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Synthesis and in vitro evaluation of α-synuclein ligands

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ABSTRACT

Accumulation of misfolded α-synuclein in Lewy bodies and Lewy neurites is the pathological hallmark of Parkinson's disease (PD). To identify ligands having high binding potency toward aggregated α-synuclein, we synthesized a series of phenothiazine derivatives and assessed their binding affinity to recombinant α-synuclein fibrils using a fluorescent thioflavin T competition assay. Among 16 new analogues, the in vitro data suggest that compound **11b** has high affinity to α-synuclein fibrils (K_i = 32.10 ± 1.25 nM) and compounds **11d**, **16a** and **16b** have moderate affinity to α-synuclein fibrils ($K_i \approx$ 50–100 nM). Further optimization of the structure of these analogues may yield compounds with high affinity and selectivity for aggregated α-synuclein.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD). Its main clinical features include rest tremor, bradykinesia, rigidity and loss of postural reflexes.[1] The motor symptoms of PD are caused by degeneration of dopaminergic neurons in the substantia nigra, which is accompanied by Lewy bodies (LBs) and Lewy neuritis (LNs). In addition to motor symptoms, individuals with PD also have a high risk of dementia[2] and postmortem analysis has demonstrated a correlation between dementia and LBs in cortical neurons.[3–6] To date, the correlation between the density of LBs/LNs in substantia nigra and the severity of these diseases is not clear.[7] Mechanisms responsible for initiation and progression of pathogenic processes in PD are still not entirely understood. Aggregated α-synuclein is the major component of LBs and LNs.[8–10] α-Synuclein, a presynaptic terminal protein containing 140 amino acids,[11] plays an important function in the central nervous system (CNS) by regulating synaptic vesicle recycling and synthesis, vesicular storage and release of neurotransmitter.[12–15] One analysis approach suggests that aggregated α-synuclein outside LBs and LNs is 10-fold higher than the amount of aggregated α-synuclein inside LBs and LNs.[16] The formation of α-synuclein aggregates may result in synaptic dysfunction and neuronal cell death.[17–20] Quantifying α-synuclein aggregation in vivo will be very useful to improve early diagnosis of PD and to monitor disease progression. Furthermore, the ability to quantify aggregated α-synuclein in vivo may be useful to monitor therapeutic efficacy in early clinical studies of disease-modifying treatments. Therefore, small molecules that have suitable pharmaceutical properties as fibrillar α-synuclein ligands and that can be labeled with position emission tomography (PET) radionuclides such as C-11 and F-18, will have great opportunity to serve as PET probes for quantifying α-synuclein aggregation in the brain. In addition, inhibition of the progress of α-synuclein protein aggregation may be a potential strategy for treating PD and its associated diseases. Thus, investigators have attempted to identify highly potent ligands for α-synuclein fibrils.[21–24] To achieve the goal of developing highly potent α-synuclein ligands, we focused on exploring the derivatives of phenothiazine. Herein, we report our initial work on the synthesis of new analogues of phenothiazine and the analysis of their binding affinity toward α-synuclein fibrils. Our current work was inspired by (1) to date, no small molecular ligand for α-synuclein fibrils has been reported to have the capability to prevent α-synuclein accumulation in vivo; (2) no suitable PET tracer has been reported that can be used to assess aggregated α-synuclein accumulation in the brain in vivo; (3) phenothiazine structure has been proposed as a promising pharmacophore for the therapy of neurodegenerative diseases[25,26] by protecting the dopaminergic neurons against oxidative stress; the representative

Abbreviations: PD, Parkinson's disease; AD, Alzheimer's disease; LBs, Lewy bodies; LNs, Lewy neuritis; CNS, central nervous system; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ThT, thioflavin T; AFU, arbitrary fluorescence units.

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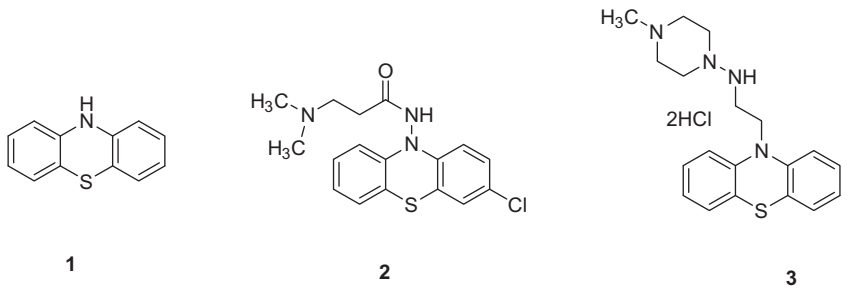


Figure 1. Three chemical structures reported previously for neuronal protection.

structures include phenothiazine (**1**), *N*-acylaminophenothiazine (**2**) and *N*-alkylphenothiazine (**3**) (Fig. 1). Furthermore, it was reported that phenothiazines inhibit α-synuclein filament assembly with IC_{50} values in the low micromolar range.[21]

2. Results and discussion

2.1. Chemistry

Based on our structure–activity relationship analysis, we optimized the structure of phenothiazine and synthesized a series of new analogues as shown in Schemes 1–3.

Using copper iodide as catalyst, in the presence of L-proline and potassium carbonate, compound **4** was reacted with 1-bromo-4-methoxybenzene in DMSO to afford bis(4-methoxyphenyl)amine (**5**) following Ullman reaction (Scheme 1). The target compound 3,7-dimethoxy-10H-phenothiazine (**6**) was obtained by cyclization of intermediate **5** with sulfur at 150 °C using dichlorobenzene as solvent and iodine as the catalyst.

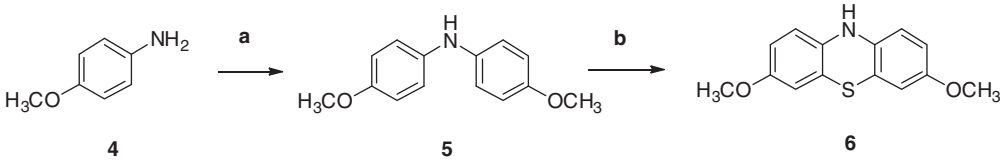
The synthesis of compounds **11a**–**e** was achieved as shown in Scheme 2. Hydrolysis of compound **8a** in the presence of potassium hydroxide afforded 2-amino-5-methoxybenzothiol, which was coupled with either 4-chloro-3-nitrobenzonitrile (**7a**) or 1-chloro-2,4-dinitrobenzene (**7b**) to obtain the substituted diphenylsulfide intermediate **9a** or **9b** under mild acidic condition. Acetic anhydride was used to protect the amino group in **9a** and **9b** to afford acetamides **10a** and **10b** which were cyclized via Smile rearrangement[27] under the strong basic conditions to afford the target compounds **11a** and **11b**. Following the similar synthetic procedure followed for **9a** and **9b**, compound **9c** was obtained starting with **7b** and 2-amino-benzothiol (**8b**) in the presence of sodium hydroxide. Compound **9c** was acylated with acetic anhydride to obtain **10c**. Compound **10c** was treated with *N*-bromosuccinimide (NBS) in dimethylformamide (DMF) or iodine monochloride (ICl) in acetic acid to afford **10d** or **10e**, respectively. Cyclization of **10c**, **10d** and **10e** followed the similar procedure described for **11a** to obtain target compounds **11c**, **11d** and **11e**.

To test how the proton on the nitrogen of the middle ring affects the binding potency of analogues to α-synuclein fibrils, a methyl group or an acetyl group was substituted on the nitrogen. Analogues **12**, **13a**–**c**, **14a**–**b**, **15** and **16a**–**c** were synthesized as shown in Scheme 3. Compound **12** was directly obtained by the N-methylation of **11b** using methyl iodide in DMF in the presence of sodium hydride. Compound **13a** was generated by reducing compound **12** via hydrogenation under hydrogen atmosphere in the presence of 10% palladium on activated carbon in ethanol. Further N-methylation of **13a** using one or two equivalents of methyl iodide in acetonitrile yielded compounds **13b** and **13c**. Compounds **14a** and **14b** were obtained via N-acetylation of compound **11b** or **11c** using acetyl chloride. Removing the methyl group from **14a** with boron tribromide afforded the corresponding phenol analogue **15**. O-Alkylation of compound **15** using 1-bromo-2-fluoroethane, 3-bromopropyne or 3-bromo-1-iodopropene, followed by hydrolysis in the presence of 3 N HCl aqueous solution gave the compounds **16a**, **16b** and **16c**. To obtain enough quantity of target compounds for in vitro studies, some reactions discussed above were repeated and scaled-up. However, in the current work, we did not optimize the reaction conditions and determine the scalability of reactions. Compound **1** was purchased from Sigma-Aldrich Co., and compounds **2** and **3** were synthesized following the literature reported procedures[28,29] and converted them to corresponding oxalate salts.

