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Research Article

Structure-Activity Relationships for a Series of Compounds that Inhibit Aggregation of the Alzheimer's Peptide, Aβ42

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Inhibiting aggregation of the amyloid-beta (Aβ) peptide may be an effective strategy for combating Alzheimer's disease. As the high-resolution structure of the toxic Aβ aggregate is unknown, rational design of small molecule inhibitors is not possible, and inhibitors are best isolated by high-throughput screening. We applied high-throughput screening to a collection of 65 000 compounds to identify compound D737 as an inhibitor of Aβ aggregation. D737 diminished the formation of oligomers and fibrils, and reduced Aβ42-induced cytotoxicity. Most importantly, D737 increased the life span and locomotive ability of transgenic flies in a Drosophila melanogaster model of Alzheimer's disease (J Biol Chem, 287, 2012, 38992). To explore the chemical features that make D737 an effective inhibitor of Aβ42 aggregation and toxicity, we tested a small collection of eleven analogues of D737. Overall, the ability of a compound to inhibit Aβ aggregation was a good predictor of its efficacy in prolonging the life span and locomotive ability of transgenic flies expressing human Aβ42 in the central nervous system. Two compounds (D744 and D830) with fluorine substitutions on an aromatic ring were effective inhibitors of Aβ42 aggregation and increased the longevity of transgenic flies beyond that observed for the parent compound, D737.

Key words: amyloid-beta aggregation inhibitors, biologic screening, chemical biology, chemical structure, drug discovery

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Alzheimer's disease (AD) is a progressive and fatal brain disorder afflicting over five million Americans[a]; yet there are currently no effective treatments that target the underlying molecular causes of the disease (1). Pathologically, the AD brain at end stage is characterized by atrophy of the hippocampus and cerebral cortex, and an accumulation of extracellular proteinaceous plaques composed of amyloid fibrils that result from the uncontrolled aggregation of the amyloid-beta peptide (Aβ). Because the appearance of insoluble plaque is tightly linked to neurotoxicity and disease (2–9), amyloid fibrils were initially thought to be the molecular culprit responsible for AD. Recent studies, however, indicate a more decisive correlation between the levels of soluble Aβ oligomers and the extent of synaptic loss and cognitive impairment (10–14). Irrespective of the exact structure and oligomeric state of the toxic species, the overall link between AD and Aβ aggregation suggests that inhibitors of the aggregation process may lead to effective therapeutics (14–25).

Structural studies of Aβ have advanced significantly in recent years (26–30). These studies provide a foundation for structure-based design of compounds that bind Aβ oligomers and/or inhibit their formation (31). However, as the high-resolution structure of the toxic Aβ oligomer is not known, high-throughput screening (HTS) followed by analysis of structure-activity relationships (SAR) remain productive approaches for identifying inhibitors of Aβ aggregation.

In previous studies, we described a novel high-throughput method to enable rapid screening of compounds for their abilities to inhibit the aggregation of Aβ42 (24). More recently, we implemented this screen on a library of 65 000 compounds, and discovered several small molecules that reduced aggregation substantially. Among these 'hits', a compound designated **D737** ($C_{25}H_{20}N_2O$, Figure 1) was studied in depth and shown to (i) inhibit the formation of Aβ oligomers, (ii) reduce Aβ-induced cytotoxicity, and (iii) enhance the longevity and climbing ability of transgenic fruit flies expressing human Aβ42 in their central nervous system (32).

Compound **D737** has several properties considered important for Aβ binding. These include hydrophobicity and the presence of aromatic groups (15–22). For this study, we probed structure/activity relationships for a small collection of eleven commercially available analogues of the original HTS hit (Figure 1). This study has two goals: (i) To identify

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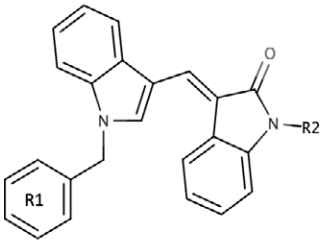

Name	R1	R2
D737:	Ph	CH_3
D830:	Ph(*m*-F)	CH_3
D745:	Ph(*p*-Br)	CH_3
D831:	Et	CH_3
D744:	Ph(*p*-F)	CH_3
D519:	Ph	H
D979:	Ph(*p*-F)	Ph
D742:	Ph(*o*-F)	CH_3
D739:	Ph(*o*-Cl)	CH_3
D746:	Ph(*p*-Cl)	CH_3
D1079:	Ph(*m*-F)	Ph
D999:	H	H

