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New neurogenic lipoic-based hybrids as innovative Alzheimer's drugs with sigma-1 agonism and beta-secretase inhibition

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Abstract

Background: Neurogenic agents emerge as innovative drugs for the treatment of Alzheimer's disease (AD), whose pathological complexity suggests strengthening research in the multi-target directed ligands (MTDLs) strategy. **Results:** By combining the lipoic acid structure with *N*-benzylpiperidine or *N*,*N*-dibenzyl(*N*-methyl)amine fragments, new MTDLs were obtained that act at three relevant targets in AD: sigma-1 receptor (σ_1R), beta-secretase-1 (BACE1), and acetylcholinesterase (AChE). Moreover, they show potent neurogenic properties, good antioxidant capacity, and favourable central nervous system permeability. Molecular modelling studies on σ_1R and BACE1 highlight relevant drug-protein interactions that may contribute to the development of new disease-modifying drugs. **Conclusion:** New lipoic-based σ_1 agonists endowed with neurogenic, antioxidant, cholinergic and amyloid β-peptide-reducing properties have been discovered for the potential treatment of AD.

List of abbreviations

Aβs, beta-amyloid peptides; AChE, acetylcholinesterase; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, beta-secretase-1; BuChE, butyrylcholinesterase; CNS, central nervous system; DBMA, *N*,*N*-dibenzyl(*N*-methyl)amine fragment; FRET, fluorescence resonance energy transfer assay; GPCRs, G protein-coupled receptors; LA, lipoic acid; LA-DBMA, lipoic – *N*,*N*-dibenzyl(*N*-methyl)amine hybrids; LA-NBP, lipoic – *N*-benzylpiperidine hybrids; MTDLs, multi-target-directed ligands; NBP, *N*-benzylpiperidine fragment; MD, molecular dynamics; NMDA, *N*-methyl-D-aspartate receptor; ORAC, oxygen radical absorbance capacity; PAMPA-BBB, parallel artificial membrane assay for the blood-brain barrier; σ_1R, sigma-1 receptor.

Key terms
beta-Secretase-1 (BACE1) is a protease involved in the formation of abnormal beta-amyloid peptides that ultimately cause amyloid plaques **Sigma-1 receptor (σ_1R)** is an intracellular protein engaged in many physiological processes, including neuroprotection and neurogenesis **Lipoic acid (LA)** is a natural antioxidant that captures free radicals, possesses chelating properties and regenerates other biogenic antioxidants **Neurogenesis** is the process by which neuronal cells are generated from neural stem-cells

Executive summary

Background

Multi-target directed ligands (MTDLs) could be an important advance for the treatment of Alzheimer's disease (AD), which is a highly complex pathology that affects an increasing population percentage. Recently, neurogenesis has emerged as an innovative AD-therapeutic approach, able to refurbish death neuronal cells by healthy ones.

Results & discussion

New lipoic – *N*-benzylpiperidine and lipoic – *N*,*N*-dibenzyl(*N*-methyl)amine hybrids have been obtained in good yields. These MTDLs interact with three AD-related targets: sigma-1 receptor (σ_1R), beta-secretase-1 (BACE1) and acetylcholinesterase (AChE). They also show potent neurogenic properties, good antioxidant capacity and favourable central nervous system permeability. *In silico* studies have shown the most important drug-protein interactions that could help to develop improved σ_1R and BACE1 ligands.

Conclusion

New neurogenic lipoic-based hybrids endowed with σ_1 agonism, antioxidant, cholinergic and amyloid β-peptide-reducing properties have been developed as potential AD-drugs

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with intricate pathological cascades that lead to the accumulation of abnormal deposits of beta-amyloid peptides (Aβs) and hyperphosphorylated tau protein, as well as a massive loss of neurons and synapses, especially in the cholinergic system [1]. Four drugs are currently marketed for the palliative treatment of AD: three acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine, and galantamine) and one antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, memantine [2]. These drugs relieve symptoms and could delay disease progression in the short-term, but they do not counteract the long-term degeneration and neuronal loss [3]. Therefore, there is an urgent need to find new therapeutic agents that actually could remedy AD rather than temporarily address its symptoms. Due to the highly complexity of AD, in the last years a great amount of multi-target directed ligands (MTDL) have been developed, following the idea that targeting different AD-objectives should produce better therapeutic rewards than one-target drugs [4-6].

Production and aggregation of Aβs in the brain result in amyloid plaques, both in familial and sporadic AD. Pathologic Aβs are the consequence of an abnormal cleavage of the amyloid precursor protein (APP), consecutively produced by β- and γ-secretase [7]. Even if these two proteins have been actively used as therapeutic targets, in recent years **β-secretase (BACE1)** has gained a great prominence due to two recent outstanding findings. Firstly, genetic evidences revealed that a mutation in the APP gene results in a 40% reduction in the formation of amyloidogenic Aβ, and in an effective protection against AD and cognitive decline in the elderly [8]. Secondly, several human clinical tests have showed a correlation between inhibition of BACE1 and lower levels of pathologic Aβs [9]. Moreover, the fact that BACE1 is involved

upstream in the neurotoxic cascade makes its inhibitors interesting disease-modifying drugs that can actually halt the degenerative process [10].

Beyond the classic, but still valid, AD targets (AChE, NMDA, BACE1, etc.), in recent years new therapeutic objectives have emerged, such as the **sigma receptors (σRs)** [11,12]. These proteins have received a great attention because of their involvement in several biological functions of therapeutic interest in neurodegenerative diseases [13]. They were originally misclassified as a subtype of opioid receptor in the 1970s, but nowadays σRs are considered as a unique type of intracellular proteins, different from the G protein-coupled receptors (GPCRs) and ionotropic receptors. In humans, σRs and in particular the subtype-1 (σ_1R) are expressed in regions of the central nervous system (CNS) related to motor, emotional, and cognitive functions: hippocampus, cerebral cortex, substantia nigra, dentate gyrus of hippocampus, and cerebellum, suggesting their involvement in numerous physiological processes [14]. For instance, activation of σ_1R elicits neuroprotection by different mechanisms: modulation of voltage-dependent calcium channels, which in turn controls calcium homeostasis [15]; attenuation of the production of reactive oxygen and nitrogen species, by increasing levels of endogenous antioxidant proteins [16]; and preservation of mitochondrial function in ischemic stress conditions, by increasing mitochondrial respiration and ATP synthesis [17].

Moreover, several studies have evaluated the potential usefulness of σ_1R agonists in AD models. The aminotetrahydrofuran derivatives anavex1-41 and anavex2-73 demonstrated an effective protection against the cell loss induced by Aβ toxicity in areas of hippocampus involved in memory and cognition, through a synergic activation of muscarinic and σ_1 receptors [18,19]. Another study reported that anavex2-73 was able

to reduce $A\beta_{25-35}$-induced tau hyperphosphorylation by restoration of levels of the active form of protein kinase B (PKB) [20]. It has successfully completed phase 1 clinical trial and is currently being evaluated in a phase 2a clinical study for AD [21].

