Modulation of the Extracellular Matrix in Advanced Atherosclerosis

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4 Development of a Selective Matrix Metalloproteinase-9 Inhibitor by Combinatorial Peptide Synthesis

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Abstract

Remodeling of the extracellular matrix is an important process in the pathophysiology of many disabling diseases, such as rheumatoid arthritis and atherosclerosis. Amongst others, the gelatinase matrix metalloproteinase-9 (MMP-9) has been implicated in the pathobiology of plaque rupture and could make an interesting target for plaque stabilizing therapies.
In the present study we describe the development of a selective MMP-9 inhibitor by combinatorial peptide synthesis. Earlier studies by Koivunen et al. identified the parent peptide CTTHWGFTLC as a highly specific gelatinase inhibitor. However, this decamer appeared to be a relatively poor inhibitor (IC50: 100 and 40 µM for MMP-2 and -9 respectively) and thus lacks the potency for pharmaceutical purposes. Therefore, we systematically modified this prototype peptide with respect to its secondary structure by introducing intramolecular disulfide bridges and β-turn inducers. To identify the minimal essential motif for MMP-9 specific inhibition with submicromolar affinity, the parent decapeptide was modified by truncation and the function of individual amino acids was studied by alanine scanning. These modifications showed that intramolecular cyclization is not required for gelatinase inhibition. To explore the effect of zinc-binding both intramolecular and terminal zinc-binders were introduced, but this did not notably add to MMP-9 affinity.
In conclusion, this process yielded several interesting peptide sequences with micromolar affinity that, however, still need to be optimized. Extended specificity analysis will ultimately be followed by testing the in vivo properties of the novel MMP-9 inhibitor in mouse models of atherosclerosis and restenosis.

Introduction

Remodeling of the extracellular matrix in both healthy and pathological conditions enables the organism to adapt to the ever-changing environmental factors and to repair damaged tissues. Moreover, the extracellular matrix (ECM) is actively involved in cellular behavior and survival.[1, 2] Changes in matrix composition and turnover are mediated by a large heterogeneous family of proteolytic enzymes and their physiological inhibitors. Matrix metalloproteinases (MMPs) are probably the best characterized to date and form an extensive family of zinc-dependent proteases that are extremely important in tissue repair and remodeling and in many pathological conditions.[3]

Currently, the MMP family counts more than 20 members that, based on substrate specificity, can be categorized to five subgroups: interstitial collagenases, gelatinases, stomelysins, membrane-type MMPs and others. The gelatinases MMP-2 and -9 are involved in degradation of collagen type IV, laminin and other constituents of the pericellular matrix enabling cells to proliferate and migrate through the surrounding ECM.[4] In addition, gelatinases, particularly MMP-9, can activate a range of other proteases.[5] Through these actions, MMP-9 not only participates in matrix turnover, but also influences release of matrix-bound bioactive molecules including cytokines and growth factors.[2] Hence, depending on the environmental context, MMP-9 can be actively involved in matrix remodeling and in cellular proliferation, migration, recruitment, neo-angiogenesis and apoptosis. A dysbalance between MMP-9 activity and its physiological inhibitors is instrumental of many pathological conditions, such as cardiac remodeling after myocardial infarction, heart failure, atherosclerotic plaque progression, rheumatoid arthritis, multiple sclerosis, carcinogenesis and tumor metastasis.[6-11] Because the exact pathobiological mechanisms by which MMP-9 acts at different stages of disease progression are largely unclear for many conditions, it may be useful to develop a selective inhibitor that can contribute to map the pathogenic role of MMP-9 and could possibly form the basis for diagnostic and therapeutic modalities in the future.

