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Discovery of the First-in-Class GSK-3 $\beta$ /HDAC Dual Inhibitor as Disease-Modifying Agent To Combat Alzheimer's Disease.

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# Discovery of the First-in-Class GSK 3 $\beta$ /HDAC Dual Inhibitor as Promising Disease-modifying Agent to Combat Alzheimer's Disease

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**ABSTRACT:** Several evidences pointed out the role of epigenetics in Alzheimer's disease (AD) revealing strictly relationships between epigenetic and "classical" AD targets. Based on the reported connection among histone deacetylases (HDACs) and glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), herein we present the discovery and the biochemical characterization of the first-in-class hit compound able to exert promising anti-AD effects by modulating targeted proteins in the low micromolar range of concentration. Compound **11** induces an increase in histone acetylation and a reduction of tau phosphorylation, it is non-toxic and protective against H<sub>2</sub>O<sub>2</sub> and 6-OHDA in SH-SY5Y and in CGN cell lines. Moreover, it promotes neurogenesis and displays immunomodulatory effects. Compound **11** shows no lethality in a wt-zebrafish model (< 100  $\mu$ M) and high water solubility.

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Alzheimer's disease (AD) represents the most common cause of dementia and it is a pathological condition for which a cure is not available. Unfortunately, it is also the disease with the highest attrition rates in drug discovery (99.6% of failure rate). Indeed, since Memantine's approval no candidates have successfully completed the clinical trials and no new drugs are on the horizon.<sup>1</sup> In this context it is not surprising the decision of some big company to end R&D on new anti-AD drugs.<sup>2</sup> The failures of intense decades of research from both industry and academia may be due to the not complete knowledge of the physiopathological events which lead to the onset of the pathology.<sup>3</sup> Due to the complexity of AD, it is now widely accepted that it could be better contrasted by a chemical entity able to simultaneously modulate multiple targets involved in the onset of the disease. It is an accepted belief that a single compound able to fulfill such an intricate scenario should more significantly impact the course of disease progression.<sup>4</sup> However, the development of multi-target ligands is a huge challenge in terms of target selection, molecules design and

physical-chemical properties requirements of the designed molecules.<sup>5</sup>

Plenty of ligands have been developed so far hitting a single or multiple targets. These targets act on different and not strictly related pathways believed to be involved in AD pathogenesis, such as acetylcholinesterase, monoamino oxidases, oxidative stress, metal dyshomeostasis etc.<sup>6, 7</sup> Besides the afore mentioned classical and deeply explored targets, some other proteins have been recognized as validated target for AD, like glycogen synthase kinase (GSK-3 $\beta$ ), which is considered one of the most attractive one, due to its pivotal role in this disorder.<sup>8</sup> It represents a key signaling enzyme involved in many pathways related to AD and it is one of the best candidates to modulate tau hyperphosphorylation. On the other hand, since the epigenetic modification is considered a new promising trend to ride for the study and treatment of AD,<sup>9</sup> the interest in regulating histone acetylation by modulating histone deacetylases (HDACs) activity is rising up. The emerging role of HDACs as AD target is also supported by the evidences of the

role of histone acetylation in rescuing learning and memory impairment.<sup>10</sup>

Particularly, several connections between GSK-3 $\beta$  and HDACs have been highlighted. D’Mello and coworkers have shown that the neurotoxic effects of HDAC1 depend on GSK-3 $\beta$  activity and, as a consequence, the pharmacological blockage of such activity prevents HDAC1-induced neuronal death in cerebellar granule neurons (CGNs).<sup>11</sup> In hippocampal neurons GSK-3 $\beta$  and HDAC6 were found in the same protein complex, in which GSK-3 $\beta$  phosphorylates HDAC6 enhancing its activity.<sup>12</sup> It is worth noting that elevated HDAC6 activity increases tau phosphorylation interfering with its propensity to aggregate and the HDAC6-selective inhibitor tubacin attenuates tau phosphorylation.<sup>13</sup> Furthermore, both GSK-3 $\beta$  and class I-II HDAC inhibitors are able to shift microglia from the neurotoxic activation (M1, proinflammatory) to the neuroprotective (M2, antiinflammatory) phenotype.<sup>14</sup> Moreover, it is reported that combined inhibition of GSK-3 $\beta$  and HDACs induces synergistic neuroprotective effects compared to the single drug with a potential improved therapeutic selectivity.<sup>15-17</sup> Based on the above considerations, herein we report the design, synthesis and preliminary biological evaluations of the first class of dual GSK-3 $\beta$ /HDACs inhibitors (Figure 1). Specifically, compound **11** shows the best biochemical profile opening up the road for the development of new groundbreaking anti-AD agents.

