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Design, Synthesis and Bioevaluation of Tacrine Hybrids with Cinnamate and Cinnamylideneacetate Derivatives as Potential Anti-Alzheimer Drug Candidates

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ABSTRACT

A series of novel tacrine-cinnamate and tacrine-cinnamylidene acetate hybrids have been designed, synthesized and evaluated as multitarget compounds for the treatment of Alzheimer´s disease. Results from the assessment of their inhibitory activity against acetylcholinesterase (AChE), antioxidant activity and self-induced β-amyloid (Aβ) aggregation are reported. These hybrid compounds showed promising results: all presented high activity against AChE, with IC_{50} values between low micromolar and nanomolar range of concentrations. The molecular modeling studies suggested dual interaction with both the catalytic and peripheral sites. Some compounds presented good antioxidant activity (DPPH free radical method) in the low micromolar range, namely the cinnamate derivatives with hydroxyl substituents and extended allyl-conjugation. Moreover, these compounds showed good neuroprotective effects by rescuing neuroblastoma cells stressed with β-amyloid peptide and hydrogen peroxide. Overall, our results suggest that some of these new hybrids have good potential as multifunctional drug candidates for AD treatment.

1. INTRODUCTION

Alzheimer's disease (AD) is a serious neurodegenerative disorder characterized by progressive cognitive decline and irreversible memory loss. Although the etiology of AD is not well understood, there is an amount of evidences about the existence of multiple factors that may play important roles in the disease pathophysiology, such as the deposit of aberrant proteins (*eg* extracellular β-amyloid (Aβ) and intracellular τ-protein), the low levels of acetylcholine (ACh), oxidative stress in the central nervous system and dyshomeostasis of biometals.[1-3] Despite the great research efforts on the development of new AD therapies, aimed at targeting the main pathological hallmarks of the disease, including the amyloid plaque formation, to date they have not yet resulted in clinically effective treatments. The only approved treatments for AD patients are currently based on acetylcholinesterase inhibitors (AChEIs) (*eg* tacrine, galanthamine, donepezil and rivastigmine) which improve the cholinergic neurotransmission and the symptomatic cognitive loss, but are ineffective in advanced stages of the disease.[4]

The complex multi-etiological nature of AD is believed to be responsible for the absence of disease-modifying drugs. In order to address this issue a new multitarget-based strategy has recently emerged. Thereby, we and other researchers have recently been involved in the development of molecular entities with ability to simultaneously affect multiple disease pathways instead of only one.[5-6] Many multipotent compounds have been designed and studied based on repositioning well known AChEI classical drugs, such as tacrine which has been hybridized with a variety of functional moieties, including two cinnamate derivatives.[7-11]

Following the identical multitargeting design strategy, and based in our previous results on hydroxycinnamate hybrids with anti-oxidant and anti-neurodegenerative properties[12] as well as tacrine hybrids,[8,9] we have developed herein a new series of tacrine hybrids bearing moieties with a set of derivatives of cinnamic and cinnamylidene acetic acid (see Figure 1). Following this strategy we aimed at develop new agents able to simultaneously address symptomatic and etiological routes in a possible AD therapy, such as the cholinergic impairment, the oxidant stress, the Aβ aggregation. Some derivatives of cinnamic acid and cinnamylidene acetic acid (5-phenyl-2,4-pentadienoic acid) are of natural origin,[13] and have important biological properties such as antimicrobial and anti-cancer activity.[14,15] Phenolic derivatives, such as caffeic acid and other hydroxycinnamic derivatives, have been considered as multifunctional antioxidants because, besides the more usual the radical scavenging roles by electron or hydrogen-donation to the existing radicals, they are also able to chelate redox-active metal ions, thus disabling their participating in Fenton reaction.[16]

In our design strategy was considered a main purpose of extending the double-bonded conjugation of the cinnamate systems aimed at enhancing the anti-oxidant activity due to expected stabilization of the phenoxyl radical formed, as well as extra interactions with the Aβ peptides. The size of the linker between the two main aromatic moieties was selected to enable a bimodal AChE-ligand binding interaction with the two major AChE sub-sites (the catalytic anionic site, CAS and the peripheral anionic site, PAS) and, ultimately improve their inhibitory activity as compared with the simple tacrine. Different aromatic substituents at the cinnamoyl moiety were included to provide some differentiation and balance among the antioxidant properties and the interactions with enzyme's active site. It should be mentioned that while this research project was ongoing,[17] other studies on tacrine-cinnamate hybrids were published.[18,19] Also an

interesting work was very recently reported, as a synthetic renewal in tacrine-ferulic acid hybrids, with an extrafunctionalization at the amide point of attachment by a multicomponent synthetic approach.[20]

