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**Multitarget Drug Discovery for Alzheimer's Disease: Triazinones as BACE-1 and GSK-3β Inhibitors****

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**Abstract** Cumulative evidence strongly supports that the amyloid and tau hypotheses are not mutually exclusive, but concomitantly contribute to neurodegeneration in Alzheimer's disease (AD). Thus, the development of multitarget drugs which are involved in both pathways might represent a promising therapeutic strategy. Accordingly, reported here in is the discovery of 6-amino-4-phenyl-3,4-dihydro-1,3,5-triazin-2(1H)-ones as the first class of molecules able to simultaneously modulate BACE-1 and GSK-3β. Notably, one triazinone showed well-balanced in vitro potencies against the two enzymes (IC50 of (18.03±0.01) μM and (14.67±0.78) μM for BACE-1 and GSK-3β, respectively). In cell-based assays, it displayed effective neuroprotective and neurogenic activities and no neurotoxicity. It also showed good brain permeability in a preliminary pharmacokinetic assessment in mice. Overall, triazinones might represent a promising starting point towards high quality lead compounds with an AD-modifying potential.

Alzheimer's disease (AD) is the major unmet medical need in neurology,[1] and despite massive investments, there are no effective treatments. Amyloid beta (Aβ) aggregates and hyperphosphorylated neurofibrillary tangles (NFT) of tau represent the two main pathological hallmarks of AD. These distinctive features have fostered the proposal of two etiological hypotheses which have inspired most of the AD drug discovery efforts.[2] Aβ is generated by the cleavage of the membrane-associated amyloid precursor protein (APP). A key enzyme has been identified as being responsible for Aβ formation, namely, the aspartyl protease β-secretase (BACE-1).[3] BACE-1 inhibitors are in clinical development, with the most advanced ones in phase III of clinical trials.[4] As for the tau hypothesis, glycogen synthase kinase-3β (GSK-3β) has been recognized as one of the key players. GSK-3β is responsible for tau hyperphosphorylation, which causes tau to be detached from the microtubules and precipitate as tangle aggregates.[5] Therefore, the development of selective GSK-3β inhibitors has been recently pursued.[6] Notably, these molecules also enhance neurogenesis and could be promising from a regenerative medicine standpoint.[7] Furthermore, since GSK-3β is involved in neuroinflammatory cascades, its inhibitors might hamper the production of inflammatory mediators and neurotoxic factors.[8] Thus far, neither approach has led to effective drugs to combat AD. Notably, although in the past the two hypotheses have been considered to be at odds, recent evidence suggests that they concomitantly contribute to neuronal death in AD.[9] So, although drug development has been focused on targeting Aβ and tau pathology independently, a disease-modifying treatment for AD should address both amyloid and tau cascades.[10] In light of these considerations, multitarget (MT) drugs, namely small molecules which are able to hit multiple targets responsible for the disease, emerge as promising therapeutic tools.[11]

Herein we report on the development of the first class of MT molecules which are able to modulate BACE-1 and GSK-3β activity simultaneously. We envisaged that such dual-target inhibitors, which intervene at two crucial points in the neurotoxic pathways, might represent a breakthrough in the quest for clinical efficacy.

In MT drug discovery, fragment-based strategies play a pivotal role.[12] On these premises,[13] we utilized a fragment-based approach to design dual inhibitors against BACE-1 and GSK-3β, two enzymes ancestrally quite divergent and sharing a sequence identity of only 19 %. We exploited a ligand-based approach by combining the pharmacophoric features responsible for binding to BACE-1 and GSK-3β, such as a guanidino motif and a cyclic amide group, respectively, into a single scaffold (Figure **1**).[14] As a result, we identified the 6-amino-4-substituted triazinone scaffold as potential starting point (Figure **1**). We subsequently performed structure-based docking simulations[15] to study the interactions of triazinone-based compounds with the catalytic pocket of both enzymes (Figure **2**). We found that 6-amino-4-phenyl-3,4-dihydro-1,3,5-triazin-2(1*H*)-one (**1**; Scheme **1**) should efficiently bind to both targets.

