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COMMUNICATION

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Crown ethers attenuate aggregation of Amyloid beta of Alzheimer's diseaseYanli Tian^{1,2§}, Xueli Zhang^{1,3,4§}, Yuyan Li^{1,3}, Timothy M. Shoup⁵, Xin Teng⁵, David R. Elmaleh⁵, Anna Moore^{1*}, and Chongzhao Ran^{1*}Received 00th January 2012,
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In this report, we reasoned that non-covalent modification of amyloid beta (A β) by crown ethers could inhibit its aggregation. We demonstrated that PiB-C, a conjugate PiB and crown ether, could significantly reduce the aggregation in vitro. Additionally, two-photon imaging showed that PiB-C could efficiently label A β plaques and CAAs in AD mice.

No cure is currently available for Alzheimer's disease (AD). Reducing amyloid beta (A β) loading is one of the main goals for AD drug development^{1, 2}. In past decades, several categories of A β -reducing agents have been developed, and some of them had advanced into clinical trials. These categories included aggregating inhibitors such as tramiprosate, humanized monoclonal antibodies such as bapineuzumab, β -secretase inhibitors LY2811376 and the γ -secretase inhibitor Flurizan. Unfortunately, these clinical trials, by and large, failed to demonstrate their efficacy and safety²⁻⁴.

All these failures clearly imply that new strategies for developing drugs for AD are urgently needed. In this report, we propose a new strategy to attenuate the aggregation of A β s through a non-covalent modification at its surface. We reasoned that crown ethers could be used to "neutralize" positive charges of the amino groups of A β s through the formation of hydrogen bonds. To specifically target A β s with the purpose of reducing aggregation, we proposed a conjugate (PiB-C) of a 12-crown-4 ether and PiB (Pittsburg Compound B, the widely used PET ligand for imaging A β s⁵⁻⁷).

Crown ethers are well known for their capability to form complexes with alkali ions such as K⁺, Na⁺ and Li⁺. Apart from their high affinities for alkali ions, crown ethers also could form stable complexes with protonated amines through hydrogen bonds, a property that has been adapted for various applications of crown ethers. Beauchamp et al, Julian et al., and others developed the SNAPP (selective non-covalent adduct protein probing) technique using crown ether to probe protein sequences, which is based on the selectivity of crown ethers towards basic amino acids such as lysine, arginine, and histidine⁸⁻¹⁰. Using theoretical electronic structure calculations, Chen et al. recently demonstrated that crown ethers could form non-covalent complexes with lysine, arginine and

histidine¹¹. Clemmer et al. used crown ethers as shift reagents for ion mobility spectrometry¹². Moreover, molecular recognition capacity of crown ethers has recently been utilized for the investigation of the protein-folding process^{8, 13, 14}. We believe that non-covalent alteration of the folding property of the aggregation-prone protein/peptide could be a new approach for designing new anti-aggregation drugs.

Under physiological pH, basic amino acids of A β are partially positively charged. Yoshiike et al. reported that positively charged A β fibrils/protofibrils are highly cytotoxic, and neutralization of the charges could reduce neuronal toxicity^{15, 16}. Moreover, covalent modification of the charged amino groups of A β peptides through glycation and acylation could significantly reduce cytotoxicity of A β species¹⁵. In addition, Yang et al. showed that surface coating of A β s with open-chain conjugates of polyether-thioflavin analogues could reduce the adhesion of A β s towards Anti-A β IgG, and could ameliorate dendritic spine density and improve cognitive function in an AD mouse model^{17, 18}.

Studies suggested that K16 and K28 are crucial amino acids for the mis-folding of A β s, because K16 could form inter-sheet salt bridges and K28 can form intra-peptide salt bridges with Aspartate23 (D23) leading to the stabilization of the misfolded peptides^{19, 20}. In this report, we hypothesized that crown ethers can form non-covalent complexes with A β peptides through the formation of hydrogen bonds with positively charged basic amino acids such as R5 (arginine-5), K16, K28 (lysine-16, 28), and H13, H14 (histidine-13, 14). We reasoned that crown ether has the capability to break down the salt bridge, and thus to attenuate the aggregation process of A β s.

12-crown-4 ether was chosen over other crown-ethers in our studies, due to the following reasons: 1) it has insignificant disturbance of the homeostasis of the physiological ions such as K⁺, and Na⁺²¹; 2) its low molecular weight can be beneficial for BBB penetration; 3) it can effectively form complexes with charged amino acids¹². In our preliminary studies we used a non-conjugated 12-crown-4 ether to demonstrate its anti-aggregating properties in A β solution.

