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Aftins Increase Amyloid-β_{42}, Lower Amyloid-β_{38}, and Do Not Alter Amyloid-β_{40} Extracellular Production *in vitro*: Toward a Chemical Model of Alzheimer's Disease?

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Abstract

Increased production of amyloid-β (Aβ)$_{42}$ peptide, derived from the amyloid-β protein precursor, and its subsequent aggregation into oligomers and plaques constitutes a hallmark of Alzheimer's disease (AD). We here report on a family of low molecular weight molecules, the Aftins (Amyloid-β Forty-Two Inducers), which, in cultured cells, dramatically affect the production of extracellular/secreted amyloid peptides. Aftins trigger β-secretase inhibitor and γ-secretase inhibitors (GSIs) sensitive, robust upregulation of Aβ$_{42}$, and parallel down-regulation of Aβ$_{38}$, while Aβ$_{40}$ levels remain stable. In contrast, intracellular levels of these amyloids appear to remain stable. In terms of their effects on Aβ$_{38}$/Aβ$_{40}$/Aβ$_{42}$ relative abundance, Aftins act opposite to γ-secretase modulators (GSMs). Aβ$_{42}$ upregulation induced by Aftin-5 is unlikely to originate from reduced proteolytic degradation or diminished autophagy. Aftin-5 has little effects on mitochondrial functional parameters (swelling, transmembrane potential loss, cytochrome *c* release, oxygen consumption) but reversibly alters the ultrastructure of mitochondria. Aftins thus alter the Aβ levels in a fashion similar to that described in the brain of AD patients. Aftins therefore constitute new pharmacological tools to investigate this essential aspect of AD, in cell cultures, allowing (1) the detection of inhibitors of Aftin induced action (potential 'anti-AD compounds', including GSIs and GSMs) but also (2) the identification, in the human chemical exposome, of compounds that, like Aftins, might trigger sustained Aβ$_{42}$ production and Aβ$_{38}$ down-regulation (potential 'pro-AD compounds').

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Keywords

Aftins; Alzheimer's disease; amyloid-β; $A\beta_{38}$; $A\beta_{40}$; $A\beta_{42}$; γ-secretase; γ-secretase modulators; mitochondria

INTRODUCTION

Following its description by Aloïs Alzheimer at the beginning of the 20th century, the neurodegenerative disease bearing the name of the German neuropathologist has been the object of numerous studies. Despite extensive investigation, the causes and mechanisms underlying the initiation and development of this disease still remain poorly understood (reviewed in [1, 2]). There is only a relatively mild consensus on the respective roles and importance of the two best described events associated with Alzheimer's disease (AD), namely (i) the release of amyloid-β (Aβ) peptides by proteolytic processing of the amyloid-β protein precursor (AβPP) and their extracellular aggregation in oligomers and ultimately in plaques [3–5] and (ii) hyperphosphorylation of the microtubule-binding protein tau by various kinases and subsequent intracellular aggregation as paired helical filaments [reviewed in 6–8].

Aβ peptides are the endproducts of two successive proteolytic actions catalyzed first by β-secretase (BACE 1, β-site AβPP cleaving enzyme 1) and then by γ-secretase, a complex of four proteins: presenilin-1/2 (PSEN1/PSEN2) (the catalytic subunit, an aspartyl protease), nicastrin, anterior pharynx-defective 1, and presenilin enhancer 2 (PEN-2) (reviewed in [9]). The action of β-secretase on AβPP leads to a soluble extracellular fragment (sAβPPβ) and a membrane bound fragment (C99 or βCTF, β-carboxyl-terminal fragment). γ-Secretase then acts on this C-terminal fragment of AβPP, leading to the possibly stepwise [10] generation of Aβ peptides of various lengths, such as $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, $A\beta_{43}$, and the AβPP intracellular domain (AICD). $A\beta_{40}$ is the main Aβ peptide. However, there is considerable data showing that generation of $A\beta_{42}$ is strongly correlated with the onset and development of AD. In autosomal dominant forms of early onset AD, which represents less than 1% of all AD cases, mutations affect AβPP, PSEN1, and PSEN2 (reviewed in [11]) and all lead to enhanced production of $A\beta_{42}$ or increase in the $A\beta_{42}/A\beta_{40}$ ratio, a critical factor in the onset of AD [12]. $A\beta_{42}$ is more toxic than $A\beta_{40}$ and this appears to be linked to its higher stability and ability to oligomerize and aggregate in plaques [13].

In a previous article, we reported on the properties of a family of tri-substituted purines, the Aftins (Amyloid-β Forty-Two Inducers) [14]. These low molecular weight compounds induce robust production of $A\beta_{42}$ in cultured cells while leaving $A\beta_{40}$ levels unaffected. They bind specific mitochondrial proteins and reversibly alter mitochondrial structure. They constitute new pharmacological tools to investigate the molecular mechanism underlying the modified $A\beta_{42}/A\beta_{40}$ ratio associated with AD.

We have synthesized and tested a small library of 52 Aftins and related purine analogues. In this article, we report on an optimized Aftin (Aftin-5) which lacks cellular toxicity at high doses, while dramatically modifying extracellular $A\beta_{38}/A\beta_{40}/A\beta_{42}$ production in cultured N2a-AβPP695 and other cells (primary neuronal cells, HEK293 cells expressing AβPP,

normal N2a cells). Aftin-5 triggers massive upregulation of $A\beta_{42}$ and parallel down-regulation of $A\beta_{38,}$ while $A\beta_{40}$ levels remain stable. Intracellular levels of these amyloids remain stable. These effects are sensitive to pharmacological inhibitors of β-secretase or γ-secretase (GSIs) and to a γ-secretase modulator (GSM). We show that upregulation of $A\beta_{42}$ by Aftin-5 is unlikely to be due to reduced degradation by proteases or diminished autophagy. Aftin-5 has no significant effects on mitochondrial functional parameters such as swelling, transmembrane potential loss, cytochrome *c* release, and oxygen consumption (complex I or II activation). Mitochondria ultrastructure is modestly, but reversibly, affected. Aftin-5 thus appears to alter Aβ relative abundance in cells in a fashion similar to that described in the brain of AD patients [15–17]. Aftin-5 therefore constitutes a new pharmacological tool that could be used to investigate this essential aspect of AD in cell cultures. The established cellular system allows (i) the detection of inhibitors of this Aftin-5 induced action (potential 'anti-AD compounds') but also (ii) the identification of other compounds like Aftins that might trigger massive $A\beta_{42}$ production and $A\beta_{38}$ down-regulation (potential 'pro-AD compounds'). Aftin-5 may also be a first step toward the generation of a chemically-induced animal model of AD.

MATERIAL AND METHODS

Synthesis of Aftins and γ-secretase modulator

N6-dimethylaminopurine (N6DA) (1) was obtained from Sigma Aldrich (Lyon, France). Aftins of Fig. 1 were synthesized as follows. Compound (2) was obtained upon heating 2-amino-6-chloropurine with N-methylbenzylamine in 2-propanol. Aftin-1 (3) and Aftin-2 (4) were prepared from compound (2). Aftin-3 (5) was also obtained in a two steps procedure starting from 2-amino-6-chloropurine. Aftin-4 (6) and Aftin-5 (7) were obtained in a three steps procedure starting from 2,6-dichloropurine. (R)-Roscovitine (8) was synthesized as previously described [18]. The 'Torrey Pines' compound (9) was synthesized following a published procedure [19]. Detailed preparation procedures and characterization of these products are provided in the Supplementary data (available online: http://www.j-alz.com/issues/35/vol35-1.html#supplementarydata01). All compounds were solubilized as 100 mM stock solutions in 100% dimethylsulfoxide (DMSO) and diluted just prior to use. Aftin-5 is available from ManRos Therapeutics, 29680-Roscoff, France (E-mail: meijer@manros-therapeutics.com).

Other reagents

DMSO, Nonidet P-40, Tween-20, bovine serum albumin (BSA), Na_2CO_3, $NaHCO_3$, citric acid monohydrate, $Na_2HPO_4.2H_2O$, H_2O_2, thiorphan, captopril, quinaprilat, phosphoramidon, digitonin, and rapamycin were purchased from Sigma Aldrich. Protease inhibitors mix (Complete) was obtained from Roche (Boulogne-Billancourt, France). Streptavidin-horseradish peroxidase (HRP) conjugate was purchased from Thermo Scientific Pierce (Brebières, France). o-Phenylenediamine dihydrochloride (OPD) tablets were from Invitrogen (St. Aubin, France). DAPT (N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester), BMS299897, 3-methyladenine and bafilomycin A1 were obtained from Tocris Bioscience (Lille, France). Paraformaldehyde (16% stock) was

purchased from Electron Microscopy Sciences (EMS, Hatfield, USA). β-secretase inhibitor IV was from Calbiochem (Molsheim, France).

Cell culture

N2a-AβPP695 cells (Rockefeller University) were cultured in Dulbecco's minimal essential medium (DMEM)/OptiMEM (1 : 1 v/v; Gibco, InVitrogen, St. Aubin, France) containing 0.2 mg/mL Geniticin (Geneticin Selective Antibiotic, Gibco) and 5% fetal bovine serum (FBS, Gibco, EU approved origin) in a humidified, 5% CO_2 incubator. Cells were split routinely every 4/5 days. They were first rinsed with PBS (Gibco) and detached from the plate bottom using 4 mL Versene (Gibco) at room temperature (RT) for 3–4 min. Eight mL of fresh medium were added to the cell suspension, and the mix was centrifuged for 3 min at 1,000 rpm. The cell pellet was resuspended in fresh medium before seeding (1/10 dilution) in new flasks.

