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Posted Date: 13 June 2024

doi: 10.20944/preprints202406.0901.v1

Keywords: alpha-Synucleinopathy; Adenosine A1 receptor; 1-Aminoindan; Caffeine; Neuroprotection; Biodistribution; PET-Imaging; Protein Misfolding



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Article

The Bifunctional Dimer Caffeine-Indan Attenuates α -Synuclein Misfolding, Neurodegeneration and Behavioural Deficits after Chronic Stimulation of Adenosine A1 Receptors

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Abstract: We previously found that chronic adenosine A1 receptor stimulation with N⁶-Cyclopentyladenosine increased α -synuclein misfolding and neurodegeneration in a novel α -synucleinopathy model, a hallmark of Parkinson's disease. Here, we aimed to synthesize a dimer caffeine-indan linked by a 6-carbon chain to cross the blood-brain barrier and tested its ability to bind α -synuclein, reducing misfolding, behavioural abnormalities, and neurodegeneration in our rodent model. Behavioural tests and histological stains assessed neuroprotective effects of the dimer compound. A rapid synthesis of the ¹⁸F-labelled analogue enabled Positron Emission Tomography and Computed Tomography imaging for biodistribution measurement. Molecular docking analysis showed that the dimer binds to α -synuclein N- and C-termini and the non-amyloid- β -component (NAC) domain, similar to 1-aminoindan, and this binding promotes a neuroprotective α -synuclein "loop" conformation. The dimer also binds to the orthosteric binding site for adenosine within the adenosine A1 receptor. Immunohistochemistry and confocal imaging showed the dimer abolished α -synuclein upregulation and aggregation in the substantia nigra and hippocampus, and the dimer mitigated cognitive deficits, anxiety, despair, and motor abnormalities. The ¹⁸F-labelled dimer remained stable post-injection and distributed in various organs, notably in the brain, suggesting its potential as a Positron Emission Tomography tracer for α -synuclein and adenosine A1 receptor in Parkinson's disease therapy.

Keywords: α -Synucleinopathy; Adenosine A1 receptor; 1-Aminoindan; Caffeine; Dimer; N⁶-cyclopentyladenosine; Neuroprotection; Biodistribution; PET-Imaging; Protein misfolding.

Background

Dysregulation of adenosine homeostasis has been linked to aging-related neurodegenerative diseases [1,2]. We recently reported that a 7-day chronic intraperitoneal (i.p.) injection of the adenosine A1 receptor (A1R) agonist N⁶-Cyclopentyladenosine (CPA) produced similar levels of α -synuclein (α -Syn) upregulation and neurodegeneration as in 5-week daily i.p. CPA injections [3,4]. Therefore, the 7-day CPA chronic injection represented a convenient α -synucleinopathy model to test

novel compounds with neuroprotective potential and drugs that could increase the CPA-induced neurotoxicity. Some neuroprotective drugs have been found to bind to α -Syn and prevent further aggregation, including caffeine, nicotine, 1-aminoindan and metformin [5]. Conversely, there are other drugs such as methamphetamine, cocaine, 2-aminoindan and the herbicides, paraquat and rotenone, which appear to be neurotoxic because they increase α -Syn misfolding and can be correlated with a higher incidence of PD [6–8]. Nonetheless, even with drugs such as caffeine or nicotine, there are several problems including being unsafe at high doses and causing numerous side effects [9]. However, it was argued that linking these compounds and other similar neuroprotective drugs in dimers or trimers would decrease the misfolding and aggregation of α -Syn without risking the safety of the patients [10]. These compounds can be also referred to as bifunctional drugs [11]. They are connected via a six-carbon alkyl linker to minimize solubility issues and retain the flexibility that will allow further binding to α -Syn. Their synthesis and purity have been previously described [10].

The dimers were tested in vitro using techniques such as nanopore analysis and it was established that they bind to α -Syn and do not cause a compact conformation [10]. Focusing on the caffeine-indan (C₈-6-I) dimer, the isothermal titration calorimetry results indicated that the dimer has 1 binding site with α -Syn and a binding constant of $5.3 \times 10^4 \text{ M}^{-1}$, thus it appears to be neuroprotective [10]. Furthermore, the C₈-6-I dimer was successful in rescuing one and two-copied α -Syn-Green Fluorescent Protein (GFP) yeast strain from the α -Syn-induced cell death under the control of a galactose promoter. This dimer was also effective in preventing large α -Syn foci in the one and two-copied α -Syn-GFP yeast [10]. Present in vitro studies of the C₈-6-I dimer were conducted using molecular docking simulations [12]. This technique allows the prediction of the binding conformation of the eight in vitro α -Syn structures published from the Chen group [13,14] with our dimer. This technique is helpful in further elucidating the effect of the dimer on the misfolding patterns of α -Syn. Moreover, in the present study, the C₈-6-I was tested in combination with the A1R agonist CPA. Following 7-day i.p. treatments with vehicle control or with CPA (3mg/kg) in absence or presence of C₈-6-I (3 or 5 mg/kg), the animals underwent a series of behavioural tests such as Y-maze, open field, and forced swim test. We hypothesized that systemic administration of the dimer would inhibit the CPA-induced upregulation and aggregation of α -Syn similar to the previously reported neuroprotective effects of the A1R antagonist DPCPX and 1-aminoindan [3,4]. We will use molecular docking to determine whether the dimer interacts with α -Syn to induce a neuroprotective “loop” conformation, which could explain reduced neurodegeneration and improved behavioural outcomes of animals.

To test the blood-brain barrier permeability and distribution of the dimer we also performed an in vivo study with CD-11 albino mice using PET imaging. Before PET imaging, the dimer was radiolabeled with fluoride-18 [¹⁸F] radioisotope [11]. Therefore, PET imaging allows the quantitation of drug concentration, distribution, and pharmacokinetics of radiotracers [15], as well as providing information about physiology such as metabolism, receptor concentration, and transport across cell membranes [16,17], as a result, PET is a useful tool in drug development [18–20]. Since the pharmacological activity of a drug depends on the concentration reaching the tissue of interest, establishing the concentration of free drug reaching organs/tissues of interest and its full biodistribution is an important starting point for early phase development [17]. PET can be used to non-invasively infer molecule distribution in vivo and brain exposure [17,21]. Recent advances in radiochemistry now mean many drug molecules can be rapidly labelled with radionuclides such as ¹¹C or ¹⁸F and used for biodistribution studies [22,23]. Radiotracers are commonly used in non-clinical studies to assess the drug absorption, distribution, metabolism and excretion (ADME) profile of a drug [24]. To measure the biodistribution of C₈-6-I, we have now developed a rapid and robust synthesis of the ¹⁸F-labelled analogue (as seen in **Figure 1**) for PET imaging and biodistribution studies. Here we also report the radiosynthesis, purification, and in vivo PET imaging and ex vivo biodistribution of ¹⁸F- C₈-6-I in healthy CD-1 mice.

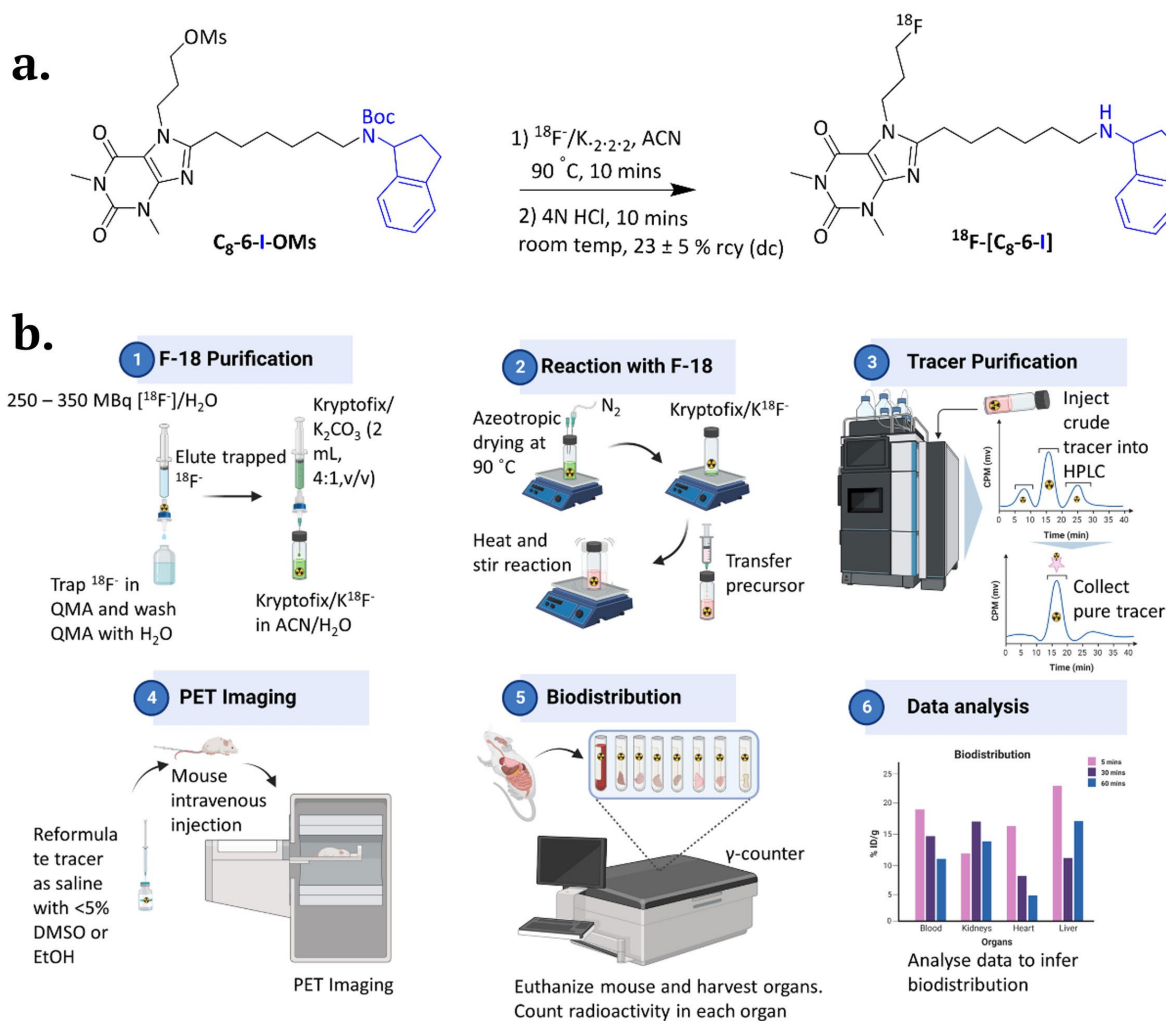


Figure 1. Radiosynthesis of the C₈-6-I dimer and in vivo studies with the CD-1 mice. a. Formation reaction of ^{18}F -C₈-6-I from C₈-6-I-OMs in $23 \pm 5\%$ rcy (decay corrected). **b.** All steps involved in the b.1 radiosynthesis of ^{18}F -C₈-6-I from [^{18}F]fluorine purification, b.2 reaction with Kryptofix/ K^{18}F and b.3 further purification to b.4 PET-imaging and b.5 biodistribution of the ^{18}F -C₈-6-I in major organs. The data was then analyzed and graphed using GraphPad Prism 8 (San Diego, CA, USA). Created using BioRender.com.

2. Results

2.1. Caffeine-Indan Dimer Decreased the Behavioural Deficits after Chronic A1R Stimulation

We assessed the effects of the dimer compound in the recently described α -synucleinopathy model [3,4] involving daily administration of A1R agonist to Sprague-Dawley rats for 7 days or daily for 5 weeks, and we measured α -Syn expression and aggregation, neurodegeneration and behavioural deficits. Male Sprague-Dawley rats were tested for hippocampal-dependent spatial learning deficits using the Y-maze, for anxiety using the open field test, and for helplessness and motor behaviour using the forced swim test [4,25]. The 7-day chronic injections with CPA induced hippocampal-dependent spatial memory deficits that were assessed using the Y-maze test (see **Figure 2 a**). The co-administration of either 3 or 5 mg/kg C₈-6-I with 3 mg/kg CPA attenuated the learning deficits caused by CPA as shown by a recovery of the percentage of time spent in the novel arm closer to the vehicle control values. The 5 mg/kg C₈-6-I injection significantly ameliorated the cognitive deficits caused by CPA. Another noteworthy observation was the tendency of rats to remain in the start arm when co-administered with CPA + 3 mg/kg C₈-6-I. as seen in **Figure 2 a**. These rats spent

approximately 45% of their time in this arm. A higher percentage of total start arm exploration indicates a possible sign of anxiety.