The molecule **1** was then explored in terms of preliminary structure–activity relationships (SARs), and a first set of molecules (**2**–**7**) was synthesized and tested against the two enzymes. Experiments were also carried out at cellular levels. Finally, for one of the most promising compounds, **3**, we generated pharmacokinetics data in mice. Anticipating our results, it turned out that triazinones are promising fragments for developing lead compounds endowed with a BACE-1 and GSK-3β dual-inhibition profile, and are potentially useful as AD modifying agents.

The two-step synthetic route to the target compounds **1**–**7** is depicted in Scheme **1**. First, the acid-catalyzed hydration of the 1-cyanoguanidines **8** and **9** provided the guanylurea **10** and (*N*'-ethylcarbamimidoyl)urea **11**, respectively, in excellent yield. Subsequently, **10** and **11** were coupled to the aromatic aldehydes of interest (**12**–**17**) through a condensation reaction to provide **1**–**7**.[16]

The compounds **1**–**7** showed high aqueous kinetic solubility (ca. 250 µM in PBS, pH 7.4), which enabled further investigation. The ability to inhibit both enzymes was investigated in comparison to inhibitor IV and SB415286, which were used as reference compounds for BACE-1 and GSK-3β, respectively (Table **1**).[17]

The compounds **1**, bearing no substituents, and **2**, bearing a dimethylamino group on the aromatic ring, did not show any significant effect in BACE-1 inhibition assay. The methyl derivative **5** showed modest activity. However, the halogenated derivatives **3**, **4**, **6**, and **7** resulted in moderate inhibition. This inhibition could be due the F, $CF_3$, and Br substituents establishing polar and hydrophobic interactions with a cage made by Leu30, Ile118, Phe108, and Trp115 at subsite P1. Remarkably, the fluorinated **3** and **7** were the most active of the series, with $IC_{50}$ values of 18.03 and 16.05 µM, respectively. Importantly, their enzymatic activity was mirrored by a moderate reduction of cellular Aβ and no cytotoxicity (Figure **3**). Figure **3 a** shows that exposing neuroglioma cells, overexpressing APP harboring the Swedish mutation, for 24 hours to **3** and **7** did not affect viability. Furthermore, after the same time frame **3** and **7** slightly reduced Aβ(1–40) levels, compared to results obtained with inhibitor IV (Figure **3 b**).

With regards to GSK-3β, all compounds modulated the enzyme activity with similar potency, ranging from 7.11 to 37.78 µM. As a result, **3** showed a well-balanced in vitro BACE-1/GSK-3β inhibitory profile ($IC_{50}$ values of 18.03 µM and 14.67 µM).

To explore the enantioselectivity of these compounds, enantiomer separation by HPLC using a chiral stationary phase was performed. The compound **7** was selected for these studies because of its higher solubility in the solvents used for the normal-phase HPLC method. (−)-**7** and (+)-**7** turned out to be equally potent against BACE-1 and GSK-3β, that is, there is no enantiomer discrimination.

From a MT drug discovery standpoint, the most promising result of the present study is the remarkably similar enzymatic activities of **3**, despite the two-digit micromolar range. In fact, where connections exist

between two targets, as it seems to be the case for GSK-3β and BACE-1, inhibitors with only moderate potency are expected to produce superior in vivo effects compared to higher-potency single-target compounds, and fewer side effects.[18], [19] Therefore, despite the moderate activity, we propose that **3**, due to its structural simplicity and low molecular weight, might be a more suitable binder than reference inhibitors. In fact, it exhibits a ligand efficiency (LE=0.32) which is higher than those of inhibitor IV (0.19) and SB415286 (0.29). In addition, **3** showed a promising lipophilic efficiency (LipE; see Table S1 in the Supporting Information).

