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Effective Mast Cell Degranulating Peptide Inhibitors of the IgE/FcεRI Receptor Interaction

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Previous studies with mast cell degranulating (MCD) peptide have shown that peptide [Ala^{12}]MCD 8 was an inhibitor of IgE binding to mast cell receptors. In an attempt to produce increased inhibition, analogs were synthesized that maintained the alanine residue in position 12 in the MCD peptide sequence and were further modified at both termini. Analogs modified at the C-terminus were [Ala^{12},desLys^{21}]MCD 2 and [Ala^{12},D-Lys^{21}]MCD 4. N-terminus modifications were [desLys^{6}–Arg^{7}–His^{8},Ala^{12}]MCD 1, [Ala^{6}, Ala^{12}]MCD 6, and [Val^{6}, Ala^{12}]MCD 7. To assess the role of the Proline^{12}, analogs [D-Ala^{12}]MCD 3 and [Meleu^{12}]MCD 5 were also synthesized. The analogs were tested for binding to the IgE receptor in cultured mast cells. Inhibitory activity of IgE-caused degranulation was measured using a β-hexosaminidase assay. Circular dichroism (CD) and molecular modeling of selected analogs were used to follow possible structural differences among these analogs. All analogs showed binding affinity to the IgE receptor and inhibition of IgE-induced mast cell degranulation at different levels. Differences in inhibition were most likely because of diverse interactions of the analogs with the receptor as inferred by the CD and modeling studies. Based on the results of the β-hexosaminidase assay, analog [Val^{6}, Ala^{12}]MCD 7 proved to be an excellent inhibitor of IgE-mediated mast cell degranulation.

Key words: FcεRI receptor, fluorescence binding, IgE, MCD peptides, β-hexosaminidase

Abbreviations: Fc, constant domain region of IgE; FcεRI, high-affinity receptor for IgE; IgE, monoanti-dinitrophenyl-mouse IgE; MeLeu, *N*-α-methyl-l-leucine; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid: TFE, 2,2,2-trifluoroethanol; TEAP, triethylammonium phosphate.

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Allergic disorders are a major health problem of epidemic proportions (1). Nearly 20% of the population suffers from some form of allergy (2). The body's response to allergens is an abnormal production of immunoglobulin E (IgE) molecules. The main target cells for IgE in the body are mast cells. Mast cells act as 'collectors' for circulating IgE that binds to the high affinity FcεRI receptors on the mast cell surface. When adjacent IgE molecules are cross-linked through the binding of multivalent allergens, the FcεRI receptors are aggregated. Subsequent activation of mast cells results in their release of granule-stored inflammatory mediators such as histamine, proteases, and cytokines (3). The release of these mediators initiates the type I immediate hypersensitivity reaction and the first phase in the allergic inflammatory pathway (4).

The broad spectrum of allergies and side effects caused by many anti-allergic drugs allow many alternative therapeutic strategies. One promising strategy is to find molecules that prevent IgE/FcεRI binding and thus IgE receptor activation. For this purpose, monoclonal antibodies that bind IgE in the circulation have been used to prevent IgE binding to mast cells and showed considerable therapeutic potential for certain allergies (5). Similarly, as an alternative strategy, small molecules such as peptides could be useful in blocking IgE binding to the FcεRI receptor.

Over time, various peptides from different sources were synthesized in order to block IgE/FcεRI binding and showed some promising results (6–9). Importantly, in these studies, it became apparent that disulphide-bonded peptides showed higher inhibition than linear ones. In our search for IgE/FcεRI binding inhibitors, mast cell degranulating (MCD) peptide which derives from a pool of biologically active peptides in bee venom, offered a natural choice because of its relative stability and modulatory action. [Pro^{12}]MCD **9** contains two disulphide bonds and multiple basic amino acids in its sequence. It is a non-immunological stimulus, i.e., it releases histamine at low concentrations in the absence of IgE. On the other hand, it also acts as an inhibitor of IgE-driven histamine release at concentrations higher than those at which it releases histamine (10). Synthetic MCD peptide has been found to bind to mast cell receptors and, to some degree, inhibit IgE binding (11). Consequently, over the years, our efforts to find better inhibitors of the IgE/FcεRI interaction led to many biological and structural studies with MCD peptide analogs (12 and references cited therein). Eventually, alanine scans of the MCD peptide sequence (13,14) resulted in [Ala^{12}] MCD peptide **8** in which alanine was substituted for the proline residue in position 12 (see sequence in Materials and Methods). This analog has been found to compete effectively with IgE binding to the FcεRIα subunit of the human IgE receptor at μM