On the other hand, **lipoic acid (LA)** is a natural occurring substance with antioxidant properties. LA is rapidly absorbed from diet and easily reaches the blood stream and tissues where is likely converted to its reduced form, dihydrolipoic acid (DHLA). LA and DHLA achieve some of the most important requirements to be considered good antioxidants. They are able to quench free radicals in aqueous and lipid phases, possess chelating properties and are able to regenerate other biogenic antioxidants [22]. LA has been identified as a cofactor for several enzymes, important in energetics and mitochondrial metabolism [23]. A great number of studies have demonstrated the beneficial properties of LA derivatives in several models of oxidative stress and neuroprotection [24,25]. Indeed, positive effects of LA on cognitive parameters and disease progression of AD patients has been reported in several studies [26]. Besides its antioxidant properties, BACE1 inhibitory activity has been also reported for molecules bearing LA such as lipocrine, a hybrid between LA and the AChE inhibitor tacrine [27].

Recently, **neurogenesis** has emerged as an innovative approach to combat AD and other neurological diseases [28]. During the embryonic development neurogenesis is a widespread process, but in the adult brain it becomes vestigial and only two stem-cells niches preserve a significant neurogenic action: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the hippocampus. In the last years, different molecular targets and signalling pathways involved in neurogenic processes have been identified and, as a consequence, different drugs have been tested in neuronal plasticity [29]. For instance, it has been demonstrated that several antioxidants increase

the number of connections between neurons [30] and that activation of σ_1R has positive effects on neurogenic processes in the SGZ [31].

Continuing with our interest in the development of new molecules for the potential treatment of neurodegenerative diseases [32-38], we recently focused our efforts on three important targets related to AD namely, AChE, BACE1, and σ_1R. With the purpose of developing new effective inhibitors for these proteins, we specifically combined the LA structure with *N*-benzylpiperidine (NBP) or *N*,*N*-dibenzyl(*N*-methyl)amine (DBMA) fragments, which are present in the well-known AChE inhibitors donepezil and 3-(4-((benzyl(methyl)amino)methyl)phenyl)-6,7-dimethoxy-2*H*-chromen-2-one (AP2238), respectively [39-41] (Figure 1).

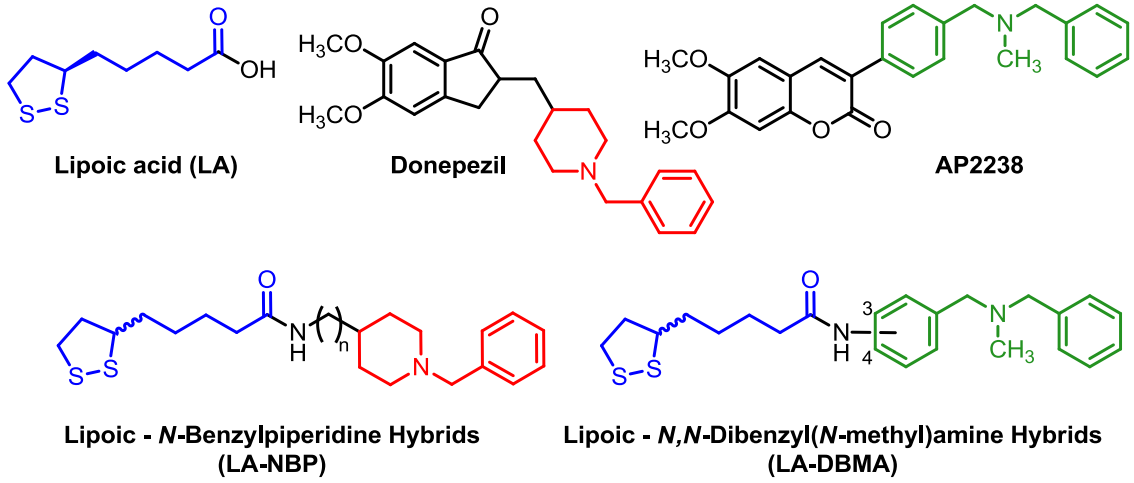

Figure 1. Structures of lipoic acid (LA), donepezil, AP2238, and new LA-based hybrids

Accordingly, in this article we describe the synthesis of new LA-based hybrids, namely lipoic – *N*-benzylpiperidine (LA-NBP) and lipoic – *N*,*N*-dibenzyl(*N*-methyl)amine (LA-DBMA) derivatives. We report their biological assessment on AChE, butyrylcholinesterase (BuChE), BACE1, and σRs (binding constants for σ_1R

and σ_2R; and functional activity in σ_1R), as well as their evaluation *in vitro* as potential neurogenic agents, using murine neural stem-cells. Their oxygen radical absorbance capacity (ORAC) and their CNS permeation predicted by the *in vitro* PAMPA-BBB assay are also evaluated. Lastly, molecular modelling studies on the interactions of these hybrid drugs with BACE1 and σ_1R provide a rationale for the binding behaviour of these novel LA-based hybrids, and may constitute the starting point for the development of further neurogenic drugs acting at important AD protein targets, such as AChE, BACE1, and σ_1R.

Results & discussion

Chemistry

New LA-based hybrids were obtained via coupling reaction between LA and the corresponding amine derivative (Figure 2). In the NBP series, the required intermediates 1-benzylpiperidin-4-amine (n = 0) and 2-(1-benzylpiperidin-4-yl)ethanamine (n = 2) are commercially available, whereas *N*-benzylpiperidine amines with different linker lengths (n = 1 and 3) were synthesized by two different methods depending on the length of the hydrocarbon chain. The treatment of piperidine-4-carbonitrile with benzyl bromide in toluene at reflux for 3h gave 1-benzylpiperidine-4-carbonitrile (**1**) in excellent yield (92%). Its subsequent overnight reduction with $LiAlH_4$ in ethyl ether at rt provided amine **2** in 85% yield. On the other hand, the Knoevenagel-Doebner reaction of 1-benzylpiperidine-4-carbaldehyde and cyanoacetic acid in refluxing pyridine for 3h yielded a mixture of unsaturated nitriles, which were subjected to a Pd-catalysed hydrogenation in EtOH at rt overnight, yielding the required nitrile **3** (87%). Reduction

of the above nitrile with $LiAlH_4$ at rt gave the corresponding amino derivative **4** in good yield (75%). In the DBMA series, intermediates **5** and **6** were obtained in high yields (95-97%) by the reaction between *N*-methyl-benzylamine and the corresponding nitro benzyl bromide in refluxing toluene for 3h. Their reduction by Pd-catalyzed hydrogenation gave the corresponding amines **7** and **8** in almost quantitative yields (97-98%) (Figure 2) (see supplementary information for further details).

