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Potent Inhibitors of β-Tryptase and Human Leukocyte Elastase Based on the MCoTI-II Scaffold

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Abstract: MCoTI-II is a member of a class of microproteins known as cyclotides that possess a macrolactam−cystine knot scaffold imparting exceptional physiological stability and structural rigidity. Modification of residues in the active loop and engineered truncations have resulted in MCoTI-II analogues that possess potent activity against two therapeutically significant serine proteases: β-tryptase and human leukocyte elastase. These results suggest that MCoTI-II is a versatile scaffold for the development of protease inhibitors against targets in inflammatory disease.

Cyclotides are a unique class of macrocyclic cystine knotted microprotein composed of 26−37 amino acids. They display a wide range of biological activities ranging from anti-HIV, uteronic, neurotensin antagonistic, hemolytic, antibacterial, and insecticidal activity to protease inhibition.[1−3] In contrast to many naturally occurring backbone-cyclic peptides, cyclotides are true gene products and are synthesized ribosomally in vivo.[4,5] Subsequent processing of a precursor protein by putative proteases/ligases and oxidative refolding results in a well-defined three-dimensional structure whereby two disulfide bonds form an embedded ring in an N-to-C cyclic backbone, which is penetrated by the third disulfide bond. The resulting scaffold is exceptionally rigid and highly resistant to thermal and enzymatic degradation.[6]

Tryptase and human leukocyte elastase (HLE,[a] also known as human neutrophil elastase) are serine proteases that play significant roles in a range of pathological conditions. The former is a homotetrameric serine protease generated mainly by mast cells and is implicated in the allergic response, including asthma.[7,8] Similarly, HLE has a key role in the tissue destruction associated with pulmonary emphysema, rheumatoid arthritis, cystic fibrosis, adult respiratory distress syndrome, chronic bronchitis, and pancreatitis.[9−11] Given their potential as drug targets, much effort has been made to develop inhibitors of each of these enzymes, with a view to therapeutic applications.[12−14] Peptidic and nonpeptidic inhibitors have been reported that target tryptase and HLE.

Cyclic peptides are generating increasing interest as potential drugs and drug scaffolds.[15] Cyclotides have recently been shown to possess excellent biological stability and can also be modified to display defined structural motifs.[16,17] Furthermore,