concentrations and to have low histamine-releasing activity. It also inhibited mast cell degranulation caused by IgE showing a 50% inhibition of β-hexosaminidase secretion at a 100 μM concentration (15).

Based on findings with the [Ala^{12}] peptide **8**, we sought to improve its properties using peptide strategies to produce antagonism (16).

Because a single proline substitution in position 12 switched agonism to antagonism (15), at first, we changed only this position by means of amide backbone modifications, either by reversing the direction of the peptide backbone (D-Ala) or by reducing the peptide backbone (MeLeu). Second, we prepared analogs that maintained the alanine in position 12 in the sequence in combination with side chain substitutions or deletion of positive charged amino acids in order to enhance hydrophobicity, which might increase antagonism, yet, minimize histamine release (17). We hypothesized that an additive effect resulting from these modifications would improve the peptide's inhibitory properties.

Materials and Methods

Peptide synthesis

The peptide analogs were derived from [Ala^{12}]MCD peptide **8** with the sequence: I K C^{3} N C^{5}K R H V I K A^{12} H I C^{15} R K I C^{19} G K N-NH_2; the disulphide bonds connected at C^{3,15} and C^{5,19}. Analogs were synthesized and analyzed according to a well-established protocol as previously described and used for all MCD peptide analogs reported by us (13 and references cited therein).

HPLC of analogs **1**–**7** in gradient (A) 10–40% of 0.1% TFA and 0.1% TFA in acetonitrile : t_R min: **1** = 13.2; **2** = 12.9; **3** = 12.8; **4** = 12.8; **5** = 13.4; **6** = 13.5; **7** = 13.9. In gradient (B), 10 mM TEAP/5 mM butanesulfonate/5%1-propanol and 10 mM TEAP/5 mM butanesulfonate/15% 1-propanol: t_R min: **1** = 13.5; **2** = 13.3; **3** = 13.3; **4** = 13.3; **5** = 13.7; **6** = 14.1; **7** = 14.5.

Mass (Micromass Q-Tof spectrometer) for analogs **1**–**7** was: **1 =** 2139.4 (calcd. 2139.7); **2** = 2433.2 (2433.1); **3** = 2561.2 (2161.5); **4** = 2489.9 (2490.1); **5** = 2635.1 (2635.3); **6** = 2504.0 (2504.2); **7** = 2532.4 (2532.2).

Histamine release

Histamine-releasing activity of the analogs was assayed using purified rat peritoneal mast cells; histamine release was detected fluorometrically and data analyzed as described previously (18).

β-Hexosaminidase assay

Rat basophilic leukemia cultured mast cells (RBL-2H3) were used (19) and the enzyme assay was performed as described previously for the [Ala^{12}] analog (15). Briefly, attached cells to well plates loaded with various concentrations of peptide/IgE mixtures or only IgE and containing the same amount of 0.460 μg/mL IgE were incubated for 90 min at 37 °C. After several washing with buffer, the IgE was cross-linked with dinitrophenyl human serum albumin and aliquots of the released enzyme in the cell supernatants were treated with the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidase. The degree of enzyme release in the supernatants was measured as the changes in OD as detected using a plate reader. Data were analyzed as described previously (15).