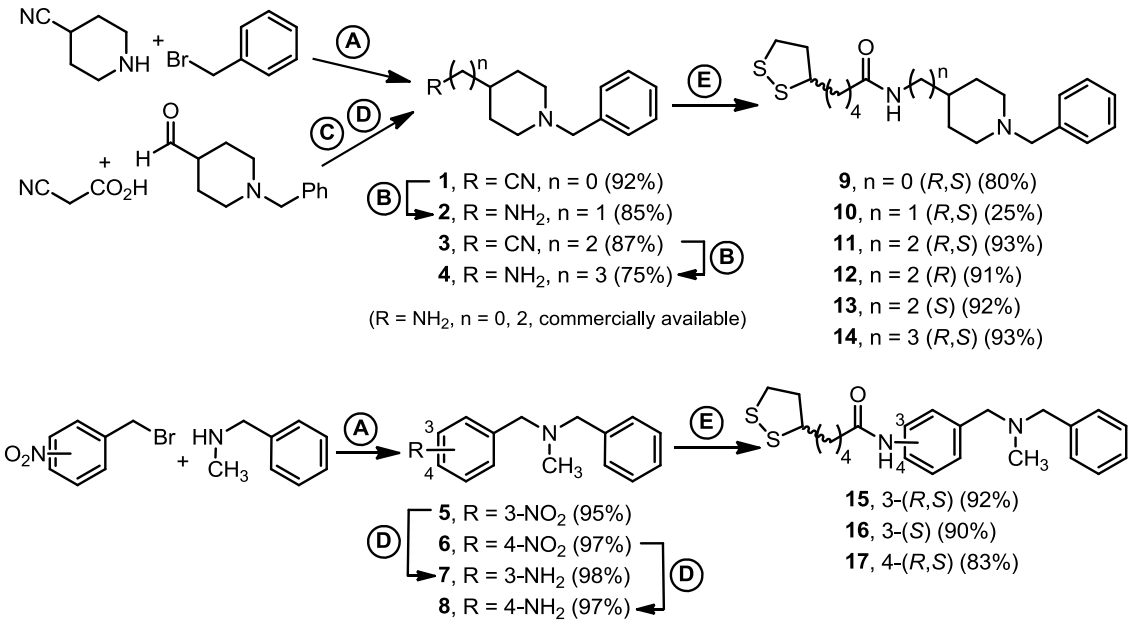

Figure 2. Synthetic routes to LA-NBP and LA-DBMA hybrids. Conditions: **(A)** Et_3N, toluene, reflux 3h; **(B)** $LiAlH_4$, ethyl ether, rt overnight; **(C)** Pyridine, reflux 3h; **(D)** H_2, Pd/C (5%), EtOH rt overnight; **(E)** Lipoic acid, CDI, THF, mw 120°C.

Finally, LA-based hybrids **9**-**17** were obtained by a coupling reaction between the corresponding amine derivative and LA in a microwave oven at 120 ºC, using 1,1'-carbonyldiimidazole (CDI) as activating agent and THF as solvent. In general, hybrids

were isolated in high yields (80-93%), although in the case of **10** an unexpected low 25% was obtained despite several attempts of improving the reaction work-up (Figure 2).

All new LA-NBP and LA-DBMA hybrids were purified in silica gel cartridges using an automatized chromatographic equipment (IsoleraOne, Biotage), and were characterized by their analytical (HPLC, HRMS) and spectroscopic data (^{1}H NMR, ^{13}C NMR). Complete NMR assignment of their hydrogen and carbon atoms (see supplementary information for details) were made by ^{1}H – ^{13}C two-dimensional diagrams, mainly HSQC (heteronuclear single quantum correlation) and HMBC (heteronuclear multiple bond correlation).

Inhibition of human cholinesterases (h-ChEs) and beta-aminosecretase (h-BACE1). Evaluation of the oxygen radical absorbance capacity and prediction of the CNS-permeation

Firstly, all LA-based hybrids were evaluated as inhibitors of human AChE and BuChE (h-AChE and h-BuChE), following the Ellman method [42] (Table 1). In general, compounds demonstrated a moderate inhibition of human ChEs, with IC_{50} in the range micro- and submicromolar. For the NBP series and in relation to the hAChE inhibition, the chain length connecting LA and NBP fragments was found to be a critical parameter; the best activity results were obtained for n = 2 (i.e., -$(CH_2)_2$-). Racemic (*R*,*S*)-**11** and its enantiomers (*R*)-**12** and (*S*)-**13** showed IC_{50} values for hAChE in the sub-micromolar range, without any noticeable difference between racemic mixture and pure enantiomers. However, in the case of h-BuChE, both enantiomers (*R*)-**12** and (*S*)-**13** displayed better inhibition (around one order of magnitude) than the corresponding

racemic mixture (*R*,*S*)-**11**. Replacement of the NBP fragment with DBMA did not improve the inhibitory potency of compounds but shifted the selectivity between the two h-ChEs, displaying a slight tendency for h-BuChE. Hybrids **15** and **16**, bearing a *meta*-substituted intermediate ring, maintained their sub-micromolar inhibition for h-BuChE, unlike their para-substituted counterpart **17**.

Table 1. Inhibition of h-AChE, h-BuChE, and h-BACE1 (IC_{50}, µM), evaluation of the oxygen radical absorbance capacity (ORAC, trolox equiv.), and measure of the permeability by the PAMPA-BBB assay (P_e,10^{-6} cm s^{-1}) with prediction of the CNS-penetration[a]

Compd.	h-AChE	h-BuChE	h-BACE1	ORAC	PAMPA-BBB
9	6.81 ± 0.25	7.99 ± 0.35	27%[b]	0.94 ± 0.07	nd
10	31%[b]	<20%[b]	6.33 ± 0.21	nd	nd
11	0.39 ± 0.03	2.13 ± 0.14	5.65 ± 0.26	0.96 ± 0.02	nd
12	0.43 ± 0.11	0.79 ± 0.20	8.11 ± 0.26	1.04 ± 0.09	33.0 ± 3.1 (cns +)
13	0.21 ± 0.09	0.63 ± 0.09	9.92 ± 0.39	0.80 ± 0.08	26.6 ± 0.7 (cns +)
14	3.75 ± 0.91	0.93 ± 0.3	7.91 ± 0.77	0.63 ± 0.01	nd
15	46%[b]	0.53 ± 0.21	28%[b]	1.00 ± 0.08	nd
16	48%[b]	0.33 ± 0.12	25%[b]	0.79 ± 0.09	nd
17	2.43 ± 0.12	4.87 ± 0.23	38%[b]	0.73 ± 0.11	37.6 ± 0.3 (cns +)
Donepezil	0.01 ± 0.002	2.5 ± 0.07	0.17[c]	nd	nd
AP2238	0.044[d]	48.9[d]	nd	nd	nd

[a]Results are expressed as mean ± SEM (n =3). [b]Inhibition percentage at 10 µM. [c]Taken from ref **[43]**. [d]Taken from ref **[41]**. nd: Not determined.