So far, the development of a specific MMP-9 inhibitor has been proven to be rather difficult.[12-16] Most synthetic inhibitors of gelatinase activity do not discriminate between MMP-9 and -2 or any other MMPs and often even display higher affinity for MMP-2. In 1999, Koivunen et al. identified several specific gelatinase inhibitors by phage display.[12] Cyclic decapeptides, containing the histidine-tryptophan-glycine-phenylalanine (HWGF) core motif, were found to selectively inhibit MMP-2 and -9. The prototype peptide CTTHWGFTLC inhibits cell migration in vitro as well as tumor angiogenesis in vivo. However, the non-HWGF motif residues are unlikely to be optimal and do not provide significant specificity for either MMP-2 or -9. The present study explores the characteristics of the parent decapeptide to identify the residues that are essential for MMP-9 inhibition and investigates the effect of hydroxamate zinc-binders on MMP-9 affinity. This yielded several new compounds that are of interest and could selectively inhibit MMP-9 activity in vitro. Future modifications are expected to further optimize their inhibiting capacity.

Materials and Methods

Materials

Mass spectra were recorded on a Perkin Elmer/PerSpective Biosystems Voyager-DE-RP MALDITOF MS. Analysis was performed on a Jasco HPLC system equipped with an Alltech C18 column (250x10 mm) connected to a Perkin Elmer SCIEX API 165 Single Quadrupole LC/MS instrument. pyBOP was purchased from MultiSyntech. Di-isopropyl-ethylamine piperidine and NMP were purchased from Biosolve and are peptide grade. Fmoc protected amino acids were obtained from Multitech, Fluka, Acros or Sigma Aldrich.

General peptide synthesis

All described peptides were synthesized on a Multisyntech Syro Multiple Peptide Synthesizer using Fmoc chemistry protocols. Parallel synthesis of 48 peptides was performed on a 10 µmol scale using pyBOP as a coupling agent and dipea as the base. Tentagel S RAM resin (capacity 0.25 mmol/g) was treated with 5 equivalents of the preactivated Fmoc amino acids in a total reaction volume of 300 µL. Fmoc deprotection was effected by treatment with a 20% piperidine solution 3 times, followed by extensive washing yielding a total cycle/deprotection time of approximately 1½ hours. Cleavage from the resin and deprotection of the immobilized peptides was effected by a mixture of trifluoro acetic acid (95%), tri-isopropyl-silane (TIS) (2.5%) and water (2.5%). For cysteine containing peptides 2% ethane-di-thiol was used in stead of TIS. The average cleavage/deprotection time was 3 hours. The resulting crude peptides were analyzed by MALDITOF MS and subsequently purified on a BIOCAD VISION automated HPLC system using an acetonitrile gradient and 0.1% TFA in water (column either Alltima C18 5u 250x10 mm or a Prosphere C18 300A 10 uM 150X22mm). Disulfides were generally formed prior to purification. Next, the peptides were lyophilized followed by LC-MS analysis. Peptides more then 95% pure were then dissolved in dimethylsulphoxide (10 mM) for use in the MMP activity assays. Specific groups like the internal zinc binding hydroxamate were introduced as previously described.[17] The synthesis of the Fmoc protected sugaramino acid (Saa, Figure 2) was performed by Kriek et al.[18] Next, the peptides were lyophilized followed by LC-MS analysis. Peptides more then 95% pure were then dissolved in dimethylsulphoxide (10 mM) for use in the MMP activity assays

MMP activity assays

MMP activity was measured using the internally quenched fluorogenic peptide substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys(Fluorescein)-Ala-Lys-NH2). Samples were tested at concentration ranging from 100 to 0.1 µM in MMP assay buffer (50 mM Tris, 5 mM $CaCl_2$, 250 mM NaCl, 1 µM $ZnCl_2$, 0.02% NaN_3 and 0.01% Brij-35, pH 7.5). Enzymatic cleavage of TNO211-F (5 µM) was assessed in presence of recombinant human MMP-9 or -2 (Amersham) using 5 µM BB94 (a broad spectrum MMP inhibitor) as a positive control. Fluorescence was monitored real-time for 4 hrs at 30°C using a Cytofluor 4000 apparatus (Applied Biosystems, Foster City, CA). The difference in the initial rate of substrate conversion between recombinant MMP with or without BB94 addition was used as a measure of 100% MMP activity. The inhibitory capacity of the samples is expressed relative to this value.

