Research report

Identification and in vitro characterization of C05-01, a PBB3 derivative with improved affinity for alpha-synuclein

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HIGHLIGHTS

• C05-01 is an analogue of PBB3 displaying higher affinity for α-synuclein and was successfully labelled with ³H.
• The binding of C05-01 to α-synuclein was evaluated using various techniques, such as fluorescence and autoradiographic evaluation in fresh frozen tissue, autoradiography in tissue microarrays (TMAs), and in vitro binding assays using human brain homogenates and fibrils.
• [³H]C05-01 showed specific binding in tissue with α-synuclein pathology from PD and MSA patients, but also in tissue with amyloid-β and tau pathology.
• [³H]C05-01 displayed off-target binding that could not be attributed to MAO-A, MAO-B, or Sigma 1 and that requires further examination and a need to improve the physicochemical properties and the relative affinity of C05-01 for further development as PET radioligand.
• TMAs are useful tools for evaluating the binding of test compounds to misfolded proteins.

ARTICLE INFO

Keywords:
Alpha synuclein
PBB3
Aturoadiography and in vitro binding assay
Tissue microarrays

ABSTRACT

The neuropathological hallmark of Parkinson's disease, multiple system atrophy and dementia with Lewy bodies is the accumulation of α-synuclein. The development of an imaging biomarker for α-synuclein is an unmet need. To date, no selective α-synuclein imaging agent has been identified, though initial studies suggest that the tau tracer [¹¹C]PBB3 displays some degree of binding to α-synuclein. In this study, a series of compounds derived from the PBB3 scaffold were examined using fluorescence imaging and tissue microarrays (TMAs) derived from brain samples with different proteinopathies. One compound, C05-01, was selected based on its higher fluorescence signal associated with Lewy body aggregates compared with other PBB3 analogues. In vitro binding assays using human brain homogenates and recombinant fibrils indicated that C05-01 had higher affinity for α-synuclein (Kᵦ/Kᵢ 25 nM for fibrils, Kᵦ 3.5 nM for brain homogenates) as compared with PBB3 (Kᵦ 58 nM). In autoradiography (ARG) studies using fresh frozen human tissue and TMAs, [³H]C05-01 displayed specific binding in cases with α-synuclein pathology.

C05-01 is the first PBB3 analogue developed as a potential compound targeting α-synuclein. Despite improved affinity for α-synuclein, C05-01 showed specific binding in AD tissue with Amyloid β and tau pathology, as well as relatively high non-specific and off-target binding. Additional efforts are needed to optimize the pharmacological and physicochemical properties of this series of compounds as ligands for α-synuclein. This study also showed that the construction of TMAs from different proteinopathies provides a tool for evaluation of fluorescent or radiolabelled compounds binding to misfolded proteins.
1. Introduction

Proteinopathies are neurodegenerative diseases characterized by pathological accumulation of amyloid-β (Aβ), tau, α-synuclein, and TDP-43 (Harada et al., 2018). Compounds that bind to β-sheet structures can be developed as potential agents for imaging of proteinopathies using positron emission tomography (PET). High affinity, selectivity, and low nonspecific binding are important pre-requisites for a small molecule to be developed as PET imaging agent. Several tracers have already been developed for imaging of Aβ and tau (Gjerum et al., 2020; Harada et al., 2018; Higuchi, 2019; Wang & Edison, 2019).

Imaging of aggregated α-synuclein is of key importance for accurate diagnosis of synucleopathies, such as Parkinson’s disease (PD), multiple system atrophy (MSA) and dementia with Lewy bodies (DLB), as well as for monitoring of therapeutic interventions and to support drug development programs. However, the development of a PET tracer for α-synuclein continues to be a major challenge. Like other amyloids, α-synuclein acquires the cross-β-sheet structure in the seeded nucleation process (Liberis and Ironside, 2015). The similarity of the beta-sheet core structure makes it difficult to develop small molecules that bind α-synuclein with high selectivity. In addition, relatively high affinity for α-synuclein is likely required to image Lewy body pathology at a clinical stage corresponding to the onset of the disease, when the load of α-synuclein in the brain is expected to be low.