LA-based hybrids were evaluated as inhibitors of the human recombinant BACE1 protein in a fluorescence resonance energy transfer (FRET)-based assay [37,43]. Firstly, compounds were tested at a single concentration (10 µM) and those displaying an inhibition percentage above 50% were re-evaluated in a concentration range comprised between 0.1 µM and 100 µM to calculate the IC_{50} values listed in Table 1. Whereas compounds **9** and **15**-**17** showed low percentage of h-BACE1 inhibition at 10 µM, hybrids **10**-**14** were found to be good h-BACE1 inhibitors with IC_{50} values in the low micromolar range. Thus, compounds containing the NBP moiety were more potent than DBMA derivatives. In the NBP series, hybrids **10**-**14** (n = 1-3) showed little variations in their IC_{50} values (5.7 – 9.9 µM).

Radical scavenging properties were evaluated with the ORAC assay [44,45]. Trolox, the scavenging part of vitamin E, was used as internal standard with the arbitrary value of ORAC = 1.0. Results are expressed as trolox equivalents (trolox mmol / tested compd mmol) in a comparative scale. All tested LA-based hybrids showed radical scavenging properties close to vitamin E and thus, they could be considered as good antioxidant agents (Table 1).

Derivatives **12**, **13** and **17** were next selected as representative compounds of the two families to evaluate their ability to cross the blood-brain barrier in the *in vitro* parallel artificial membrane assay (PAMPA-BBB) [37,46]. Experimental permeability data were validated by comparison with previously reported values for several commercial drugs (see supplementary information). All evaluated compounds were predicted to reach the CNS according to this *in vitro* model (Table 1).

Studies on sigma receptors

The affinities of new lipoic-based hybrids for σ_1R and σ_2R were determined in competition experiments with radioligands. All compounds were tested against σ_1 and σ_2 receptors of animal origin obtained from guinea pig brain (σ_1) and rat liver (σ_2), respectively. Two well-known sigma binding site ligands, (+)-pentazocine and 1,3-di-o-tolylguanidine (DTG), were also tested for comparative purposes (Table 2).

Table 2. Affinities and selectivities towards σ_1R and σ_2R of compounds **11**-**13**, **15** and **17**. The affinity of pentazocine and DTG is reported as reference compounds.

Compd	**σ_1R[a]**	**σ_2R[a]**	**Selectivity *vs.* σ_1R[b]**
11	8.90 ± 0.45	232 ± 27	26
12	7.56 ± 0.98	205 ± 42	27
13	15.1 ± 1.4	289 ± 51	19
15	25.3 ± 1.9	1200 ± 170	48
17	21.0 ± 2.6	1400 ± 230	67
Pentazocine	15.0 ± 3.0	-	-
DTG	-	54 ± 8	-

[a]Results are expressed as K_i (nM) and are the mean ± SEM of the experiments repeated in triplicates. [b]Selectivity *vs.* σ_1R was calculated as $K_i\sigma_2$R/ $K_i\sigma_1$R

As shown in table 2, tested compounds showed good affinities for σRs, with K_is comprised between the low-micromolar and the low-nanomolar scale. In all cases, lipoic-based hybrids exhibited a remarkable preference for the σ_1R subtype since their experimental σ_1R affinities are in the range of low-nanomolar concentration with a selectivity ratio against the σ_2R greater than at least 19 times.

New compounds were then functionally tested at σ_1R, using a basic assay to define their agonistic or antagonistic character. The general consensus is that σ_1R antagonists provoke a high cytotoxic effect leading to cell death, whereas σ_1R agonists preserve cell survival [47]. For defining the agonist/antagonist profile of the new derivatives we tested their cytotoxicity on the SH-SY5Y neuroblastoma cell line by adapting the approach originally proposed by Zeng et al. based on the MTT assay [48]. To this aim, the cytotoxicity of the novel σ_1R ligands was tested in SH-SY5Y cells and compared to that measured for NE-100 and pentazocine, two commonly accepted σ_1R antagonist and agonist, respectively.

Figure 3 shows cytotoxicity data of σ_1R ligands at 50 μM, expressed as a percent relative to the cytotoxicity of NE-100 at the same concentration, as obtained from the cell viability assay. Conversely, at the same concentration, pentazocine showed only limited toxicity (2%) to the SH-SY5Y cells. As inferred from figure 3, the moderate or low cytotoxicity of all lipoic-based hybrids is more consistent with a σ_1R-agonist, rather than with a σ_1R-antagonist activity. Both DBMA derivatives (*R*,*S*)-**15** and (*R*,*S*)-**17** could be define as full agonists, as demonstrated by a cytotoxic level under 10%, comparable to the prototypical agonist pentazocine. Instead, the NBP derivative (*R*,*S*)-**11** and its pure enantiomers (*R*)-**12** and (*S*)-**13** behaved as partial agonists with a cytotoxicity comprised between 40-60%, in any case lower compared to the antagonist NE-100. This fact could be ascribed to their smaller selectivity against the σ_1R subtype as illustrated in table 2.

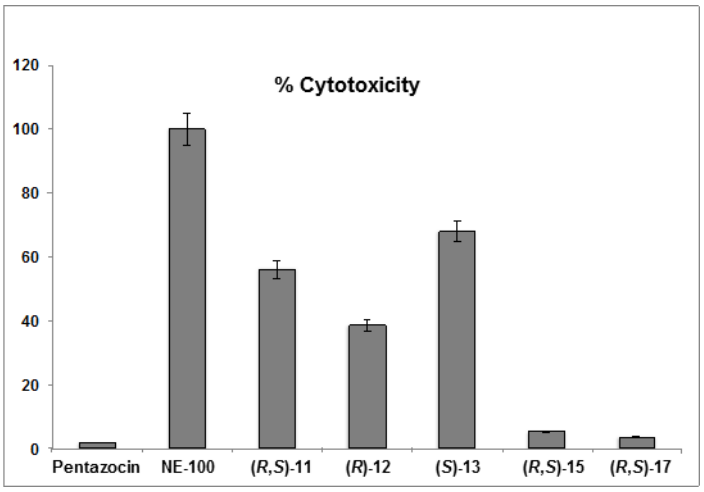

Figure 3. Cytotoxicity of σ_1R ligands as obtained from the MTT assay. SH-SY5Y cells were treated with different σ_1R ligands (50 μM) for 48h. MTT assay was then performed, cytotoxicity of compounds was determined, and data were reported as % of NE-100 cytotoxicity at 50 μM (100%). The bars represent the mean ± SD from three independent experiments performed in triplicate.