Fluorometric receptor-binding assay

Rat basophilic leukemia cells RBL-2H3 stably transfected with the human α, β, and γ subunits of the human FcεRI receptor (RBL SX – 38) were a generous gift from J. P. Kinet (Harvard Medical School). RBL SX-38 cells were grown in Eagle's minimal essential medium (EMEM) with glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 mg/mL geneticin (G418) (all Invitrogen, Carlbad, CA, USA) in a humidified atmosphere of 5% CO_2 in air at 37 °C (20). The medium was changed until the cells reached 80% confluence. The adherent cells were than treated overnight with dexamethasone (Sigma, St Louis, MO, USA) at a 10 μM final concentration in order to induce FcεRI expression (21). The adherent cells were washed with fresh medium, detached from the plates by gentle scraping and adjusted to a density of 5×10^4 cells/100 μL in EMEM. The concentrations of the peptides ranged from 10 nM to 1 μM. 100 μL cells and peptides were incubated overnight in 96-well black plates with a flat black bottom (BD Biosciences, Franklin Lakes, NJ, USA). After aspiration of the medium and several washings with EMEM, 4 μL (~20 nM stock) fluorescent IgE (Accurate Chemicals, Westbury, NY, USA) in a final volume of 100 μL EMEM was added to each well and the adherent cells were incubated for another 2–3 h at 37 °C. After aspiration the cells were washed four times with warm phosphate-buffered saline (PBS). Then 100 μL PBS was added to each well and the fluorescence of the adherent cell was measured on a fluorescence plate reader (Bio-TEK, Winooski, VT, USA). The filters used were 485 nm for excitation and 530 nm for emission. Using the same procedure, comparative binding was performed with [Ala^{12}]MCD previously tested using anisotropy (15) as a blank and with unlabeled IgE. The ID_{50}, was determined by nonlinear regression using ORIGIN software (Microcal, Amherst, MA, USA).

CD spectroscopy

Analogs were analyzed in 50% TFE and water at room temperature from 190 to 250 nm. Circular dichroism (CD) spectra in TFE were recorded with an AVIV 62-DS spectrophotometer (path length 1.0 mm). CD spectra in water were recorded with a Jasco J 810 instrument (path length 0.1 mm). Accumulation time was 15 seconds and bandwidth was 1 nm. The spectra were expressed in units of molar residue ellipticity. The α-helical content of the peptides was calculated using a known algorithm (22).

Modeling and simulation methods

Structure modeling: MCD peptide structures were modeled based on the experimentally determined structure of tertiapin (Data Bank: PDBid, 1TER) (23). The Modeller-9v1 program was used to model the MCD peptides (24). Five putative models were generated and the model with the lowest global DOPE (Discrete Optimized Protein Energy) score in the key residues was used for further struc-

Table 1: Binding affinities of MCD peptide analogs to the FcεRI human mast cell receptor and histamine-releasing potency

No	Analog/peptides	Histamine release ED_{50} (10^{-5} M)[a]	Binding IC_{50} (μM)[b]
1	[desLys^6–Arg^7–His^8,Ala^{12}]MCD	1.70	0.61 ± 0.06
2	[Ala^{12},desLys^{21}]MCD	0.78	0.15 ± 0.01
3	[D-Ala^{12}]MCD	0.26	0.12 ± 0.07
4	[Ala^{12},D-Lys^{21}]MCD	0.64	0.17 ± 0.02
5	[NmeLeu^{12}]MCD	0.25	0.60 ± 0.08
6	[Ala^6,Ala^{12}]MCD	0.32	0.64 ± 0.02
7	[Val^6,Ala^{12}]MCD	0.66	0.60 ± 0.05
8	[Ala^{12}]MCD	21.24[c]	0.04 ± 0.002
9	[Pro^{12}]MCD standard	0.16	–
	IgE	–	0.01 ± 0.001

[a]Binding inhibition was performed using transfected rat basophilic leukemia RBL SX-38 cells using fluorescent IgE and various peptide concentrations in micro well plates. The fluorescence of the adherent cells was read using a plate reader. ID_{50} is the concentration of peptides displacing 50% of receptor bound fluorescent IgE (±SEM, n = 4).
[b]Histamine release was done using peritoneal mast cells, ED_{50} is the concentration of peptides at 50% maximal histamine release. It was calculated using a sigmoidal dose–response curve fit where the bottom of the curve was 0% and the top 100%. R2 for each curve fitting was 0.99 (n = 6).
[c]Data taken from Ref. (13).