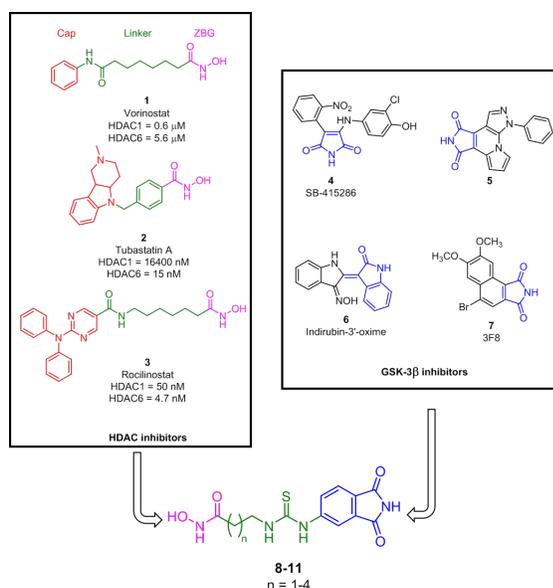


Figure 1. Drug design strategy leading to compounds **8-11**. On the top-left the pharmacophore features of HDACs known inhibitors are highlighted: a cap group, a linker and a zinc-binding group (ZBG) (colored in red, green and magenta, respectively). On the top-right some GSK-3 $\beta$  inhibitors containing the phthalimide-like function (depicted in blue).

## Results and Discussion

**Design.** In order to design dual HDACs/GSK-3 $\beta$  inhibitors, we sought to combine in a single chemical entity the pharmacophoric groups responsible for binding to GSK-3 $\beta$  and HDACs (Figure 1). Looking at known HDAC inhibitors (i.e. **1-3** in Figure 1),<sup>18-20</sup> it emerges that the following pharmacophore features are present: the hydroxamic acid as ZBG to chelate the Zn<sup>2+</sup> located in HDACs active site, an aromatic

moiety, named cap group, which occludes the entrance of the pocket and a linker connecting the two moieties. Particularly, polycyclic aromatic compounds are quite selective towards HDAC6 (see **2** and **3**), due to its larger substrate binding pocket.<sup>20</sup> As regards the GSK-3 $\beta$ , many inhibitors, for instance **4-7**,<sup>21-24</sup> possess a phthalimide-like scaffold, like the aryl-maleimide or the aryl-pyrrolinone, which are acknowledged to competitively bind to the ATP binding site of the enzyme. Bearing in mind these observations, we thought to design a small series of molecules possessing a hydroxamic acid as ZBG and the phthalimide moiety as cap group. In fact, this rather large aromatic moiety might possibly fit in both HDAC1 and HDAC6 and bind the GSK-3 $\beta$  enzyme. The hydroxamic acid has been connected to the phthalimide moiety through polymethylene linkers of different length (**8-11**), with the aid of a thiourea group. The choice of the thiourea group, among several connecting units, was due to its synthetic accessibility.

