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Original article

Design, synthesis and biological evaluation of indane-2-arylhydrazinylmethylene-1,3-diones and indol-2-aryldiazenylmethylene-3-ones as β-amyloid aggregation inhibitors

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ABSTRACT

Biological screening of (hetero)aromatic compounds allowed the identification of some novel inhibitors of $A\beta_{1-40}$ aggregation, bearing indane and indole rings as common scaffolds. Molecular decoration of lead compounds led to inhibitors exhibiting a potency, measured by the Thioflavin T fluorimetric assay, ranging from high to low micromolar IC_{50}. The 2-(*p*-isopropylphenyldiazenylmethylene)indolone derivative **6c** resulted as the most potent aggregation inhibitor exhibiting an IC_{50} of 1.4 μM, with complete lack of fibril formation as confirmed by transmission electron microscopy. Structure–activity relationships suggested that binding to the Aβ peptide may be largely guided by π-stacking and hydrogen bond interactions.

1. Introduction

Amyloid aggregates are the hallmarks of many neurodegenerative disorders, including Alzheimer's (AD), Huntington's and Parkinson's diseases [1]. Morphological and biochemical characterization of the plaques in AD indicated beta-amyloid proteins $A\beta_{1-40}$ and $A\beta_{1-42}$ as their main constituents. The formation of neuronal plaques is a lengthy, albeit yet undefined, biochemical process. The key steps of this process imply an initial conformational transition from the natural unfolded structure to a β-sheet, a nucleation process and the formation of low-oligomeric aggregates which evolve into the high-oligomeric species protofibrils and fibrils. The aggregation of fibrils with other biological components finally leads to the formation of extracellular neuronal plaques.

Diverse strategies have been envisaged to identify inhibitors of β-amyloid protein folding and aggregation, including the rational design of synthetic peptides and peptidomimetics [2,3], the search for natural Aβ-binding proteins [4–7] and the biological screening of a large number of molecular libraries [8].

Over the years small organic molecules containing single or multiple (hetero)aromatic residues very often with phenolic hydroxyls, have been extensively tested for their ability to inhibit Aβ fibrillization in vitro (Chart 1). The detected inhibitors include Congo red [9], furan [10], indole [11–13], and bis-styrylbenzene derivatives [14], stilbenes [15], curcumin [16], quercetin [17], and many other diverse synthetic and natural compounds [18–20].

Several studies have suggested that interactions between aromatic units may play an important role in many areas of chemistry and biochemistry, most notably in molecular recognition and self-assembly [21–24]. The attractive non-bonded interactions between planar aromatic rings are referred to as π–π interactions or π-stacking. The steric constrains associated with the formation of these ordered stacking structures play a fundamental role in self-assembly processes leading to the formation of supramolecular structures [21–27]. The hypothetical role of π-stacking interactions in amyloid fibril formation suggests that molecules capable of blocking π-stacking may be potential candidates for the control of amyloid diseases [26].

Selection and screening, by an in vitro assay with Thioflavin T (ThT) [28], of (hetero)aromatic compounds synthesized in our laboratory allowed the identification, among others, of 2-[(2-(4-chlorophenyl) hydrazinyl) methylene]-1*H*-indene-1,3(2*H*)-dione **3f** [29] (Table 2) as a novel inhibitor of amyloid aggregation (IC_{50} = 23 μM). The good activity of this compound, originally

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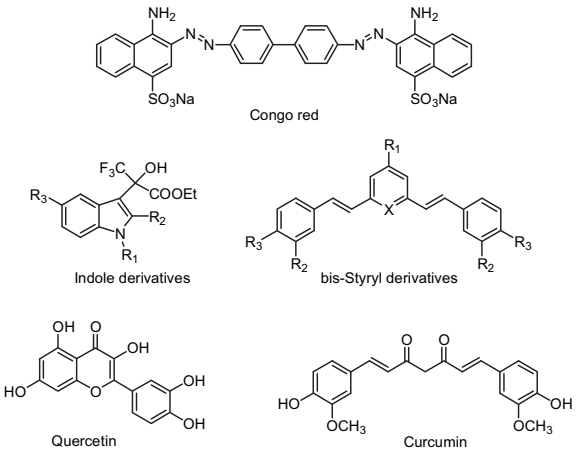


Chart 1. Known amyloid aggregation inhibitors.

reported as a benzodiazepine receptor ligand [29–32], prompted us to design and synthesize a series of arylhydrazine derivatives – **2**, **3** (Scheme 1) and **4** (Fig. 1) – and the indole analogues **6a**–**m** (Scheme 2) and **8** (Fig. 1).

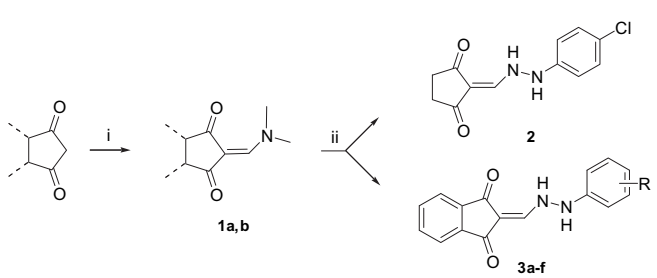
2. Chemistry

Compounds **2** and **3a**–**f** were prepared starting from 1,3-cyclopentanedione and 1,3-indanedione respectively, according to the synthetic pathway shown in Scheme 1.

1,3-diketones were refluxed in *N,N*-dimethylformamide dimethylacetal (DMFDMA) to yield the corresponding dimethylaminomethylene intermediates which were then reacted with suitable arylhydrazines in an ethanol/acetic acid solvent mixture at 0 °C to produce **2** and **3a**–**f**. Hydrazone **4** (Fig. 1) was obtained by condensing ninhydrin with 4-chlorophenylhydrazine hydrochloride in tetrahydrofuran at room temperature.

Indole derivatives **6a**–**m** were prepared starting from commercially available 3-acetoxyindoles according to the sequence of reactions shown in Scheme 2.

The reaction of 3-acetoxyindoles with DMFDMA yielded the (2*Z*)-2-(dimethylaminomethylene)-indol-3-ones **5a**–**b** [33]. The *Z* configuration was assigned to intermediates **5a**–**b** on the basis of ^{1}H NMR spectral data. Unlike 2-aminomethylene-1,3-dicarbonyl derivatives **1a**–**b**, the ^{1}H NMR spectra of **5a**–**b** showed only one signal (singlet) for the two methyl groups of the dimethylamino moiety (Table 1), that are magnetically equivalent owing to their free rotation around the C–N(Me)$_2$ single bond. The hindered rotation around the same bond in compounds **1a**–**b** determined a different pattern of signals for the two methyls of the dimethylamino group, whose ^{1}H NMR spectra showed indeed two separate singlets, in full agreement with literature data [34,35].



Scheme 1. Reagents and conditions: (i), DMFDMA, reflux; (ii), R–C$_6$H$_4$NHNH$_2$, ethanol, acetic acid, 0 °C.

