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Exploration of α/β/γ-Peptidomimetics Design for BH3 Helical Domain

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Systematic incorportation of ring-constrained β- and γ-amino acid residues to α-helix mimetics engenders stable helical secondary structures. In this paper, functional α/β/γ-helical peptidomimetics were explored for the mimicry of BH3 helical domain, Bim as a pioneering study. The Bim-based α/β/γ-peptides in an αγααβα-hexad repeat with five helical turns inhibited the interaction between Bak and Bcl-x_L with excellent resistance against proteolytic digestion. Further optimization of the α/β/γ-backbone strategy will considerably expand the utilities of functional α/β/γ-peptidomimetics especially with its prominent stability against proteolysis.

Fine-tuned modulation of protein-protein interactions (PPIs) involving helical contacts with helical peptidomimetics is highly desirable for pharmacological applications.[1] Natural α-amino acid-based helical peptidomimetics have critical limitations with respect to biomedical applications, mainly for their rapid proteolytic degradation under physiological conditions. Foldamers with a heterogeneous backbone containing α- and β^3-residues can mimic a canonical α-helix with enhanced proteolytic stability, and further, improved activity was achieved by selective incorporation of five-membered ring-constrained β-amino acid residues (β^c) for their pre-organization of the helical backbone (Fig. 1). [2-4]

γ-Amino acid residues have been explored as additional chemical moieties for distinct structural peptidomimetics. Wilson and Aitken et al. have described the sequence-based design of hexameric α/β/γ-peptides with either αγβγβγ or an

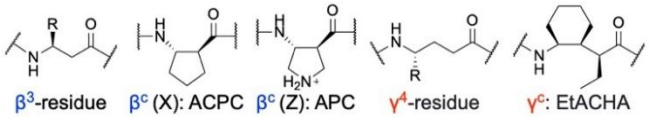

Fig. 1 Chemical structures of acyclic-/cyclic- β and γ residues used in the current study. APC was used instead of ACPC when a charge was needed.

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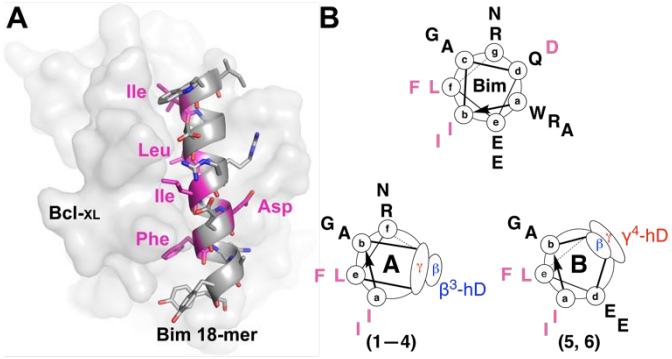

Fig. 2 (A) Co-crystal structure of Bim and Bcl-x_L (PDB 3FDL). A partial Bim sequence (18-mer) is shown here. Important residues for the binding to Bcl-x_L are highlighted in magenta. (B) Helical wheel diagrams of Bim 15-mer (all α heptad repeat) and α/β/γ-peptides with two αγααβα hexad registers. Key residues involved in binding to Bcl-x_L are highlighted in magenta.

αγβγβα backbone that function as specific inhibitors of the p53-*h*DM2 interaction.[5] In this study as well as other group's study including ours, γ^4-residues, despite the increased conformational freedom of the peptide backbone with two extra methylene units, have appeared to be favorable for helical folding (Fig. 1).[5-11] Further studies involving various ring-constrained γ-residues for the pre-organization of helical peptide backbones led to the creation of a *cis*-cyclohexyl-containing γ-amino acid, EtACHA, which showed adaptive helical folding when incorporated in γ-, α/γ-, β/γ-, and α/β/γ-peptides in solution as well as crystalline states (Fig. 1).[6,12-15]

Based on the information about the ring-constrained EtACHA and γ^4 amino acids, we previously set out for α/β/γ-peptide design for 'α → β or γ' replacement in an iso-atomic manner: the number of backbone atoms in an αααααααα heptad making about two helical turns in a canonical α-helix, is the same as that in a 4:1:1 α:β:γ hexad in an α/β/γ-peptide.[6,7,12] The *de novo* designed α/β/γ-peptides with an αγααβα hexad pattern containing cyclic β- and cyclic/acyclic γ-residues formed canonical α-helix-like structures in an aqueous buffer, which was demonstrated through X-ray and NMR studies.[6,12]

Herein, we further explored the designability and application of α/β/γ-peptides in αγααβα hexad repeats for PPIs modulation using a well-studied model system, the interaction