A series of phenyl/pyridinyl butadienyl benzothiazoles/benzothiazoliums (PBBs) have been developed as potential tau PET ligands. Among those, 2-((1E,3E)-4(6([11 C]methylamino)pyridin-3-yl)buta-1,3-zoliums (PBBs) have been developed as potential tau PET ligands. These compounds were synthesized by several research groups and have been extensively evaluated using in vivo imaging of tau pathology in the human brain (Maruyama et al., 2013; Hashimoto et al., 2014). Recent studies conducted either on postmortem tissue from patients with synucleinopathies (Koga et al., 2017) or in patients expected to have high load of α-synuclein pathology (Perez-Soriano et al., 2017) suggested that [11 C]PBB3 also binds to α-synuclein. However, the postmortem data presented by Koga et al. (2017) implied that α-synuclein pathology in Lewy body disease was undetectable by [11 C]PBB3, whereas the pathology could be visualized in a subset of MSA cases with high density of glial cytoplasmic inclusions that carry a high load of α-synuclein. Based on these initial findings, a library of compounds structurally related to PBB3 was developed for further evaluation using fluorescence imaging and tissue microarrays (TMAs). TMAs were produced by taking small punches from a series of paraffin-embedded donor tissue blocks and transferring these tissue cores into a positionally encoded array in a recipient paraffin block (Fowler et al., 2011). Several tissue cores may be taken without consuming the tissue block, allowing the donor block to be returned to its archive for any additional studies.

These constructs have several advantages over conventional whole histological sections for research. Tissue from multiple patients can be examined on the same slide and multiple sections (100–300) can be cut from a single array block, allowing for hundreds of analyses per microarray. TMAs can be used for multiple purposes including immunohistochemistry, immunofluorescence, in situ hybridization, and conventional histochemical staining, as well as with autodiagnostic or, quantum dot preparations (Rabinovich et al., 2006). To the best of our knowledge, this approach has not been used for semi-quantitative examination of fluorescence compounds.

The aim of the present study was first to evaluate the fluorescence signal of a selected number of PBB3 analogs in Lewy aggregates using a semi-quantitative approach on TMAs. The initial evaluation lead to the selection of one compound, C05-01 for further analysis (Fig. 1). The second aim was to characterize the binding properties of C05-01 using in vitro binding techniques on brain homogenates and α-synuclein fibrils and with postmortem autodiography.

2. Results

2.1. Evaluation of fluorescence compounds using tissue microarrays

A selected library of 44 compounds structurally similar to PBB3 was evaluated with fluorescence microscopy at the National Institutes for Quantum and Radiological Science and Technology (QST, Chiba, Japan). The initially screened 44 compounds consisted of 18 amino-phenyl-butadienyl-benzothiazole derivatives (e. g. C01-30), nine amino-pyridinyl-butadienyl-benzothiazole derivatives (e. g. PBB3), five amino-phenyl-butadienyl-benzene derivatives, four amino-pyridinyl-butenyl-benzothiazole derivatives (e. g. C05-01), four amino-pyridinyl-butadienyl-benzoxazole derivatives, two amino-pyridinyl-butenyl-quinoline derivative, one amino-phenyl-ideneamino-benzothiazole derivative, and one amino-phenyl-alllylidenediamino-benzothiazole derivative.

Compounds were initially tested using a qualitative approach, in which the strength of the fluorescent signal corresponding to α-synuclein, Aβ and Tau aggregates was measured. The analysis was done by assigning a qualitative score to the strength of the fluorescence signal observed in tissue from dementia with Lewy bodies (DLB) and AD patients containing Lewy bodies, Lewy neurites, Aβ plaques and tau aggregates. A total of 5 compounds (including PBB3) were selected from the initial evaluation, based on their relatively higher signal than PBB3 for aggregates of α-synuclein and similar or relatively lower signal than PBB3 for Aβ plaques and tau aggregates. The 5 compounds were evaluated using a pre-characterized set of tissues representing neurodegenerative disease with deposits of Aβ, phospho-Tau and α-synuclein (see material and methods for more details). The methods and workflow were established to semi-quantitatively examine the individual fluorescence signal relative to the one of PBB3.

Fig. 2 shows the flow chart showing a schematic step protocol for quantitative image analysis of ligand binding. All ligands bound to α-synuclein, Aβ, and phospho-Tau aggregates within the tissues, although providing different mean fluorescent intensity for each type of pathological aggregates. Variable levels of non-specific binding to the tissues were also observed outside of the areas with fibrillary deposits.

From the chosen compounds tested in TMA with this fluorescence/IHC validated technique, C05-01 and C01-30 were the compounds displaying the best properties compared with PBB3. Quantitative image analysis of C05-01 in PD tissue showed similar binding to α-synuclein aggregates as C01-30 but higher than the one observed for PBB3 (Fig. 3A). In the same tissue, the background signal of C05-01 was lower than the one observed for C01-30 (Fig. 3A). Finally, in AD tissue, the fluorescence intensity of C05-01 for Aβ and Tau was lower than the one observed for C01-30 (Fig. 3B and 3C).