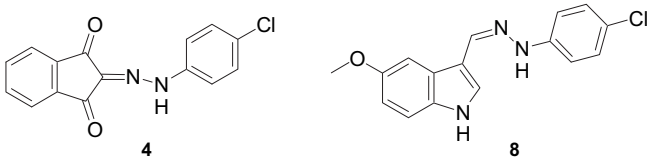


Fig. 1. Structure of compounds **4** and **8**.

Intermediates **5** reacted with diverse arylhydrazines to yield the dehydrogenated derivatives **6a**–**m** instead of the expected arylhydrazines deriving from the nucleophilic substitution of the dimethylamino group. The structural assignments of **6a**–**m** were accomplished on the basis of elemental analysis, ESI-MS and ^{1}H NMR spectral data.

The oxidation reaction leading to compounds **6** might involve the formation of the tautomeric intermediate **7(B)** that in the air might be oxidated to the C-2 hydroxylated derivative, which in turn might easily dehydrate to yield final compounds **6** (Scheme 3).

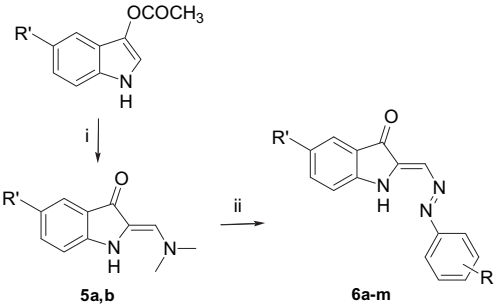
To confirm the proposed oxidation mechanism we carried out the reaction of **5b** with 3-chlorophenylhydrazine, under an argon atmosphere, in an ethanol/acetic acid 97/3 solvent mixture previously degassed with helium. Under these experimental conditions, TLC monitoring (light petroleum ether/ethyl acetate 65:35 v/v as eluent mixture) showed only one spot at R_f 0.50 compared with **6k** (R_f 0.80). However, the work-up of the reaction always led to **6k** instead of the corresponding and readily oxidizable arylhydrazine **7k**.

A sample of **7k** was isolated by condensing 5-bromo-3-hydroxy-1*H*-indole-2-carbaldehyde, prepared by hydrolysis of the enamine **5b** with KOH [36], with 3-chlorophenylhydrazine in an ethanol/acetic acid 97/3 solvent mixture under an argon atmosphere. ESI-MS and ^{1}H NMR spectral data were in good agreement with the proposed arylhydrazine structure.

Oxidation of **7k** to **6k** was monitored in acetone-d_6 solution by recording ^{1}H NMR spectra over time (Fig. 2).

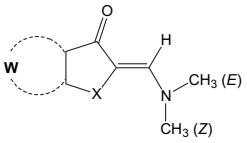
Indole compounds **6a**–**m** might exist in two tautomeric forms, namely hydrazone-imine form **C** and diazenyl-enamine form **D** (Scheme 4). Their ^{1}H NMR spectra in DMSO-d_6 suggested a high prevalence of tautomeric form D. In fact, signals at δ = 7.80–8.00 ppm could be attributed to 2′ and 6′ protons, and doublet close to δ 7.10 ppm might be assigned to the H-7 proton, easily distinguishable from the H-4 signals in the 5-Br derivatives **6g**–**m**. Moreover, singlets close to δ 7.4 and δ 11 ppm could be assigned to CH and NH protons respectively. This assignment was in full agreement with the ^{1}H NMR data reported in the literature for highly similar compounds [37].

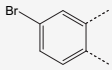
In order to further discriminate between the hydrazone-imine and the diazenyl-enamine forms we decided to run a phase-sensitive 2D-NOESY experiment on a DMSO-d_6 solution of compound **6b**. Interestingly, a strong cross-peak was seen between NH and the



Scheme 2. Reagents and conditions: (i), DMFDMA, reflux, 1 h; (ii), R–C$_6$H$_4$NHNH$_2$, ethanol, acetic acid, 0 °C. **5a**, R′ = H; **5b**, R′ = Br.

Table 1
Identificative chemical shifts (^{1}H NMR, DMSO-d_6) of enaminoketones **1a–b** and **5a–b**.



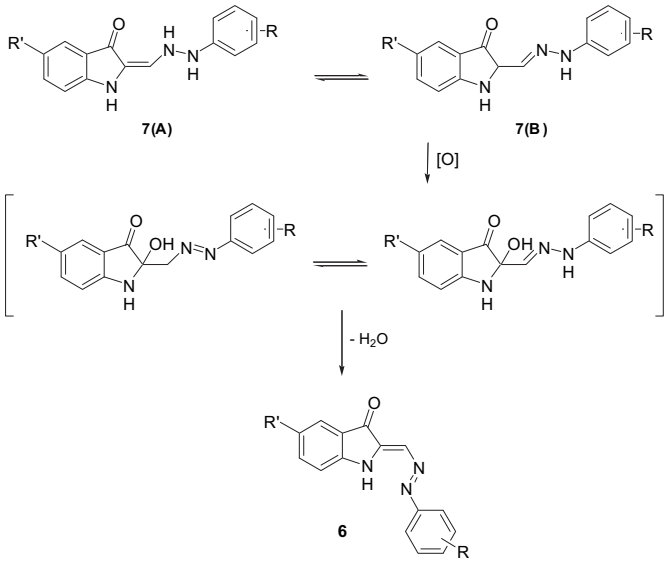
Compound	W	X	δ C=C–H	δ CH_3 (*E*)	δ CH_3 (*Z*)	δ N–H
1a	–	C=O	7.40	3.50	3.60	–
1b		C=O	7.54	3.37	3.67	–
5a		NH	6.97	3.16		8.80
5b		NH	7.02	3.18		9.07

indole H-7 at δ 7.13, and this may occur only in the diazene–enamine form. In addition, a weaker cross-peak was also observed between NH and the arylhydrazinic CH at δ 7.83 (see Supporting Information). The latter would be consistent with a *Z* geometry of the exocyclic enaminic bond which would justify, at the same time, the strong downfield shift observed for NH (δ 10.98) most probably due to the formation of a six-membered intramolecular hydrogen bond.

Finally, compound **8** (Fig. 1), which is structurally related to **6f** and **6l**, was prepared by reacting 5-methoxyindole-3-carbaldehyde with 4-chlorophenylhydrazine hydrochloride.

3. Fluorescence spectroscopy

In vitro inhibition of $A\beta_{1-40}$ aggregation was studied following a recently reported improved fluorescence-based method [28]. The ThT fluorimetric assay [38] in phosphate-buffered saline was modified using DMSO (10%) as a co-solvent to allow poorly water-soluble compounds to be tested even at high concentrations (up to 200 μM). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was also used as a co-solvent (2% v/v), because it dramatically accelerates the aggregation process, as already reported [39]. The modified experimental conditions allowed us to perform medium-throughput screening of small chemical libraries, either available commercially or originating from our laboratories, in much less time compared with the co-solvent-free ThT assay. Anti-aggregating activities were measured after 2 h of incubation at 25 °C instead of the 2–3 weeks required in co-solvent-free experiments. This method was validated by satisfactorily reproducing inhibition data of known anti-aggregating molecules [28].



Scheme 3. Possible pathway of the air oxidation of **7** to **6**.