2.2. Thioflavin T fluorescence assay for α-synuclein fibrils

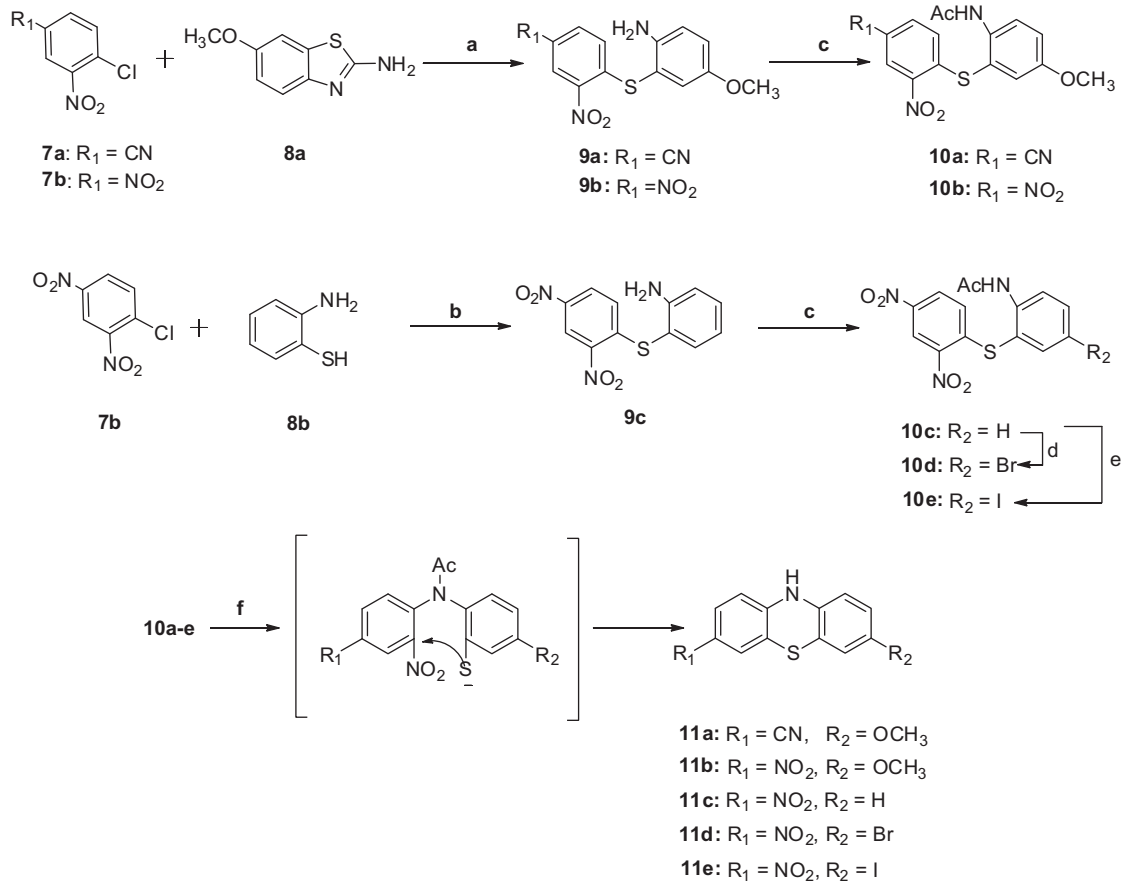
At present, no existing standard protocol can be used to measure the binding affinity of compounds for α-synuclein fibrils. Determining the binding affinities of compounds toward proteins with radioligands has higher accuracy and sensitivity than with fluorescent probes. Nevertheless, until today, no radioligand was reported to be suitable for determining binding affinities of compounds toward aggregated α-synuclein. Moreover, thioflavin S has been used to determine the binding affinity of compounds toward β-amyloid, tau and α-synuclein aggregates.[30–33] In the current work, we used fluorescent methodology to determine the relative binding potency of compounds toward α-synuclein fibrils and to identify potential ligands that can be radiolabeled with I-125 or H-3 to further characterize their binding properties.

Thioflavin T (ThT) is a fluorescent dye with an identified chemical structure. It is weakly fluorescent in the presence of



Reagents and conditions:
a) 1-bromo-4-methoxybenzene, CuI, L-proline, K_2CO_3, DMSO, 90°C; b) S, I_2, 1,2-dichlorobenzene, 150°C

Scheme 1. Synthesis of compound **6**.



Reagents and conditions:

a) i: KOH/H_2O, reflux, overnight; ii: AcOH, EtOH/H_2O, ambient temperature, 2h; b) NaOH, EtOH/H_2O, ambient temperature, 2h; c) pyridine, Ac_2O, ambient temperature, 3h; d) NBS, DMF, 100°C, overnight; e) ICl, AcOH, reflux, 3h; f) KOH, Acetone/EtOH, reflux, 2h;

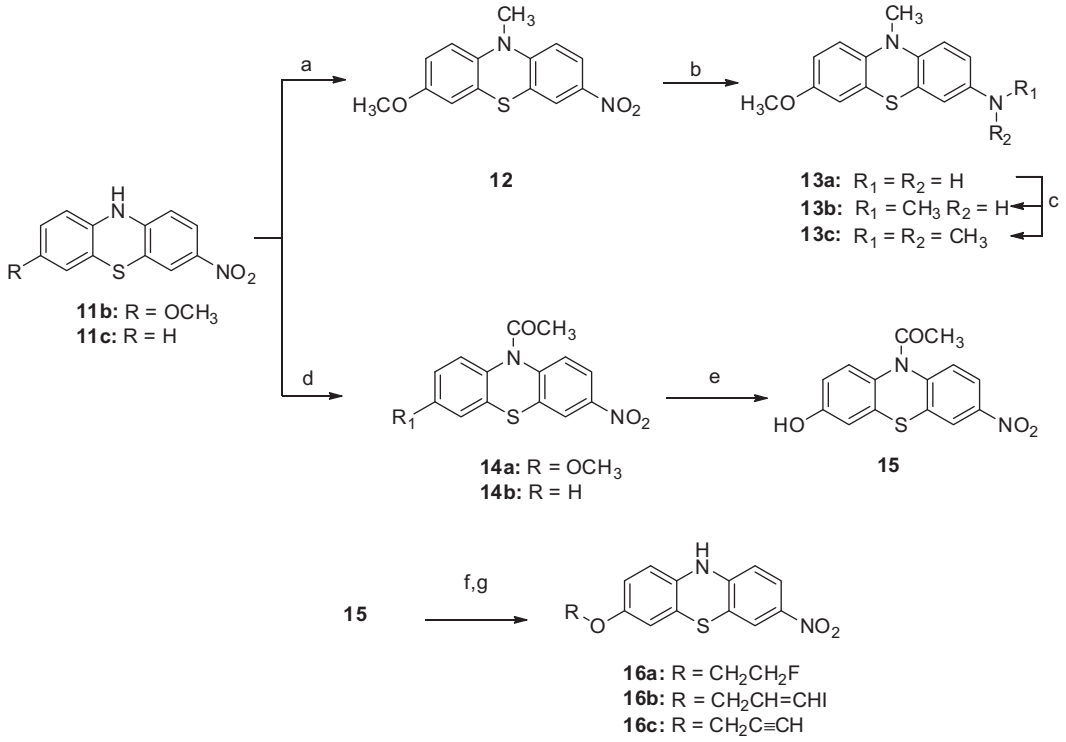
Scheme 2. Synthesis of compounds **11a–e**.

monomeric α-synuclein or in an α-synuclein fibril-free system. In the presence of α-synuclein fibrils, ThT fluorescence intensity increases by several orders of magnitude at the emission wavelength maximum (λ_{em} = 483 nM).[31] To determine the binding affinity of compounds toward α-synuclein fibrils, first, the ThT fluorescent emission spectrum was confirmed to be consistent with previously reported data (Fig. 2, left); second, we incubated ThT with α-synuclein fibrils and measured ThT's maximum fluorescent emission wavelength (λ_{em} = 485 nM) under its excitation wavelength (λ_{ex} = 440 nM). No increase in fluorescent emission was observed when ThT was incubated in the presence of monomeric α-synuclein fibrils or in α-synuclein fibril-free buffer. Furthermore, the ratio of ThT's fluorescence intensity in the presence of α-synuclein fibrils to ThT's fluorescence intensity in either monomeric α-synuclein fibrils or α-synuclein fibril-free buffer is about 30-fold. Therefore, the fluorescence intensity of ThT in either monomeric α-synuclein or α-synuclein free buffer is negligible and unlikely to interfere with the measurement of α-synuclein fibril binding affinity for compounds.