Based on these considerations, **3** was selected for additional cellular studies aimed at assessing its effect on neuroprotection and neurogenesis, two crucial properties for AD-modifying drugs. GSK-3β overactivity results in increased microglia activation, thus leading to the neuronal cell death in AD. Furthermore, GSK-3β regulates inflammatory tolerance in astrocytes.[20] Accordingly, we explored the potential neuroprotective activity of **3** in primary cultures of astrocytes and microglia by evaluating nitrite production. Glial cells were first incubated with **3** (10 µM) for 1 hour, and then cultured for another 24 hours with lipopolysaccharide (LPS), a potent inducer of inflammatory reaction and of a cascade of intracellular events involved in neuronal death. We observed an important induction of nitrite production, which was significantly reduced by treatment with **3** (Figure **4**). Similar results were obtained by co-treatment of primary rat glial cells with LPS and **3** (data not shown).

GSK-3β activity promotes inducible nitric oxide synthase (iNOS) induction,[21] thus resulting in the elevated NO concentrations associated with the neurodegenerative pathology.[22] Moreover, iNOS is considered a marker of the inflammatory M1 microglial phenotype, and iNOS inhibitors have been shown to exert beneficial anti-inflammatory properties.[23]

Therefore, we evaluated the effect of **3** on iNOS expression in parallel cultures of astrocytes and microglia. When cells were stimulated with LPS, we observed the expected iNOS induction, which was significantly reduced by **3**, in a dose dependent manner (Figure **5 a–d**). In addition, the modulatory action of **3** was assessed by studying the microglial M1/M2 phenotypes through the evaluation of the phagocytic receptor TREM2.[24] Compounds that induce microglia switch from inflammatory M1-type to anti-inflammatory M2-type have been proposed to attenuate neuroinflammation and bolster neuronal protection and recovery.[25] Remarkably, **3** was able to shift microglia phenotype from M1 to M2, with a decrease in iNOS expression, but there were no changes in the positive microglial phagocytic activity, as assessed by TREM2 expression (Figure **5 d,e**).[26] Altogether these results indicate that **3** is a highly promising anti-inflammatory and neuroprotective agent, thereby decreasing the neurotoxic microglial activation without affecting the neuroprotective one. Moreover, **3** does not display any toxicity in glial and neuronal cells (see Figure S1).

To evaluate the potential neurogenic effect of **3**, we analyzed whether its addition to neurospheres of primary rat neural stem cells could regulate differentiation toward a neuronal phenotype. As shown in Figure **6**, some neurogenic effects were evident after treatment with **3**. Particularly, when compared to the control, the number of MAP-2 positive cells (mature neuronal marker) was increased in treated cultures. Notably, this underlines the ability of **3** to differentiate neural stem cells into mature neurons.

Finally, pharmacokinetic (PK) analyses in mice showed that **3** has good oral bioavailability and blood-brain-barrier (BBB) penetration. After dosing **3** intravenously and orally, we monitored plasma concentrations for 8 hours. After intravenous dosing (3 mg $kg^{-1}$), we measured a maximal plasma concentration of 713 ng $mL^{-1}$. The half-life of **3** for the elimination phase was 31 minutes. The compound **3** showed a volume of distribution of 3.4 L $kg^{-1}$ and disappeared from the systemic circulation with a clearance of 77 mL min $kg^{-1}$. After oral administration (10 mg $kg^{-1)}$, the maximal plasma concentration of **3** was observed 30 minutes after dosing (665 ng $mL^{-1}$). The oral bioavailability of **3** was 66 %. Furthermore, **3** showed good BBB penetration, thus reaching a maximum concentration in 1 mL of brain homogenate of 0.613 ng $mg_{protein}^{-1}$, 30 minutes after oral administration (see Table S2). Accordingly, **3** reaches a total cerebral concentration of

0.62 µM (see the Supporting Information). Although below the in vivo target engagement levels, this represents an attractive starting point towards a promising hit optimization campaign.

