A preliminary electron microscopic investigation into the interaction between Aβ_{1-42} peptide and a novel nanoliposome-coupled retro-inverso peptide inhibitor, developed as a potential treatment for Alzheimer's disease.

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Abstract. Alzheimer’s disease (AD) is a progressive neurodegenerative condition that results in severe cognitive and functional decline in sufferers and for which there are currently no effective treatments to halt or reverse disease progression. AD is the most common form of dementia and age is the major risk factor for this disease. With worldwide population structures changing as increasing number of individuals survive into old age, there is urgent need for novel disease modifying treatments for this condition, which has profound effects upon sufferers in addition to those around them. Some of us have previously developed a peptide inhibitor of Aβ_{1-42} aggregation (RI-OR2-TAT) that has been shown to reduce Aβ_{1-42} pathology in vivo in mouse models of AD.

~1690 copies of RI-OR2-TAT have been covalently attached to nanoliposome carrier particles forming Peptide Inhibitor NanoParticles (PINPs), and this study investigated the effect of PINPs upon Aβ_{1-42} aggregation at the molecular level. Our results show that PINPs are able to reduce Aβ_{1-42} aggregation and do so by binding early (oligomers) and late (fibrillar) stage aggregates. These results highlight the ability of PINPs to disrupt the formation of multiple Aβ_{1-42} aggregates capable of causing neurotoxicity and thus provide a strong case for PINPs to be carried forward into early stage clinical trials as a novel therapeutic option for the treatment of AD.

1. Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disease and is the leading cause of dementia [1]. The disease is listed as one of the most common causes of death in many countries, causing numerous symptoms such as impaired memory, cognitive decline, difficulty in
communication, and ultimately in the inability to perform activities of daily living. The major risk factor for AD is age, with the late-onset form (responsible for approximately 95% of cases) affecting individuals over the age of 65 [1]. Population structures continue to change as increasing numbers of people are surviving into old age and as a result, the need to address the lack of treatments available for diseases of old age such as AD is clear. AD affects, on average, 1 in 9 individuals over the age of 65 and this figure rises to 1 in 3 individuals over the age of 85 [2]. The characteristic pathological features of AD are the formation of extracellular senile plaques and intracellular neurofibrillary tangles. The senile plaques are formed by self-aggregation of the amyloid-$\beta$ (A$\beta$) peptide, which is cleaved from a precursor protein – APP – by $\beta$- and $\gamma$- secretases, whilst the neurofibrillary tangles are formed from hyperphosphorylated Tau protein. Aggregation of the A$\beta_{1-42}$ peptide primarily occurs via amino acid residues 16-22 which comprise the sequence KLVFFAE [3,4]. A$\beta$ assembles into toxic aggregates of varying sizes, however it is thought that the most neurotoxic forms are aggregates consisting of a small number of peptides, referred to as oligomers [5]. A multitude of evidence exists to suggest that at least some of the neurodegeneration observed in AD is due to the A$\beta$ peptide. Current therapies available to sufferers of AD are aimed at alleviating the symptoms of the disease rather than impacting upon disease progression. Although these treatments have been shown to reduce the rate of cognitive decline in AD sufferers, their lack of effect upon the underlying cause of the disease means that their beneficial effect is only temporary. In order to prevent the progression of AD, disease modifying therapies (DMTs) are required which address the underlying pathological mechanisms of the disease and thus prevent, or maybe even reverse, the effects of AD. As of yet, no DMTs have progressed past phase III trials and, therefore, there are none available for the treatment of AD.

Previously, we have developed a brain-penetrating retro-inverso peptide inhibitor (RI-OR2-TAT, Ac-rGffvlkGrrrrqrrkkrGy-NH2) that blocks the in vitro formation and toxicity of the A$\beta$ oligomers whilst also stimulating neurogenesis [6]. Previous studies have indicated that the aggregation inhibiting activity of this peptide is due to competitive binding between RI-OR2-TAT and A$\beta_{1-42}$ monomers for the KLVFF region of the A$\beta_{1-42}$ peptide [4]. In this project RI-OR2-TAT has been attached to the surface of liposomes to attempt to produce a multivalent inhibitor of A$\beta_{1-42}$ toxicity (termed Peptide Inhibitor NanoParticles – PINPs), and we have analyzed the interaction of the PINPs with A$\beta_{1-42}$ at the molecular level by transmission electron microscopy (TEM). It is hoped that RI-OR2-TAT will prove to be a successful DMT for AD, thus providing a more positive prognosis for the tens of millions of people suffering from this disease worldwide [7].

2. Materials and Methods

2.1. PINPs
PINPs were produced by Dr M Gregori and Dr M Masserini at the University of Milano-Bicocca, Milan, Italy. Liposomes consist of 1:1 cholesterol:sphingomyelin with 5% maleimide polyethylene glycol (PEG). RI-OR2-TAT is attached to the PEG via the maleimide group by ‘click-chemistry’ to form PINPs. PINPs were solubilized in either PBS or MilliQ water.

2.2. Transmission Electron Microscopy
Samples were imaged using a JEOL JEM 1010 transmission electron microscope at an operating voltage of 80kV. Samples were placed on 300-mesh carbon/formvar coated copper grids (Agar Scientific, UK) and negatively stained with 2% (w/v) phosphotungstic acid (PTA) pH 7.4.

