

Polyamine Conjugation as a Promising Strategy To Target Amyloid Aggregation in the Framework of Alzheimer's Disease

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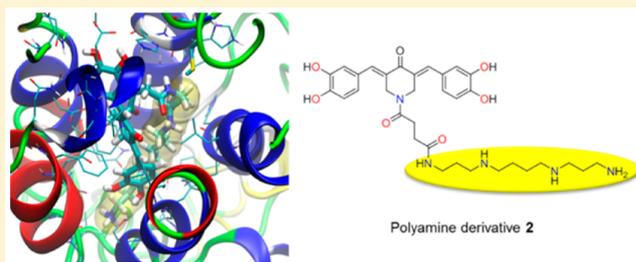
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S Supporting Information

ABSTRACT: Spermine conjugates 2–6, carrying variously decorated 3,5-dibenzylidenepiperidin-4-one as bioactive motives, were designed to direct antiaggregating properties into mitochondria, using a polyamine functionality as the vehicle tool. The study confirmed mitochondrial import of the catechol derivative 2, which displayed effective antiaggregating activity and neuroprotective effects against A β -induced toxicity. Notably, a key functional role for the polyamine motif in A β molecular recognition was also unraveled. This experimental readout, which was supported by in silico studies, gives important new insight into the polyamine's action. Hence, we propose polyamine conjugation as a promising strategy for the development of neuroprotectant leads that may contribute to decipher the complex picture of A β toxicity.



KEYWORDS: Alzheimer's disease, amyloid, mitochondria, polyamines

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, with a complex interplay of genetic and biochemical factors contributing to the cognitive decline. Among all pathological features, AD is traditionally characterized by the presence of extracellular plaques composed of aggregated amyloid β peptides (A β). Recent years have witnessed tremendous efforts in developing therapeutic strategies to decrease A β production, aggregation, and toxicity.¹ However, the mechanistic connection between protein aggregation and tissue degeneration as well as the different roles for A β monomeric and oligomeric forms in the amyloidogenic pathway are not yet fully understood.² Intracellular imbalances, such as mitochondrial dysfunction and oxidative stress, have been recognized as hallmarks of A β -induced neuronal toxicity.³ Oligomeric A β is supposed to enter cell organelles and generate a feedback loop that might ultimately lead to neuronal damage and cognitive decline.⁴ Thus, whereas A β pathology was previously seen as primarily extracellular, the recent literature strongly supports a dominant role for the intracellular toxic A β species in the generation of molecular and biochemical abnormalities prior to neuritic plaque formation.⁵ The heightened interest for the role of

intracellular A β and the need to highlight the molecular mechanisms behind A β toxicity call for the development of new pharmacological tools targeting the cellular compartment.

In 2010, we pursued polyamine conjugation of antioxidant features to gain an efficient intracellular uptake and mitochondria targeting by virtue of electrostatic forces. In particular, variously decorated 3,5-dibenzylidenepiperidin-4-one (DBP) frameworks were used as bioactive motives, with the polyamine derivative 1 emerging as the most promising candidate of the series.⁶ Herein, we sought to apply the same targeting strategy to drive antiaggregating agents at intracellular/mitochondrial level. To this aim, we further exploited the versatility and chemical accessibility of the DBP scaffold, and by using spermine as vehicle tool, we designed and synthesized compounds 2–6 (Figure 1). The aryl substitution patterns were selected finding inspiration from natural polyphenols, which are a rich source of agents able to reduce A β production and toxicity through different mechanisms.⁷ In

