Inhibition of Toxicity in the β-Amyloid Peptide Fragment β-(25–35) Using N-Methylated Derivatives

A GENERAL STRATEGY TO PREVENT AMYLOID FORMATION

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β-(25–35) is a synthetic derivative of β-amyloid, the peptide that is believed to cause Alzheimer’s disease. As it is highly toxic and forms fibrillar aggregates typical of β-amyloid, it is suitable as a model for testing inhibitors of aggregation and toxicity. We demonstrate that N-methylated derivatives of β-(25–35), which in isolation are soluble and non-toxic, can prevent the aggregation and inhibit the resulting toxicity of the wild type peptide. N-Methylation can block hydrogen bonding on the outer edge of the assembling amyloid. The peptides are assayed by Congo red and thioflavrin T binding, electron microscopy, and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) toxicity assay on PC12 cells. One peptide (Gly25N-methylated) has properties similar to the wild type, whereas five have varying effects on prefolded fibrils and fibril assembly. In particular, β-(25–35) with Gly38N-methylated is able to completely prevent fibril assembly and to reduce the toxicity of prefolded amyloid. With Leu34N-methylated, the fibril morphology is altered and the toxicity reduced. We suggest that the use of N-methylated derivatives of amyloidogenic peptides and proteins could provide a general solution to the problem of amyloid deposition and toxicity.

Alzheimer’s disease (AD)1 is the most common form of senile dementia. β-Amyloid (Aβ), a 39–43-amino acid β-sheet peptide, aggregates in the brain to form the major component of characteristic deposits known as senile plaques (1–4). X-ray diffraction data have shown that the conformation of Aβ is characterized by an antiparallel cross-β-pleated sheet (5), although more recent solid state NMR evidence suggests that the peptide has a parallel β-sheet structure (6). Nevertheless, aggregation occurs because of hydrogen bonding between β-strands, and the resulting fibrils have axes perpendicular to the β-strand and parallel to the cross-linking hydrogen bonds (5).

Of all of the Aβ derivatives studied so far, β-(25–35), sequenGSKNGAIIGLM, is the shortest fragment that exhibits large β-sheet fibrils and retains the toxicity of the full-length peptide (2, 7–9). It has been proposed that β-(25–35) represents the biologically active region of Aβ. In vitro studies have shown that it does not require aging to aggregate and become toxic (8–10), unlike the full-length peptide. As with Aβ-β, toxicity is dependent on the aggregation state of the peptide, because β-(25–35) that has been solubilized and unfolded in 35% acetonitrile (AcN), 0.1% trifluoroacetic acid is nontoxic (8, 11). In this study, β-(25–35) has been chosen as a model for full-length Aβ because it retains both its physical and biological properties, while its short length readily allows derivatives to be synthesized and studied.

A great deal of evidence, much of which comes from studying hereditary forms of the disease, supports the view that Aβ aggregation is implicated in AD (1–4, 12–16). Controversy has raged, however, over whether these fibrils are actually a cause or a consequence of the disease. Although many mutations in the amyloid precursor protein gene have been linked to premature onset of AD, the amount of amyloid deposited in the brain does not necessarily correlate with disease severity. A resolution of this apparent paradox may be that it is a β-sheet-protofilament, which subsequently forms fibrils and then plaques, that is the pathogenic element (17, 18). What is clear is that the most important factor influencing Aβ toxicity is its aggregation state, because only the non-soluble fibrillar form is neurotoxic (2, 11, 19). In view of the role fibril formation plays in AD and other diseases, it has been proposed that a valid therapeutic strategy would be to administer compounds that can block fibril or protofilament formation by binding to their ends (20, 21). Designed peptides have already been shown to act as “β-sheet breakers,” inhibiting amyloid formation and lowering toxicity in Alzheimer’s peptides and prion proteins (20, 22–27). Our proposal is that N-methylated peptide derivatives can also act as β-sheet breakers.

Six N-methylated (NMe) derivatives of β-(25–35) were synthesized. We demonstrate that these can both prevent the aggregation of fibrils and inhibit the resulting toxicity. These peptides may have the potential to fold correctly and remain soluble in either monomeric or dimeric form.