4. Transmission electron microscopy (TEM)

TEM analysis was performed for the most active compound **6c**, compared with a free incubation sample of Aβ. Incubation conditions were suitably modified [40], without use of HFIP and with ethanol as co-solvent (10%) in pH 8 phosphate buffer and incubation at 37 °C, in order to achieve the complete aggregation within 7 days.

5. Results and discussion

The anti-aggregating activities of the examined compounds on $A\beta_{1-40}$ fibril formation by the ThT fluorimetric assay are listed in Tables 2 and 3 as IC_{50} or, for less active compounds, as the percentage of aggregation inhibition at 100 μM. Even though we are aware about the higher aggregating propensity and neurotoxicity of $A\beta_{1-42}$ protein, we performed our inhibition experiments on the more studied and readily accessible $A\beta_{1-40}$. Quercetin, a known inhibitor of β-amyloid aggregation [17], was included in our assay as the reference compound. Under our experimental conditions an IC_{50} of 0.8 μM was measured for quercetin, a value quite similar to those reported in the literature derived from traditional ThT assays (0.5–2.0 μM) [17, 28]. The effects of indanedione derivatives **3a–f** and **4** and cyclopentanedione analogue **2** on Ab_{1-40} aggregation are reported in Table 2.

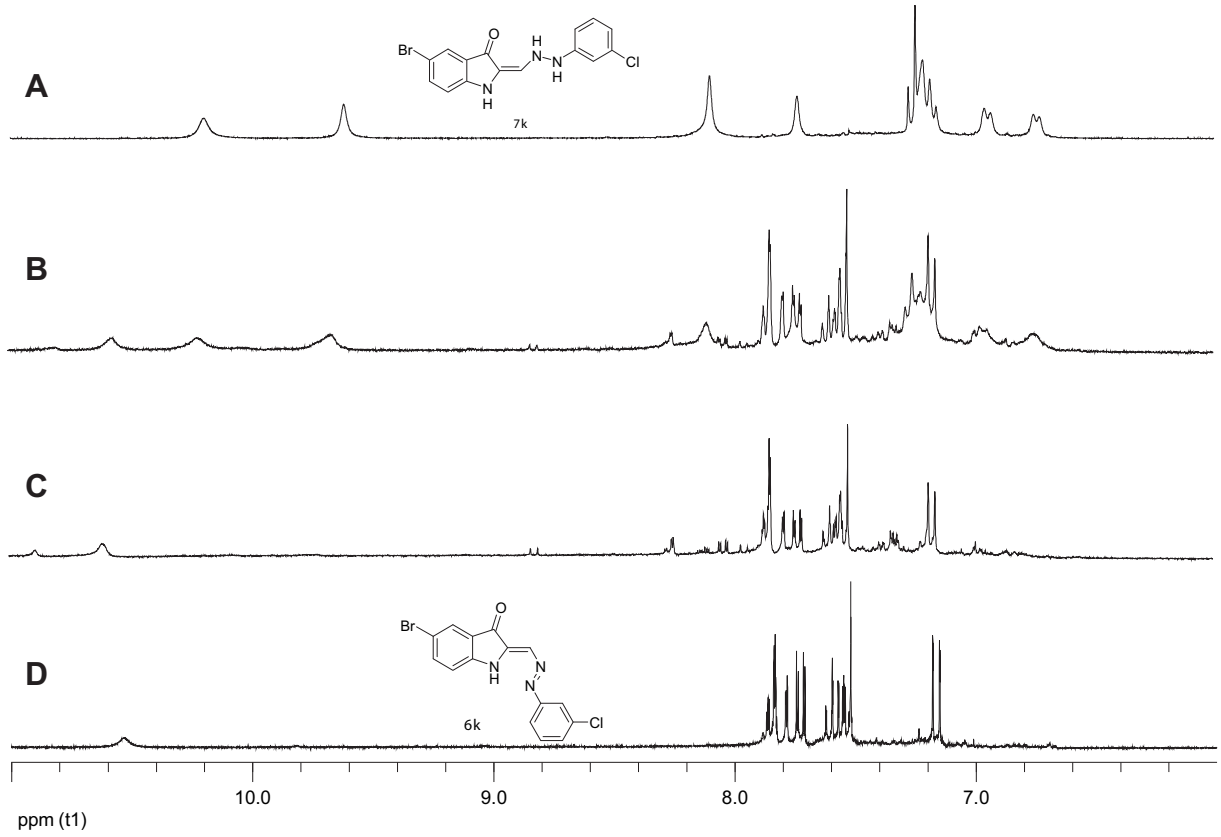


Fig. 2. Air oxidation of **7k** (*A*) to **6k** (*D*) in CD_3COCD_3 after 4 (*B*) and 30 days (*C*).

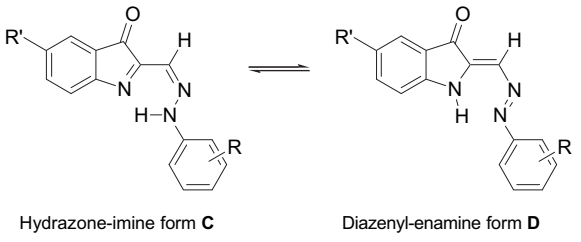
Analysis of biological data from Table 2 revealed that all the examined compounds inhibited, to a different extent, the Aβ aggregation process. Compounds **3c–f** displayed good IC_{50} values equal to 18, 32, 18 and 23 μM, respectively. Removal or substitution with a methyl group of either lipophilic chlorine or isopropyl *para*-substituents yielded compounds **3a–b** with reduced inhibitory activity.

Removal of the aromatic moiety of indanedione (see compound **2**) or shortening of the linker (see compound **4**) also induced a significant lessening of activity compared to compounds **3c–f**.

These findings revealed that the distance between the two aromatic nuclei is an essential determinant of high activity.

To elaborate structure–activity relationships and learn more about the nature of binding interactions with the Aβ peptide, indole derivatives **6a–m** and **8** were prepared and tested (Table 3). Biological data indicated that simply altering the position of the chloro group (**6d–f** and **6j–m**), determined noteworthy effects on potency. In particular, *ortho* and *meta*-monosubstitution (**6d–e** and **6j–k**) led to highly active inhibitors, whereas the *para*-chlorosubstituted derivatives **6f**, **l**, **m** resulted unexpectedly inactive. On the contrary compounds *para*-monoalkylsubstituted **6b–c** showed a considerable increase of the activity with the increase of substituent lipophilicity (**6c** > **6b** > unsubstituted **6a**). Since no other evidences can be proposed to explain the low activity of **6f**, **l**, **m**, further studies will be undertaken by exploring a larger series of analogs. Moreover, to carry on through the biological data discussion, the substitution with bromine at position 5 of the indole moiety decreased inhibitory activity of the unsubstituted and *para*-alkylsubstituted compounds (compare **6g–i** vs. **6a–c**). Finally, compound **8**, containing a *p*-chlorophenylhydrazonomethylene substituent at C-3 of the indole ring, showed a significantly enhanced inhibitory potency compared to the analog **6f** (IC_{50} > 100 and 23 μM for compounds **6f** and **8**, respectively).