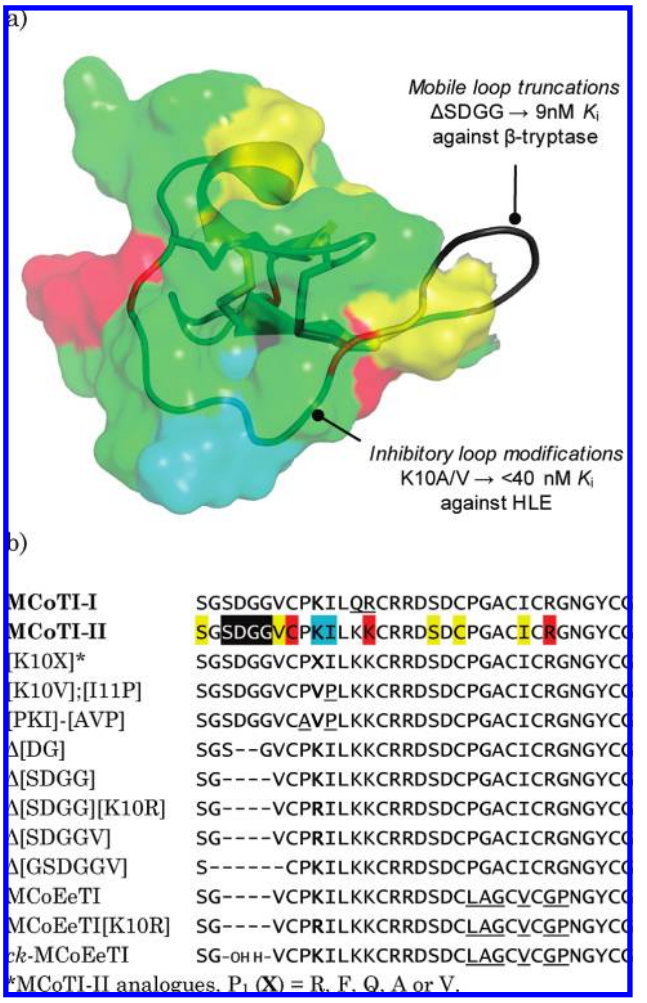

Figure 1. (a) NMR structure (ribbon and surface) of MCoTI-II.[23] The residues truncated in Δ[SDGG]MCo-TI-II are black. The residues with significant changes in chemical shift on going from MCoTI-II to Δ[SDGG]MCo-TI-II are colored yellow ($0.5 > \Delta\delta > 0.25$ ppm) and red ($\Delta\delta > 0.5$ ppm) (see Figure 3 and Supporting Information for data). The P_1 and P_1' residues are in cyan. (b) Sequence alignment of naturally occurring MCoTI-I and -II and the 15 other MCoTI analogues used in this study. The P_1 residue of the trypsin-binding loop is marked in bold, and significant sequence changes from MCoTI-II are underlined. Note that *ck*-MCoEeTI retains the cystine knot motif, with Val7 and Gly2 becoming free N and C termini, respectively.

recent research by our group[2,18,19] and others[20−22] has shown that they are readily amenable to chemical and microbial synthesis. We have previously reported the first total syntheses of MCoTI-I and -II by a thia-zip/native chemical ligation/refolding cascade reaction[19] and also by a protease-mediated ring-closure process.[18] As part of these studies, the P_1 position of the MCoTI-II scaffold was re-engineered to redirect its inhibitory activity toward chymotrypsin, subtilisin, or a chymotrypsin-like viral 3C cysteine protease.[2]

We sought to establish whether the impressive adaptability of the MCoTI cyclotides could be exploited to generate potent and selective inhibitors of HLE and tryptase. Here we report the design and chemical synthesis of a set of analogues of the MCoTI family trypsin inhibitor cyclotides (Figure 1) and their

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[a] Abbreviations: HLE, human leukocyte elastase; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

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low-nanomolar inhibitory activity against the therapeutically significant serine proteases tryptase and HLE.

Accordingly, a first set of MCoTI analogues varying at the P_2-P_1' positions were designed and synthesized by SPPS of a linear thioester peptide precursor followed by a one-pot thia-zip/NCL/refolding reaction (see Supporting Information for experimental details). Testing against the target enzymes (Table 1) revealed striking inhibition profiles. While potency was not retained against the highly trypsin-homologous enzyme tryptase, with $K_i > 100$ nM for all compounds tested, re-engineering the P_1 position in the MCoTI-II scaffold to Ala or Val ([K10A] or [K10V]) was found to impart low-nanomolar inhibition toward HLE. However, attempts to exploit a previously identified preference of HLE for Pro at P_1' were unsuccessful,[24] with these analogues showing much reduced activity. Finally, none of the analogues displayed any inhibitory activity against thrombin at the concentrations tested (0−2 μM, data not shown).

To investigate the potential origin of relatively poor tryptase inhibition by MCoTI-II, a molecular model was generated to examine the likely binding pose of MCoTI-II by taking advantage of the high homology between trypsin and the tryptase monomer. A composite backbone alignment was performed between the crystal structure of tetrameric tryptase bound to a small molecule inhibitor (PDB code 2FS9) and a MCoTI-II binding orientation derived from the closely analogous cystine knot protein EETI in complex with trypsin (PDB code 1H9I). The composite model indicated that the flexible loop in MCoTI-II that contains Asp4 is likely to be placed in proximity to the negatively charged neck of the tryptase tetramer pore (Figure 2).

From this model a second set of five MCoTI-II scaffolds truncated in the Asp4 loop were designed and synthesized by thia-zip/NCL/refolding chemistry (Figure 1b). Gratifyingly, MCoTI-II-Δ[SDGG] was found to be a 9 nM tryptase inhibitor, a 60-fold improvement over the parent compound, with K10 (P_1 = Lys) and K10R (P_1 = Arg) analogues showing identical activity (Table 2). Comparison of the TOCSY and NOESY NMR spectra of MCoTI-II with the Δ[SDGG] truncate (Supporting Information) and quantification of the change in chemical shift for NH and CH α-protons (Figure 3) indicated that the backbone is only moderately perturbed and at sites distant from the active loop, suggesting that the improvement in activity may indeed be due to the removal of Asp4. Interestingly, truncates with smaller or larger deletions showed significantly worse activity, presumably due to unfavorable conformational changes in the adjacent tryptase-binding loop.