**Synthesis.** Detailed descriptions of the synthetic sequences are reported in the SI.

**In vitro enzymes inhibition.** Based on the expected activity profile, compounds **8-11** underwent to parallel evaluation for their ability to inhibit GSK-3 $\beta$ , HDAC1 and HDAC6 in comparison to compounds **12**, which bears the maleimide moiety responsible for GSK-3 $\beta$  inhibition (see SI for structure), **4**, a known GSK-3 $\beta$  inhibitor, and Vorinostat (**1**), a known HDAC inhibitor. All the new compounds are able to inhibit the activity of the three enzymes in the low micromolar range of concentration. As reported in Table 1, an interesting trend can be defined as compound **11** is more active than compounds **8-10** and **12** against GSK-3 $\beta$ . Compound **11** is also the most active inhibitor of HDAC6 of the series with an IC<sub>50</sub> similar to that of **1** (3.19  $\pm$  0.08 vs 5.6  $\mu$ M) and the inferior homologues are characterized by lower IC<sub>50</sub> values (**11** < **9** < **10** < **8**). Conversely, compound **11** is the weakest inhibitor within the series against HDAC1 with the most active of the series being **9** (IC<sub>50</sub> 2.24  $\pm$  1.17 vs 12.78  $\pm$  0.11  $\mu$ M of **11**). These effects clearly reflect the importance of the length of the spacers separating the hydroxamic acid and the phthalimide moieties. It is important to note that **1**, a well-known HDAC inhibitor, is not active against GSK-3 $\beta$ . In the same way, compound **12**, bearing the phthalimide moiety responsible for GSK-3 $\beta$  recognition, is not active against HDACs.

Table 1. Effects of compounds **1**, **4** and **8-12** on GSK-3 $\beta$ , HDAC1 and HDAC6 activity.

Compounds	n	GSK-3 $\beta$ IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	HDAC1 IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	HDAC6 IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>
<b>1</b>		n.a. <sup>b</sup>	0.6 (0.31-3.22) <sup>c, d</sup>	5.6 (5.31-7.45) <sup>c, d</sup>
<b>4</b>		0.05 $\pm$ 0.01	n.d. <sup>e</sup>	n.d. <sup>e</sup>
<b>8</b>	1	19.96 $\pm$ 1.76	3.75 $\pm$ 0.58	18.13 $\pm$ 0.17
<b>9</b>	2	9.85 $\pm$ 1.00	2.24 $\pm$ 1.17	12.58 $\pm$ 0.96
<b>10</b>	3	4.11 $\pm$ 0.01	5.02 $\pm$ 1.2	14.71 $\pm$ 0.19
<b>11</b>	4	2.69 $\pm$	12.78 $\pm$ 0.11	3.19 $\pm$ 0.08

		0.01		
<b>12</b>		20.22 ± 0.40	n.a. <sup>f</sup>	n.a. <sup>f</sup>

<sup>a</sup> IC<sub>50</sub> values are defined as the drug concentration which reduces by 50% the target activity and are reported as a mean value of three or more determinations. <sup>b</sup> n.a.: not active up to 50 μM. <sup>c</sup> Data from Ref. 25. <sup>d</sup> 95% confidence interval. <sup>e</sup> not determined. <sup>f</sup> not active up to 30 μM.

Compound **11** has been selected for a deeper biological investigation due to its high activity against GSK-3β and the two HDAC isoforms. Interestingly, it shows balanced *in vitro* activities against the targets.