Table1. Amino acid abbreviations

Alanine	A	Ala	Methionine	M	Met
Cysteine	C	Cys	Asparagine	N	Asn
Aspartate	D	Asp	Proline	P	Pro
Glutamate	E	Glu	Glutamine	Q	Gln
Phenylalanine	F	Phe	Arginine	R	Arg
Glycine	G	Gly	Serine	S	Ser
Histidine	H	His	Threonine	T	Thr
Isoleucine	I	Ile	Valine	V	Val
Lysine	K	Lys	Tryptophan	W	Trp
Leucine	L	Leu	Tyrosine	Y	Tyr

Results

Characterization of the parent peptide CTTHWGFTLC and its core motif HWGF

Koivunens' principle inhibitor CTTHWGFTLC as well as the core motif HWGF were synthesized, purified and tested for their MMP-2 and MMP-9 inhibiting capacity. The full-length peptide CTTHWGFTLC yielded an IC50 of approximately 100 µM for MMP-2 and 40 µM for MMP-9 as determined by MMP-2 and -9 activity assays. The HWGF-motif inhibited MMP-2 by only 10% at 100 µM, while MMP-9 lost already 45% of its activity at a concentration as low as 10 µM (Fig. 1). Although the core motif by itself displayed a reasonable high specificity for MMP-9 its potency clearly is to low for pharmacotherapeutic application and thus this lead compound requires considerable optimization. Moreover, while the HWGF-motif is very selective for MMP-9, the prototype peptide CTTHWGFTLC possessed less specificity. Systematic alteration of the peptide sequence, influencing chemical and, particularly, structural properties may help to optimize the characteristics of this gelatinase inhibitor.

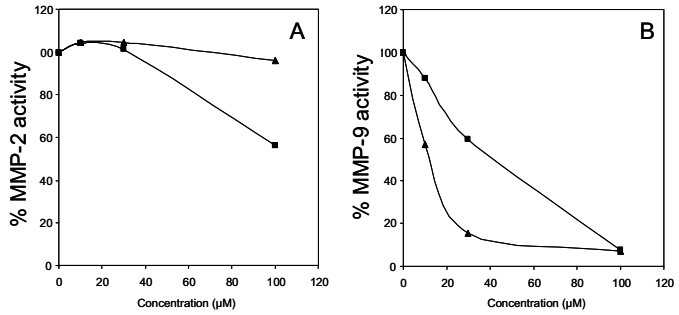

Figure 1. Percentage MMP-2 (A) or -9 (B) inhibition by CTTHWGFTLC (-■-) and by the core motif HWGF (-▲-). The activity of recombinant human MMP-2 and -9 was evaluated by monitoring the degradation of the fluorescent gelatinase substrate TNO211-F over time in the presence of the indicated concentrations.

Variations in secondary structure by cysteine scans and β-turn inducers.

We have studied the effect of the secondary structure by shifting the two cysteine residues throughout various places within the parent peptide CTTHWGFTLC. This resulted in cyclic peptides with various cycle sizes via the formation of intramolecular disulphide bridges between both cysteine residues. In addition, various commercially available β-turn inducers (Fig. 2) were introduced into the parent peptide flanking the central HWGF-box, which was kept preserved. Table 2 displays a systematic overview of both the cysteine scan and the β-turn inducer containing peptides with their respective IC50 values for MMP-9. The affinity of the peptide-analogues was in the low micromolar range. Although several sequences displayed an improved IC50 for MMP-9 activity as compared to the parent peptide, none could inhibit this protease at nanomolar concentrations.
These results could suggest however, that a C-C constraint may not be required for recognition of the gelatinase binding pocket. In addition, the N-terminal β-turn inducers aa2 and aa3 showed a negative effect on affinity, while an N-terminal aa1 considerably increased peptide affinity as compared to the parent peptide. On the C-terminal end, aa1, aa3 and aa4 increased the IC50 for MMP-9 as well.

