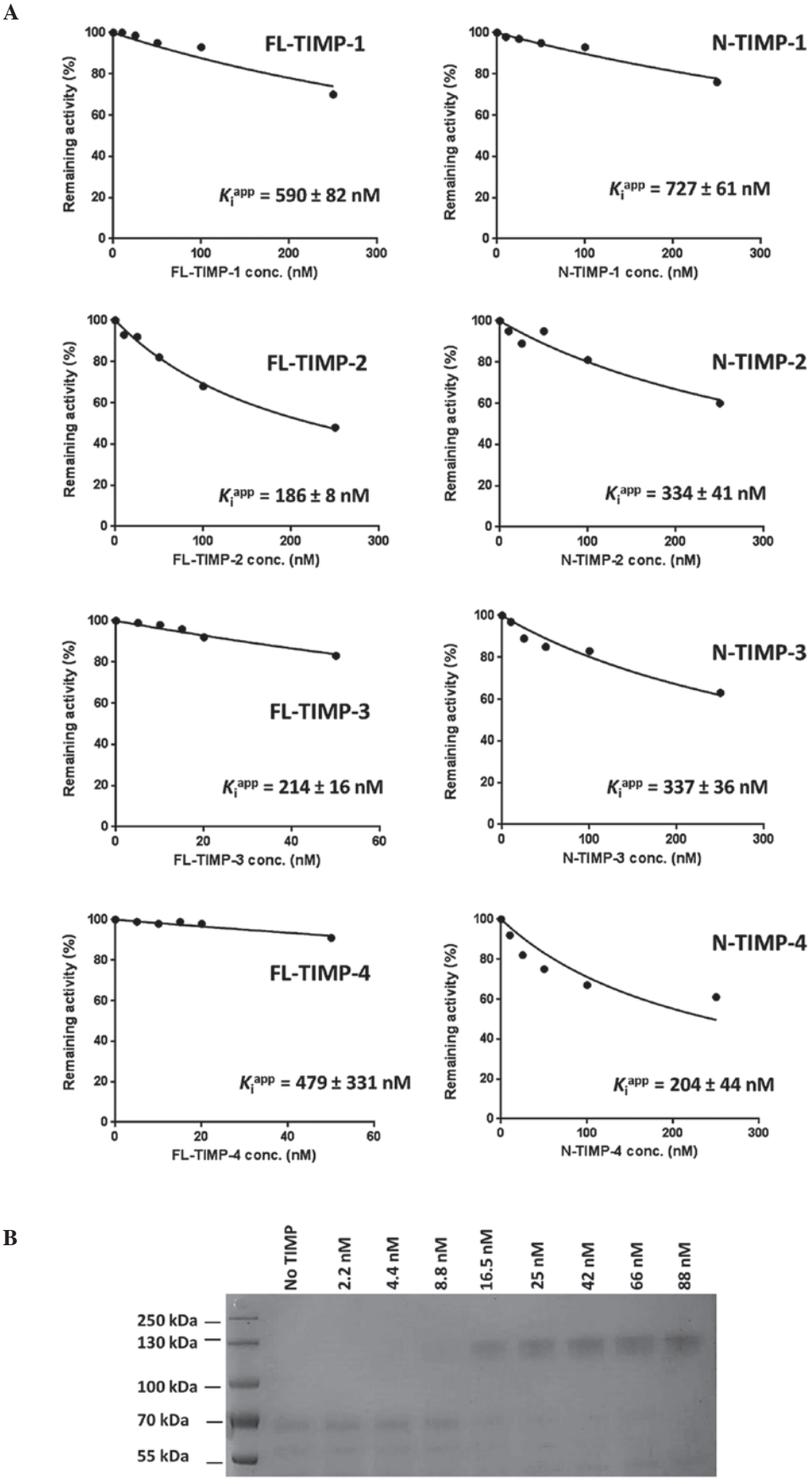

Figure 1. Inhibition profiles of a disintegrin and metalloproteinase with thombospondin type 1 motifs (ADAMTS) 13 with the full-length (FL) and N-terminal domain forms of tissue inhibitors of metalloproteinase (N-TIMP)-1, -2, -3 and -4. (A) ADAMTS13 (1.4 nM) was pre-incubated with increasing concentrations of full-length and N-TIMPs for 1 h at room temperature prior to the addition of 2.5 μM fluorescent peptide substrate von Willebrand factor 73. While FL-TIMP-2, TIMP-3 and N-TIMP-4 demonstrated a small but noticeable inhibitory effect, other TIMPs showed minimal or no activity. (B) To demonstrate the potency of FL-TIMP-3 on other ADAMTS proteinases, an inhibition study was performed with ADAMTS5 (also known as aggrecanase-2). The ability of FL-TIMP-3 to inhibit ADAMTS5 is clearly visible as the concentration of the TIMP increases, similar to a previous study (9).