We first used zeta-potential measurement to investigate whether 12-crown-4 ether can non-covalently change the surface charges of A β s. Zeta potential has been used to evaluate the potential change between the double layer and the sliding plane of particles, and has also been used for studying interactions between amyloid fibrils and its ligands^{16, 22, 23}. Our zeta-potential test suggested that 12-crown-4 ether could change the surface charges of A β s. Zeta potential of A β 40 fibrils/aggregates was significantly changed once it was mixed with 12-crown-4 ether (from -48 mV to -4 mV), suggesting that the crown ether could modify the surface charges of A β s (Fig.1a).

Next, we investigated whether 12-crown-4 ether could inhibit the aggregation A β 40 by incubating A β 40 with 12-crown-4 ether in PBS (pH 7.4) for 10 days. Thioflavin T (ThioT) was used for monitoring the aggregation of A β 40. We found that fluorescence intensities of the 12-crown-4-treated group were significantly (50%) lower than that of the control group (Fig.1b). From TEM images, fewer A β deposits could be found in the samples treated with 12-crown-4 ether (Fig.1e). Interestingly, we observed a different morphology of periodic twists in these images. Specifically, the twists of the A β fibrils were apparently rougher in the presence of 12-crown-4 ether than without it (Fig.1e right panel). These data most likely suggest that 12-crown-4 ether could affect the aggregation process. It has been reported that different morphology of fibrillar A β s could have different toxicity, and more twisted A β s fibrils may have higher toxicity²⁴. To further validate the effectiveness of 12-crown-4 ether, we used a dot blot for measuring the amount of protofibrils/fibrils in solution with A β antibody 2H4, which is more specific for fibrillar/protofibrillar A β s than for monomers/oligomers²⁵⁻²⁷. Compared to the signal from the control group, a significantly lower signal (62%) was observed for the 12-crown-4 ether group after 48 hours of incubation (Fig.1c, d), suggesting that it could attenuate the formation of protofibrils/fibrils.

The above studies indicated that 12-crown-4 ether could efficiently inhibit the aggregation of A β s; however this compound is not A β specific. For targeting, we used a well-studied A β ligand PiB, which is specific to A β s, particularly to fibrillar A β s⁵⁻⁷. A conjugate PiB-C was synthesized by attaching the 12-crown-4 ether moiety to the amino group of PiB (Fig.2a). The synthesized PiB-C showed a similar fluorescence spectrum as PiB. However, once mixed with fibrillar A β 40, it displayed a significant emission wavelength red-shift, which probably reflected polarization that was induced by the interaction of 12-crown-4 ether with the positively charged amino groups of A β s (SI Fig.1a).

Like with 12-crown-4 ether, we found that PiB-C could change the zeta potential of A β fibrils (SI Fig.1b). Before testing the anti-aggregating ability of PiB-C, we first examined the fluorescence contribution from PiB-C under the ThioT testing condition. We found that the contribution was minimal (data not shown), because the used 450nm excitation was suitable for ThioT, but not for PiB-C. To investigate the efficiency of PiB-C, we incubated PiB-C with A β 40 in PBS for 6 days. Both the ThioT testing and dot blotting indicated that PiB-C could inhibit the aggregation (Fig.2b-d). At day 6, $F_{(\text{ThioT})}$ of the control samples was 2.47-fold higher than that of the PiB-C-treated samples (Fig.2b). Dot blot with 2H4 antibody showed that the signal from the control group was 1.62-fold higher than that from the PiB-C group (Fig.2c, d). Moreover, TEM images from the PiB-C-treated samples showed fewer fibrillar A β deposits than that from the control samples. We also observed that the detailed morphology of the fibrils was different, and the fibrils from