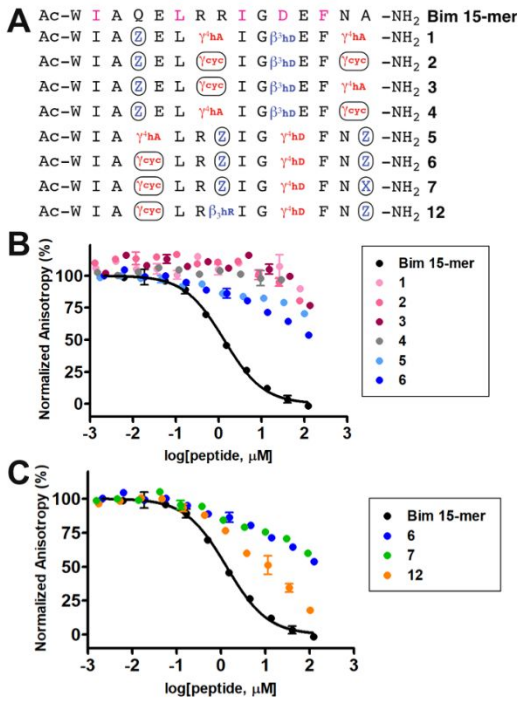

Fig. 3 (A) Sequences of α-Bim 15-mer and the analogous α/β/γ-peptides. (B, C) Inhibition curves from the Bcl-x_L competition binding FP assays using α-Bim 15-mer and α/β/γ-peptides **1**-**7** and **12** (Bim 15-mer IC_{50} = 1.7 ± 0.4 μM).

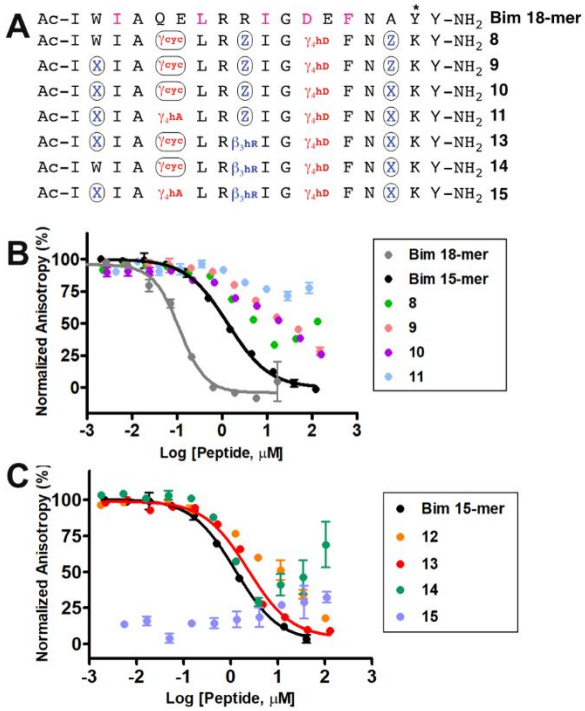

Fig. 4 (A) Sequences of α-Bim 18-mer and the analogous α/β/γ-peptides. Bcl-xL competition binding FP data converted to % inhibition (B) for α-Bim 18-mer and the analogous α/β/γ-peptides **8**-**11** (C) for α/β/γ-peptides **12**-**15** containing β^3-Arg. Inhibition curves from the Bim 15-mer is overlaid in these graphs (B and C) for the comparison with α/β/γ-peptides (Bim 18-mer IC_{50} = 0.11 ± 0.02 μM, Bim 15-mer IC_{50} = 1.7 ± 0.4 μM, α/β/γ-peptide **13** IC_{50} = 2.6 ± 0.3 μM). *A previous hydrophile scan analysis indicated that the modification of Tyr-17 to Lys does not affect the affinity of 18- mer Bim peptide to Bcl-x_L (ref 18).

between helical BH3 domain, Bim, and a hydrophobic cleft within B-cell lymphoma-extra large (Bcl-x_L) protein (Fig. 2A).[16]