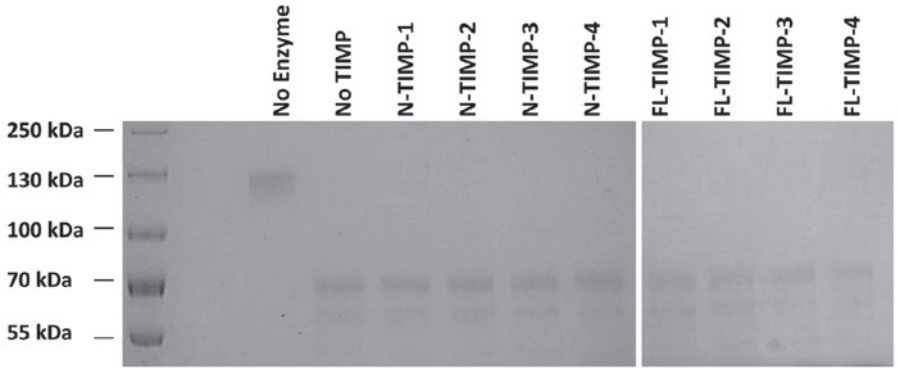

Figure 2. A disintegrin and metalloproteinase with thombospondin type 1 motifs (ADAMTS) 15 is not sensitive to the full-length (FL) or N-terminal domain forms of tissue inhibitors of metalloproteinase (N-TIMP)-1, -2, -3 and -4. ADAMTS15 (20 nM) was pre-incubated with 1 μM of FL- or N-TIMPs (enzyme:inhibitor ratio, 1:50) for 1 h at room temperature before 2 μg of recombinant human aggrecan G1-IGD-G2 was added. Incubation was continued for 4 h at 37˚C before the digestion products were analyzed on an 8% SDS-PAGE followed by Coomassie Brilliant Blue staining. TIMP-1, -2, -3 and -4 have no ability to inhibit the enzymatic activity of ADAMTS15 in the FL or N-terminal domain form.