Sample preparation (extracellular amyloids)

N2a-AβPP695 cells were seeded at 10,000 cells/well in a 96 well plate with modified media (0.5% FBS) and incubated overnight. Cells were treated with fresh media and different compounds (equal quantity of DMSO), then incubated for 18 h in a humidified, 5% CO_2 incubator. The plate was finally centrifuged to remove cell fragments before collecting supernatant samples for amyloids levels determination (see below).

Sample preparation (intracellular amyloids)

N2a-AβPP695 cells were seeded at 2×10^6 cells in a T25 flask with modified media (0.5% FBS) and incubated overnight. Cells were treated with fresh media and different compounds (equal quantity of DMSO), then incubated for 18 h in a humidified, 5% CO_2 incubator. The supernatants were collected and centrifuged before testing in an ELISA capture assay. Cells were washed with PBS (Gibco) and then scraped in 1.5 mL of PBS + protease inhibitors. Cells were centrifuged (1,000 rpm for 3 min at 4°C) and cell pellets were resuspended in PBS + protease inhibitors + 1% Nonidet P-40 and sonicated. After centrifugation (14,000 rpm for 15 min at 4°C), amyloid concentrations were determined in the supernatants by an ELISA capture assay (see below).

ELISA capture assay

$A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ levels were measured in a double antibody sandwich ELISA using a combination of monoclonal antibody (mAb) 6E10 (SIG-39320, Covance, Eurogentec, Seraing, Belgium) and biotinylated polyclonal $A\beta_{38}$ [20], $A\beta_{40}$, or $A\beta_{42}$ [21] antibodies (provided by Dr. P.D. Mehta, Institute for Basic Research in Developmental Disabilities, Staten Island, USA). Briefly, 100 µl mAb 6E10 diluted in carbonate-bicarbonate buffer (0.015 M Na_2CO_3 + 0.035 M $NaHCO_3$, pH 9.6), was coated in the wells of microtiter plates (Maxisorp, NuncTher-moFisher Scientific, Illkirch, France) and incubated overnight at 4°C. The plates were washed with PBST (PBS containing 0.05% Tween-20) and blocked for 1 h with 1% BSA in PBST to avoid non-specific binding. Each of the $A\beta_{38}$, $A\beta_{40}$, or $A\beta_{42}$ antibodies did not cross-react with the other amyloid peptides (data not shown). Standard

curves were prepared with synthetic amyloids and each of the three antibodies. Fitting was performed using a 4 parameters sigmoid equation (SigmaPlot, Systat).

Following a washing step, experimental samples were added into the wells and incubation was carried out for 2 h at RT and overnight at 4°C. Plates were washed before incubation with biotinylated polyclonal antibody diluted in PBST + 0.5% BSA at RT for 75 min. After a washing step, streptavidin-HRP conjugate, diluted in PBS + 1% BSA, was added and incubation was carried out for 45 min at RT. After washing, 100 µl OPD in citrate buffer (0.049M citric acid monohydrate + 0.1M $Na_2HPO_4.2H_2O$ + 1 mL H_2O_2 30%/L) pH 5.0 were added. The reaction was stopped after 15 min with 100 µL 1 N sulfuric acid. The optical density was measured at 490 nm in a microELISA reader (BioTek Instrument, El 800, Gen 5 software).

Cell viability

To measure cell viability, the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 -(4-sulfophenyl)-2*H*-tetrazolium) assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Charbonnières-les-bains, France) was used in the same wells as the capture ELISA assay. Briefly, 20 µL of MTS reagent was added in each well containing 100 µL of media and incubation then proceeded for 3 h (37°C, 5% CO_2, and 95% humidity). Measurements were made at OD 490Δ630 nm using a microELISA reader.

Autophagy assessment

Autophagy was assessed by counting of LC3 foci by immunofluorescence. Briefly, cells were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized using 50 µM digitonin and stained with a monoclonal anti-LC3 antibody (Enzo Life Sciences, Lausen, Switzerland). Images were acquired with a DFC310FX camera on a Leica AF6000 microscope using a 40× NA 0.85 objective (Leica Microsystems). Automated counting of LC3 foci was performed using Image J (NIH).

Mitochondria studies

Swelling, transmembrane potential loss, cytochrome *c* release, oxygen consumption (through complex I or complex II activation), and electron microscopy were carried out as described in details in the Supplementary data (see also http://www.mitologics.com).

RESULTS

Aftins, a family of roscovitine-related purines, dramatically increase production of Aβ42 in N2a cells, but not $A\beta_{40}$

In a search for compounds inhibiting the CK1 kinase [22, 23] and possibly modifying Aβ production in cultured N2a-AβPP695 cells (N2a cells stably expressing human AβPP695), we identified a sub-family of purines, related to the kinase inhibitor roscovitine (Fig. 1, compound 8) [24] and able to decrease the $A\beta_{40}/A\beta_{42}$ ratio. This family of compounds was named Aftins, which stands for Amyloid Forty Two Inducers [14]. Here we have prepared and tested a small library of 52 purines and related compounds. A selection of these

molecules is shown in Fig. 1. N6-substituted purines were synthesized and added at various concentrations to N2a-AβPP695 cells. After 18 h incubation, levels of $A\beta_{40}$ and $A\beta_{42}$ in cell supernatants were determined by an ELISA assay and cell viability was estimated by the MTS assay (see Material & Methods section). A subset of compounds massively increased the production of $A\beta_{42}$ (Fig. 2, upper panel) while $A\beta_{40}$ level remained unaffected (Fig. 2, lower panel). This screen allowed to select the most active Aftins (in terms of $A\beta_{42}$ induction) displaying the lowest cytotoxicity (MTS assay). The $A\beta_{42}$-inducing compounds have all lost the H-bonding potential at N^6 (indicated by an asterisk on roscovitine), which is key to the binding of roscovitine (8) to its kinase targets [25], either through methylation [Aftins-1 (3), -2 (4), -4 (6), -5 (7)] or replacement of the nitrogen atom by an oxygen [Aftin-3 (5)]. This is necessary but not sufficient as Aftins also require a hydrophobic substituent at position 9 [compare inactive compounds N6DA (1) and compound (2) to Aftin-1 (3) and -4 (6)]. Kinase assays showed that Aftins are kinase inactive (data not shown), while the kinase inhibiting roscovitine shows little effect on $A\beta_{42}$ levels (Fig. 2A). As for Aftin-4 [14], running Aftin-5 (10 μM) on the KinomeScan panel of 402/442 kinases revealed no interaction with any of the kinases (Supplementary Table 1). Kinase inhibition activity therefore does not seem to be involved in the biological activity of Aftins.

Non-cytotoxic Aftin-5 upregulates $A\beta_{42}$ and down-regulates $A\beta_{38}$

Among all the compounds tested, Aftin-5 (7), the purvalanol equivalent of Aftin-4 (N^6-methyl-roscovitine), did not show any cytotoxic effects on cells even at the highest concentration tested (100 μM) or at the longest exposure (6 days) (data not shown). Comparison of the effects of Aftin-4 and Aftin-5 on the survival of various cell lines illustrated the lower cell toxicity of Aftin-5 (Table 1). Dose-response curves showed that Aftin-5 gradually induced increasing levels of $A\beta_{42}$ production (up to >900% increase), while the levels of $A\beta_{40}$ remained essentially stable (maximum 40% increase) (Fig. 3A). In contrast, the levels of $A\beta_{38}$ were found to decrease (>70%) in parallel to the increase of $A\beta_{42}$. Time-course experiments showed that $A\beta_{42}$ production reached a plateau about 12 h after Aftin-5 addition and remained stable even up to 48 h (Fig. 3B). These results were confirmed with non-transfected N2a cells, with HEK293 stably expressing AβPPsw (data not shown) and with primary neuronal cell cultures (Mabondzo et al., in preparation). $A\beta_{42}$ production induced by Aftin-4 was bell-shaped, indicative of some cell toxicity at high doses. In contrast, $A\beta_{42}$ production induced by Aftin-5 was linear up to the highest dose tested (150 μM).

Aftin induced upregulation of $A\beta_{42}$ requires active β- and γ-secretases

To evaluate the importance of β-secretase in the effects of Aftin-5, we used a pharmacological inhibitor of β-secretase, β-secretase inhibitor IV. The drug inhibited production of both $A\beta_{40}$ and $A\beta_{42}$ in a dose-dependent manner (data not shown). To investigate the importance of γ-secretases in the effects of Aftin-5, we made use of two potent pharmacological inhibitors of γ-secretases (GSIs), DAPT [26] and BMS299897 [27]. Both drugs dose-dependently inhibited Aftin-5 induced production of $A\beta_{42}$ (Fig. 4). Both drugs also reduced the level of basal $A\beta_{40}$ and $A\beta_{38}$. IC_{50} values ($A\beta_{38}$/$A\beta_{40}$/$A\beta_{42}$: 112/62/43 nM and 88/56/29 nM, for DAPT and BMS 299897, respectively) were in agreement with reported IC_{50} values for these GSIs (25–56 nM and 8–300 nM,

respectively). A similar sensitivity of Aftin-4's effects to γ-secretase inhibitors has been observed (data not shown).