tural and computational studies (25). Amino- and amide-terminals were then added to the peptide with the VMD (Visual Molecular Dynamics, psfgen module) program (26). Molecular dynamics (MD): Mutants of analogs **4**, **7**, **8,** and **9** (Table 1) were generated using the same method and subsequently were minimized with 2000 conjugate gradient energy minimization steps. Each minimized peptide was solvated with a 35Å^3 TIP-3 water box and the system's charge was neutralized with chlorine ions. The whole system was then minimized for an additional 2000 steps. The solvent around the fixed peptides underwent a heating phase of 20 ps during which the temperature was slowly increased from 0 to 310 K, by rescaling the velocities every 100 steps until the 310 K target temperature was reached. Peptide restrains were gradually relaxed over the subsequent 5 ps simulation.

All molecular dynamics simulations and minimizations were run using the macromolecule simulation package with programs NAMD2.6 (27) and Charmm-27 force fields (28). Periodic boundary conditions were used to eliminate boundary surface effects. The particle Mesh Ewald (PME) method was used to increase the accuracy in the representation of the long-range electrostatics (29). The RATTLE algorithm was used to constrain all bonds involving hydrogen atoms. Each system underwent a 10 ns equilibration run and a 100 ns production run under isothermal-isobaric (NPT) conditions. The average size of all the systems was ∼6000 atoms.

Results and Discussion

Results of earlier studies with the [Ala^{12}]MCD peptide **8** (15), led us to synthesize various analogs of this peptide in order to increase inhibition of IgE-mediated mast cell degranulation. Table 1 shows the potency of the histamine-releasing activity of analogs **1**–**7** compared to MCD peptide **9**. The values obtained for histamine release by peritoneal mast cells showed that the new analogs had decreased potency compared to peptide **9,** which is the standard for all MCD peptide analogs tested in this assay. The diminished potency of the analogs is mainly because of the loss in overall basicity of peptide **9** that is generally thought to be responsible for histamine release (30). Analogs **2** and **4** showed that the loss of potency was greater when the change was in the basic amino acid Lys^{21} at the C-end either by deletion of this amino acid or by introducing its optical isomer. Interestingly, the substitutions D-Ala^{12} in analog **3** and MeLeu^{12} in analog **5** were able to simulate the role of Pro^{12} by showing almost the same potency as peptide **9.** All analogs were more potent than peptide **8.**

All analogs tested for histamine release from rat peritoneal cells displayed typical sigmoidal histamine release responses and released 100% histamine at sufficient dose, which varied among the analogs. Thus the analogs had equal efficacy in the test system, although the potency as indicated by the ED_{50} varied. Because this response is asymptotic and thus difficult to measure effectively as it approaches 100%, for precision, the 50% release doses are reported as histamine-releasing activity, as has been performed often historically (31).

With respect to their binding affinities to the FcεRI receptor (Table 1) the analogs can be ordered in two groups: group 1, comprised of analogs **1**, **5**, **6**, and **7** with changes toward the amino end of the sequence, and group 2, comprised of analogs **2**, **3**, and **4** with changes toward the carboxyl end. Group 1 showed a four-fold decrease in binding affinity compared to group 2. Peptide **8** showed a three-fold increase in binding affinity compared to that of the analogs in group 2.

The inhibition of IgE-induced β-hexosaminidase by the analogs was assayed in rat RBL-2H3 cultured mast cells. These cells are homologous to mucosal mast cells and also carry the FcεRI receptor (32). The enzyme β-hexosaminidase is a component of the mast cell granules and is useful as a measure of mast cell degranulation. Figure 1 shows the inhibition of IgE-mediated β-hexosaminidase release by the monosubstituted analogs of peptide **8** as compared to that of IgE. No changes in inhibition were achieved beyond the indicated concentrations.