Herein, we describe the design and synthesis of a set hybrid compounds based on the conjugation of tacrine with a set of cinnamate and cinnamylideneacetate derivatives, with different substituent groups and linkers, which were investigated for their anti-oxidant, anticholinesterase and anti-Aβ aggregating activities. Finally, these compounds were bioassayed with neuronal cells stressed with Aβ and hydrogen peroxide (H_2O_2) for their potential neuroprotective roles.

Figure. 1.

2. RESULTS AND DISCUSSION

2.1. Molecular modeling

The strategy followed for the design of these new inhibitors was similar to previous works,[8,12] which consisted on the selection of two main molecular scaffolds to account for the biological properties associated with Alzheimer's disease. In this case we have selected the tacrine and a set of cinnamate-based moieties (Figure. 1) to endow the hybrids with anti-AChE and anti-oxidant properties, respectively. Then, the length and type of linker (*n* and *p* parameters, Figure 1) was varied in order to tune the best interactions possible with the gorge-lining residues.

In order to confirm the expected binding modes of the new hybrid compounds with AChE and attempt to predict their relative inhibitory strength, we have performed docking studies with this enzyme. The catalytic site of this enzyme is located near the bottom of a narrow ~20 Å-deep gorge, where the substrate (acetylcholine) is hydrolyzed into acetic acid and choline.[21] This site is formed by three amino acids, Ser200, His440 and Glu327 (sequence numbering of *Torpedo Californica* AChE, *Tc*AChE), that are essential for the catalytic enzymatic activity, and are known as the 'catalytic triad.[21,22] There are two main sites of binding for the AChE inhibitors, which are the catalytic anionic site (CAS), formed by three important amino acid residues, Trp84, Glu199 and Phe330, located at the lower part of the gorge, and the peripheral anionic site (PAS), located at the entrance of the gorge, and formed by Tyr70, Asp72 and Trp279.

The docking studies were performed using GOLD program, version 5.1.[23] The crystal structure of *Tc*AChE complexed with an inhibitor (N-4'-quinolyl-N'-9"-(1",2",3",4"-tetrahydroacridinyl)-1,8-diaminooctane; PDB entry 1ODC[24] was used due to the structural similarities between the ligand in that complex and those in this study. In fact, the ligand in that crystal structure is also a bifunctional inhibitor containing tacrine and an aromatic moiety connected by a long alkylen linker. Hence, the protein conformation in that complex is expected to be similar to those with our ligands. The docking calculations were performed using ASP scoring function, since this function has previously proven to give the best docking predictions for AChE inhibitors, and followed the same protocol.[12,25]

The docking studies revealed that several aromatic interactions were established between the new compounds and the amino acid residues of the active site of AChE. The binding modes found for these inhibitors were very similar, differing only slightly from each other. Representative results of the docking studies are presented in Figure 2, which shows compounds **6a** and **9b** well inserted in the cavity of the active center, blocking the accessibility of the active site to the substrate and water molecules. The tacrine moiety was always found inserted in the bottom of the gorge of the enzyme, binding to CAS by π-π stacking with the aromatic side chains of residues Trp84 and Phe330, very similarly to the tacrine moiety of the original ligand (Figure 2a).[24] The short distances between the carbonyl oxygen of His440 and the pyridinic nitrogen of tacrine (ranging from 2.7 to 3.3 Å), indicates the existence of a hydrogen bond between the tacrine pyridinium group and the carbonyl oxygen from His440, as previously reported for other tacrine-based inhibitors;[26] this can be rationalized by the expected p*K*a value (ca 8-9) of the tacrine moiety and the shift of pyridine-pyridinium tautomeric equilibrium in the CAS.[27] Generally, the different spacers were found accommodated along the hydrophobic cavity. The moiety of the cinnamic acid derivatives and corresponding cinnamylidene analogs were always placed at the entrance of the gorge and were able of forming favorable sandwich interactions with the aromatic side chains of residues Trp279 and Tyr70 of PAS.