Figure 1: Compound **D737** and its analogues.

some of the chemical features of **D737** that are important for activity; and (ii) To search for analogues that might be more active than the original **D737** molecule. Our results show that alterations of a phenyl group at one end of **D737** and a methyl group at the other end both affect activity. Two compounds (**D744** and **D830**) with fluorine substitutions on an aromatic ring inhibited Aβ42 aggregation and increased the longevity of transgenic flies beyond that observed for compound **D737**.

Methods and Materials

Compounds

D737 and analogues were purchased from ChemDiv (San Diego, CA, USA). All compounds were first tested for intrinsic fluorescence at 512 nm (green fluorescent protein, GFP wavelength) and 570 nm (MTT wavelength), and fluorescence was negligible. DMSO concentration did not exceed 1% in any experiments.

Aβ42-GFP fusion screen

As described in previous work (24), *E. coli* strain BL21 (DE3) harboring a vector encoding the Aβ42-GFP fusion protein was grown in LB supplemented with 35 mg/mL kanamycin. When cultures reached an OD_{600} = 0.8, 100 μL of culture was transferred to the wells of 96-well plates. **D737** analogues were added to a final concentration of 50 μM, and protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Samples were incubated with gentle agitation at 37 °C. Following incubation for 5 h, the fluorescence of each well was measured at 512 nm (excitation 490 nm) using a Varioskan plate reader. Results represent the average of three wells.

Synthetic peptide

Aβ42 peptide was purchased from the Keck Institute at Yale University and purified on a C4 reverse-phase column (Vydac). After purification, the peptide was snap frozen and lyophilized. Monomeric samples were prepared by adding trifluoroacetic acid (TFA) and sonicating for 15 min. Residual TFA was removed by hexafluoroisopropanol and argon blow.

Cell toxicity assays

Rat pheochromocytoma (PC12) cells were cultured on collagen-coated-tissue-treated Petri dishes in 5% CO_2 at 37 °C in complete growth media (82.5% RPMI, 15% horse serum and 2.5% fetal bovine serum – ATCC). The cells were plated in 96-well plates to a concentration of 10 000 cells per well and allowed to attach to the plate overnight before adding peptide. Synthetic Aβ42 peptide at 200 μM was preincubated in PBS for 24 h in the presence or absence of inhibitors. Aβ42 concentration was 20 μM, and small molecule concentrations were 50 μM. Following this incubation, 10 μL Aβ42 (with or without compound) was added to cells. After 24 h at 37 °C, cell viability was evaluated using the MTT assay according to the supplier's instructions (Roche, Branchburg, NJ, USA). The lane marked 'cells' indicates the viability of the PC12 cells without added peptide. This positive control is normalized to 100%. The lane marked 'DMSO' is the negative control showing the reduced viability of cells that received Aβ42, but no added compound.

Fly longevity assay

Male flies carrying elav-Gal4 (on the X chromosome) were crossed with female flies carrying Aβ42 under UAS GAL control to produce female progeny expressing Aβ42 in the central nervous system. Positive control flies were female carriers of elav-Gal4, which do not express peptide. Flies were reared at 29 °C on medium with 20 μM **D737** analogues or an equivalent amount of DMSO. For each class, vials containing 20 female flies each were collected and fed fresh food twice a week. The number of viable flies was recorded daily posteclosion. Survival rates were analyzed using Kaplan–Meier statistics. Medial survival represents the day when 50% flies remain alive, and the student *t*-test was used to generate p values.

Fly-climbing assay

Locomotive ability was assayed as described in reference (33). Ten-centimeter vials containing 20 flies each were tapped gently on the table. The number of flies that climbed to the top of the vial was recorded after 18 seconds. The fraction of flies that climbed to the top of the vial after 18 seconds was recorded two to three times per week.