In conclusion, we have reported on the first class of BACE-1 and GSK-3β dual-target inhibitors in the search for innovative AD modifiers. One of these new derivatives (**3**) has shown balanced in vitro activities against the two targets, with potency in the low micromolar range. In this respect, it is worth noting that **3** displays fragment-like features, and has a high LE against both targets, as well as several possibilities in terms of chemical tractability and functionalization. In addition, **3** has shown a promising profile in terms of neuroprotection and neurogenesis in cells, as well as of immunomodulation, with no sign of toxicity. It has also displayed good brain exposure, a fundamental property for CNS-directed drugs. Indeed, it is widely acknowledged that combining a desirable balanced biological profile with drug-like properties is a critical aspect of MT drug discovery.[19] In light of these features, we can point to triazinones as a promising class of MT fragments able to tackle amyloid and tau pathological cascades, and they deserve to be further explored in the field of AD.

The challenge in the fragment evolution process is to transform these low affinity binders into potent leads, while maintaining reasonable LE and an attractive PK profile. Such studies have been undertaken, and progress on the development of optimized dual BACE-1/GSK-3β inhibitors derived from **3** will be reported in due course.

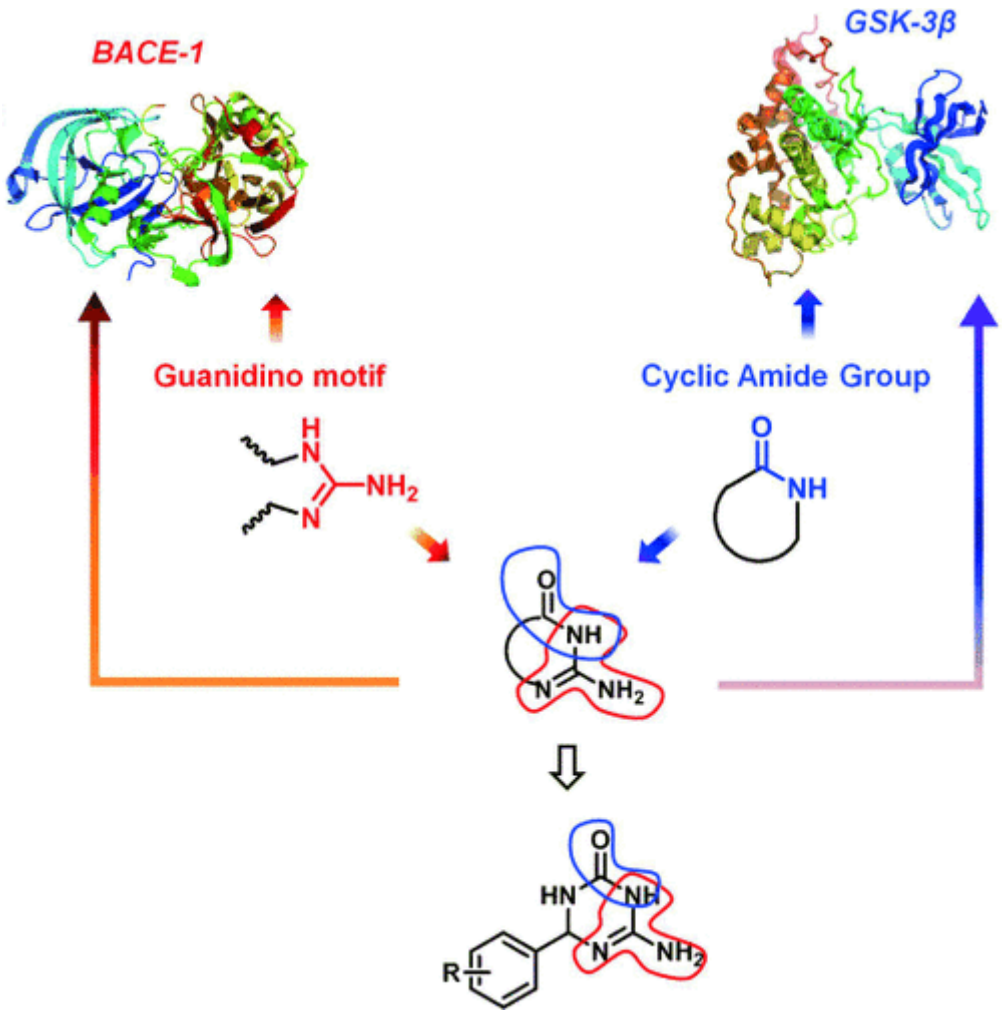



**Figure 1** Design strategy to dual BACE-1/GSK-3β inhibitors.

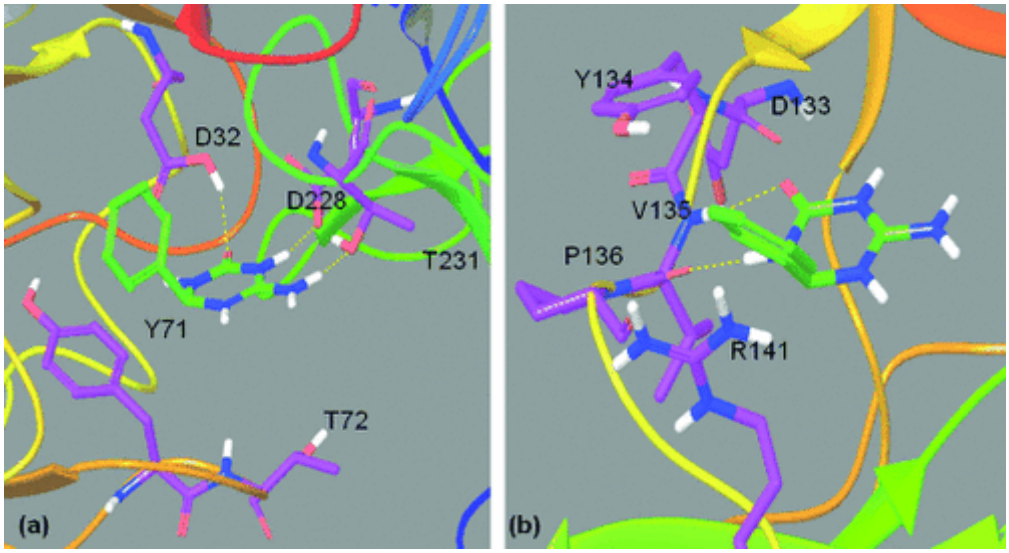



**Figure 2** (S)-1 docked into the catalytic region of BACE-1 (a) and GSK-3β (b). a) (S)-1 participated in a H-bond with D32, D228, and T231 in the BACE-1 catalytic pocket. The binding affinity associated to this pose is −7.291 kcal mol−1. b) (S)-1 participated in two H-bonds with V135 and stacking interactions with R141 (−6.372 kcal mol−1). The best orientation for (R)-1 presented similar binding affinities on both targets.

