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Amyloid peptides incorporating a core sequence from the amyloid beta peptide and gamma amino acids: relating bioactivity to self-assembly†

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A series of heptapeptides comprising the core sequence Aβ(16–20), KLVFF, of the amyloid β peptide coupled with paired N-terminal γ-amino acids are investigated in terms of cytotoxicity reduction and binding to the full Aβ peptide, both pointing to inhibition of fibrillisation for selected compounds. This is related to the self-assembly capacity of the heptapeptides.

Intense research activity is focussed on the development of treatments for Alzheimer's disease. The aggregation of the amyloid β peptide into oligomers or fibrils is now implicated as a key process associated with progression of this disease.[1–4] Several mechanisms for cytotoxicity have been suggested,[2,5,6] of which the most well known is probably the Aβ-induced formation of ion channels in cell membranes. A number of strategies are actively being pursued by research teams in academia and the pharmaceutical industry.[7–9] These include (i) development of γ-secretase inhibitors (γ-secretase is an enzyme involved in cleavage of amyloid β (Aβ) peptides from the Amyloid Precursor Protein), (ii) passive immunization based on Aβ antibodies (iii) inhibition of aggregation of oligomers.

Here, we report on the development of a series of novel compounds that show promising *in vitro* properties in binding to Aβ and in restoring viability of neuronal cells. Inhibition of oligomer aggregation has been targeted *via* use of self-recognition elements (SREs); these are molecules based on fragments of the Aβ peptide which are capable of binding to the corresponding sequence, but modified so as to disrupt β-sheet fibrillisation. Findeis *et al.* showed that compounds based on a core sequence of the Aβ peptide implicated in fibrillisation, Aβ(16–20), KLVFF, showed promise as SREs.[10] Other groups have developed related approaches based on extended or modified KLVFF sequences[11–15] or oligomers with complementary residues[16] or non-peptidic compounds designed to recognise KLVFF.[17] We have recently investigated the interaction of βAβAKLVFF (βA = β²-alanine) with Aβ(1–42) and showed that the βA-modified heptapeptide can bind Aβ(1–42) and

affect fibrillisation.[18] In related work, SREs have been developed based on core sequences of the amyloid-forming peptide α-synuclein, modified using β-alanine or γ-aminobutyric acid (GABA).[19] Reduced aggregation of α-synuclein in the presence of SREs was observed, and one of the β-alanine modified peptides studied also inhibited aggregation of Aβ(1–40). Non-natural amino acids such as β- and γ-amino acids are of interest since they are resistant to proteolysis, enabling the preparation of compounds designed to incorporate biostable motifs.

We investigated a series of γ-amino acid peptides containing the KLVFF sequence or its D-amino acid variant (Scheme 1) in cell viability studies using the MTT assay with SH-SY5Y neuronal cells. It should be noted that compound **II** is βAβAKLVFF, the subject of a previous study.[18]

We investigated the effect of compounds **I–V** on the viability of SH-SY5Y cells using an MTT reduction colorimetric assay.

Dose response curves are presented in Fig. 1. These results show that Aβ(1–42) leads to a loss of cell viability to around 30%. The γ-amino acid containing peptides alone do not cause cell death, cell viability remains above 90%. Unfortunately, **I** and **II** do not reduce the cytotoxicity of Aβ(1–42) (this conclusion for **II** is in agreement with our previous report).[18] However, compounds **III**, **IV** and **V** show a substantial
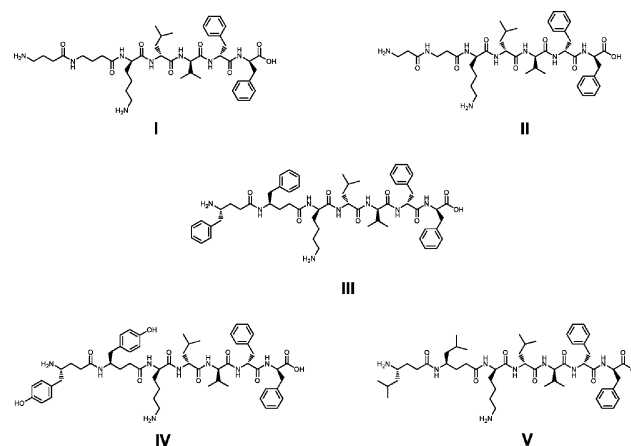
Scheme 1 Peptides studied. **I**, **III**, **IV**, **V**: N-terminal γ-amino acid modified KLVFF SRE peptides. Compound **II** contains N-terminal βA residues, this compound, studied previously by us,[18] is included as a reference sample. It may be noted that in compounds **III**, **IV** and **V**, the KLVFF sequence is based on D-amino acids. In the following, the influence of these non-natural α-amino acid residues on self-assembly is examined.