2.2.1. Immunolabeling
Briefly, 4μl of sample was added to grids for 1 minute, blotted and grids were blocked in 1:10 Goat serum:PBS*. Samples were then incubated with primary antibody (6E10, BioLegend) at room temperature for 1h, washed with PBS*, incubated with secondary antibody (G777, Sigma-Aldrich) for
1h, washed in PBS and then a further wash in MilliQ water. Grids were then negatively stained by addition 4μl of PTA stain for 1 minute.

3. Results
The purpose of this study was to investigate any interactions between PINPs and Aβ1-42 using TEM.

3.1. Investigating the effect of PINPs upon Aβ1-42 aggregation.

Aβ1-42 samples incubated at 37°C for 48 hours in the absence of PINPs revealed numerous large masses of aggregation, illustrating the tendency of Aβ1-42 to self-aggregate into the multimeric aggregates associated with toxicity in AD (Figure 1A). Interestingly, PINPs + Aβ1-42 coincubation resulted in an altered appearance of PINPs (C) and binding of PINPs to Aβ1-42 fibrils (D) following co-incubation. A proposed explanation for this finding was the binding of small oligomers to the PINP surface. The number of Aβ1-42 aggregates observed in the absence of PINPs was increased in comparison to the sample in which PINPs were present. Upon analysis, the Aβ1-42 + PINPs sample clearly showed PINPs binding to Aβ1-42 fibrils of various lengths, with binding observed along the length of the fibril as well as to fibril termini (Figure 1D).

3.2. Immunogold labelling of PINPs to evaluate their ability to bind Aβ1-42 oligomers.

Aβ1-42 samples incubated at 37°C for 48 hours in the absence of PINPs revealed numerous large masses of aggregation, illustrating the tendency of Aβ1-42 to self-aggregate into the multimeric aggregates associated with toxicity in AD (Figure 1A). Interestingly, PINPs + Aβ1-42 coincubation resulted in an altered appearance of PINPs (1B-Before, 1C-After), with PINPs appearing much ‘rougher’ than when observed in the absence of Aβ1-42. A proposed explanation for this finding was the binding of small oligomers to the PINP surface. The number of Aβ1-42 aggregates observed in the absence of PINPs was increased in comparison to the sample in which PINPs were present. Upon analysis, the Aβ1-42 + PINPs sample clearly showed PINPs binding to Aβ1-42 fibrils of various lengths, with binding observed along the length of the fibril as well as to fibril termini (Figure 1D).

Figure 1 shows the effect of PINPs upon the aggregation of Aβ1-42 following 48-hour incubation at 37°C. (A) Aβ1-42 aggregates in absence of PINPs. (B) PINPs alone. Altered appearance of PINPs (C) and binding of PINPs to Aβ1-42 fibrils (D) following co-incubation. Scale bars = (A) = 200nm (B,C,D) =100nm.

Figure 2– Immunogold labelling to detect PINP bound Aβ1-42 oligomers. (A) Immunolabeling of Aβ1-42 on a PINP surface. (B) In absence of primary antibody, no labelling is observed. Scale bar = 100nm.
To test the hypothesis that the altered appearance of PINPs in the presence of Aβ1-42 was due to binding of Aβ1-42 oligomers, immunolabeling was performed to detect any Aβ1-42 bound to the PINP surface. Results (Figure 2A) showed positive labelling (black dots) on the PINP surface indicating the presence of Aβ1-42 oligomers. Figure 2B shows that in the absence of the primary antibody, no immunolabelling is observed, illustrating that the secondary antibody only binds where the primary antibody is present, showing the specific labelling only where Aβ1-42 is present. Therefore the positive labelling on the PINP surface strongly indicates the presence of Aβ1-42.

4. Discussion
This study investigated the interactions between PINPs and Aβ1-42 at the molecular level in an attempt to evaluate the potential for PINPs to be carried forward as a novel treatment for AD.

Our results indicate that PINPs are able to suppress fibril formation and elongation as co-incubation of Aβ1-42 with PINPs resulted in much fewer dense Aβ1-42 aggregations than was observed with Aβ1-42 alone. Our results show that PINPs are able to bind directly Aβ1-42 fibrils, both along the length of the fibril and to fibril termini. These results suggest that binding of PINPs is able to inhibit further fibril elongation, providing a visual explanation for the termination of fibril elongation reported during analysis of RI-OR2 [4]. Whilst evaluating the ability of PINPs to interact with Aβ1-42, a change in PINP morphology was observed, which we hypothesized was due to the binding of Aβ1-42 oligomers. Binding of Aβ1-42 to the PINP surface was investigated and confirmed using an immunolabelling approach where 10nm gold conjugated antibodies were used to signal positive labeling, demonstrating the ability of PINPs to bind to early (oligomers) and late stage (fibrils) Aβ1-42 aggregates. Thus our results confirm that the peptide has retained the ability to interact with early and late stage aggregates despite attachment to the nanoliposomal carrier particle [4].

This study demonstrates that attachment of RI-OR2-TAT to nanoliposome carrier particles preserves the ability of the peptide to bind oligomers and mature fibrils, reducing total levels of Aβ1-42 aggregation and appearing to inhibit fibril elongation. PINPs were developed as a potential DMT for the treatment of AD. By attaching ~1690 RI-OR2-TAT peptides to the liposome surface, PINPs are able to function as a multivalent inhibitor of Aβ1-42 aggregation, with potential for use in the treatment of AD. We believe that these results demonstrate the strong potential that PINPs possess to be carried forward for further investigation, including early stage clinical trials in order to evaluate their safety and potential therapeutic efficacy in human subjects.

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