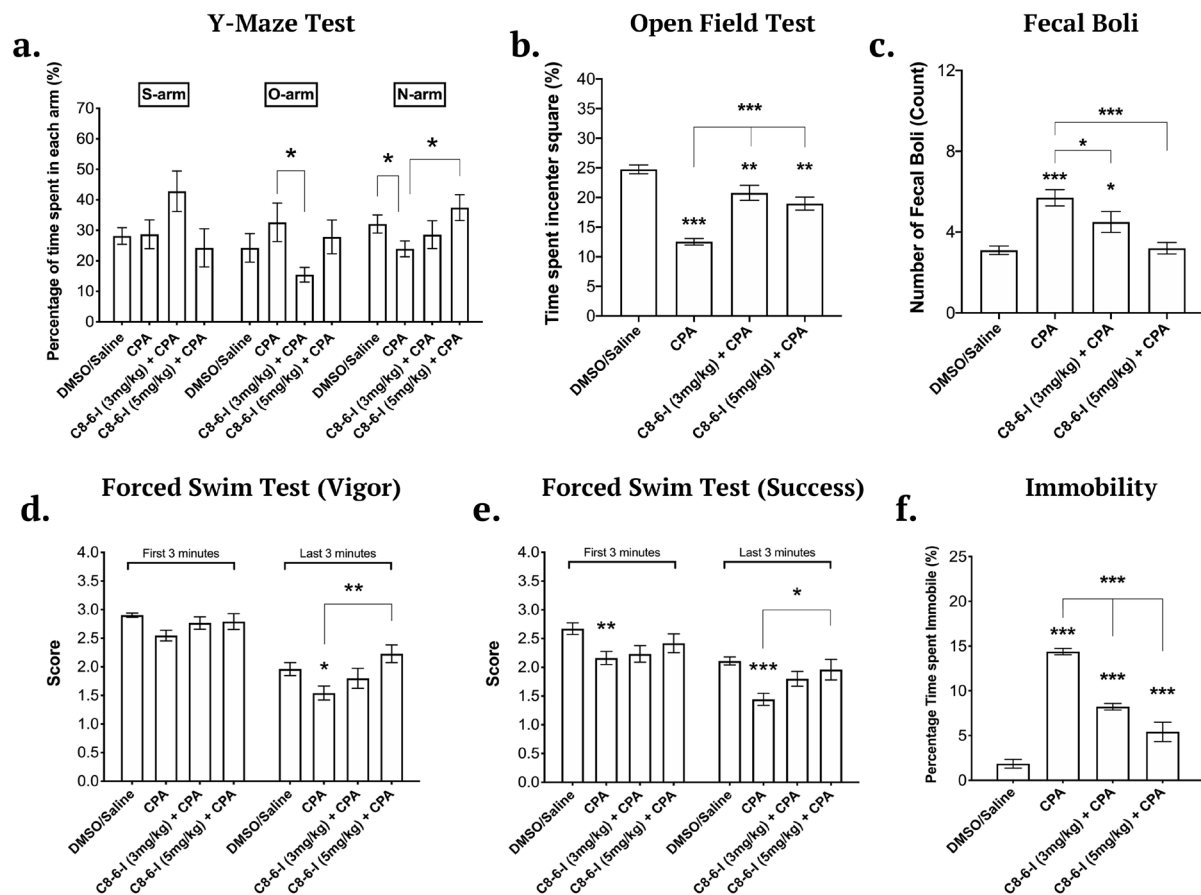


Figure 2. Behavioural tests conducted for male Sprague Dawley rats after the 7-day chronic injection of C8-6-I at 3 and 5 mg/kg. a. Y-maze test values of the 7-day chronic C8-6-I dimer (3 mg/kg and 5mg/kg) as percentage of time spend in each of the arms: S-arm ("start" arm), O-arm ("old" arm), and N-arm ("new" arm). The percentages of the time spent in each arm were calculated from the 5-minute trial. Open field test values of the 7-day chronic C8-6-I dimer (3 mg/kg and 5mg/kg) as percentage of time spend in the center square. The animal is placed in the center square of the grid and free to explore the field for 10 minutes. b. The percentage of the time spend in the red center square. c. The total fecal boli count. Forced swim test results of the 7-day chronic C8-6-I dimer (3 mg/kg and 5mg/kg) treatment groups as measurements of swimming vigorously and successfully. The animals were placed on the forced swim tank and let free to swim for 10 minutes. Once the test was conducted the animals were scored for d. Vigor, the ability to purposely swim and use all limbs and e. Success, the ability to keep their head above water. f. The total time spent immobile was also measured to assess learned helplessness and despair. Each dimer treatment was repeated at least 10 times per treatment ($n=10$) and the average of each treatment is presented in bar graphs as means $\pm$ SEM. Significances were determined using One-way ANOVA, followed by Student-Newman-Keuls multiple comparison test with ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

The open field test is commonly used to assess anxiety levels [25,26]. During the open field test, the CPA-treated rats spent significantly more time around the perimeter of the square box and less time exploring the center as shown by the significantly lower percentage of time spent in the center square (**Figure 2 b**). The co-administration of the dimer compound improved the exploratory behaviour and reduced the anxiety caused by CPA. Similar to what we previously showed with the A1R antagonist DPCPX improving anxiety, cognitive and motor behaviour of rats subjected to 5-week-CPA treatment [4], we now show that both 3 and 5 mg/kg C8-6-I doses significantly decreased

the fecal boli count, thus indicating that Cs-6-I attenuated the CPA-induced animal anxiety and improved exploratory behaviour (**Figure 2 b-c**).

Initially, when the animals are first placed in water, they swim as vigorously as they possibly can to escape the stressful situation. Often, in the first minute of being in the forced swim tank, animals dive toward the bottom of the tank to explore the corners of the apparatus for a possible exit. However, the time spent underwater exploring it is not included in the calculation of vigour or success scores. Nonetheless, towards the end of the forced swim test trial, the animals reach a point of helplessness and fatigue, which is reflected by immobility or “floating” while trying to keep their heads above the water to breathe. The forced swim test showed that CPA induced an increase in motor deficits. However, CPA in the presence of either 3 or 5 mg/kg Cs-6-I produced no significant motor deficits. However as seen in **Figures 2 d** and **e**, only the 5 mg/kg bodyweight concentration significantly improved the ability to purposely swim and use all limbs as well as the ability to keep their head above water. The immobility time calculated in percentage (a measure of helplessness behaviour) showed that animals treated with either 3 or 5 mg/kg Cs-6-I spent significantly less time being immobile compared to the CPA-treated rats (see **Figure 2 f**). This result indicates that Cs-6-I caused significant improvement of the CPA-dependent depressive-like behaviour and motor deficit.

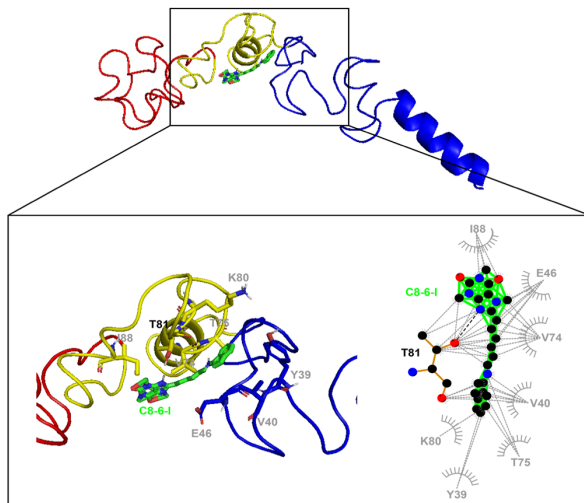
2.2. Molecular Docking Revealed Potential Binding of Cs-6-I to α -Syn and Adenosine Receptors

The α -syn is comprised of three functional domains (see **Supplementary Figure 1a**): the mostly positively charged N-terminus (blue region), the hydrophobic NAC domain (yellow), and the highly disordered negatively charged C-terminus (red). Previous reports using nanopore analysis and molecular docking showed that 1-aminoindan, caffeine, CPA and DPCPX interacted with the different domains of α -Syn [3,6,10]. Using nanopore analysis, previous reports suggested that 1-aminoindan and its derivatives [5,27] as well as the A1R antagonist DPCPX [3] interacted with α -syn in a manner that promotes a neuroprotective “loop” conformation of α -syn. Subsequent molecular docking using the eight soluble α -syn monomeric structures (clusters 1-8, C1-C8) identified by Chen et al. [13] (see **Supplementary Figure 1b**), revealed that the A1R antagonist DPCPX binds to the α -syn structures C2, C5, C7 and C8 while the 1-aminoindan binds to C1, C4, C5 and C8 structures [3]. Since the dimer compound Cs-6-I was also previously demonstrated by nanopore analysis to bind to α -syn and prevent α -syn aggregation in a yeast assay [10], we then determined using molecular docking analysis if the eight soluble monomeric structures of α -syn interacted with the dimer compound to reveal a neuroprotective “loop” conformation.

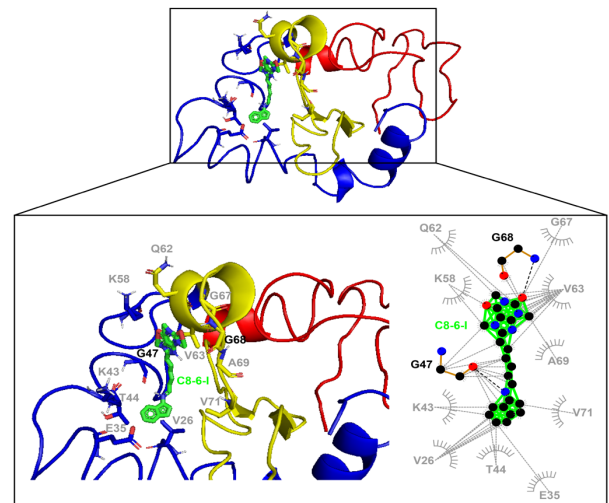
Therefore, using molecular docking and the eight α -syn structures (C1-C8) as we previously employed for 1-aminoindan, the four α -syn structures, C1, C2, C3 and C8, were selected to analyze the in silico binding of the Cs-6-I to α -syn. For the C1 structure, Cs-6-I was shown to form a hydrogen bond with threonine 81 (T81) of the NAC region as well as hydrophobic interactions with the hydrophobic side chains of tyrosine 39 (Y39) and valine 40 (V40) and the negatively charged glutamic acid (E46) of the N-terminus (**Figure 3a**). Cs-6-I also forms additional hydrophobic interactions with V74, T75, lysine 80 (K80), and isoleucine 88 (I88) in the NAC region. For the C2 structure (**Figure 3b**), Cs-6-I was shown to form hydrogen bonds with the glycine 47 and 68 (G47 and G68) in the N-terminus and NAC regions, respectively. Cs-6-I forms also hydrophobic interactions with the electrically charged cleft of the C2 N-terminus consisting of V26, E35, K43, T44, and K58 amino acids, and with the mostly hydrophobic cleft of the NAC region consisting of glutamine 62 (Q62), V63, G67, alanine 69 (A69), and V71 amino acids. For the C3 structure (**Figure 3c**), Cs-6-I was shown to form hydrogen bonds with T59 in the N-terminus and G67, A69, and V70 in the NAC region. In addition, the dimer compound forms weak interactions at the end of the N-terminus and throughout the NAC region with K60, V63, asparagine 65 (N65), G68, V71, phenylalanine 73 (F73), and V74. These three conformations indicate the formation of a “loop” conformation between the N-terminus and farthest NAC region of the protein when dimer binds to them. However, the C8 structure (**Figure 3d**) was shown to form different drug-protein complexes that may regulate fibrillation (C8) by only forming hydrogen bonds with the NAC region with V66 and T72 stopping it from misfolding and forming fibril bodies. Cs-6-I was shown to have weak interactions with K21 and T59 in the N-terminus and

with Q61, T64, N65, G67, G68, V71, and Q79 in the NAC region. C2, C3 and C8 structures form similar binding with the dimer by engaging both the caffeine and indan moieties of the dimer compound.