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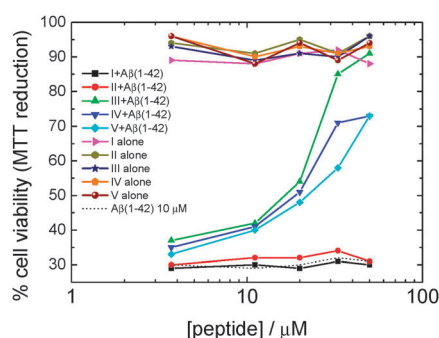
Fig. 1 Dose-response curves based on MTT cell viability assay for selected compounds in the presence or absence of Aβ(1–42).

Fig. 2 Cryo-TEM images for peptides **I** (3 wt%), **III** (2 wt%) and **IV** (5 wt%) and **V** (3 wt%). The scale bars represent 200 nm.

dose-dependent increase in cell viability, in the case of peptide **III** to around 90%, at physiologically relevant concentrations of Aβ(1–42) and peptide.

To investigate the binding of our compounds to Aβ(1–42) in more detail, circular dichroism (CD) spectroscopy experiments were performed. CD also provides information on the self-assembly of the peptides in the absence of Aβ(1–42). The resulting data are presented in ESI† Fig. 1 (measured spectra) and ESI† Fig. 2 which shows the measured spectra for mixtures of Aβ (50 μM) with **I**, **II**, **IV** and **V** at the molar ratios shown, along with calculated spectra based on addition of the measured spectra for the two components. These spectra reveal that compounds **III** and **V** show the strongest binding characteristics, in fact even at the lower molar ratio of added heptapeptide studied the measured curves deviate significantly from those calculated. At higher molar ratios, the CD spectra for these systems become rather flat pointing to loss of secondary structure upon binding of peptide to Aβ(1–42). The finding that **III** in particular binds strongly to Aβ and disrupts β-sheet structure correlates to the cytotoxicity behaviour shown in Fig. 1. Dye binding studies using thioflavin T (ESI† Fig. 3) also show binding of **III** to Aβ(1–42).

It may be noted that the CD spectrum for **III** (ESI† Fig. 1) shows significantly stronger features (in particular a maximum at 225 nm) than the other peptides. This suggests that the reduced cytotoxicity when adding **III** to Aβ(1–42) and increased binding of these species, may be related to the intrinsic self-assembly properties of **III**. These were therefore investigated in more detail, in comparison with the other peptides. The self-assembly of γ-amino acid based peptides is also attracting considerable attention at present in its own right due, for instance, to enhanced properties such as resistance to proteolysis,[20] formation of unique secondary structures[20–22] and also the ability to mimic natural secondary structures using non-natural amino acids.[23] In contrast to previous work, the incorporation of a β-sheet forming α-amino acid motif (KLVFF) along with two γ-amino acid residues in **I**, **III**, **IV** and **V** inhibits the formation of the more common helical structure of γ-amino acid peptides, and instead amyloid β-sheet structures are observed.

Fibrillisation of these peptides was directly confirmed by cryogenic-TEM. Fig. 2 shows representative images. The fibril diameter for all four peptides is approximately 10 nm.

X-Ray diffraction from stalks prepared from 1–1.8 wt% solutions (except for **I** for which stalks could not be prepared unless the concentration was increased) confirmed the presence
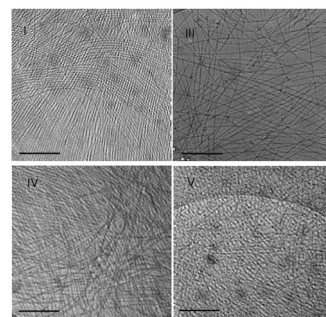
of β-sheets, since a characteristic "cross-beta" pattern[24] is observed (ESI† Fig. 4) with strong meridional reflections from the spacing of beta strands aligned perpendicular to the vertical fibril axis, along with a series of equatorial reflections from spacings within and between β-sheets.

A table listing observed reflections is provided as ESI† Table 1. An interesting feature was noted for all samples except **II**, the subject of previous papers[25,26] and **III**, in that the main meridional reflection was split into a doublet, with the strongest peak corresponding to a spacing $d = 4.94$ Å along with a lower intensity peak from a spacing $d = 4.73$–4.77 Å. This latter spacing is in the range usually associated with the inter-strand distance in a β-sheet,[27,28] however the increased spacing $d = 4.94$ Å also observed for these peptides points to an expansion of the inter-strand distance and is ascribed to folding of the γ-amino acid residues at the N terminus. This hypothesis is supported by the fact that the spacing associated with the length of the peptide (ESI† Table 1) is significantly smaller than that calculated for an α-amino acid heptapeptide.[29]