Based on the results of the β-hexosaminidase assay all analogs were to some degree competitive inhibitors of IgE and showed increasingly inhibition in the order initiated (analogs **1**–**7).** All monosubstituted analogs **1**–**7** showed inhibition in the nanomolar concentration range and improved inhibition by four orders of magnitude (10–40 nM) compared to peptide **8** which showed 50% inhibition at 100 μM (15). Analog **7** reached almost complete inhibition of IgE-caused degranulation showing 95–98% inhibition at 10 and 20 nM concentrations.

The apparent discrepancies between the values of inhibition of binding to the IgE receptor and of hexosaminidase release inhibition in RBL-2H3 cells as seen with our ligands (Table 1 and Figure 1), have also been observed previously in studies of IgE/receptor

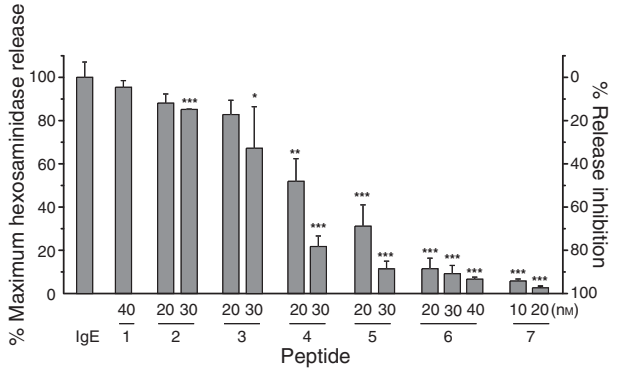

Figure 1: Inhibition of IgE-induced β-hexosaminidase release by MCD peptide analogs. The numbers refer to analogs identified in Table 1. Bars represent means ± SEM of 10 separate experiments. The assays of all analogs were performed with the same batch of cells on each single day. Secreted β-hexosaminidase was expressed as % of total cellular enzyme. The maximum release of β-hexosaminidase by IgE in the absence of peptides was taken as 100% release, i.e., 0% inhibition. *$p < 0.06$, **$p < 0.01$, ***$p < 0.0001$, obtained by one way ANOVA analysis. Peptides **8** and **9** (not shown) had 50% and 0% inhibition at 100 μM concentration (15).

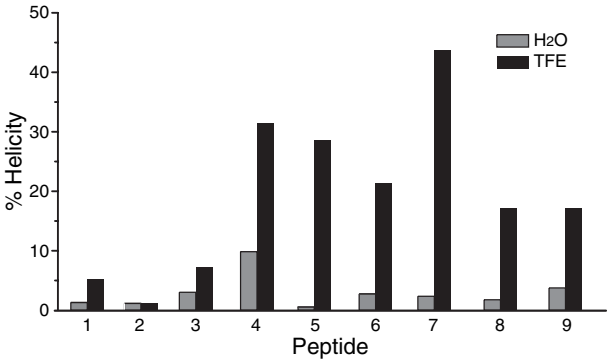

Figure 2: Helical content of MCD peptides and analogs as seen by CD spectroscopy. The numbers refer to analogs identified in Table 1. CD spectra of the analogs were taken in water and in 50% TFE at room temperature and expressed in units of molar residue ellipticity. % helicity was calculated at 222 nm and compared to these of the peptides **8** and **9.**

interactions (6,9,33). Although many compounds inhibit binding of IgE to the cell to different extents, only a small fraction of the cell receptors were required for maximal degranulation (34). The precise nature and degree of this divergence depends on the structural preferences of the receptor and the intrinsic properties of the analogs.

Structural changes in peptide **8** because of additional substitutions have occurred in the secondary and tertiary structures of the analogs as seen by CD spectroscopy and molecular modeling in this study. On the other hand, X-ray crystallography and mutagenesis studies with the FcεRI receptor showed two binding domains D1 and D2 for IgE; four surface tryptophans at the top of the D2 domain of the receptor are implicated in IgE binding (35).

