Human meprin α and β homo-oligomers: cleavage of basement membrane proteins and sensitivity to metalloprotease inhibitors

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Meprin is a zinc endopeptidase of the astacin family, which is expressed as a membrane-bound or secreted protein in mammalian epithelial cells, in intestinal leucocytes and in certain cancer cells. There are two types of meprin subunits, α and β , which form disulphide-bonded homo- and hetero-oligomers. Here we report on the cleavage of matrix proteins by hmeprin (human meprin) α and β homo-oligomers, and on the interactions of these enzymes with inhibitors. Despite their completely different cleavage specificities, both hmeprin α and β are able to hydrolyse basement membrane components such as collagen IV, nidogen-1 and fibronectin. However, they are inactive against intact collagen I. Hence the matrix-cleaving activity of hmeprin resembles that of gelatinases rather than collagenases. Hmeprin is inhibited by hydroxamic acid derivatives such as batimastat, galardin and Pro-Leu-Gly-hydroxamate, by TAPI-0 (tumour necrosis factor α protease inhibitor-0) and TAPI-2, and by thiol-based compounds such as captopril. Therapeutic targets for these inhibitors are

MMPs (matrix metalloproteases), TACE (tumour necrosis factor α -converting enzyme) and angiotensin-converting enzyme respectively. The most effective inhibitor of hmeprin α in the present study was the naturally occurring hydroxamate actinonin ($K_i = 20$ nM). The marked variance in the cleavage specificities of hmeprin α and β is reflected by their interaction with the TACE inhibitor Ro 32-7315, whose affinity for the β subunit ($IC_{50} = 1.6$ mM) is weaker by three orders of magnitude than that for the α subunit ($K_i = 1.6$ μ M). MMP inhibitors such as the pyrimidine-2,4,6-trione derivative Ro 28-2653 that are more specific for gelatinases do not bind to hmeprin, presumably due to the subtle differences in the mode of zinc binding and active-site structure between the astacins and the MMPs.

Key words: astacin, extracellular matrix, human meprin, matrix metalloprotease, protease inhibitor.

INTRODUCTION

Meprin is a mammalian zinc-dependent endopeptidase of the astacin family/metzincin superfamily of metalloproteases [1–3]. The human enzyme (hmeprin; *N*-benzoyl-L-tyrosyl-*p*-amino-benzoic acid hydrolase; EC 3.4.24.18) was originally identified in the brush border membranes of small intestinal epithelial cells [4,5]. Mouse and rat meprin were detected in the brush borders of kidney proximal tubules [6,7]. There are two related meprin subunits, α and β , which assemble to form homo- or hetero-oligomeric complexes [8,9]. Both subunits are multidomain proteins composed of an N-terminal propeptide, an astacin-like protease domain with the active-site motif HEXXHXXGFXHE [1], as well as a MAM (meprin A5 protein tyrosine phosphatase μ) domain and a MATH [meprin and TRAF (tumour necrosis factor receptor associated factor) homology] domain, both of which are thought to mediate protein–protein interactions. An intervening domain, also called ‘afterMATH’ domain, precedes an epidermal growth factor-like module and the C-terminal transmembrane and cytosolic regions; an additional (I)-domain insert in the α subunit allows for proteolytic cleavage in the secretory pathway, resulting in the loss of the membrane anchor and subsequent secretion of the enzyme [10,11], while the β subunit remains mostly membrane-bound.

Generally, meprin is secreted from or bound to the apical side of kidney and intestinal epithelial cells, and is also expressed at low levels in leucocytes of the intestinal lamina propria mucosae [1,5,6,12]. During development, a broader expression has been reported, including craniofacial regions, salivary glands, bladder and ureter [13,14]. Meprin has also been detected in certain epithelial carcinomas, such as colorectal cancer [15,16], where soluble hmeprin α is secreted not only apically but also basolaterally, thereby increasing the proteolytic potential of tumour cells for the destruction of the basement membrane [16]. Abnormal meprin secretion has also been observed in experimentally induced acute renal failure in rats [17,18]. There are various *in vitro* observations on the cleavage of basement membrane proteins, protein kinases, growth factors, cytokines and other bioactive peptides by meprin [19–23]. These findings suggest a contribution to epithelial differentiation, matrix remodelling and cell migration, and also to inflammatory processes, tumour growth and metastasis.