Upregulation of $A\beta_{42}$ and down-regulation of $A\beta_{38}$ by Aftin-5 are counteracted by γ-secretase modulator

To evaluate the mechanism of action of Aftin-5 we next made use of a recently reported γ-secretase modulator (GSM), the 'Torrey Pines' compound [28, 29]. This compound down-regulated $A\beta_{42}$ and $A\beta_{40}$ production, in a dose-dependent manner, while it triggers a parallel upregulation of $A\beta_{38}$ level (Fig. 5A) in N2a-AβPP695 cells as previously reported in HEK293/sw cells (stably expressing Swedish mutant AβPP) [29, 30]. When increasing concentrations of the 'Torrey Pines' compound were added to N2a-AβPP695 cells 1 h prior to 100 μM Aftin-5, a dose-dependent inhibition of Aftin-5 induced $A\beta_{42}$ upregulation and $A\beta_{38}$ down-regulation was observed (Fig. 5B). This shows that Aftin-5 and the GSM 'Torrey Pines' have opposite effects on the production of $A\beta_{38}$ and $A\beta_{42}$ peptides in N2a-AβPP695 cells.

Aftin-5-induced upregulation of $A\beta_{42}$ and down-regulation of $A\beta_{38}$ concerns extracellular excreted amyloids, but not intracellular amyloids

We next measured both extracellular and intracellular levels of the three amyloids in N2a-AβPP695 cells following exposure to Aftin-5. Cells were exposed for 18 h to 100 μM Aftin-5, in the absence or presence of 1 μM DAPT or 'Torrey Pines' compound. Both extracellular and intracellular amyloids were detected and quantified by the ELISA assay. The validity of the intracellular amyloid assay was confirmed by spiking (and recovering) some samples with a known amount of $A\beta_{42}$ (data not shown). Results show that Aftin-5 did not modify the levels of intracellular $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ (Fig. 6A), while the levels of the extracellular amyloids (Fig. 6B) were modified as described above. DAPT down-regulated the levels of all three extracellular amyloids but not that of intracellular amyloids. The 'Torrey Pines' compound had the expected effects on extracellular amyloids, and no effects on intracellular amyloids. These results suggest that Aftins affect the production of extracellular amyloids only. However intracellular amyloids are more difficult to detect and these results might be invalidated by other extraction and detection methods.

Aftin-5-induced upregulation of $A\beta_{42}$ is not due to down-regulation of Aβ proteolytic degradation or autophagy

Increased accumulation of $A\beta_{42}$ peptides could be due to an increased production (i.e., release from AβPP) or to a decreased disposal (proteolytic degradation or autophagy), mimicking the reported reduced clearance of amyloids in AD brain [31]. Although it is unlikely that these elimination mechanisms would specifically remove $A\beta_{42}$, leave $A\beta_{40}$ untouched, and allow an increase in $A\beta_{38}$ level, we tested their potential implications in the levels of all three amyloid peptides.

There are a number of reported amyloid-degrading enzymes (neprilysin, endothelin-converting enzyme (ECE-1, ECE-2), angiotensin converting enzyme (ACE), insulin-degrading enzyme (IDE), cathepsin B, matrix metalloproteinase) (reviewed in [32]). We therefore tested the effects of various inhibitors of these enzymes, alone or in combination

with Aftin-5, on the levels of amyloid peptides after incubation of N2a-AβPP695. An 18 h exposure to 0.1–100 μM thiorphan (neprilysin inhibitor), captopril and quinaprilat (ACE inhibitors), or phosphoramidon (IDE inhibitor) had no effects on extracellular levels of amyloid $A\beta_{38}$, $A\beta_{40}$, or $A\beta_{42}$ (data not shown). These results suggest that these amyloid elimination mechanisms are probably not significant in our cellular systems and that inhibition of amyloid peptide degradation is unlikely to be the driving mechanism of action of Aftin-5. However, other amyloid degradation pathways, not challenged by the above-mentioned inhibitors, may be involved.

An alternative explanation for Aftin-5 triggered increase in $A\beta_{42}$ level could be a reduced autophagy. It has indeed been reported that autophagy is one of the mechanisms that contribute to the elimination of amyloid peptides [33–36], that deterioration in the autophagylysosomal system participates to $A\beta_{42}$ neurotoxicity [35, 37], and that autophagy is linked to AD [38–40]. Therefore an inhibition of autophagy might increase amyloid peptides levels. Exposure of N2a-AβPP695 cells for 18 h to a classical inhibitor of autophagy (0.033–10 mM 3-methyladenine) did not interfere with Aftin-5 induced extracellular $A\beta_{42}$ production (data not shown). Furthermore, 0.01–20 μM rapamycin, a well characterized autophagy inducer, did not prevent the increase in extracellular $A\beta_{42}$ level induced by 100 μM Aftin-5 (data not shown), although it triggered a classical hallmark of autophagy, namely the accumulation of LC3 foci (Supplementary Figure 1). Neither Aftin-5 nor its inactive analog compound (2) impaired rapamycin-induced autophagy in N2a-AβPP695 cells (Supplementary Figure 1). Neither drugs alone had significant effects on autophagy (Supplementary Figure 1).

We conclude that the change in $A\beta_{38}/A\beta_{40}/A\beta_{42}$ relative abundance induced by Aftin-5 is unlikely to be dominantly due to a reduction in proteolytic or autophagic activity. This might be different in primary neurons or in an animal situation.

Mitochondrial effects

In a previous article, we reported that Aftin-4 had significant effects on the morphology of mitochondria and that several mitochondrial proteins were specifically binding to agarose immobilized Aftin-4 [14]. We therefore investigated the effects of the much less toxic Aftin-5 on mitochondria. We first tested the effects of Aftin-5 treatment (1.6 to 200 μM) on several biological parameters of mitochondria isolated from N2a-AβPP695 (Supplementary data section on mitochondria). The following parameters were measured in these isolated mitochondria: transmembrane potential loss ($\Delta\psi_m$ loss), cytochrome *c* release, oxygen consumption (through complex I or complex II activation).

Aftin-5 had no important effects when added directly to isolated mitochondria prepared from untreated N2a-AβPP695 cells (Supplementary data), only a slight stimulation of oxygen consumption was measured under conditions of complex I activation. We also investigated the mitochondrial effects of Aftin-5 on cultured N2a-AβPP695 cells. At the cellular level, the mitochondrial parameters were essentially undistinguishable between control and Aftin-5 treated cells (Supplementary data), except for global respiration functions where a slight inhibition was measured at 24 h.

We next analyzed the ultrastructure of mitochondria following Aftin-5 treatment of isolated mitochondria or intact cells. Treatment of mitochondria isolated from N2a-AβPP695 cells with 100 μM Aftin-5 did not induce any swelling or cristae remodeling, suggesting that the compound has no direct effect on mitochondrial structure (Fig. 7A). This is in accordance with the spectrofluorimetry results. As a control, calcium induced a strong swelling reversed by cyclosporin A (*CsA*), an inhibitor of mitochondrial permeability transition pore. N2a-AβPP695 cells were next treated for 6 h or 24 h with 100 μM Aftin-5. Electron microscopy analysis of cells revealed little detectable effects at 6 h, but a partial mitochondrial swelling without disruption of cristae at 24 h (Fig. 7B), without cell death induction. These effects were reversible upon washing Aftin-5 away from cells. As a positive control, doxorubicin altered the structure of cristae and induced cell death. Altogether these results suggest that Aftin-5 has some structural effects on mitochondria which, like for Aftin-4, are reversible.

DISCUSSION

In this article we confirm and extend the results described in a previous article [14]. In cell cultures (immortalized cell lines and primary neuronal cultures) Aftins robustly increase the production and extracellular release of $A\beta_{42}$ while $A\beta_{40}$ levels remain stable. In contrast, Aftins trigger important parallel down-regulation of $A\beta_{38}$. This effect on the $A\beta_{38}/A\beta_{40}/A\beta_{42}$ peptides relative abundance triggered in cells by Aftins is similar to that previously observed, yet to a much lower extent, with fenofibrate, celecoxib, and indomethacin [35]. Among all the purines derivatives and analogues tested, we have selected Aftin-5 as one of the most potent Aftins. In addition, it displays little cell toxicity (up to 100 μM) or animal toxicity (per os doses up to 150 mg/kg daily for 20 days) (data not shown). Aftin-5 is thus the pharmacological tool we recommend.

