β-Amino acid-containing hybrid peptides—new opportunities in peptidomimetics

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Hybrid peptides consisting of α-amino acids with judiciously placed β-amino acids show great promise as peptidomimetics in an increasing range of therapeutic applications. This reflects a combination of increased stability, high specificity and relative ease of synthesis.

1 Introduction

Nature has provided a vast array of bioactive peptides with which organisms control many vital processes. In so doing, researchers have been presented with remarkable opportunities to design molecules to mimic the action of these peptides thus providing new chemical therapies for a range of human diseases. Indeed, the past few decades are testament to the ingenuity of chemists in designing such chemical entities ("peptidomimetics").[1] Some of the major challenges for any peptidomimetic to function effectively *in vivo* include, inter alia: (a) stability, (b) affinity, (c) specificity and (d) efficacy. In this account we will outline our efforts to employ β-amino acids to successfully address each of these issues.

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2 Stabilisation strategies for peptidomimetics

Many strategies have been developed in order to design molecules (peptidomimetics) which mimic the structure and action of a particular peptide.[1] In order to function effectively, a peptidomimetic must reach its site of action intact. Hence *in vivo* stability is a critical issue. The human body is a particularly hostile environment for peptides, with a host of different circulating peptidases. Consequently the use of native peptides is problematic for therapeutic applications and many approaches have been developed which allow peptidomimetics to persist in the body for therapeutically acceptable periods. These include the use of cyclic peptides, and a large variety of main chain modified peptides including, inter alia, conformationally-restricted peptides, *N*-alkylated peptides, azapeptides and of direct relevance to this review, the use of β-amino acids.[1] Inevitably the introduction of one of the above stabilising elements can lead to a loss of affinity and/or efficacy. Hence no single approach constitutes a universal solution. In this account we demonstrate that the use of β-amino acids shows great potential as the stabilising element in peptidomimetics whilst maintaining excellent efficacy.

3 β-Amino acid-containing peptidomimetics

Although β-amino acids are found in Nature they are relatively rare compared to α-amino acids.[2–4] Consequently human peptidases generally don't recognize—and hence don't cleave—peptides containing β-amino acids.[5–7] Given their similarity in structure to α-amino acids this makes them an attractive moiety for inclusion in protease resistant peptidomimetics. Hence the approach we have taken is to strategically replace specific α-amino acids within a given peptide sequence with closely related β-amino acids.[8–10] (In this Account no mention of β-peptides—*i.e.* oligomers of *only* β-amino acids will be made as they are a separate field of research.[11]) As mentioned above β-amino acids are very similar in structure to α-amino acids except that they contain an "extra" carbon atom inserted between the amino and carboxy termini.[12] β-Amino acids, with any given side chain, can exist as the *R* or *S* isomers at either the α (C2) carbon or the β (C3) carbon, resulting in a total of 4 possible isomers for any particular side chain (Fig. 1). The flexibility to generate a large range of stereo- and regioisomers, together with the possibility of di- and

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Rebecca Lew obtained her PhD at the University of Virginia before migrating to Australia, where she has spent 15 years researching the biochemistry and physiology of metallopeptidases, resulting in more than 60 publications. She now divides her time between medical writing and lecturing in biochemistry at Monash University. She is the current Editor of the magazine of the Australian Society for Biochemistry and Molecular Biology, and has been guest editor of several special journal issues focussed on peptides and peptidases.

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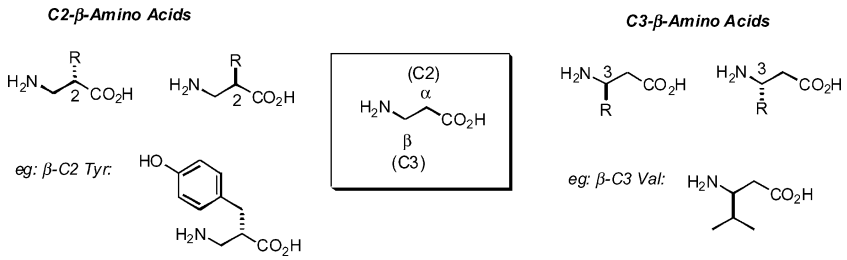

Fig. 1 Structure of β-amino acids.

poly-substitution, significantly expands the structural diversity of β-amino acids while at the same time conserving the functional group, and provides enormous scope for molecular design. It is also evident from Fig. 1 that many more isomers are available in β-amino acids than is possible for the corresponding α-amino acids.