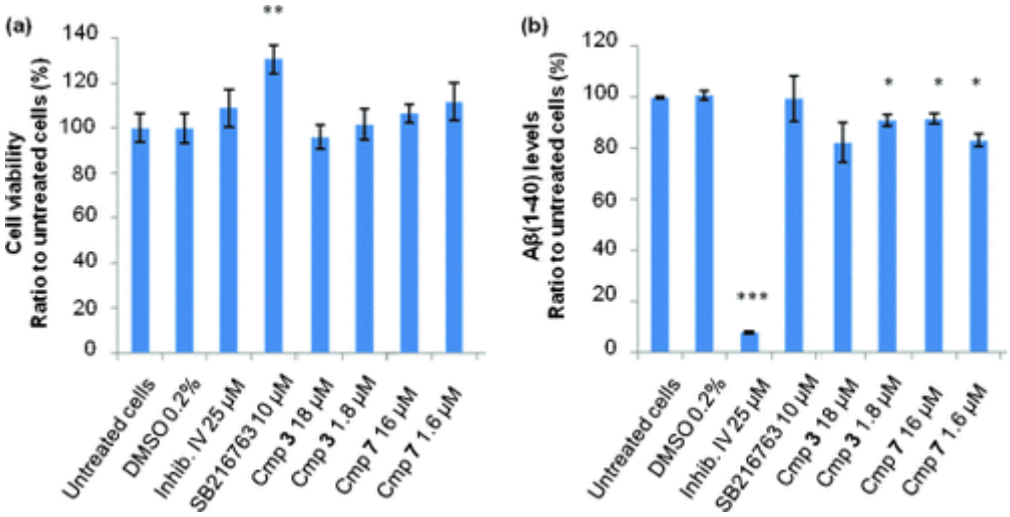



**Figure 3** H4 APPsw cells were treated with 3, 7, or reference compounds. a) Cell viability was assessed through MTT assay. Data represent mean ±SD. **p<0.01 Student t-Test compared to cell treated with vehicle alone. b) Aβ(1–40) levels in conditioned media were quantified through ELISA, and adjusted to take into account differences in cell proliferation. Data represent mean ±SD. *p<0.05, *** p<0.001, Student t-Test compared to cell treated with vehicle alone.

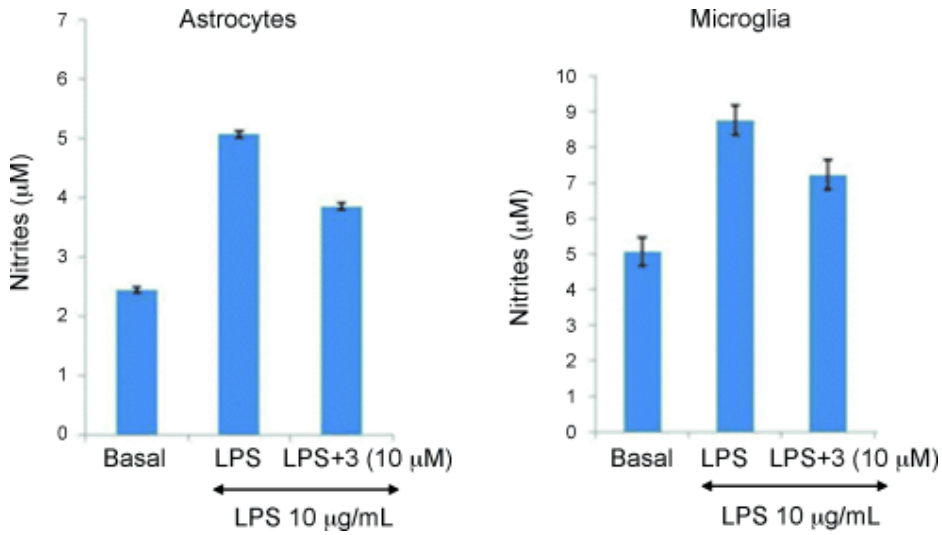



**Figure 4** Primary microglia and astrocyte cells were treated with LPS in the absence and presence of 3. The production of nitrites from the medium was measured with the Griess reaction.