In a separate set of experiments using fresh frozen tissue with pure α-synuclein pathology, the fluorescence signal of C05-01 (30 µM) co-localized with the immunohistochemistry signal to a large extent in correspondence of the Lewy bodies and to a lower extent at the level of the Lewy neurites (Fig. 4). Based on the properties observed in paraffine embedded and fresh frozen tissue, C05-01 was selected for further in vitro characterization.

Fig. 1. Chemical structure of C05-01.
2.2. In vitro binding in brain homogenates and fibrils to determine the affinity of C05-01 to α-synuclein

Fig. 5 shows different approaches to determine the binding affinity of C05-01. The dissociation \( (K_D) \) and inhibition \( (K_i) \) constants measured using α-synuclein fibrils (1 µM), showed affinity values in the range of 25–30 nM, using both saturation and competition binding assays with \([3H]C05-01\) and non-labelled C05-01. In brain homogenates obtained from a DLB amygdala sample (Fig. 5C), \([11C]PBB3\) displayed moderate affinity binding (\(K_D = 58 \text{ nM}\)), and non-labelled C05-01 inhibited the binding of \([11C]PBB3\) with high affinity (\(K_i = 3.5 \text{ nM}\)).

2.3. In vitro autoradiography using \([3H]C05-01\) in TMAs

To assess the binding of \([3H]C05-01\) to α-synuclein pathology and other misfolded proteins, autoradiography using tissue micro arrays (TMA) was performed with a low concentration (0.1 nM) of radioligand (Fig. 6A). The TMA contained core sections of several cases and brain regions from donors with PD, MSA, Pick’s disease, vascular dementia, AD, as well as from controls. All core sections were included in the same slide. As it is shown in Fig. 2A (TMA construction), a separate TMA was constructed for PD cases, which contained its own set of control samples. As it is shown in Fig. 6A, total binding of \([3H]C05-01\) was higher in MSA white matter cores than in samples from vascular dementia and controls. In all cases, the binding of \([3H]C05-01\) (Fig. 6A) was completely blocked by competition with excess of non-labelled C05-01.

Fig. 2. Work-flow for quantitative image analysis of ligand binding using tissue microarrays (TMAs). (A) Example of mini TMA containing tissue from the substantia nigra of PD and non-demented control subjects, NDC (left image) and other pathologies (right image) such as multi system atrophy (MSA), Vascular dementia (VD), Pick’s disease (pick), Alzheimer’s disease (AD) and control subjects (NDC). (B) The immunohistochemistry signal is overlaid with the fluorescence signal of the molecule binding to Lewy bodies (large inclusion displayed in yellow) or Lewy neurites (red). (C) The image is segmented in order to classify the different components: Large inclusions (red) are separated from Lewy neurites (yellow), from auto-fluorescence (blue) and background (light blue). The mean fluorescence intensity is then measured in the large inclusions.

Fig. 3. Quantitative image analysis outcome of ligand binding with C05-01 (blue dots), C01-30 (yellow dots) and PBB3 (pink dots) in PD tissue targeting α-synuclein (A) AD tissue targeting Aβ (B) and AD tissue targeting tau (C). Each point represents mean fluorescence intensity (MFI) value from one individual. Li: Large α-synuclein inclusion (α-synuclein + ligand); CP: Core of compact plaques (Aβ + ligand); pTF: pTau-tangles dual labelled with (AT8 + ligand) and Bgd: Tissue background (area with no pathology).
In each pathology, the specific binding was normalized to their respective controls (Fig. 6C). In MSA cases, normalized specific binding of \[^3H\]C05-01 was higher in the tissue from the patient with higher burden of α-synuclein pathology (Fig. 6A and 6C, white arrows and Supplementary Fig. 1A). In PD cases and AD cases (Fig. 6C), normalized specific binding of \[^3H\]C05-01 was significantly higher than in other pathologies (one-way ANOVA, with post-hoc Bonferroni, \(p < 0.001\) for PD; \(p < 0.01\) for AD). Pick’s disease cases showed lower normalized specific binding (Fig. 6C). Normalized specific binding was the lowest in cases of vascular dementia. In AD and Pick’s disease, areas of specific binding (Fig. 6A black arrows) corresponded to areas with Aβ and tau aggregates (Supplementary Fig. 1B and 1C). Finally, anterior cingulate cortex from a PD case with high α-synuclein load was examined in the same experiment as the TMA (Fig. 6D). A high total binding to a α-synuclein aggregates was observed in the grey matter of this sample.