**Binding Mode Analysis.** To gain insights into the mechanism of dual GSK-3β/HDAC inhibition and to elucidate the binding mode of the most active compound **11**, molecular docking calculations were performed using Glide 5.7 in SP mode.<sup>26</sup> As for GSK-3β, given a certain flexibility detected at the nucleotide-binding-loop, an ensemble docking using four structures (PDB codes: 1Q3D, 1Q41, 2JLD, 1UV5),<sup>23,27</sup> representative of different conformation, was carried out (see paragraph “X-ray Structures Selection” in SI). Docking of **11** in the 1Q41 structure resulted in not full convincing poses, as the maleimide moiety was mostly found not to H-bond the hinge region as expected. Differently, docking results were pretty similar and more reasonable when 1Q3D, 2JLD and 1UV5 structures were used, where the software mainly suggested two possible binding modes. In the binding mode A (Figure 2a), the maleimide core forms the two typical H-bonds with the hinge region backbone residues V135 and D133, the thiourea H-bonds, the Q185 backbone carbonyl and the long aliphatic linker allows the hydroxamic moiety to reach a really polar region of the protein formed by several arginine and an histidine. The proximity of these residues justifies the deprotonation of the hydroxamic acid and the establishment of two charge-reinforced H-bonds with the R144 and R141 side chains. In the binding mode B (Figure 2b), the maleimide establishes the same aforesaid contacts with the hinge region while the central spacer bends so that the hydroxamic acid is found under the nucleotide-binding loop forming two H-bonds with the S203 and the D200 (of the DFG motif) side chains. Looking at the **8-11** inhibitor profiles in Table 1, it is evident that the shorter the linker the lower the activity and only the binding mode A seems to be in accordance with these evidences. As regards the docking of **11** into the HDAC1 and HDAC6 (PDB codes: 4BKX and 5EDU; see SI for choice criteria)<sup>28, 29</sup> it converged toward a very similar pose in both the isoforms (see Figure 2c and 2d, respectively), where the hydroxamic acid coordinates the zinc ion and H-bonds a conserved tyrosine (Y303 in HDAC1, Y782 in HDAC6); the aliphatic spacer is found in the acetyl lysine tunnel stacked between two conserved aromatic side chain (F150 and F205 in HDAC1, F620 and F680 in HDAC6). The thiourea forms a bifurcated H-bond with the side chains of D99 in HDAC1 and of S538 in HDAC6, while the phthalimide contacts a conserved histidine (H28 in HDAC1, H500 in HDAC6) and H-bonds the E98 in HDAC1 and the D497 in HDAC6. Reasonably, the better accommodation of the phthalimide into the larger HDAC6 substrate binding pocket,<sup>20</sup> can explain the superior activity of DDD**11** toward HDAC6 with respect to HDAC1.

**In-cell Evaluations.** In order to evaluate whether the *in vitro* inhibitory activities translate into intracellular inhibition of HDACs, compound **11** was further evaluated in cell-based assay. Western blotting analysis was performed using human neuroblastoma SH-SY5Y cell line to determine the effects of compound **11** on the acetylation levels of tubulin and histone H3 at lysines 9/14 (H3K9K14ac) (Figure 3a). Cells were treated with compound **11** and its parent compounds, **1** and **12** (5 μM), for 30 h at three different concentrations (0.1, 1.0 and 5.0 μM). Compound **11** was able to induce hyperacetylation of α-tubulin although to a lesser extent than **1**. This effect was already visible at 0.1 μM and was concentration-dependent. On the opposite, it was not able to induce similar increase in the acetylation of histone H3, not even at the highest concentration tested. Since tubulin is a substrate of HDAC6 and acetylated tubulin levels function as a biochemical marker for HDAC6 cellular activity, the increase in the level of acetyl-α-tubulin compared to H3K9K14ac could be ascribed to an intrinsic cellular selectivity of compound **11** for HDAC6. Important effects on tubulin and histone H3 were not observed when cells were treated with **12**.