In the intestine, pro-hmeprin α and β are activated by trypsin [24]. Outside the gut, pro-hmeprin α subunits [25], but not pro-hmeprin β [9] can be activated by plasmin.

The subunits differ in their mode of pro-enzyme activation, and they also exhibit marked differences in peptide bond cleavage specificity [23]. In this paper we analyse the ability of soluble,

Abbreviations used: ACE, angiotensin-converting enzyme; hmeprin, human meprin; MMP, matrix metalloprotease; MMP1, matrix metalloprotease inhibitor; MT1-MMP, membrane-type 1 matrix metalloprotease; NNGH, *N*-isobutyl-*N*-(4-methoxyphenylsulphonyl)glycyl hydroxamic acid; TACE, tumour necrosis factor α -converting enzyme; TAPI, tumour necrosis factor α protease inhibitor.

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heterologously expressed hmeprin homo-oligomers to cleave constituents of the basal lamina, and identify several subunit-specific cleavage sites. This supports the concept that hmeprin might expand the capacity of migrating cells to cross the basement membrane.

MMPs [MMP (matrix metalloprotease) inhibitors] have been studied extensively during the past two decades, due to their potential in the therapy of cancer and rheumatoid arthritis. However, several recent clinical trials have resulted in a scaling down of expectations concerning successful treatment of cancer with MMPs [26]. Inhibitor development has relied in part on a rational design approach using the known three-dimensional structures of MMPs or models of related enzymes [27]. Although hmeprin has been implicated in colorectal cancer [16], no detailed studies have been carried out so far focusing on the effects of MMPs on hmeprin. A first hint has been the observation that the hydroxamic acid MMPi Pro-Leu-Gly-hydroxamate binds hmeprin α more effectively than MMP-1, and has been useful for the purification of hmeprin α [22].

Here we present kinetic data on the interaction of hmeprin α and hmeprin β with a series of metalloprotease inhibitors, and we provide structural models to explain the observed differences in inhibitor binding between the hmeprin subunits and compare these with mouse and rat meprin.

MATERIALS AND METHODS

Chemicals and enzymes

All chemicals were of analytical grade and, if not stated otherwise, were obtained from Amersham Bioscience (Freiburg, Germany), Applichem (Darmstadt, Germany), Serva (Heidelberg, Germany), Bio-Rad (Munich, Germany), Bachem (Heidelberg, Germany), Sigma/Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Expression and purification of recombinant hmeprin subunits

hmeprin α and a soluble tail switch mutant of hmeprin β (termed ProHisMeprin β^s) were expressed and purified as described previously [9,22]. In brief, the cDNA encoding the proteins was cloned into pFASTBAC1 followed by heterologous expression in BTI-TN-5B1-4 insect cells using the baculovirus system (Gibco Life Technologies, Paisley, U.K.). Purification of the inactive pro-form of hmeprin α was achieved by ammonium sulphate precipitation and gel filtration. Subsequently, the zymogen was activated by trypsin and the active enzyme was affinity-purified on immobilized Pro-Leu-Gly-hydroxamate, a procedure which also removes residual tryptic activity [22]. Pro-hmeprin β was purified by Ni^{2+} -nitrilotriacetate chromatography, preceding activation by incubation with trypsin. Tryptic activity was removed by affinity chromatography using a column containing immobilized chicken ovomucoid [9].

Proteolytic activity of hmeprin against extracellular matrix components

hmeprin α or β (50 nM) was incubated with 20 μg of collagen I or collagen IV from human placenta (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), fibronectin from human plasma (Roche Diagnostics GmbH, Mannheim, Germany) or recombinant human nidogen-1 (kindly provided by Dr R. Timpl, Max-Planck-Institute for Biochemistry, Martinsried, Germany) for 20 h at 37 °C in 0.1 M Tris/HCl, pH 7.5, in a total

volume of 50 μl . The reactions were terminated by addition of trichloroacetic acid to a final concentration of 10 % (v/v). After precipitation for 1 h on ice and centrifugation (13 000 g, 10 min, 4 °C), the pellets were washed twice in acetone and then dissolved in SDS/PAGE sample buffer containing 2-mercaptoethanol. The samples were boiled and subjected to electrophoresis on a 7.5 % (w/v) polyacrylamide gel [28]. Proteins were visualized with Coomassie Brilliant Blue G-250. For the identification of cleavage sites, protein bands were blotted on to a PVDF membrane, stained with Coomassie Brilliant Blue R-250, excised and subjected to N-terminal amino acid sequencing (SeqLab, Göttingen, Germany).