Cystein scan	IC50 (μM)	β-turn inducers	IC50 (μM)
CTTHWGFTL**C**	40		
CTTHWGFGT**C**L	60	CTTHWGF**aa1**C	6
CTTHWGF**C**TL	20	CTTHWGF**aa2**C	30
CTTHWG**C**FTL	18	CTTHWGF**aa3**C	8
CTTHW**C**GFTL	60	CTTHWGF**aa4**C	8
CTTH**C**WGFTL	40	C**aa1**HWGFTLC	8
CTT**C**HWGFTL	25	C**aa2**HWGFTLC	50
CT**C**THWGFTL	8	C**aa3**HWGFTLC	50
CCTTHWGFTL	60	C**aa4**HWGFTLC	14
TT**C**HWGFTL**C**	10	CTTHWGF[**Saa**]C	20
TTH**C**WGFTL**C**	5		
TTHW**C**GFTL**C**	25		
TTHWG**C**FTL**C**	5		
TTHWGF**C**TL**C**	40		
T**C**THWGF**C**TL	11		
TTH**C**WG**C**FTL	8		

Table 2. IC50 values for MMP-9 inhibition. Variation in intramolecular cycle sizes and β-turn inducers did not provide a rationale for inhibitory capacity or MMP-9 specificity.
The frame highlightsthe full-length parent decapeptide.

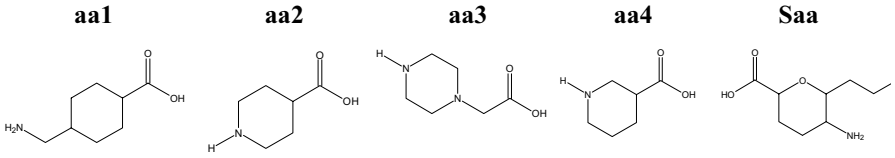

Figure 2. Chemical structures of the β-turn inducers that were utilized flanking the core-motif HWGF.

Contribution of individual side chains

From these systematic structural alterations it could not be determined if there is a rationale in the effect of the peptides' primary structure on gelatinase inhibition. This prompted additional modifications of the original prototype peptide.
To study the contribution of the individual peptide side chains consecutive amino acids were substituted by the relatively inert alanine (A). Few of these "mutations" resulted in a noteworthy improvement. The replacement of leucine (L) or phenylalanine (F) by alanine (CTTHWGFT**A**C and CTTHWG**A**TLC respectively) considerably reduced the IC50 for MMP-9 from 40 μM to 1 μM (Table 3).
It was striking that modifications in the core motif HWGF were well tolerated. This suggests that the histidine residue may not be as important for zinc binding as Koivunen et al. had postulated. Also, C/A mutations did not deteriorate gelatinase inhibition, indicating that linearized peptides are equally potent as their cyclic analogues.

Peptide truncation

Because cyclic peptides showed no additional benefit over the linearized, C- and N-terminal truncation studies were initiated to pinpoint the minimal essential motif of CTTHWGFTLC (Table 3). Considering the 500D rule of thumb, a smaller molecule can also be expected to possess better pharmaceutical properties.

Removal of the N-terminal cysteine (TTHWGFTLC) led to a more potent inhibitor (IC50 < 1 μM). The deletion of the C-terminal leucine-cysteine yielded a similar effect, adding to the hypothesis that linearized peptides hold greater affinity than cyclic molecules. In another sequence the replacement of the threonins (T) by serines (S) (SSHWGFT) resulted in a stronger inhibitor and greater specificity for MMP-9 over MMP-2 (IC50: 2 μM and 100 μM, respectively).