Molecular mechanism of action of Aftins

The action of Aftins on cells requires rather high concentrations (50–150 μM). Despite extensive chemical synthesis work, we were able to improve only slightly the potency of the initial Aftin-4 [14]. Furthermore we have not been able to establish a clear-cut structure activity relationship (SAR) in our Aftins library. The structural requirements for Aftins' biological activity can be summarized as follows: (i) the methyl (or other aryl) substituent on position N^6 is a key requirement, (ii) the N9 position can accommodate larger substituents than the corresponding kinase inhibitors, (iii) substitution at C2 is necessary, but no specific modifications could be identified. The lack of unambiguous SAR probably comes from the fact that the biological assay is a cellular assay (change in the ratio of extracellular $A\beta_{38}/A\beta_{40}/A\beta_{42}$ peptides) rather than a molecular assay, implying that multiple parameters might contribute to the measured signal (cell permeability, action on different molecular targets, off-target binding, intracellular distribution, saturation effect, intracellular degradation, etc., all of which may vary from one Aftin to the other). Affinity chromatography on immobilized Aftin-4 has already been allowed the identification of mitochondrial proteins (mitofilin, voltage-dependent anion channel (VDAC) and prohibitin) as well as PSEN and AβPP as interacting ligants. The use of photo-affinity labeled compounds would further help us uncover direct targets of Aftins, some of which might be one of these 5 candidates. The targets directly interacting with Aftins (rather than associated protein complexes) remain to

be identified. At this stage, we feel that protein kinases are quite unlikely targets of Aftins. KinomeScan analysis of Aftin-4 [14] and Aftin-5 (Supplementary Table 1) revealed no significant interaction between these Aftins with any of the 402 or 442 kinases, respectively.

The change in $A\beta_{38}/A\beta_{40}/A\beta_{42}$ peptides relative abundance induced by Aftin-5 requires active γ-secretase, as demonstrated by the complete inhibition of Aftins by γ-secretase inhibitors [14] (Fig. 4). This suggests the possibility that Aftin-5 directly binds to γ-secretase, modifying its substrate specificity toward the production of $A\beta_{42}$, at the expense of $A\beta_{38}$, while the production of $A\beta_{40}$ remains unaffected. Aftins might then also modify the cleavage sites on other substrates of γ-secretase [41]. Another possibility is that Aftins directly bind to the substrate, AβPP, and thereby alters the interaction with γ-secretase, leading to a shift of the cleavage site on AβPP. Aftins would then act on the AβPP transmembrane domain to drive γ-secretase's cleaving activity toward longer Aβ peptides at the expense of shorter versions [10]. An alternative explanation would be an interaction with mitochondrial proteins as demonstrated for Aftin-4 [14]. In this respect, the association of γ-secretase with VDAC [42, 43] and the specific binding of VDAC to immobilized Aftin-4 [14] are particularly interesting.

The effects of Aftins and the other $A\beta_{42}$ increasing agents on the levels of different $A\beta_{38}$ and $A\beta_{42}$ peptides (Fig. 3A) [14, 44] are opposite to those induced by the 'Torrey Pines' GSM (Fig. 5) [28–30, 45–47], suggesting a mutually exclusive action of these two families of γ-secretase modulating agents. GSMs action also depends on presenilin mutations [24, 45, 48]. The mechanisms of action of GSMs falls in several categories (reviewed in [49–51]: direct binding to Aβ and/or AβPP [52–55], to PEN-2 [22], and PSEN-1 [30, 46, 47, 56]. Structurally different GSMs may thus target different components of the AβPP/ γ-secretase complex. Besides the two families of γ-secretase modulating agents (GSMs and Aftins) which modify the $A\beta_{38}/A\beta_{40}/A\beta_{42}$ peptides ratio in opposite ways, mutations in the AβPP transmembrane domain also appear to modulate the proportions of amyloid peptides species [57–59]. In particular Lysine 624 (Lys28 in the Aβ peptides) appears to be a critical determinant for the length of the Aβ peptide released by presenilins as mutation to an Alanine or Glutamine leads to a decrease in the production of $A\beta_{40}$ and $A\beta_{42}$ peptides in favor of the production of shorter Aβ peptides ($A\beta_{33}$, $A\beta_{34}$, $A\beta_{37}$, $A\beta_{38}$, $A\beta_{39}$) [55]. These mutations partially mimic the effects of GSMs. In fact the effects of GSMs also depend on the sequence of the AβPP transmembrane segment [60].

Cellular mechanism of action of Aftins

Comparison of intracellular and extracellular levels of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ following exposure to Aftin-5 suggests that, at least under the extraction and assays conditions used here, Aftin-5 modifies the ratio of extracellular $A\beta_{38}/A\beta_{40}/A\beta_{42}$ (Fig. 6A), but not that of intracellular amyloid peptides (Fig. 6B). This extracellular production/release of Aβ and $A\beta_{42}$ in particular is clearly relevant to what happens in AD [61].

Besides a direct interaction with γ-secretase or its substrate AβPP, we also investigated the possibility that increased $A\beta_{42}$ peptide level induced by Aftins could be due to globally reduced degradation or disposal of amyloid peptides. Although we cannot formally exclude a decreased proteolytic degradation by one or several of the numerous proteases that target

amyloid peptides [26], we feel this is an unlikely explanation for the action of Aftins. Indeed, if this was the case we would expect an upregulation of $A\beta_{38}$ and $A\beta_{40}$ which, respectively, partially disappears or remains stable. Furthermore, addition of inhibitors of such degradation systems did not lead to massive increases of $A\beta_{42}$ (data not shown). Similarly, down-regulation of autophagy is unlikely to provide an explanation for the increase in $A\beta_{42}$, and even less so for the down-regulation of $A\beta_{38}$.

Although Aftin-4 has some clear yet reversible effects on mitochondria structure, Aftin-5 also affects the mitochondria though to a lesser extent. Aftin-5 has no detectable functional effects on isolated mitochondria and mitochondria isolated from Aftin-5 treated cells also display normal physiological characteristics. In contrast when the ultrastructure of cells exposed to Aftin-5 is analyzed, some alterations are seen, which are less obvious than those seen with Aftin-4 (Fig. 7). These results suggest that Aftin-5, like Aftin-4, also interacts with mitochondria in a reversible way. When Aftin is washed away from treated cells or when mitochondria are isolated from treated cells (a procedure that includes numerous washes), mitochondria regain a normal structure. We believe that these modest morphological changes are not dependent on $A\beta_{42}$ upregulation or $A\beta_{38}$ down-regulation.

Aftins, new pharmacological tools to investigate amyloid peptides production: Toward a chemical animal model of AD?

Aftins, and particularly Aftin-5, constitute new pharmacological tools to investigate the generation of amyloid peptides from AβPP and particularly the regulation of the balance between $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ peptides. Aftin-5 allows a shift in the Aβ peptides relative abundance which is reminiscent of that observed in AD. Aftins' effects on amyloid peptide levels have been observed in several cell lines whether or not they overexpress AβPP, as well as in primary neuronal cultures [14] (Mabondzo et al., in preparation). We are currently investigating the molecular mechanism of action of Aftins. We are also investigating the effects of Aftin-5 in animals, both wild-type mice and AD models such as AβPP/PS1 mice. The overall goal is to establish a chemically induced AD model, similar to the MPTP-induced Parkinsonism model [62, 63].

Altogether we believe that Aftin-5 will contribute to the understanding of the pathological mechanisms that favor the γ-secretase dependent production of $A\beta_{42}$ and subsequent neurological disorders.

Cellular screens for 'anti-AD' and 'pro-AD' compounds

The ELISA assay we have set up allows simple, cheap, and fast detection of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ peptides released extracellularly from cultured cells exposed to Aftin-5. This simple set-up can be turned to a large scale, cell based assay to screen for inhibitors of $A\beta_{42}$ production. This would allow the easy detection of β-secretase and γ-secretase inhibitors (Fig. 4) or GSMs (Fig. 5). It might also allow the detection of compounds acting through other pathways (interaction between presenilin and other partners of the γ-secretase complex, interaction with AβPP, interaction with the elements composing the lipid rafts where γ-secretase is enclosed, interaction with intra-cellular proteins involved in the trafficking of AβPP, γ-secretase, and the amyloid peptides, interaction with protein involved

in the degradation or withdrawal of amyloids, etc.). All these compounds have a potential as possible ‘anti-AD’ drugs that would deserve further investigation.

The very same cell culture and amyloid detection system allows the detection of Aftin-related compounds (in terms of biological activity rather than structure), namely compounds that upregulate $A\beta_{42}$ and down-regulate $A\beta_{38}$. These compounds represent potential hazards as possible ‘pro-AD’ compounds that deserve careful handling, and controlled/regulated exposure. We envisage the testing of a set of natural products but also of anthropic molecules humans are exposed to (the so-called ‘human chemical exposome’). We believe our simple screening test, using Aftin-5 as a positive, reference compound, should be included in safety screens designed to detect potentially hazardous molecules among the large number of compounds that are already on or being brought to the market. These molecules may constitute environmental factors contributing to the onset, development, and acceleration of AD. Their detection and identification would constitute a first step in a preventive action to reduce or even eradicate late onset AD which accounts for more than 99% of all AD cases.