Results and Discussion

Analogues of D737 inhibit aggregation

In previous work, we described a high-throughput screen to search for compounds that inhibit the aggregation of Aβ (24). This screen uses GFP as a reporter for the solubility (non-aggregation) of Aβ: Briefly, the 42-residue alloform of Aβ is linked upstream of GFP, and the Aβ42-GFP fusion protein is expressed in *E. coli*. In the absence of inhibition, the Aβ42 portion of the fusion aggregates rapidly and causes the entire fusion protein to misfold and aggregate into an insoluble precipitate that does not fluoresce. However, inhibition of Aβ42 aggregation allows GFP to fold into its native green fluorescent structure (34). Compounds such as **D737**, which inhibit Aβ aggregation, yield green fluorescence, while compounds that are inactive (or toxic) do not produce fluorescence (24,34,35).

We used this assay to test analogues of **D737** for their efficacy as inhibitors of Aβ aggregation. Assays were performed using *E. coli* cells transformed with a plasmid directing expression of the Aβ42-GFP fusion protein, as described previously (24,34,35). Isopropyl-β-D-thiogalactopyranoside was added to induce expression, and cells were grown in 96-well plates containing 50 μM compound or DMSO control. After 5 h of growth at 37 °C, GFP fluorescence was measured (Figure 2). Higher fluorescence indicates a compound inhibits Aβ aggregation, thereby enabling the folding and fluorescence of the Aβ42-GFP fusion (24,34,35). As shown in Figure 2, most of the analogues inhibit aggregation, albeit at lower levels than **D737**. One compound, **D830**, has similar activity as the **D737** parent compound.

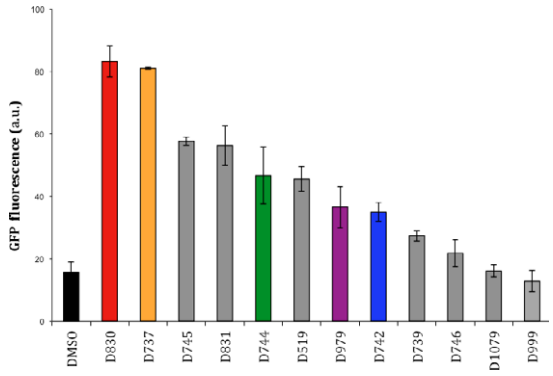

Figure 2: Fluorescence was measured for *E. coli* cells expressing the Aβ-green fluorescent protein fusion protein. Compounds are color-coded consistently for comparison of Figures 2–6.

Our SAR studies focused on two parts of the **D737** scaffold: the phenyl group at R1 and the methyl group at R2 (Figure 1).

Modification or replacement of the phenyl group at R1

Halogen substitutions on aromatic rings are known to affect the binding properties of small molecules (36,37). To probe the effect of halogen substitutions on the inhibitory activity of **D737**, fluorine, chlorine and bromine were incorporated at the ortho, meta and para positions of the R1 aromatic ring. As shown in Figure 2, while a fluorine substitution to the R1 phenyl group in the meta position (**D830**) retains essentially the same inhibition activity as **D737**, a para substitution (**D744**) slightly decreases inhibition of aggregation and inhibition is greatly reduced by a fluorine substitution in the ortho position (**D742**) of R1. The position of fluorine substitutions greatly affects the inhibition activity of **D737** (m–F > p–F > o–F), which suggests that there are specific interactions that allow the aromatic R1 to fit into the chemical landscape of Aβ assemblies.

Next, we assayed compounds with bromine or chlorine on the phenyl group. Chlorine substitutions to the R1 phenyl group in the ortho position (**D739**) and para position (**D746**) do not significantly inhibit aggregation, which suggests that the larger chlorine atom may be unfavorable for peptide binding and inhibition. Given that chlorine substitutions are less active, one would expect that a larger bromine substitution would also be unfavorable for inhibition. Surprisingly, **D745** (p-Br) is a very effective inhibitor. This suggests that in addition to size, factors such as solubility, electronegativity and sterics may influence inhibitory activity.

Lastly, in compound **D831**, an ethyl group replaces the aromatic phenyl group of **D737**. Although **D831** still shows an ability to inhibit Aβ aggregation, it permits less GFP fluorescence than **D737**, which suggests that the R1 phenyl ring is indeed involved for interrupting critical protein–protein interactions that allow Aβ assembly. These results demonstrate that both the identity and the position of halogen atoms on the R1 aromatic ring influence the activity of analogues of **D737**.