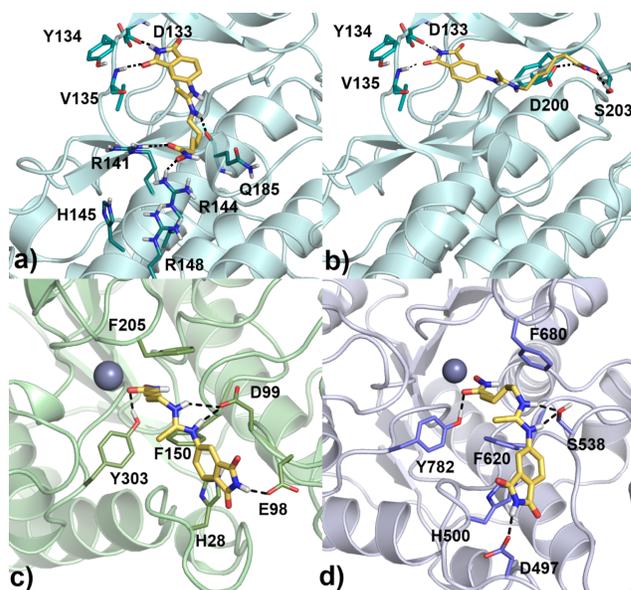


Figure 2. Binding mode A (a) and B (b) of **11** (golden sticks) in the active site of GSK-3β shown as a cyan cartoon and deep teal sticks. In c) and d) binding poses of **11** into the HDAC1 (green cartoon) and HDAC6 (violet cartoon) catalytic domains are depicted. All interacting residues are colored by atom type, whereas the H-bonds are represented as black dashed lines.

In order to evaluate the effect of compound **11** on tau-hyperphosphorylation, the protein's phosphorylation was induced in differentiated SH-SY5Y cells upon incubation with copper. Copper induced a time-dependent tau phosphorylation in differentiated SH-SY5Y cells, with a peak after 16h of incubation. Conversely, the level of tau phosphorylation returned to control cell value after 24h of incubation, probably due to compensatory mechanisms (data not shown). Based on these data, 400 μM copper for 16h was chosen as experimental condition. Following, the ability of the compounds to counteract copper-induced tau phosphorylation was examined. LiCl was used as reference control (Figure 3b). As reported, compound **11** (10 μM) completely counteract copper mediated tau