Although our in vitro ThT fluorimetric assay does not provide any experimental evidence about which step leading to Aβ aggregation is inhibited by our compounds, it seems reasonable to hypothesize that prevention of cross-β-sheet strand formation and/or disruption might occur. In either case, efficient binding at the Aβ peptide guided by π-stacking and hydrogen bond interactions



Scheme 4. Tautomeric equilibria of compounds **6**.

Table 2
Inhibition data of $Aβ_{1–40}$ fibrillogenesis of indane and cyclopentane derivatives **2**, **3a–f**, **4**.

Compound	R	IC_{50}, μM[a] (% inhibition @ 100 μM ± SEM)
2		(44 ± 5)
3a	H	(49 ± 2)
3b	4-CH_3	(44 ± 4)
3c	4-$CH(CH_3)_2$	18
3d	2-Cl	32
3e	3-Cl	18
3f	4-Cl	23
4		(38 ± 5)

[a] SEMs of IC_{50}s are within ±15% of the reported values.

Table 3
Inhibition data of $A\beta_{1-40}$ fibrillogenesis of indole derivatives **6a–m** and **8**.

Compound	R	R′	IC_{50}, μM[a] (% inhibition @ 100 μM ± SEM)
6a	H	H	8.8
6b	4-CH_3	H	3.6
6c	4-$CH(CH_3)_2$	H	1.4
6d	2-Cl	H	13
6e	3-Cl	H	2.5
6f	4-Cl	H	(33 ± 1)
6g	H	Br	35
6h	4-CH_3	Br	51
6i	4-$CH(CH_3)_2$	Br	23
6j	2-Cl	Br	7.8
6k	3-Cl	Br	4.4
6l	4-Cl	Br	(20 ± 3)
6m	3-Cl, 4-Cl	Br	(23 ± 2)
8			23
QUR			0.8

[a] SEMs of IC_{50}s are within ±15% of the reported values. QUR:quercetin.

should play a key role. Moreover, the highest activity of compound **6c**, which contains the isopropyl substituent on the phenylhydrazine ring, suggests that additional hydrophobic interactions could significantly strengthen binding to the Aβ peptide.

Since fibrils ultimately form after extended incubation times, we performed a TEM assay to qualitatively validate the inhibitory potency of most active compound **6c** (Fig. 3). To ensure the highest reproducibility in comparison with other literature data, we decided to shift the incubation conditions to those described by Bartolini et al. [40], where fibrillization process is achieved in 10% ethanol/phosphate buffer pH 8 at 37 °C (ethanol was preferred to DMSO as co-solvent). Aliquots were removed at various time points during aggregation and analyzed by TEM assay at 60,000-fold magnification. $A\beta_{1-40}$ was not aggregated at time point 0 (Fig. 3A) but formed protofibrils with a diameter as small as 10–20 nm by day 3 (Fig. 3B). These filaments gave amorphous amyloid aggregates after 7 days (Fig. 3C). The effects of compound **6c** on the $A\beta_{1-40}$ assembly are depicted in Fig. 3D–F. At time point 0 (Fig. 3D) $A\beta_{1-40}$ showed no aggregation; after 3 day of incubation (Fig. 3E) **6c** induced only a very reduced formation of isolated protofibrils. Data obtained after 7 days of incubation (Fig. 3F) were consistent with the inhibition of the extended fibril formation.

6. Conclusions

The ThT fluorescence and TEM analysis results clearly indicated that indane and indole derivatives inhibit fibrillization of $A\beta_{1-40}$.

Structural optimization led to novel and potent $A\beta_{1-40}$ aggregation inhibitors **2–4**, **6a–m** and **8**, showing IC_{50} values in the micromolar range. Some of them exhibited considerable potency comparable to the reference compound quercetin.

Structure-activity relationships suggested that both distance and substitution of the two aromatic moieties of the examined compounds play a key role, along with the possible hydrogen bond formation, in binding to the Aβ peptide.

Though in-depth biochemical and biophysical studies are necessary to clarify the effect of our compounds on $A\beta_{1-40}$ fibrillization and then establish their anti-aggregating mechanism, this work highlights new chemical entities eliciting high inhibitory activity against amyloid aggregation.

7. Experimental

7.1. Chemistry

Melting points (mp) were taken on a Gallenkamp MFB 595010 M apparatus and are uncorrected. Elemental analyses were performed on a Carlo Erba 1106 analyser for C, H, N; experimental results agreed to within ±0.40% of the theoretical values. IR spectra were recorded using potassium bromide disks on a Perkin Elmer

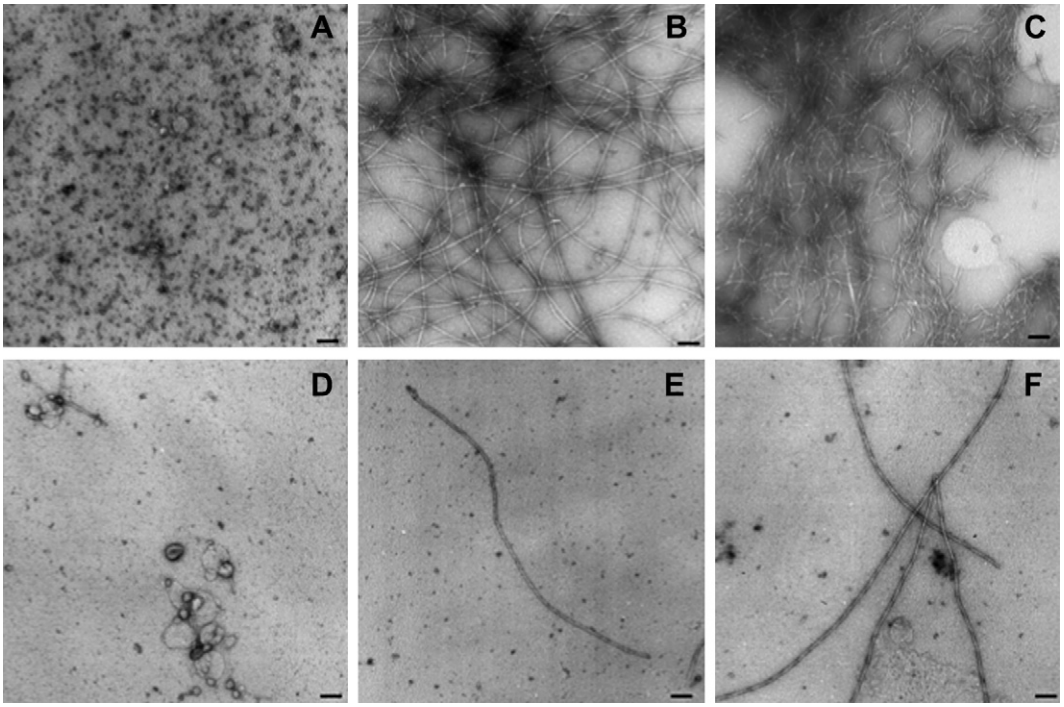


Fig. 3. Effect of fibrillization inhibitor **6c** on $A\beta_{1-40}$ aggregation. $A\beta_{1-40}$ was incubated under "fibrillization conditions" alone (A–C) or in the presence of 50 μM **6c** (D–F). Aliquots of each reaction were assayed by TEM (60.000-fold magnification) at various time points. In the control reaction: (A) at time point 0; (B) at day 3; (C) after 7 days. In the presence of **6c**: (D) at time point 0; (E) at day 3; (F) after 7 days. The black scale bars are 100 nm.