Deletion or replacement of a methyl group

To determine whether the methyl group in the R2 position is optimal for activity, we replaced it with yet another aromatic phenyl group or hydrogen. In compound **D519,** the R2 methyl group is replaced with hydrogen and inhibition activity is slightly decreased. The diminished activity of

compound **D519** suggests that the methyl group on **D737** may be more favorable for disrupting aggregation.

The methyl group of R2 is replaced with a phenyl group in compounds **D979** and **D1079**; additionally, the R1 components of **D979** and **D1079** have p-F and m-F substitutions, respectively. This comparison serves a dual purpose to determine whether increased aromaticity favors or disfavors binding, and to confirm whether halogen additions to the aromatic ring in the R1 component do indeed require precise orientations. Interestingly, **D1079** (*m*-F) allows for slightly less GFP fluorescence than Aβ42-GFP fusion without drug. On the other hand, compound **D979**, which has a phenyl group in the R2 position and a p-F substitution in the R1 component, inhibits Aβ aggregation. Somehow, the fluorine in the para position allows for more favorable protein–drug interactions and inhibition of aggregation. In comparison, **D745**, which has only one addition to **D737**, p-Br in the R1 component, is an active aggregation inhibitor. This result suggests that those compounds with substitutions in the para position of the R1 could be creating a more favorable environment, critical enough to balance the unfavorable environment created by adding a second aromatic group of R2.

Finally, **D999** replaces both the R1 and R2 components of **D737** with hydrogen, and these substitutions seem to abrogate activity. Overall, we conclude that the aromatic group at the R1 position is critical for inhibition of Aβ aggregation, and a methyl group in the R2 component may be the optimal size for fitting into the Aβ structure (Figure 2).

Analogues of D737 reduce Aβ42 toxicity in neuronal cells

Inhibiting aggregation of the Aβ peptide has been suggested as an approach for reducing cytotoxicity (38–40). Therefore, we probed the ability of **D737** and its analogues to prevent Aβ-induced toxicity in cultured neuronal cells. Because Aβ oligomers are thought to be more toxic than fibrils, we allowed the peptide to aggregate at 37 °C without shaking, conditions known to favor the formation of soluble oligomers (15,16,41).

Synthetic Aβ42 peptide (20 μM) was incubated with 50 μM of **D737** or its analogues for 24 h and then added to cultured PC12 cells (Compounds were also tested alone for toxicity to PC12 cells and showed no apparent toxicity.). Following incubation for an additional 24 h, cell viability was determined by the MTT assay (42). As shown in Figure 3, compounds that inhibited Aβ aggregation in the Aβ42-GFP assay (Figure 2) also reduced Aβ-induced cytotoxicity, thereby increasing cell viability. For example, the parent compound, **D737**, increased cell viability by about 30% at 100 μM and about 20% at 50, 20 or 5 μM concentrations (Figure 3 and Figure S1). Additionally, compounds with fluorine substitutions on the R1 ring, including **D744** (*p*-F) and

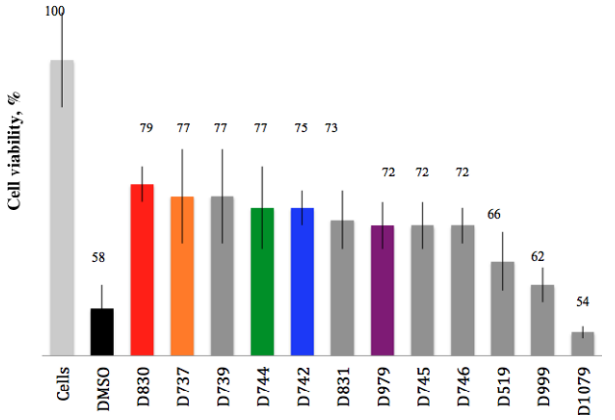

Figure 3: Analogues of compound **D737** rescue PC12 cells from Aβ42-induced toxicity.

D830 (*m*-F), also increase cell viability by 20–30%. In contrast, **D999** and **D1079**, which were less effective at inhibiting aggregation in the Aβ GFP screen (Figure 2), are also less effective at reducing Aβ-induced toxicity (Figure 3).