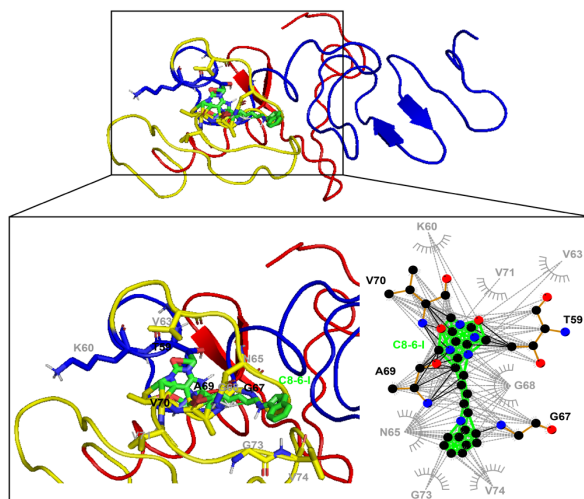
a. C1 α -Syn-C8-6-I Interaction



b. C2 α -Syn-C8-6-I Interaction



c. C3 α -Syn-C8-6-I Interaction



d. C8 α -Syn-C8-6-I Interaction

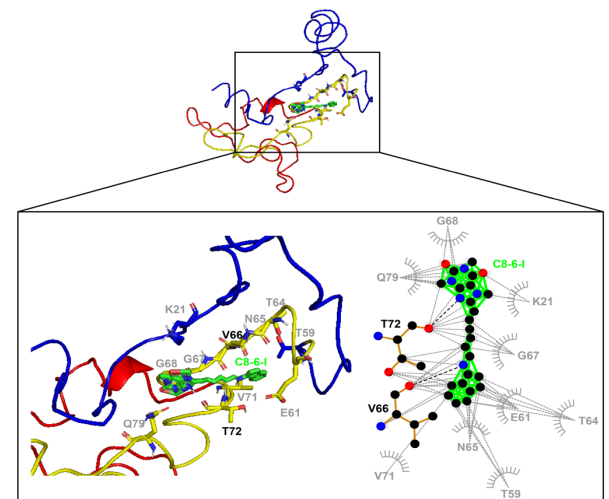
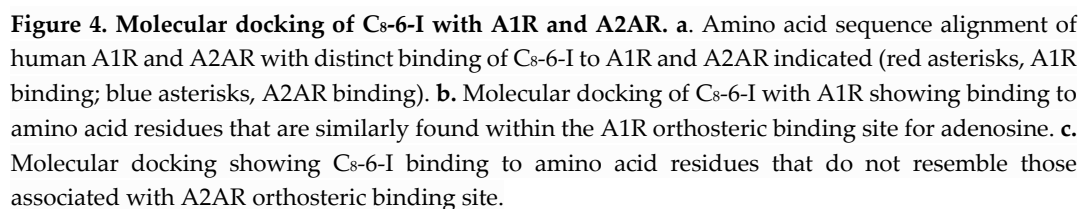


Figure 3. Molecular docking simulation of α -Syn structures a. C1, b. C2, c. C3, and d. C8 bound to C8-6-I. Bold black dashed lines and amino acid residues indicate hydrogen bonding, while the grey dashed lines and amino acid residues indicate hydrophobic interactions. **a.** The bifunctional dimer compound forms hydrogen bond with T81 located in the NAC region of α -Syn, and additional hydrophobic interactions are found with the α -Syn N-terminus and NAC region. **b.** The dimer compound interacts via hydrogen bonding to α -Syn N-terminus, and additional hydrophobic interactions occur with amino acid residues in N-terminus and NAC region. **c.** The dimer compound binds via hydrogen bonds to amino acid residues in α -Syn N-terminus and NAC region, and additional hydrophobic binding occurs with amino acids located in the distal N-terminus and NAC region. **d.** The dimer compound binds via hydrogen bonding with NAC amino acid residues (V66, T72) and through hydrophobic interactions with amino acid residues in N-terminus and NAC domain. C1, C2, C3 and C8 α -Syn structures bind to the dimer compound which is predicted to form a “loop” conformation of α -Syn.

Also, since caffeine is a non-selective antagonist of A1R and A2AR, we determined whether the caffeine moiety-containing C8-6-I still retained binding activity to A1R or A2AR. Using alpha fold molecular docking of C8-6-I to A1R and A2AR, we showed that C8-6-I was still capable of binding to



Although the C₈-6-I made hydrophobic interactions with A2AR (see **Figures 4a** and **c**), these interactions did not contact amino acid residues that reside within the orthosteric binding pocket of A2AR. The C₈-6-I formed hydrophobic interactions with residues in TM1 (G23, L26, V27, A30), TM7 (V282, P285, F286, A289, T290), and C-terminus (F299, F295). These residues do not match those found in the orthosteric binding site of A2AR, which binds the agonist adenosine and the naturally occurring antagonist caffeine. The orthosteric binding site for A2AR has been well characterized and includes amino acid residues that bind both agonists and antagonists (F168, M177, L249, N253, I274) and residues that bind only agonist (T88 in TM3 and S277/H278 in TM7) [30].

2.3. C₈-6-I Decreased CPA-Induced α -Synuclein Expression and Aggregation in Substantia Nigra and Hippocampus

Brain slices from the 7-day chronic injection model were randomly selected from 6-well plates containing 40 μ m coronal brain slices, and fixed hippocampal slices (bregma -3.80 and -4.16 mm) as well SN slices (bregma -5.30 and -5.60 mm) were stained with DAPI, anti- α -Syn and anti-tyrosine hydroxylase (see **Figure 5**), and in parallel slices with DAPI, anti- α -Syn and Thio-S to detect aggregation levels. With C₈-6-I co-administration with CPA, we found comparable decreased pattern levels of α -Syn in both the SNc and CA1 regions of the hippocampus (**Figures 5** and **6**). The α -Syn levels in both the SNc and CA1 hippocampal region markedly increased after CPA treatment alone, however, C₈-6-I co-treatment with CPA prevented this α -Syn upregulation. Interestingly, a significant increase of Thio-S levels was observed in both the CPA alone treatment and the 3 mg/kg C₈-6-I + CPA-treated rats compared to control levels in SNc (**Figure 5b,c**), while 3 mg/kg C₈-6-I + CPA treatment abolished the CPA-induced Thio-S levels in CA1 hippocampus (**Figure 6b**). However, the Thio-S levels of the 5 mg/kg C₈-6-I + CPA-treated rats reached control levels in the SNc while a moderate, but significant Thio-S level increase was observed in the hippocampus.

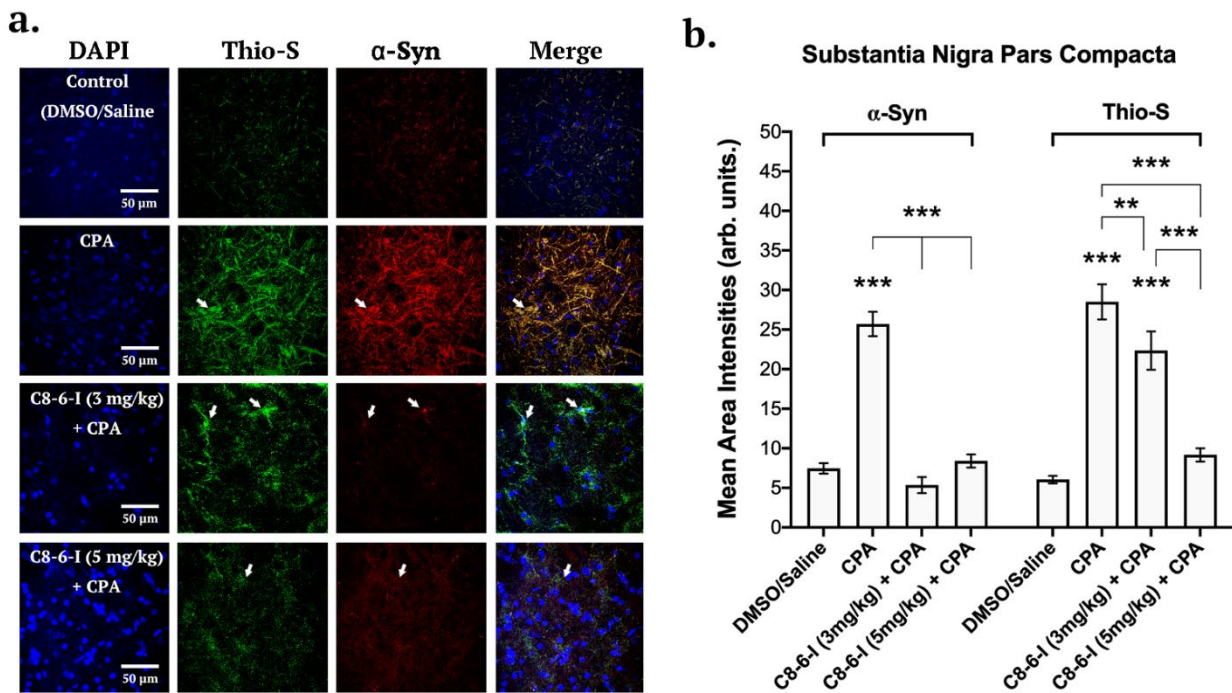


Figure 5. Summary of the surface area analysis of the dimer study's pars compacta region of the substantia nigra of DAPI, TH, and α -Syn. **a.** Representative images of 40 μ m pars compacta region of substantia nigra taken with 63X oil immersion objective of a confocal microscope (126 times magnification). Separate channels and montages of 7-day chronic intraperitoneal injections with 3 mg/kg of the following treatments: Control (DMSO/Saline), CPA, C8-6-I (3 mg/kg) + CPA, and C8-6-I (5 mg/kg) + CPA. Slices were probed for DAPI (Blue), TH (Green, Alexa Fluor 555), and α -Syn (Red, Alexa Fluor 647). **b.** Representative images from 40 μ m nigral brain slices of rats after probing for

DAPI, anti- α -Synuclein and Thioflavin S taken at 63 times magnification with a confocal microscope. Scale 50 μ m. c. Bar charts showing the mean area intensities of α -Syn and Thioflavin S in the pars compacta region of the substantia nigra of the eight treatments, respectively. Similar areas of 100 by 100 μ m ROI coordinates for lateral pars compacta of SN were quantified respectively for each slice and normalized by subtracting F0 (50 by 50 μ m ROI coordinates) values of the background (non-cell body bottom area). Average intensity values and correlation coefficients in bars represent the average mean $\pm$ SEM from $n=4$ independent experiments. Significances were determined using One-way ANOVA, followed by Student-Newman-Keuls multiple comparison test with ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