Figure 2b displays the structure of **9b**-AChE complex resulting from the docking simulation, which reveals the same π-π stacking interactions between the quinoline ring of tacrine and the CAS residues, as observed for the other ligands. At the other end, the benzene ring of the cinnamate moiety could also establish π-π stacking interactions with the same aromatic residues of PAS. The major differences in the described interactions

arise from the substitution of the cinnamate benzene ring with methoxy groups, which may establish extra Van der Waals interactions with the some of the residues around PAS. Furthermore the docking studies also suggest a possible hydrogen bond between an NH group of the chain spacer and a hydroxy group of Tyr121. These interactions may explain the better results of AChE inhibition for the compounds **9(a-c)** (Table 1). The four compounds with OH groups in the benzene ring displayed the same main interactions mentioned above, but with the further ability to establish a hydrogen bond with Ile275 residue above PAS (Figure S1). However, some of the favorable hydrophobic interactions with the methoxy groups are not possible with compounds **11**.

Besides the key ligand-enzyme interactions described above, some differences on the inhibitory activities may be mainly explained by different ring-substituent groups of cinnamic moiety and the spacer sizes. However, the docking calculations with compounds with longer spacers, (*e.g.* with four carbon atoms, n = 4, in the alkylenediamine chain) evidenced that the cinnamic moiety may potentially outstretch towards the bulk solvent, which may result in a lower interaction between the compounds and the enzyme, and concomitantly higher IC_{50} values (Table 1).

Globally, since the binding modes found for these compounds were so similar to each other, their relative potency as inhibitors would be difficult to predict with confidence. More importantly, the modeling studies allowed us confirming the expected ability of the compounds to interact with both the CAS and PAS of AChE, thus explaining some of the strong inhibitory activities observed toward AChE.

Figure 2.

2.2. Chemistry

In this study, fourteen tacrine-cinnamate based conjugates, (**5-9)(a-c**) were firstly synthesized as shown in Scheme 1. The tacrine derivatives were obtained from 9-chloro-1,2,3,4-tetrahydroacridine **3**, which was prepared from anthranilic acid, as previously reported.[28,29] The 9-aminoalkyl-tacrines **4(a-c)** were obtained from the reaction of **3** with excess of the corresponding alkylendiamines in the presence of phenol and a catalytic amount of KI, at 180 °C. Finally, the free amino group of these aminoalkylene-bearing tacrines **4(a-c**) was condensed with the carboxylic group of the

cinnamic acid derivatives, in the presence of T3P (propylphosphonic anhydride acid) and NMM (*N*-methylmorpholine), under N_2 atmosphere at room temperature, affording the corresponding tacrine-cinnamate hybrid compounds (**5-9**). Among them, four compounds containing methylenodioxy-cinnamate moieties, **7(a**,**b)** and **8(a**,**b**), were further submitted to acetal cleavage to obtain the corresponding compounds with two free hydroxyl groups. For that purpose very mild conditions were used, namely the mixture of the Lewis acid boron trichloride (BCl_3) with anhydrous tetra-*n*-butylammonium iodide (*n*-Bu_4NI) in CH_2Cl_2 under nitrogen atmosphere and low temperature (-78 °C).[30]

Scheme 1.

2.3. AChE inhibition

The results of AChE inhibitory activities obtained for the set of hybrid compounds and tacrine, as reference, are summarized in Table 1; this assessment was based on an adaptation of the protocol described by Ellman.[8,25] In general, all the compounds displayed high inhibitory activities with IC_{50} values, mostly in sub-micromolar range, as would be expected from enclosing a tacrine moiety. Some of the newly synthesized compounds, **9(a,b)**, **7(a,b)** and **8b,** revealed better inhibitory activities (IC_{50} = 0.09-0.18 μM) than tacrine. The best inhibitors were **9a** and **9b** (IC_{50} = 0.09 μM) with 3,4-dimetoxy substitutions in the cinnamate unit; compounds **7a** and **7b** with methylenedioxy substituents (O-CH_2-O) also exhibited high inhibitory potency (IC_{50} = 0.18 and 0.13 μM, respectively). Regardless the effect of the linkers, these results confirm that these nonpolar groups allow favorable interactions with some PAS hydrophobic amino acids and enhance the stability of the protein-ligand complex, as predicted by the modeling studies. The difference between **7a** and **7b** could be due to size of spacer, namely the spacer with 3 carbon atoms which may provide the optimal length for the interaction of the cinnamic moiety with PAS residues.