Although, overall, we observed a good correlation between the ability of a compound to inhibit aggregation in the Aβ42-GFP assay, and its efficacy in reducing Aβ-induced cytotoxicity in PC12 cells (Figure 4), two analogues – **D739** and **D746** – were dramatically less active in one assay than another (Figure 3). For instance, **D746** (p-Cl) was not shown to significantly inhibit aggregation in the screen (Figure 2), but increases cell viability by 8–14% at 100, 50, 20 and 5 μM concentrations (Figure S1). Likewise, **D739** (o-Cl) is effective at high concentrations 21%, 19% and 11% at 100, 50 and 20 μM, respectively, but actually reduces cell viability at 5 μM unlike **D737**, which greatly reduces Aβ-induced toxicity at 5 μM (Figure S1). This result implies that **D739** and **D742** are only effective exceeding stoichiometric levels not employed during the HTS.

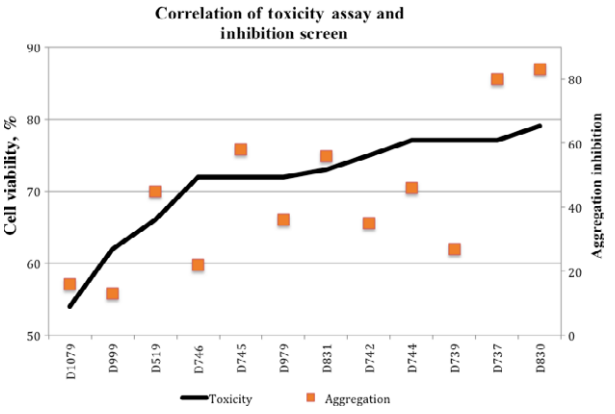

Figure 4: Correlation between the ability of a compound to inhibit Aβ42 aggregation and its efficacy in preventing Aβ42-induced toxicity.

Analogues of D737 increase the longevity of transgenic flies expressing Aβ42

Perhaps the most important activities for SAR studies are those monitored *in vivo*. To test whether analogues of **D737** are active *in vivo*, we used transgenic *Drosophila melanogaster* that express human Aβ42 in their central nervous system. In these flies, Aβ42 is expressed under control of a Gal4-UAS system. Male stocks carry the pan-neuronal elav-Gal4 on their X chromosome, while female stocks are homozygous for the autosomal UAS-regulated Aβ42 transgene. Both the male and female stocks are asymptomatic and can be propagated normally. Crossing these flies, however, produces female progeny that expresses Aβ42 in their CNS and displays disease phenotypes, including reduced longevity and a gradual decline of locomotive ability (43,44).

We used these transgenic flies to assess the activity *in vivo* of **D737** and four analogues. Three of these analogues carry substitutions on the R1 phenyl group: Compounds **D744**, **D830** and **D742** contain fluorine at the *para, meta* and *ortho* positions of this aromatic ring. The fourth compound, **D979**, also has methyl to phenyl substitution at R2, was chose for comparison.

Flies were fed 20 μM compounds (or DMSO as a control) continuously from the embryonic stage through adult life. In our earlier studies, we had determined that this concentration was optimal for **D737** in extending life span, while higher doses slightly impaired longevity of the flies. As summarized in Table 1, all five compounds enhanced longevity beyond the DMSO control. Compound **D737** increased the median life span by 1 week, while **D744**, **D830**, **D979** and **D742** increased life span by 9, 8, 5 and 3 days, respectively (Table 1). As shown in Figure 5, the most effective compound (**D744**) produced a life span curve for the transgenic flies that is fairly close to that of the positive control (female carriers that do not express Aβ42).

Compounds **D742**, **D744** and **D830** differ from one another only by the position of the fluorine on the R1 aromatic ring, yet they have different impacts on life span (Table 1 and Fig. 5). This is consistent with results presented above using Aβ42-GFP to probe inhibition of aggregation (Figure 2), and PC12 cells to measure inhibition of Aβ-induced cytotoxicity (Figure 3). In those assays, compound **D742** was less active than **D744**, **D830** or the parent compound **D737**. Likewise, in the *Drosophila* longevity assay, compounds **D744**, **D830** and **D737** increase median life span by 9, 8, 7 days, respectively, while **D742** increases life span by only 3 days (Table 1).