FTIR and CD were used to investigate the secondary structure of the peptides, as a function of concentration (at higher concentration than the CD data in ESI† Fig. 1 and ESI† Fig. 2). Fig. 3 shows CD and FTIR spectra measured in in two concentration ranges. The FTIR spectra in the amide I′ region in more dilute solution (Fig. 3a) show that only **III** forms β-sheets in dilute solution since for this sample there is a peak at 1618 cm^{-1} (with a shoulder at 1626 cm^{-1}). All spectra also show a peak at 1672 cm^{-1}
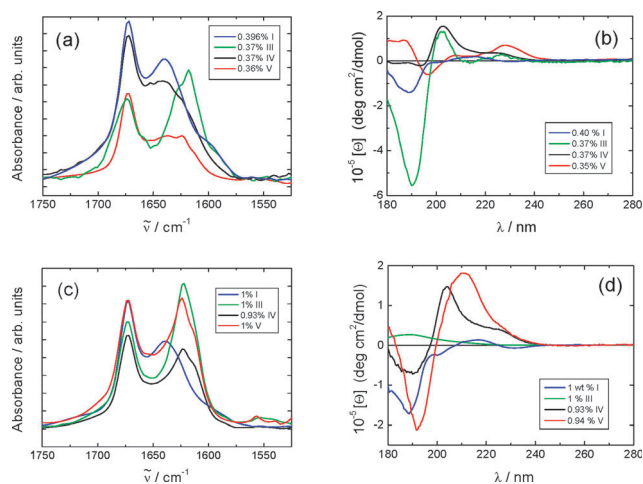

Fig. 3 FTIR and CD spectra. (a, b) FTIR and CD spectra for 0.4 wt% solutions, (c,d) FTIR and CD spectra for 1 wt% solutions.

due to TFA counterions.[27] For **III**, the absence of a peak around 1680 cm^{-1} indicates that the β-sheets have a parallel configuration. Samples **I** and **IV** show a peak at 1640 cm^{-1} which is characteristic of either random coil or polyproline II structure (as discussed elsewhere,[30] the two are difficult to distinguish based on standard FTIR spectroscopy alone). Sample **V** shows mainly the 1640 cm^{-1} peak, with a shoulder indicating some contribution from β-sheet structures.

The FTIR spectra assist in assignment of the CD spectra (Fig. 3b), which are often difficult to interpret for short peptides that do not exhibit classical α-helical or β-sheet spectra. Consistent with the FTIR data, **III** shows a distinct spectrum with a maximum just above 200 nm and a minimum at 213 nm as expected for β-sheets. The spectra for all samples (except **I**) also contain a peak or shoulder at 230 nm which is ascribed to phenylalanine stacking interactions.[31] The spectra suggest random coil rather than PPII structure is predominant. Turning to Fig. 3c,d, when concentration is increased to 1 wt%, FTIR shows clear features of (parallel) β-sheets for all peptides except **I** which retains a random coil conformation. Interestingly, the CD data for samples at 1 wt% do not show clear β-sheet like features, *i.e.* a minimum in the range 215–220 nm. We believe that contributions from aromatic residues at the C and N termini of the spectra predominate in these CD spectra.[31–33] At still higher concentration (2–3 wt%, comparable to that used in the cryo-TEM studies), FTIR confirmed the presence of features associated with β-sheets in the amide I′ region for all samples (ESI† Fig. 5). A further interesting observation for several of these compounds was the formation of self-supporting hydrogels at sufficiently high concentration. ESI† Fig. 6 shows photographs of representative systems. Formation of these hydrogels is associated with β-sheet features in the FTIR spectra and suggests they comprise a fibrillar network structure. These hydrogels might be useful in the development of slow release systems.

In summary, we have discovered a promising lead compound (**III**) containing two γ-amino acid residues [(R)-(4-amino-5-phenyl]pentanoyl and the D-amino acid version of KLVFF (sometimes denoted klvff).[10] This material shows a favorable dose-response curve, reducing the cytotoxicity of Aβ in a physiologically relevant concentration range. In turn we show by CD spectroscopic and ThT dye fluorescence studies that this peptide binds to Aβ. This led us to examine whether the bioactivity of this peptide could be related to its self-assembly propensity. Indeed, we find that peptide **III** forms β-sheet structures at lower concentration than the other samples examined. Another unanticipated finding was the splitting of the β-strand spacing in the fibre XRD patterns of several of the peptides, a feature we associate with the inability of the N-terminal γ-amino acid residues to fit into the regular H-bonding pattern of the α-amino acid residues. This was not observed for **III**, possibly contributing to the enhanced fibrillisation properties of this peptide. These findings provide insight into the self-assembly of this novel class of peptides. The combination of γ-amino acids with an Aβ SRE motif (KLVFF) provides a new class of fibrilisation inhibitor.

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