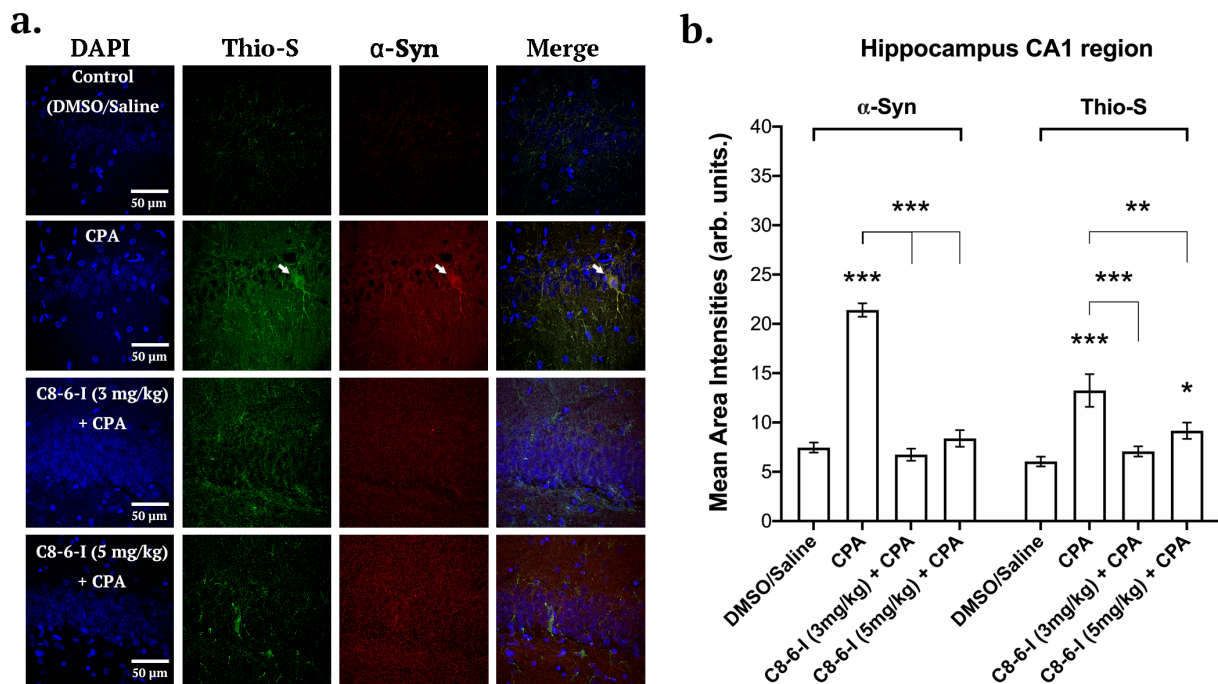


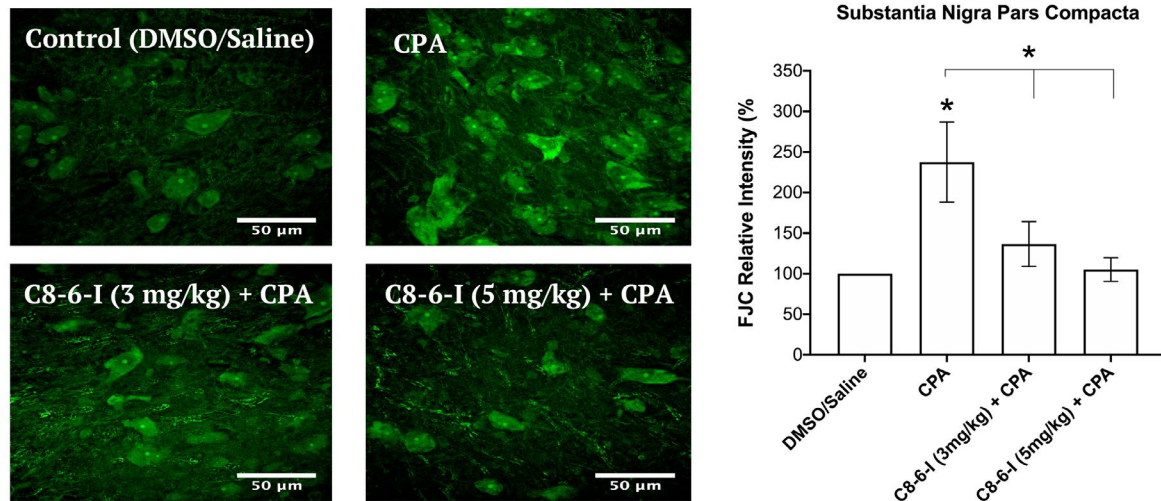
Figure 6. Summary of the surface area analysis of the CA1 region of the hippocampus of DAPI, α -Syn and Thio-S. a. Representative images from 40 μ m hippocampal rat brain slices after probing for DAPI, anti- α -Synuclein and Thioflavin S taken at 63 times magnification with a confocal microscope for the following treatments: Control (DMSO/Saline), CPA, C8-6-I (3 mg/kg) + CPA, and C8-6-I (5 mg/kg) + CPA. Scale 50 μ m. b. Bar charts showing the mean area intensities of α -Syn and Thioflavin S in the CA1 region of the hippocampus of the eight treatments. Fluorescence intensities from a 100 by 100 μ m ROI from the CA1 pyramidal cell layer of the hippocampus were quantified using a similar method employed for pars compacta region. Average intensity values and correlation coefficients in bars represent the average mean $\pm$ SEM from $n=4$ independent experiments. Significances were determined using One-way ANOVA, followed by Student-Newman-Keuls multiple comparison test with ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

2.4. C8-6-I Decreased CPA-induced Neurodegeneration in Substantia Nigra and Hippocampus

We recently reported that chronic administration of CPA contributed to increased neuronal damage and selective inhibition of A1Rs and administration of 1-aminoindan both attenuated neurodegeneration and α -Syn aggregation [3]. We then tested C8-6-I, which contains both the caffeine and indan moieties, to determine if this dimer exhibited neuroprotective properties *in vivo*. We used the same FJC protocol [3] as previously established to test for neurodegeneration in the brain. Similar anatomical brain slices like those used in **Figures 5-6** were utilized in this study (nigral slices -5.30 to -5.60 mm from the bregma as well as hippocampal slices -3.80 to -4.16 mm from the bregma). The SNc representative images indicate that CPA alone increased the levels of FJC fluorescence compared to the control (as summarized in **Figure 7 a**). In contrast, both doses of the C8-6-I (3 mg/kg) + CPA and C8-6-I (5 mg/kg) + CPA did not significantly increase FJC staining compared to the vehicle control

slices. Similar results were observed in the CA1 region of the hippocampus (as summarized in **Figure 7 b**), where treatments with CPA demonstrated much higher levels of degenerating CA1 pyramidal neurons compared to the control, as previously observed [3], or with co-administered C₈-6-I. Similar to the nigral slices, the co-administration of C₈-6-I (3 mg/kg) or C₈-6-I (5 mg/kg) with CPA prevented neurodegeneration in hippocampal slices.

a.



b.

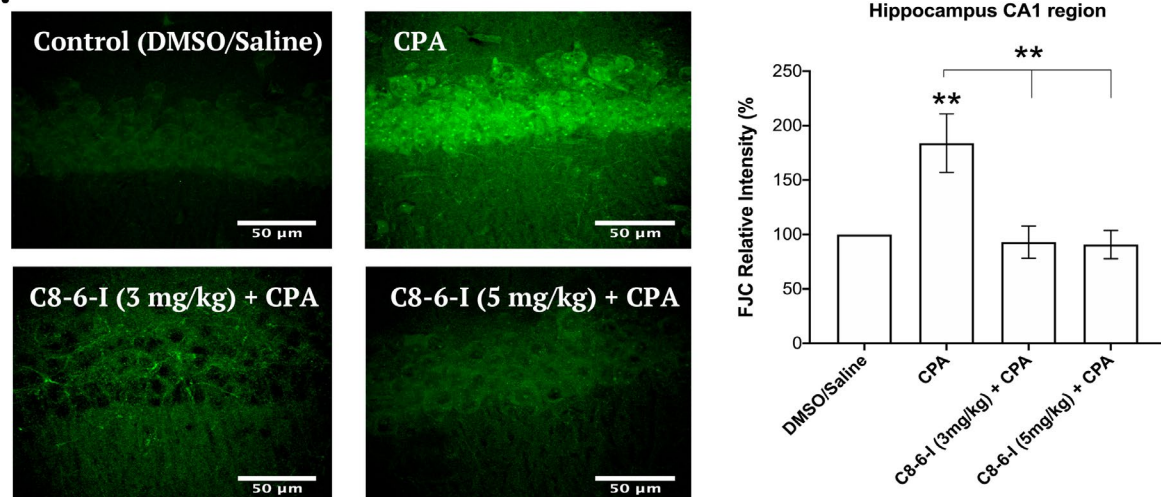


Figure 7. Fluoro-Jade C (FJC) staining in the SN pars compacta and in the hippocampus CA1 region of rats with 7-day chronic intraperitoneal injection of Control (DMSO/saline), CPA, C₈-6-I (3 mg/kg) + CPA, and C₈-6-I (5 mg/kg) + CPA. Representative images with 50 μ m scale bar for the a. SN pars compacta and b. the CA1 region of the hippocampus. FJC fluorescence intensity in a 100 $\times$ 100 μ m² region was normalized to the control group (100%). Values are shown as mean $\pm$ SEM. The average FJC fluorescence values were obtained from $n = 4$ independent experiments. ns, non-significant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ (one-way ANOVA followed by Student-Newman-Keuls post-hoc multiple comparison test).

2.5. ¹⁸F-Labelled C₈-6-I and its Potential Therapeutic Use for In Vivo Studies

To synthesize the ¹⁸F-C₈-6-I, we envisaged that S_N2 displacement of the mesylated precursor C₈-6-I-OMs with [¹⁸F]fluoride [21] would yield the desired radiotracer (as seen in **Figure 1**). We anticipated that the secondary amine would require protection with tert-butyloxycarbonyl (Boc) to prevent the formation of unwanted byproducts during radiolabelling. Boc could then be rapidly

removed using trifluoroacetic acid (TFA) or hydrochloric acid (HCl) under short reaction times required for ^{18}F -radiochemistry. Although rapid incorporation of ^{18}F (acetonitrile, 90 °C) was observed at high yields as judged by HPLC, N-Boc deprotection with TFA was unsuccessful with the reaction producing major side products. Despite several attempts to optimize the Boc deprotection step by varying reaction temperature and time, < 1% of the radioactive compounds were identified as ^{18}F -C₈-6-I. Alternatively, we decided to use 4 N HCl in dioxane for our N-Boc deprotection at room temperature and this led to an increase in our total radiochemical yields (rcy) from < 1% (using TFA) to $23 \pm 5\%$ ($n = 7$, using 4 N HCl), decay corrected, with a radiochemical purity $\geq 98\%$ suitable for PET imaging in healthy mice.

2.6. ^{18}F -Labelled C₈-6-I In Vivo Metabolism and Distributed in the Major Organs Including the Brain

To further investigate the pharmacokinetics of ^{18}F -C₈-6-I and its metabolic stability *in vivo*, a metabolic stability study was carried out via radio HPLC analysis on extracts of liver, pancreas, and brain at 40 min p.i. Previous knowledge of the *in vitro* metabolic stability profiles of ^{19}F -C₈-6-I and C₈-6-I in human, mouse and rat liver microsomes [11] suggested that a significant percentage of the parent ^{19}F -C₈-6-I remained after 1 h incubation, which may allow *in vivo* radio metabolite analysis of ^{19}F -C₈-6-I in various tissues including the brain, liver and GI tract. However, the *in vivo* metabolic stability of ^{18}F -C₈-6-I has not been analyzed. Therefore, it is crucial to determine whether significant levels of the parent ^{18}F -C₈-6-I remain in the blood plasma and lead to higher uptake in the brain for a 1h to 2h PET imaging. The radioactivity measured in our studies was mostly due to radioactive emission from ^{18}F -C₈-6-I (see **Figure 8a**). Using reverse phase HPLC with UV detection for ^{19}F -C₈-6-I and radio detection for ^{18}F -C₈-6-I, we determined that the ^{18}F -C₈-6-I was stable with little or fewer metabolites compared to ^{19}F -C₈-6-I (**Figure 8a**). Representative radio-HPLC chromatograms from the liver and pancreas (**Figure 8b-c**) showed that the parent compound ^{18}F -C₈-6-I accounted for $\geq 95\%$ of the radioactivity signal. We were unable to identify the radioactivity in the brain due to low uptake in the brain and poor sensitivity using radio HPLC. However, the ^{18}F -C₈-6-I was detectable in major organs, including the liver and pancreas where a single peak was found to correspond to the ^{18}F -C₈-6-I signal around 8.813 min (**Figure 8b-c**).