N-Methylation is known to promote β-sheet formation by locking the residue into a β conformation (28), and it has been shown to generate soluble monomeric β-sheet peptides (29). By N-methylating the amide NH groups at the outer edges of the β-sheet and so prevent intermolecular hydrogen bonding, both aggregation and toxicity should be prevented. Because the NMe derivatives are homologous to β-(25–35), they are expected to bind to the peptide but prevent the further addition of β-(25–35) monomers.
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**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—β-(25–35) and the six NMe derivatives, NMeGly29, NMeGly29, NMeAla30, NMeLeu31, NMeGly33, and NMeLeu34 were synthesized on an Applied Biosystems 431A peptide synthesizer using standard solid phase Fmoc (N-9-fluorenylmethoxycarbonyl) L-amino acid chemistry and Fmoc-Met Wang resin. Coupling was carried out in most cases using the standard chemistry of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxy-7-azabenzotriazole (HOBt) in dimethylformamide with double coupling after addition of the NMe residue. The exception was NMeIle31, which in addition to double coupling required the use of [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium] (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) in dimethylformamide because of the difficulty of coupling following a β-branched NMe residue. The synthesis of NMeIle31 proved impossible despite numerous attempts. Purification was carried out by reverse-phase high pressure liquid chromatography on a Hewlett Packard 1100 using a Resource RPC 3-ml column and a linear gradient of 5–40% ACN in H2O with 0.1% trifluoroacetic acid. Purified peptides were then analyzed by electrospray mass spectrometry generating the expected masses of 1060 for β-(25–35) and 1074 for the NMe derivatives. Once purified, peptides were stored in lyophilized form at -20 °C.

**Peptide Preparation**—For each assay, peptides were prepared by solubilizing in either H2O or 35% ACN, 0.1% trifluoroacetic acid. They were lyophilized following any mixing and resolubilized in 20 mM MOPS at pH 7. Peptides were then incubated for 1 week at 37 °C prior to assay. For Congo red binding, thioflavin T fluorescence, and electron microscopy, peptides were either alone or in 1:1 combinations of β-(25–35) to each NMe derivative with the exception of NMeGly29.

**Quantitative Congo Red**—Quantitative measurement of Congo red (CR) binding was carried out essentially as described in Refs. 30–32. Readings were taken on a Kontron Uvikon 922 spectrophotometer at 480 and 540 nm, and the amount of CR bound was calculated as follows: CR (bond) (M) = (Δabs/29285) – (Δabs/46306). Error bars were calculated from the range between duplicate readings.

**Thioflavin T (ThT) Fluorescence**—ThT fluorescence was carried out as described in Refs. 32 and 33. Measurements were carried out in triplicate on a Perkin-Elmer luminescence spectrophotometer LS50B using FLWINLAB software and an integration time of 5 s. All data were scaled using β-(25–35) alone as 100%. Scaled figures were plotted using the standard deviation to generate the error bars.

**Electron Microscopy**—Electron microscopy (EM) was carried out to determine whether the NMe derivatives were able to alter wild type peptide morphology. Peptides were solubilized at 500 μM (1 mm total peptide concentration for 1.1 combinations), and 50–500 μL drops were applied to glow discharged, carbon-coated, 400-mesh copper grids. Peptides were then negatively stained with 2% uranyl acetate before being viewed on a Philips EM 301 electron microscope at 100 kV using a magnification of 75,000.

**Toxicity Assay**—MTT reduction was carried out as described in Refs. 9 and 34 using rat pheochromocytoma (PC12) cells, with cells subcultured 50/50 1 day prior to assay. Prior to peptide application, cells were treated with 1 mg/mL DNase to break up any clumps, a feature of PC12 cell growth. This allows for a more accurate cell count. An overnight incubation at 37 °C followed mixing of peptides and cells before the addition of MTT, and after a further 2-h incubation cells were lysed and read at 550 nm. All readings were carried out in triplicate. Positive controls consisted of addition of 20 mM MOPS, pH 7, to cells, and negative controls consisted of the addition of 0.1% Triton X-100, which lyses cells and abolishes MTT reduction. Assay values for positive controls consisted of the addition of 0.1% Triton X-100, which lyses cells and abolishes MTT reduction. Assay values for positive.