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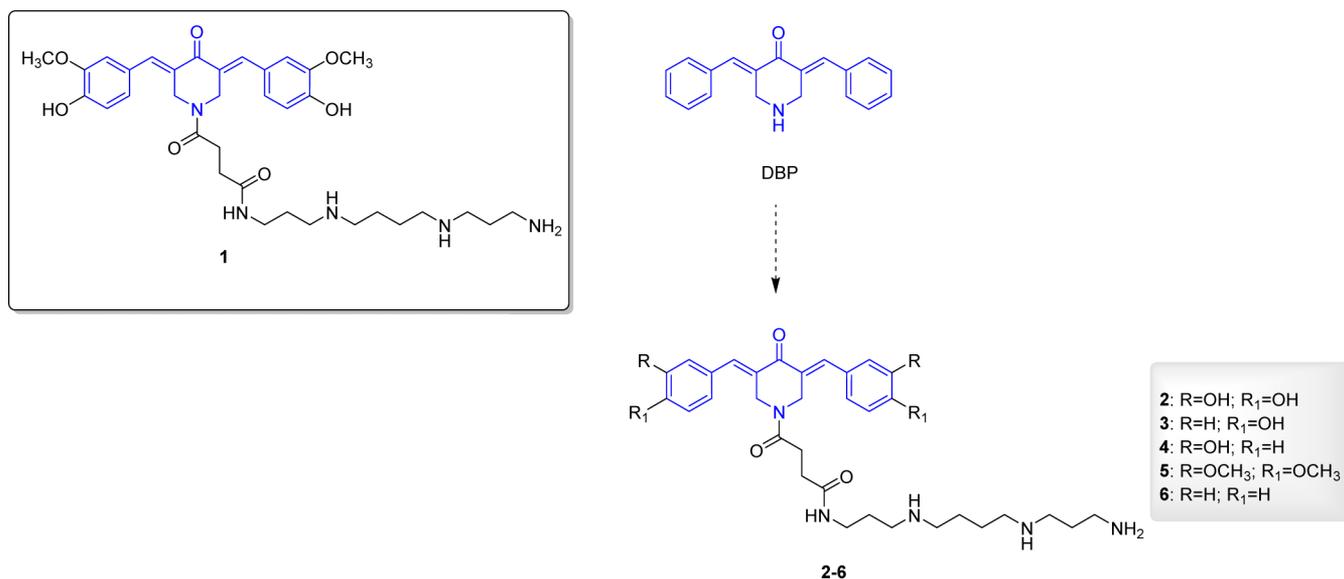
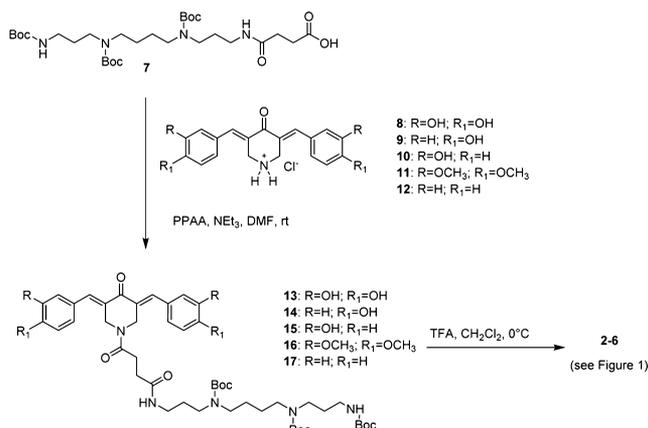


Figure 1. Design strategy for compounds 2–6. DPB stands for 3,5-dibenzylidenepiperidin-4-one.

particular, we focused on the catechol moiety, which turned out to be a key structural feature to inhibit the $A\beta_{42}$ self-aggregation process.^{8,9}

Compounds 2–6 were synthesized through a convergent synthetic approach as outlined in Scheme 1. To join the

Scheme 1. Reaction Procedure for the Synthesis of Compounds 2–6



spermine chain to the DBP scaffold, intermediate **7**⁶ was condensed in the presence of propylphosphonic anhydride (PPAA) with **8–12**, which were previously synthesized through an automated procedure (see SI), to give **13–17**. This protocol for amidation via mixed phosphoric anhydrides^{6,10} consents an excellent selectivity for *N*- versus *O*-(phenolic) acylation. Deletion of *tert*-butyloxycarbonyl (Boc) groups with trifluoroacetic acid (TFA) in CH_2Cl_2 gave the final compounds **2–6** as trifluoroacetate salts. All compounds were tested in biological assays using their TFA salt forms.