Spectrum One FT-IR spectrophotometer, only the most significant and diagnostic absorption bands being reported. ^{1}H NMR spectra were recorded in DMSO-(d_6), unless otherwise specified, on a Varian Mercury 300 spectrometer. Chemical shifts are expressed in δ (ppm) and the coupling constants J in Hz. The following abbreviations were used: s, singlet; d, doublet; t, triplet; ep, eptuplet; dt, double triplet; m, multiplet. Exchange with deuterium oxide was used to identify OH and NH protons, which in some cases gave broad signals (br) widely spread on the base line and thus very difficult to detect. Chromatographic separations were performed on silica gel 63-200 (Merck). Commercial reagents and solvents were purchased from Sigma–Aldrich.

ESI-MS was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MSD Trap System Agilent, Palo Alto, CA). The sample was infused via a KD Scientific syringe pump at a rate of 10 μL/min. Ionization was achieved in the negative ion mode. The pressure of the nebulizer gas was 15 psi. The drying gas was heated to 350 °C at a flow of 5 L/min. Full-scan mass spectra were recorded in the mass/charge (m/z) range of 50–800 amu.

Compounds **1a**, **b** and **3a** were prepared according to Schenone et al. [34]. Compound **3f** was previously described in ref. [29].

Indole enaminone **5b** was prepared as described for **5a** [33], which had yields and spectroscopic data in very good agreement with those reported; both were used without further purification.

*7.1.1. General procedure for the synthesis of compounds **2**, **3b–e** and **6a–m***

Suitable arylhydrazine (12 mmol) was dissolved in ethanol (10 mL) and slowly added with stirring at 0 °C to a solution of intermediates **1** [34] or **5** [33] (10 mmol) in ethanol (30 mL) and acetic acid (1.5 mL). After stirring overnight, the resulting precipitate was filtered and crystallized or the reaction mixture was evaporated to dryness and the residue purified by column chromatography.

*7.1.1.1. 2-((2-(4-Chlorophenyl)hydrazinyl)methylene)cyclopentane-1,3-dione **2**.* Yield: 70%; ^{1}H NMR δ 2.42 (s, 4H, $2CH_2$), 6.76 (d, 2H, J = 8.8 Hz, H-2′ and H-6′), 7.25 (d, 2H, J = 8.8 Hz, H-3′ and H-5′), 7.62 (s, 1H, CH–N), 9.22 (br, 1H, NH; NH signal not detectable). MS (ESI), m/z = 248.9 (100%) $[M - H]^-$, 250.8 (34%) $[M - H]^-$ + 2. IR (KBr): 3230, 1690, 1640 cm^{-1}. Anal. calcd for $C_{12}H_{11}ClN_2O_2$: C, 57.50; H, 4.42; N, 11.17. Found: C, 57.56; H, 4.45; N, 11.04. mp 197–9 °C from chloroform/methanol 95:5 (v/v).

*7.1.1.2. 2-((2-p-Tolylhydrazinyl)methylene)-1H-indene-1,3 (2H)-dione **3b**.* Yield: 55%; ^{1}H NMR δ 2.20 (s, 3H, CH_3), 6.79 (d, 2H, J = 8.3 Hz, H-2′ and H-6′), 7.00 (d, 2H, J = 8.3 Hz, H-3′ and H-5′), 7.50–7.80 (m, 5H, H-4, H-5, H-6, H-7 and CH–N), 8.60 (s, 1H, NH), 10.90 (br, 1H, NH). MS (ESI), m/z = 276.9 (100%) $[M - H]^-$. IR (KBr): 3265, 3200, 1700, 1650 cm^{-1}. Anal. calcd for $C_{17}H_{14}N_2O_2$: C, 73.37; H, 5.07; N, 10.07. Found: C, 73.32; H, 5.14; N, 9.97. mp 187–9 °C from ethanol.

*7.1.1.3. 2-((2-(4-Isopropylphenyl)hydrazinyl)methylene)-1H-indene-1,3(2H)-dione **3c**.* Yield: 40%; ^{1}H NMR δ 1.13 (d, 6H, J = 6.9 Hz, $2CH_3$), 2.78 (ep, 1H, J = 6.9 Hz, CH), 6.69 (d, 2H, J = 8.4 Hz, H-2′ and H-6′), 7.09 (d, 2H, J = 8.4 Hz, H-3′ and H-5′), 7.68–7.71 (m, 5H, H-4, H-5, H-6, H-7 and CH–N), 8.59 (s, 1H, NH), 10.70 (br, 1H, NH). MS (ESI), m/z = 304.9 (100%) $[M - H]^-$. IR (KBr): 3235, 3168, 1645, 1468 cm^{-1}. Anal. calcd for $C_{19}H_{18}N_2O_2$: C, 74.49; H, 5.92; N, 9.14. Found: C, 74.36; H, 5.93; N, 9.08. mp 212–4 °C from ethanol.

*7.1.1.4. 2-((2-(2-Chlorophenyl)hydrazinyl)methylene)-1H-indene-1,3(2H)-dione **3d**.* Yield: 60%; ^{1}H NMR δ 6.82 (d, 1H, J = 7.8 Hz, H-6′), 6.87 (t, 1H, J = 7.8 Hz, H-4′),7.22 (t, 1H, J = 7.8, H-5′) 7.36 (d, 1H, J = 7.8 Hz, H-3′), 7.60–7.70 (br, 1H, CH–N), 7.70–7.80 (m, 4H, H-4, H-5, H-6 and H-7), 8.60 (s, 1H, NH), 10.90 (br, 1H, NH). MS (ESI), m/z = 296.9 (100%) $[M - H]^-$, 298.8 (35%) $[M - H]^-$ + 2. IR (KBr): 3240, 1700, 1650 cm^{-1}. Anal. calcd for $C_{16}H_{11}ClN_2O_2$: C, 64.33; H, 3.71; N, 9.38. Found: C, 64.02; H, 3.83; N, 9.12. mp 207–9 °C from ethanol.

*7.1.1.5. 2-((2-(3-Chlorophenyl)hydrazinyl)methylene)-1H-indene-1,3(2H)-dione **3e**.* Yield: 65%; ^{1}H NMR δ 6.65–6.75 (m, 2H, H-2′ and H-6′), 6.80–6.90 (m, 1H, H-4′), 7.23 (t, 1H, J = 8.0 Hz, H-5′), 7.57–7.65 (br, 1H, CH–N),7.66–7.75 (m, 4H, H-4, H-5, H-6 and H-7), 9.00 (s, 1H, NH), 10.90 (br, 1H, NH). MS (ESI), m/z = 296.9 (100%) $[M - H]^-$, 298.8 (34%) $[M - H]^-$ + 2. IR (KBr): 3240, 1700, 1645 cm^{-1}. Anal. calcd for $C_{16}H_{11}ClN_2O_2$: 64.33; H, 3.71; N, 9.38. Found: C, 64.20; H, 3.74; N, 9.30. mp 192–4 °C from ethanol.