Neurogenic studies

These studies were performed to assess the potential ability of new LA-based compounds to promote differentiation of brain stem-cells into a neuronal phenotype. Derivatives **12** and **17**, covering different structural features and biological activities, were selected for these experiments. The LA-NBP compound **12** showed low micromolar and sub-micromolar inhibition of h-ChEs and h-BACE1, an ORAC value similar to vitamin E, and was found to be the most potent σ_1R agonist of this work. In contrast, the LA-DBMA hybrid **17** showed worse inhibition of h-ChEs, no activity at h-BACE1, an ORAC value under the trolox value, but displayed the most selective σ_1R agonism compared to σ_2R. Adult mice neural stem-cells were isolated from the SGZ of

the dentate gyrus of the hippocampus, and cultured as neurospheres (NS) following described protocols [34,49,50]. NS were incubated in the presence of **12** and **17** during 7 days and then adhered on a substrate to allow differentiation for 3 days. Immunocytochemical analysis using β-III-tubulin (clone TuJ1) and MAP-2 (microtubule-associated protein 2) antibodies were used to visualize early proliferation and neuronal maturation, respectively. As shown in Figure 4, treatment with **12** and **17** clearly enhanced neurogenic activity on cultured NS. Both compounds were able to induce the expression of early neurogenesis markers like β-III-tubulin and also promoted neuronal maturation, increasing the number of MAP-2 expressing cells. Interestingly, compound **12** appeared to be more effective not only promoting early neurogenesis but also stimulating neuronal maturation, showing a great neurogenic effect.

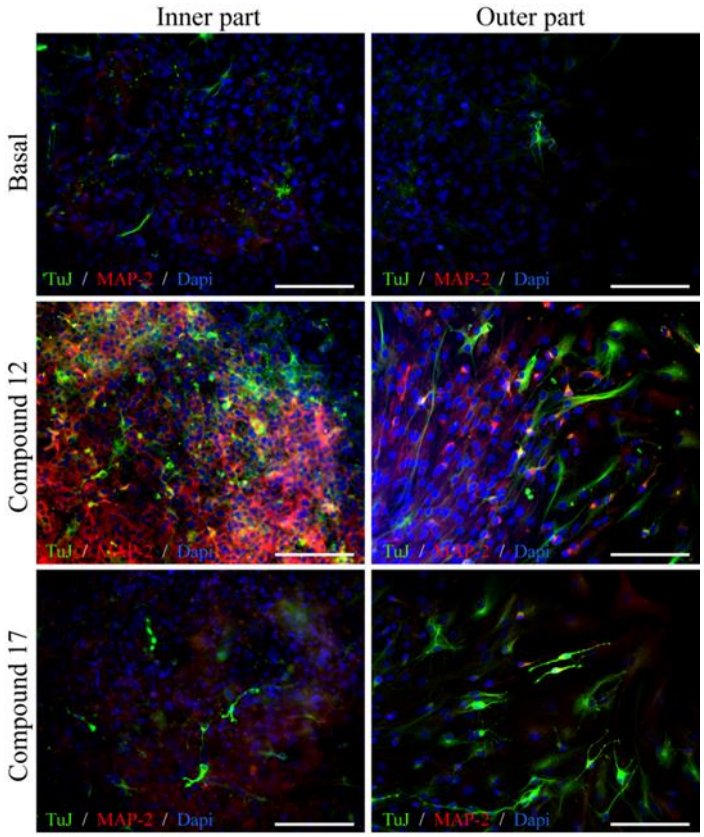

Figure 4. *In vitro* neurogenic effect of compounds **12** and **17** on murine hippocampal SGZ-derived neurospheres. Neural stem-cells enriched neurospheres (NS) were grown for 7 days in culture in the presence of **12** or **17** (10 µM). Later on, NS were adhered on a substrate to allow differentiation for 3 days in the presence of compounds. Representative images show the expression of β-III-tubulin (TuJ clone; green) and MAP-2 (red) inside the NS (inner part) and in the distal area (outer part). DAPI was used for nuclear staining. Scale bar, 200 µm.

Finally, to learn about the possible binding mode of new LA-NBP and LA-DBMA hybrids in both BACE1 and σ_1R, two molecular modelling studies were performed.

Modelling studies in h-BACE1

BACE1 is an aspartic protease, which acts in the first step of the pathway leading to the production and deposition of Aβs. It is a structurally challenging protein target, which displays a pronounced induced-fit conformational adjustment upon ligand interaction. Indeed, a detailed comparison of the available X-ray structures suggests that both the flap open region of the enzyme (residues 68–74 forming a β hairpin loop) and the 10s-loop (residues 9–14) located near the S3 site undergo a substantial rearrangement upon ligand binding [51]. Accordingly, BACE1 adopts a bilobal structure with the inhibitor binding in the substrate binding pocket between the N-terminal and C-terminal lobes of the enzyme. Catalytic aspartates D32 and D228 are located in the centre of this pocket, and form part of an extensive hydrogen bonding network within the protein active site [52].

A challenge in the design and discovery of BACE1 inhibitors is posed by the large size of its substrate pocket. However, the development of large inhibitors is of poor practical use, given the known pharmacokinetics and pharmacodynamics problems such drugs may encounter *in vivo*. Concomitantly, small-molecule inhibitors would hardly fill the binding pocket adequately and, as such, are not endowed with great inhibitory potency. A way to overcome this issue can consist in increasing the affinity of a given inhibitor for the BACE1 active site by potentiating its interactions with the residues lining the enzyme binding cavity.

In this context, we have carried out a molecular modelling study aimed at providing insights into the binding mode of the new LA-NBP and LA-DBMA hybrids onto BACE1. Preliminary predictions suggest that these derivatives show good blood-brain barrier penetration and CNS activity [53], in good agreement with our experimental *in vitro* PAMPA-BBB model. A three-step computational procedure was followed. In the

first stage, we performed docking studies on the whole target protein surface. In a second stage, the best determined binding mode was further refined by molecular dynamics (MD) simulations. Finally, binding free energy (ΔG_{bind}) calculations in the MM-PBSA framework of theory [54] were carried out to gain insight into thermodynamics and the nature of the stabilizing interactions for each drug/protein complex (Table 3).

Table 3. Binding free energies ΔG_{bind} and its components for **12**, **13**, (*R*)-**17** and (*S*)-**17** in complex with BACE1[a]

Compd.	ΔH	$T\Delta S$	ΔG_{bind}
(*R*)-**12**	-47.50 (0.21)	-29.16 (0.27)	-18.34 (0.35)
(*S*)-**13**	-47.26 (0.23)	-29.10 (0.26)	-18.16 (0.33)
(*R*)-**17**	-32.11 (0.21)	-23.42 (0.29)	-8.69 (0.37)
(*S*)-**17**	-32.09 (0.19)	-23.28 (0.28)	-8.81 (0.44)

[a]Values are expressed in kcal/mol and errors are given in parenthesis as standard errors of the mean.