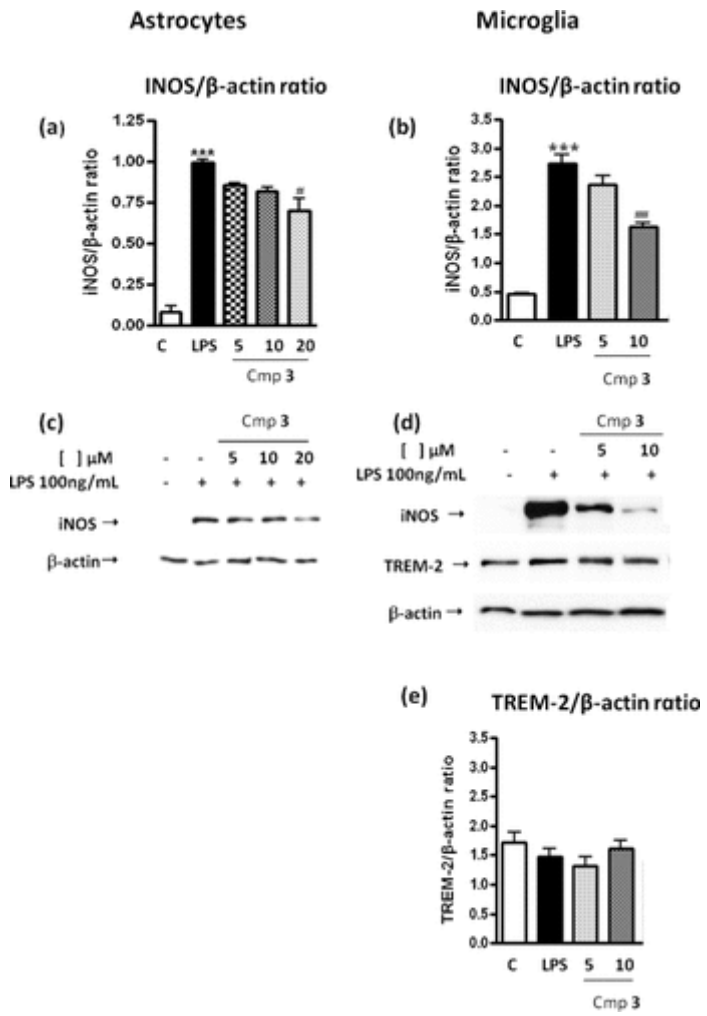



**Figure 5** Primary rat microglia and astrocyte cells were treated with LPS in the presence or absence of 3. a–d) Immunomodulatory activity of 3 in glial cells was evaluated through Western blot analysis of iNOS expression, using β-actin as control. d,e) The modulatory action of 3 on M1/M2 phenotypes was assessed by studying TREM2 expression in microglia.