MCoEeTI, a cystine knot MCoTI-II/EETI hybrid generated by recombinant expression that lacks the SDGG motif,

Table 1. K_i Values for MCoTI-II Analogues against Tryptase and Human Leukocyte Elastase[a]

cyclotides [Pro_9(P2)], [Lys_{10}(P1)], [Ile_6(P1′)]	K_i (pM), trypsin	K_i (μM), tryptase	K_i (nM), HLE
MCoTI-I	29 ± 2	1.6 ± 0.1	NI
MCoTI-II	75 ± 5	0.6 ± 0.3	NI
[K10R]	85 ± 7	0.1 ± 0.5	NI
[K10F]	$>10^5$	> 50	> 7500
[K10Q]	$>10^6$	> 50	NI
[K10A]	$>10^9$	> 50	32 ± 13
[K10V]	$>10^9$	> 50	21 ± 7
[PKI]-[AVP]	ND	> 50	500 ± 50
[K10V];[I11P]	ND	> 50	520 ± 50

[a] See Supporting Information for details of synthesis and assays. The values for trypsin are from ref 2. ND = not determined. NI = no inhibition observed.

Table 2. K_i Values for MCoTI-II Truncates and MCoEeTI Analogues against Tryptase and Trypsin[a]

cyclotides	K_i (nM), tryptase	K_i (nM), trypsin
Δ[DG]	600 ± 20	4.3 ± 1
Δ[SDGG]	9 ± 0.5	2.6 ± 0.5
Δ[SDGG][K10R]	10 ± 0.6	18 ± 4
Δ[SDGGV]	> 3000	130 ± 10
Δ[GSDGGV]	> 2000	155 ± 15
ck-MCoEeTI	110 ± 9	99 ± 9
MCoEeTI	33 ± 7	48 ± 5
MCoEeTI[K10R]	28 ± 2	8.8 ± 0.3

[a] Error values are SD across independent triplicate repeats.

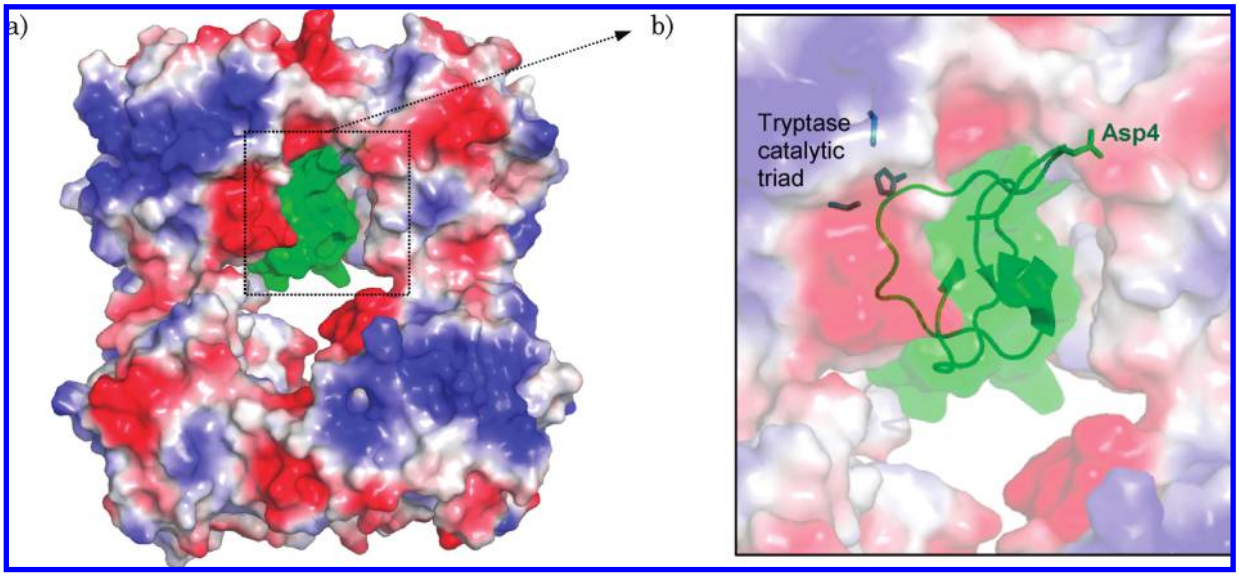

Figure 2. (a) MCoTI-II (green) docked at an active site of the tryptase tetramer (electrostatic potential surface): (blue) positive charge; (red) negative charge. (b) Inset showing Asp4 of MCoTI-II. Active site residues of the docked tryptase monomer (His57, Asp102, Ser197) are shown in black.