Determination of inhibition constants

The following inhibitors were tested for their inhibitory effects on hmeprin α and β : epigallocatechin gallate, NNGH [*N*-isobutyl-*N*-(4-methoxyphenylsulphonyl)glycyl hydroxamic acid], TAPI-0 (tumour necrosis factor α proteinase inhibitor-0; Biomol GmbH, Hamburg, Germany), TAPI-2 (from Dr R. A. Black, Amgen, Seattle, WA, U.S.A.), amastatin, Pro-Leu-Gly-hydroxamate (Bachem), galardin (ilomastat, GM 6001; Calbiochem-Novabiochem GmbH, Schwalbach, Germany), bestatin, captopril, phosphoramidon (Fluka, Sigma-Aldrich Chemie GmbH), α_2 -macroglobulin, actinonin (Sigma-Aldrich Chemie GmbH) and enalapril maleate (STADA Arzneimittel AG, Bad Vilbel, Germany). Batimastat was synthesized as described in [28a]. Ro 28-2653 {5-biphenyl-4-yl-5-[4-(4-nitro-phenyl)-piperazine-1-yl]pyrimidine-2,4,6-trione} was synthesized according to [28a,29]. Ro 32-7315 was prepared as described in [30].

Meprin activities were determined using *N*-benzoyl-L-tyrosyl-p-aminobenzoic acid as substrate [5]. The substrate concentration was 40 mM, and the enzyme concentration was always at least 10 times below K_i . Inhibitors were employed in a concentration range from 5 pM to 5 mM, depending on their inhibitory effect. Each inhibitor was tested over a concentration range covering at least ten different concentrations from $K_i/5$ to $5 \times K_i$. All reactions were carried out at 37 °C in 50 mM Tris/HCl, pH 7.5, and 0.5 mM MgCl_2 .

Determination of the inhibition constant K_i was performed by non-linear regression analysis using GraFit 4.0 (Erithacus Software) by plotting the ratio of the inhibited and uninhibited enzyme activities against the inhibitor concentration and fitting the data to the following eqn [31]:

$$\frac{v_i}{v_0} = 1 - \frac{E_0 + I_0 + K_i - \sqrt{(E_0 + I_0 + K_i)^2 - 4 \cdot E_0 \cdot I_0}}{2 \cdot E_0}$$

In the case of weak inhibition or restricted availability of inhibitors, IC_{50} values were obtained from plots of the relative activity against $-\log [I]$ and calculated using GraFit 4.0.

Molecular modelling

Homology models of the meprin protease domains based on the X-ray crystal structure of astacin [32] were built on an Indigo² Impact Workstation (Silicon Graphics Inc., Mountain View, CA, U.S.A.) using HOMOLGY and energy-minimized with DISCOVER embedded in the INSIGHT II 97.0 software package (Molecular Simulations, San Diego, CA, U.S.A.). Co-ordinates were obtained from the EBI Protein Data Bank (accession numbers 1AST and 1QJJ for astacin [32,33], 1IAF for a theoretical model of the mouse meprin α subunit [34], and 1JK3 for MMP-12 complexed with batimastat [35]). The three zinc-binding histidine

Figure 1 Proteolytic activity of hmeprins against components of the extracellular matrix

Portions of 20 μ g of collagen IV (**A**), collagen I (**B**), fibronectin (**C**) and nidogen-1 (**D**) were incubated with 50 nM purified hmeprin α or β for 20 h at 37 °C in 0.1 M Tris/HCl, pH 7.5, in a total volume of 50 μ l. Afterwards, samples were separated on 7.5% (w/v) polyacrylamide gels and stained with Coomassie Brilliant Blue. Controls (lanes C) contained substrates only. In the gels, lower-case letters indicate proteolytic fragments for which unambiguous N-terminal sequences could be obtained by protein sequencing (see Figure 2).

N ϵ atoms and the methionine of the Met-turn were used as anchor groups for the superposition of the catalytic domains of these metzincin proteases. Numbering of residues relies on a structure-

based sequence alignment of the members of the astacin family [34].