Analysis of the binding mode of (*R*)-**12** and (*S*)-**13** into the BACE1 enzyme revealed that the NBP scaffold locates below the flap region, allowing the protonated nitrogen of the piperidine to interact with both catalytic aspartic acids D32 and D228 [52,55], and with T231 via electrostatic and H-bond interactions (Figure 5). The benzylic group establishes favorable π-π stacking contacts with the side chain of Y198 (S2' pocket) and hydrophobic interactions with V332, I226, and T329 in the entry region of the binding pocket. The piperidine ring sits in the S1' subsite, defined by residues D32, D228, G34, S35 and T231. The hydrophobic side chains of L30, Y71, F108, W115, and L118 in the S1 site nicely accommodate the linker portion of the compounds, with a hydrogen bond

formed between the amide carbonyl and the flap backbone NH of Q73. This is an important finding since hydrogen bonds to the backbone NH of the flap are essential to exhibit activity [56,57]. Additionally, the amide proton is hydrogen bonded to the backbone nitrogen of G230. Also in this case, the presence of an amide moiety that occupies the S1/S3 pockets of BACE1 and engages G230 proved to be a successful strategy to significantly increase BACE1 potency [58]. The chain of the LA fragment extends deep into the S3 pocket, thereby establishing hydrophobic contacts with I110, W115, G11, G13, and Q12 side chains, while the dithiolane ring docks in the hydrophobic subpocket formed by the side chains of Y14, S229, T232, and A335. All together, these interactions may account for the micromolar inhibitory potency of (*R*)-**12** and (*S*)-**13**. Traditionally, appropriate substituents in S3 contribute significantly to potency [59,60]; moreover, the S3 pocket can accommodate large hydrophobic ligands [61,62]. Thus, the chirality on the LA fragment is expected to have a minor impact. In fact, and in line with the experimentally determined IC_{50} values, we did not detect any significant difference in the binding mode of the two enantiomers (*R*)-**12** and (*S*)-**13**.

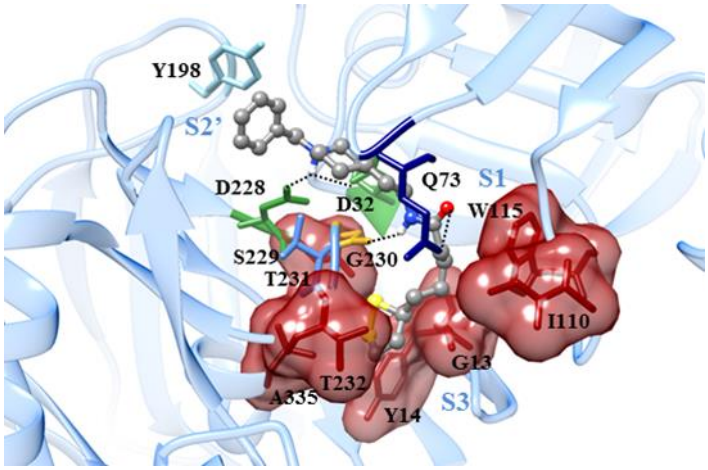

Figure 5. Details of compound (*S*)-**13** (in atom-colored sticks-and-balls: grey, C; blue, N; red, O; yellow, S) in the binding pocket of BACE1. The secondary structure of the protein is portrayed as a light-blue ribbon. Hydrogen bonds are highlighted as black dotted lines. Hydrogen atoms, water molecules, ions and counter ions are omitted for clarity.

In all complexes analyzed, BACE1 assumes a closed conformation; the flap adopts a conformation complementary to the shape and nature of the ligand bound in the active site, while Y71 is hydrogen bonded to W76 side chain, which physically separates the S1 and S2′ sites. However, some differences have been found regarding the DBMA derivative **17** (Figure 6). The protonated nitrogen of the ligand is able to reach the acid environment formed by the catalytic dyad D32 and D228, but less efficiently (larger and, hence, weaker H-bonds, see Figure 6, right panel) than the couple of derivatives **12** and **13**, whereas the *N*-benzyl ring establishes π interaction with Y198, its position inside the active site resembling that of **12** and **13**. Nevertheless, the presence of the rigid aromatic core induces a severe clash with the flap: indeed, the flap residue Y71 breaks the hydrogen bond with W76 that is not counterbalanced in energy by the formation of π interactions with the aromatic ring of the ligand. Moreover, as a consequence of this flap conformational change, the H-bond between the amide and Q73 is weaker than for the couple **12** and **13**, as revealed by the corresponding per residue binding enthalpy deconvolution (Figure 6, right panel). Overall, the complex is less stable and, accordingly, shows a higher ΔG_{bind} value than the NBP derivatives. Thus, even if the ligand somehow interacts with both key catalytic aspartic acids and the LA is also well accommodated in the hydrophobic cavity S3, the opening and destabilization of the flap due to the aromatic core could account for the lower potency of the DBMA derivatives.

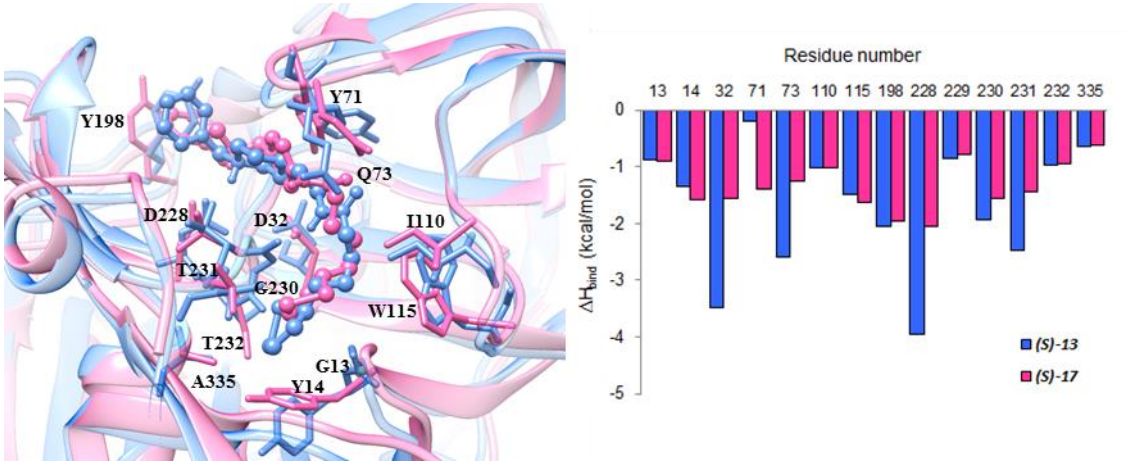

Figure 6. (Left) Comparison between the optimized MD binding conformations within the BACE1 receptor putative binding site of compounds (*S*)-**13** (blue) and (*S*)-**17** (pink). (Right) Per residue enthalpy decomposition for the same system.

Finally, we noted that the presence of a basic amine group (p$K_a \geq 6$) as in NBP or DBMA, seems crucial for BACE1 inhibition since the replacement by a 1,2-dimethoxybenzene group results in a drop of activity (not shown). Indeed, our calculations were unable to isolate a stable complex along the pertinent MD simulation trajectory.