2.4. In vitro autoradiography using radiolabeled \[^3H\]C05-01 in fresh frozen brain tissue

Postmortem autoradiography with \[^3H\]C05-01 in amygdala, cingulate cortex and substantia nigra from a single case of PD with pure α-synuclein pathology showed specific binding in all three regions (Fig. 7A). However, displaceable binding was observed also in the cingulate cortex and substantia nigra of a control case and in the parietal cortex of an AD case (Fig. 7A). To examine the relative binding to α-synuclein and Aβ pathology, a single PD case with mixed pathology was included in the analysis. The immunohistochemistry analysis of this case showed a high level of α-synuclein aggregates in the grey matter, which corresponded to areas of high specific binding (Supplementary Fig. 1B and 1C). The inhibition of radioligand binding by PBB3 and C05-01 was described by 1-site models, and parameters resulting from curve fits are indicated. Data are presented as mean ± standard error.

Fig. 5. \[^3H\]C05-01 dissociation and inhibition constant measurement (\(K_0\) and \(K_i\), respectively) using α-synuclein fibrils (1 μM). (A) Saturation binding of \[^3H\]C05-01 using increasing concentrations of \[^3H\]C05-01 and a fixed concentration of blocker (ThT 50 μM). (B) Competition binding using a fixed concentration of \[^3H\]C05-01 (2 nM) and displaced with serial dilution concentrations of the cold compound (from –3 to –14 M). Results are expressed as specific binding in counts per minute (CPM). (C) Specific binding of 5 nM of \[^11C\]PBB3 in a DLB amygdala sample blocked homologously by non-labelled PBB3 (red circle) and heterologously by non-labelled C05-01 (blue circle). Inhibition of radioligand binding by PBB3 and C05-01 was described by 1-site models, and parameters resulting from curve fits are indicated. Data are presented as mean ± standard error.
case showed two separated and well-defined areas with pure α-synuclein pathology and with Aβ deposits, with negligible Tau pathology (Fig. 7B). Autoradiography with [3H]C05-01 showed specific binding to both α-synuclein and Aβ at concentrations as low as 0.03 nM. The specific binding was higher in PD tissue compared to the control tissue (supplementary Fig. 2).

In order to further understand the nature of the binding that could be displaced by homologous blocking in control tissue, [3H]C05-01 was co-incubated with several blockers, specific to different targets, i.e. MAO-A, MAO-B, Aβ, tau, and Sigma-1 receptors. None of the compounds in this battery blocked the binding of [3H]C05-01 in control tissue to the same as extent as non-labelled C05-01 at a saturating concentration (10 µM) (Supplementary Figs. 3 and 4).

### 3. Discussion

In this study, we report the in vitro characterization of a compound, C05-01, derived from a library of analogues of PBB3. C05-01 was selected based on a qualitative evaluation using fluorescence microscopy and on a semiquantitative evaluation of its fluorescence signal corresponding to Lewy aggregates using TMAs. In vitro binding assays using recombinant fibrils and brain homogenates were performed to measure the affinity of C05-01 for α-synuclein. Further characterization was done with autoradiography performed with TMAs and fresh frozen tissue.

As shown in double staining immunofluorescence studies in tissue with pure Lewy pathology, C05-01 showed positive immunoreactivity that co-localized with α-synuclein inclusions. These findings were confirmed in TMAs in which C05-01 showed mean fluorescence values measured in large inclusions (Lewy bodies) that were higher than corresponding values obtained from quantification in dense Aβ plaques or tau aggregates.

The affinity of [3H]C05-01 measured using recombinant fibrils and brain homogenates was in a similar nM range as other α-synuclein ligands recently reported (Hsieh et al., 2018). The $K_D$ measured in brain homogenates was lower than the one measured using α-synuclein fibrils, probably due to experimental variance or to differences in the tertiary structure between fibrils and tissue aggregates. In brain homogenates, however, the $K_D$ of C05-01 was lower than the $K_D$ of PBB3, suggesting higher affinity for α-synuclein. Previous studies in postmortem tissue from DLB and MSA patients have shown that [11C]PBB3 binds to tissue with high load of α-synuclein. In addition, PBB3 at a high concentration (32.3 µM) can bind to diverse α-synuclein pathology forms, including Lewy bodies, Lewy neurites, spheroids, glial cytoplasmic and neuronal inclusions (Koga et al., 2017). In the present study, the $K_D$ of PBB3 was measured in brain tissue for the first time. The results confirm previous findings that PBB3 has low affinity for α-synuclein.