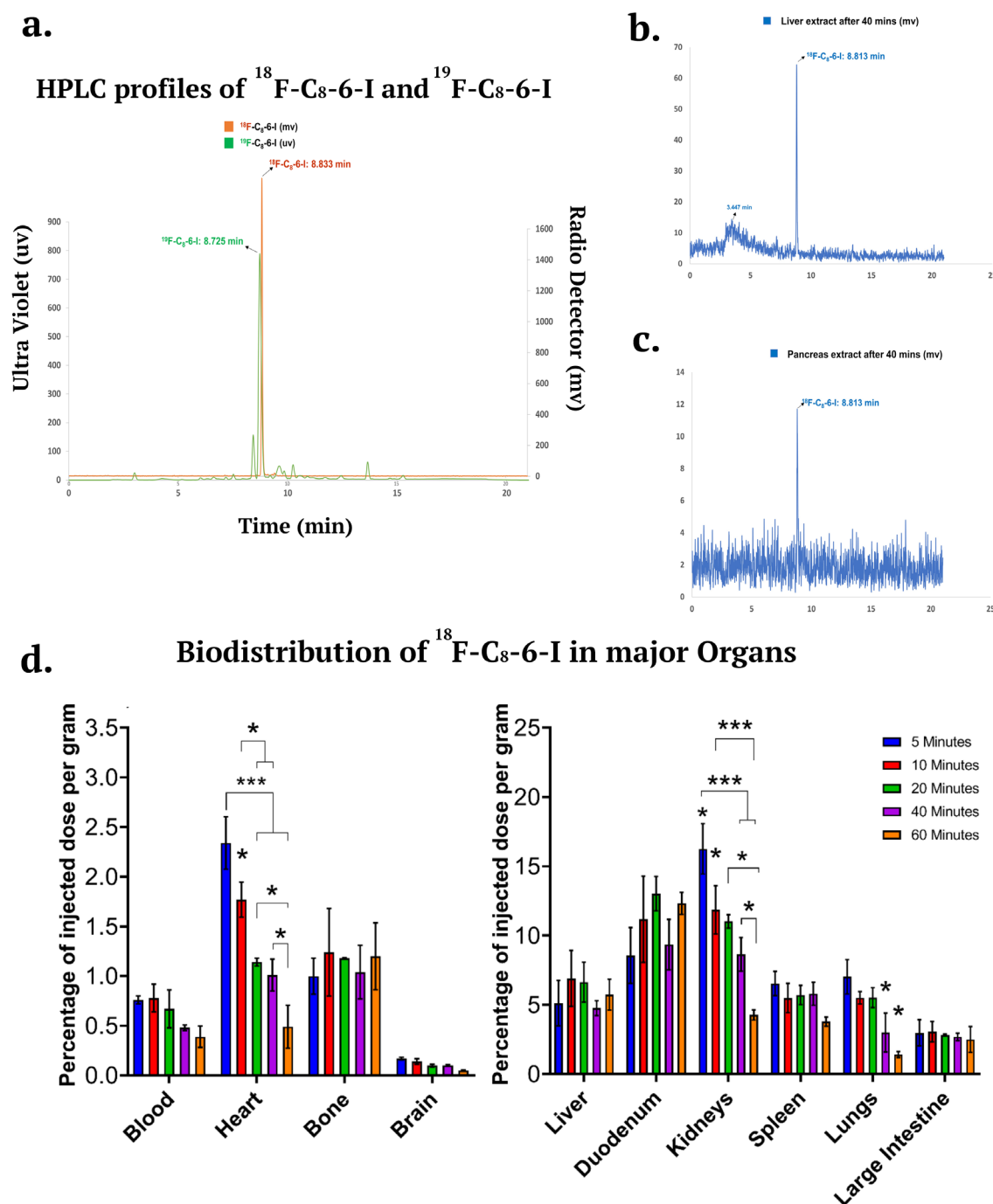


Figure 8. Ex vivo stability and biodistribution in CD-1 mice at five different time points (5, 10, 20, 40, and 60 min) and major organs. **a.** HPLC co-registration profiles of ^{18}F -C₈-6-I and ^{19}F -C₈-6-I based on a radio detector and ultraviolet detector, respectively. Analytical radio-HPLC chromatograms in mouse **b.** liver and **c.** pancreas extracts at 40 mins after injection of ^{18}F -C₈-6-I. **d.** The distribution of ^{18}F -C₈-6-I was calculated as percentage of the injected dose per gram of tissue (% ID/g) and results were displayed into two groups: Lower distribution—Blood, heart, bone, and brain (left panel) and higher distribution—Liver, duodenum, kidneys, spleen, lungs, and large intestine (right panel). The data were obtained from $n = 3$ independent animals. Values are presented as mean $\pm$ SEM.

^{18}F -C₈-6-I exhibited a moderate CNS multiparameter optimization (MPO) score (4.2), reasonable topological surface area (68.25 Å) and proper lipophilicity (cLogP: 3.46 and cLogD: 1.26) [31]. However, ^{18}F -C₈-6-I has a high molecular weight (455.57) and high pKa (9.49). To evaluate the pharmacokinetics of ^{18}F -C₈-6-I, whole body ex vivo biodistribution was performed with healthy female CD1 mice at 5, 10, 20, 40, and 60 mins p.i. The results of the biodistribution studies are shown

in **Figure 8 d**. The data is also summarized in **Table 2**. Notably, ¹⁸F-C₈-6-I displayed low initial brain uptake ($0.17 \pm 0.014\%$ ID/g) at 5 min p.i. and slow washout over a period of 60 min ($0.05 \pm 0.004\%$ ID/g) as seen in **Figure 8 d**. The slow clearance of ¹⁸F-C₈-6-I from the brain ($\text{brain}_{5\text{min}}/\text{brain}_{20\text{min}} = 1.7$, and $\text{brain}_{5\text{min}}/\text{brain}_{60\text{min}} = 3.4$) may explain why chronic i.p. injections of C₈-6-I over the 7 days in the Sprague-Dawley rats indicated not only decreased expression of α -Syn and its aggregates as seen in **Figure 5-6** but also significantly lowered the levels of neurodegenerations as seen in **Figure 7**. Thus, the dimer has desirable properties as a therapeutic for PD. The bone uptake of ¹⁸F-C₈-6-I ($\leq 1.20\%$ ID/g, **Figure 8 d**) was also low indicating little defluorination occurred *in vivo*. The amount of radioactivity in the blood was also low with initial uptake ($0.76 \pm 0.04\%$ ID/g) at 5 min p.i. and very slow elimination ($\text{blood}_{5\text{min}}/\text{blood}_{20\text{min}} = 1.1$, and $\text{blood}_{5\text{min}}/\text{blood}_{60\text{min}} = 1.9$) until 60 min ($0.39 \pm 0.10\%$ ID/g).

Table 2. Biodistribution of ¹⁸F-C₈-6-I in healthy CD-1 mice. Data shown are percentage of injected dose per gram of tissue (%ID/g). The values are presented as mean $\pm$ SEM, *n* = 3.

Organ	5 min	10 min	20 min	40 min	60 min
Blood	0.76 ± 0.040	0.78 ± 0.14	0.67 ± 0.19	0.48 ± 0.029	0.39 ± 0.11
Liver	5.12 ± 1.65	6.91 ± 2.02	6.64 ± 1.44	4.76 ± 0.54	5.74 ± 1.12
Small intestine	8.56 ± 2.02	11.18 ± 3.11	13.02 ± 1.24	9.35 ± 1.82	12.33 ± 0.81
Kidney	16.26 ± 1.82	11.86 ± 1.74	11.02 ± 0.48	8.65 ± 1.21	4.27 ± 0.37
Spleen	6.54 ± 0.88	5.49 ± 1.05	5.71 ± 0.69	5.80 ± 0.83	3.79 ± 0.33
Lung	7.03 ± 1.24	5.51 ± 0.44	5.52 ± 0.72	3.00 ± 1.40	1.42 ± 0.22
Heart	2.34 ± 0.26	1.77 ± 0.17	1.14 ± 0.040	1.01 ± 0.16	0.49 ± 0.018
Large intestine	2.98 ± 0.94	3.06 ± 0.73	2.83 ± 0.068	2.69 ± 0.26	2.50 ± 0.93
Bone	1.00 ± 0.18	1.24 ± 0.44	1.18 ± 0.005	1.04 ± 0.27	1.20 ± 0.34
Brain	0.17 ± 0.014	0.14 ± 0.027	0.10 ± 0.013	0.10 ± 0.011	0.05 ± 0.005

As shown in **Figure 8 d**, several organs including the kidneys, lungs, spleen, duodenum, and liver showed high initial uptakes greater than 5% of injected dose per gram (%ID/g) of ¹⁸F-C₈-6-I at 5 min p.i.. After high initial uptake, the radioactivity in the kidney, spleen, and lungs displayed a much faster washout than other organs. The kidneys displayed the highest uptake ($16.26 \pm 1.82\%$ ID/g) of radiotracer at 5 min p.i., with fast elimination over a period of 60 mins. The liver showed an uptake of $5.12 \pm 1.64\%$ ID/g at 5 min p.i. and $6.91 \pm 2.02\%$ ID/g at 10 min p.i.. The activity in the liver decreased until 40 min ($4.76 \pm 0.54\%$ ID/g) before showing an increase again at 60 min ($5.74 \pm 1.11\%$ ID/g). A similar pharmacokinetic profile is observed for the duodenum where activity uptake at 5 min p.i. is $8.56 \pm 2.01\%$ ID/g and this increases until 20 min (13.02 ± 1.23) before decreasing to $9.35 \pm 1.81\%$ ID/g at 40 min p.i. However, an increase in uptake ($12.33 \pm 0.80\%$ ID/g) is observed at 60 min p.i. similar

to that observed for the liver at 60 min (see **Figure 8 d, right panel**). These results of a parallel increase in liver and duodenum at 60 min are consistent with ^{18}F -Cs-6-I undergoing hepatobiliary elimination.

2.7. *In Vivo* PET/CT Imaging in CD-1 Mice

We performed 60 min ($n = 2$) to 120 min dynamic imaging in healthy female CD-1 mice (see **Figure 9 a-c**). Mice were administered with 1-2 pg/kg of ^{18}F -Cs-6-I using tail-vein injections. PET dynamic imaging indicates that ^{18}F -Cs-6-I accumulates in the liver, followed by uptake in the gastrointestinal tract. The dynamic image combined with our biodistribution further suggests that ^{18}F -Cs-6-I undergoes hepatobiliary elimination. The 120 PET summation images suggest some uptake in the thyroid and lymph nodes. Negligible uptake is observed in the bone which validates little defluorination occurs for this radiotracer *in vivo*. However, the image suggests very low brain uptake consistent with our biodistribution studies. Quantification of activity concentration in the brain indicated a maximum of 0.47 standardized uptake value (SUV) in the whole brain and this slowly decreased to 0.21 after 50 minutes. The *in vivo* brain uptake is lower than will be ideal for CNS imaging (peak > 1 SUV), however, the slow decrease from 0.47 to 0.21 SUV after 50 minutes indicates why the chronic i.p. injection of the dimer makes it suitable as a potential therapeutic for PD. In addition to the whole brain, specific anatomical regions were also measured to establish the uptake levels in the cortex, hippocampus and midbrain (see **Figure 9d**). This demonstration that ^{18}F -Cs-6-I accumulates in the brain correlates with the observed *in vivo* neuroprotective effects of Cs-6-I (see **Figure 7**), reduced α -Syn aggregation (**Figures 5-6**), and improved behavioural outcomes (**Figure 2**) in our 7-day chronic i.p. CPA injection α -synucleinopathy model.