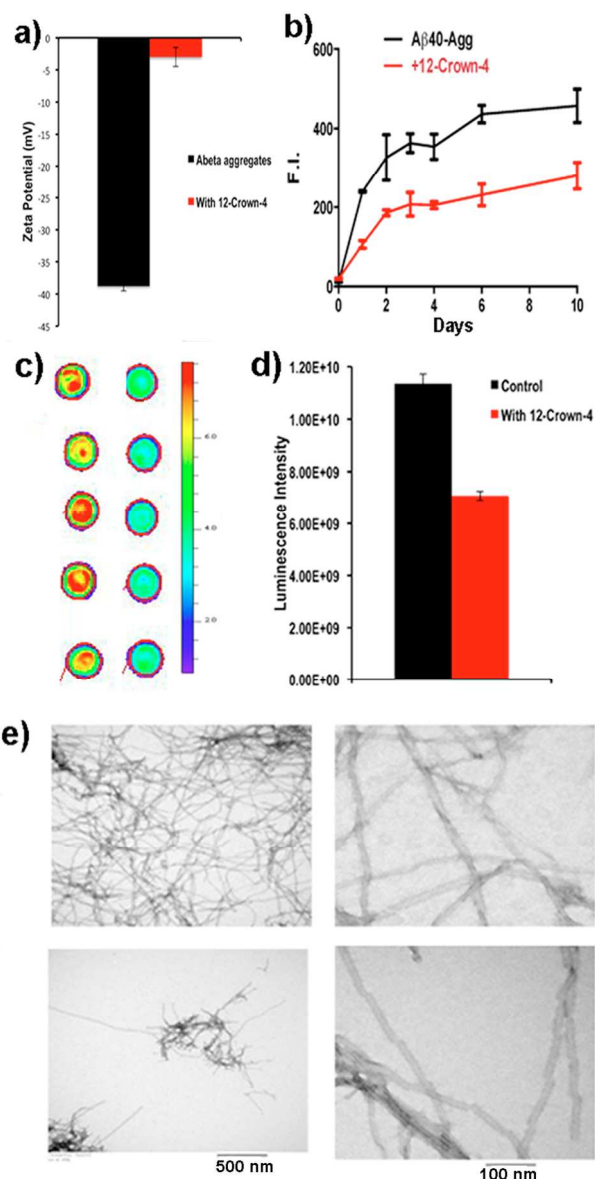


Fig.1 Anti-aggregation testing of 12-crown-4 ether for A β 40 in solutions. (a) Zeta potential measurements of A β 40 aggregates with and without 12-crown-4 ether (n=3). (b) Change in fluorescence intensities of ThioT as a measurement of A β 40 aggregation at different time points with and without 12-crown-4 ether (n=3). (c) Dot blotting of A β 40 incubated without (left) and with (right) 12-crown-4 ether for 48-hours (n=5). (d) Quantitative analysis of the dots in (c). (e) Representative TEM images of A β 40 without (upper) and with (bottom) 12-crown-4 ether on day10. Two sets of images are shown with different scale bars.

PiB-C samples were less twisted (SI Fig.2). We also used non-conjugated PiB as a control. Although PiB showed apparent aggregation inhibitory effects from ThioT testing and dot blotting, it was obviously weaker than PiB-C (Fig.2b, c, d), indicating that the crown-ether moiety of PiB-C contributes to the inhibitory effects.

In addition, Sengupta et al showed that the intrinsic fluorescence of tyrosine (Y10) of A β s could be used to estimate the critical concentration for initializing aggregation (or thermodynamic saturation concentration (C_{sat}))²⁸. In this method, the fluorescence ratio of tyrosine of the supernatant after centrifuging at 0 h ($F(0)$)

and a certain time point ($F(t)$) was used to find C_{sat} . Our data indicated that C_{sat} was between $10\mu\text{M}$ and $15\mu\text{M}$ after incubation for 120 hours for the samples without PiB-C. This result was similar to the reported data²⁸. However, no significant ratio changes were observed with PiB-C, suggesting PiB-C can effectively reduce aggregation (SI Fig.3a).

To examine whether 12-crown-4 ether and PiB-C treatment can reduce $A\beta$ toxicity, we treated SH-SY5Y neuronal cells with $A\beta 42$ ($20\mu\text{M}$) for 4 hours in the absence or presence of 12-crown-4 ether and PiB-C ($40\mu\text{M}$). The cells were treated under five different conditions, including 1) pure cell culture medium, 2) the vehicle that was consisted of 1% DMSO and 9% PBS and 90% cell culture medium, 3) $A\beta 42$ and the vehicle, 4) 12-crown-4 ether, $A\beta 42$ and the vehicle, 5) PiB-C, $A\beta 42$ and the vehicle, and 6) PiB and the vehicle. The cell viability from the pure culture medium treated group was considered to be 100%. Our results indicated that both 12-crown-4 ether (green bar) and PiB-C (red bar) could lower the toxicity of $A\beta 42$ (black bar) (Fig.2e). However, no apparent neuroprotective effect was observed with PiB, indicating that the neuroprotective capacity of PiB-C was not originated from PiB. Although ThioT has been reported as a potential neuroprotective compound²⁹, the ineffectiveness of PiB (analogue of ThioT) is probably due to its structural difference from ThioT.