Further characterization of [3H]C05-01 by autoradiography using TMA sections and fresh frozen tissue confirmed the presence of specific binding of the tracer in PD and MSA tissue. Specific binding was however observed also in tissue from AD and Picks disease, indicating that [3H]C05-01 binds to Aβ and tau. Further development is therefore needed to improve the selectivity for α-synuclein.

[3H]C05-01 displayed binding that could be displaced by non-labelled C05-01 also in control tissue. This suggests the presence of off-target binding. Experiments conducted using different blocking agents...
for known brain targets, such as pyrilindole (MAO-A), L-deprenyl (MAO-B), AZ2184 (Aβ), THK-5351 (Tau/MAO-B), haloperidol (D2/Sigma 1 receptors) and BD-1047 (Sigma 1 receptors) showed that none of these compounds could block the binding of [3H]CO5-01 to the same extent as non-labelled CO5-01. Therefore, the nature of the off-target binding is still unknown.

CO5-01 is the first compound structurally related to PBB3 that shows improved affinity for α-synuclein. It is important to notice that a finding of this study is that amino-pyridinyl-butenynyl-benzothiazole derivatives, including CO5-01, yielded stronger fluorescence labeling of α-synuclein lesions than PBB3, implying the significance of the butenyn versus butadien linker for the enhanced ligand reactivity with α-synuclein fibrils.

The compound binds to both Lewy bodies and Lewy neurites, and displays specific binding in PD and MSA tissue. These findings suggest that chemical modifications of the PBB3 scaffold can increase affinity for α-synuclein. However, further optimization is needed in order to increase the selectivity for α-synuclein and reduce the off-target binding. The approach used in this study also shows that TMAs are a useful tool for the evaluation of ligands in tissue from different proteinopathies, using both fluorescence and autoradiographic assays.

4. Materials and methods

4.1. Radiolabeling

For [3H]CO5-01 radiolabeling (Fig. 8), 200 µL of DMSO were added to the mixture of precursor (2 mg) and potassium hydroxide (7–10 mg) and mixed by a vortex for 15 min. A [3H]methyl iodide solution in toluene (40 MBq) was added to the mixture, which was heated at 90 °C for 10 min, after which 200 µL of water was added. Analysis and purification were performed by radio-HPLC on an ACE 5 C18 HL column (250x100mm). The product was eluted by a mixture of acetonitrile and ammonium formate (0.1 M) (30/70, v:v) at a flow rate of 5 mL/min. The effluent from the HPLC column was monitored for UV absorbance (254 nm) and radioactivity. After repeated syntheses and combination of collected fractions, solvents were removed by rotary evaporation under reduced pressure, and the [3H]CO5-01 was dissolved in a mixture of ethanol and water (50/50, v:v). The product solution was analysed and identified by HPLC using the abovementioned conditions. [3H]CO5-01 was obtained as a stock solution (97 MBq in 1.7 mL of ethanol/water), in a concentration of 70.7 µM and a molar activity of 810 GBq/mmol.

Preparative chromatography and spike test was carried out using an ACE 5 µm C18_HL 250x10mm column with the following gradient method: Mobile phase: 0–5 min: TFA (0.1%):MeCN (70:30, v:v) and then TFA:MeCN,(40:60) until 10 min at a flowrate of 5 mL/min. The retention time for C-05–01 in this system was 14 min. An additional spike test was carried out using the following isocratic method: ACE 5 µm C18_HL 250x10mm column, mobile phase TFA (0.1%):MeCN, (40:60, v:v) at a flow rate of 5 mL/min. The retention time for C-05–01 in this system was 7.7 min.

Radiochemical purity analysis was carried out using ACE 5 µm C18_HL 250x4.5 mm column, with a mobile phase corresponding to TFA (0.1%):MeCN (50:50, v:v), at a flow rate of 2 mL/min. The retention time for C-05–01 in this system was 5.9 min. Radiochemical purity was greater than 97%. Low concentrations of chemical impurities were observed in the product solution. Of these, the major chemical impurity was the unlabeled precursor, which was present in threefold lower concentration than the radioligand (Supplementary Fig. 5). [11C]PBB3 was synthesized as described elsewhere (Ono et al., 2017).
4.2. Tissue/fibrils included in the study