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References

1. Huang Y, Mucke L. Alzheimer mechanisms and therapeutics strategies. Cell. 2012; 148:1204–1222. [PubMed: 22424230]
2. Selkoe, DJ.; Mandelkow, E.; Holtzman, DM. The Biology of Alzheimer Disease. Cold Spring Harbor Press; Cold Spring Harbor, New York: 2012. p. 511
3. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer’s disease: Progress and problems on the road to therapeutics. Science. 2002; 297:353–356. *Erratum in: Science* 297, 2209. [PubMed: 12130773]
4. Benilova I, Karran E, De Strooper B. The toxic Aβ oligomer and Alzheimer’s disease: An emperor in need of clothes. Nat Neurosci. 2012; 15:349–357. [PubMed: 22286176]
5. Karran E, Mercken M, De Strooper B. The amyloid cascade hypothesis for Alzheimer’s disease: An appraisal for the development of therapeutics. Nat Rev Drug Discov. 2011; 10:698–712. [PubMed: 21852788]
6. Iqbal K, Liu F, Gong CX, Grundke-Iqbal I. Tau in Alzheimer disease and related tauopathies. Curr Alzheimer Res. 2010; 7:656–664. [PubMed: 20678074]
7. Lee VM, Brunden KR, Hutton M, Trojanowski JQ. Developing therapeutic approaches to tau, selected kinases, and related neuronal protein targets. Cold Spring Harb Perspect Med. 2011; 1:a006437. [PubMed: 22229117]
8. Obulesu M, Venu R, Somashekhar R. Tau mediated neurodegeneration: An insight into Alzheimer’s disease pathology. Neurochem Res. 2011; 36:1329–1335. [PubMed: 21509508]
9. De Strooper B, Vassar R, Golde T. The secretases: Enzymes with therapeutic potential in Alzheimer disease. Nat Rev Neurol. 2010; 6:99–107. [PubMed: 20139999]
10. Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y. gamma-Secretase: Successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. J Neurosci. 2009; 29:13042–13052. [PubMed: 19828817]

11. Bateman RJ, Aisen PS, De Strooper B, Fox NC, Lemere CA, Ringman JM, Salloway S, Sperling RA, Windisch M, Xiong C. Autosomal-dominant Alzheimer's disease: A review and proposal for the prevention of Alzheimer's disease. Alzheimers Res Ther. 2011; 3:1. [PubMed: 21211070]
12. Kuperstein I, Broersen K, Benilova I, Rozenski J, Jonckheere W, Debulpaep M, Vandersteen A, Segers-Nolten I, Van Der Werf K, Subramaniam V, Braeken D, Callewaert G, Bartic C, D'Hooge R, Martins IC, Rousseau F, Schymkowitz J, De Strooper B. Neurotoxicity of Alzheimer's disease Aβ peptides is induced by small changes in the Aβ42 to Aβ40 ratio. EMBO J. 2010; 29:3408–3420. [PubMed: 20818335]
13. McGowan E, Pickford F, Kim J, Onstead L, Eriksen J, Yu C, Skipper L, Murphy MP, Beard J, Das P, Jansen K, Delucia M, Lin WL, Dolios G, Wang R, Eckman CB, Dickson DW, Hutton M, Hardy J, Golde T. Abeta42 is essential for parenchymal and vascular amyloid deposition in mice. Neuron. 2005; 47:191–199. [PubMed: 16039562]
14. Bettayeb K, Oumata N, Zhang Y, Luo W, Bustos V, Galons H, Greengard P, Meijer L, Flajolet M. A chemical route towards Alzheimer's disease? FASEB J. 2012; 26:5115–5123. [PubMed: 22972917]
15. Findeis MA. The role of amyloid beta peptide 42 in Alzheimer's disease. Pharmacol Ther. 2007; 116:266–286. [PubMed: 17716740]
16. Pannee J, Portelius E, Oppermann M, Atkins A, Hornshaw M, Zegers I, Höjrup P, Minthon L, Hansson O, Zetterberg H, Blennow K, Gobom J. A selected reaction monitoring (SRM)-based method for absolute quantification of Aβ38, Aβ40, and Aβ42 in cerebrospinal fluid of alzheimer's disease patients and healthy controls. J Alzheimers Dis. 2013; 33:1021–1032. [PubMed: 23076076]
17. Blennow K, Zetterberg H, Fagan AM. Fluid biomarkers in Alzheimer disease. Cold Spring Harb Perspect Med. 2012; 2:a006221. [PubMed: 22951438]
18. Oumata N, Ferandin Y, Meijer L, Galons H. Practical synthesis of roscovitine and CR8. Org Process Res Dev. 2009; 13:641–644.
19. Cheng, S.; Comer, DD.; Mao, L.; Balow, GP.; Pleynet, D. World Intellectual Property Organization. 2004. compounds and uses thereof in modulating amyloid beta. Pub. No. WO/2004-110350
20. Mehta PD, Pirttila T. Increased cerebrospinal fluid A beta38/A beta42 ratio in Alzheimer disease. Neurodegener Dis. 2005; 2:242–245. [PubMed: 16909004]
21. Potempska A, Mack K, Mehta P, Kim KS, Miller DL. Quantification of sub-femtomole amounts of Alzheimer amyloid beta peptides. Amyloid. 1999; 6:14–21. [PubMed: 10211407]
22. Flajolet M, He G, Heiman M, Lin A, Nairn AC, Greengard P. Regulation of Alzheimer's disease amyloid-beta formation by casein kinase I. Proc Natl Acad Sci U S A. 2007; 104:4159–4164. [PubMed: 17360493]
23. Oumata N, Bettayeb K, Ferandin Y, Demange L, Lopez-Giral A, Goddard M-L, Myrianthopoulos V, Mikros E, Flajolet M, Greengard P, Meijer L, Galons H. Roscovitine-derived, dual-specificity inhibitors of cyclin-dependent kinases (CDKs) and casein kinase 1 (CK1). J Med Chem. 2008; 51:5229–5242. [PubMed: 18698753]
24. Meijer L, Raymond E. Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. Accounts Chem Res. 2003; 36:417–425.
25. Bettayeb K, Oumata N, Echalier A, Ferandin Y, Endicott JA, Galons H, Meijer L. CR8, a potent and selective, roscovitine-derived inhibitor of cyclin-dependent kinases. Oncogene. 2008; 27:5797–5807. [PubMed: 18574471]
26. Dovey HF, John V, Anderson JP, Chen LZ, de Saint Andrieu P, Fang LY, Freedman SB, Folmer B, Goldbach E, Holsztynska EJ, Hu KL, Johnson-Wood KL, Kennedy SL, Kholodenko D, Knops JE, Latimer LH, Lee M, Liao Z, Lieberburg IM, Motter RN, Mutter LC, Nietz J, Quinn KP, Sacchi KL, Seubert PA, Shopp GM, Thorsett ED, Tung JS, Wu J, Yang S, Yin CT, Schenk DB, May PC, Altstiel LD, Bender MH, Boggs LN, Britton TC, Clemens JC, Czilli DL, Dieckman-McGinty DK, Droste JJ, Fuson KS, Gitter BD, Hyslop PA, Johnstone EM, Li WY, Little SP, Mabry TE, Miller FD, Audia JE. Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J Neurochem. 2001; 76:173–181. [PubMed: 11145990]

27. Zhao B, Yu M, Neitzel M, Marugg J, Jagodzinski J, Lee M, Hu K, Schenk D, Yednock T, Basi G. Identification of gamma-secretase inhibitor potency determinants on presenilin. J Biol Chem. 2008; 283:2927–2938. [PubMed: 18032377]

28. Kounnas MZ, Danks AM, Cheng S, Tyree C, Ackerman E, Zhang X, Ahn K, Nguyen P, Comer D, Mao L, Yu C, Pleynet D, Digregorio PJ, Velicelebi G, Stauderman KA, Comer WT, Mobley WC, Li YM, Sisodia SS, Tanzi RE, Wagner SL. Modulation of gamma-secretase reduces beta-amyloid deposition in a transgenic mouse model of Alzheimer's disease. Neuron. 2010; 67:769–780. [PubMed: 20826309]

29. Kretner B, Fukumori A, Gutsmiedl A, Page RM, Luebbers T, Galley G, Baumann K, Haass C, Steiner H. Attenuated Abeta42 responses to low potency gamma-secretase modulators can be overcome for many pathogenic presenilin mutants by second-generation compounds. J Biol Chem. 2011; 286:15240–15251. [PubMed: 21357415]

30. Ebke A, Luebbers T, Fukumori A, Shirotani K, Haass C, Baumann K, Steiner H. Novel γ-secretase enzyme modulators directly target presenilin protein. J Biol Chem. 2011; 286:37181–37186. [PubMed: 21896486]

31. Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, Bateman RJ. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science. 2010; 330:1774. [PubMed: 21148344]

32. Miners JS, Barua N, Kehoe PG, Gill S, Love S. Aβ-degrading enzymes: Potential for treatment of Alzheimer disease. J Neuropathol Exp Neurol. 2011; 70:944–959. [PubMed: 22002425]

33. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B, Wyss-Coray T. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest. 2008; 118:2190–2199. [PubMed: 18497889]

34. Ling D, Song HJ, Garza D, Neufeld TP, Salvaterra PM. Abeta42-induced neurodegeneration via an age-dependent autophagic-lysosomal injury in Drosophila. PLoS One. 2009; 4:e4201. [PubMed: 19145255]

35. Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T. Regulation of amyloid precursor protein processing by the Beclin 1 complex. PLoS One. 2010; 5:e11102. [PubMed: 20559548]

36. Tian Y, Bustos V, Flajolet M, Greengard P. A small-molecule enhancer of autophagy decreases levels of Abeta and APP-CTF via Atg5-dependent autophagy pathway. FASEB J. 2011; 25:1934–1942. [PubMed: 21368103]