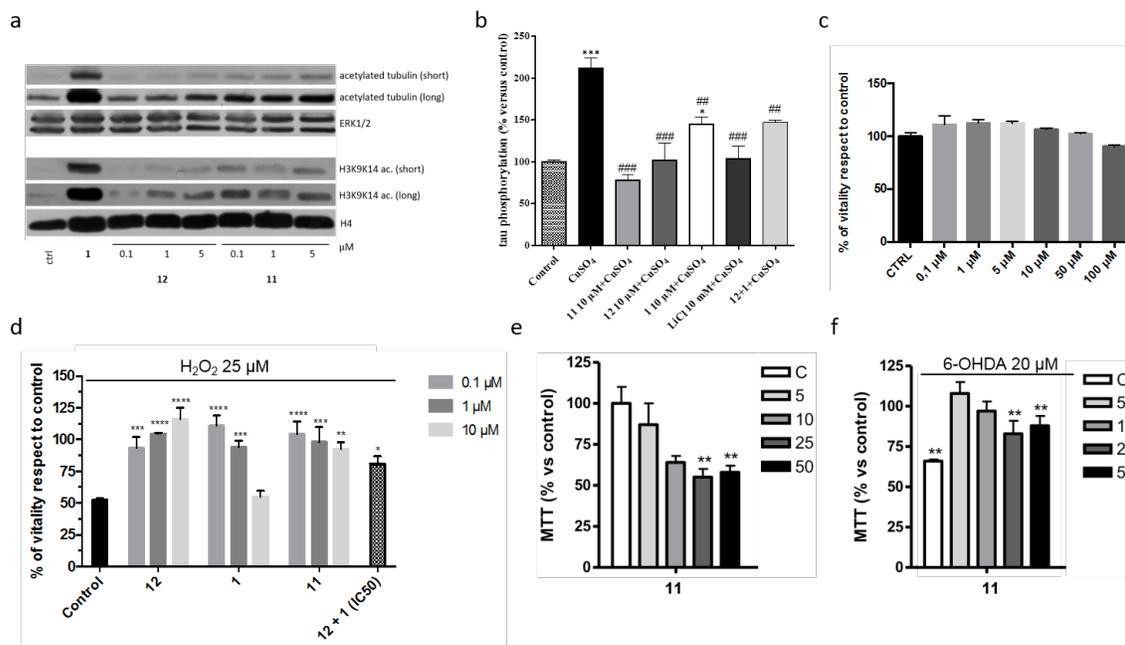


Figure 3. Cell-based Assay. a) Western blot probing for acetylated tubulin and histone H3K9K14ac in the SH-SY5Y cell line after 30 h treatment with **1**, **11** and **12**. b) Effects of the compounds on tau phosphorylation. The cells were pre-treated with LiCl (10 mM), **1**, **11**, **12** (10  $\mu$ M) or **1+12** for 1 h and then, with Copper (400  $\mu$ M) for 16 h. Following incubation time, cells were harvest by scraping and the pellet was obtained. Subsequently, the extent of tau phosphorylation was determined by immunoenzymatic assay. Absorbance was measured at 450 nm. Values represent the mean  $\pm$  SEM; \* $p$ <0.05, \*\*\* $p$ <0.001 vs control; # $p$ <0.05, ### $p$ <0.01, #### $p$ <0.001 vs cells treated with copper. c) Effect of **11** on SH-SY5Y cell viability after 24 h exposure at 37°C by MTS assay. Data are expressed as % of vitality respect to control. d) Effects of **1**, **11**, **12** and **12+1** on SH-SY5Y challenged with H<sub>2</sub>O<sub>2</sub>. The effect of drugs (0.1 – 10  $\mu$ M) on SH-SY5Y cells challenged for 24h with H<sub>2</sub>O<sub>2</sub> (25 and 50  $\mu$ M) was evaluated by MTS assay. Data are expressed as % of vitality respect to control. \* $P$  < 0.05, \*\* $P$  < 0.005, \*\*\* $P$  < 0.001, vs H<sub>2</sub>O<sub>2</sub>. e) and f) Neurotoxicity and neuroprotection on 6-OHDA-induced neurotoxicity in primary differentiated CGNs of compound **11**. Results are the mean  $\pm$  S.E. of at least 3 different experiments in quadruplicate; \*\*  $p$  < 0.01, \*\*\* $p$ <0.001 relative to untreated CGNs; ##  $p$  < 0.01, #### $p$ <0.001 relative to differentiated CGNs treated with 6-OHDA, Bonferroni's post-hoc test following one-way ANOVA.

phosphorylation in major extent compared to LiCl (10 mM), **12** and **1** (10  $\mu$ M). In order to assess if a mixture of compounds **1** and **12** could exhibit synergic effects on the block of tau phosphorylation, the cells were treated with compounds **1** and **12**. When combined together, no significant additive/synergic effect on phospho-tau levels was noticed with respect to single-treated cells (Figure 3b). Moreover, the percentage of phospho-tau inhibition was markedly lower with respect to that elicited by the dual-target compound **11**. These data suggest that a dual-target molecule may offer advantages in term of pharmacokinetics and cellular localization that enable an enhancement of its efficacy.

Since HDAC inhibitors are used in therapy as anticancer drugs, compound **11** was evaluated for its potential toxicity in SH-SY5Y cell line in comparison to **1** and compound **12** using MTS assay (Figure 3c and SI). Compound **11** did not show any toxic effects up to 100  $\mu$ M, while **1** showed a consistent decrease of the cell viability already at 10  $\mu$ M (see SI). Furthermore, compounds **1**, **11** and **12** were tested for their ability to counteract oxidative stress-induced neuronal death (Figure 3d). When compounds **1**, **11** and **12** were administered, the viability was restored at almost control value excepted for **1**. For compound **11** this effect was evident already at the concentration of 0.1  $\mu$ M. Cotreatment of **1** plus **12**, at their respective IC<sub>50</sub>s concentration, did show any synergic effect in con-

trasting H<sub>2</sub>O<sub>2</sub>-induced cell death. Compound **11** turned out to be more efficient than this combination. To further elucidate the activity of compound **11**, the levels of p53 protein were measured after treatments with H<sub>2</sub>O<sub>2</sub> in presence or in absence of compound **11** (see SI). Interestingly, in SH-SY5Y cells treated with H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M), p53 expression increases of about 30% and the treatment with compound **11** (10  $\mu$ M) restored the levels of p53 to control levels. Potential toxic and protective effects of compound **11** were also evaluated on primary differentiated CGNs and it was found able to completely counteract toxic stimuli induced by 6-hydroxydopamine (6-OHDA) already at 5  $\mu$ M (Figure 3e, f). Also in this case, compound **11** showed superior activity respect to the combination of **1** plus **12** in contrasting 6-OHDA-induced cell death (see SI).