4.2.1. Postmortem human brain tissues

The experimental use of brain tissue from donors with different pathologies was approved by the Ethics Committee of the Stockholm region. Postmortem human brains were obtained from various sources depending on the experimental procedure and processed accordingly. For binding studies using brain homogenates, human brains were obtained from autopsies carried out at the Department of Neuroscience of the Mayo Clinic on patients with DLB (Table 1). Tissues for homogenate binding assays were frozen. For in vitro autoradiography using fresh frozen tissue, blocks from Lewy body disease (LBD), Parkinson’s disease (PD), Alzheimer’s disease (AD) and controls were obtained from the Netherlands brain bank (Table 1), sectioned accordingly and stored until use. Other tissue from these and other pathologies was used for both autoradiography and immunohistochemistry studies using both fresh frozen tissue and paraffin embedded for the generation of tissue micro arrays (TMA). Two types of mini-TMAs were produced (Table 2 for demographics), both containing their own controls. One TMA was generated from a series of paraffin-embedded tissue blocks of the substantia nigra from patients with Parkinson’s disease and from controls. The second one was generated from a series of paraffin-embedded tissue blocks of different brain regions and neurodegenerative diseases (Multi system atrophy, Pick’s disease, Vascular dementia, Alzheimer’s disease) and matched controls. Demographics are shown in Table 2.

| Code | Diagnose          | Brain region | Age | Gender | PMD |
|------|-------------------|--------------|-----|--------|-----|
| DLB  | Dementia with Lewy bodies | AMY          | 57  | M      | –   |
| LBD  | Lewy body disease  | MTG          | 76  | M      | 6   |
| PD   | Parkinson’s disease | AMY, CG, SN  | 85  | F      | 15  |
| PD   | Parkinson’s disease | CG           | 82  | M      | 6   |
| MSA  | Multi system atrophy | CB           | 69  | F      | 4   |
| MSA  | Multi system atrophy | CB           | 66  | M      | 5   |
| AD   | Alzheimer’s disease | PC           | 88  | F      | 5   |
| Control | Non demented control | SN, CG      | 35  | M      | 22  |
| Control | Non demented control | SN, CG      | 82  | M      | 21  |
| Control | Non demented control | SN, CG      | 68  | M      | 10  |
| Control | Non demented control | CG           | 78  | F      | 7   |

4.2.2. Expression, purification and preparation of α-synuclein

Alpha-synuclein (wild type) was kindly donated from Umeå University, Faculty of Science and Technology, Department of Chemistry. The construct for human α-synuclein was used from GenScript (NJ, USA) and cloned into a pET-3a vector according to the previously published procedure (Nasstrom et al., 2020).

For the fibrils formation, lyophilized stock solutions of α-synuclein were dissolved in 20 mM phosphate buffer at pH 7.4 containing 140 mM NaCl and filtered (0.22 μm). The concentration of α-synuclein in the stock was 70 μM. A magnet was included in the vial to enhance the fibril formation and incubated at 37 °C for at least 72 h using constant shaking (450 RPM). No additional sonication or centrifugation steps were included in the protocol.

For determination and quantification of α-synuclein fibrils, Thioflavin T (ThT) assay was carried out. All samples contained 20 μM ThT; fluorescence was measured at 480 nm (excitation 440 nm) every 10 min. The final α-synuclein concentration on each well for binding experiments was 1 μM.

4.3. Immunohistochemistry

4.3.1. Histological examination in fresh frozen tissue

Fluorescence and immunohistochemical labeling were performed using 20-μm-thick fresh frozen sections post-fixed in 4% paraformaldehyde solution derived from DLB brains. For fluorescence labeling with C05-01, section was incubated in 50% ethanol containing...
30 µM of C05-01 at room temperature for 30 min. The samples were rinsed with 50% ethanol for 5 min, dipped into distilled water twice for 3 min, and mounted in non-fluorescent mounting media (Vectashield; Vector Laboratories). Fluorescence images were captured using a DM4000 microscope (Leica) equipped with custom filter cube (excitation band-pass at 391–437 nm and suppression low-pass with 458 nm cutoff). Following fluorescence microscopy, sections were autoclaved for antigen retrieval, and immunostained with anti-phosphorylated α-synuclein antibody (pS129, ab59264, abcam). Adjacent sections were also stained with anti-amyloid beta antibody (6E10, SIG-39321-1000, Covance) and anti-phosphorylated tau antibody (AT8, MN1020, Thermo Fisher). Immunolabeling was then examined using DM4000.

4.3.2. Histological examination in paraffin-embedded tissue – TMA
4.3.2.1. Dual assay in TMA format. This study was performed with the aim to facilitate a ligand screening campaign on tissue sections from the human brains in a tissue micro array format (TMA) and selected samples from pathological tissue (PD, AD, LBD, VD, Pick, NDE, PD) and control. The tissue included consisted of human brain samples from the Netherlands brain bank (NBB), Human brain MSA and MSA control from Banner Institute of Health (demographics are shown in Tables 1 and 2). Construction of the Tissue MicroArray was performed essentially as in Kononen et al., 1998.