**RESULTS**

**Detection of Amyloid Using ThT Fluorescence and CR Binding**—Formation of β-(25–35) amyloid was measured at 500 μM, alone and in 1:1 combinations with various NMe derivatives using ThT fluorescence and quantitative CR binding. Preparing and mixing peptides in water looks at the ability of NMe peptides to break down preformed β-(25–35) fibrils, whereas preparing and mixing peptides in 35% ACN is a way to observe their ability to prevent fibril assembly. We found that β-(25–35) alone showed the characteristic binding of CR (30), whereas the NMe derivatives in isolation do not, with the exception of NMeGly29. NMeGly29, NMeGly33, and NMeLeu34 were then analyzed by electrospray mass spectrometry generating the expected masses of 1060 for β-(25–35) fibrils, whereas the NMe derivatives in isolation formed ribbon-like fibrils visible under EM using 500 magnification of 75,000.

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**RESULTS**

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**Reduction of β-(25–35) Toxicity as Measured by MTT Assay**—Aggregation of Aβ and the related peptide β-(25–35) is known to be an important factor in toxicity because non-fibrillar soluble forms are nontoxic (11). We studied toxicity using the
FIG. 2. Congo red binding of β-(25–35), NMe peptides in isolation, and NMe peptides mixed with unfolded β-(25–35). Following mixing, peptides are lyophilized and resolubilized in 20 mM MOPS, pH 7, conditions that promote wild type (WT) aggregation.

FIG. 3. ThT fluorescence at Ex 450/Em 482 of β-(25–35), NMe peptides in isolation, and NMe peptides mixed with prefolded β-(25–35). Following mixing, peptides are lyophilized and resolubilized in 20 mM MOPS, pH 7. All data are scaled with β-(25–35) alone, representing 100% fluorescence. WT, wild type.

FIG. 4. ThT fluorescence at Ex 450/Em 482 of β-(25–35), NMe peptides in isolation, and NMe peptides mixed with unfolded β-(25–35). Following mixing, peptides are lyophilized and resolubilized in 20 mM MOPS, pH 7, conditions that promote wild type (WT) aggregation. The figure shows the results from two separate experiments, each of which was carried out in triplicate. All data are scaled with β-(25–35) alone representing 100% fluorescence.

established MTT assay, which measures the redox potential of cells, in this case PC12 cells, and thus monitors cell condition (9). Healthy cells reduce MTT, turning the redox dye from yellow to purple/blue, whereas unhealthy cells show less of a color change. This assay is specific for Aβ toxicity, and both Aβ and β-(25–35) inhibit the cellular reduction of MTT (35).

All peptides were prepared and mixed as described above using a concentration range of 20 nM-400 μM β-(25–35). Wild type peptide was assayed alone and in combination with 100 μM NMe peptides to see whether the ED₅₀ for the β-(25–35) toxicity curve was altered. Interestingly, at 400 μM β-(25–35) toxicity is reduced compared with slightly lower concentrations, consistent with protofibrils being the toxic form rather than highly aggregated and structured amyloid. It is possible that at this concentration aggregation is near completion, and it may be the process of aggregation that is toxic rather than the presence of the aggregates themselves (36). Apart from NMeGly29, none of the NMe peptides are toxic in isolation. On mixing with β-(25–35) we found that the NMe peptides had different effects depending on the mixing conditions. NMeGly25 shows the same toxicity profile as wild type, and NMeAla30 and NMeLeu34 have little effect using either of the mixing conditions, whereas NMeGly29 and NMeLeu34 have some inhibitory effect on toxicity when mixed with unfolded wild type. NMeGly29, however, appears to inhibit toxicity when premixed with either folded or unfolded β-(25–35) but with a greater effect when mixed with the unfolded peptide. Fig. 6 shows examples of some of these results, and Tables I and II provide a summary of the results.

DISCUSSION

The purpose of this study is to assess whether N-methylated derivatives of β-(25–35) can successfully inhibit fibril formation and the resultant toxicity of this amyloidogenic peptide. An important factor in the success of inhibition is the structural state of β-(25–35) when it is mixed with the potential inhibitor. Premixing peptides with unfolded wild type is generally more effective than premixing with aggregated wild type, because the latter requires the inhibitor to insert into and break up existing fibrils. We demonstrate that inhibition can be achieved using these NMe derivatives and that the outcome depends on which residue has been N-methylated.

NMeGly29 is a β-sheet peptide that aggregates and has the same toxicity profile as the wild type peptide. Fibrils typical of wild type are seen under EM, and the same level of CR binding occurs. It does not have the same effect upon ThT fluorescence, however, and generates a very small amyloid-induced peak. The addition of an NMe group at the N terminus of β-(25–35) still allows the peptide to fold, aggregate, and thus become toxic, but it clearly affects the mechanism of ThT binding. As with CR, the binding mechanism of ThT to amyloid is unknown (20, 23), but these results suggest that they must be binding different parts of the peptide because for NMeGly29, CR binding is typical of amyloid, whereas ThT binding is not. If the mechanism of binding of these dyes could be understood, it might give some insight into the structure of amyloid.