Alanine-scan	IC50 (μM)	Truncated peptides	IC50 (μM)
CTTHWGFTLC	40		
ATTHWGFTLC	30	CTHWGFTC	13
C**A**THWGFTLC	40	CHWGFC	15
CT**A**HWGFTLC	60	HWGF	15
CTT**A**WGFTLC	15	CTTHWGFTL	12
CTTH**A**GFTLC	20	CTTHWGFT	1
CTTHW**A**FTLC	20	CTTHWGFS	5
CTTHWG**A**TLC	1	CTTHWGF	25
CTTHWGF**A**LC	25	TTHWGFTLC	<1
CTTHWGFT**A**C	1	THWGFTLC	40
CTTHWGFTL**A**	25	HWGFTLC	50
		TTHWGFT	30
		SSHWGFT	2
		THWGFT	1
		HWGFT	50
		HWGFTTC	10

Table 3. IC50 values for MMP-9 inhibition. Alanine-scanning showed that the absence of the phenylalanine or leucine side-chain may augment inhibitory capacity. SSHHWGFT also featured a relatively high specificity for MMP-9 as compared to MMP-2, and therefore was used for further optimization.

Zinc binders

The most potent derivative from the alanine-scan, CTTHWGFTAC, was explored with respect to its zinc binding properties. To achieve this, successive amino acids were substituted by aspartate (D) with or without a zinc binding hydroxamate (NHOH). Whereas the zinc-binding properties of the histidine residue (H) in the HWGF-motif could very well be responsible for MMP inhibition, substitution of this amino acid by the zinc-binding aspartyl-hydroxamate surprisingly increased its IC50 from 1 μM to 15 μM (table 4). While the replacement of histidine and threonine with aspartyl-hydroxamate deteriorated the MMP-9 inhibiting capacity, the effect of substituting phenylalanine (F) or alanine by this zinc-binder was indifferent.
Taken together, the insertion of intramolecular zinc binders was not well tolerated or at least showed no additional benefit on MMP-9 affinity. This turned our attention to the introduction of a hydroxamate at the N- or C-terminal end. However, this did not yield additional selective MMP-9 inhibition when introduced in the two promising leads SSHWGFT and TTHWGFTLC (table 4). Also, the introduction of a PEG-spacer between the peptides and the incorporated zinc-binders could not enhance their inhibiting activity. Therefore it may be concluded that zinc-binders do not notably add to the activity of the peptides. We even noticed a negative effect on MMP-9 specificity, which prompted us to leave this line of investigation and explore the introduction of other C- and N-terminal substitutions.

AspNHOH scan (Zinc-binders)	IC50 (μM)	N-terminal zinc-binders	IC50 (μM)
CTTHWGFTLC	40		
CTTHWGFTAC	1		
CTT**D***WGFTAC	15	SSHWGFT	2
CTT**D**WGFTAC	3	X-SSHWGFT	1
CTTH**D***GFTAC	3	X-PEG-SSHWGFT	1
CTTH**D**GFTAC	3		
CTTHW**D**FTAC	4	TTHWGFTLC	1
CTTHWG**D***TAC	2	X-TTHWGFTLC	1
CTTHWG**D**TAC	1	X-PEG-TTHWGFTLC	1
CTTHWGF**D***AC	13		

Table 4. IC50 values for MMP-9 inhibition. The introduction of hydroxamate (*) zinc-binders in the CTTHWGFTAC peptide did not enhance its inhibiting potency. Truncation of the prototype peptide resulted in several interesting leads with high gelatinase affinity. N-terminalzinc-binders did not result in a significantly improved inhibition.

Discussion

A dysbalance in matrix homeostasis is involved in many pathological conditions, including atherothrombosis.[19] The zinc-dependent protease MMP-9 has been subject to intensive study over the past decades, but its exact role in atherogenesis and plaque rupture is still under debate. Although MMP-9 expression is clinically associated with unstable plaques and acute coronary syndromes,[19-22] direct evidence of a causal relation is still lacking. In fact, ApoE/MMP-9 deficient mice feature a less stable plaque phenotype than their MMP-9 expressing littermates.[23] Conversely, chapter 3 of this thesis demonstrates that MMP-9 overexpression could indeed lead to plaque instability in advanced complex lesions and proposes a context dependent effect of this enzyme, pointing to the importance of the local micro-environment to predict the overall effect of MMP-9. This highlights the significance of a targeted therapeutic strategy. In this respect it should also be noted that specific MMP inhibitors possibly induce less adverse effects than, for instance, the broad-spectrum inhibitor batimastat. Although designed to prevent cancer[24], batimastat treatment led to the induction of secondary liver tumors in immunodeficient in mice, indicating that wide-ranging MMP inhibition should be approached with utmost care.[25]