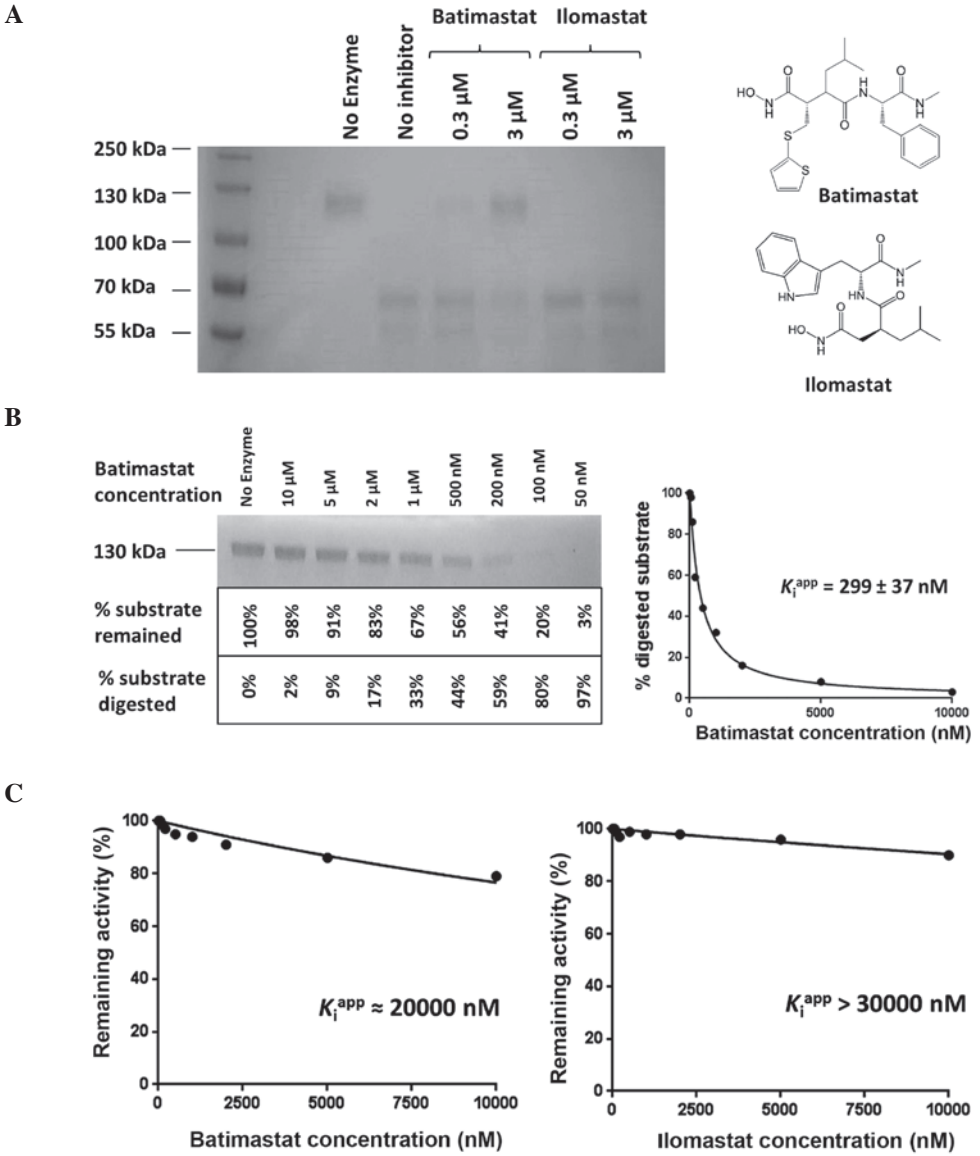

Figure 3. Effect of the broad spectrum hydroxamate inhibitors batimastat and ilomastat on a disintegrin and metalloproteinase with thombospondin type 1 motifs (ADAMTS) 13 and ADAMTS15. (A) While batimastat is a good inhibitor against ADAMTS15, ilomastat has no ability to inhibit the enzyme. ADAMTS15 (20 nM) was pre-incubated with two doses (300 nM or 3 μM) of batimastat and ilomastat prior to the addition of aggrecan substrate G1-IGD-G2. The inhibitory effect of batimastat can be clearly discerned from the undigested 130 kDa G1-IGD-G2 bands. (B) Calculation of the K_i^{app} value of batimastat by densitometric analysis of the undigested G1-IGD-G2 bands on a 12% SDS-PAGE. (C) The impacts of batimastat and ilomastat on ADAMTS13 activity in fluorogenic assays. In contrast to ADAMTS15, batimastat and ilomastat have no effect on ADAMTS13, in spite of the excessive doses.

Under the assay conditions described in the present study, the K_i^{app} of batimastat was determined to be 299±37 nM, which is the indication of a good inhibitor for a small hydroxamate molecule. Despite the potency of batimastat on ADAMTS15, neither batimastat nor ilomastat showed any signs of activity on ADAMTS13. The inhibition curves of batimastat and ilomastat for ADAMTS13 are shown in Fig. 3C.

Discussion

The present findings are significant for two reasons. First and foremost, the data suggest that the topology of the catalytic clefts of ADAMTS13 and 15 is likely to be markedly different from those of the MMPs and ADAMs. Among the ADAMTS proteinases isolated to date, ADAMTS1, 4 (aggrecanase-1) and 5 (aggrecanase-2) are known to be responsive to the inhibitory activity of the TIMPs, in particular TIMP-3 (8,9). The fact that TIMP-3 is able to inhibit ADAMTS1, 4 and 5, as well as the majority of the MMPs and ADAMs, hinted a structural similarity among the active sites of these enzymes. Subsequent delineation of the metalloproteinase domains of ADAMTS1, 4 and 5 by X-ray crystallography revealed an overall structural similarity to those of the MMP and ADAM proteinases (PDB 3Q2G; 2RJP; and 2RJQ) (18,19). The inability of the TIMPs to form a tight binary complex with ADAMTS13 and 15 indicates that the 2 ADAMTS proteinases may have a significantly different active site configuration from those of the MMPs, ADAMs, and ADAMTS1, 4 and 5.

Furthermore, the present findings also confirm the notion that TIMPs are not part of the regulatory mechanisms that modulate ADAMTS13 and 15 activities. Thus far, ADAMTS13 has been shown to be susceptible to proteolysis by thrombin and furin, as well as inhibitory autoantibodies (20-24). These findings, taken together with the results of the present study, suggest that TIMPs have no role to play in its regulation. In comparison to ADAMTS13, much less is known with regards to how ADAMTS15 is regulated at present, as there have been extremely few biochemical studies on the enzyme. As shown in the present study, none of the four native TIMPs had the true ability to inhibit the proteinase. Therefore, future studies should uncover the physiological regulator of ADAMTS15.

Another aspect that warrants further investigation is the discrepancy in potency between batimastat and ilomastat in ADAMTS15 inhibition. The two are small molecules that inhibit a wide range of MMPs and ADAM proteinases; however, only batimastat is able to bind to ADAMTS15. Without a crystal structure of the ADAMTS15 metalloproteinase domain, it is difficult to speculate the reason for the discrepancy. The inhibitor could, however, be of use in protein stabilization if the metalloproteinase domain of ADAMTS15 is crystalized in the future. In conclusion, the present data confirm that ADAMTS13 and 15 are not the subjects of a typical metalloproteinase regulatory mechanism in which TIMPs have a central and indispensable role.

Acknowledgements

The authors thank the National Natural Science Foundation of China (grant no. 31270850) and the Research Development Fund, XJTLU, for their generous funding of the present study.

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