Initially, compounds **2–6** were studied by a thioflavin-T (ThT)-based fluorometric assay, an *in vitro* test that is commonly used to monitor $A\beta$ fibril formation and evaluate the inhibitory potency of new potential antiaggregating agents.^{11,12} Unfortunately, a preliminary analysis showed a significant interference (quenching of the ThT signal in the

presence of preformed amyloid fibrils), which strongly impacted the validity of the results. Thus, an orthogonal method, i.e., a previously optimized electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS) approach, was used.¹³ Since during $A\beta$ fibril formation the amount of $A\beta$ monomers ($A\beta_{42m}$) progressively decreases because of their inclusion into the growing oligomeric species, the MS approach allows to monitor $A\beta$ assembly and its inhibition by monitoring the changes in the amount of $A\beta_{42m}$ over time. In detail, amyloid aggregation was studied by evaluating the $A\beta_{42m}$ decrease after 24 h incubation at 30 °C in the presence and absence of inhibitor, using reserpine as internal standard. In the absence of any inhibitor, a progressive decrease in the monomer content is observed, due to inclusion of $A\beta_{42m}$ into growing stable oligomers¹⁴ (residual $A\beta_{42m}$ after 24 h incubation = 17.1%; Ctrl 24 h vs Ctrl t0, Figure 2).

The study of **2–6** clearly demonstrates a strong influence of the aryl substitution pattern on the ability to prevent $A\beta_{42}$ self-assembly process. Indeed, when $A\beta_{42}$ was coincubated with the catechol derivative **2** at 10 μM , residual $A\beta_{42m}$ was 53.0%, meaning that **2** significantly retarded monomer inclusion into

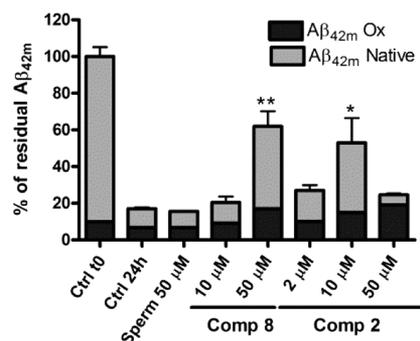


Figure 2. Inhibition of $A\beta_{42}$ aggregation by **2**, **8**, and spermine (Sperm) as determined by ESI-IT-MS. The $A\beta_{42}$ monomer ($A\beta_{42m}$) content in the absence (Ctrl) of inhibitor was considered as 100%. The $A\beta_{42m}$ content is displayed as the sum of the native ($A\beta_{42m}$ Native) and oxidized ($A\beta_{42m}$ Ox) forms of $A\beta_{42}$. * $p < 0.05$, ** $p < 0.01$, versus Ctrl 24 h (total $A\beta_{42m}$); Dunnett's multiple comparison test.

the growing fibrils (Figure 2). Any modification of the catechol unit of **2**, either the removal or masking into a methoxy-function of one or both hydroxyl substituents, as in **3–6** and in **1**, resulted in a complete loss of the antiaggregating efficacy (inhibition < 10% for **3–6** and **1**). This clearly highlights the importance of the catechol moiety of **2** in amyloid recognition.

To verify if the appropriately decorated DBP scaffold is, *per se*, sufficient to determine **2**'s antiaggregating efficacy, the catechol fragment **8** was also studied. In the same conditions, **8** did not show any significant inhibition of the aggregation process (residual $A\beta_{42m}$ at 24 h = 20.5%, not significantly different from Ctrl 24 h).