Table 1: Median survival and p value of Kaplan–Meier curves for transgenic flies treated with **D737**, analogues or the DMSO control

Treatment	Median Survival	p Value
DMSO	34 days	
D744	43 days	0.00146
D830	42 days	0.02365
D737	41 days	0.00484
D979	39 days	0.25604
D742	37 days	0.06431

Compound **D979**, with its methyl to phenyl substitution at R2, does not improve fly longevity significantly (p value = 0.25604). This is consistent with results shown above (Figures 2–4), which suggested that replacing this methyl with an aromatic ring does not increase activity relative to **D737**.

In this study, we learned that those compounds shown previously to inhibit Aβ42 aggregation and reduce Aβ42-induced cytotoxicity also improve the life span of flies expressing Aβ42. More importantly, we showed that our compounds are active *in vivo*, as both the parent compound, **D737**, and its analogues affect the life span and behavior of Drosophila. Remarkably, although compound **D744** (*p*-F) was not the most effective in either the HTS or the cell viability assays, it was the most effective in increasing the life span of transgenic flies. This effect probably occurs because many other factors affect bioavailability when protein is expressed *in vivo* and in higher organisms such as challenges in crossing the blood brain barrier. Possibly, the increased molecular hydrophobicity of **D744** is responsible for improved efficacy.

Analogues of D737 enhance the climbing ability of transgenic flies expressing Aβ42

The locomotive ability of transgenic flies expressing Aβ42 was monitored in the presence or absence of **D737** and the four analogues described above. Flies were fed the indicated compound (or DMSO) continuously from the embryonic stage through adult life. The flies were then tested for locomotive ability every few days. Flies (20 per vial) were gently tapped to the bottom of the vial, and after 18 seconds, the fraction of flies climbing to the top of the vial was recorded. As shown in Figure 6, flies receiving the DMSO control began to show a sharp decline in locomotive ability around 31 days posteclosion. For example after 1 month, only 23% of the control flies had the ability to climb (Figure 6). Compound **D737** and all four analogues improved climbing ability: After 31 days, the following percentages and statistical significances of flies retained the ability to climb: **D737** (45%, p value = 0.0001), **D744** (40%, p value = 0.001), **D830** (40%, p value = 0.018), **D742** (38%, p value = 0.244) or **D979** (33%, p value = 0.034). In agreement with the results on fly longevity (Table 1), **D737**, **D744** and **D830** were more active, while compounds **D742** and **D979** showed the least activity in this assay for mobility in a simple animal model of AD.

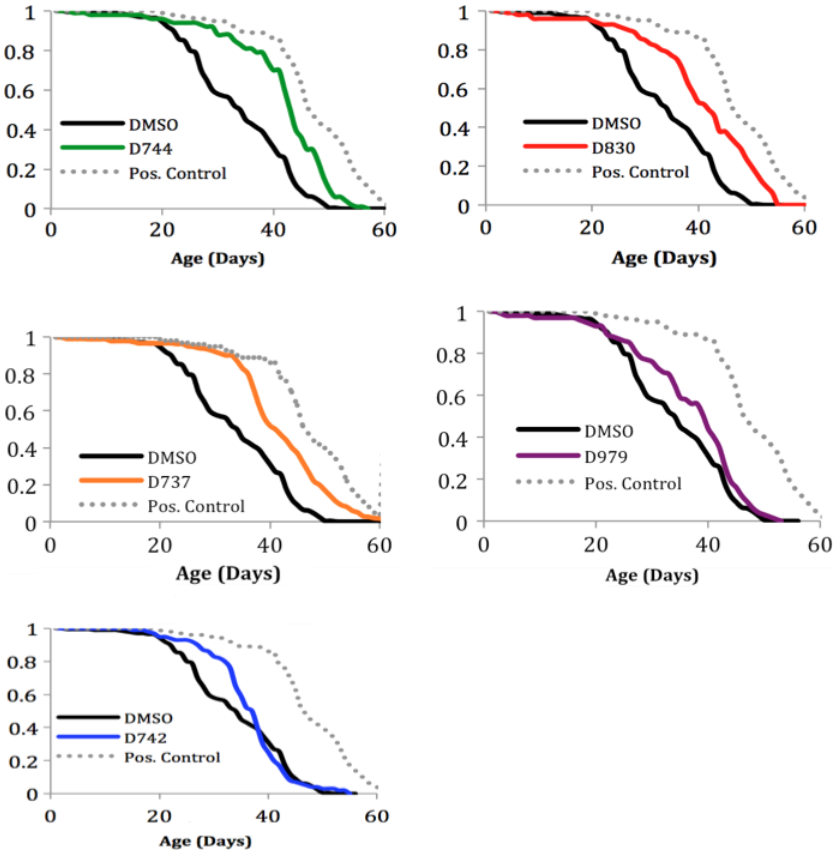

Figure 5: The effect of compound **D737** and analogues on the life span of transgenic *Drosophila* expressing Aβ42 in the CNS.