The remaining NMe peptides are all non-aggregating and nontoxic in isolation, demonstrating that the amide NH groups of residues 29, 30, 31, 33, and 34 are all essential to amyloid formation. These peptides show different levels of effectiveness as inhibitors of β-(25–35) aggregation and toxicity, and this does not appear to be related to secondary structure. Analysis of these peptides in isolation using circular dichroism (data not shown) shows that whereas NMeAla30, NMeGly29, and
NMeGly$_{33}$ in aqueous solution are predominantly random coil, only NMeGly$_{33}$ is an effective inhibitor of both aggregation and toxicity. NMeIle$_{31}$ and NMeLeu$_{34}$ both show elements of β-sheet structure in isolation, but only NMeLeu$_{34}$ is effective at altering wild type fibrils and inhibiting toxicity. Clearly, the position of the NMe group is most important and determines how the derivative interacts with the wild type peptide.

NMeGly$_{29}$, NMeAla$_{30}$, and NMeIle$_{31}$ are not effective inhibitors of wild type aggregation and toxicity. When mixed with aggregated β-(25–35), no differences in behavior are apparent and fibril size and morphology, as measured by EM, are comparable with wild type alone. On mixing with unfolded β-(25–35), there is a reduction in the amyloid-induced ThT fluorescence peak. Some inhibition of toxicity occurs, but the peptides must be present in at least 5-fold molar excess of the wild type before this is apparent.

For NMeLeu$_{34}$, the picture is much the same apart from fibril morphology. CR binding and ThT fluorescence are not affected when NMeLeu$_{34}$ is mixed with aggregated β-(25–35), and no inhibition of toxicity is seen. Fibril morphology as measured by EM is altered, however, demonstrating that it is possible for changes to occur without affecting the binding abilities of these dyes. This highlights the importance of using these assays in conjunction with both the EM and the MTT assay to obtain a complete picture. It may have been concluded otherwise that NMeLeu$_{34}$ has no effect on β-(25–35). NMeLeu$_{34}$ premixed with unfolded wild type exhibits this same altered fibril morphology under EM, but the effects on CR binding, ThT fluorescence, and toxicity of β-(25–35) are different. Toxicity is inhibited with NMeLeu$_{34}$ in 5-fold molar excess, and the ThT fluorescence peak is reduced. CR binding, however, is slightly greater than that of wild type alone. These results illustrate that although fibril morphology can appear the same under EM, differences in biochemical behavior still occur.

NMeGly$_{33}$ is the most effective inhibitor of β-(25–35) aggregation and toxicity. When mixed with aggregated wild type, although CR binding and ThT fluorescence are comparable with wild type alone, EM shows that fibrils are much smaller and finer in appearance. There is also inhibition of toxicity, not seen with any of the other NMe peptides using these mixing conditions. When premixed with unfolded β-(25–35), the effects are more dramatic. CR binding is abolished, the ThT fluorescence peak falls to less than 10% of the wild type alone, and no fibrils are visible under EM. Most importantly, NMeGly$_{33}$ can inhibit β-(25–35) toxicity when present in equimolar amounts. The midpoint of the toxicity curve is shifted from 30 to 120 μM β-(25–35). It should be noted, however, that when wild type peptide is completely unfolded in 35% AcN, at lower peptide concentrations, aggregation, and thus toxicity, does not reproducibly return during the time scale of the experiment. If it did return to the levels typical of those when wild type is solubilized in water, then NMeGly$_{33}$ would have shifted the midpoint of the curve from 3 to 120 μM. This means that PC12 cells can tolerate the presence of 40-fold more β-(25–35) with 100 μM NMeGly$_{33}$ than without it.

Mixing NMeGly$_{33}$ and NMeLeu$_{34}$ with unfolded wild type again highlights the differences in the binding mechanisms of CR and ThT. Although NMeGly$_{33}$ causes a reduction in the effect of β-(25–35) on both dyes, NMeLeu$_{34}$ increases the effect on CR binding but decreases the effect on ThT fluorescence. With NMeLeu$_{34}$, the long fibril structures observed under EM must permit CR to bind but alter the binding of ThT. With NMeGly$_{33}$, the wild type fibrils must either be reduced to a size too small to bind either dye or be observable under EM or they must disrupt fibril formation altogether.

