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# Design and study of peptide-based inhibitors of amylin cytotoxicity

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ABSTRACT

The incidence of type II diabetes is on the increase each year and the World Health Organisation (WHO) predicts there to be over 360 million diabetic patients worldwide by the year 2030. Deposits consisting mainly of a small protein, called islet amyloid polypeptide (amylin), which aggregates into oligo-/polymeric beta sheet structures is responsible for cytoxicity to the pancreatic β-cells, thus inhibition of this process has been explored as a potential prevention or treatment. N-Methylated and non N-methylated peptides spanning the length of amylin$_{1-37}$ were synthesised and evaluated for their inhibition of full length amylin mediated cytoxicity to RIN-5F cells. The non N-methylated peptides were very effective in inhibiting the cytotoxicity while the N-methylated peptides were not. Both the N-methylated and non N-methylated versions of the 29-34 region were equally effective.



The incidence of type II diabetes also referred to as non-insulin-dependent diabetes *mellitus* is on the increase each year and the World Health Organisation (WHO) predicts there to be over 360 million diabetic patients worldwide by the year 2030.[1]

From as early as 1901, a link between the deposition of fibrillar material in the Islets of Langerhans in the pancreas and manifestation of type II diabetes had been established.[2] It was found that the deposits consisted mainly of a small protein, called islet amyloid polypeptide (amylin), which aggregates into amyloid fibrils and type II diabetes was thus classified as an amyloid disease.[2–4]

Islet amyloid polypeptide, also referred to as amylin or diabetes associated peptide, is a 37-amino acid long polypeptide and is released from the β-granules of pancreatic β-cells together with insulin in a constant molar ratio of 20:1 (insulin/amylin). It is believed to play a role in maintaining glucose homeostasis and has found to be released by the body following an increase in blood glucose level, as is the case after a meal.[5,6]

Soluble monomeric amylin is stabilised when it self-associates into oligomeric states and can further associate through hydrogen bonding into parallel, insoluble β-sheet-rich amyloid fibrils.[7,8] The exact nature of the toxics species is controversial with some workers arguing that amyloid fibres are toxic[9–17] and others arguing that the pre-amyloid intermediates exert the toxic effect.[18–22]

In 2002, it was found that the introduction of N-alkylated amino acids or ester functionalities into peptide sequences allowed the peptide to act as β-sheet inhibitors and prevented toxicity.[23] Single strands form β-sheets by hydrogen bonding between NH- and O-moieties that point out of the peptide backbone. Peptides containing N-alkylated amino acids are able to bind to the native protein and prevent the attachment of any further peptide strands by disrupting the hydrogen-bonding capacity and by providing steric hindrance.

In 1990, Westermark et al. found that the 20–29 region alone was able to form amyloid fibrils and for many years it was believed that this was the only region that was crucial for aggregation into cytotoxic fibrils.[24] Kapurniotu et al. (2000) identified the 22–27 region as the shortest fragment of amylin that could form the typical cytotoxic amyloid fibrils. In 2001, Fraser and co-workers demonstrated that the 8–20 fragment is capable of forming fibrils in the β-conformation.[25] More recently, Abedini and Raleigh synthesised the 8–37 region of amylin, substituting residues at positions 17, 19 and 30 for proline and showed that the modified peptide had a significant lack of aggregating potential as compared to its wild type peptide.[26] They asked the question 'Is there a critical amyloidogenic domain in human Iapp'.

The first inhibitor of amylin-mediated cytotoxicity was SNNF(*N*-Me)GA(*N*-Me)ILSS which was reported by Kapurniotu et al. and was shown to inhibit toxicity of the 20–29 region of amylin.[15] It was also reported that the presence of N-methylations allowed the peptide to exist in an ordered β-sheet structure and

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was crucial if the peptide was to be used as an inhibitor.[15] However, to date only two other modified peptides have shown to inhibit the aggregation of full length amylin and its subsequent cytotoxicity. The first of these two peptides made use of the full length amylin with N-methylations in positions 24 and 26 and the second was a hexapeptide homologous to the 22–27 region with N-methylations in the 23 and 25 positions.[16,27]