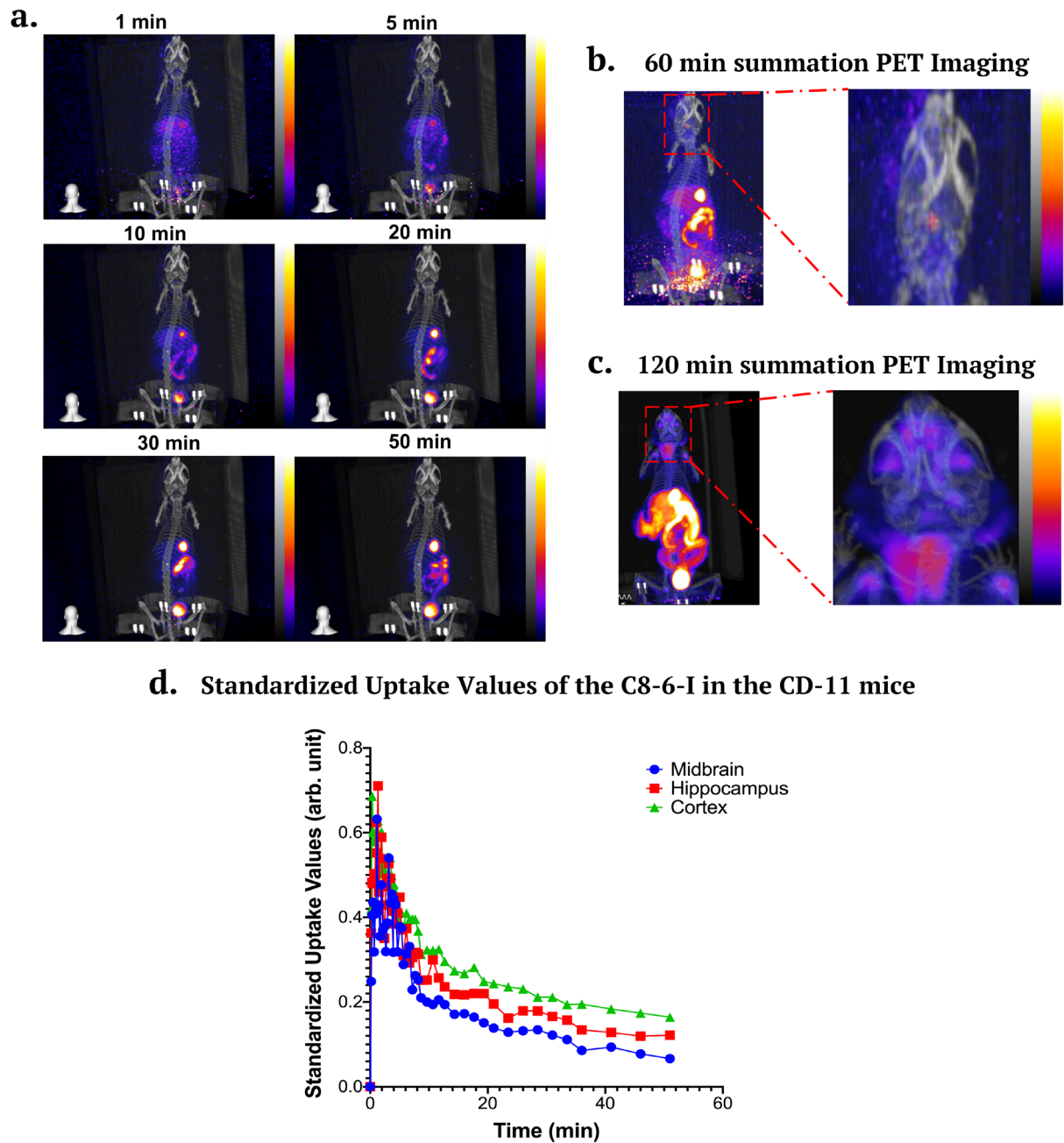


Figure 9. Representative PET/CT images in CD-1 mice at different time points throughout an hour as well as time activity curve in the brain. a. PET images at different time points (1 min, 5 min, 10 min, 20 min, 30 min, and 50 min), b. PET summation images for 60 min and c. 120 min dynamic imaging. d. Time-activity curve (TAC) of ^{18}F -C8-6-I for whole brain and three region consisting of the cortex, midbrain, and hippocampus from the PET/CT imaging. Values are presented as standardized uptake value (SUV). The data were obtained from $n = 3$ independent animals.

Discussion

Many epidemiological and multipurpose drug studies have presented interesting observations, such as the consumption of coffee or tobacco significantly decreasing the risk of PD [32–37]. Recently it was shown that caffeine as well as nicotine are neuroprotective against wild-type and mutant parkin proteins [9]. The substitution of glutamic acid to lysine (E28K) in the ubiquitin-like binding domain of the parkin protein is strongly associated with early onset PD but also with several cancers like onset melanoma [38,39]. Caffeine and nicotine were shown to bind directly to the 28th amino

acid residue of both parkin and E28K mutant [9]. These two drugs show neuroprotective effects by decreasing the misfolding of the two proteins. Another interesting finding was the unexpected effects of metformin, a drug used to treat type 2 diabetes, which has been reported to be neuroprotective against PD in long-term studies [40–42]. Another study looked at the role of metformin in reducing mitochondrial hyperactivity [43]. The dysfunction of the branched-chain amino acid (BCAA) metabolism pathway was linked to PD-like motor deficits and neurodegeneration. This neurodegeneration was linked directly to hyperactivity of the mitochondria; however, metformin decreased the mitochondria respiration levels. Mitochondria dysfunction has been previously linked with PD in numerous studies [44–46]. Moreover, as previously mentioned, 1-aminoindan, a metabolite of rasagiline, is also shown to be neuroprotective [47–49]. 1-aminoindan was shown to bind directly to α -Syn, thereby promoting a neuroprotective “loop” conformation which attenuates the α -Syn misfolding and aggregation in a yeast model overexpressing α -Syn or in an in vivo rodent model of α -Synucleinopathy [3,5,6,27].

Although drugs such as caffeine, nicotine or 1-aminoindan would be clinically relevant for reducing the risk of developing PD, these drugs may be highly toxic at high concentrations [50–52]. In addition, there are several side effects of consuming high doses of caffeine or nicotine such as anxiety and nervousness, fast heart rate, heartburn, high blood pressure, cancer and others [53–55]. Therefore, linking these compounds and other similar neuroprotective drugs into dimers or trimers may be a more effective strategy to reduce the misfolding and aggregation of α -Syn without risking the safety of the patients [10,11,56]. These compounds were optimized with a six-carbon alkyl linker, to minimize solubility issues and retain the flexibility that will allow further binding to α -Syn and protect the protein from misfolding and aggregating [10]. Thus, the present study investigated the role of C₈-6-I dimer as a promising neuroprotective agent in our established in vivo 7-day CPA injection α -synucleinopathy model, which displayed increased α -Syn expression, aggregation, and neurodegeneration [3]. Determining the dimer concentration is difficult to attain confidently via conventional approaches, especially for tissue compartments with specialized epithelial or endothelial cell barriers such as the central nervous system (CNS) [17]. For example, the blood-brain barrier (BBB) prevents compounds, typically hydrophilic, from entering the brain and is a major obstacle that must be overcome during drug development. A lack of adequate brain exposure to drugs often leads to the failure of CNS drug candidates [22,57]. Lipophilic compounds that can passively diffuse across membranes may also be substrates of efflux transporters (such as P-glycoproteins, organic anion transporters, and multidrug-resistance-associated proteins) at the BBB resulting in low brain penetration of pharmacologically active drug molecules [22]. To then predict the BBB permeability of ¹⁸F-C₈-6-I, we calculated the CNS multiparameter optimization (MPO) desirability score using the compound's physicochemical properties [58]. For the CNS MPO score, six physicochemical parameters (calculated LogP, calculated LogD, molecular weight, topological polar surface area, hydrogen bond donor, and acidity constant) are normalized and converted to a function ranging from 0 to 1, and the sum of all the normalized physicochemical properties produce the CNS MPO score ranging from 0 to 6. Generally, therapeutic agents with preferable BBB permeability show CNS MPO scores of 4 or higher [59–64]. The CNS MPO score for ¹⁸F-C₈-6-I was calculated as 4.2. Based on these initial results from binding studies with isothermal titration calorimetry and in vitro yeast assay indicating binding to α -Syn [10], in vitro metabolic studies [11], kinetic measurement and pharmacokinetic predictions [31], and CNS MPO desirability score, we synthesized ¹⁸F-C₈-6-I for in vivo evaluation in healthy mice. We found that ¹⁸F-C₈-6-I was distributed in the brain regions, including the hippocampus and midbrain, which supports the reduced α -Syn expression and aggregation and neurodegeneration observed in these regions. Although our PET-CT studies showed ¹⁸F-C₈-6-I biodistribution and slow elimination in different organs, including the brain, it is yet unclear whether this PET-tracer compound is binding directly to α -Syn or other targets in the brain to mediate the neuroprotection and mitigate behavioural deficits. However, our present molecular docking results and previous reports using nanopore analysis [10] confirm that the C₈-6-I dimer compound likely binds to α -Syn to promote a neuroprotective “loop” conformation that reduces α -Syn misfolding and aggregation. Moreover, our molecular docking results also indicate

that the C₈-6-I compound could bind to other targets, including adenosine receptors (see below), which further contributes to the observed neuroprotective effects of the dimer in the hippocampus and SN brain regions.

In previous studies, we demonstrated that chronic A1R stimulation with 5 mg/kg CPA for 5 weeks showed a significant increase in α -Syn in the midbrain region and produced motor and cognitive deficits in male Sprague-Dawley rats [4]. Recently, we also reported a novel 7-day i.p. injection model of 3 mg/kg CPA indicating similar findings in increased α -Syn expression and aggregation in both the SN pars compacta and the hippocampus [3]. This was demonstrated using confocal images as well as Western blotting techniques. We also showed that chronic CPA treatments increased neurodegeneration levels, as shown by the FJC staining. However, the co-administration of DPCPX or 1-aminoindan prevented the CPA-induced α -Syn aggregation and neurodegeneration [3]. Interestingly, we showed comparable results with the C₈-6-I dimer. We tested two doses of the dimer, 3 and 5 mg/kg. Following the 7th day of injections, the Sprague-Dawley rats were tested for cognitive dysfunction using the Y-maze test, anxiety levels using the open field test, and kinetic/despair behaviour using the forced swim test. We showed that chronic administration of CPA induced hippocampal-dependent spatial memory deficit, which was attenuated by co-administration with both doses of the dimer. During the open field test, the CPA group spent significantly less time exploring the center as shown by the significantly lower percentage of time spent in the center square. The co-administration of the dimer compound improved the exploratory behaviour and reduced the anxiety caused by CPA. Moreover, the forced swim test indicated that the CPA-treated rats exhibited reduced vigour and success scores, indicating reduced motor behaviour possibly due to increased despair/helplessness behaviour. Both doses of C₈-6-I used (3 and 5 mg/kg) were effective in restoring cognitive function, anxiety levels and motor activity, and these results are consistent with the observed decrease in neurodegeneration in the hippocampus and SN pars compacta. We did not test the effects of higher doses due to the difficulty in synthesizing and obtaining a high yield of the dimer. However, the initial dose of 3-5 mg/kg C₈-6-I dimer was sufficient to produce neuroprotective benefits in our α -synucleinopathy model. It is also important to note that the C₈-6-I dimer is a racemic mixture, and it is not known whether one or both enantiomers are responsible for the activity.

In addition, previous studies with C₈-6-I and ¹⁸F-C₈-6-I indicated that these compounds were promising as CNS PET tracers [11,31]. We previously determined that C₈-6-I directly interacted with α -Syn in vitro and rescued yeast cells from α -Syn fibrillation mediated toxicity [10]. In the present study, we reported that both the 3 and 5 mg/kg doses of the dimer prevented the CPA-induced α -Syn accumulation and aggregation in both the SN pars compacta and hippocampal CA1 region. This coincides with the significant reduction of FJC neurodegeneration levels in both the dopaminergic cells of the SN pars compacta and the pyramidal neurons of the CA1 region of the hippocampus. We further synthesized ¹⁹F-C₈-6-I and determined that ¹⁹F-C₈-6-I possessed a similar metabolic profile to C₈-6-I in mouse, rat, and human liver microsomes and the metabolites are 1-aminoindan hydroxylation, 1-aminoindan *N*-dealkylation, *N*3 and *N*1-demethylation with the exception of alkyl hydroxylation only found in ¹⁹F-C₈-6-I [11]. No defluorination was observed as this is important for the development of PET probes since fluoride ions can accumulate in the skull and bones, cause toxicity and confound the interpretation of PET images [11]. To further understand the in vivo parameters of C₈-6-I and ¹⁹F-C₈-6-I, we measured and reported the unbound intrinsic clearance, CL_{int}, in mouse liver microsomes and extrapolated to predict in vivo clearance in mouse plasma, CL_p using the well-stirred model. We reported a predicted CL_p of 36.0 ml/min/kg for ¹⁹F-C₈-6-I compared to 34.3 ml/min/kg for C₈-6-I [31]. These metabolic stability studies indicated that similar metabolites were observed in mouse, rat and human microsomes, suggesting that mice and rats are appropriate models for future animal studies of ¹⁸F-C₈-6-I. Emerging evidence supports the key role of the gut-brain connection in the pathogenesis of PD [65–67]. Our pharmacokinetics studies demonstrated a relatively high level of intestinal localization of ¹⁸F-C₈-6-I dimer probes which further suggests the potential applicability of our probe in gut-brain studies.