Modelling studies in sigma-1 receptor

During the past years several three-dimensional pharmacophore models for σ_1R ligands have been published [63-67], and all these models propose a basic amino group and at least two hydrophobic substituents at the N-atom. However, the last-generation of σ_1R pharmacophore models is characterized by an additional requirement: a heteroatom, such as O or S, in the scaffold of the molecule that can form hydrogen bond interactions with a counterpart in the receptor binding cavity [66,67]. From a qualitative point of view, both NBP and DBMA scaffolds match the pharmacophore requirements to

efficiently bind the σ_1R. Actually, all tested derivatives demonstrated low nanomolar affinities for the σ_1R (Table 2). As for the BACE1 receptor, to describe at molecular level the binding mechanism of these new derivatives, compounds (*R*)-**12** and (*S*)-**13** as well as both enantiomers of compounds **15** and **17** were docked in the putative binding site of our well validated 3D-model of σ_1R [68,69]. Consequently, the corresponding ligand/receptor free energies of binding (ΔG_{bind}) were calculated by applying a validated molecular dynamics (MD) procedure [68,69] based on MM/PBSA calculations [54] (Table 4). Lastly, to investigate in detail the binding mode of the inhibitors to the target, a deconvolution of the enthalpic component (ΔH_{bind}) of ΔG_{bind} into contributions from each protein residue was carried out.[69]

Table 4. Binding free energies ΔG_{bind} and its components for (*R*)-**12**, (*S*)-**13**, (*R*)-**15**, (*S*)-**15**, (*R*)-**17** and (*S*)-**17** in complex with the σ_1R[a]

Compd.	ΔH	$T\Delta S$	ΔG_{bind}
(*R*)-**12**	-39.07 (0.19)	-28.62 (0.26)	-10.45 (0.32)
(*S*)-**13**	-39.42 (0.21)	-28.76 (0.28)	-10.66 (0.35)
(*R*)-**15**	-37.95 (0.21)	-27.68 (0.26)	-10.27 (0.34)
(*S*)-**15**	-38.41 (0.17)	-27.98 (0.25)	-10.43 (0.33)
(*R*)-**17**	-38.23 (0.20)	-27.84 (0.27)	-10.39 (0.34)
(*S*)-**17**	-38.39 (0.18)	-27.88 (0.29)	-10.51 (0.34)

[a]Values are expressed in kcal/mol and errors are given in parenthesis as standard errors of the mean.

The results of our computational methodology confirmed the experimental data about the binding capability of the new lipoic-based hybrids. As shown in Table 4, NBP

and DBMA derivatives established a stable complex with σ_1R and this is translated in a favourable ΔG_{bind} values, less than -10 kcal/mol for each complex.

Taking compound (*S*)-**13** as a proof of concept, the analysis of the binding mode revealed the classical main interactions involved in the stabilization of the ligand/receptor complexes, in line with our previous findings on other similar σ_1R ligands [47,67-70]. As we can see from Figure 7A, during the MD simulation (*S*)-**13** performed stable salt bridge and H-bond interaction with the side chain group of residue D126 and T151 respectively. Additionally, the *N*-benzyl ring established π interactions with R119 and W121 while the bulky dithiolanpentanamide moiety is perfectly encased in the typical hydrophobic pocket of σ_1R surrounding residues I128, F133 Y173 and L186 in which also the aliphatic portion of the piperidine group plays a role in the stabilization of the complex.

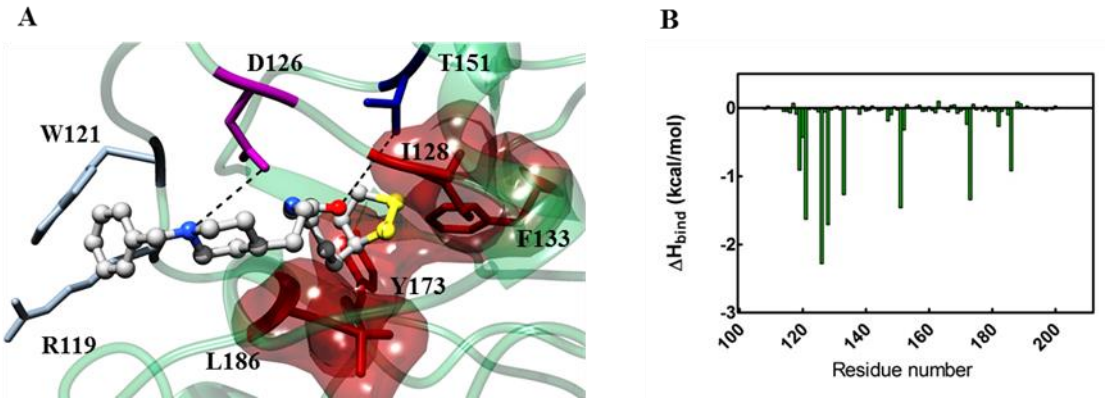

Figure 7. (A) Details of compound (*S*)-13 (in stick-and-ball representation) in the binding pocket. Salt bridge and hydrogen bond are highlighted as black broken lines. (B) Per residue binding free energy decomposition for the σ_1R in complex with (*S*)-13. Only σ_1R amino acids from position 100 to 200 are shown, as for all the remaining protein residues the contribution to ligand binding is irrelevant.

Even the deconvolution of the free energy of binding (Figure 7B) supported the binding mode of (*S*)-**13**: the interaction spectra, in fact, shows that the major contributions to the binding are afforded by the aforementioned σ_1R ligands.

For the purpose to compare the results obtained for (*S*)-**13** with the remaining compounds, we assessed the role of the chirality and the effect of the substitution of the NBP group with the DBMA moiety on the interactions in the σ_1R binding site. Concerning the first point, as expected from previous evidences on this topic [71,72], we did not detect any significant difference in the binding mode of the two enantiomers (Figure 8A); in fact, the flexible nature of the σ_1R binding site enables the receptor to easily and efficiently accommodate the (*R*)-configuration of compound **12** that is able to perform similar interactions with the same σ_1R residues (Figure 8C).