4 β-Amino acid derived enzyme inhibitors

4.1 Microbial aminopeptidase P (APP)

Replacing an α-amino acid with the corresponding C3-β-amino acid at the scissile bond of a peptide can dramatically increase the new, hybrid peptide's affinity for targeted active sites in enzymes.

Mammalian APP (EC 3.4.11.9) is a manganese-dependent enzyme which cleaves the N-terminal amino acid from polypeptides where the second residue is proline. It is present in a variety of tissues, particularly the brushborder membranes of kidneys and lungs.[13] The precise role of mammalian APP is not clear but it is thought to be involved in cardiovascular function[14–16] and inflammation.[17] Our work in this area has so far involved the use of *microbial* APP as a model for mammalian APP and, as such, provides an excellent example of the ability of β-substituted peptides to maintain their affinity with respect to that of the "native" substrate.[18] In this study we prepared a series of β-substituted dipeptides based on the sequence Pro-Pro or Pro-Leu. The sequences of all dipeptides are listed in Table 1.

All peptide inhibitors displayed competitive inhibition against the cleavage of bradykinin, a substrate peptide inactivated by APP (Fig. 2). β-Amino acid incorporations into certain peptides resulted in enhanced inhibitory potency, with some compounds exhibiting nanomolar K_is. With the Pro-Pro series, incorporation of β-Pro in position one had little effect while β-Pro in position 2 resulted in a 200-fold decrease in the inhibitory activity, and β-Pro in both positions caused a 10-fold decrease in inhibition. Remarkably different results were observed for the Pro-Leu series. Incorporation of β-Pro in position one resulted in a 500-fold increase in the inhibitory activity to give a K_i of **7nM**. β-Leu in position 2 caused a 5-fold decrease in inhibition, while a β-amino acid in both positions caused little change in inhibition.

Preliminary analysis demonstrated that all the dipeptides were completely stable to peptidases in kidney membranes after 24 hours. Should these inhibitors prove to be stable to a wider range of peptidases, such as those found in plasma (as is the case with other β-amino acid-substituted peptides), they will be used to further define the physiological role of mammalian APP. In the longer term these new peptides may well act as lead molecules in the development of therapeutic agents in the treatment of cardiovascular diseases.

Table 1 Sequences and K_is for beta-substituted dipeptides based on Pro-Pro and Pro-Leu

Entry	Peptide	K_i (μM)
1	P–P	0.52
2	P–P	0.27
3	P–P	115.8
4	P–P	7.22
5	P–L	1.28
6	P–L	0.007
7	P–L	6.78
8	P–L	0.87

= L-α-amino acid, = L-C3-β-amino acid.

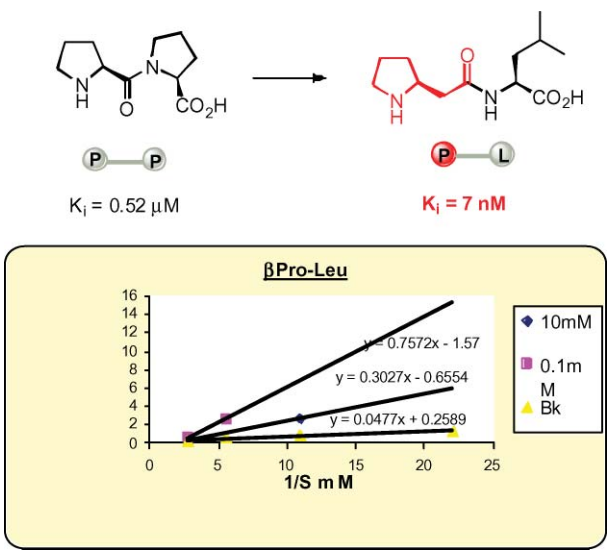

Fig. 2 Lineweaver–Burk plot indicating β-Pro-Leu competitive inhibition of bradykinin (Bk) degradation by microbial APP.