It is well known that GSK-3 $\beta$ /HDAC inhibition promotes neurogenesis *in vitro* and *in vivo*. To assess whether compound **11** induces neurogenesis in SH-SY5Y cell line, the cells were treated with compound **11** for 24 h and at the end of the treatment the mRNA expression of recognized markers of neurogenesis including GAP43, N-myc and MAP-2 were assessed by RT-PCR analysis. The same experiments were performed by using **1**, **12** and retinoic acid (RA) as positive control (Figure 4a).

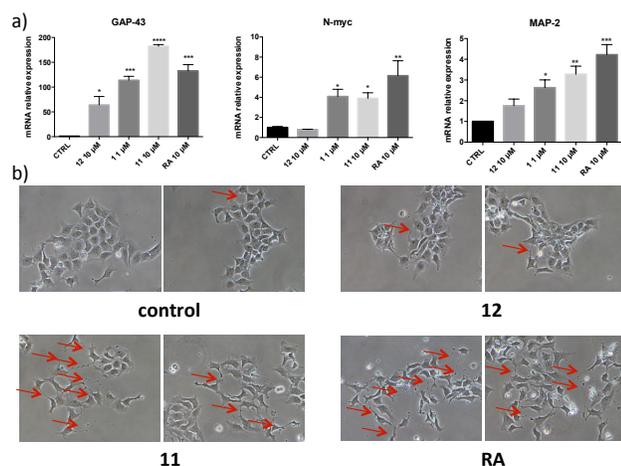


Figure 4. a) Effect of **11**, **12**, RA (10  $\mu$ M) and **1** (1  $\mu$ M) on neurogenesis markers expression (GAP43, N-myc and MAP-2) in SH-SY5Y after 24h-treatment. The data are expressed as fold of control. b) Effects of **11**, **12** and RA (10  $\mu$ M for 72h) on neurite outgrowth. Red arrows indicate cells bearing neurites. Pictures were taken at 20x magnification.

All the compounds were used at the concentration of 10  $\mu$ M with the exception of **1**, which was added at the final concentration of 1  $\mu$ M based on the vitality assay results. All the treatments were able to up-regulate GAP-43 and MAP-2 while N-myc was up-regulated by compound **11**, **1** and RA but not by compound **12**. Compound **11** increased the expression of MAP-2 and GAP-43 at higher levels than **1**. As expected, RA induced the expression of all the three markers. To confirm the obtained results, the morphology of the differentiated neuronal neurite outgrowth was assessed after cell treatment for 72 h with compounds **11**, **12** and RA (10  $\mu$ M) used as positive control. As shown in Figure 4b, compound **12** showed a moderate effect in stimulating neurite outgrowth, whereas compound **11** induced a substantial neurite outgrowth comparable to that caused by RA. It was not possible to analyze the effect of **1** due to compound toxicity after 72h of treatment. Furthermore, we evaluated the ability of compound **1**, **11** and **12** to modulate the microglial phenotypic switch from the M1 (pro-inflammatory) to the M2 (anti-inflammatory) type thereby decreasing neuroinflammation (Figure 5). Indeed, modulation of microglial activation seems to be a valid therapeutic approach in AD and compounds able to modulate the switch from the M1 to M2 phenotype would determine a decrease of neuroinflammation with a parallel increase of neuronal protection and recovery.<sup>30</sup>

The immunomodulatory effects of compounds **1**, **11** and **12** were evaluated on pure primary cultures of microglia by evaluating the expression of NOS2 and MRC1, which are both markers of M1 microglia, or TREM2, marker of M2 microglia. Western blot analysis clearly shows a more evident decrease in both NOS2 and MRC1 expression in cells treated with the compound **11** than in those exposed to **1** and **12**, while TREM2 is unchanged, thus indicating a shift of microglial cells from the M1 neurotoxic to the M2 neuroprotective phenotype and therefore an immunomodulatory activity of compound **11**.