RESULTS

Cleavage of matrix components by hmeprin

For the investigation of the activity of hmeprin α and β against components of the extracellular matrix, homo-oligomers instead of hetero-oligomers were used to facilitate clear separation between the cleavage specificities of the two subunits. Protein substrates were incubated with 50 nM hmeprin α or β at 37 °C for 20 h and the resulting cleavage products were separated by SDS/PAGE, followed by transfer to PVDF membranes (Figure 1).

Human collagen IV was degraded completely by both subunits; no defined products could be detected (Figure 1A). In contrast, collagen I was not cleaved at all (Figure 1B). Human fibronectin (Figure 1C) and nidogen-1 (Figure 1D) were proteolytically cut by both meprin subunits at several sites. Some of these sites could be identified by N-terminal sequencing of distinct protein bands from Coomassie Blue-stained PVDF membranes (Figure 2). The numbering of human fibronectin and nidogen-1 corresponds to that of the full-length sequences starting from the initiator methionine [Swiss-Prot/TrEMBL (<http://us.expasy.org>) entries P02751 and P14543 respectively]. Accordingly, hmeprin α cleaved fibronectin at positions Tyr²⁹⁴ ↓ Gln²⁹⁵ and Asn⁷⁰⁹ ↓ Thr⁷¹⁰, corresponding to the linker regions between fibronectin type I repeats 5 and 6 and between fibronectin type III repeats 1 and 2 respectively. hmeprin β cleaved fibronectin at Leu⁷²¹ ↓ Val⁷²² and Arg⁹⁰³ ↓ Ser⁹⁰⁴, in the linker regions between fibronectin type III repeats 1 and 2 and between fibronectin type III repeats 3 and 4 respectively (Figure 2). Sequencing of the breakdown products of human nidogen-1 revealed limited proteolysis by hmeprin α at Asp⁶⁷ ↓ Arg⁶⁸ within the globular domain G1 and between Gln³⁶³ and His³⁶⁴, which corresponds to the flexible linker region between G1 and G2. Several other minor bands were also subjected to N-terminal sequencing, and turned out to represent secondary degradation products of major bands or mixtures, which did not reveal unambiguous sequences.

Figure 2 Cleavage sites in human fibronectin and nidogen-1 for hmeprin α and β

N-terminal sequencing identified the fragments obtained in Figure 1, and the corresponding cleavage sites in the domain structure of fibronectin (FN) and nidogen-1 have been localized and assigned.

Inhibition studies

The proteolytic activity of hmeprins against basement membrane components resembled that of certain MMPs, e.g. gelatinases (MMP-2 and MMP-9) and MT1-MMP (membrane-type 1 MMP; MMP-14), suggesting structural similarities in the substrate binding region. Hence the question arose as to whether hmeprin subunits would likewise be sensitive to MMPIs. Individual inhibitor profiles for the α and β subunits were recorded using synthetic MMPIs, inhibitors of TACE (tumour necrosis factor α -converting enzyme), ACE (angiotensin-converting enzyme) and aminopeptidases, and in addition the non-specific plasma proteinase inhibitor α_2 -macroglobulin. Inhibition constants (K_i) were determined by non-linear regression analysis [31] (Figure 3).

Neither the α nor the β subunit of hmeprin was inhibited by α_2 -macroglobulin, the aminopeptidase inhibitors amastatin and bestatin, or phosphoramidon. These results confirm previous observations on the mouse and rat enzymes [1,36,37]. In this context, 'no inhibition' implies that no decrease in enzymic activity was observed with inhibitor concentrations of up to 1 mM.

The ACE inhibitor captopril has been described previously to inhibit hmeprin [5]. In that study, no distinction was possible between the α and β subunits. We determined K_i values of 0.7 mM (hmeprin α) and 0.4 mM (hmeprin β), which are in a range similar to that of small thiol-containing inhibitors of astacin [38]. In contrast, enalapril, a more specific anti-hypertensive compound, did not lower the activity of hmeprin.