37. Ling D, Salvaterra PM. Brain aging and Aβ1-42 neurotoxicity converge via deterioration in autophagylysosomal system: A conditional Drosophila model linking Alzheimer's neurodegeneration with aging. Acta Neuropathol. 2011; 121:183–191. [PubMed: 21076961]

38. Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, Wolfe DM, Martinez-Vicente M, Massey AC, Sovak G, Uchiyama Y, Westaway D, Cuervo AM, Nixon RA. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell. 2010; 141:1146–1158. [PubMed: 20541250]

39. Nixon RA, Yang DS. Autophagy failure in Alzheimer's disease-locating the primary defect. Neurobiol Dis. 2011; 43:38–45. [PubMed: 21296668]

40. Yang DS, Stavrides P, Mohan PS, Kaushik S, Kumar A, Ohno M, Schmidt SD, Wesson DW, Bandyopadhyay U, Jiang Y, Pawlik M, Peterhoff CM, Yang AJ, Wilson DA, St George-Hyslop P, Westaway D, Mathews PM, Levy E, Cuervo AM, Nixon RA. Therapeutic effects of remediating autophagy failure in a mouse model of Alzheimer disease by enhancing lysosomal proteolysis. Autophagy. 2011; 7:788–789. [PubMed: 21464620]

41. Beel AJ, Sanders CR. Substrate specificity of gamma-secretase and other intramembrane proteases. Cell Mol Life Sci. 2008; 65:1311–1334. [PubMed: 18239854]

42. Wakabayashi T, Craessaerts K, Bammens L, Bentahir M, Borgions F, Herdewijn P, Staes A, Timmerman E, Vandeker-ckhove J, Rubinstein E, Boucheix C, Gevaert K, De Strooper B. Analysis of the gamma-secretase interactome and validation of its association with tetraspanin-enriched microdomains. Nat Cell Biol. 2009; 11:1340–1346. [PubMed: 19838174]

43. Hur JY, Teranishi Y, Kihara T, Yamamoto NG, Inoue M, Hosia W, Hashimoto M, Winblad B, Frykman S, Tjernberg LO. Identification of novel γ-secretase-associated proteins in detergent-resistant membranes from brain. J Biol Chem. 2012; 287:11991–12005. [PubMed: 22315232]
44. Kukar T, Murphy MP, Eriksen JL, Sagi SA, Weggen S, Smith TE, Ladd T, Khan MA, Kache R, Beard J, Dodson M, Merit S, Ozols VV, Anastasiadis PZ, Das P, Fauq A, Koo EH, Golde TE. Diverse compounds mimic Alzheimer disease-causing mutations by augmenting Abeta42 production. Nat Med. 2005; 11:545–550. [PubMed: 15834426]
45. Page RM, Baumann K, Tomioka M, Pérez-Revuelta BI, Fukumori A, Jacobsen H, Flohr A, Luebbers T, Ozmen L, Steiner H, Haass C. Generation of Abeta38 and Abeta42 is independently and differentially affected by familial Alzheimer disease-associated presenilin mutations and gamma-secretase modulation. J Biol Chem. 2008; 283:677–683. [PubMed: 17962197]
46. Ohki Y, Higo T, Uemura K, Shimada N, Osawa S, Berezovska O, Yokoshima S, Fukuyama T, Tomita T, Iwatsubo T. Phenylpiperidine-type γ-secretase modulators target the transmembrane domain 1 of presenilin 1. EMBO J. 2011; 30:4815–4824. [PubMed: 22002539]
47. Crump CJ, Fish BA, Castro SV, Chau DM, Gertsik N, Ahn K, Stiff C, Pozdnyakov N, Bales KR, Johnson DS, Li YM. Piperidine acetic acid based γ-secretase modulators directly bind to Presenilin-1. ACS Chem Neurosci. 2011; 2:705–710. [PubMed: 22229075]
48. Czirr E, Cottrell BA, Leuchtenberger S, Kukar T, Ladd TB, Esselmann H, Paul S, Schubenel R, Torpey JW, Pietrzik CU, Golde TE, Wiltfang J, Baumann K, Koo EH, Weggen S. Independent generation of Abeta42 and Abeta38 peptide species by gamma-secretase. J Biol Chem. 2008; 283:17049–17054. [PubMed: 18426795]
49. Wolfe MS. γ-Secretase inhibitors and modulators for Alzheimer's disease. J Neurochem. 2012; 120(Suppl 1):89–98. [PubMed: 22122056]
50. Oehlrich D, Berthelot DJ, Gijsen HJ. γ-Secretase modulators as potential disease modifying anti-Alzheimer's drugs. J Med Chem. 2011; 54:669–698. [PubMed: 21141968]
51. Borgegard T, Juréus A, Olsson F, Rosqvist S, Sabirsh A, Rotticci D, Paulsen K, Klintenberg R, Yan H, Waldman M, Stromberg K, Nord J, Johansson J, Regner A, Parpal S, Malinowsky D, Radesater AC, Li T, Singh R, Eriksson H, Lundkvist J. First and second generation γ-secretase modulators (GSMs) modulate amyloid-β (Aβ) peptide production through different mechanisms. J Biol Chem. 2012; 87:11810–11819. [PubMed: 22334705]
52. Richter L, Munter LM, Ness J, Hildebrand PW, Dasari M, Unterreitmeier S, Bulic B, Beyermann M, Gust R, Reif B, Weggen S, Langosch D, Multhaup G. Amyloid beta 42 peptide (Abeta42)-lowering compounds directly bind to Abeta and interfere with amyloid precursor protein (APP) transmembrane dimerization. Proc Natl Acad Sci U S A. 2010; 107:14597–14602. [PubMed: 20679249]
53. Botev A, Munter LM, Wenzel R, Richter L, Althoff V, Ismer J, Gerling U, Weise C, Koksch B, Hildebrand PW, Bittl R, Multhaup G. The amyloid precursor protein C-terminal fragment C100 occurs in monomeric and dimeric stable conformations and binds γ-secretase modulators. Biochemistry. 2011; 50:828–835. [PubMed: 21186781]
54. Kukar TL, Ladd TB, Bann MA, Fraering PC, Narlawar R, Maharvi GM, Healy B, Chapman R, Welzel AT, Price RW, Moore B, Rangachari V, Cusack B, Eriksen J, Jansen-West K, Verbeeck C, Yager D, Eckman C, Ye W, Sagi S, Cottrell BA, Torpey J, Rosenberry TL, Fauq A, Wolfe MS, Schmidt B, Walsh DM, Koo EH, Golde TE. Substrate-targeting gamma-secretase modulators. Nature. 2008; 453:925–929. [PubMed: 18548070]
55. Kukar TL, Ladd TB, Robertson P, Pintchovski SA, Moore B, Bann MA, Ren Z, Jansen-West K, Malphrus K, Eggert S, Maruyama H, Cottrell BA, Das P, Basi GS, Koo EH, Golde TE. Lysine 624 of the amyloid precursor protein (APP) is a critical determinant of amyloid β peptide length: Support for a sequential model of γ-secretase intramembrane proteolysis and regulation by the amyloid β precursor protein (APP) juxtamembrane region. J Biol Chem. 2011; 286:39804–39812. [PubMed: 21868378]
56. Lleó A, Berezovska O, Herl L, Raju S, Deng A, Bacskai BJ, Frosch MP, Irizarry M, Hyman BT. Nonsteroidal anti-inflammatory drugs lower Abeta42 and change presenilin 1 conformation. Nat Med. 2004; 10:1065–1066. [PubMed: 15448688]
57. Munter LM, Voigt P, Harmeier A, Kaden D, Gottschalk KE, Weise C, Pipkorn R, Schaefer M, Langosch D, Multhaup G. GxxxG motifs within the amyloid precursor protein transmembrane

sequence are critical for the etiology of Abeta42. EMBO J. 2007; 26:1702–1712. [PubMed: 17332749]

58. Kienlen-Campard P, Tasiaux B, Van Hees J, Li M, Huysseune S, Sato T, Fei JZ, Aimoto S, Courtoy PJ, Smith SO, Constantinescu SN, Octave JN. Amyloidogenic processing but not amyloid precursor protein (APP) intracellular C-terminal domain production requires a precisely oriented APP dimer assembled by transmembrane GXXXG motifs. J Biol Chem. 2008; 283:7733–7744. Erratum in: *J Biol Chem* 283, 12680. [PubMed: 18201969]
59. Sagi SA, Lessard CB, Winden KD, Maruyama H, Koo JC, Weggen S, Kukar TL, Golde TE, Koo EH. Substrate sequence influences γ-secretase modulator activity, role of the transmembrane domain of the amyloid precursor protein. J Biol Chem. 2011; 286:39794–39803. [PubMed: 21868380]
60. Gong P, Vetrivel KS, Nguyen PD, Meckler X, Cheng H, Kounnas MZ, Wagner SL, Parent AT, Thinakaran G. Mutation analysis of the presenilin 1 N-terminal domain reveals a broad spectrum of gamma-secretase activity toward amyloid precursor protein and other substrates. J Biol Chem. 2010; 285:38042–38052. [PubMed: 20921220]
61. Jonsson T, Atwal JK, Steinberg S, Snaedal J, Jonsson PV, Bjornsson S, Stefansson H, Sulem P, Gudbjartsson D, Maloney J, Hoyte K, Gustafson A, Liu Y, Lu Y, Bhangale T, Graham RR, Huttenlocher J, Bjornsdottir G, Andreassen OA, Jönsson EG, Palotie A, Behrens TW, Magnusson OT, Kong A, Thorsteinsdottir U, Watts RJ, Stefansson K. A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. Nature. 2012; 488:96–99. [PubMed: 22801501]
62. Prediger RD, Aguiar AS Jr, Moreira EL, Matheus FC, Castro AA, Walz R, De Bem AF, Latini A, Tasca CI, Farina M, Raisman-Vozari R. The intranasal administration of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP): A new rodent model to test palliative and neuroprotective agents for Parkinson's disease. Curr Pharm Des. 2011; 17:489–507. [PubMed: 21375482]
63. Fox SH, Brotchie JM. The MPTP-lesioned non-human primate models of Parkinson's disease. Past, present, and future. Prog Brain Res. 2010; 184:133–157. [PubMed: 20887873]