There are only five reports that describe the testing of the synthesised fragments as inhibitors of full length amylin cytotoxicity. Scrocchi et al. focussed on the 20–29 region of amylin and observed that amylin$_{20–25}$ reduced toxicity by 25% whereas amylin $_{24–29}$ did not.[28] However, it should be noted that amylin alone was only 35-40% toxic to RIN-1056 cells. In a later report, Scrocchi et al. also tested the amylin$_{12-17}$ and amylin$_{15-20}$ fragments as potential inhibitors of toxicity on the RIN-1056 cell line and found that neither of the two peptides were sucessful.[29] Potter et al. highlighted that amylin$_{13–18}$ could completely prevent amylin-mediated cytotoxicity on RIN-1056A cells.[30] They also found that when freshly cultured human islets were exposed to full length amylin, 25% of the cells were apoptotic whereas amylin$_{13–18}$ reduced the amount of apoptotic cells to 12.5%.[30] Potter et al. also observed that amylin$_{20-25}$ reduced amylin toxicity by 50%.[30] The modified peptide analogue NF(*N*-Me)GA(*N*-Me)IL was demonstrated, by Tatarek-Nossol et al., to significantly reduce amylin-mediated cell damage and death of RIN-5fm cells.[16] Yan et al. synthesised full length amylin that contained *N*-methylated amino acids at positions 24 and 26 and found that this modified peptide could also inhibit cytotoxicity of RIN-5fm cells.[27]

When working with amyloid proteins, it is difficult to compare results between reports. In this communication we report the synthesises short N-methylated and non N-methylated peptide analogues spanning the full length of amylin (Fig. 1) and evaluated these via a cytotoxicity assay in a single study. It was also decided to use as many N-methylated amino acids in each peptide as synthetically possible since it was recently reported by Arvidsson et al. that a hexapeptide with five N-methylations was effective in inhibiting toxicity mediated by A$\beta_{1–42}$ protein.[31]

Circular dichroism (CD) spectroscopy measurements were taken of each peptide to determine its secondary structure. CD is based on the optically active molecules ability to preferentially absorb either left- or right-handed circularly polarised light. The non-methylated peptide fragments appear to be primarily unstructured in solution. All of the spectra exhibit a minimum around 200 nm and lack significant intensity between 210 and 230 nm (Fig. 2).

However, all of the peptide fragments containing N-methylated amino acids displayed an ordered structure with maxima at approximately 200 nm with a minima just after 220 nm (Fig. 3).

Prior to testing, it was essential to start with completely disaggregated amylin. Although previous studies have recommended the use of either DMSO or HFIP alone for the purpose of amylin dis-

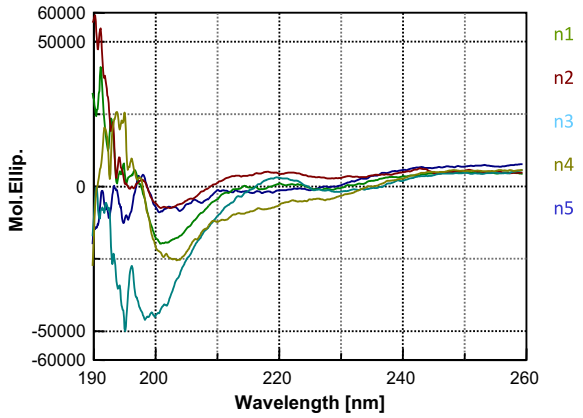


**Figure 2.** Figure showing the CD spectra of peptides n1–n5. Mol. Ellip. = molecule ellipticity measured in deg cm$^2$ dmol$^{-1}$.

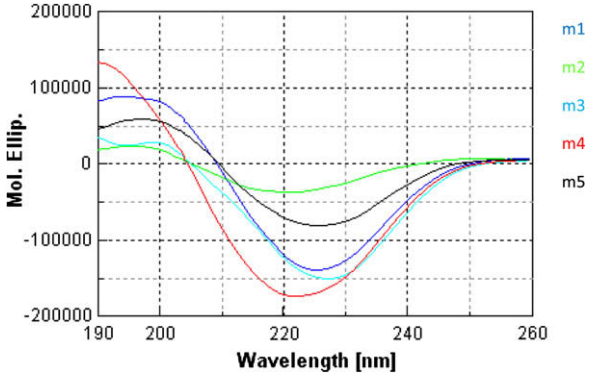


**Figure 3.** Figure showing the CD spectra of peptides m1–m5. Mol. Ellip. = molecule ellipticity measured in deg cm$^2$ dmol$^{-1}$.

aggregation, in the current study the following optimal procedure was employed. This was achieved by dissolving amylin in 50:50 (v/v) HFIP/TFA and sonicating for five minutes. Samples were left to stand overnight at room temperature and solvents were removed under vacuum. Neat HFIP was added to the sample and removed under vacuum. This was repeated twice to eliminate all traces of TFA. The freshly disaggregated amylin was dissolved in buffer to give a final concentration of 45 μM. Inhibitor peptides (5 equiv, final concentration of 225 μM) were added to the amylin solution and immediately used for the cytotoxicity testing by adding 40 μL of the sample to each well. As seen in Figure 4, we were able to obtain reproducible data of approximately 70% toxicity of amylin to RIN-5F cells and this is attributed to the use of high purity