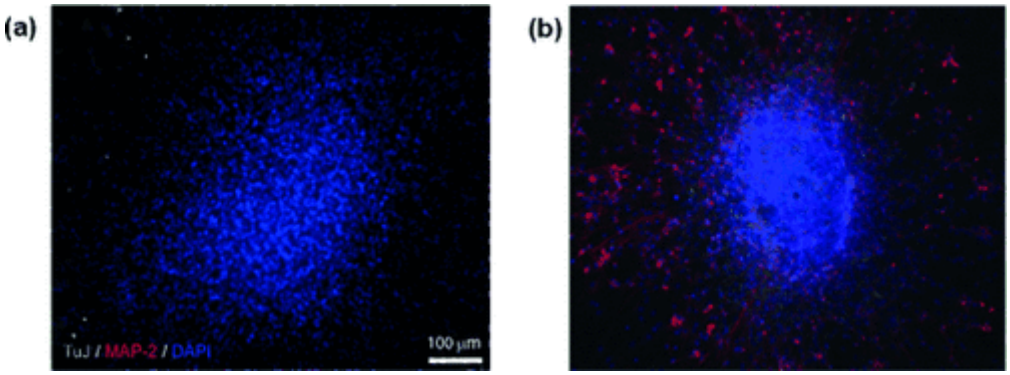
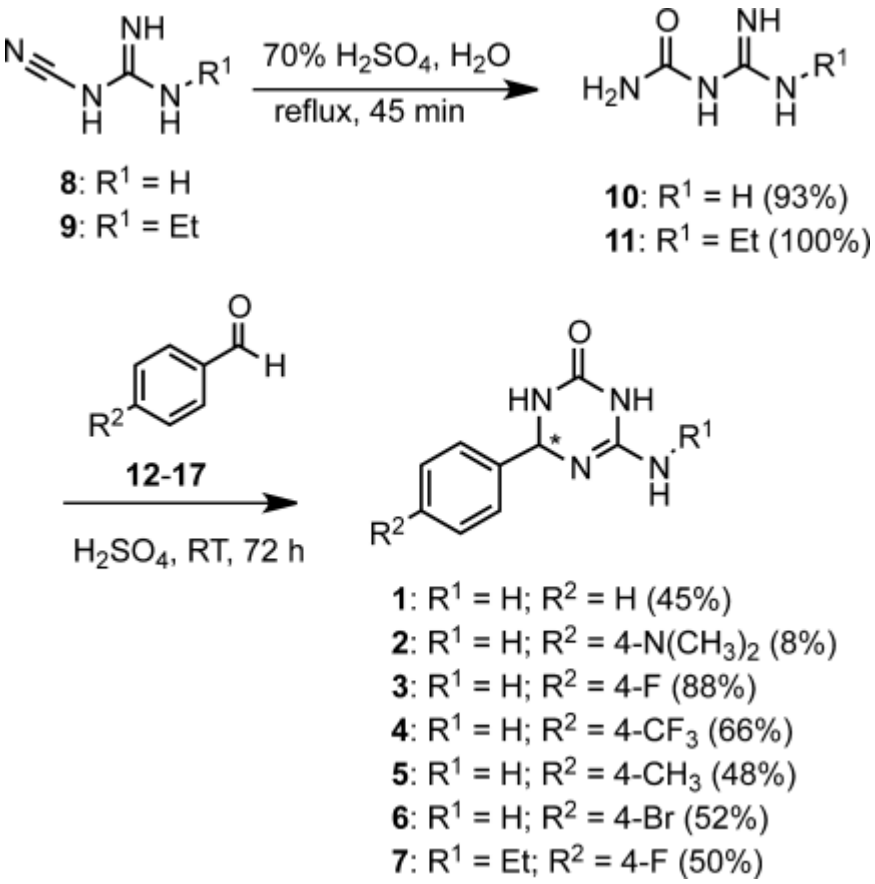



**Figure 6** The neurospheres were cultivated in the absence (a) or presence (b) of 3 during a week. After that, they were first incubated with anti-β-tubulin and anti-MAP2 antibodies, and treated with the corresponding Alexa-labeled secondary antibodies (green and red labels to reveal β-tubulin and MAP2 respectively). DAPI staining (blue) was used as nuclear marker.




**Scheme 1** Synthesis of 1–7.

| Compound | $S_{kinetic}$ [µM] | BACE-1 $IC_{50}$ [µM][a] ±S.E.M. | GSK-3β $IC_{50}$ [µM][a] ±S.E.M. |
|---|---|---|---|
| **1** | >250 | n.a. | 30.91±0.50 |
| **2** | 249 | n.a. | 26.44±0.01 |
| **3** | >250 | 18.03±0.01 | 14.67±0.78 |
| **4** | >250 | 61.53±14.2 | 37.78±4.50 |
| **5** | 247 | 222.37±60.2 | 27.37±6.13 |
| **6** | >250 | 41.24±1.29 | 25.75±0.26 |
| **7** | >250 | 16.05±0.64 | 7.11±0.37 |
| (+)-**7** | – | 14.23±0.01 | 12.69±0.44 |
| (−)-**7** | – | 12.55±0.01 | 16.00±0.84 |
| Inhibitor IV | – | 0.02±0.00 | – |
| SB415286 | – | – | 0.05±0.01 |

[a] IC50 values are reported as a mean value of three or more determinations. n.a.=not active up to a concentration of 100 µM.

**Table 1.** Aqueous kinetic solubility (Skinetic) and inhibitory potencies (IC50) of 1–7 and reference compounds against BACE-1 and GSK-3β.

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