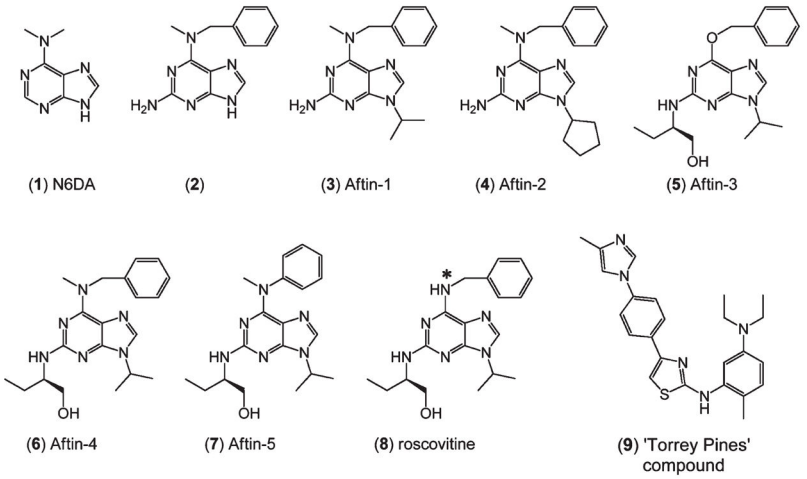

Fig. 1.

Structure of the compounds used in this study. N^6-diamino-purine (N6DA) (1), compound (2), Aftin-1 (3), Aftin-2 (4), Aftin-3 (5), Aftin-4 (6), Aftin-5 (7), (R)-roscovitine (8) and "Torrey Pines" compound (9).

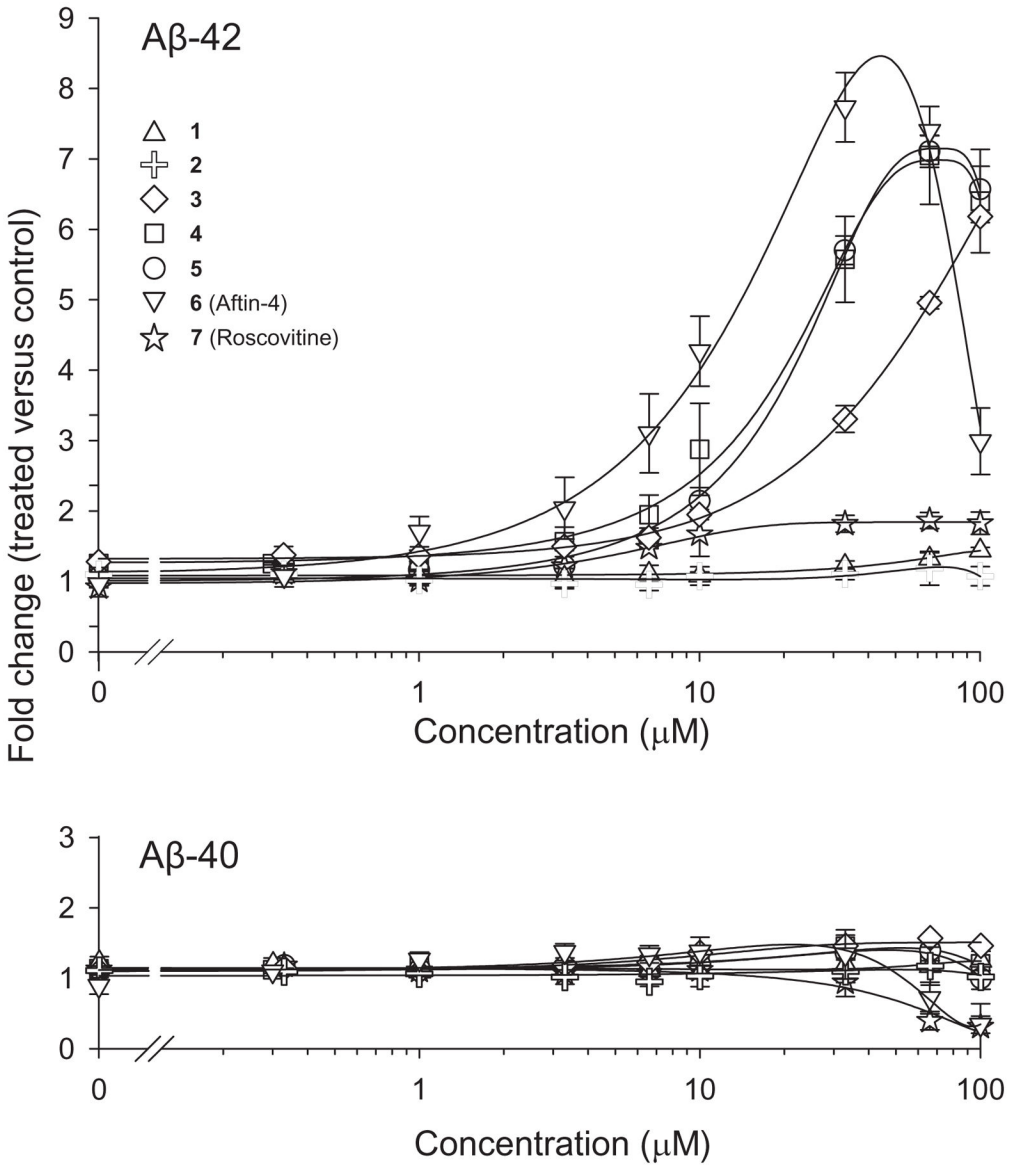

Fig. 2.

Dose-dependent effect of various purines on extracellular $A\beta_{42}$ and $A\beta_{40}$ production in N2a-AβPP695 cells. Cells were exposed to various concentrations of compounds 1–6, 8 for 18 h. Extracellular $A\beta_{42}$ (upper panel) and $A\beta_{40}$ (lower panel) levels were measured by an ELISA assay and are expressed as fold change over the level of control, vehicle-treated cells. Representative of two independent experiments, errors bars represent standard deviation of triplicate values.

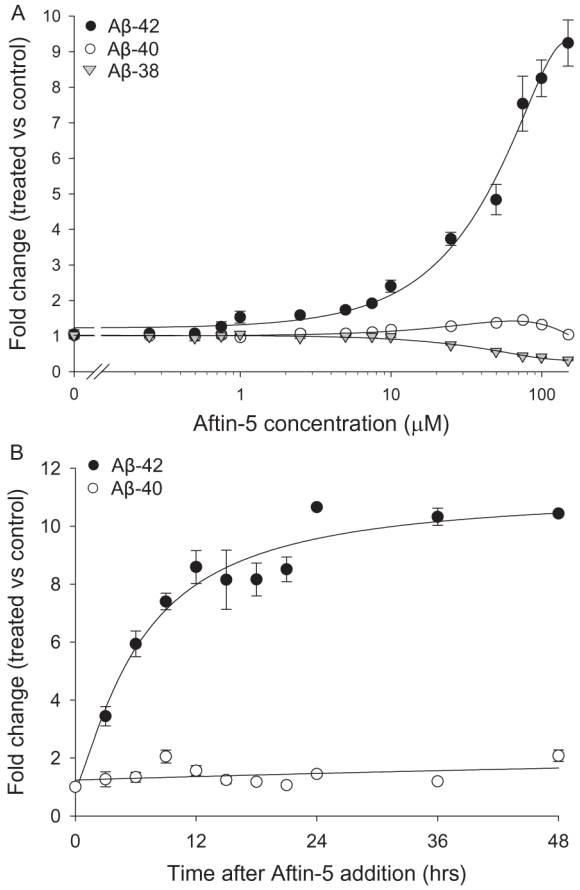

Fig. 3.

Effect of Aftin-5 on extracellular Aβ production in N2a-AβPP695 cells. A) Aftin-5 dose-dependent effect on extracellular amyloids levels. Cells were exposed to various concentrations of Aftin-5 for 18 h. Extracellular $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ levels were measured by an ELISA assay and are expressed as fold change over the level of control, vehicle-treated cells. B) Aftin-5 time-dependent effect on $A\beta_{40}$ and $A\beta_{42}$ levels. Cells were exposed to 100 μM Aftin-5 for various periods of time and extracellular $A\beta_{40}$ and $A\beta_{42}$ levels were measured by an ELISA assay. Representative of three independent experiments, errors bars represent standard deviation of triplicate values.