To assess that inhibition was concentration dependent, lower (2 μ M) and higher (50 μ M) concentrations of **2** were also assayed, while, due to the lack of activity at 10 μ M, **8** was assayed only at 50 μ M. When tested at 50 μ M, **8** showed a significant antiaggregating activity (residual $A\beta_{42m}$ at 24 h = 61.9%; inhibition of $A\beta_{42}$ aggregation = 54.1%). As expected, a lower inhibitory activity was detected when **2** was assayed at 2 μ M (residual $A\beta_{42m}$ = 27.0%, inhibition of $A\beta_{42}$ aggregation = 12.0%), while an unexpected loss of antiaggregating efficacy was observed at 50 μ M.

We sought to also verify if the spermine tail of **2** had by itself the ability to limit $A\beta$ fibrilization. As shown in Figure 2, no significant effect was observed after treatment with 50 μ M spermine, proving that the polyamine alone was not able to affect $A\beta_{42}$ aggregation.

The peculiar antiaggregating profile of **2** prompted us to deepen insight of its mode of action at a molecular level. Previous studies on the natural polyphenol myricetin showed it to inhibit $A\beta_{42}$ aggregation both by preventing the inclusion of the native monomers ($A\beta_m$ Native) into the fibrils and by leading to the formation of an oxidized form of $A\beta$ ($A\beta_m$ Ox), which is known to be less prone to aggregation.¹⁴ Myricetin pro-oxidant properties toward $A\beta_{42}$ were explained by the well-accepted attitude of polyphenols to act as either antioxidant or pro-oxidant agents, depending on environmental conditions.¹⁵ The residue M35 was identified as the specific site of oxidation.¹⁶

With these concepts in mind, we sought to assess if oxidizing mechanisms could partially mediate **2**'s antiaggregating activity. As shown in Figure 2, a small percentage of $A\beta_{42}$ Ox is always present in $A\beta_{42}$ commercial samples (~10%, Ctrl t0), and in agreement with the $A\beta_{42}$ Ox lower inclination to aggregate, the initial content of the oxidized $A\beta$ just slightly decreases after 24 h incubation.¹⁴

Compared to the control sample, both **2** and **8** induced a significant dose-dependent increase of $A\beta_{42m}$ Ox, with **2** being significantly more effective. In particular, concerning fragment **8**, no significant effect was detected at 10 μ M, while at 50 μ M a significant increase of $A\beta_{42m}$ Ox was observed (2.56 times higher than Ctrl 24 h). In the same settings, **2** at 2, 10, and 50 μ M determined an increase of $A\beta_{42m}$ Ox of 1.46, 2.14, and 2.79 times compared to Ctrl 24 h, respectively. On this basis, it might be concluded that the antiaggregating ability of **2** (as well as of **8**) is complemented by an oxidizing action. However, the oxidizing mechanism, even if significant, contributes to the overall inhibitory activity only at a low extent (less than 10%).

Oxidative stress is a prominent feature of AD, and a strong correlation exists between ROS overproduction and $A\beta$ toxicity.¹⁷ Interestingly, a wide body of evidence has recently emerged that provides clues to link this crucial partnership to the mitochondrial compartment.¹⁸ On this basis, we sought to

verify if $A\beta$ oxidation induced by **2** derives from a nonspecific pro-oxidant effect. Accordingly, **2** and derivative **3**, which conversely does not cause any significant $A\beta$ oxidation (MS analysis), were tested *in vitro* to evaluate their redox profile.

After treatment of T67 cells with **2** and **3** at 1–10 μ M, the fluorogenic probe MitoSOX Red was used to selectively measure mitochondrial ROS formation.