4.2 EP24.11, EP24.15 and EP24.16

Replacing an α-amino acid with the corresponding C3-β-amino acid at the scissile bond of a peptide dramatically increases the new, hybrid peptide's resistance to proteolysis whilst, in most cases retaining good to excellent target affinity.

A second example comes from our modification of "CFP" (*N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-amino-benzoate), a widely-studied inhibitor of the metalloprotease EP24.15 (Fig. 3). EP24.15 is of great interest as it has been proposed that it is involved in the degradation of neuropeptides[19,20] and has been implicated in the hypothalamic regulation of pituitary function in the reproductive axis[21] and also in blood pressure regulation.[22] Furthermore, it has also been suggested that EP24.15 may be involved in processing Aβ protein associated with Alzheimer's disease.[23,24] We were interested, inter alia, in investigating the possibility of generating hybrid analogues of CFP which would be resistant to proteolysis by neprilysin—an enzyme related to EP24.15 and which readily cleaves CFP at the Ala-Tyr bond—but still have high affinity for EP24.15.

Some clear trends emerged (Table 2). First, replacement of the C-terminal *p*-aminobenzoic acid with β-Gly had little effect on its inhibitory activity against EP24.15 or the closely-related EP24.16.[25,26] Second, substitution of the residue on either side of the scissile bond with a β-amino acid eliminated degradation by neprilysin whilst in many cases significant affinity for the active site in both EP24.15 and EP24.16 was maintained. Third, replacing the scissile Ala with its β-analogue led to significant reduction in affinity for both enzymes but with very promising levels of selectivity for EP24.16 over EP24.15 (entry 3). Interestingly we found that substituting the same Ala with *rac*-C2β-Ala gave much improved affinities but with complete loss of selectivity (entry 4). In fact the best performing analogue in the small library we prepared contained both a *rac*-C2β-Ala on the N-terminal side of the scissile bond and Phe replacing the Tyr on the C-terminal side of the scissile bond (entry 7).

Hence a single substitution can lead to complete loss of affinity for neprilysin whilst maintaining good affinity for EP24.15 and EP24.16. (Degradation assays subsequently established that the introduction of one or more β-amino acids completely abolishes

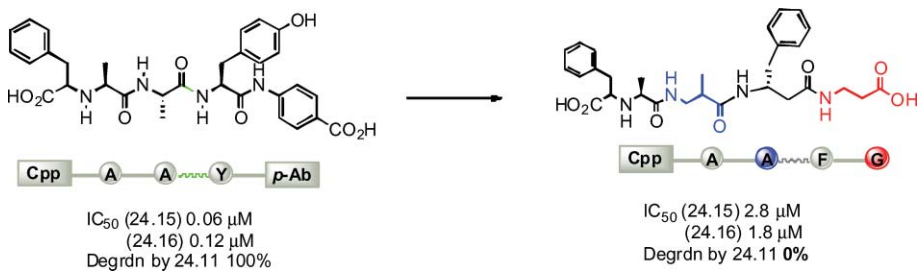

Fig. 3 Modification of "CFP" with β-amino acids yields a strong inhibitor of EP24.15 and 16 whilst providing complete stability against neprilysin.

Table 2 Selected sequences of CFP analogues, inhibitory constants (K_i) and % degradation by 24.11.

Entry	Peptide	IC_{50} (μM) EP 24.15	IC_{50} (μM) EP 24.16	% Degradation by EP 24.11
1	Cpp A A Y *p*-Ab "CFP"	0.06	0.12	100
2	Cpp A A Y G	0.12	0.18	100
3	Cpp A A Y G	>500	72	0
4	Cpp A A Y G	6.3	6.3	0
5	Cpp A G Y G	5.6	4.8	0
6	Cpp A A Y G	25	14	0
7	Cpp A A F G	2.8	1.8	0
8	Cpp A P Y G	—	—	0

= L-α-amino acid, = L-β3-amino acid, = D-β3-amino acid, = (±)-β2-amino acid, = proteolytically stable bond, = proteolytically labile bond.