The α/β/γ-peptides in αγααβα hexad pattern were designed based on the helical Bim peptide sequence, aiming to precisely mimic the side chain projections of five key residues (Leu, Phe, Asp and two Ile residues) critical for binding Bcl-x_L.[17,18] In the X-ray crystal structure of Bim and Bcl-x_L complex (PDB 3FDL), the hydrophobic residues of Leu, Phe and the two Ile of Bim are displayed along the same side of the helical face recognizing the hydrophobic cleft of Bcl-x_L. The Asp of Bim is located at the opposite face of the hydrophobic residues and forms a critical salt-bridge with the side chain of Arg in Bcl-x_L (Fig. 2).[17,18] Keeping these key structural features intact, we designed two αγααβα hexad registers **A** and **B** (Fig. 2B). For the carboxylate group of Asp in α/β/γ-peptides, we incorporated either acyclic β^3-homo-Asp (β^3-hAsp) or γ^4-homo-Asp (γ^4-hAsp), for their corresponding β- or γ-residues, respectively. These design features resulted in four helical turns of 15-mer-Bim-mimicking 13-mer-α/β/γ-peptides **1–4** containing β^3-hAsp in register **A**, and peptides **5** and **6** containing γ^4-hAsp in register **B** (Fig. 3A). Different numbers of cyclic γ-residues were incorporated in these peptides, to test whether the substitution of acyclic-to-cyclic γ residues affects peptide activity by promoting the helical conformations in α/β/γ-peptides.[6,7] To compensate for the hydrophobicity increase caused by the incorporation of EtACHA and to maintain the overall characteristics of the peptides, we used a positively charged cyclic β-amino acid, APC (Z) for α/β/γ-peptides **1–6**.

To evaluate the PPI modulation of the α/β/γ-peptides against the Bak and Bcl-x_L interaction, we performed a fluorescence polarization (FP) competition assay. A fluorescence-labeled pro-apoptotic Bak-derived 16-mer peptide (Bodipy-GQVGRQLAIIGDDINR-NH_2, tracer-Bak) bound to Bcl-x_L was released by titrating Bim-derived α/β/γ-peptides (Fig. S1 and Table S3).[3,4] The FP assay data indicated that α/β/γ-peptides **5** and **6** in register **B** were more promising candidates for inhibiting Bak/Bcl-x_L interaction, compared to α/β/γ-peptides **1–4** in register **A** (Fig. 3B).

With register **B**, we investigated the effect of introducing one additional helical turn by adding three residues mimicking α-Bim 18-mer, resulting in 16-mer-α/β/γ-peptides (Fig. 4) Previous studies suggested that the Bim 18-mer displayed tighter binding to Bcl-x_L than its analogous 15-mer α-peptide (Fig. 3A and 3B), possibly due to the higher helical population of Bim 18-mer than that of Bim 15-mer (Fig. S2).[3,4] Importantly, the enhanced binding affinity of the extended peptides would indicate that the designed α/β/γ-peptides fold into secondary structures similar to that of the native α-helix.

The hydrophobic residue extension involving Ile and Trp near the N-terminus raised an aggregation issue (α/β/γ-**8**) thus, Trp was replaced with ACPC (X). The resulting 16-mer α/β/γ-peptide **9** showed an improved affinity for Bcl-x_L compared to 13-mer α/β/γ-peptide **6** (Fig. 3, 4B). Residue extension promoted the better affinities of α/β/γ-peptides to Bcl-x_L but the limited activities of α/β/γ-peptides **9** and **10** required further design optimizations (Fig. 4B).

The low inhibitory ability of α/β/γ-peptides **9** and **10** may be caused by the loss of the side chain of Arg, replaced by cyclic β- or γ- residues bearing no side chains. The previous "hydrophile scan" analysis with anionic mutations on the Bim sequence indicated that the two Arg residues in the middle of Bim were important for binding to Bcl-x_L.[18] We therefore restored the side chain by incorporating β^3-hArg in place of APC (Z) in α/β/γ-peptide **12** (Fig. 3A). Previously, without β-residue ring-constraints, α/β/γ-peptides generally showed diminished helical propensities.[7] Surprisingly, however, Bim 15-mer derived α/β/γ-peptide **12** showed a significantly improved binding affinity to Bcl-x_L compared to peptide **6**, suggesting a favored interaction involving Arg side chains (Fig. 3C).

Further Arg restoration was tested in the longer version of α/β/γ- peptides **13**–**15** that are Bim 18-mer analogues (Fig. 4A). In these α/β/γ-peptides, ACPC (X) was used in place of APC (Z) near the C-terminus, since peptide **10** containing ACPC (X) showed slightly better activity than peptide **9**. The final α/β/γ-peptide **13** showed an improved affinity for Bcl-x_L comparable to that of the α-Bim 15-mer (Fig. 4B, IC_{50} of Bim 15-mer ≈ 1.7 μM, IC_{50} of **13** ≈ 2.6 μM). This indicates that the side chain of β^3-hArg in **13** promotes suitable projections of Arg residues to interact with Bcl-x_L, whereas cyclic β residue APC (Z) in **10** cannot induce these favorable contacts (Fig. 4C).