The toxicity of β-(25–35) is dependent on fibril morphology.

**FIG. 5.** Electron micrographs. A, β-(25–35) alone; B, NMeGly$_{29}$ mixed with unfolded β-(25–35); C, NMeLeu$_{34}$ mixed with prefolded β-(25–35); D, NMeGly$_{33}$ mixed with prefolded β-(25–35). Total magnification, ×129,600. Scale = 100 nm.
and aggregation levels (11). This conclusion is supported by our results, but it should be noted that not all changes to fibril morphology result in a reduction of toxicity. With NMeGly\textsuperscript{33}, the disruption of fibrils does indeed lead to an inhibition of wild type toxicity. In the case of NMeLeu\textsuperscript{34}, however, the morphological change is not great enough to have an inhibitory effect, illustrating that morphology must be altered in particular ways for the NMe peptide to be an effective inhibitor. Both NMeGly\textsuperscript{33} and NMeLeu\textsuperscript{34} alter the wild type fibrils under both conditions, but NMeGly\textsuperscript{33} is clearly the better inhibitor of wild type toxicity and is most effective when fibrils are abolished or reduced to levels not visible under EM. It appears that these NMe derivatives are not simply binding to the end of a growing $\beta$-(25–35) fibril and blocking further addition of monomers but
that their structural effects are clearly more complicated. If it is the protofilament precursors of amyloid fibrils that are neurotoxic (17, 18), then it is possible that the N-methylated peptides are exerting their inhibitory effects via these elements rather than fully formed fibrils. Nevertheless, the effectiveness of NMeGly33 in particular does validate this strategy of using NMe derivatives of β-(25–35) and full-length Aβ to prevent aggregation and toxicity.

β-(25–35) is a particularly intractable peptide because it aggregates rapidly, unlike the full-length Aβ peptide, which requires aging for up to a week before it aggregates and becomes toxic (2). β-(25–35) is very toxic and, according to previous work, requires only nanomolar levels to have an effect (2), although we find toxicity is apparent only above 1 μM. It is therefore not surprising that although some inhibition is seen when NMeGly33 is added to aggregated β-(25–35), showing that the peptide must be in a dynamic state of equilibrium between the folded and unfolded state, inhibition works best when added to unfolded β-(25–35) before applying conditions that promote aggregation. Previous work done with β-sheet breaker peptides on full-length Aβ has proved effective to varying degrees (20, 22–27, 37, 38), and this provides encouragement that if NMe derivatives of β-(25–35) are added to β-(1–42) they will be able to disrupt the aggregation and toxicity of this peptide also. Future work will involve testing these derivatives and possibly NMe derivatives of Aβ itself on the full-length AD peptide.

We believe that the use of NMe derivatives could provide a general strategy for inhibiting the aggregation and toxicity of amyloid. There are more than 20 diseases that are the result of amyloid-like aggregation of particular peptides and proteins, including prion diseases, type II diabetes mellitus, Huntington’s disease, and Parkinson’s disease (39–41). The use of peptides as therapeutic agents, however, is problematic in two main ways. First, they must be able to cross the blood-brain barrier, and second, they must be able to avoid degradation by proteases in the brain. Previous work has shown that not only are the d-α-amino acid forms of Aβ and β-(25–35) equally toxic to their l-α-amino acid counterparts but that short d-α-amino acid β-sheet breaker peptides can bind to either α or β forms of Aβ (42). D-Amino acids are not susceptible to the natural proteolytic processes in the brain (37, 42), and therefore constructing NMe derivatives from d-amino acids could overcome one of the hurdles of creating an effective drug from these peptides. The use of small derivatives may also overcome the problem of getting these peptides to cross the blood-brain barrier (37), particularly if, like NMeGly33, the peptide is amphipathic. With these facts in mind, it is possible that the NMe derivatives of amyloidogenic peptides and proteins could provide a general and valid lead to therapeutic drugs for a large number of diseases that are currently untreatable.

Acknowledgments—We thank the Leukaemia Research Fund, University of Manchester Institute of Science and Technology for providing materials and facilities for cell culture, Sue Slack for guidance in cell culture techniques, and Drs. Mark Rosenberg and Stuart Ruffle for assistance with EM. We thank Dr. Russell Mortishire-Smith (Merck Sharp and Dohme) for helpful discussions.

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