To determine the binding affinity for new compounds toward α-synuclein fibril, we first evaluated the saturation binding curve of ThT fluorescence intensity in the presence of α-synuclein fibrils, from which 60 min was chosen as the optimized incubation time (Fig. 2, right). In our system, we obtained the K_d value of 948 ± 271 nM for ThT binding to α-synuclein fibrils, which is different from other reported values, 15 μM and 588 ± 2 nM.[31,32] Differences in K_d values may be explained by differences in α-synuclein fibril preparations and incubation conditions.[34,35] Importantly, we observed consistent α-synuclein fibril binding affinity when using different batches of fibrils in our experimental protocol.

2.3. In vitro evaluation of binding affinities of phenothiazine compounds to α-synuclein fibrils

Compounds were incubated with α-synuclein fibrils to determine whether any fluorescence emission was present at λ_{em} = 485 nM, which would have permitted binding affinity (K_d) values to be directly assessed using the same procedure as ThT. All of the analogues reported here had no significant fluorescence, and their binding affinity for α-synuclein fibrils was determined using an indirect ThT competition assay, in which the competition of ThT binding to α-synuclein fibrils was determined at various compound concentrations. This approach enabled IC_{50} and K_i values of the new compounds to be determined and compared. Results of the competitive binding assays for each compound are shown in Supplementary Figure 1 and Supplementary Table 1. The corresponding K_i values are listed in Table 1. Although, fluorescence quenching can potentially interfere with measurement of competitive binding, the data for the individual compounds closely fit a competitive binding model. Absorbance spectra were measured at the IC_{50} concentration for each compound. Absorbance



Reagents and conditions:
a) NaH, CH_3I, DMF, 0°C, overnight; b) H_2, Pd-C (10%), 90 psi, ambient temperature, overnight, c) CH_3I, Na_2CO_3, MeCN, ambient temperature, overnight; d) CH_3COCl, DCM, ambient temperature, overnight; e) BBr_3/DCM (1M), DCM, -78°C, overnight. f) 1-bromo-2-fluroethane, 3-bromopropyne or 3-bromo-1-iodopropene, Na_2CO_3, MeCN; g) HCl (3 M), H_2O, reflux, 5h;

Scheme 3. Synthesis of N-substituted analogues **12**, **13a–c**, **14a–b**, **15**, **16a–c**.

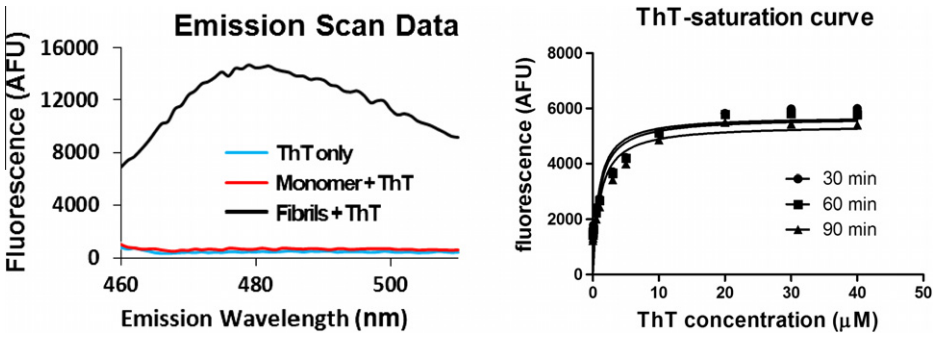


Figure 2. Left: Fluorescence emission spectra of ThT in the corresponding buffer alone (blue), monomer (red) and fibrils (green) at λ_{ex} = 440 nM; right: saturation curve of ThT (3 μM) for α-synuclein fibrils (1.5 μM) in Tris buffer (30 mM, pH 7.4) at different incubation times: 30 min (circle), 60 min (square), 90 min (triangle) at room temperature. The K_d for ThioT binding to fibrils was 948 nM and the B_{max} was 5672 afu.

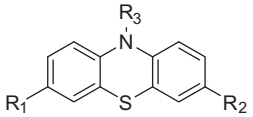
was less than 0.001 in the range of 400–500 nM for all of the compounds including **11b** as shown in Supplementary data Figure 3, indicating that absorbance at the excitation or emission wavelengths did not interfere with the fluorescence assay.

Based on in vitro data generated by the protocol described in the experimental section, it was found that the dimethoxy substituted phenothiazine analogue **6** had a K_i value of 121.8 ± 5.1 nM. When a cyano group was used to replace one of the methoxy groups in **6**, the binding affinity (K_i value) for **11a** was decreased 2.8-fold to 346 ± 37 nM**;** contrarily, when a nitro group was used to replace one methoxy group in **6**, the binding affinity (K_i value) for **11b** was increased about 3.8-fold to reach 32.1 ± 1.3 nM. Compound **11b** is the most potent compound in our current work. In addition, the calculated Log*P* value using ACD/log D program is 3.79 which suggests the lipophilicity of **11b** is a little higher than the desired range (range from 1 to 3), but it is still acceptable with a high possibility of crossing the blood–brain-barrier.

To test the possibility of improving binding affinity of compounds to α-synuclein fibrils, compounds **11c–e** were synthesized by replacing the methoxy group in the structure of compound **11b** using H, Br and I, respectively. Compared to compound **11b** (K_i = 32.1 ± 1.3 nM), the binding affinities of all three compounds toward α-synuclein fibrils, **11c**, **11d** and **11e** were decreased; the K_i values were decreased to 116.5 ± 1.9, 75.3 ± 8.4, 106.0 ± 9.3 nM for **11c**, **11d** and **11e**, respectively. The order of binding potency to α-synuclein fibrils is $-OCH_3 > -Br > -I > -H$.

To confirm if the free proton on the nitrogen of the middle ring is necessary for having high α-synuclein fibril binding potency,

Table 1
Binding affinity ($K_i \pm SD$, nM)[a] of synthesized compounds determined by ThT competition assay



Compound #	R_1	R_2	R_3	K_i (nM)	Log$P$[b]
1	H	H	H	>500	4.15
2	H	H	$N_2C_5OH_{11}$	>500	2.29
3	H	H	$N_3C_7H_{16}$	>500	2.85
6	OCH_3	OCH_3	H	121.8 ± 5.1	3.98
11a	OCH_3	CN	H	346 ± 37	3.50
11b	OCH_3	NO_2	H	32.1 ± 1.3	3.79
11c	H	NO_2	H	116.5 ± 1.9	3.88
11d	Br	NO_2	H	75.3 ± 8.4	4.65
11e	I	NO_2	H	106.0 ± 9.3	4.91
12	OCH_3	NO_2	CH_3	>500	4.14
13a	OCH_3	NH_2	CH_3	>500	3.12
13b	OCH_3	$NHCH_3$	CH_3	>500	3.78
13c	OCH_3	$N(CH_3)_2$	CH_3	>500	4.52
14a	OCH_3	NO_2	$COCH_3$	>500	1.86
14b	H	NO_2	$COCH_3$	>500	2.76
15	OH	NO_2	$COCH_3$	>500	1.93
16a	OCH_2CH_2F	NO_2	H	49.0 ± 4.9	4.02
16b	$OCH_2CH{=}CHI$	NO_2	H	57.9 ± 2.7	5.72
16c	$OCH_2C{\equiv}CH$	NO_2	H	>500	3.83

[a] K_i values (mean ± SEM) were determined in at least three experiments and were calculated using K_d of 948 nM for ThT binding to α-synuclein fibrils as shown in Figure 2.
[b] Calculated value at pH 7.4 with ACD/Lab, version 7.0 (Advanced Chemistry Development, Inc., Canada)

compounds **12**, **13a–b**, **14a–b**, **15** were made; the competitive binding data demonstrated that none of these compounds displayed higher α-synuclein fibril binding affinity (K_i > 500 nM) compared to compounds **6** and **11a–e** that have a free proton on the nitrogen of the middle ring.