The mitochondrial ROS measured by MitoSOX indicated a slight dose-dependent pro-oxidant activity of both **2** and **3** (Figure 3). As first outcome, this result corroborated the

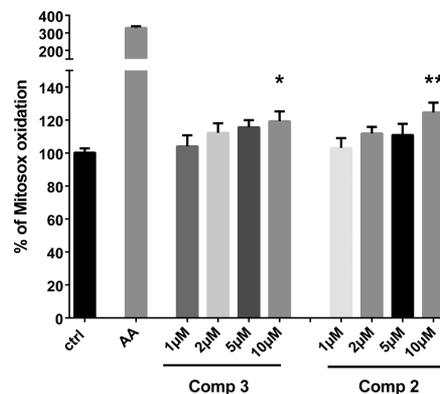


Figure 3. Mitochondrial superoxide production as determined by MitoSOX fluorescence in T67 cell line. Cells were treated with different concentrations of **2** and **3** for 4 h. Antimycin A (10 μ M) was used as positive control. Statistically significant values relative to control are indicated (one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test). * $p \leq 0.05$, ** $p \leq 0.01$ ($n = 8$).

intracellular uptake and mitochondria targeting of these compounds. Second, the redox profile in T67 cell line of **2** and **3** does not parallel the pro-oxidative activity on $A\beta$ found in MS-based experiments. Indeed, **3**, which is endowed with the same moderate pro-oxidant effect of **2** on T67 mitochondria, did not affect $A\beta$ aggregation either directly or contributing to $A\beta$ oxidation. On this basis, we might speculate that $A\beta$ oxidation requires a specific catechol-driven molecular interaction and cannot be simply ascribed to pro-oxidative properties, *per se*.

As we previously found that compound **1** was able to decrease ROS formation induced by *tert*-butyl hydroperoxide,⁶ we tested the redox properties of **2** and **3** in T67 cells treated with this radical initiator. Notably, while **1** confirmed its antioxidant efficacy, **2** and **3** did not show significant antioxidant properties (Figure S1). This observation confirms the aromatic substitution pattern to have a key role also in determining the redox behavior.

To gain further insights into the binding mode of **2** to $A\beta$, molecular dynamic (MD) simulations were performed on **2**, intermediate **8**, and spermine. During the whole MD simulation (600 ns) performed with helical monomers of $A\beta_{42}$, **2** kept a compact conformation (the end of the spermine tail located close to the catechol ring) and formed several H-bonds by means of the catechol moiety and spermine tail, mostly with the surrounding α -helices of $A\beta_{42}$ monomers (Figure 4a; for binding details see SI). Conversely, **8** in complex with $A\beta_{42}$ (Figure S2a; for binding details see SI) established a much smaller number of H-bonds than **2**, but a new H-bond with the charged nitrogen of its piperidone ring is observed. In this case, helices are mostly in their unfolded form (they were converted