*7.1.1.6. (2Z)-2-{[(E)-Phenyldiazenyl]methylene}-1,2-dihydro-3H-indol-3-one **6a**.* Yield: 80%; ^{1}H NMR δ 6.99 (t, 1H, J = 7.5 Hz, H-5), 7.12 (d, 1H, J = 8.0 Hz, H-7), 7.39 (s, 1H, CH=N), 7.50–7.63 (m, 5H, H-3′, H-5′, H-4, H-6 and H-4′), 7.90 (d, 2H, J = 8.2 Hz, H-2′ and H-6′), 11.0 (s, 1H, NH). MS (ESI), m/z = 247.9 (100%) $[M - H]^-$. IR (KBr): 3430, 2920, 1690, 1630 cm^{-1}. Anal. calcd for $C_{15}H_{11}N_3O$: C, 72.28; H, 4.45; N, 16.86. Found: C, 72.38; H, 4.81; N, 16.72. MP 207–9 °C from ethyl acetate/light petroleum ether 20:80 (v/v).

*7.1.1.7. (2Z)-2-{[(E)-(4-Methylphenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one **6b**.* Yield: 81%; ^{1}H NMR δ 2.38 (s, 3H, CH_3), 6.98 (t, 1H, J = 7.4 Hz, H-5), 7.12 (d, 1H, J = 8.2 Hz, H-7), 7.36 (d, 2H, J = 8.2 Hz, H-3′ and H-5′), 7.37 (s, 1H, CH=N), 7.55–7.62 (m, 2H, H-4 and H-6), 7.82 (d, 2H, J = 8.2 Hz, H-2′ and H-6′), 10.97 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ 21.01 (CH_3), 112.1 (C-7), 120.0 (C-3a), 120.6 (C-5), 122.6 (C-6′and C-2′), 124.0 (CH–N), 124.4 (C-4), 129.8 (C-5′ and C-3′), 137.2 (C-6), 138.9 (C-4′), 141.7 (C-1′), 151.5 (C-2), 152.9 (C-7a), 187.6 (C=O). MS (ESI), m/z = 261.9 $[M - H]^-$. IR (KBr): 3350, 1686, 1590, 1115 cm^{-1}. Anal. calcd for $C_{16}H_{13}N_3O$: C, 72.99; H, 4.98; N, 15.96. Found: C, 73.15; H, 5.07; N, 16.11. mp 199–201 °C from ethyl acetate/light petroleum ether 30:70 (v/v).

*7.1.1.8. (2Z)-2-{[(E)-(4-Isopropylphenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one **6c**.* Yield: 55%; ^{1}H NMR δ 1.22 (d, 6H, J = 6.9 Hz, $2CH_3$), 2.48 (ep, 1H, J = 6.9 Hz, CH), 6.97 (t, 1H, J = 7.8 Hz, H-5), 7.12 (d, 1H, J = 8.0 Hz, H-7), 7.37 (s, 1H, CH–N), 7.42 (d, 2H, J = 8.2 Hz, H-3′ and H-5′), 7.54–7.62 (m, 2H, H-4 and H-6), 7.83 (d, 2H, J = 8.2 Hz, H-2′ and H-6′), 10.96 (s, 1H, NH). MS (ESI), m/z = 289.9 (100%) $[M - H]^-$. IR (KBr): 3221, 1678, 1623, 1594, 1120 cm^{-1}. Anal. calcd for $C_{18}H_{17}N_3O$: C, 74.20; H, 5.88; N, 14.42. Found: C, 73.92; H, 6.04; N, 14.28. mp 159–60 °C from light petroleum ether/ethyl acetate 70:30 (v/v).

*7.1.1.9. (2Z)-2-{[(E)-(2-Chlorophenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one **6d**.* Yield: 66%; ^{1}H NMR δ 7.01 (t, 1H, J = 7.4 Hz, H-5), 7.12 (d, 1H, J = 7.8 Hz, H-7), 7.42 (s, 1H, CH–N), 7.45–7.70 (m, 5H, H-3′, H-5′, H-4, H-6 and H-4′), 7.86 (d, 1H, J = 7.4 Hz, H-6′), 11.25 (s, 1H, NH). MS (ESI), m/z = 281.9 (100%) $[M - H]^-$, 283.8 (32%) $[M - H]^-$ + 2. IR (KBr): 3430, 1685, 1600 cm^{-1}. Anal. calcd for $C_{15}H_{10}ClN_3O$: C, 63.50; H, 3.55; N, 14.81. Found: C, 63.41; H, 3.44; N, 15.01. mp 182–4 °C from ethanol.

*7.1.1.10. (2Z)-2-{[(E)-(3-Chlorophenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one **6e**.* Yield: 56%; ^{1}H NMR δ 7.00 (t, 1H, J = 7.6 Hz, H-5), 7.12 (d, 1H, J = 8.0 Hz, H-7), 7.39 (s, 1H, CH–N), 7.55–7.63 (m, 4H, H-4, H-6, H-4′ and H-5′), 7.90 (d, 1H, J = 7.1 Hz, H-6′), 7.94 (s, 1H, H-2′), 11.18 (s, 1H, NH). MS (ESI), m/z = 281.8 (100%) $[M - H]^-$, 283.8 (35%) $[M - H]^-$ + 2. IR (KBr): 3435, 1670, 1599, 1464 cm^{-1}. Anal. calcd for $C_{15}H_{10}ClN_3O$: C, 63.50; H, 3.55; N, 14.81.

Found: C, 63.75; H, 3.62; N, 14.49. mp 157–8 °C from light petroleum ether/ethyl acetate 70:30 (v/v).

7.1.1.11. (2Z)-2-{[(E)-(4-Chlorophenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one ***6f***. Yield: 60%; ^{1}H NMR δ 7.00 (t, 1H, J = 7.3 Hz, H-5), 7.12 (d, 1H, J = 6.8 Hz, H-7), 7.39 (s, 1H, CH–N), 7.55–7.67 (m, 4H, H-3′, H-5′, H-4 and H-6), 7.91 (d, 2H, J = 8.8 Hz, H-2′ and H-6′), 11.10 (s, 1H, NH). MS (ESI), m/z = 281.9 (100%) $[M - H]^-$, 283.9 (34%) $[M - H]^- + 2$. IR (KBr): 3380, 2925, 1700, 1605 cm^{-1}. Anal. calcd for $C_{15}H_{10}ClN_3O$: C, 63.50; H, 3.55; N, 14.81. Found: C, 63.81; H, 3.75; N, 14.69. mp 207–9 °C from chloroform/methanol 95:5 (v/v).

7.1.1.12. (2Z)-5-Bromo-2-{[(E)-phenyldiazenyl]methylene}-1,2-dihydro-3H-indol-3-one ***6g***. Yield: 55%; ^{1}H NMR δ 7.09 (d, 1H, J = 8.4 Hz, H-7), 7.40 (s, 1H, CH–N), 7.52–7.59 (m, 3H, H-3′, H-4′ and H-5′), 7.72 (d, 1H, J = 8.4 Hz, H-6), 7.76 (br, 1H, H-4), 7.90 (d, 2H, J = 8.0 Hz, H-2′ and H-6′), 11.15 (s, 1H, NH). MS (ESI), m/z = 325.9 (95%) $[M - H]^-$, 327.7 (100%) $[M - H]^- + 2$. IR (KBr): 3310, 1685, 1625, 1600 cm^{-1}. Anal. calcd for $C_{15}H_{10}BrN_3O$: C, 54.90; H, 3.07; N, 12.80. Found: C, 55.03; H, 3.25; N, 12.77. mp 200–1 °C from ethanol.