Since compound **11b** displayed relatively high affinity (32.10 ± 1.25 nM), the methoxy was replaced by fluoroethoxy, (3-iodoallyl)oxy and prop-2-yn-1-yloxy to synthesize compounds **16a–c**. The in vitro data indicated that both compounds **16a** and **16b** had relatively high affinities with K_i value of 49.0 ± 4.9 and 57.9 ± 2.7 nM, respectively, which are comparable to the affinity of compound **11b** (32.1 ± 1.3 nM. Log$P$ = 3.79). However, compound **16c** displayed significantly lower affinity (K_i > 500 nM). The structures of compound **16a** and **16b** contain fluorine or iodine atom, providing positions for labeling with F-18 or I-125.

We also measured the binding affinities for three previously described phenothiazine analogues shown in Figure 1. Compound **1** (phenothiazine) had low affinity (K_i > 500 nM) indicating that the substitutions described in this study are important to confer binding affinity to the phenothiazine pharmacophore structure. Compounds **2** and **3** also had low affinity (K_i > 500 nM), consistent with the low affinities observed for other analogues with substitution on the nitrogen of the middle ring. To further evaluate the specificity of the ThT competition assay, we tested eticlopride hydrochloride, a known selective ligand to the dopamine D_2 receptor, and observed no significant competition for ThT in the assay (Supplementary data). Finally, we evaluated the possibility that the observed changes in ThT fluorescence in the binding assays could result from fibril dissociation rather than competition for ThT binding to α-synuclein fibrils. We used SYPRO Ruby staining to quantify α-synuclein fibrils in SDS–PAGE gels following centrifugation of fibrils at 100,000g and conversion to monomer by boiling in SDS sample buffer. We observed no change in the mass of α-synuclein fibrils during incubation with each of compounds **11b**, **16a**, or **16b** at a concentration of 3 μM. Since each compound produces greater than 90% inhibition of ThT fluorescence at a concentration of 3 μM, the lack of fibril dissociation indicates that changes in fluorescence reflect competition for ThT binding.

Collectively, based on our structure–activity relationship studies, we found that: (1) there is a high possibility of identifying highly potent ligands for imaging α-synuclein fibril aggregates by optimizing the structure of phenothiazine; (2) the presence of an electron withdrawing nitro group in one of the aromatic rings favors the α-synuclein fibril binding affinity; (3) the free proton of the amino group in the middle ring is essential for maintaining high binding affinity of compounds toward α-synuclein fibrils; otherwise, the binding affinities of compounds will drop dramatically. Compounds having moderate binding affinity for α-synuclein fibrils (K_i < 60 nM) will be assessed for their in vitro binding affinity toward $A\beta_{1-40/42}$, tau proteins or other neurotransmitters, receptors, transporters, enzymes, and ion channels to determine their binding specificity for α-synuclein fibrils in future studies. Based on previous studies in the development of PET/SPECT ligands for imaging Aβ amyloid in vivo, we anticipate that compounds with binding affinities less than 10 nM for α-synuclein fibrils will need to be obtained.[36,37] Further optimizing the phenothiazine analogues to identify ligands that have high α-synuclein potency (K_i < 10 nM) and specificity, suitable lipophilicity (Log$P$ ranges from 1 to 3) and other biological properties is necessary to achieve the goal of identifying a candidate PET/SPECT ligand for imaging α-synuclein fibril aggregation in the brain.

3. Conclusion

In this work, we successfully optimized the structure of phenothiazine for binding α-synuclein fibrils by synthesizing a series of new analogues and assessing their α-synuclein fibril binding affinity in a ThT competition assay. Based on the in vitro binding affinity screening data, several lead compounds, **11b**, **11d**, **16a** and **16b** were identified with high potency for α-synuclein fibrils with K_i < 100 nM. Particularly, compounds **11b**, **16a** and **16b** have K_i values as 32.10 ± 1.25, 48.96 ± 4.94 and 57.94 ± 2.67 nM, respectively. After further validating the binding specificity of these three compounds toward α-synuclein fibrils, the radioactive [^{11}C]**11b**, [^{18}F]**16a** will be synthesized for further study to test the feasibility of assessing α-synuclein fibril aggregation in vivo; [^{125}I]**16b** could be used as radioactive ligand to establish radioactive methodology to screen the binding affinity of other compounds toward the α-synuclein fibrils. In addition, the in vitro data reported here will provide very useful SAR information to guide further design and synthesis of new analogues to achieve the goal of identifying highly potent small molecules that have high affinity and selectivity for α-synuclein fibrils.

4. Experimental

All reagents and chemicals were purchased from Sigma-Aldrich Corporation (Milwaukee, WI) or VWR international, Inc. (Earthy city, MO) and used without further purification unless otherwise stated. The solvent 'hexane' means '*n*-hexane' unless otherwise stated. The air and water sensitive reactions were carried out under nitrogen. The melting points of all the intermediates and final compounds were determined on Hake-Buchler melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian-300 MHz and ^{13}C NMR spectra were recorded on Varian-400 MHz which were maintained by the Chemistry Department of Washington University in St. Louis. Spectra are referenced to the deuterium lock frequency of the spectrometer. The chemical shifts (in ppm) of residual solvents were found to be at 7.26 for $CHCl_3$ and at 2.50 for DMSO. The following abbreviations were

used to describe peak patterns when appropriate: br s = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Elemental analysis or HPLC methods (>95%) were used to determine the purity of the target compounds.

Plate reader & software was used for fluorescence scan: TECAN infinite M100 plate reader, i-control 1.7 TECAN software was used to run plate reader. Plate reader & software used for binding assay: Biotek synergy 2 plate reader, Gen 5 software was used to run plate reader, fluorescence filters: Excitation 440/30, emission 485/20. Optical setting top 50% and sensitivity = 60. Absorbance scans were performed in quartz cuvettes in a Beckman Coulter DU 800 spectrophotometer.

4.1. Chemistry

4.1.1. Bis(4-methoxyphenyl)amine (5)

4-Aminoanisole (300 mg, 2.5 mmol), 4-bromoanisole (360 mg, 2 mmol), CuI (75 mg, 0.4 mmol), L-proline (95 mg, 0.8 mmol) and K_2CO_3 (1.1 g, 8 mmol) were placed in a 50 mL flask and the DMSO (10 mL) was added. The reaction mixture was stirred and heated at 100 °C for 2 d. The reaction mixture was quenched by adding water (50 mL) and extracted with ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified on a silica gel column using ethyl acetate/hexane (1/4, v/v) to yield white solid (0.15 g, 33%). ^{1}H NMR ($CDCl_3$): δ 3.76 (s, 6H), 5.29 (br s, 1H), 6.81 (d, J = 9.0 Hz, 4H), 6.93 (d, J = 9.0 Hz, 4H). mp 92.4–95.0 °C.

4.1.2. 3,7-Dimethoxy-10H-phenothiazine (6)

Compound **5** (150 mg, 0.655 mmol), sulfur (91 mg, 2.3 mmol) and I_2 (29 mg, 0.1 mmol) were added into 1,2-dichlorobenzene (10 mL). The reaction mixture was heated at 150 °C for 12 h. the reaction mixture was cooled down to room temperature and purified on a silica gel column using ethyl acetate/hexane (1/4, v/v) as mobile phase to yield yellow solid (50 mg, 29%).^{1}H NMR ($CDCl_3$): δ 3.64 (s, 6H), 6.57–6.61 (m, 6H), 8.14 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 55.8, 111.9, 113.6, 115.2, 117.4, 136.7, 154.7. Anal. Calcd for $C_{14}H_{13}NO_2S$: C, 64.84; H, 5.05; N, 5.40. Found: C, 64.57; H, 5.15; N, 5.21. mp 194.2–196.1 °C.