While evaluating the binding of Bim-derived α/β/γ-peptides to Bcl-x_L using FP assays, **8** and **14**, which contain Trp near the N-terminus, showed FP signal back-up at higher concentrations (> 10 μM) (Fig. 4 and S3). To confirm the aggregation of α/β/γ-peptide **8**, a direct binding FP assay with Bcl-x_L was performed with a fluorescein-labeled derivative of the α/β/γ-peptide **8**. High FP values were observed at all concentrations of Bcl-x_L, indicating the self-aggregation of **Flu-8** (Fig. S3B). We could not identify any α/β/γ-peptides that did not aggregate when the α/β/γ-peptide was derived from the Bim 18-mer containing Trp, indicating the incorporation of hydrophilic cyclic γ-amino acids could be useful for future research. [15]

To evaluate the relationship of α/β/γ-peptides activity and helicity, we subjected peptides **10**, **13**, and **14** in circular dichroism (CD) spectroscopy (Fig. 5). We have previously shown that the CD spectrum with a single minimum in the 203-205 nm region is indicative of helical structure in the α/β/γ-peptide.[6,7,12,19] The most active α/β/γ-peptide **13** showed a good helical population with a minimum at 205 nm in both PBS and 50% MeOH/PBS buffers (Fig. 5). Since **13** had better activity than **10,** but showed similar helical folding, this suggests that the guanidinium groups on Arg or β^3-hArg in **13** formed a positive contact with the binding partner, Bcl-x_L. It is noteworthy that without a ring-constrained γ-residue near the N-terminus, α/β/γ-peptides **5**, **11** and **15** showed significantly decreased Bak inhibition, and this could be due to the flexible backbone and low helical propensity.

Since α/β/γ-peptide **14** showed reasonable binding at non-aggregating concentrations (< 8 μM), we were interested in its folding behavior (Fig. 4C). The α/β/γ-peptide **14** also showed a minimum at 205 nm in aqueous solutions indicating an α-helical folding (Fig. 5).[20, 21] The helicity data of **14** suggest that future optimization addressing the aggregation issue of α/β/γ-peptides containing Trp residue may lead to higher affinity to Bcl-x_L.

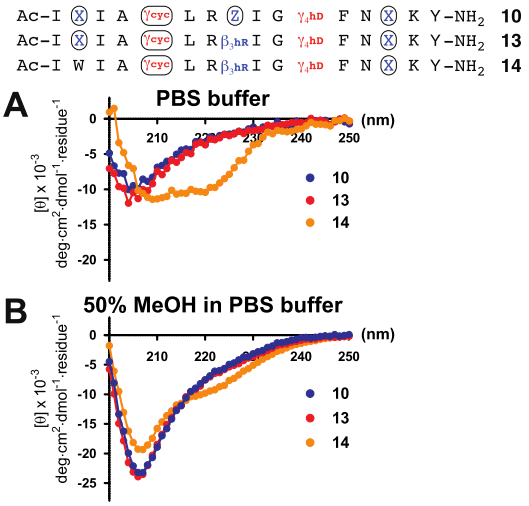

Fig. 5 Circular dichroism (CD) spectra of α/β/γ-peptides **10**, **13** and **14**. (A) in PBS buffer pH 7.4 at 20 °C and (B) in 50 % MeOH/ 50 % PBS buffer at 20 °C. Concentrations of the α/β/γ-peptides are 50 μM.

The heterogeneous backbone in peptides containing non-natural amino acid residues offers therapeutic promise owing to its α-helix-like folding propensity and its proteolytic stability in physiological environments.[2-4] To test the susceptibility of the Bim-derived α/β/γ-peptides to enzymatic cleavage, α-Bim and the analogous α/β/γ-peptides were incubated with a promiscuous protease, proteinase K, and the cleavage process was monitored by HPLC. As expected, α-Bim 15-mer and 18-mer were rapidly degraded in the presence of proteinase K, with a half-life of approximately 2-3 min (Table 1, Fig. S4).[4] On the other hand, we observed substantial proteolytic resistance of α/β/γ-peptides. No significant degradation was detected for α/β/γ-peptides **6** and **10** even after 20 h and 90 h incubation with proteinase K, respectively (Fig. S5 and S6). The most active α/β/γ-peptide **13** showed minimal degradation at 90 h post incubation with proteinase K (Fig. S6 and S7) but its half-life is 300-fold better than the α-analogue and further 4-fold greater than the reported value for the α/β-analogue containing five cyclic-β residues (Table S2).[4] This prominent proteolytic stability highlights that the α/β/γ-heterogeneous backbone is useful as a chemical moiety for the design of peptide-mimics which display a long-term activity *in vivo*.