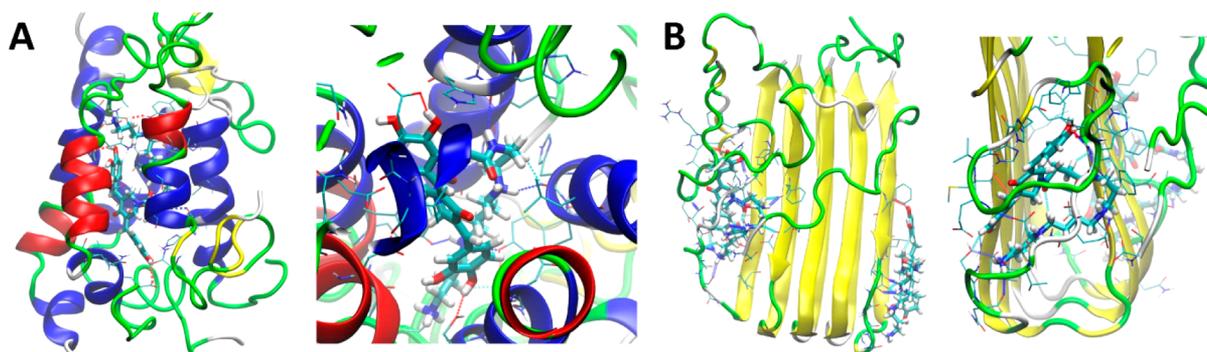


Figure 4. (A) Complex of **2** and five helical monomers of $A\beta_{42}$ after 600 ns MD simulation. Left panels, side view; right panels, top view. Colors used: α -helix, blue; π -helix, red; β -sheet and β -bridge, yellow; β -turn, green; coil, white. Compounds are shown as thick sticks, while amino acid residues in the vicinity of compounds are shown as thin sticks. Hydrogen bonds are shown as dashed lines. (B) Complex of **2** and a fibril of $A\beta_{42}$ after 600 ns MD simulation. Two copies of each compound were located at each side of the fibril.

into β -turns) with a small number of π -helices (two helix turns).

Spermine (Figure S2b), which is highly positively charged, established H-bonds with the $A\beta$ helices, but interestingly, its final position is much different from that observed for **2** and **8**. In particular, spermine is reoriented perpendicularly to the main axes of $A\beta$ helices from its parallel initial position. The whole structure is much less compact than those observed for **2** and **8**, and the helices are not parallel to each other with large distances among them.

We also performed 600 ns MD simulations of the interaction between **2**, **8**, and spermine and a fibril composed of five $A\beta_{42}$ monomers. Each compound was placed at both ends of the fibril to test both sides since they may behave differently. The distance between both compounds was large enough to have no contact to one another and also changes made at one end of the fibril did not propagate to the other end. The final conformation of **2** at both ends of the fibril is compact (Figure 4b) and similar to the conformation observed in a helix bundle (Figure 4a). Both copies are positioned perpendicularly to the β -sheet of the amyloid fibril. Intermediate **8** forms a much smaller number of H-bonds than **2** (Figure S3a) at both ends of the fibril and establishes π - π interactions with three amino acids. At one end of the fibril **8** is positioned parallel to β -sheet, while at the other end it is positioned perpendicularly. Moreover, spermine binds to $A\beta_{42}$ fibril in the extended conformation (Figure S3b), creating several H-bonds with main and side chains of the amino acids.

The interaction pose of spermine is completely changed (from parallel to perpendicular to amyloid β -sheets) when it is linked to the DBP moiety as for **2**. Such perpendicular pose and the large number of interactions of **2** might be responsible for its antiaggregating activity. However, at higher concentration, intermolecular interactions between two compounds at the same end of the fibril might occur leading to more extended conformations, which might cause the observed drop in activity.

Noteworthy, MD simulations showed only for compound **2** a close contact of the catechol moiety with M35 additionally involving residue H14. This was observed both in helix bundle and in complex with the amyloid fibril (Figure S4). The spermine tail of **2** seems to contribute to the proper alignment of the three-ring system and to the stabilization of **2**'s active conformation. Thus, a strategic functional role emerges for spermine in addition to its vehicle properties.

Motivated by the promising *in vitro* results, we investigated the potential neurotoxicity of **2** and **8** and defined a suitable range of concentrations for investigating their biological profile in a cellular context. For these studies, we chose mixed rat neuronal cultures, a validated model to study the neuroprotective efficacy of antiaggregating compounds against $A\beta$ -induced neuronal death.¹⁹ To examine neuronal toxicity, mixed cultures of cortical cells were exposed to **2** or **8** at 0.25–50 μ M for 48 h, and neuronal damage was quantitatively assessed by counting dead neurons stained with Trypan blue. Both **2** and **8** were not toxic up to 50 μ M (Figure S5).