4.1.3. 4-((2-Amino-5-methoxyphenyl)thio)-3-nitrobenzonitrile (9a)

Compound **8a** (500 mg, 2.8 mmol) was suspended in 10 mL of aqueous KOH (50%) solution and refluxed overnight. The reaction solution was cooled down to room temperature and added dropwise to the solution of **7a** (510 mg, 2.8 mmol) in ethanol (20 mL)/AcOH (50 mL) in ice-water bath. The reaction mixture was stirred for additional 3 h. The precipitate was filtrated and washed with water/ethanol (1/1, v/v) to afford red solid. (510 mg, 60%). ^{1}H NMR ($CDCl_3$): δ 3.77 (s. 3H), 3.99 (br s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 6.98 (m, 3H), 7.58 (d, J = 8.7 Hz, 1H), 8.56 (s, 1H). mp 163.6–165.1 °C.

4.1.4. 2-((2,4-Dinitrophenyl)thio)-5-methoxyaniline (9b)

Synthetic procedure described for **9a** was followed starting with **8a** (5 g, 28 mmol) and **7b** (6.7 g, 28 mmol), yellow solid (7.3 g, 81%). ^{1}H NMR ($CDCl_3$): δ 3.76 (s. 3H), 4.00 (br s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 6.96–7.06 (m, 3H), 8.18 (d, J = 9.0 Hz, 1H), 9.13 (s, 1H). mp 169.4–171.7 °C.

4.1.5. 2-((2,4-Dinitrophenyl)thio)aniline (9c)

Compound **7b** (10 g, 49 mmol) in ethanol was added dropwise into the solution of **8b** (6.8 g, 54 mmol), NaOH (2.16 g, 54 mmol) in ethanol (50 mL). The reaction mixture was stirred at ambient temperature for 2 h. the precipitate was filtered and washed by ethanol to obtain yellow solid (11.4 g, 88%). ^{1}H NMR ($CDCl_3$): δ 4.28 (br s, 2H), 6.87 (m, 2H), 7.03 (d, J = 9.0 Hz, 1H), 7.42 (m, 2H), 8.17 (d, J = 9.0 Hz, 1H), 9.12 (s, 1H). mp 150.7–151.8 °C.

4.1.6. *N*-(2-((4-Cyano-2-nitrophenyl)thio)-4-methoxyphenyl)acetamide (10a)

Acetic anhydride (10 mL) and pyridine (2 mL) were added into a flask containing compound **9a** (0.5 g, 1.66 mmol). The solution was stirred for 3 h at ambient temperature and quenched by pouring into ice-cold water. The precipitate was filtrated and washed with water to afford yellow solid (0.46 g, 81%) ^{1}H NMR ($CDCl_3$): δ 2.91 (s. 3H), 3.82 (s, 3H), 6.89 (d, J = 8.4 Hz, 1H), 7.08 (s, 1H), 7.15 (d, J = 9.3 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 7.66 (br s, 1H). 8.32 (d, J = 9.0 Hz, 1H). 8.58 (s, 1H). mp 195.0–198.9 °C.

4.1.7. *N*-(2-((2,4-Dinitrophenyl)thio)-5-methoxyphenyl)-acetamide (10b)

Synthetic procedure described for **10a** was followed starting with **9b** (0.3 g, 0.93 mmol) to afford yellow solid (0.31 g, 95%). ^{1}H NMR ($CDCl_3$): δ 2.05 (s, 3H), 3.81 (s, 3H), 6.95 (d, J = 9.0 Hz, 1H), 7.09 (s, 1H), 7.16 (d, J = 9.0 Hz,1H), 7.65(br s, 1H), 8.19 (d, J = 9.0 Hz,1H), 8.33 (d, J = 8.7 Hz, 1H), 9.14 (s, 1H). mp 124.6–125.6 °C.

4.1.8. *N*-(2-((2,4-Dinitrophenyl)thio)phenyl)acetamide (10c)

Synthetic procedure described for **10a** was followed starting with **9b** (1.1 g, 3.8 mmol) to afford yellow solid (1.25 g, 99%). ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3H), 6.88 (d, J = 8.7 Hz,1H), 7.26 (t, J = 6.3 Hz,1H), 7.59 (m, 2H), 7.94 (br s, 1H), 8.19 (d, J = 9.0 Hz,1H), 8.54 (d, J = 8.4 Hz,1H), 9.14 (s, 1H). mp 182.7–184.0 °C.

4.1.9. *N*-(5-Bromo-2-((2,4dinitrophenyl)thio)phenyl)acetamide (10d)

Compound **10c** (2.0 g, 6 mmol) and NBS (4.0 g, 24 mmol) was dissolved in DMF (5 mL). The reaction mixture was heated at 100 °C overnight. The reaction solution was quenched in water (100 mL). The precipitate was filtered and purified on silica gel column chromatography using ethyl acetate/hexane(1/2, v/v) as mobile phase to get yellow solid (2.3 g, 90%). ^{1}H NMR ($CDCl_3$): δ 2.11 (s, 3H), 6.91 (d, J = 9.0 Hz, 1H), 7.73 (m, 2H), 7.91 (br s, 1H), 8.25 (d, J = 9.0 Hz, 1H), 8.51 (d, J = 9.3 Hz, 1H), 9.16 (s, 1H). mp 193.7–195.7 °C.

4.1.10. *N*-(2-((2,4-Dinitrophenyl)thio)-5-iodophenyl)-acetamide (10e)

Compound **10c** (0.5 g, 1.5 mmol) was dissolved in acetic acid (20 mL). ICl (1 M) (10 mL) was added into above solution under nitrogen atmosphere. The mixture was refluxed for 3 d and quenched in water (200 mL). After filtration, the residue was purified on silica gel column chromatography using ethyl acetate/hexane (1/2, v/v) as mobile phase to yield yellow solid (220 mg, 32%). ^{1}H NMR ($CDCl_3$): δ 2.08 (s, 3H), 6.90 (d, J = 9.3 Hz, 1H), 7.87 (m, 2H), 7.99 (br s, 1H), 8.23 (d, J = 8.7 Hz, 1H), 8.31 (d, J = 8.4 Hz, 1H), 9.11 (s, 1H). mp 224.1–226.0 °C.

4.1.11. General procedure A

Potassium hydroxide (98 mg, 1.74 mmol)) was added into the solution of compound **10a** (300 mg, 0.87 mmol) in acetone (20 mL) in portion under reflux. The reaction solution was refluxed for 2 h and quenched in ice-cold water. The precipitate was filtrated and recrystalized in acetone/water (3/2, v/v) to achieve the corresponding product **11a**. Compounds **11b**–**11e** were prepared by the same procedure.

4.1.12. 7-Methoxy-10*H*-phenothiazine-3-carbonitrile (11a)

Yellow solid (100 mg, 45%). ^{1}H NMR (DMSO-d_6): δ 3.67 (s, 3H), 6.62 (m, 4H), 7.34 (m, 2H), 9.02 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ

55.8, 102.7, 112.1, 113.7, 114.3, 116.1, 117.0, 117.0, 119.5, 129.9, 132.6, 133.4, 146.7, 155.8. Anal. Calcd for $C_{14}H_{10}N_2OS$: C, 66.12; H, 3.96; N, 11.02. Found: C, 66.13; H, 3.86; N, 11.03. mp 198.0–198.9 °C.

4.1.13. 3-Methoxy-7-nitro-10H-phenothiazine (11b)

Synthetic procedure described for **11a** was followed starting with **10b** (100 mg, 280 mmol). Violet solid (61 mg, 79%) ^{1}H NMR (DMSO-d_6): δ 3.66 (s, 3H), 6.58–6.64 (m, 4H), 7.71 (s, 1H), 7.83 (d, J = 9.0 Hz, 1H), 9.39 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 55.8, 112.0, 113.3, 113.8, 116.2, 116.6, 116.8, 122.1, 125.2, 132.2, 140.8, 148.4, 156.3. Anal. Calcd for $C_{13}H_{10}N_2O_3S$: C, 56.92; H, 3.67; N, 10.21. Found: C, 56.64; H, 3.54; N, 10.07. mp 168.8–170.1 °C.