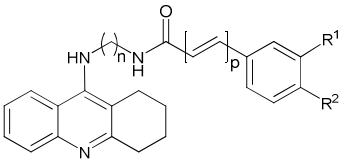
A more detailed analysis of the inhibitory results reveal that the activity is lower for the tacrine-cinnamate hybrids unsubstituted at position 3 and 4 or substituted by hydroxyl groups. Additionally, for all spacers (n = 2, 3 and 4), the presence of 3- and 4-methoxy substituent groups lead to IC_{50} values lower than the corresponding non-substituted analogues. Another factor that influences the AChE inhibition is the extension of double

bond between the benzene ring and carboxyl group that allows stronger hydrophobic interactions between the ligands and AChE.

The compounds containing free OH groups, **10(a,b)** and **11(a**,**b)**, display higher IC_{50}, which may be rationalized by their increased polar character that reduce the interactions within the active center of AChE and therefore exhibit lower enzyme inhibitory capacity. However, the corresponding IC_{50} are still in the low micromolar range and, although less potent than tacrine or their less polar derivatives, they can still be considered quite active AChE inhibitors with potential interest (especially taking into account other important associated properties).

In summary, the new hybrid compounds revealed interesting properties as AChE inhibitors, in some cases with higher activity than the reference drug (tacrine). Moreover, since they possess other potentially active functional groups, they may reveal more beneficial and efficient agents against AD than tacrine.

Table 1. Biological properties of the synthesized compounds. AChE inhibition and antioxidant activity (DPPH method).



Compds	n	p	R_1	R_2	AChE inhibition IC_{50} (μM)±SD[a]	Antioxidant Activity % (1 mM)[b]	Aβ aggreg. inhibition[f]
5a	2	1	H	H	0.77±0.02	7.9	
5b	3	1	H	H	0.38±0.08	16.3	
5c	4	1	H	H	0.85±0.1	22.5	
6a	2	2	H	H	0.32±0.2	15.6	
6b	3	2	H	H	0.29±0.04	16.9	
6c	4	2	H	H	0.62±0.1	23.0	
7a	2	1	OCH_2O		0.18±0.05	12.2	30.9
7b	3	1	OCH_2O		0.13±0.04	15.4	8.27

7c	4	1	OCH_2O		0.73±0.1	18.6	
8a	2	2	OCH_2O		0.30±0.03	26.2	
8b	3	2	OCH_2O		0.13±0.03	20.8	72.2
9a	2	1	OCH_3	OCH_3	0.09±0.02	8.8	19.6
9b	3	1	OCH_3	OCH_3	0.09±0.01	16.8	56.5
9c	4	1	OCH_3	OCH_3	0.39±0.1	16.1	
10a	2	1	OH	OH	0.84±0.07	12.3 μM[c]	
10b	3	1	OH	OH	0.98±0.13	11.3 μM[c]	
11a	2	2	OH	OH	1.09±0.2	9.5 μM[c]	
11b	3	2	OH	OH	0.48±0.02	9.1 μM[c]	
Tacrine					0.19±0.02	>1[d]	22.8[g]
Cinnamic ac.					9900±700[e]	665[h]	
Caffeic acid					5551[e]	24.8[h]	32.3[g]

[a] The values presented are the mean of three independent experiments ± SD (standard deviation). [b] Percentage of inhibition of antioxidant activity for 1 mM concentration. Standard deviation is within 10% of the values. [c] Antioxidant activity EC_{50} in μM, standard deviation is within 10% of the values. [d] Literature value, $EC_{50} > 1$ mM.[19] [e] Literature value.[12] [f] Inhibition of self-mediated $A\beta_{42}$ aggregation (%). The thioflavin-T fluorescence method was used, and the measurements were carried out in the presence of inhibitor (80 μM); SEM < 10%. [g] Literature value for $A\beta_{40}$, in presence of inhibitor (20 μM).[19] [h] Literature value, EC_{50} (μM).[12]

2.4. Antioxidant Activity

The antioxidant activity or the free radical scavenging capacity of the synthesized tacrine-cinnamate hybrids was assessed through their interaction with stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).[31]

Analysis of the results of anti-oxidant activity (see Table 1) shows that it is quite dependent on the cinnamate substituents. In particular, the compounds with two free hydroxyl groups (**10a**, **10b, 11a, 11b)** showed the highest antioxidant activity (EC_{50} in low micromolar range). Although the compounds **10(a,b)** have been recently studied,[19] our aim was beyond that, namely to study the effect of an extra conjugated double bond on the cinnamate derivatives and lead to the development of the homologous **11(a**,**b)**. These results, showed that the presence of a second double bond generally lead to a slight improvement of the anti-oxidant activity. Regarding the hybrids with non-

substituted benzene rings or with protected hydroxyl substituents, only moderate antioxidant activity was found (%AA in the millimolar range), similarly to previously reported for several cinnamic acid derivatives.[12] Among these compounds, **8a,** which resulted from the extension of the conjugated double bond of compound **7a,** presented the highest activity (26.2%). Except for hydroxyl groups, the other cinnamate substituents groups do not sensibly affect the antioxidant activity.