Therefore, our results may be attributable to diverse binding of the analogs to the one or the other domain of the receptor. The analogs might be positioned within or between these two binding domains and at different distances from the D2 tryptophan location. In this respect, analog **6** and especially analog **7** with their hydrophobic amino ends may interact through hydrophobic interactions with the tryptophan residues in D2 in such a way as to produce maximum inhibition. Furthermore, the results of the assays indicated that full receptor occupancy is not required for triggering a maximal biological response (9). The properties of peptide **8** also support this assumption [Table 1 and Ref. (15)].

CD studies and partial molecular modeling of selected analogs were performed to ascertain whether there is a structural rationale for the inhibitory properties of the analogs. As observed previously with CD (36) and with NMR studies (37) of MCD peptide analogs, a helical part at the C-terminus between Ile^{14} and Lys^{21} is included in the structure of MCD peptide **9** that influences activity (36).

Figure 2 shows the helical content of the analogs and of peptides **8** and **9** calculated from CD spectra. In water, the analogs and peptides were mainly unstructured with little helical propensity. However 50% TFE conferred flexibility and significantly increased the helical content of some of these analogs.

The low helical content of analog **1** with three deletions in the N-terminus may be due to changes in hydrogen bonding of the helix between the C- and N- termini (37). Analog **2** in which Lys^{21} is deleted from the helix is, as expected, devoid of helical content. CD spectra obtained in both water and TFE indicate that peptide **8** with L-Ala^{12} has a higher helical content than analog **3** with D-Ala^{12} (38).

In contrast, analog **4** with D-Lys^{21} exhibited enhanced helicity compared to peptide **8** with L-Lys^{21} (39). Analog **5** with $MeLeu^{12}$ showed no helical content in water but assumed helicity in TFE that is higher than that of its counterpart analog **3** with D-Ala^{12}, and peptides **8** with L-Ala^{12}, and **9** with Pro^{12}. Analogs **6** and **7** showed the same helical content in water. In TFE analog **7** is higher in helical content compared to analog **6** and all the other analogs tested. Analogs **7** showed the highest inhibitory effect. Peptides **8** and **9** had similar helical contents. However, they showed lower helicity and inhibitory activity (15) compared to the strongest inhibitors, analogs **4**, **5**, **6**, and **7.** There seems to be a correlation between inhibition and secondary structure with respect to the Ala^{12} monosubstituted analogs. The observation that the inhibitory effect increases with increasing helicity indicates that the α-helix is important for inhibition.

To further understand the three-dimensional structure of the analogs, we modeled analogs **4**, and **7** and peptides [Ala^{12}]MCD **8** and [Pro^{12}]MCD **9**, all shown in Figure 3. Because no complete NMR structure of the parent peptide **9** is available, it was