After pathological evaluation of tissues, the assembly of 26 autopsy cores (diameter = 0.8 mm – 2 mm) from selected tissues with clearly identified deposits of fibrillar Aβ, Tau and α-synuclein deposits were integrated into one single recipient TMA paraffin block.

As negative controls, a total of 4 autopsies with no or minimal fibrillar deposits was inserted in the TMA. The TMA was reconfirmed for the presence of fibrillar Abeta, pTau and α-synuclein deposits within the inserted tissue autopsies using specific IHC staining in combination with binding of fluorescent reference compound (FSB) with specific binding capacities to Aβ, ptau and α-synuclein. A semi quantitative method was developed to measure mean fluorescence intensity from each label (pathological hallmarks α-synuclein, Aβ and pTau over compounds). All tissues were formaldehyde fixed (4%) and embedded into paraffin blocks.

4.3.2.2. Validation of assays conditions set up of parameters
4.3.2.2.1. Immunohistochemical staining. IHC was performed as in Lund et al., 2013. Briefly, IHC staining for Aβ, pTau or α-synuclein was performed using a robotic platform for IHC staining (Ventana Discovery XT) followed by manual application and incubation with the chemical ligands. The dual staining was in tissue sections from the TMA only paraffin embedded sections were used for IHC/ligand dual staining because of better tissue morphology and decreased background staining compared with fresh frozen tissue. The reagents were primary antibodies purchased from Signet laboratories (Abeta 6E10, Abeta 4G8), Abcam (Anti-Alpha-synuclein antibody [LB 509]) and Thermo Fisher Scientific (AT-8 Phospho-PHF-Tau).

4.3.2.2.2. Histochemical assay for FSB and all ligands. Visualization of Aβ plaques with FSB were performed as described previously (Maeda & Kanda, 2013). Briefly, 0.01% FSB and 5 reference ligands in 50% EtOH were incubated over slide sections in the darkness. This was followed by incubation in 50% EtOH and washing. All slides were mounted in prolong gold mounting media (Invitrogen, Molecular Probes).

4.3.2.3. Construction and execution of the assay for quantitative image analysis
4.3.2.3.1. Workflow for image analysis. The stained tissue sections were initially scanned digitally in fluorescence using a whole slide scanner (3D Histech Panoramic 250 flash II), a 20x objective (equivalent to 38 X due to camera and adaptor) and five different filter channels (DAPI, PBB3, FITC, Cy3, Cy5). A low-resolution version of whole slide image files of digitally scanned glass slides was imported into a software for image analysis (VIS, Visiopharm) and specific areas were outlined (Regions of Interest; ROI) for further analysis (Fig. 1). High resolution images were acquired from each ROI. Images were selected for the automatic processing and further reviewed and adjusted manually if necessary (post processing). The software program was trained to specifically recognize the labels (α-synuclein, Aβ or pTau) and bound ligand. Full analysis was run on selected tissues, the data exported to Microsoft Excel and the output data from each tissue summarized into one single value. Data was transferred for data visualization to GraphPad prism program (version 7.00).

4.3.2.3.2. Test/validation of assay using five reference ligands. As means to validate the assay, five compounds with known binding properties were analyzed. The binding of each reference compound as measured by their emission of fluorescence (mean intensity of fluorescence; MIF) to tissue structures IHC-labelled for α-synuclein, Aβ or pTau was calculated.

4.4. In vitro binding techniques
4.4.1. In vitro autoradiography
ARG experimental procedures were previously described elsewhere (Veldman et al., 2017) and in brief carried out as follows; slides were thawed at room temperature and incubated with radioligand in binding buffer (50 mM Tris HCL 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, at pH 7.4) at 0.3 nM for 1 h. The binding was displaced on adjacent section with cold compound at 10 µM. After incubation, the slides were washed three times in washing buffer (50 mM Tris HCl , pH 7.4) followed by a brief wash in distilled water. The slides were dried and exposed to new phosphor imaging plates (FujiFilm Plate BAS-TR2025, Fujifilm, Tokyo, Japan). Tritium micro scales standards (American Radiolabeled Chemicals Inc.) were placed in cassettes together with the sections for calibration and quantification of the binding density.

For image analysis, the phosphor imaging plates were exposed for approximately ninety hours. Then, the films were scanned, and the resulting images were processed in a FujiFilm BAS-5000 phosphor imager (Fujifilm, Tokyo, Japan). Manual delineation of each region of interest (ROI) was performed visually on each digital image using three- to fourfold magnification. Mean pixel values of the ROIs from each section were transformed into radioactivity values using the tissue standards used for creating a calibration standard curve and recalculated as binding density (fmol/mg protein). Based on these measurements, specific binding values were calculated in the absence or presence of the inhibitor (total binding – non-specific binding).