Therefore, as anticipated, the presence of the phenolic hydroxyl groups considerably increased the antioxidant activity, with EC_{50} values in low micromolar range in close similarity to those reported for **10a** and **10b** (14.1 and 11.1 μM, respectively).[19] This may be due to the fact that these antioxidants possess one extra mechanism of action, as compared with the hydroxyl protected analogues. In fact, similarly to reported for the analogue caffeic acid, the hybrids containing *cis*-phenolic (or catechol) groups, **10(a,b)** and **11(a,b)** can form chelates with trivalent (Fe, Al) and divalent (Cu, Zn) metal ions.[32-34] However, especially relevant for their anti-oxidant role is their high chelating capacity for the redox-active metal ions, such as iron and copper, well-known catalysts of oxidative stress.

Conversely, the deprotection of the phenolic groups is expected to decrease the lipophilicity of the compounds and decrease their ability to penetrate the blood–brain barrier (BBB) and the cell membranes. Hence, these two factors, together with the AChE inhibitory activity, must be well balanced. This kind of tradeoff is common in the development of this type of drugs in order to make them valuable as potential anti-neurodegenerative drugs.

2.5. Pharmacokinetic Properties

To predict the potential of new compounds as eventual drugs, some descriptors of their pharmacokinetic profile were determined, through the use of QikProp program, v.2.5.[35] Parameters such as the lipo-hydrophilic character (*c*log *P*), the ability to cross the blood brain barrier (log *BB*), the ability to be absorbed through the intestinal tract to the blood (Caco-2 cell permeability) and the verification of *Lipinski's rule of five* were calculated in order to analyze their *druglikeness* for a potential oral used as anti-AD agents.

Analyzes of the results of *in silico* screening (Table S1), indicates moderate to high octanol/water log *P* coefficient (*c*log *P*) for all the compounds. The high lipophilic

character (>5.0) calculated for some compounds, namely those containing hydroxyl protected groups or longer chain spacers, may difficult their transport through the blood strain. However, their molecular weights are lower than 500 $gmol^{-1}$ and lead to only one violation of the *Lipinski's rule of five (clog P>5)*.[36] The Caco-2 permeability rates (> 500 nms^{-1} is considered good) indicate that the absorption through the intestinal tract to the blood is possible. Also the compounds with negative log *BB* values should have good blood brain permeability. In fact according to the limits given by the Qikprop program, the range between -3.0 to +1.0 are acceptable; however values of log BB less than -1.0 are considered poor whereas drugs with 0.3 are considered good and are able to penetrate the blood brain barrier. Regarding the drug activity in the CNS, the Qikprop program does not supply accepted limits, but categorizes the compounds on a - 2 (inactive) to +2 (active) scale. Thus, these results indicate that, notwithstanding the generally high lipophilicity of most of these compounds, they fulfill most of the drug-like criteria to be considered as drug candidates as for oral administration and possible brain penetration.

2.6. Inhibition of self-mediated $A\beta_{42}$ aggregation

To test the capacity of a selection of the new synthesized hybrids to inhibit the Aβ aggregation, in vitro assays with thioflavin T (ThT) were carried out, since ThT interacts with Aβ structures and may compete for binding to Aβ fibrils. To monitor the presence of fibrils the ThT fluorescence emission was followed at λ_{em} = 490 nm (λ_{ex} = 446 nm).[19,37] Within this wavelength range the compounds do not any absorption (see Fig.2 suplementary). The studies were carried out by incubating $A\beta_{42}$ (40 μM) in the presence and in the absence of the selected compounds (80 μM) at 37 °C for 24 h. Each solution was further incubated for 5 min with a ThT solution and afterwards the respective fluorescence emission was registered.