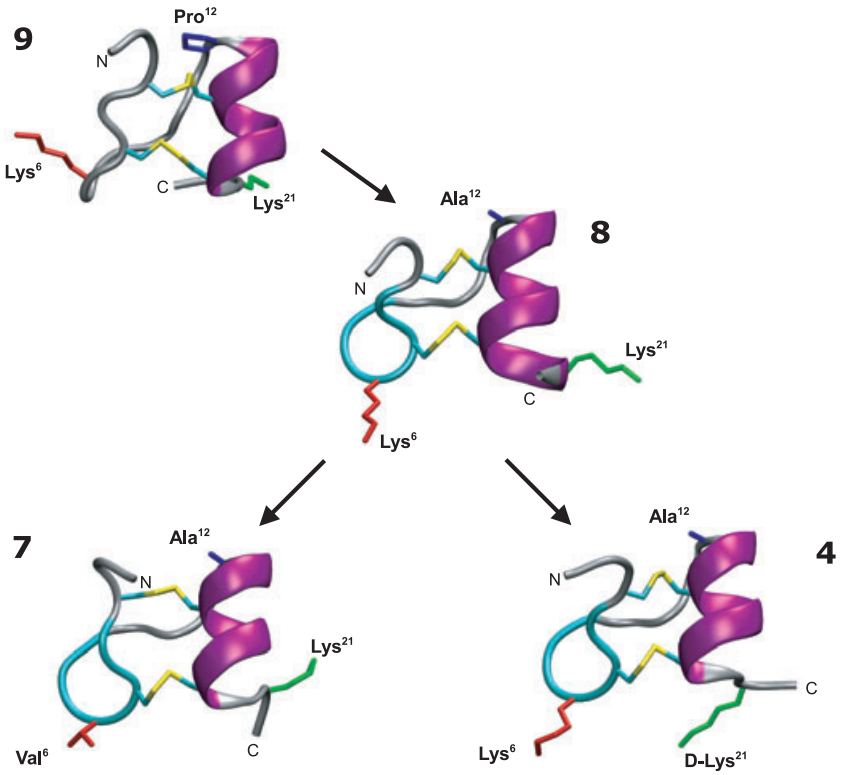

Figure 3: Tertiary structure of MCD peptides and analogs. Structures of peptides: **9**: [Pro^{12}]MCD; **8**: [Ala^{12}]MCD; and of analogs **4**: [Ala^{12},D-Lys^{21}]MCD; and :**7** [Val^{6}, Ala^{12}]MCD. The C and N letters denote the carboxyl and amino ends, respectively. Lys^{21}, green; Lys^{6}, red; Ala^{12}, and Pro^{12}, blue. Models were based on the structure of **9** and were constructed as described in Materials and Methods.

modeled based on the structure of tertiapin in an aqueous medium (22). Based on the peptide **9** model, peptide **8** was modeled as well as its monosubstituted analog **4** with the highest ratio of binding to inhibition and analog **7** with the highest inhibition. In the models, the Lys^{6} at the amino end and the Lys^{21} at the carboxyl end are used as orientation markers for the positions of the two termini.

Significant differences in the overall structure of peptides **8** and **9** are observed. Differences had already been observed previously using Monte Carlo simulations (15). Structural differences are also apparent between peptide **8** and analog **4**. These differences are small at the amino end but are significant at the carboxyl end. Comparing analog **7** and peptide **8**, the differences are greater at both ends. In these models, most of the C-terminal region is structurally conserved as an α-helix. The N-terminal region appears more variable and therefore may cause deviations at the C-terminus as a result of the spatial relationship between the N- and C-termini. The models suggest that the N- and C- termini in analogs **4** and **7** are not similarly oriented as in peptide **8**. This spatial orientation is important for receptor recognition and binding in MCD peptide analogs (35).

The order of increase in peptide binding affinity is analogs **7** < **4** < **8** (Table 1) while the order of increase in inhibition is **8** < **4** < **7** (Figure 1). Because the potencies of the analogs are attributed to the helix [Ref. (34) and this study], it is tempting to assume that the C-terminus is important for activity and that the N-terminus represents the binding site. The variable N-terminal site and its approach to the receptor may be decisive for the degree of activity of the analogs, i.e., inhibition.

Conclusion

Monosubstituted analogs derived from [Ala^{12}]MCD peptide **8** were found to compete with IgE binding to the FcεRI receptor and to inhibit release of β-hexosaminidase, a known mediator of mast cell degranulation, i.e., allergic reaction. Their effects were additive to the inhibitory activity of peptide **8** and thus greatly improved the inhibition of IgE-induced mast cell degranulation. Notably, analog [Val^{6}, Ala^{12}] **7** almost completely inhibited mast cell secretion in the β-hexosaminidase assay and represents a significant improvement over the ones previously reported. Furthermore, a relative dissociation between histamine-releasing potency and inhibition of release was achieved with the analogs. This is significant because therapeutically inhibitors with negligible histamine-releasing potency are required. Thus, the biological and structural studies of these peptide analogs may promote the search for still stronger peptide inhibitors of the IgE/FcεRI interaction with little or no histamine-releasing potency.

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