4.4.2. In vitro binding assay
4.4.2.1. Saturation and competition binding using α-synuclein fibrils. For radioligand saturation and competition binding assays, α-synuclein fibrils were used as binding substrate (1 µM final concentration) in all cases. These α-synuclein fibrils were prepared previously described (section 4.2.2). In case of saturation studies, a serial range of [3H]C05-01 dilutions (0 – 400 nM) with a single concentration of competitor (10 µM) were combined with α-synuclein fibrils in 30 mM Tris-HCL pH 7.4, 0.1% BSA, in a total volume of 150 µL.

In case of competition studies, different concentrations of competitor compounds, ranging from 1 nM to 1000 nM were combined with α-synuclein fibrils and 2 nM [3H]C05-01 in 30 mM Tris-HCL pH 7.4, 0.1% BSA, in a total volume of 150 µL.

The binding mixtures were incubated at 37 °C for 2 h in 96 well plates. Bound and free radioligand were then separated by vacuum filtration through 1.0 µm Glass Fiber filters in 96-well filter plates (Millipore), followed by three 200 µL well washes with cold assay buffer. Filters containing the bound ligand were mixed with 150 µL of Optiphase Supermix scintillation cocktail (PerkinElmer) and counted.
after overnight incubation. All data points were performed in triplicate. Data were analyzed using Graphpad Prism 7 software to obtain EC50 values by fitting the data to the equation Y = bottom + (top–bottom)/(1 + 10(−logEC50)). Ki values were calculated from EC50 values using the equation $K_i = EC50/(1 + [\text{radioligand}]/K_d)$.

4.4.2.2. Competition binding using human brain homogenates. Frozen tissues derived from the amygdala of a DLB patient were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing protease inhibitor cocktail (cOmplete™ EDTA-free; Roche), and stored at −80 °C pending analyses. To assay radioligand binding with homologous blockade, homogenates (100 μg tissue) were incubated with 5 nM $[^{11}C]$PBB3 (specific radioactivity: 133.5 GBq/µmol) in the absence or presence of unlabeled PBB3 or C05-01 at varying concentrations ranging from $10^{-11}$ to $10^{-6}$ M in Tris-HCl buffer containing 10% ethanol, pH 7.4, for 30 min at room temperature. Non-specific binding of $[^{11}C]$PBB3 was determined in the presence of $5 \times 10^{-6}$ M PBB3 and $2.5 \times 10^{-8}$ M C05-01, respectively. Samples were run in quadruplicates and specific radioligand binding was determined as pmol/g tissue. Inhibition constant (Ki) was determined by using non-linear regression to fit a concentration-binding plot to one-site binding models derived from the Cheng-Prusoff equation with GraphPad Prism version 5.0 (GraphPad Software), followed by F-test for model selection. Dissociation constant (Kd) were calculated from homologous competitive binding using this function:

$$K_D = K_i = IC50 - [\text{Radioligand}]$$

where IC50 and [Radioligand] are concentration of the competitor inducing 50% inhibition and radioligand concentration, respectively.

**CRediT authorship contribution statement**

**Patricia Miranda-Azpiazu:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Marie Svedberg:** Conceptualization, Methodology, Visualization. **Makoto Higuchi:** Conceptualization, Methodology, Data curation, Funding acquisition. **Maiko Ono:** Data curation, Investigation. **Zhisheng Jia:** Data curation, Resources. **Dan Sunnemark:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Software, Data curation, Visualization. **Charles S. Elmore:** Data curation, Resources. **Magnus Schou:** Conceptualization, Funding acquisition. **Andrea Varrone:** Conceptualization, Validation, Writing - original draft, Supervision, Project administration, Funding acquisition.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. MH and MO filed a patent (PCT/JP2020/002607) related to C05-01.

**Acknowledgements**

This study was supported by funds from Stockholm Brain Institute, Vinnova, Sweden (Dnr. 2017-03093), the Michael J Fox Foundation, USA (ID# 10908), Hjärnfonden (FO2018-0082) and Astra Zeneca, Sweden. MS was supported by the Knut and Alice Wallenberg Foundation (Dnr: 2018.0066). The authors thank Fredrik Almqvist and Jörgen Ådén from the University of Umeå for providing the α-synuclein fibrils for the in vitro binding experiments.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.brainres.2020.147131](https://doi.org/10.1016/j.brainres.2020.147131).

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