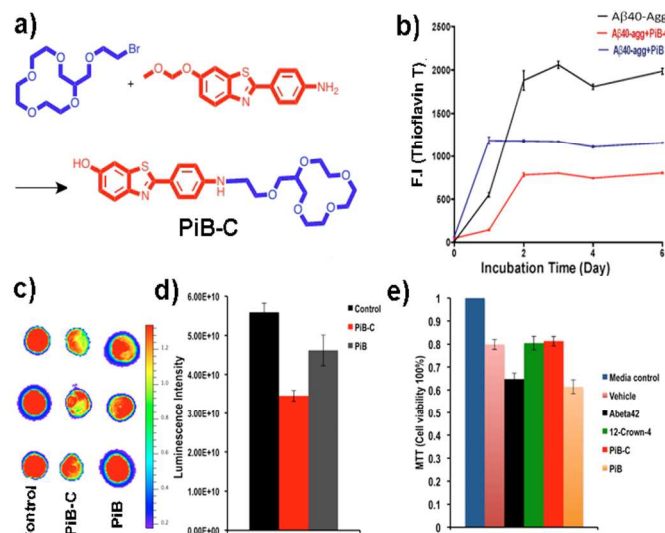


Fig.2 Anti-aggregating studies with PiB-C. (a) The synthetic route for PiB-C. (b) Change in fluorescence intensities of ThioT as a measure of $A\beta 40$ aggregation at different time points without (black line) and with PiB-C (red line), and with PiB (blue line) ($n=3$). (c) Dot blotting of $A\beta 40$ incubated without (left) and with PiB-C (middle), and PiB (right) for 48-hours ($n=3$). (d) Quantitative analysis of the dots in (c). (e) MTT Testing of cell viability under different treatment conditions.

To investigate whether PiB-C could specifically interact with $A\beta$ s in a biologically relevant environment, we first incubated PiB-C with a mouse AD brain slice with characteristic $A\beta$ plaques. As expected, PiB-C could specifically label the plaques (Fig.3a, b). To further investigate whether PiB-C could potentially be used for in vivo studies, we first tested its BBB penetration in a wild type mouse using two-photon imaging. Our results indicated that PiB-C, like PiB, could efficiently cross BBB (SI Fig.3b). PiB-C reached its maximum in brain around 10 minutes after i.v. injection, and then slowly washed out. To further test whether PiB-C could specifically

interact with $A\beta$ plaques in vivo, we used two-photon imaging to validate. As expected, PiB-C can clearly highlight the plaques (Fig.3c, d). Similar to PiB³⁰, PiB-C could also efficiently label CAAs (cerebral amyloid angiopathy). Taken together, all these data indicated that PiB-C could be used for future in vivo therapeutic studies.

In summary, in this report we propose a novel approach to inhibit the aggregation of $A\beta$ s through neutralizing positive charges on amino acids of $A\beta$ s. Our approach is different from traditional approaches that include high throughput screening and other organic dye-based structure modification. Introduction of 12-crown-4 ether would not only neutralize positive charge, but could also change the hydrophobicity of the $A\beta$ surface, which probably also contributed to the efficiency of 12-crown-4 ether. One of the limitations of PiB is its binding to white matter in the brain³¹. PiB-C may increase its specificity towards $A\beta$ s over white matter, because PiB-C has much higher hydrophilicity due to the crown ether moiety. In addition, PiB-C could be considered as a theranostic agent, because it could be potentially used for both therapy and imaging. Although results from $A\beta$ antibody therapy for AD indicated that removing plaques and anti-aggregation could increase the concentrations of neurotoxic $A\beta$ oligomers³²⁻³⁵, whether small anti-aggregation molecules will behave similarly is not clear. Therefore, we propose further in vivo studies in mice to investigate whether PiB-C can reduce both insoluble and soluble $A\beta$ s.

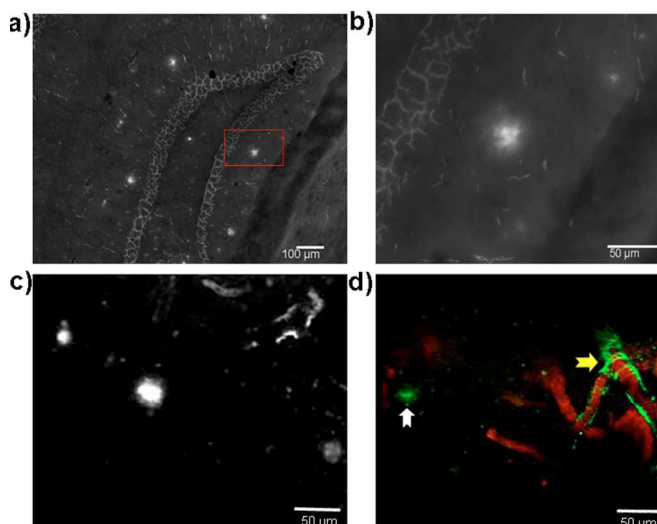


Fig.3 Microscopic imaging of amyloid deposits with PiB-C in vitro and in vivo. (a-b) Microscopic images of PiB-C showing stained plaques in a hippocampus area of a brain slice from a 10-month APP-PS1 mouse. (b) Zoomed-in image of a plaque in the red box in (a). (c-d) Representative two-photon images of PiB-C in a 12-month old APP-PS1 mouse. (c) $A\beta$ plaques were highlighted by PiB-C; (d) both CAAs (yellow arrow) and plaques (white arrow) were highlighted by PiB-C (green). The blood vessels were labeled with Texas Red-Dextran conjugates (MW 70,000) (red).

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Notes

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