Thus far, it has been proven to be rather difficult to synthesize a gelatinase inhibitor that is highly specific for either MMP-2 or -9. The identification of a novel gelatinase inhibiting peptide by Koivunen et al.[12] paved the way to develop such a selective inhibitor as the core motif HWGF was seen to display high specificity for MMP-9. With the present study we aimed to optimize the peptide leads in terms of potency and specificity by modification of the parent peptide CTTHWGFTLC through combinatorial peptide synthesis.

Alteration of the secondary structure by introducing intramolecular cycles and β-turns or by replacing individual amino acids did not affect the potency of the peptide. This led us to conclude that MMP-9 inhibition by these peptides did not so much depend on their three dimensional structure, but probably more on the chemical characteristics of the individual amino acid side groups. The finding, that replacement of the C-terminal leucine by alanine resulted in a 40-fold gain in affinity, might support this hypothesis. In turn, truncation studies showed that linearized

peptides are at least equally potent as their cyclic analogues. Removal of the N-terminal cysteine resulted in a potent gelatinase inhibitor, but with lower selectivity for MMP-9 as compared to MMP-2. Interestingly, replacement of the N-terminal threonins by serine-residues restored MMP-9 specificity at a slightly lower potency (i.e. IC50: 2 μM).

However, further optimization strategies, by inserting intramolecular hydroxamate zinc-binders at various sites in the decapeptide CTTHWGFTAC failed to enhance its affinity. The alanine-scanning studies may suggest that the histidine site is possibly not critical in zinc-chelation, indicating that the actual binding pocket of the classical MMP inhibitors and the HWGF derivatives might differ. In general, the introduction of a zinc-binder did not result in a significant improvement of MMP-9 inhibition. While the inhibiting properties of SSHWGFT had only marginally improved with the introduction of N-terminal hydroxamate, either with or without PEG spacers, the relatively potent inhibitor TTHWGFTLC did not benefit from an N-terminal zinc-binder at all. These observations demonstrated that that the zinc-binding site is probably quite remote from the peptide binding site and that a different optimization strategy may be more favorable.

From the various truncated peptides, SSHWGFT was selected for further C- and N-terminal modifications. This peptide combined MMP-9 selectivity with a high potency. C- and N-terminal modifications of SSHWGFT are currently being explored in order to reach IC50 values in the nanomolar range. In addition, the possibilities to add a radioactive or fluorogenic label will be explored in future studies to enable detection in *in vitro* assays or *in vivo* imaging techniques.

At present, additional studies need to establish the applicability of our candidate peptides. This includes specificity profile, *in vitro* cell invasion assays, *in vivo* distribution experiments and, in a final stage, the effect on neointima and plaque formation could be studied in validated models of restenosis and atherosclerosis.

Once the specificity of the compounds can be confirmed, MMP-9 inhibiting peptides could already be utilized in, for instance, MMP activity assays. Other research applications can be found in various *in vitro* and *in vivo* models of disease in which the role of MMP-9 is studied. Furthermore, it is conceivable that MMP-9 inhibition will be a viable therapeutic modality for the stabilization of high-risk vulnerable plaques or as an adjuvant to prevent or delay restenosis. Finally, binding of a labeled MMP-9 inhibitor to regions of high MMP-9 activity could be a useful diagnostic tool to visualize vulnerable plaques. High MMP-9 activity in atherosclerotic plaques is not only deemed to be associated to plaque rupture[26], but also to the subsequent acute thrombosis[27] leading to clinical syndromes such as myocardial infarction or stroke. Early detection of vulnerable lesions might help to identify those patients that could benefit from plaque stabilizing therapy and thus contribute to an individually tailored management of atherosclerosis.

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