4.1.14. 3-Nitro-10*H*-phenothiazine (11c)

Synthetic procedure described for **11a** was followed starting with **10c** (300 mg, 0.9 mmol). Violet product (160 mg, 73%). ^{1}H NMR (DMSO-d_6): δ 6.69 (m, 2H), 6.84 (t, J = 7.2 Hz, 1H), 6.93 (d, J = 7.2 Hz, 1H), 7.02 (t, J = 7.2 Hz, 1H), 7.73 (s, 1H), 7.85 (d, J = 8.7 Hz, 1H), 9.51 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 113.8, 115.7, 115.8, 117.3, 122.0, 124.1, 125.0, 126.7, 128.5, 139.2, 141.4, 148.2. Anal. Calcd for $C_{12}H_8N_2O_2S$: C, 59.00; H, 3.30; N, 11.47. Found: C, 59.02; H, 3.26; N, 11.33. mp 219.1–219.6 °C.

4.1.15. 3-Bromo-7-nitro-10*H*-phenothiazine (11d)

Synthetic procedure described for **11a** was followed starting with **10d** (240 mg, 0.6 mmol) to afford violet solid (87 mg, 45%). ^{1}H NMR (DMSO-d_6): δ 6.60 (d, J = 8.4 Hz, 1H), 6.67 (d, J = 8.4 Hz, 1H), 7.17 (m, 2H), 7.73(s, 1H), 7.85(d, J = 9.0 Hz, 1H), 9.59 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 114.0, 114.8, 116.7, 117.2, 118.4, 122.1, 125.2, 128.6, 131.0, 138.7, 141.7, 147.6. Anal. Calcd for $C_{12}H_7BrN_2O_2S$: C, 44.60; H, 2.18; N, 8.67. Found: C, 44.54; H, 2.26; N, 8.56. mp >250 °C.

4.1.16. 3-Iodo-7-nitro-10*H*-phenothiazine (11e)

Synthetic procedure described for **11a** was followed starting with **10e** (100 mg, 0.22 mmol) to afford violet solid (290 mg, 36%). ^{1}H NMR (DMSO-d_6): δ 6.45 (d, J = 8.7 Hz, 1H), 6.64 (d, J = 9.3 Hz, 1H), 7.22 (s, 1H), 7.30 (d, J = 9.3 Hz, 1H), 7.69 (s, 1H), 7.82 (d, J = 8.7 Hz, 1H), 9.54 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 86.0, 114.0, 116.9, 117.6, 118.4, 122.1, 125.1, 134.0, 136.9, 139.1, 141.6, 147.6. HRMS (ESI) m/z Calcd for $C_{12}H_7IN_2O_2S$ [M] 369.9276. Found: 369.9269. HPLC purity: 97%. mp >250 °C.

4.1.17. 3-Methoxy-10-methyl-7-nitro-10*H*-phenothiazine (12)

Sodium hydride (60%) (29 mg, 0.73 mmol) was added into the solution of compound **11b** (100 mg, 0.36 mmol) in DMF (10 mL) at 0 °C. the reaction mixture was stirred under nitrogen atmosphere for 30 min and warmed to room temperature. CH_3I (103 mg, 0.73 mmol) in DMF (2 mL) was added to the above solution and stirred for additional 2 h. The reaction mixture was quenched by adding water (100 mL) and extracted with ethyl acetate. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated on rotary evaporator. The residue was purified on the silica gel column chromatography using ethyl acetate/hexane (1/2, v/v) as mobile phase to afford red solid (90 mg, 87%). ^{1}H NMR (DMSO-d_6): δ 3.28 (s, 3H), 3.63 (s, 3H), 6.71–6.76 (m, 2H), 6.87–6.94 (m, 2H), 7.84 (s, 1H), 7.96 (d, J = 9.3 Hz, 1H). ^{13}C NMR (DMSO-d_6): δ 36.3, 55.9, 112.9, 113.7, 114.0, 116.9, 122.1, 122.3, 124.7, 136.7, 141.7, 151.8, 156.5. Anal. Calcd for $C_{14}H_{12}N_2O_3S$: C, 58.32; H, 4.20; N, 9.72. Found: C, 58.11; H, 4.11; N, 9.75. mp 174.7–175.5 °C.

4.1.18. 7-Methoxy-10-methyl-10*H*-phenothiazin-3-amine (13a)

Compound **12** (500 mg, 1.7 mmol) and 10% Pd on activated carbon (20 mg) were suspended in ethanol (15 mL). Under H_2 (90 psi), the reaction mixture was stirred at ambient temperature overnight. The reaction solution was filtrated and concentrated in vacuum. The product was purified on the silica gel column chromatography using ethyl acetate/hexane (1/2, v/v) as mobile phase to afford yellow solid (350 mg, 80%). ^{1}H NMR (DMSO-d_6): δ 3.15 (s, 3H), 3.69 (s, 3H), 4.79 (br s, 2H), 6.42–6.45 (m, 2H), 6.64 (d, J = 9.3 Hz, 1H), 6.73–6.80 (m, 3H). ^{13}C NMR (DMSO-d_6): δ 35.3, 55.8, 112.8, 112.9, 112.9, 113.4, 114.6, 115.2, 122.8, 123.9, 136.1, 140.4, 144.4, 154.6. Anal. Calcd for $C_{14}H_{14}N_2OS$: C, 65.09; H, 5.46; N, 10.84. Found: C, 65.29; H, 5.53; N, 10.59. mp 141.6–142.9 °C.

4.1.19. 7-Methoxy-*N*,10-dimethyl-10*H*-phenothiazin-3-amine (13b) and 7-methoxy-*N*,*N*,10-trimethyl-10*H*-phenothiazin-3-amine (13c)

Methyl iodide (280 mg, 2 mmol) in acetonitrile (2 mL) was added into the solution of compound **13a** (200 mg, 0.77 mmol) in acetonitrile (10 mL) with Na_2CO_3 (210 mg, 2 mmol). The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C overnight. The reaction mixture was cooled to room temperature and partitioned between ethyl acetate and aqueous phase. The organic extract was purified by silica gel column chromatography using ethyl acetate/hexane (1/3, v/v) as mobile phase to yield two yellow solids, compound **13b** (32 mg, 15%) and compound **13c** (57 mg, 26%). **13b**: ^{1}H NMR (DMSO-d_6): δ 2.58 (s, 3H), 3.66 (s, 3H), 3.73 (m, 4H), 6.39 (m, 2H), 6.74 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 30.6, 35.3, 55.8, 110.5, 111.1, 112.8, 112.9, 114.7, 115.2, 123.1, 123.9, 136.0, 140.4, 146.1, 154.7. Anal. Calcd for $C_{15}H_{16}N_2OS$: C, 66.15; H, 5.92; N, 10.29. Found: C, 66.36; H, 6.09; N, 10.24. mp 170.9–171.7 °C. **13c**: ^{1}H NMR (DMSO-d_6): δ 2.80 (s, 6H), 3.70 (s, 3H), 3.76 (m, 3H), 6.62 (m, 2H), 6.80 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 35.3, 41.1, 55.8, 111.9, 112.4, 112.8, 113.0, 114.8, 115.1, 123.1, 123.8, 136.7, 140.2, 147.0, 154.8. Anal. Calcd for $C_{16}H_{18}N_2OS$: C, 67.10; H, 6.33; N, 9.78. Found: C, 67.36; H, 6.25; N, 9.79. mp 185.0–186.5 °C.

4.1.20. 1-(3-Methoxy-7-nitro-10*H*-phenothiazin-10-yl)ethanone (14a)

Acetyl chloride (850 mg, 11 mmol) was added into the solution of compound **11b** (1 g, 3.6 mmol) in dichloromethane (20 mL). The reaction mixture was stirred overnight at ambient temperature. The solvent and excessive acetyl chloride was removed in vacuum. The residue was dissolved into ethyl acetate and washed by water and saturated NaCl solution. The organic extract was dried by anhydrous Na_2SO_4 and purified on silica gel column chromatography using ethyl acetate/hexane (1/2, v/v) as mobile phase to yield yellow solid (0.4 g, 89%).^{1}H NMR ($CDCl_3$): δ 2.23 (s, 3H), 3.83 (s, 3H), 6.90 (d, J = 9.0 Hz, 1H), 6.98 (s, 1H), 7.32 (d, J = 8.7 Hz, 1H), 7.72 (d, J = 8.7 Hz, 1H), 8.18 (d, J = 8.7 Hz, 1H), 8.29 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 22.9, 55.7, 112.7, 114.0, 122.0, 122.9, 127.4, 127.9, 130.7, 133.2, 134.3, 144.7, 145.6, 158.5, 169.2. Anal. Calcd for $C_{15}H_{12}N_2O_4S$: C, 56.95; H, 3.82; N, 8.86. Found: C, 56.72; H, 3.89; N, 8.70. mp 155.9–156.8 °C.