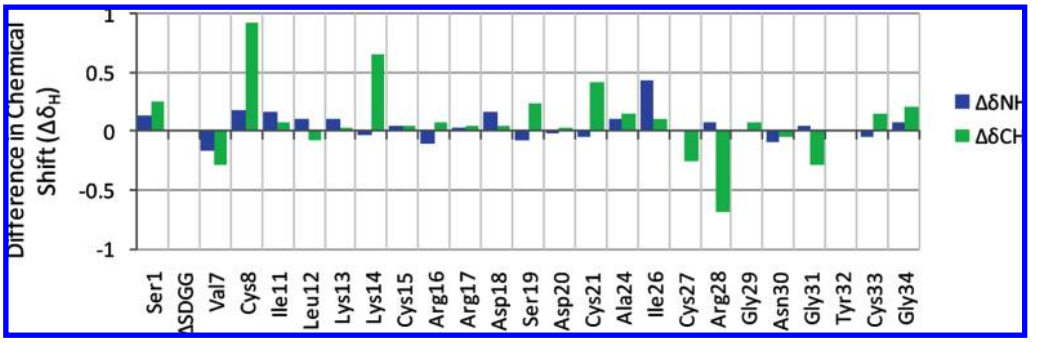

Figure 3. Change in chemical shift for backbone N−H ($\Delta\delta_{NH}$) and C−H α ($\Delta\delta_{CH}$) protons for Δ[SDGG]MCoTI-II relative to MCoTI-II (see Supporting Information for spectral data). Only signals assigned with high confidence are shown. ΔSDGG marks the Ser3 to Gly6 deletion in the mobile loop.

was recently reported to show submicromolar activity against tryptase.[21,25]

By way of comparison with our current study we synthesized the previously unknown cyclotide (backbone cyclic) form of MCoEeTI, along with the cystine knot analogue corresponding to the previously reported recombinant MCoEeTI. Linear knotted MCoEeTI shows tryptase inhibition over an order of magnitude weaker than the most active MCoTI truncates, while the cyclotide form shows only 3-fold higher K_i, indicating the importance of the cyclic backbone for efficient inhibition.[6,26] The previous study suggested that MCoEeTI attains 4-fold occupancy of the tryptase tetramer, though no supporting experimental evidence was provided.[21] The current study casts doubt on this assumption: all active cyclotides show inhibition compatible with a 1:1 complex, and our homology model suggests that significant structural rearrangement of enzyme and cyclotide would be required to allow access to the remaining tryptase active sites via the pore. Instead, it seems plausible that binding of one cyclotide is sufficient to block substrate access to the remaining three sites by occluding the pore.

The MCoTI-II truncates presented here further illustrate the impressive adaptability of the cyclotide scaffold. Together with our previous work, we have now shown that the MCoTI-II scaffold can be varied between 28 and 36 residues without affecting its ability to cyclize and fold under suitable conditions. Furthermore, protease inhibition by MCoTI-II can be readily reengineered to target β-tryptase, HLE, or chymotrypsin-like proteases[2] with excellent selectivity between enzymes. Notably, none of the cyclotides described here show inhibitory activity against thrombin,[27] an abundant serum protease. The well-established biostability of the cyclotides coupled with their potent activity suggests that they may represent a new class of potential therapeutics for the treatment of allergic disorders. Studies are ongoing to investigate the activity of MCoTI analogues in cell-based disease model systems.

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Supporting Information Available: Experimental procedures and characterization data (RP-HPLC, MALDI-ToF MS) for the preparation of MCoTI analogues, NMR data for MCoTI-II and the MCoTI-II Δ[SDGG] truncate, and inhibition assay details for trypsin, HLE, and tryptase. This material is available free of charge via the Internet at http://pubs.acs.org.

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