We also tested whether C₈-6-I could act on other targets, including A1Rs and A2ARs, since this bifunctional compound contains the caffeine moiety which could retain its binding affinity for

adenosine receptors. Whereas caffeine non-selectively binds to the respective orthosteric adenosine binding sites in A1Rs and A2ARs [29,30], the C₈-6-I dimer appears to bind preferentially to the A1R orthosteric site. Using molecular docking, we found that C₈-6-I interacted with the A1R orthosteric binding site and additional putative allosteric binding sites were also identified. In contrast, C₈-6-I did not make similar contacts with the orthosteric binding site or allosteric binding site for A2AR. Important amino acid residues in the second extracellular loop (ECL2) of A1R have been identified to play a crucial role in A1R allosteric modulation. Previous studies using an all-atom Gaussian accelerated molecular dynamics (GaMD) simulation using an inactive A1R structure (PDB: 5UEN) supported a key role of residue E172 (in the ECL2 region) as an important binding determinant for the two positive allosteric modulators (PAMs), PD71723 and VCP171. Alanine substitution of E172 decreased the binding affinity of two allosteric modulators, PD81723 and VCP171 [68,69]. The residues E171 (also in ECL2) and N254 on TM6 are known to interact with orthosteric antagonists DU172 and PSB36 [68] as well as endogenous agonist, adenosine (PDB:6D9H) [28]. In the present study, we observed the binding of C₈-6-I to the PAM-binding residue E172 and the orthosteric agonist/antagonist binding residue F171. Since we found that C₈-6-I was effective in preventing neurodegeneration of hippocampal and substantia nigral neurons, which likely depended on preserving A1R expression, this suggests that C₈-6-I may act as an A1R PAM that promotes less A1R desensitization compared to A1R orthosteric agonists, adenosine, and CPA. However, the C₈-6-I binding to the orthosteric site F171 also suggests that C₈-6-I may reduce agonist binding and A1R activity. Although the influence of C₈-6-I allosteric binding on A1R binding and function has yet to be investigated, it is also possible that the allosteric mechanism may involve decreasing the rate of orthosteric agonist dissociation and enhancing agonist binding and function [70]. This potential A1R allosteric ligand property of C₈-6-I may be therapeutically beneficial and warrants further studies due to potential therapeutic implications. If C₈-6-I is an allosteric modulator of A1R, it will be important to carry out further concentration-response analysis, as some A1R PAMs have been shown to act as inhibitors at high concentrations and cause a functional response in the absence of an agonist (e.g., T62) while some compounds (e.g., PD81723 derivative with bridged 3- and 4- positions) exhibited A1R antagonism at low concentrations, but PAM activity at higher concentrations [68,71].

In summary, we showed further evidence that a 7-day chronic CPA i.p. injection rat model generated increased α -synuclein expression and aggregation, which provides a convenient model to assay several neuroprotective drugs, including the bifunctional compound C₈-6-I, without waiting several months to observe the presence of α -synucleinopathies normally seen in genetic mouse models of PD (e.g., A53T mouse model). Our results indicate that the C₈-6-I dimer compound promotes neuroprotection by decreasing the CPA-induced α -synucleinopathy and neurodegeneration in the hippocampus and SN pars compacta. This likely involves C₈-6-I directly binding to α -Syn domains that promote the neuroprotective "loop" conformation that prevents α -Syn misfolding and aggregation. Moreover, directly linking caffeine to 1-aminoindan also appears to increase the caffeine moiety's preferential binding to the A1R adenosine orthosteric binding site. This implies that the observed biodistribution of C₈-6-I dimer inside the CNS makes it an ideal therapeutic for PD, as this bifunctional drug promotes neuroprotection by inhibiting α -Syn misfolding and aggregation and by suppressing chronic adenosine A1 receptor stimulation which is expected to prevent α -Syn over-expression. Finally, this study indicates that C₈-6-I may be an appropriate PET-CT tracer that can be used for imaging diagnostics and treatment of PD.

Methods

In Vivo Animal Protocol

Our in vivo studies were designed to test the effects of C₈-6-I on α -Syn aggregation and neurodegeneration induced by 7 days of i.p. injections of CPA (3mg/kg) in male Sprague-Dawley rats (28-30 days old, 250-300 g) which were then assessed by behavioural and histological experiments. To test for the biodistribution and pharmacokinetics of radiolabeled C₈-6-I, female Swiss Albino CD-1 mice (34-46 g) were used. All animals were housed and treated humanely following the guidelines from

the following governing bodies: National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (Washington DC, 2011); Canadian Council on Animal Care (CCAC); and the University of Saskatchewan Animal Research Ethics Board (AREB) that approved our Animal Use Protocol (#20070090 and #20170079). The animals were housed in cages of two, with access to food pellets and water ad libitum. The four treatments of the Sprague Dawley rats consist of **1.** Control (0.1% DMSO in 0.9% saline), **2.** CPA, **3.** Cs-6-I (3 mg/kg) + CPA, and **4.** Cs-6-I (5 mg/kg) + CPA. Drugs were dissolved at 3 mg/ml in DMSO, and each drug was administered to the animals by daily i.p. injections (3 mg/kg or 5 mg/kg body weight) for 7 consecutive days. After the first injections with Cs-6-I 3 mg/kg or Cs-6-I 5 mg/kg, the animals were returned to their cages for 30 min before a subsequent CPA injection was administered. Then on days 8 and 9 following the final injections, the animals were subjected to several behavioural tests.

Reagents

CPA was purchased from Abcam (Toronto, Canada), and DPCPX from Tocris (Burlington, Canada). Formic acid (LC-MS grade purity), 4 N HCl in dioxane, and Kryptofix 222 were purchased from Sigma Aldrich (Oakville, Canada), anhydrous acetonitrile from Thermo Fisher Scientific (Waltham, MA, USA), and sodium bicarbonate from EM SCIENCE (affiliate of Merck, KGaA, Darmstadt, Germany). As seen in **Figure 1 b.1**, [¹⁸F]Fluoride was produced using the ¹⁸O(p,n)¹⁸F reaction with 24 MeV protons in an Advanced Cyclotron System Inc (ACSI) high-current TR-24 cyclotron at Saskatchewan Centre for Cyclotron Sciences (Saskatoon, SK, Canada). C8-6-I-OMs precursor was synthesized according to our previously described synthetic procedure [11].

Behaviour Tests

Y-Maze

The Y-maze or the modified Y-maze is a rodent behaviour test used to assess short-term spatial memory based on the innate inclination of animals to explore new areas not previously seen [4]. In this study, the Y-maze test is used to assess short-term memory, especially hippocampal-dependent spatial memory [25,26]. The maze is shaped like a “Y” with each arm oriented at a 120° angle from one another. The arms are 50 cm in length, 15 cm in width and 35 cm in depth. For the first trial, which lasts 15 minutes, the animal is placed at the beginning of the “Start arm” and is free to explore this arm and another one (referred to as the “Old arm”) while the remaining arm (“New arm”) is blocked. This trial should give the animal time to explore and become acquainted with the maze’s two arms. After a resting period of 90 minutes, the blocked arm is open, and the animal is placed again at the “Start arm”. The animal is free to explore all arms including the “New arm” for 5 minutes. This trial is then used to calculate the percentage of time the animals spend in each arm. A video camera was used to record the animal movement in both trials. Each experiment was repeated at least fifteen times per treatment and the average of each treatment is presented in the bar graphs as average means ± standard error of the mean (SEM).

Open Field Test

The open field is a general locomotor test that detects anxiety and willingness to explore [25,26]. The test is conducted in a white open cube measuring 50 cm in length, 50 cm in width and 35 cm in depth. The bottom field is grided in 4*4 squares at 12.5 cm with a center square in the middle of the field. The animal is placed in the center square and is free to explore the entirety of the field for 15 minutes. The first 5 minutes are considered an acclimation to the maze therefore are not calculated as part of the behaviour test. This trial is then used to calculate the percentage of time the animals spent in the center zone (red square) and the total fecal boli count. A video camera was used to record the animal movement in this test. The open field test was conducted after 1 hour resting period from the time the Y-maze second trial is completed. Each experiment was repeated at least ten times per treatment and the average of each treatment is presented in the bar graphs as average means ± SEM.

Forced Swim Test

Forced swim test is used to assess depressive behaviour such as despair and learned helplessness [4]. The test is also used to assess the vigor and the ability of the animal to keep its head above water in which the animals swim [72,73]. In addition, the first and last three minutes of the test were manually scored for the ability of the rat to swim with all its four limbs. **Vigor:** swim with four limbs—Score 3; occasionally float—Score 2.5; floating more than swimming—Score: 2; occasionally swimming—Score 1.5; occasionally swimming with hind limbs—Score: 1; no use of limbs—Score: 0. In addition, the ability of the animal to keep its head above water was scored as well. **Success:** entire head above water—Score 3; ears below water—Score 2.5; eyes below water—Score: 2; entire head below water for 3 seconds—Score 1.5; entire head below water for 6 seconds—Score: 1; in the bottom of tank for more than 10 seconds—Score: 0.

The forced swim test apparatus is plexiglass cuboid measuring at 30 cm in length, 30 cm in width and 60 cm in depth. The tank is then filled two-thirds with lukewarm water not exceeding 25 °C. Afterwards, the animal will be left in the cylinder for 10 minutes where its time spent immobile as well as its ability to swim and keep its head above water will be analyzed. This trial is then used to calculate the percentage of time the animals spend immobile, as well as the vigor and success in which the animal swims. A video camera was used to record the animal movement in this test. The forced swim test was conducted the following day (day nine) giving the animals a sufficient resting period from the Y-maze and open field tests. Each experiment was repeated at least fifteen times per treatment and the average of each treatment is presented in the bar graphs as average means $\pm$ SEM.

Structural Modeling and Molecular Docking

The structures of soluble α -Syn monomers (C1-C8, see **Supplementary Figure 1B**) have previously been identified and were recently applied to predict the binding of A1R ligands and 1-aminoindan to α -Syn [13]. The C1-C8 α -Syn structures were used in our molecular docking simulations to predict the dimer-protein complexes, and these structures were obtained from PDB-DEV (Entry: PDBDEV_00000082) [14]. The molecular docking study was carried out using the Autodock Vina module implemented in the PyRx tool [3,12]. Protein and ligand interactions were analyzed and visualized through Pymol and LigPlot+.

Immunohistochemistry

Anesthetized rats were transcardially perfused with 0.9% saline, and then fixed with 4% paraformaldehyde. The extracted brains were put in 30% cryoprotected sucrose solution for 48 h prior to slicing. The brains were initially frozen at -40°C (BFS-30 mp controllers) and sliced with the help of a microtome (Leica SM2010 R Sliding controller). Coronal slices of 40 μm were then washed three times in 0.1 M phosphate buffered saline followed by 1-h blocking at room temperature with blocking buffer. The buffer solution components have been previously described [74]. The slices were then incubated overnight at 4°C with the following primary antibodies: 1:200 mouse monoclonal to α -synuclein (Abcam Inc, Toronto, Canada) and 1:200 rabbit polyclonal to tyrosine hydroxylase (TH) (Millipore-Sigma, Oakville, Canada). Subsequently, slices were then incubated for 1 h in the dark at room temperature with the following secondary antibodies: AlexaFluor-555-conjugated anti-mouse and AlexaFluor-647-conjugated anti-rabbit (1:1000) purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA). Slices were then treated with Thioflavin-S (see further details below). Lastly, the slices were incubated for 5 min at room temperature with DAPI (2mg/ml) from Invitrogen (Thermo Fisher Scientific, Waltham, MA) and images were taken using a Zeiss LSM700 confocal microscope (Carl Zeiss Group, Canada) and analyzed with ImageJ (Public Domain). Images of the hippocampal CA1 pyramidal layer and the SN pars compacta were obtained using the Zeiss Plan-Apochromat 63X/1.4 oil objective lens (Carl Zeiss Group, Canada). Images were acquired as Z-stack images of hippocampal or SN regions with 12–13 Z-stack images taken at 1- μm intervals near the middle of brain slices. Two Z-stack images were taken along the hippocampal CA1 or SN pars

compacta region for each slice, and immunofluorescence signals were averaged using densitometry analysis.

Thioflavin-S

Thioflavin-S (Thio-S) is a fluorescent marker (Sigma-Aldrich, Oakville, Canada) that detects α -Syn aggregates and amyloid plaques. Coronal slices of 40 μ m were firstly treated with 0.3% KMnO₄ for 4 min, followed by a 30-min incubation with 1 M phosphate buffered saline at 4 °C. These slices were then stained with 0.05% Thioflavin-S in 50% ethanol in the dark for 8 min, rinsed with 80% ethanol twice, followed by three rinses with ultra-pure water for 30 s. Finally, the slices were incubated again with 1 M phosphate buffered saline for 30 min at 4 °C before starting the DAPI stain. The FITC filter (488 nm laser line) was used to image Thioflavin-S using a Zeiss LSM700 confocal microscope (Carl Zeiss Group, Canada) and images were analyzed with ImageJ (Public Domain).