4.1.21. 1-(3-Nitro-10*H*-phenothiazin-10-yl)ethanone (14b)

Synthetic procedure described for **14a** was followed starting with **11c** (150 mg, 0.6 mmol) to afford yellow solid (110 mg, 62%). ^{1}H NMR (DMSO-d_6): δ 2.18 (s, 3H), 7.36 (t, J = 7.2 Hz, 1H), 7.45 (t, J = 7.2 Hz, 1H), 7.61 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 7.8 Hz, 1H), 7.86 (d, J = 7.8 Hz, 1H), 8.24 (d, J = 8.4 Hz, 1H), 8.42 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 23.1, 122.7, 123.3, 127.7, 127.9, 128.3, 128.5, 128.6, 131.2, 134.3, 138.1, 144.5, 145.8, 168.7. Anal. Calcd for $C_{14}H_{10}N_2O_3S$: C, 58.73; H, 3.52; N, 9.78. Found: C, 58.60; H, 3.61; N, 9.62. mp 144.0–145.8 °C.

4.1.22. 1-(3-Hydroxy-7-nitro-10*H*-phenothiazin-10-yl)ethanone (15)

The solution of BBr_3 (1 M) in dichloromethane (1 mL) was added dropwise into the solution of compound **11a** (100 mg, 0.32 mmol) in dichloromethane (10 mL) at −78 °C. the reaction solution was stirred overnight at ambient temperature. The solvent was removed in vacuum. The residue was partitioned between ethyl acetate and water. The organic extract was dried by anhydrous Na_2SO_4 and purified on silica gel column chromatography using ethyl acetate/hexane (1/2, v/v) as mobile phase to yield yellow solid (79 mg, 81%). ^{1}H NMR (DMSO-d_6): δ 2.15 (s, 3H), 6.82 (d, J = 9.0 Hz, 1H), 6.93 (s, 1H), 7.47 (d, J = 9.0 Hz, 1H), 7.82 (d, J = 9.0 Hz, 1H), 8.22 (d, J = 9.0 Hz, 1H), 8.39 (s, 1H), 10.00 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 23.0, 114.3, 115.3, 122.6, 123.2, 128.4, 128.5, 129.3, 132.3, 134.2, 145.1, 145.6, 156.7, 169.1. HRMS (ESI) m/z Calcd for $C_{14}H_{10}N_2O_4S$ [M+1] 303.0440. Found: 303.0435. HPLC purity: 98%. mp 202.3–205.1 °C.

4.1.23. 3-(2-Fluoroethoxy)-7-nitro-10*H*-phenothiazine (16a)

Compound **15** (120 mg, 0.4 mmol) was dissolved in anhydrous DMF (10 mL), NaH (24 mg, 0.6 mmol) was added under 0 °C and stirred for 30 min. 1-Bromo-2-fluoroethane (150 mg, 0.6 mmol) was added and stirred overnight at room temperature. The reaction mixture was quenched with water (100 mL) and extracted with ethyl acetate. After removal of solvent, the residue was suspended in aqueous HCl solution (3 M) in methanol/water (1:1) and refluxed for 5 h. the reaction mixture was quenched in water and extracted with ethyl acetate, the organic extract was dried by anhydrous Na_2SO_4 and purified on silica gel column chromatography using ethyl acetate/hexane (1.2, v/v) as mobile phase to get violet solid (40 mg, 33%). ^{1}H NMR (DMSO-d_6): δ 4.09 (t, J = 3.6 Hz, 1H), 4.19 (t, J = 3.6 Hz, 1H), 4.60 (t, J = 3.6 Hz, 1H), 4.76 (t, J = 3.6 Hz, 1H), 6.64 (m, 4H), 7.72 (s, 1H), 7.83 (d, J = 9.3 Hz, 1H), 9.41 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 67.8, 68.0, 81.6, 83.3, 112.8, 113.4, 114.6, 116.3, 116.6, 116.9, 122.1, 125.2, 132.6, 140.9, 148.4, 155.1. Anal. Calcd for $C_{15}H_{10}N_2O_3S$: C, 60.39; H, 3.38; N, 9.39. Found: C, 60.15; H, 3.50; N, 9.15. mp 189.3–191.4 °C.

4.1.24. (*E*)-3-(3-Iodoallyloxy)-7-nitro-10*H*-phenothiazine (16b)

Prop-2-yn-1-ol (0.7 g, 12 mmol), $Pd(Ph_3P)_2Cl_2$ (42 mg, 0.06 mmol) and $HSnBu_3$ (3 g, 10 mmol) was dissolved in anhydrous tetrahydrofuran and stirred at ambient temperature for 1 h. The solvent was removed in vacuum. The residue was purified on the silica gel column using ethyl acetate/hexane (1/9, v/v) as mobile phase to yield colorless liquid, (*E*)-3-(tributylstannyl)prop-2-en-1-ol (1.2 g, 34%) as intermediate.

Iodine solution in chloroform (0.1 M) was added into the solution of (*E*)-3-(tributylstannyl)prop-2-en-1-ol (200 mg, 0.58 mmol) in $CHCl_3$ (10 mL) and stirred at ambient temperature for 2 h. The reaction mixture was quenched by aqueous $Na_2S_2O_5$ (5%) and extracted with ethyl acetate. The organic extract was concentrated and purified on silica gel column using ethyl acetate/hexane (1/3, v/v) as mobile phase to yield colorless liquid ((*E*)-3-iodoprop-2-en-1-ol) 85 mg, 79%. ^{1}H NMR ($CDCl_3$): δ 2.04 (t, J = 5.7 Hz, 1H), 4.08 (t, J = 5.7 Hz, 2H), 6.39 (d, J = 14.4 Hz, 1H), 6.68 (d, J = 14.4 Hz, 1H).

Triphenylphosphine (133 mg, 0.51 mmol) was added into the solution of (*E*)-3-iodoprop-2-en-1-ol in CH_2Cl_2 (10 mL) at 0 °C and stirred for 1 h, following by the addition of CBr_4 (186 mg, 0.56 mmol) and stirred for additional 2 h at ambient temperature. The solvent was removed in vacuum. The residue was purified by silica gel column chromatography using ethyl acetate/hexane (1/9, v/v) as mobile phase to get colorless liquid, (*E*)-3-bromo-1-iodoprop-1-ene (40 mg, 35%). ^{1}H NMR ($CDCl_3$): δ3.87 (d, J = 7.8 Hz, 2H), 6.54 (d, J = 14.4 Hz, 1H), 6.71 (d, J = 14.4 Hz, 1H).

Compound **15** (50 mg, 0.17 mmol), (*E*)-3-bromo-1-iodoprop-1-ene (50 mg, 0.2 mmol) and Na_2CO_3 (200 mg, 0.2 mmol) was mixed in DMF (10 mL) and stirred for 3 h at ambient temperature. The reaction mixture was quenched in water (50 mL) and extracted with ethyl acetate. The organic extract was concentrated in vacuum. The residue was suspended in HCl (3 M) /MeOH (1:1, v/v) and refluxed for 5 h. the precipitate was filtrated to get violet solid.**16b** (25 mg, 36%).^{1}H NMR (DMSO-d_6): δ 4.41 (s, 2H), 6.61–6.71 (m, 6H), 7.71(s, 1H), 7.83 (d, J = 9.0 Hz, 1H), 9.41 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 69.9, 82.6, 113.0, 113.4, 114.7, 116.2, 116.6, 116.9, 122.1, 125.2, 132.6, 140.8, 141.2, 148.4, 154.7. Anal. Calcd for $C_{15}H_{11}IN_2O_3S$: C, 42.27; H, 2.60; N, 6.57. Found: C, 42.46; H, 2.72; N, 6.38. mp >250 °C.