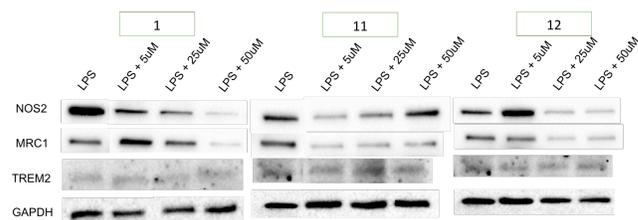


Figure 5. Western blot analysis of NOS2, MRC1 and TREM2 expression in primary microglial cells after a 24h treatment with LPS in presence or absence of compounds **1**, **11** and **12**. GAPDH was used as an endogenous control to normalize data.

**In vivo wt-Zebrafish evaluation.** Compound **11** was further profiled for its *in vivo* activity in wild-type zebrafish (*Danio rerio*) (Figure 6). Indeed, zebrafish represents an established model system for the *in vivo* validation of GSK-3 $\beta$  inhibitors and assessing drug safety and toxicity. The compounds were added 5 hpf (50% epiboly) and the embryos allowed to grow in chemical compound solution up to 2-4 days. Compound **11** showed no lethality in our concentration range (< 100  $\mu$ M). Furthermore, it showed no effects on embryos at the concentration of 25  $\mu$ M while at the concentration of 50  $\mu$ M a stunted and crooked tail was observed (Figure 6B). This phenotypic effect was more emphasized at 75  $\mu$ M (Figure 6C). At this concentration, some effects on the eyes formation could be observed. The detected effects are consistent with an *in vivo* GSK-3 $\beta$  inhibition.<sup>31</sup> Indeed, the perturbed zebrafish development induced by compound **11** is correlated to the Wnt/ $\beta$ -catenin pathway including GSK-3 $\beta$ . Also, the zebrafish embryo assay provides evidence of exposure and cell penetration of our derivative.



Figure 6. Effects on wild-type zebrafish embryos by compound **11**. (A) Control embryo in 2% DMSO. (B) Embryo treated with **11** at 50  $\mu$ M. (C) Embryo treated with **11** at 75  $\mu$ M.

**PhysicoChemical Properties of 11.** One of the main challenges in designing multiple ligands is related to the physical-chemical properties of the designed molecules. Pleasingly, compound **11** is characterized by very good water solubility of 53.76  $\mu$ g/ml (153.42  $\mu$ M) and fulfill the requirements of the Lipinski “rule of 5” (see SI).

## Conclusions.

To sum up, by exploiting a classical design strategy we have obtained the first-in-class small molecule able to exert anti-AD properties by modulating epigenetic and tau-related targets, such as HDAC1, HDAC6 and GSK-3 $\beta$ . Due to its activity profile and thanks to its low MW and high solubility, compound **11** might be considered a promising hit compound to develop an innovative disease-modifying agent. Hit-to-lead optimiza-

tion studies are currently ongoing and will be reported in due course.

## ASSOCIATED CONTENT

### Supporting Information.

Synthetic procedures, characterization of compounds, biological procedures and PDB coordinates of described complexes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ABBREVIATIONS

GAP-43, Growth Associated Protein 43; LPS, lipopolysaccharide; MAP-2, Microtubule-associated protein 2; MRC1, Mannose Receptor type C 1; N-myc, N-myc proto-oncogene protein; NOS2, nitric oxide synthase 2; TREM, Triggering Receptor Expressed on Myeloid cells 2.

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