Fluoro-Jade C

Fluoro-Jade C (FJC) is a fluorescent marker for neurodegeneration (Millipore-Sigma, Oakville, Canada). Coronal slices of 40 μ m were mounted on 5% gelatin-coated super-frost plus microscope slides (Thermo Fisher Scientific, Waltham, MA) and dried overnight at 4 °C. Initially, the microscope slides were immersed in 1% NaOH/80% ethanol for 5 min followed by 2-min immersion in 70% ethanol. The slides were then rinsed for 2 min with ultra-pure water. The microscope slides were further immersed in 0.06% KMnO₄ for 10 min, followed by an additional rinse for 2 min with ultra-pure water. The slides were then stained with 0.004% FJC in 0.1% acetic acid for 20 min with gentle shaking on an orbital shaker. Lastly, the slides were rinsed three times in ultra-pure water for 1 min each, making sure to remove all the excess water after each rinse. The slides were then rinsed in xylene and allowed to dry overnight at 4 °C. Then they were treated with Prolong Gold Antifade Reagent from Invitrogen (Thermo Fisher Scientific, Waltham, MA), and respective images were taken using a Zeiss LSM700 confocal microscope (Carl Zeiss Group, Canada) and analyzed with ImageJ (Public Domain). FJC fluorescence was obtained by exciting the dye with 488 nm laser.

Radiochemistry

¹⁸F-C₈-6-I was synthesized from the corresponding mesylate precursor, C₈-6-I-OMs using a direct S_N2 displacement with [¹⁸F]fluoride (see **Figure 1 a**). [¹⁸F]Fluoride was trapped on a quaternary methyl ammonium (QMA) cartridge purchased from Waters (MA, USA) and eluted off with 2 mL of Kryptofix 2.2.2./K₂CO₃ solution (16.0 mg of Kryptofix 2.2.2 and 3.45 mg of K₂CO₃ in 2 mL acetonitrile/water, 4/1, v/v) into a clean glass vial (see **Figure 1 b.2**). The solvent was then removed at 90 °C under a stream of nitrogen gas, and the residue was azeotropically dried with anhydrous acetonitrile (2 × 1 mL) at 90 °C. The vessel was cooled to room temperature, and a solution of the mesylate precursor, C₈-6-I-OMs (1.5–2.0 mg) in anhydrous acetonitrile (0.5 mL) was added. The reaction mixture was heated to 90 °C and stirred for 10 min. The vessel was then cooled again to room temperature, and 4 N HCl in dioxane (0.25 mL) was added. The reaction mixture was stirred at room temperature for another 10 min (see **Figure 1 b.2**). Saturated NaHCO₃ solution (0.55 mL) was added slowly to quench the reaction followed by the purification of the reaction mixture with Ultra-High-Performance Liquid Chromatography (UHPLC) (see **Figure 1 b.3**).

HPLC purification and analyses of the radiotracer were performed on a Thermo Fisher Vanquish UHPLC system equipped with a Chromeleon 7 communication software, Thermo/DIONEX UltiMate AFC-3000 fraction collector, an Agilent Zorbax Eclipse XDB-C18 Semi Prep Column (5 μ m C18, 9.4 × 250 mm reverse phase column, PN-990967-202, flow rate 5.00 mL/min), and an Eckert & Ziegler flow-count radio-HPLC detection system. To purify ¹⁸F-C₈-6-I, the reaction mixtures were separated on UHPLC using gradient elution with a binary solvent system as described in Table 1. A sample of pure ¹⁹F-C₈-6-I was used as an authentic standard to determine the expected retention times of ¹⁸F-C₈-6-I. Compound elution was monitored using both absorbance at 280 nm and radiodetection (20k max counts per minute (cpm) and 5 sec integration time) with all chromatography done at room

temperature. The purified fractions containing ¹⁸F-C₈-6-I were rapidly evaporated using the V-10 solvent evaporator at 36 °C, and the concentrate reconstituted in sterile saline solution containing 5% DMSO for animal studies. The total synthesis time was about 0.5 h. The radiochemical yield (rcy) was 23 ± 5% (*n* = 7, decay corrected), and the radiochemical purity was ≥ 98% as judged by UHPLC.

Table 1. HPLC gradient elution profile for the chromatographic purification of ¹⁸F-C₈-6-I radiotracer. Mobile Phase A—0.1% (*v/v*) formic acid in water and mobile phase B—0.1% (*v/v*) formic acid in acetonitrile.

Time (min)	Flow Rate (ml/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	5	90	10
3.00	5	90	10
7.00	5	60	40
10.00	5	40	60
15.00	5	10	90
17.00	5	10	90
18.00	5	90	10
21.00	5	90	10

Micro-PET/CT Imaging

Female CD-1 mice (34–46 g) were anesthetized with isoflurane (1.5–2.5%) in oxygen at a flow rate of 1–2 L/min and tail vein injected with ¹⁸F-C₈-6-I (5.27–5.52 MBq, 100 µL) in sterile saline containing < 5% DMSO via tail vein (see **Figure 1 b.4**). MicroPET dynamic imaging was acquired on a Sofie GNEXT PET/CT scanner following injection of ¹⁸F-C₈-6-I. The field of view (FOV) was 12 cm transaxial and 10.4 cm axial with a spatial resolution of < 1 mm. Acquisition of dynamic PET data (10 × 10, 8 × 15, 6 × 20, 6 × 30, 4 × 60, 1 × 100, 4 × 100, 6 × 150, 4 × 300) was started immediately after radiotracer injection, followed by a 1 min CT scan. PET/CT images were registered and reconstructed automatically by the GNEXT system. PET images were reconstructed using a 3D-ordered Subset Expectation Maximization algorithm with 24 subsets and 3 iterations. CT images were reconstructed using a Modified Feldkamp algorithm. All data were analyzed by Vivoquant software (IviCRO). CT images were used to define anatomical regions of interest (ROIs) through PET/CT image co-registration. Quantification of activity concentration in the brain was done by drawing ROIs and the mean and maximum standard uptake values (SUVs) were determined using the formula: SUV = [(MBq/mL) × (animal wt. (g))/injected dose (MBq)].

In Vivo Metabolic Stability and Biodistribution

In vivo, the metabolic stability of ¹⁸F-C₈-6-I was carried out following a protocol by Zhang and colleagues [75]. After a tail vein injection of ¹⁸F-C₈-6-I, the mice were euthanized (anesthesia followed by cervical dislocation) at 40 min post injection (p.i.) (see **Figure 1 b.5**). The liver, pancreas, and brain were collected, washed with saline, placed in water/acetonitrile, and homogenized using the Fisherbrand™ 150 Handheld Homogenizer (Thermo Fisher Scientific, Waltham, MA). The samples were centrifuged at 14000 relative centrifugal force (rcf) for 5 min (AccuSpin Micro 17, Thermo Fisher Scientific, Waltham, MA). The supernatants were collected and filtered using 0.22 µm organic Millipore-Sigma filters (Oakville, Canada). The clarified filtrate was injected into the HPLC and radioanalytes separated by a reverse phase column (5 µm C18, 9.4 × 250 mm reverse phase column, PN-990967-202, flow rate 5.00 mL/min) using the same gradient elution as described in **Table 1**. The elution of radioactive metabolites was detected using the radiation detector as described above. As seen in **Figure 1 b.5**, biodistribution was performed in healthy female CD-1 mice (54 ± 5.2 g). The purified ¹⁸F-C₈-6-I (about 400 kBq–700 kBq/100 µL) in sterile saline containing < 5% DMSO was injected into mice (five groups, *n* = 3 for each group) via tail vein. The mice were euthanized

(anesthesia followed by cervical dislocation followed by cardiac puncture and blood draw) at 5, 10, 20, 40, and 60 min p.i. and different organs were harvested (blood, liver, small intestine, kidney, spleen, lung, heart, large intestine, bone, and brain), washed and placed in pre-weighed tubes. The radioactivity in each organ/tissue was measured using a calibrated automatic gamma counter (2480 Perkin Elmer, Waltham MA, USA) and the tissue uptake was expressed as a percentage of injected dose (ID) per gram (% ID/g). All radioactivity measurements were corrected for decay. Data are shown as average mean $\pm$ SEM were calculated using replicated for each sample.

Statistical Analyses

For behavioural studies, histological, radiotracer biodistribution and Western blot analyses, statistical analyses were conducted with GraphPad Prism 8 software (San Diego, CA) with one-way ANOVA followed by Student-Neuman-Keuls multiple comparison *post hoc* test. The significances are indicated as: ns, non-significant; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, **Supplementary Figure 1.** a. The α -synuclein domains are denoted as follows: Blue—N-terminus; Yellow—NAC region; Red—C-terminus. b. Summary of the eight α -synuclein structures as determined by discrete molecular dynamics simulations and further confirmed by far-UV circular dichroism and cross-linking mass spectrometry [13]. **Supplementary Figure 2. Molecular docking simulation of α -Syn structures a. C4 and b. C6 bound to C8-6-I.** These structures show the dimer compound forming only hydrogen bonds with the caffeine moiety to C-terminus at Y133 and Y125, respectively, but they also show weak interaction with the N-terminus of the protein. The C4 structure surrounds the dimer compound by hydrophobic interactions with ten amino acid residues located in the C4 N-terminus (Supplementary Figure 1a). In contrast, the C6 structure interacts with the dimer compound via additional five C-terminal and one N-terminal amino acid residues. Based on this binding pattern, both C4 and C6 structures could also adopt a “loop” conformation by binding to the dimer compound. **Supplementary Figure 3. Molecular docking simulation of α -Syn structures a. C5 and b. C7 bound to C8-6-I.** These structures showed only binding to the N-terminus, which predicts a “knot” conformation which contradicts predictions from previous nanopore analysis suggesting the dimer compound adopts a “loop” conformation [5].

Authors' Contributions: E.J. performed and analyzed the animal chronic injections, behavioural tests, and postmortem histological and biochemical experiments. O.P.A. and K.J.H.A. performed the synthesis, pharmacodynamics, and toxicological work. E.J., O.P.A. and F.S.C. wrote the manuscript; C.T., O.P.A., O.M. and E.S.K. helped revise the manuscript. All authors read and approved the final manuscript.

Funding: This research was funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada Grant (FSC), the Saskatchewan Health Research (SHRF) Foundation Collaborative Innovation and Development Grant (FSC), the Heart and Stroke Foundation of Canada (FSC), and Canada Foundation for Innovation Leaders Opportunity Fund (FSC). EK and MB received NSERC Discovery Grants.

Availability of Data and Materials: All the data generated or analyzed during this study are included in this manuscript. Original raw data are available from the University of Saskatchewan (Department of Surgery and Department of Chemistry) and can be readily furnished upon request.

Acknowledgments: This work was supported by funding from Heart and Stroke Foundation of Canada, NSERC Discovery Grant, SHRF Foundation Grant, and College of Medicine (University of Saskatchewan) to F.S.C. E.J. received Doctoral Award from College of Medicine (University of Saskatchewan). O.M. received Undergraduate Student Research Award from the NSERC and the Biomedical Summer Research Project Award from College of Medicine (University of Saskatchewan).

Ethical Approval and Consent to Participate: Not applicable.

Compliance with Ethical Standards: All the biosafety and approvals for the ethical and humane use of animals were obtained prior to the start of the study. All procedures performed in this study were in accordance with the ethical standards of the University of Saskatchewan. The study was approved by the Animal Review and Ethics Board (AREB) of the University of Saskatchewan (Animal Use Protocol # 20070090).

Consent for Publication: Not applicable.

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Conflicts of Interests/Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

Abbreviations

A1R: adenosine A1 receptor; α -syn: Alpha-Synuclein; C8-6-I: Caffeine-indan dimer linked with a 6-carbon chain; CPA: N⁶-Cyclopentyladenosine; FJC: Fluoro-Jade C; TH: Tyrosine hydroxylase; Thio-S: Thioflavin S.

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