4.1.25. 3-Nitro-7-(prop-2-yn-1-yloxy)-10*H*-phenothiazine (16c)

Synthetic procedure described for **16b** was followed starting with **15** (70 mg, 0.23 mmol) and 3-bromopropyne (38 mg, 0.25 mmol) to afford violet solid (55 mg, 86%). ^{1}H NMR (DMSO-d_6): δ 3.58 (s, 1H), 4.71 (s, 2H), 6.62–6.67 (s, 4H), 7.73 (s, 1H), 7.84 (d, J = 9.0 Hz, 1H), 9.43 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 56.2, 78.7, 79.5, 113.2, 113.5, 115.0, 116.2, 116.5, 116.8, 122.1, 125.2, 133.1, 148.4, 154.1. Anal. Calcd for $C_{14}H_{10}N_2O_3S$: C, 54.89; H, 3.62; N, 9.15. Found: C, 54.87; H, 3.54; N, 8.92.mp 226.4–227.0 °C.

4.2. Thioflavin T fluorescence assay for α-synuclein fibrils

4.2.1. Production of purified recombinant α-synuclein protein

α-Synuclein recombinant protein was produced in *Escherichia. coli*.[38,39] BL21(DE3)RIL *E. coli* were transformed with a pRK172 bacterial expression plasmid containing the human α-synuclein coding sequence. Freshly transformed BL21 colonies were inoculated into 2 L baffled flasks containing 250 mL sterilized TB (1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) with 50 μg/ml ampicillin, and incubated overnight at 37 °C with shaking. Overnight cultures were pelleted by centrifugation at 3900*g* for 10 min at 25 °C. Bacterial pellets were resuspended in 20 mL osmotic shock buffer (30 mM Tris–HCl, 2 mM EDTA, 40% Sucrose, pH 7.2) by gentle vortexing and incubated at room temperature for 10 min. The cell suspension was then centrifuged at 8000*g* for 10 min at 25 °C and the pellet was resuspended in 22.5 mL cold H_2O before adding 9.4 μL 2 M $MgCl_2$ to each tube. The suspension was incubated on ice for 3 min prior to centrifugation at 20,000*g* for 15 min at 4 °C. The supernatant was transferred to a fresh tube, streptomyocin was added to a final concentration of 10 mg/mL and then centrifuged at 20,000*g* for 15 min at 4 °C. The supernatant from this step was collected and dithiothreitol (DTT) and Tris–HCl were added to final concentrations of 1 mM and 20 mM, respectively, before boiling for 10 min to precipitate heat-sensitive proteins, which were pelleted at 20,000*g* for 15 min at 4 °C. The supernatant was collected and filtered through a 0.45 μm surfactant free cellulose acetate filter (Corning) before loading onto a 1 mL DEAE Sepharose column equilibrated in 20 mM Tris–HCl pH 8, 1 mM EDTA, and 1 mM DTT. The DEAE column was washed with 20 mM Tris–HCl pH 8, 1 mM EDTA, 1 mM DTT before eluting α-synuclein protein in 20 mM Tris–HCl, pH 8, buffer with 1 mM EDTA, 1 mM DTT and 0.3 M NaCl. The purified α-synuclein protein was dialyzed overnight in 10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 1 mM DTT. Preparations contained greater than 95% α-synuclein protein as determined by SDS–PAGE and BCA assay with a typical yield of 30 mg protein per 250 ml culture.

4.2.2. Fibril preparation

The purified, recombinant α-synuclein monomer (2 mg/mL) was incubated in Tris–HCl (20 mM), NaCl (100 mM) at 37 °C while shaking at 1000 rpm in an Eppendorf Thermomixer in a 37 °C

temperature-controlled room for 72 h. To determine the concentration of fibrils, fibril reaction mixture (100 μL) was centrifuged at 18,000 xg for 10 min to separate fibrils from monomer. The concentration of α-synuclein monomer in the supernatant was determined in a bicinchoninic acid (BCA) protein assay along with a bovine serum albumin (BSA) standard curve. The measured decrease in monomer concentration was used to determine the concentration of fibrils in the 72 h fibril reaction mixture.

To prepare fibrils for binding assays, the fibril mixture prepared above was centrifuged at 18,000*g* for 10 min. The supernatant was discarded and the fibril pellet was resuspended in Tris–HCl buffer (30 mM, pH = 7.4) to achieve the desired concentration of fibrils for use in the assay.

4.2.3. Determination of maximum excitation/emission wavelength of ThT-α-synuclein fibrils

The ThT solution (6.0 μM) in Tris–HCl buffer (30 mM, pH = 7.4, 40 μL) was added into each of three wells containing α-synuclein fibrils suspension (3.0 μM) in Tris–HCl buffer (30 mM, pH = 7.4, 40 μL) in a 96 well plate for fluorescence detection. The mixture was incubated at room temperature for 1 h with gentle shaking. The reaction plate was scanned by the excitation wavelength range from 430 to 465 nM. The maximum excitation wavelength (λ_{ex}) was determined according to the fluorescent intensity-excitation wavelength curve. At $\lambda_{ex,}$ the emission wavelength was scanned to get maximum emission wavelength (λ_{em}). λ_{ex} and λ_{em} for the free ThT and ThT-monomeric α-synuclein was determined by the same procedure described above.

4.2.4. Measurement of the dissociation constant of ThT-α-synuclein fibrils (K_d)

ThT solutions of various concentration from 10 nM to 40 μM in Tris–HCl buffer (30 mM, pH = 7.4, 40 μL) were added into the 96 well plate containing α-synuclein fibrils (3.0 μM) in Tris–HCl buffer (30 mM, pH = 7.4, 40 μL). The mixture was incubated at room temperature for 1 h with shaking. The fluorescence intensity for each well was measured at λ_{ex} and λ_{em}, using a Biotek fluorescence plate reader with a 440/30 excitation filter and 485/20 emission filter. The ThT-α-synuclein fibrils saturation binding curve and K_d value were analyzed using Prism 5 software (Graphpad).

4.2.5. Measurement of compounds' affinity to α-synuclein fibrils (K_i)

Twenty microlitre ThT solution (12.0 μM) in Tris–HCl buffer (30 mM, pH = 7.4,) was added into a 96 well plate containing 20 μL α-synuclein fibrils (6.0 μM) in Tris–HCl buffer (30 mM, pH = 7.4,) plus 40 μL compound at different concentration in Tris–HCl buffer (30 mM, pH = 7.4,) plus 10% DMSO. The final concentration of ThT in the assay was 3 μM, approximately three times the K_d, which predicts approximately 75% occupancy of the ThT binding sites. The mixture was incubated at room temperature for 1 h with shaking. The fluorescence intensity for each well was measured at λ_{ex} and λ_{em}. IC_{50} values for each compound were determined by fitting the data to the equation $Y = Bottom + (Top - Bottom)/(1 + 10^{(X-Log\,IC50)})$ using nonlinear regression by Kaleidagraph software, where Top and Bottom are the Y values for the top and bottom plateaus of the binding curve. We derived the K_i values from the IC_{50} values using the Cheng-Prusoff equation,[40] $K_i = IC_{50}/(1+[ThT]/K_d)$.

4.2.6. Measurement of α-synuclein fibril dissociation in the presence of phenothiazine compounds

Phenothiazine compounds were added to reactions containing α-synuclein fibrils (1.5 μM) in 30 mM Tris–HCl buffer pH 7.4 with 3 μM ThT solution in a total volume of 800 μL and incubated at room temperature for 1 h. Separate reactions contained (1) no phenothiazine compound, (2) 3 μM compound **11b**, (3) 3 μM compound **16a**, and (4) 3 μM compound **16b**. Following the incubation period the reactions (n=3 for control and each compound) were centrifuged at 100,000*g* for 20 min at 4 °C to pellet α-synuclein fibrils. The supernatants were removed and the fibrils in the pellet were resuspended in SDS–PAGE sample buffer (recipe), sonicated in a (sonicator model) sonicator at room temperature, and then incubated at 95 °C for 10 min to solubilize the fibrils by dissociation to monomeric α-synuclein. Fifteen microlitre of solubilized fibrils from each reaction were loaded onto an SDS–PAGE (Bio-Rad Criterion) gel and electrophoresed for 1 h at 175 V. Protein was quantified by staining with SYPRO Ruby protein gel stain (Invitrogen) and then imaged with a Kodak Imager Station 440 using UV excitation. A standard curve was included on the gel to ensure that the reactions samples contained levels of α-synuclein within the linear range of SYPRO Ruby detection.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.023.

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