Table 3 Proteolytic degradation of SIINFEKL and SIIN(β-F)EKL

Entry	Peptide	% Degradation by proteases			
		Serum[a]	Pronase[b]	PK[b]	Pepsin[b]
1		95	>85	75	75
2	**"Wild type" antigen surrogate** 	70	<50	0	0

[a] After 2 h. [b] After 6 h. = L-α-amino acid, = L-C3-β-amino acid.

binding to neprilysin.) In addition subtle differences in the nature of the β-amino acid employed also lead to useful differences in specificity. For example substituting the Ala adjacent to the scissile bond with the corresponding C3-β-Ala led to the abolition of binding to EP24.15 whilst maintaining reasonable affinity for EP24.16 (see entry 5). This work provides a remarkable example of the ability of single β-amino acid substitutions to "switch off" affinity for one, in this case problematic, enzyme whilst maintaining affinity for the desired enzyme(s).

5 MHC binding peptides

The use of peptide-based vaccines has proved problematic due to relatively poor stability, bioavailability and rapid modification of the peptide under conditions of formulation and delivery.[27] Hence this provides an ideal opportunity to evaluate the efficacy of β-amino acid stabilized peptides as vaccines. The rationale underlying this approach is based on the cellular immune system's ability to recognise processed forms of antigens which are displayed on the surface of antigen presenting cells complexed with major histocompatibility complex (MHC) molecules.

Given the plausible link between immunogenicity and proteolytic stability of peptides derived from antigens[28] we chose to investigate the incorporation of β-amino acids into T cell epitope mimetics of a model antigen, SIINFEKL—the immunodominant T cell epitope derived from chicken ovalbumin—in C57BL/6 mice, and examine the resultant efficacy of these mimetics (which we have termed "betatopes").[27,29] Each of the amino acids in SIINFEKL was sequentially replaced with the corresponding C3 β-amino acid. Some analogues displayed similar MHC binding and superior protease stability in comparison to the native epitope. Replacement of α-amino acids at positions 4, 5, 6, and 8 with their corresponding β-amino acid resulted in binding equivalent to or greater than that of the wild-type peptide, whereas the remaining substitutions disrupted binding. Of these the 5, 6 and 8 mono-substituted peptides were the most stable. Table 3 compares the stability of SIINFEKL to SIINβ-FEKL clearly showing the enhanced stability conferred on the peptide by the introduction of the β-Phe at residue 5. Similar results were obtained with the other two "betatopes".

Importantly, these analogues were able to effectively mimic the native peptide by maintaining their immunogenicty in an array of *in vitro* cellular assays.[27] Moreover when these betatopes were used to immunise C57BL/6 mice they generated cross-reactive cytotoxic T lymphocytes that were capable of lysing tumor cells that expressed the unmodified epitope as a surrogate tumor antigen. Remarkably, cytotoxic T cells generated by immunization with position 5 and position 8 substituted betatopes (βPhe and βLeu) demonstrated superior cytotoxicity (killing) of tumor cells and enhanced production of key inflammatory cytokines such as interferon-γ in response to recognition of tumor cells that expressed the unmodified epitope as a surrogate tumor antigen (Fig. 4). This provides tantalizing evidence that use of proteolytically stable mimics of natural T cell epitopes results in augmented immunity and paves the way for a new generation of chemically defined peptide based vaccines.

6 A structural basis for the use of β-amino acids in peptidomimetics

The crystal structures of each of these peptides bound to murine MHC class I molecule H-2K^b were determined and compared with the previously reported structure of the wild-type complex (Fig. 5). (These appear to be the first crystal structures reported of *any* β-amino acid-modified peptides bound to a large protein.) We selected β-amino acid replacements at "anchor residue" positions 5 and 8 because these performed well in our functional analysis and proved most stable against proteases. Structural analysis of these peptides revealed that the only perturbation occurred in the region where the "extra" methylene had been inserted in the peptide. For the 5-substituted SIINFEKL, in this region of the complex it is clear that the main chain conformation is essentially unaltered however the peptide's β-Phe side chain has moved deeper into the hydrophobic pocket of H-2K^b explaining the enhanced binding affinity of this betatope for this MHC class I molecule. For the 8-substituted analogue little perturbation of the main chain occurred and once again the side chain of the β-Leu is buried more deeply in its hydrophobic pocket.