Thus, we evaluated the neuroprotective effects of compounds **2** and **8** against $A\beta$ -induced toxicity. In our model, $A\beta$ oligomers induced a substantial increase in the number of dead neurons (about 250%) after 72 h of exposure to $A\beta_{42}$ (1 μ M). To assess whether **2** exerts neuroprotective effects against $A\beta$ toxicity, 100 μ M $A\beta_{42}$ samples were incubated for 72 h in the absence or in the presence of a 5-fold molar excess of **2** or **8** and then added to neuronal cultures for additional 72 h (final concentration of **2** or **8** = 5 μ M). In control cultures, the number of dead neurons was 27.7 ± 0.9 . This number increased to 71.7 ± 3.1 after treatment with preincubated $A\beta_{42}$. Interestingly, dead neurons significantly decreased to 46.5 ± 2.4 in cultures challenged with $A\beta$ oligomers coincubated with **2**. The same result was not observed for compound **8** (Figure 5). Compounds **2** and **8** were also added to neuronal cultures treated with previously formed $A\beta$ oligomers. Again, a reduction in $A\beta$ toxicity was observed for **2**, and not for **8**, even if the concentration required (25 μ M) was significantly higher. Overall, these data suggest that **2** combines major antiaggregating effects to an appreciable neuroprotective activity against $A\beta$ toxicity.

In conclusion, mitochondrial $A\beta$ is emerging as a relevant facet of the $A\beta$ -driven AD network. Hence, we designed a small set of spermine conjugates of variously decorated DBP motives, in the attempt to specifically convey antiaggregating properties to this cellular compartment. Interestingly, substituents on the aromatic moieties allowed strategic tuning of the pharmacological profile, as only the derivative **2** efficiently inhibited $A\beta$ fibrilization. This points to the catechol motif as a key recognition fragment in amyloid binding. In mixed cultures of cortical cells, compound **2** did not exert any significant toxicity up to 50 μ M and showed neuroprotective properties against $A\beta$ toxicity. Most importantly, our study unravels a key functional role for the polyamine motif in the $A\beta$ recognition process as,

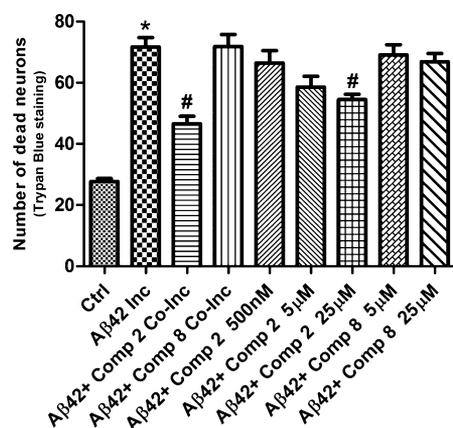


Figure 5. Effects of **2** and **8** on A β toxicity as determined by cell counting after trypan blue staining. Cortical neurons were treated with compounds coincubated with A β ₄₂. Alternatively, compounds were administered to neuronal cultures separately from A β oligomers previously formed after a 72 h incubation. Cell counts were performed in three random microscopic fields/well. Values are the means \pm SEM of nine determinations. * p < 0.05 vs control (Ctrl), # p < 0.05 vs A β incubated alone (one-way ANOVA + Bonferroni's test).

for polyamine derivative **2**, the antiaggregating and neuroprotective effects were significantly boosted. This experimental readout, which was supported by computational evidence, adds new value to the polyamine's favorable contribution. Based on these findings, compound **2** emerges as a promising molecule for neuroprotectant lead discovery. Moreover, this study suggests that polyamine conjugation may represent a valuable strategy to decipher the molecular mechanisms potentially involved in mitochondrial A β injuries.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsmchemlett.6b00339](https://doi.org/10.1021/acsmchemlett.6b00339).

Details for synthesis, biological studies, computational studies, and supplementary figures (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AD, Alzheimer's disease; A β , amyloid β ; DBP, 3,5-dibenzylidene-piperidin-4-one; PPAA, propylphosphonic anhydride; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; Th-T, thioflavin-T; ESI-IT-MS, electrospray ionization-ion trap-mass

spectrometry; A β _{42m}, amyloid β monomers; A β _m Native, amyloid β native monomers; A β _m Ox, amyloid β oxidized monomers; ROS, reactive oxygen species; MitoSOX, mitochondrial superoxide; MD, molecular dynamics

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