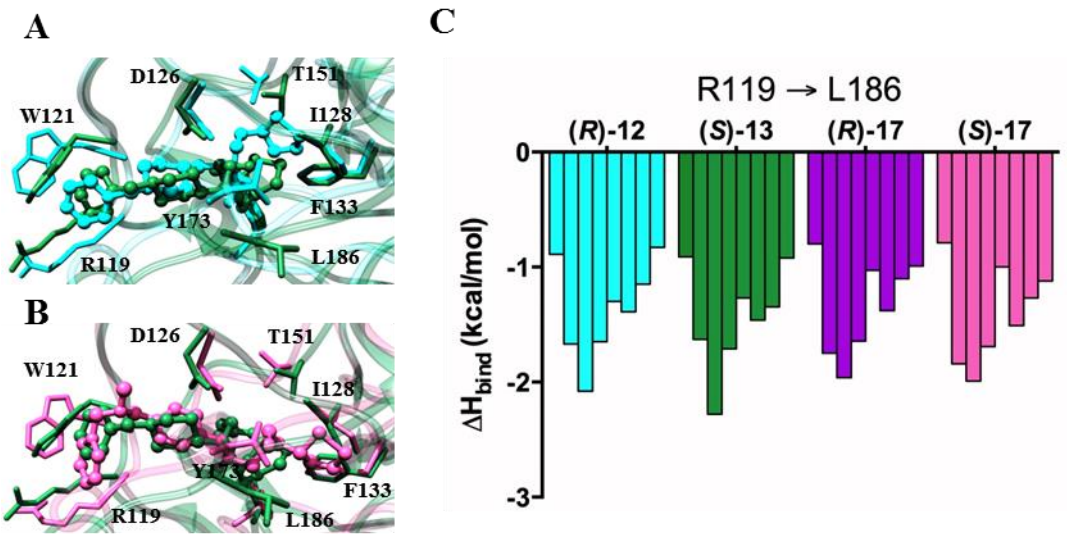

Figure 8. (A) Comparison between the optimized MD binding conformations within the σ_1R putative binding site between compounds (*R*)-**12** (cyan) and (*S*)-**13** (green). (B) Comparison between the optimized MD binding conformations within the σ_1R putative binding site between compounds (*S*)-**13** (green) and (*S*)-**17** (hot pink). (C) Per residue enthalpic contribution to binding for the σ_1R in complex with (*R*)-**12**, (*S*)-**13**, (*R*)-**17** and (*S*)-**17**. Only σ_1R amino acids critical for receptor binding are shown.

Even in the comparison between the NBP and DBMA derivatives our computational approach confirmed the experimental results, the DBMA group of compounds **15** and **17** can interact with the external part of the binding site in the same way as the NBP scaffold without affecting the correct arrangement of the dithiolanpentanamide part of the molecule in the hydrophobic cavity of σ_1R (Figures 8B and 8C, and Figure SI).

Conclusions

In this work, we have obtained a new series of LA-based compounds that stimulated the differentiation of SGZ-derived stem-cells to a neuronal phenotype and thereby, they could contribute to the brain auto-repair processes. Moreover, these new derivatives were active in three important targets involved in the pathogenesis of AD, namely AChE, BACE1 and σ_1R. By successful inhibition of AChE, these new hybrid compounds could alleviate learning and memory symptoms observed in AD patients, similarly to the drugs currently used in AD treatments. Through their BACE1 inhibitory properties, they could prevent the formation of toxic Aβs, which are considered important triggers of the pathological cascades leading to neuronal death. Last but not least, their σ_1R agonism could promote neuroprotective effects through several mechanisms, including the preservation of endoplasmic reticulum health and mitochondrial bioenergetics. In addition, LA-based hybrids showed good antioxidant properties and were predicted to be CNS-permeable, thereby reaching their biological targets.

Despite the values obtained for σ_1R are not entirely comparable with data obtained for h-AChE and h-BACE1, we can draw an idea about the way our compounds behave against each target and allow us to make some conclusions about their potential as anti-AD drugs. An important issue to be considered for future *in vivo* experiments is the potency differences for each AD-related target. For example, derivative **12** showed affinity values ranging from low-nanomolar to low-micromolar concentrations, i.e.: K_i (σ_1R) = 7.56 nM, IC_{50} (AChE) = 430 nM, IC_{50} (BACE1) = 8.11 μM, respectively. Considering these differences of affinity, we must assume that a low concentration will be required to activate σ_1R and a higher concentration might be needed to inhibit

BACE1; therefore, the question of what level of dose should be considered to use in *in vivo* experiments arises. In this regard, even though more experiments are undoubtedly required to determine the mode of action of our compounds, we believe they have a great potential for several reasons. Despite the relationship between level of BACE1 inhibition, amyloid burden and cognitive impairments is not fully understood, some genetic studies have demonstrated that a modest reduction of Aβ production may exert protective effects against AD [8]. On the other hand, BACE1 inhibition might be important in early stages of AD, when Aβ burden is not significant; in late stages, when senile plaques are largely distributed, inhibiting BACE1 might be of little therapeutic value. In this way, we consider these new LA-NBP and LA-DBMA hybrids could combine neuroprotective effects by agonizing σ_1R and by preventing amyloid production in patients suffering mild- to moderate AD.

Finally, it has been demonstrated that targeting simultaneously cholinergic system and σ_1R could be a successful strategy. For example, anavex2-73 combining σ_1R and muscarinic agonist effects (0.86 μM and 5.2 μM respectively) [20], has efficaciously completed phase 1 studies and has demonstrated to be effective improving cognitive markers in electrophysiological studies in a phase 2a clinical trial with AD patients. Additionally, a combination of anavex2-73 and a low dose of donepezil, named anavex-plus, has been tested in some AD models and is planned to be evaluated in the same phase 2a study [21]. The compounds obtained in this work, may synergistically combine the same abilities in a single small molecule.

Molecular modelling studies performed at BACE1 and σ_1R yielded a sensible molecular rationale for the interactions of LA-based molecules with these fundamental proteins and offered interesting clues for improving Aβ-reducing and neuroprotective

properties for these new drugs. Thus, these new LA-based neurogenic agents could be considered as interesting prototypes in the research of new therapeutics for the treatment of AD.

Future perspective

While the initial cause of AD remains unknown, hardly a true effective therapy could be found. The current marketed drugs acting at cholinergic or glutamatergic systems relieve symptoms and some of them slow down the memory decline in the first 12-18 months. These outcomes are certainly of great therapeutic value for such devastating disease, but do not counteract the neurodegenerative process in the long-term.

In the last years, the highly pathological complexity of AD has encouraged the research of multi-target directed ligands (MTDL), because their simultaneous interaction with two or more complementary targets could represent an important advance for AD treatment. Moreover, an increased number of AD-related targets have been continuously identified [73,74], such as sigma receptors (σRs) [11,75]. Activation of σ_1R elicits neuroprotection by different mechanisms and two σ_1 agonists, anavex1-41 and anavex2-73, are currently being studied in clinical trials for AD [18,19].

On the other hand, the existence of stem-cell niches in the adult brain offers the possibility of using drugs to stimulate the endogenous production of new neural cells, which could replace damaged or death ones. Thus, neurogenic agents could become a remedy for unmet neurological pathologies in which a massive loss of neuronal populations is observed, such as AD. Precisely, it has been demonstrated that the activation of σ_1R stimulates brain stem-cell niches and neurite outgrowth [31,76].

Combining the above therapeutic approaches, neurogenic agents endowed with a multifunctional AD-directed profile will be increasingly investigated in the future. These are the main characteristics of the new molecules here presented, which will be assayed in a murine model of AD soon.

Supplementary Data

Experimental full details can be found at the journal website www.future-science.com

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