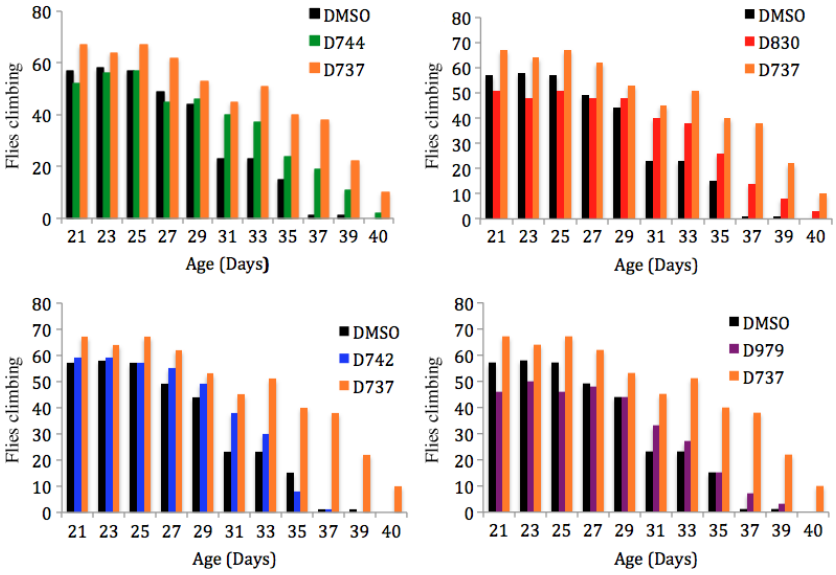

Figure 6: **D737** analogues improve the locomotive ability of transgenic flies expressing Aβ42. Bar graphs represent weeks 3–5.

Conclusions

We chose 11 commercially available analogues to probe the importance of functional groups at the R1 and R2 positions of **D737**, a molecule that had been discovered in a HTS for inhibitors of Aβ aggregation. Five of these compounds (including **D737**) were also tested *in vivo* in a *drosophila* model of AD. Overall, we observed good correlations between inhibition of Aβ42 aggregation, reduction of Aβ42-induced cytotoxicity and improved life span and locomotive ability of flies expressing Aβ42. In particular, assays *in vivo* suggest that a simple substitution

in which fluorine is included on an aromatic ring can improve the efficacy of the original compound in a *drosophila* model of Alzheimer's disease.

Acknowledgments

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Note

[a]http://www.alz.org/downloads/facts_figures_2013.pdf

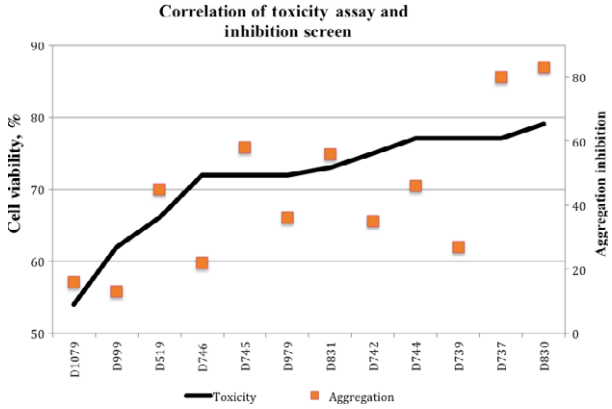
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Analogues of compound **D737** rescue PC12 cells from Aβ42 induced toxicity at concentrations 5-100 μM.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.



Eleven analogues of a novel inhibitor of Aβ42 aggregation (**D737**) were tested. Overall, the ability of a compound to inhibit Aβ aggregation was a good predictor of its ability to reduce Aβ-induced cell toxicity and prolong the life span and locomotive ability of the transgenic fly model for Alzheimer's disease.