The naturally occurring hydroxamate actinonin proved to be the strongest inhibitor of hmeprin α in the present study ($K_i = 20$ nM). It also inhibited hmeprin β in the micromolar range ($K_i = 1.7$ μ M). The hydroxamate-based metalloprotease inhibitors TAPI-0, TAPI-2, Pro-Leu-Gly-hydroxamate, batimastat, NNGH, galardin and Ro 32-7315 bound to hmeprin with inhibition constants in the micromolar range (Figure 3). Generally, hmeprin α was inhibited more strongly than the β subunit. This discrimination was most evident in the case of the TACE inhibitor Ro 32-7315, which inhibits the α subunit ($K_i = 1.6$ μ M) three orders of magnitude more efficiently than the β subunit ($IC_{50} = 1.6$ mM). No inhibition of either hmeprin subunit was observed with the pyrimidine-2,4,6-trione derivative Ro 28-2653, an inhibitor of MMP-2, MMP-9 and MMP-14 (MT1-MMP), which reflects the improved selectivity of this class of MMPIs [29]. Epigallocatechin gallate, a weak MMPI from green tea extracts, also showed no inhibitory effects on hmeprin [39].

Homology modelling

Homology models of the hmeprin subunits have been derived using the structure of astacin inhibited by a phosphinic transition-state analogue as a template [33] (Figure 4). The models were superimposed on the crystal structures of astacin complexed with Pro-Leu-Gly-hydroxamate [33], which binds into the S1, S2 and S3 subsites of the active-site cleft, and of MMP-12 complexed with batimastat [35], which binds into the S1', S2' and S3' subsites (Figure 4).

Based on these models, both inhibitors co-ordinate the catalytic zinc of hmeprin α and β with their hydroxamic acid moieties, and their backbones align in an antiparallel orientation along the outer β -strand ('edge-strand') of the upper subdomain [29,32–34].

DISCUSSION

Cleavage of basement membrane components by hmeprin

The observation of the basolateral secretion of hmeprin in colorectal cancer [16] and of mouse meprin in experimentally

Inhibitors	Meprin α	Meprin β
Actinonin	K_i $2.0 \cdot 10^{-8}$ M $\pm 2.3 \cdot 10^{-9}$ M	K_i $2.0 \cdot 10^{-6}$ M $\pm 2.3 \cdot 10^{-7}$ M
Batimastat	K_i $4.4 \cdot 10^{-6}$ M $\pm 4.6 \cdot 10^{-7}$ M	K_i $1.8 \cdot 10^{-5}$ M $\pm 2.2 \cdot 10^{-6}$ M
Galardin	K_i $1.4 \cdot 10^{-7}$ M $\pm 9.9 \cdot 10^{-9}$ M	K_i $8.9 \cdot 10^{-6}$ M $\pm 7.1 \cdot 10^{-7}$ M
NNGH	K_i $4.0 \cdot 10^{-7}$ M $\pm 3.7 \cdot 10^{-8}$ M	K_i $7.4 \cdot 10^{-6}$ M $\pm 7.6 \cdot 10^{-7}$ M
PLG-NHOH	K_i $5.3 \cdot 10^{-7}$ M $\pm 5.1 \cdot 10^{-8}$ M	K_i $1.4 \cdot 10^{-5}$ M $\pm 1.2 \cdot 10^{-6}$ M
Ro 28-2653	n. i.	n. i.
Ro 32-7315	K_i $1.6 \cdot 10^{-6}$ M $\pm 2.6 \cdot 10^{-8}$ M	IC_{50} $1.6 \cdot 10^{-3}$ M $\pm 1.2 \cdot 10^{-4}$ M
TAPI-0	K_i $2.2 \cdot 10^{-6}$ M $\pm 3.5 \cdot 10^{-8}$ M	IC_{50} $4 \cdot 10^{-4}$ M $\pm 1 \cdot 10^{-4}$ M
TAPI-2	K_i $1.5 \cdot 10^{-6}$ M $\pm 2.7 \cdot 10^{-7}$ M	IC_{50} $2 \cdot 10^{-4}$ M $\pm 1 \cdot 10^{-4}$ M
Captopril	K_i $7.4 \cdot 10^{-4}$ M $\pm 6.0 \cdot 10^{-6}$ M	K_i $4.1 \cdot 10^{-4}$ M $\pm 1.2 \cdot 10^{-5}$ M

Figure 3 Projection formulae and inhibition constants of hmeprin inhibitors

The K_i values were determined using *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (PABA-peptide) as substrate [5], and plotting the ratio of the inhibited and uninhibited enzyme activities against the inhibitor concentration. Non-linear regression analysis as described in [31] and calculation of IC_{50} values were performed using GraFit 4.0 (Erithacus Software). PLG-NHOH, Pro-Leu-Gly-hydroxamate; n.i., no inhibition.