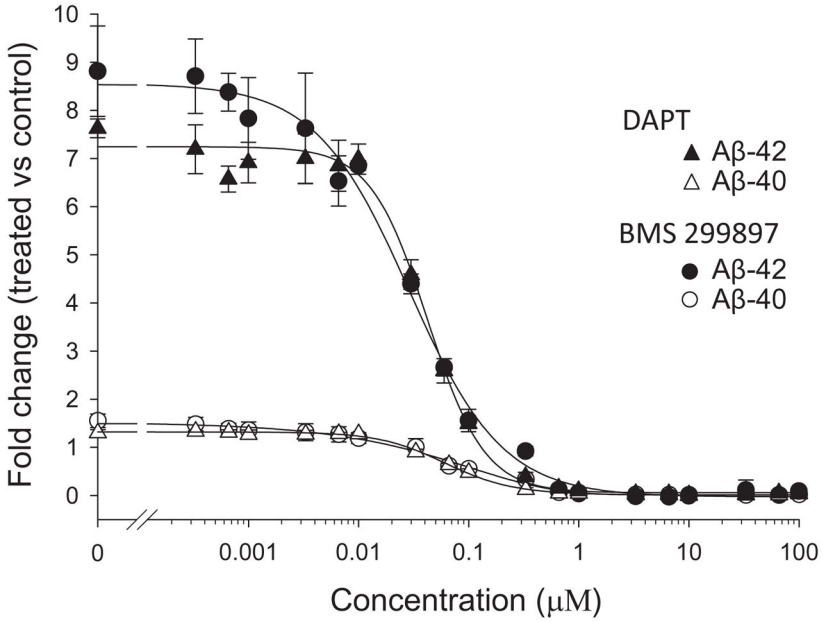

Fig. 4.

Extracellular $A\beta_{42}$ production induced by Aftin-5 and basal level of $A\beta_{40}$ are inhibited by γ-secretase inhibitors. Cells were exposed to increasing concentrations of DAPT or BMS 299897 and 1 h later to 100 μM Aftin-5. Extracellular $A\beta_{40}$ and $A\beta_{42}$ levels were measured after 18 h and are expressed relative to their levels produced by Aftin-5 treated cells in the absence of γ-secretase inhibitor. Representative of two independent experiments, errors bars represent standard deviation of triplicate values.

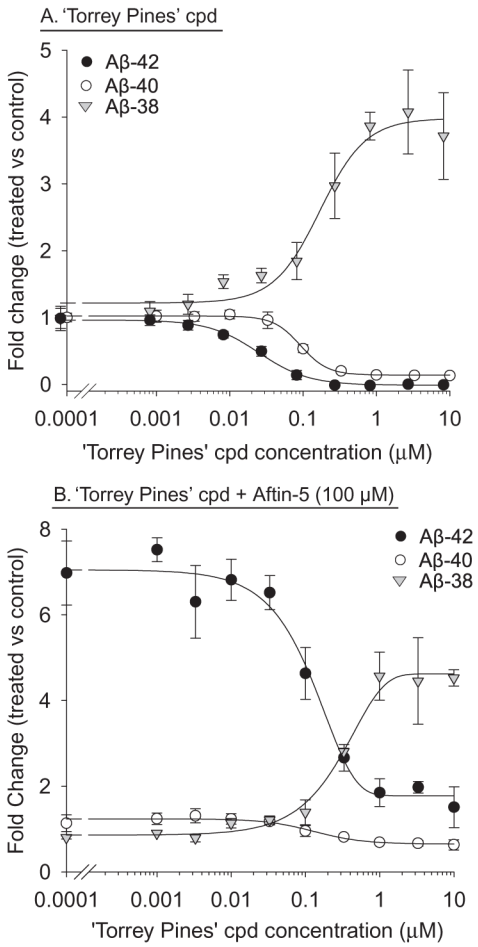

Fig. 5.

γ-Secretase modulator 'Torrey Pines' counter-balances Aftin-5 effects. A) GSM 'Torrey Pines' dose-dependent effect on extracellular $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ levels in N2a-AβPP695 cells. Cells were exposed to various concentrations of 'Torrey Pines' compound for 18 h. Extracellular Aβ levels were measured by an ELISA assay and are expressed as fold change over the level of control, vehicle-treated cells. B) GSM 'Torrey Pines' dose-dependently inhibits Aftin-5 upregulation of $A\beta_{42}$ and down-regulation of $A\beta_{38}$. Cells were exposed to various concentrations of 'Torrey Pines' compound and 1 h later to 100 μM Aftin-5. Incubation was carried out for 18 h. Extracellular Aβ levels were measured by an ELISA assay and are expressed as fold change over the level of control, Aftin-5 treated cells. Representative of three independent experiments, errors bars represent standard deviation of triplicate values.

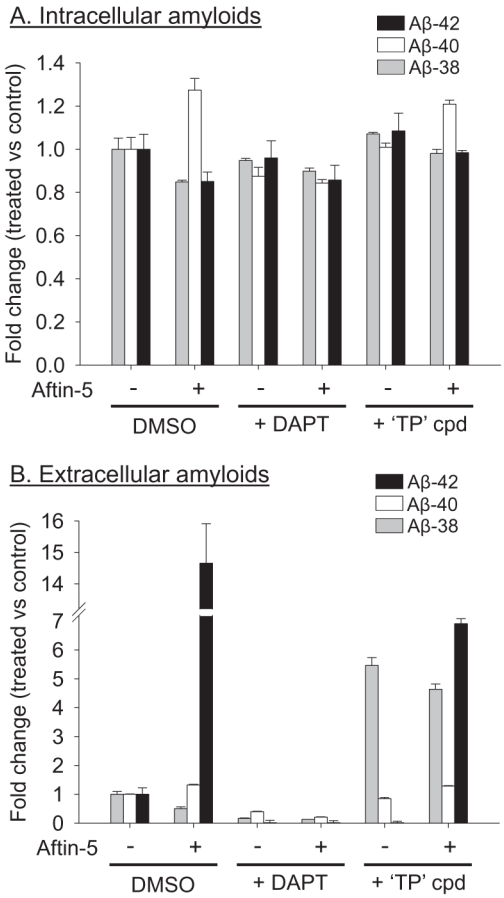

Fig. 6.

Aftin-5 modifies extracellular but not intracellular $A\beta_{38}/A\beta_{40}/A\beta_{42}$ ratios. Cells were exposed to 100 μM Aftin-5 for 18 h, in the absence or presence of 1 μM DAPT or 1 μM 'Torrey Pines' compound. Cell supernatant (A) and cell pellets (B) were recovered and $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ amyloid levels were measured by an ELISA assay. Amyloid levels are expressed as fold change over their concentrations in control, non-treated cells. Basal extracellular (A) and intracellular (B) levels of amyloids were, respectively, 61.9 and 83.5 pg/mL ($A\beta_{38}$), 471 and 48.9 pg/mL ($A\beta_{40}$), 28.9 and 33.6 pg/mL ($A\beta_{42}$). Representative of two independent experiments, errors bars represent standard deviation of triplicate values.

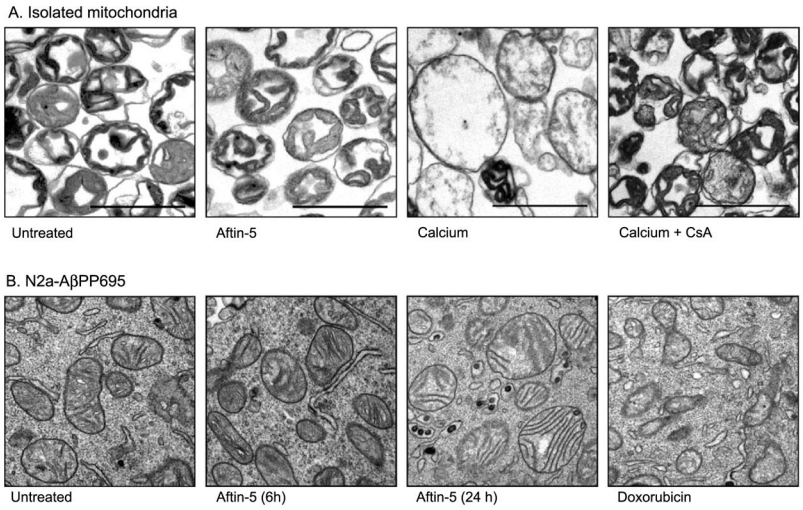

Fig. 7.

Aftin-5 has modest effects on mitochondrial structure. A) Ultrastructure of isolated mitochondria exposed to 100 μM Aftin-5, 50 μM Calcium, or 50 μM Calcium + Cyclosporin A (CsA). B) Ultrastructure of mitochondria within cells exposed to 100 μM Aftin-5 for 6 or 24 h, or to 5 μM doxorubicin. Representative of two independent experiments.

Table 1

Compared cytotoxicity of Aftin-4 and Aftin-5. The different cell lines were exposed to increasing concentrations of each compound and their viability was assessed 24 h later by the MTS assay as described in the Material & Methods section. IC_{50} values were determined from the dose-response curves and are presented in μM

Cell line	Aftin-4	Aftin-5
SH-SY5Y	106	180
HT22	74	194
N2a	100	178
N2a-AβPP695	90	150