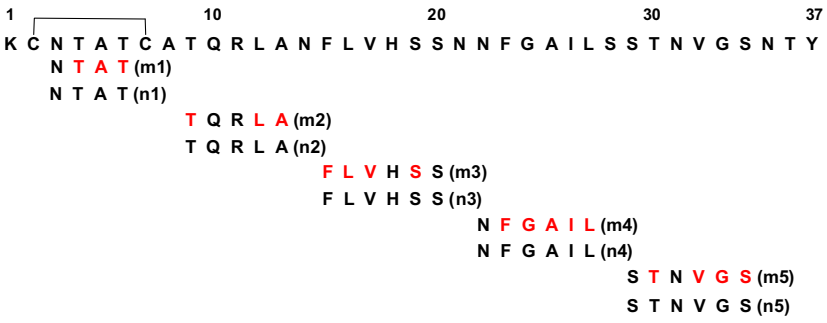


**Figure 1.** Primary structure of full length human amylin and the methylated and non-methylated peptides that were synthesised as potential inhibitors. Single letter notation used for amino acids and N-methylated amino acids are in red. m1–m5 are novel N-methylated peptides, n1–n5 are their non-methylated analogues. All peptides were amidated at the C-terminus without any modifications to the N-terminus.

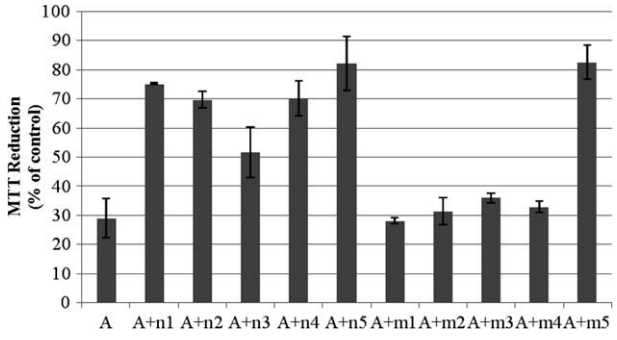


**Figure 4.** Graph showing the cytotoxic effect of amylin in the presence or absence of peptide analogues on RIN-5F cells ($7.5 \times 10^4$ cells/well after 24 h incubation with amylin). Data are percentages of control values and are the mean (±SD) of five determinants. A = amylin, m1–m5 = N-methylated peptide analogues of amylin, n1–n5 = non-methylated peptides analogues of amylin.

protein. In a control study, the peptides did not show any significant toxicity to the RIN-5F cells. In the presence of non N-methylated peptide fragments n1, n2, n4, and n5, the toxic effect of amylin was drastically reduced ($p$ <0.001). Amylin$_{3-6}$ reduced the toxic effect from 70% to 25%, while amylin$_{9-13}$ and amylin$_{22-27}$ decreased toxicity by approximately 50%. Of the non N-methylated peptides, amylin$_{29-34}$ seems to be the best inhibitor and resulted in a decrease in toxicity from 70% to approximately 18% ($p$ <0.001) whereas amylin$_{15-20}$ decreased toxicity by only 20% ($p$ <0.01). It was found that the N-methylated peptide fragments m1–m4 did not reduce the toxic effect of amylin ($p$ >0.05) while, m5 (which is homologous to amylin$_{29-34}$ and contains four N-methylated amino acids) proved to be just as effective as its non-methylated analogue and resulted in a decreased toxicity from 70% to just 18% ($p$ <0.001). Since both versions of amylin$_{29-34}$ could significantly reduce toxicity, this could be interpreted as a critical region for designing peptides for potential therapeutic inhibitors.

This is the first report describing the effect of peptides spanning the length of amylin$_{1-37}$ for their ability to inhibit toxicity of full length amylin. Amylin$_{3-6}$, amylin$_{9-13}$, amylin$_{22-27}$, amylin$_{29-34}$, and ST(*N*-Me)NV(*N*-Me)G(*N*-Me)S(*N*-Me) were identified as potential inhibitors of amylin-mediated cytotoxicity. These results are consistent with previous reports and are important since all compounds were compared in a single study. The only drawback to the non N-methylated versions would be their biological instability and for this reason the N-methylated peptide corresponding to the 29–34 region becomes significant.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.004.

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