Figure 4 Substrate binding regions of the protease domains of hmeprin α and β

Homology-based models of the protease domains of hmeprin α (A) and β (B) were built with HOMOLGY and energy minimized with DISCOVER (INSIGHT II 97.0; Molecular Simulations). The images (in stereo view) were prepared by an overlay of the crystal structures of astacin complexed with Pro-Leu-Gly-hydroxamate [33], MMP-12 complexed with batimastat [35] and Connolly surface models (rolling sphere of 1.2 Å) of the catalytic domains of hmeprin α and β . The surface is colour-coded according to the electrostatic potential: negative charges are shown in red, and positive charges in blue. Residues are numbered as described in [34]. Pro-Leu-Gly-hydroxamate binds left-handed to the zinc into the non-primed subsites (S1–S3), and batimastat binds right-handed into the primed subsites (S1'–S3'). Both inhibitors co-ordinate the catalytic zinc ion (white sphere in the centre) via their hydroxamic acid moieties. Inhibitor colour code: carbon, green; nitrogen, blue; oxygen, red; sulphur, yellow. The thienylthiomethyl-succinyl group of batimastat (see Figure 3) is buried behind the prominent protease residues Tyr¹²² (hmeprin α) and Arg¹²³ (hmeprin β). In hmeprin β , the side chain of Arg⁸⁵ forms the bottom of the S1' pocket, which harbours the P1' side chain of batimastat.

induced renal failure [17,18] raised questions about the roles of this protease in the migration of cells across the basement membrane and in the destruction of the extracellular matrix. In the present paper, evidence is provided that both hmeprin subunits cleave collagen IV (Figure 1A), fibronectin (Figure 1C) and nidogen-1 (Figure 1D). Fibronectin is cut within the proteolytically vulnerable linker regions between fibronectin type III repeats 1 and 2, 3 and 4, and 5 and 6 (Figure 2) [40]. With nidogen-1, which connects laminins with the collagen IV network [41], we observed several cleavages in the globular G1 domain and in the linker between the G1 and G2 domains (Figure 2). There are subtle deviations, such as the occurrence of basic, acidic or polar amino acid residues in the P1 and P1' positions of some hmeprin α cleavage sites, and a lack of acidic side chains in the P1' position of hmeprin β sites. However, these deviations fall within the range of variations reported for the specificity of mouse meprin, which has been based on the cleavage of a dodecamer peptide library [23].

One might argue that these deviations may be caused by aberrant oligomerization of the recombinant human enzyme as compared with that of the rodent proteins obtained from their natural sources. However, according to electron microscopic studies and size-exclusion chromatography, the human subunits behave exactly like the rodent enzymes [8], i.e. hmeprin α oligomerizes to form large aggregates (chains and rings), whereas hmeprin β forms dimers [9]. It has been considered that the state of oligomerization should not alter the specificity of the individual active sites [8,23]. Accordingly, the specificities of homo-oligomers and hetero-oligomers most probably result from the combination of the distinct specificities of the α and β subunits.

Taken together, and also considering earlier observations on the cleavage of laminin-1 and laminin-5 [22], it is clear that hmeprin is capable of hydrolysing major components of the basement membrane, and therefore may enable cells to migrate across this barrier. Generally, in their cleavage of basement membrane proteins and their failure to cut collagen I, hmeprin resembles gelatinases (MMP-2, MMP-9) rather than collagenases (e.g. MMP-1, MMP-8).

Binding of inhibitors to hmeprin

All components identified in the present study as inhibitors of hmeprin are either hydroxamic acid derivatives or thiol reagents such as the anti-hypertensive drug captopril. It has been shown by X-ray crystal structure analysis that the hydroxamate-based inhibitor Pro-Leu-Gly-hydroxamate binds to those subsites of metzincin peptidases that interact with substrate side chains N-terminal of the cleaved peptide bond [33] (Figure 4). In this region, the so-called 'non-primed side', there is a high degree of similarity, even between metzincins of different families such as the astacins and the MMPs [2,3]. An important anchoring function in this interaction is played by the niche-like S3 subsite of the β -edge strand, which harbours the inhibitor's proline ring [33,42].