7.1.1.13. (2Z)-5-Bromo-2-{[(E)-(4-methylphenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one ***6h***. Yield: 45%; ^{1}H NMR δ 2.38 (s, 3H, CH_3), 7.08 (d, 1H, J = 8.4 Hz, H-7), 7.35–7.38 (m, 2H, H-3′ and H-5′),7.39 (s, 1H, CH–N), 7.71 (d, 1H, J = 8.4 Hz, H-6), 7.75 (br, 1H, H-4),7.81 (d, 2H, J = 8.3 Hz, H-2′ and H-6′), 11.08 (s, 1H, NH). MS (ESI), m/z = 339.8 (98%) $[M - H]^-$, 341.7 (100%) $[M - H]^- + 2$. IR (KBr): 3436, 2926, 1626, 1599, 1465 cm^{-1}. Anal. calcd for $C_{16}H_{12}BrN_3O$: C, 56.16; H, 3.53; N, 12.28. Found: C, 55.88; H, 3.34; N, 12.12. mp 193–5 °C (dec.) from light petroleum ether/ethyl acetate 70:30 (v/v).

7.1.1.14. (2Z)-5-Bromo-2-{[(E)-(4-isopropylphenyl)diazenyl]-methylene}-1,2-dihydro-3H-indol-3-one ***6i***. Yield: 30%; ^{1}H NMR δ 1.22 (d, 6H, J = 6.9 Hz, $2CH_3$), 2.48 (ep, 1H, J = 6.9 Hz, CH), 7.09 (d, 1H, J = 8.4 Hz, H-7), 7.39 (s, 1H, CH–N), 7.43 (d, 2H, J = 8.5 Hz, H-3′ and H-5′), 7.71 (d, 1H, J = 8.4 Hz, H-6), 7.74 (br, 1H, H-4), 7.84 (d, 2H, J = 8.2 Hz, H-2′ and H-6′), 11.08 (s, 1H, NH). MS (ESI), m/z = 367.9 (94%) $[M - H]^-$, 369.8 (100%) $[M - H]^- + 2$. IR (KBr): 3335, 1682, 1620, 1587, 1465 cm^{-1}. Anal. calcd for $C_{18}H_{16}BrN_3O$: C, 58.39; H, 4.36; N, 11.35. Found: C, 58.33; H, 4.74; N, 11.31. mp 195–7 °C from ethyl acetate/light petroleum ether 10:90 (v/v).

7.1.1.15. (2Z)-5-Bromo-2-{[(E)-(2-chlorophenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one ***6j***. Yield: 83%; ^{1}H NMR δ 7.10 (d, 1H, J = 8.5 Hz, H-7), 7.43 (s, 1H, CH–N), 7.44–7.56 (m, 2H, H-3′ and H-5′), 7.64–7.79 (m, 3H, H-4′, H-4 and H-6), 7.84 (dd, 1H, Jm = 2.0 Hz, Jo = 7.7 Hz, H-6′), 11.35 (s, 1H, NH). MS (ESI), m/z = 359.8 (75%) $[M - H]^-$, 361.7 (100%) $[M - H]^- + 2$, 363.7 (25%) $[M - H]^- + 4$. IR (KBr): 3370, 1690, 1620, 1600 cm^{-1}. Anal. calcd for $C_{15}H_9BrClN_3O$: C, 49.69; H, 2.50; N, 11.59. Found: C, 49.43; H, 2.60; N, 11.61. mp 171–3 °C from ethanol.

7.1.1.16. (2Z)-5-Bromo-2-{[(E)-(3-chlorophenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one ***6k***. Yield 90%; ^{1}H NMR δ 7.10 (d, 1H, J = 8.5 Hz, H-7), 7.41 (s, 1H, CH–N), 7.56–7.64 (m, 2H, H-4′ and H-5′), 7.74 (dd, 1H, Jm = 2.0, Jo = 8.5, H-6), 7.77 (d, 1H, Jm = 2.0, H-4), 7.88 (dt, 1H, Jm = 2.0, Jo = 7.0, H-6′), 7.94 (d, 1H, Jm = 2.0, H-2′), 11.30 (s, 1H, NH). MS (ESI), m/z = 359.9 (74%) $[M - H]^-$, 361.8 (100%) $[M - H]^- + 2$, 363.7 (28%) $[M - H]^- + 4$. IR (KBr): 3436, 1685, 1601, 1465, 1171 cm^{-1}. Anal. calcd for $C_{15}H_9BrClN_3O$: C, 49.69; H, 2.50; N, 11.59. Found: C, 49.66; H, 2.19; N, 11.23. mp 168–9 °C from chloroform/light petroleum ether 70:30 (v/v).

7.1.1.17. (2Z)-5-Bromo-2-{[(E)-(4-chlorophenyl)diazenyl]methylene}-1,2-dihydro-3H-indol-3-one ***6l***. Yield: 80%; ^{1}H NMR δ 7.10 (d, 1H, J = 8.5 Hz, H-6), 7.40 (s, 1H, CH–N), 7.62 (d, 2H, J = 8.6 Hz, H-3′ and H-5′), 7.71 (dd, 1H, Jm = 2.2 Hz, Jo = 8.5 Hz, H-6), 7.74 (d, 1H, Jm = 1.6 Hz, H-4) 7.90 (d, 2H, J = 8.6 Hz, H-2′ and H-6′), 11.21 (s, 1H, NH). MS (ESI) m/z = 359.9 (74%) $[M - H]^-$, 361.7 (100%) $[M - H]^- + 2$, 363.7 (25%) $[M - H]^- + 4$. IR (KBr): 3317, 1686, 1602, 1463, 1264 cm^{-1}. Anal. calcd for $C_{15}H_9BrClN_3O$: C, 49.69; H, 2.50; N, 11.59. Found: C, 49.57; H, 2.39; N, 11.20. mp 241–3 °C from chloroform/light petroleum ether 80:20 (v/v).

7.1.1.18. (2Z)-5-Bromo-2-{[(E)-(3,4-dichlorophenyl)diazenyl]-methylene}-1,2-dihydro-3H-indol-3-one ***6m***. Yield: 60%; ^{1}H NMR δ 7.09 (d, 1H, J = 8.2 Hz, H-7), 7.39 (s, 1H, CH–N), 7.71–7.89 (m, 3H, H-6′, H-5′ and H-6), 7.74 (br, 1H, H-4), 8.10 (s, 1H, H-2′), 11.29 (s, 1H, NH). MS (ESI), m/z = 393.7 (51%) $[M - H]^-$, 395.7 (100%) $[M - H]^- + 2$, 397.6 (42%) $[M - H]^- + 4$, 399.6 (6%) $[M - H]^- + 6$. IR (KBr): 3284, 1690, 1598, 1466 cm^{-1}. Anal. calcd for $C_{15}H_8BrCl_2N_3O$: C, 45.37; H, 2.03; N, 10.58. Found: C, 45.18; H, 2.28; N, 10.25. mp 226–7 °C from ethyl acetate/light petroleum ether 30:70 (v/v).