Table 1 Susceptibility of the Bim BH3 domain and selected α/β/γ-analogues to degradation by proteinase K.

		$t_{1/2}$
Bim 15-mer	Ac–W I A Q E L R R I G D E F N A –NH_2	3.1 min
Bim 18-mer	Ac–I W I A Q E L R R I G D E F N A Y Y–NH_2	1.7 min[a]
6	Ac–W I A γcyc L R Z I G γ4hD F N Z –NH_2	>10 h
10	Ac–I X I A γcyc L R Z I G γ4hD F N X K Y–NH_2	>45 h
13	Ac–I X I A γcyc L Rβ3hR I G γ4hD F N X K Y–NH_2	>15 h

[a] Data is from ref 4 (the same proteolysis condition). The $t_{1/2}$ values are based on the last hours tested.

To probe the activity gap between 18-mer Bim and α/β/γ-peptide **13**, we further performed an *in silico* study with 18-mer Bim and α/β/γ-peptide **13** bound Bcl-x_L to assess the projections

of key residues for Bcl-x_L binding (Figs. S8-S10). The helical structures of 18-mer Bim and **13** were first generated from the X-ray structure data of the Bim·Bcl-x_L complex (PDB 3DFL), followed by minimization and conformational search of the peptides in a frozen Bcl-x_L. The lowest energy conformers of 18-mer Bim and α/β/γ-peptide **13** were overlaid and the side chain RMSD of the four important residues for Bcl-x_L binding, namely Leu, Arg, Ile, and Asp, was about 0.79 Å indicating a good alignment (Fig. S9). When relative conformers within 4 kJ/mol from the lowest energy of α/β/γ-peptide **13** were overlaid, and the helical region containing the four residues (Leu, Arg, Ile and Asp) was found to be conserved, implying that the helical conformation of the middle region of **13** is rigid and stable (Fig. S10). However, the C-terminus of α/β/γ-peptide **13** was out of the helical registry, and this may be responsible for its low binding affinity to Bcl-x_L compared to the native 18-mer α-Bim (Fig. S10, Supplementary data). Perhaps the iso-atomic approach for α/β/γ-peptide design caused the distortion of the helix due to the different dihedral angles involving amide bonds and rings from the cyclic β- and γ- residues.[6] Because the pattern of β- and γ-incorporation in α/β/γ-peptides does not require the use of a specific replacement pattern, it may be worth optimizing Bim-derived α/β/γ-peptides by placing β- and γ-residues in different patterns from the αγααβα hexad repeat in future studies.[7]

In conclusion, we explored the αγααβα hexad repeat to design α/β/γ-peptides that functionally mimic α-helices, using a model interaction between Bcl-x_L and the helical BH3 domain peptide, Bim. We optimized the binding of α/β/γ-peptides to Bcl-x_L by incorporating cyclic β-/γ-residues and acyclic β-/γ-residues with side chains. The most promising α/β/γ-peptide **13**, showed comparable activity to α-Bim 15-mer and showed a prominent helical propensity in aqueous buffer with great stability towards the proteolytic degradation. We suggest following as the future directions for α/β/γ-peptidomimetics; 1) exploration of diverse patterns of α/β/γ-heterogeneous backbones to minimize the helical backbone deviation from the native α-helix, 2) incorporation of various hydrophilic cyclic/acyclic γ-amino acids to enhance the solubility of the peptides, 3) restoration of N-terminus Trp in Bim by addressing aggregation problem of the α/β/γ-peptides to achieve an enhanced binding to Bcl-x_L in case of the current model PPI. Our study suggests that α/β/γ-foldamer can be developed as an alternative general platform for the sequence-based peptide design researches especially with prominent resistance to enzymatic digestion.

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Conflicts of interest

There are no conflicts to declare.

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		$t_{1/2}$
Bim 15-mer	Ac–W I A Q E L R R I G D E F N A–NH_2	**3.1 min**
Bim 18-mer	Ac–I W I A Q E L R R I G D E F N A Y Y–NH_2	**1.7 min**[a]
α/β/γ - 6	Ac–W I A γ_{cyc} L R (Z) I G γ^4hD F N (Z)–NH_2	**> 10 h**
α/β/γ - 10	Ac–I (X) I A γ_{cyc} L R (Z) I G γ^4hD F N (X) K Y–NH_2	**> 45 h**
α/β/γ - 13	Ac–I (X) I A γ_{cyc} L R β_3hR I G γ^4hD F N (X) K Y–NH_2	**> 15 h**

[a] Data is from ref 4 (the same proteolysis condition). The $t_{1/2}$ values are based on the last hours tested.