The 'primed side' region is much more variable between different astacins and also within the metzincin superfamily [2,3]. In the MMPs, for example, primed-side inhibitors bind via backbone interactions to the double-S-shaped bulge structure protruding from the N-terminal β -sheet [35]. In addition, side chains are bound in the S1' and S2' subsites. Astacins such as meprin lack the double S-turn, which prevents hydrogen bonding between

backbone carbonyl and amide groups. Therefore binding is restricted to side-chain interactions [2]. It is known from the crystal structure of astacin complexed with a transition-state analogue that the S1' subsite is most critical for inhibitor binding [33]. In astacin, the ring of Pro¹⁷⁶ seals this subsite, which is the reason why the crayfish proteinase prefers small, uncharged residues in this position [43]. In meprin α this proline is replaced by a glycine and the subsequent residue, corresponding to Tyr¹⁷⁷ in astacin, is deleted [34], which would explain the preference for bulky and long side chains in the P1' position of substrates and inhibitors. According to the K_i value of 20 nM, this interaction is obviously most favourable between hmeprin α and actinonin, which contains a long pentanyl side chain in the P1' position. In contrast, the binding of hmeprin α and β to batimastat is much weaker (Figure 3). A possible reason for this difference might be the fact that the backbone carbon atom following the carbonyl of the hydroxamate group is unoccupied in actinonin, but carries an extended 2-thienylthiomethyl-succinyl residue in batimastat (Figure 3). The additional residue might collide with the lower rim of the active-site cleft, which is much narrower in astacins than in other metzincins such as MMPs [2,3].

This idea is supported by the astacin-based molecular models of hmeprin α and β (Figure 4). In meprin, the side chains of Tyr¹²² (hmeprin α) and Arg¹²³ (hmeprin β), which protrude from the lower rim into the cleft (Figure 4), might even increase these steric constraints. This region of the active-site cleft might also be responsible for the striking differences between hmeprin α and hmeprin β in their binding to Ro 32-7315. In this inhibitor, the long and bulky 4-phenyl-3-butenyl group occupies the critical position next to the hydroxamate moiety (Figure 3). hmeprin α binds this inhibitor about 1000 times more tightly than the β subunit. A possible reason for this behaviour could be the more voluminous side chain of Arg¹²³ in hmeprin β as compared with the phenol ring of Tyr¹²² in hmeprin α (Figure 4). Substrate kinetics and modelling studies on the mouse meprin subunits by Bertenshaw et al. [23] corroborate the importance of the respective enzyme subsites for cleavage specificity. Further support has been provided recently by site-directed mutations in mouse meprin subunits [44]. In their study, Villa et al. [44] have collected strong evidence for the pivotal role of Lys¹⁸⁵ (corresponding to Arg¹²³ in hmeprin β) and Tyr¹⁹⁹ (corresponding to Tyr¹²² in hmeprin α) for the cleavage specificity of the mouse subunits.

The compounds discussed so far are considered 'first generation' inhibitors. They are rather non-specific, since they bind not only to their target enzyme, but also to other metalloproteases. Second generation MMPIs have increased selectivity due to a change of the zinc-chelating group and improved side-chain interactions [29]. An example is Ro 28-2653, a non-hydroxamic acid, pyrimidine-2,4,6-trione-based MMPI [45], which does not show any inhibitory effect against hmeprin, but is specific for MMP-2, MMP-9 and MMP-14 (MT1-MMP). The structure of Ro 28-2653 (Figure 3) would most probably collide with the lower rim of the cleft of hmeprin.

Interference of metalloprotease inhibitors with the function of meprin

Generally, the observed inhibition constants are in a physiologically relevant range, implying that the application of un-specific MMPIs would also influence the physiological function of meprins. This may not be a disadvantage in a scenario such as colorectal cancer. On the other hand, hmeprins are inhibited by broad-range MMPIs, but remain unaffected by highly specific compounds such as Ro 28-2653. Hence the application of specific MMPIs in cancer may not be sufficient for complete inhibition

of matrix-degrading activities if other proteases, such as meprin, are involved in addition to MMPs. This appears to be remarkable in view of only 60–70% inhibition of tumour growth in most animal models [26]. In addition, the question arises as to whether the known side-effects of certain inhibitors, such as kidney failure [46] and intestinal malfunctions [47] in the case of captopril [48], might be related to the functions of meprin.

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