Hence we have shown that incorporation of β-amino acids at single positions in a model peptide antigen, SIINFEKL, enhances the stability of the entire peptide, retains the original peptide's immunogenicity, and generates functional cross-reactive T cells with anti-tumor cytotoxic activity. Additionally, our studies suggest that replacement of MHC anchor residues with corresponding β-amino acids may represent a generic strategy for the augmentation of proteolytically susceptible peptide antigens.

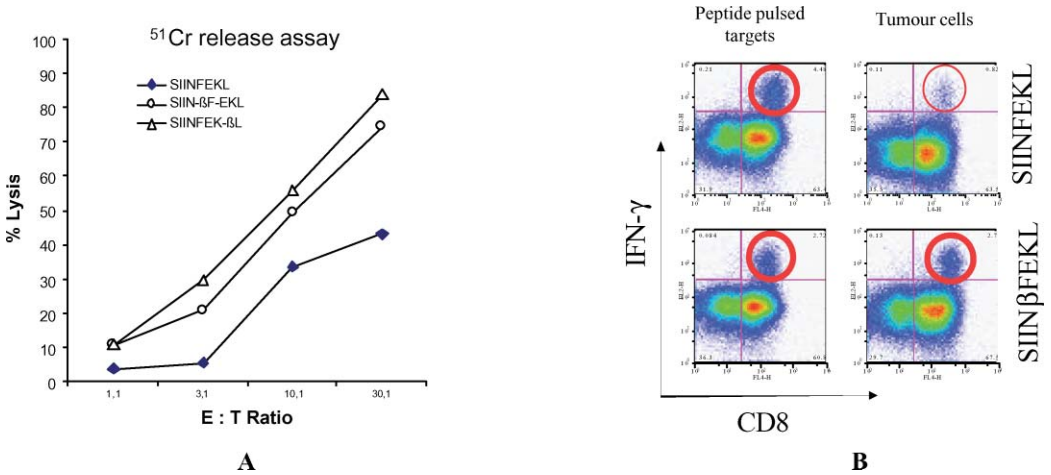

Fig. 4 CD8+ T lymphocytes from mice immunised with SIINFEKL betatopes show enhanced cytolytic activity (**A**) and cytokine production (**B**) in response to ovalbumin expressing tumor cells, but not peptide pulsed target cells in an intracellular cytokine staining experiment.

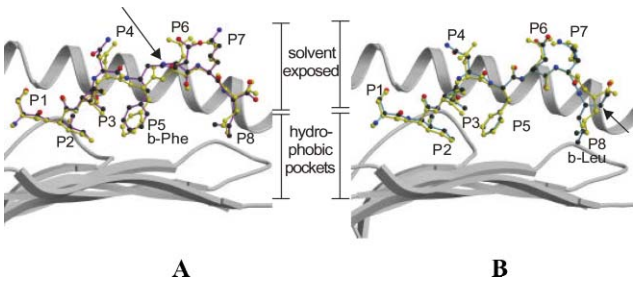

Fig. 5 Structures of H-2K^b bound to "betatopes". Peptide orientation of both β-Phe5 (**A**) and β-Leu8 (**B**) superimposed onto the wild-type peptide containing structure (yellow) is shown. The peptide ligands in all complexes have very similar conformation (arrows indicate the site of the additional methyl moiety in the peptide backbone of the β-amino acid containing analogs).

7 Conclusions

The judicious substitution of an α-amino acid with a β-amino acid in peptides of interest leads to mimetics which show good to excellent stability, affinity and function. These results hold great promise for the development of new, peptide-based therapies—a field which has long been neglected or dismissed but is now attracting renewed interest.[33] Our approach so far has been mostly limited to β-amino acids (largely the C3 family) bearing natural side-chains. We are currently focussing our efforts on extending this approach to the synthesis and incorporation of C2 and C3 β-amino acids containing customised, unnatural side chains.[30–32]

Acknowledgements

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