7.1.2. Synthesis of compounds ***4***, ***7k*** *and* ***8***

7.1.2.1. 2-[(4-Chlorophenyl)hydrazono]indane-1,3-dione ***4***. Ninhydrin (0.089 g, 0.5 mmol) and 4-chlorophenylhydrazine hydrochloride (0.072 g, 0.5 mmol) in tetrahydrofuran (3 mL) were stirred at room temperature for 24 h. The precipitate was filtered and crystallized (51% yield). ^{1}H NMR δ 7.48 (d, 2H, J = 9.0 Hz, H-2′ and H-6′), 7.66 (d, 2H, J = 9.0 Hz, H-3′ and H-5′), 7.88 (br, 4H, H-4, H-5, H-6 and H-7), 13.08 (s, 1H, NH). MS (ESI), m/z = 282.8 (100%) $[M - H]^-$, 284.8 (31%) $[M - H]^- + 2$. IR (KBr): 3160, 1715, 1675, 1590 cm^{-1}. Anal. calcd for $C_{15}H_9ClN_2O_2$: C, 63.28; H, 3.19; N, 9.84. Found: C, 63.59; H, 3.37; N, 9.80. mp 246–8 °C (dec.) from ethanol.

7.1.2.2. 3-Hydroxy-1H-indole-2-carbaldehyde(3-chloro-phenyl)-hydrazone ***7k***. 3-Chlorophenylhydrazine (0.17 g, 1.2 mmol) was dissolved in ethanol (6.5 mL) and slowly added, under an argon atmosphere, with stirring at 0 °C to a solution of 5-bromo-3-hydroxy-1*H*-indole-2-carbaldehyde [36] (0.26 g, 1.0 mmol) in ethanol (3.7 mL) and acetic acid (0.21 mL). After stirring overnight, the resulting precipitate was filtered to give the crude product (14% yield). ^{1}H NMR (Acetone-d_6) δ 6.74 (d, 1H, J = 7.5 Hz, H-6′), 7.00 (d, 1H, J = 7.8 Hz, H-4′), 7.19–7.28 (m, 4H, H-2′, H-5′, H-6 and H-7), 7.40 (s, 1H, CH–N), 8.10 (br, 2H, H-4 and NH), 9.62 (br, 1H, NH), 10.20 (br, 1H, NH), MS (ESI), m/z = 361.8 (78%) $[M - H]^-$, 363.7 (100%) $[M - H]^- + 2$, 365.7 (23%) $[M - H]^- + 4$. IR (KBr): 3413, 1596, 1479 cm^{-1}.

7.1.2.3. 5-Methoxy-1H-indole-3-carbaldehyde (4-chlorophenyl)hydrazone ***8***. 5-methoxyindole-3-carbaldehyde (0.088 g, 0.5 mmol) and 4-chlorophenylhydrazine hydrochloride (0.072 g, 0.5 mmol) in methanol (3 mL) were stirred at room temperature for 48 h. Evaporation of solvent gave **8** as a yellow solid (40% yield). ^{1}H NMR δ 3.81 (s, 3H, CH_3), 6.81 (dd, 1H, Jm = 2.6 Hz, Jo = 8.6 Hz, H-6), 7.00 (d, 2H, J = 9.0 Hz, H-2′ and H-6′), 7.22 (d, 2H, J = 9.0 Hz, H-3′ and H-5′), 7.29 (d, 1H, J = 8.6 Hz, H-7), 7.58 (d, 1H, J = 2.6 Hz, H-2), 7.72 (d, 1H, J = 2.6 Hz, H-4), 8.10 (s, 1H, CH=N), 9.95 (br, 1H, NH), 11.20 (s, 1H, NH). MS (ESI), m/z = 297.9 (100%) $[M - H]^-$, 299.8 (35%) $[M - H]^- + 2$. IR (KBr): 3113, 1652, 1486, 1230 cm^{-1}. Anal. calcd for $C_{16}H_{14}ClN_3O$: C, 64.11; H, 4.71; N, 14.02. Found: C, 64.10; H, 4.75; N, 13.63. mp 202–3 °C from ethanol.

7.2. Inhibition of β-amyloid aggregation

A spectrofluorimetric method modified from that of LeVine [38], based on fluorescence emission of ThT, was followed. In

order to obtain batches of $A\beta_{1-40}$ free from preaggregates, commercial peptide (purity >95%; Anaspec, USA) was dissolved in HFIP, lyophilized and stored at −20 °C as described by Liu [41]. The solution of ThT (25 μM) used for fluorimetric measures was prepared in phosphate buffer 0.025 M, pH 6.0, filtered through 0.45 μm teflon filters and stored at 4 °C. Inhibitors were first tested at 100 μM; test samples were prepared in phosphate-buffered saline (PBS; 0.01 M, NaCl 0.1 M, pH 7.4), 30 μM Aβ peptide concentration, and contained 2% HFIP and 10% DMSO. Incubations were run in triplicate at 25 °C for 2 h. Fluorimetric measures were performed in a 700 μL cuvette with a Perkin–Elmer LS55 spectrofluorimeter, using FLWinlab program. 470 μL of ThT solution were mixed with 30 μL of sample, and the resulting fluorescence measured with parameters set as follows: excitation at 440 nm (slit 5 nm); emission at 485 nm (slit 10 nm); integration time 2 s. Biological activity was determined as percent of inhibitory activity V_i for each compound according to the formula:

$$V_i = 100 - [(F_i - F_b)/F_0] \times 100$$

where F_i is the fluorescence value of the sample, F_b its blank value, and F_0 the fluorescence value for free aggregation of a sample of $A\beta_{1-40}$ incubated in the same buffer/HFIP/DMSO system and in absence of inhibitors. For most active inhibitors, IC_{50}s were determined by testing in triplicate 5–7 concentrations in three independent experiments; statistics were calculated within GraphPad Prism 4.0 software.

7.3. TEM studies

Samples for TEM analysis were prepared in phosphate buffer 0.217 M pH 8.0 with 10% ethanol as co-solvent and incubated for up to 7 days at 37 °C. Final concentrations of $A\beta_{1-40}$ and **6c** were 30 μM and 50 μM respectively. For each sample, a little drop (20 μL) of incubated sample solution was applied to carbon-coated copper/rhodium grid (400 mesh; TAAB Laboratories Equipment Ltd, Aldermaston, Berks, England). The coated grid was floated for 2 min on the sample drop and rinsed with 200 μL of double distilled water. Negative staining was performed with 200 μL of 2% w/v uranyl acetate solution (TAAB Laboratories Equipment Ltd). After draining off the excess of staining solution by means of a filter paper, the specimen was transferred for examination in a Philips Morgagni 282D transmission electron microscope, operating at 60 kV. Electron micrographs of negatively stained samples were photographed on Kodak electron microscope film 4489 (Kodak Company, New York, USA).

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Appendix. Supporting Information

2D-NOESY and ^{13}C spectra of compound **6b**, and dose/response inhibition curve of **6c**, can be found in the online version at doi:10.1016/j.ejmech.2009.12.029.

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