The selection of the compounds for this study was based on their best inhibitory activity against AChE. The results (Table 1) revealed that these compounds are able to induce a decreasing on ThT fluorescence, thus suggesting that they might interfere with fibril formation. Compound **8b** presents the best activity against $A\beta_{42}$ aggregation (72.2%) while the homologous compound **7b** presents the lowest value (8.3%). This result may suggest that the extra-double bond of **8b** may account for that difference on the interaction with Aβ and the inhibition of its aggregation. The insertion of an extra

methylene group in the linker leads to a somehow identical trend on **9b** (56.5%) as compared with **9a** (19.6%). However, this effect appears less relevant than that of the extra-double bond, and in the **7a** and **7b** analogues that trend is even not observed, thus suggesting that other features, such as the cinnamate substituent groups, may account for that interaction with Aβ. Therefore, the double bond extension seems to really contribute to the increase of the interaction with Aβ, eventually enabling some extra π-π stacking interaction, although other factors should be considered. The fact both the tacrine and the caffeic acid are known to have a mild inhibitory effect on the amyloid self-aggregation (Table 1 [19]) raise our expectations that the fusing of both moieties in this set of hybrids could account for their capacity to inhibit the Aβ self-aggregation.

Table S1 – Summary of some calculated pharmacokinetic descriptors.

2.7. Cell viability and effect in preventing Aβ42- and H_2O_2-induced toxicity on neuroblastoma cells

The new hybrids were firstly submitted to an evaluation of the dose-response effect on neuroblastoma cells (SH-SY5Y), to select the highest non-toxic concentration to use. Compounds with extended double conjugation (p = 2), (**6a**, **6b**, **8a** and **8b)** appear in general more toxic than the corresponding non extended analogues (p = 1) (**5a**, **5b**, **7a** and **7b**) (*see* Supplementary Figure 3S). Also, compounds containing a methylenodioxy cinnamate substitution (**7(a-c)** and **8(a,b**) appeared more toxic than the corresponding analogues with other substituents. Both the double conjugation extension and the methylenodioxy substitution are associated to an increasing of the lipophilic character (clog P >5, Table S1), which may account for toxicity enhancements. Identical toxicity increase was not observed for the compounds **11(a,b)**, as compared with the analogues **10(a,b**), eventually due to the hydroxyl cynnamate substituents with counterbalanced some lipophilicity increase. Interestingly, preliminary exploratory screenings on the toxicity of some of these compounds against ovarian cancer cells showed also toxicity in μM range, and the corresponding IC_{50} values following identical trend: **8b** (2.50), **7b** (5.82), **9a** (10.4), **9b** (19.5).[38]

The following selection of compound concentrations was used for the studies in cells: 2.5 μM for **5(a-c)**, **7a**, **7c** and **9(a-c)**; 1.5 μM for **6(a-c)**, **7b** and **8(a,b)**; 5μM for **10(a,b)**

and **11(a,b)**. Even so, the compounds **5b**, **6a**, **6c** and **8a** show a non-significant change in the cell viability, when compared to the control.

The potential therapeutic action of these compounds was then assessed on neuroblastoma cells treated with $A\beta_{42}$ peptide, which is a reliable *in vitro* cellular model to screen new disease-modifying drugs, as it mimics some of the aspects of AD neurodegeneration. The quantitative colorimetric method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction accounted for the viability of neuroblastoma cells, with or without compounds and/or $A\beta_{42}$ peptides treatment.

Our results show that the 24 h treatment with $A\beta_{42}$ peptide (1 μM) results in a significant decrease on cell viability, when compared to the control. Most of the compounds were able to reduce the $A\beta_{42}$ peptide toxic effect and increase the cell viability. Among them, compounds **5c** (2.5 μM) and **9a** (2.5 μM) evidenced the most significant protection against $A\beta_{42}$ induced toxicity in neuroblastoma cells (Fig. 3-A and B).

Figure 3.

Due to the previous results obtained, the compounds **5c**, **9a-c**, **10(a,b)** and **11(a,b)** were further selected to examine their neuroprotective effect against oxidative stress induced by hydrogen peroxide (H_2O_2). Our results show that 24 h treatment with H_2O_2 (50 μM) induced a significant decrease on cell viability, when compared to the control. Most of the compounds tested (**9(a-c)**, **10b** and **11(a,b)**) were able to reduce H_2O_2 toxic effect and increase the cell viability. Among them, compounds **9a** (2.5 μM), **9b** (2.5 μM), 1**0b** (5 μM) and **11a** (5 μM) evidenced the most significant neuroprotective effect against H_2O_2 induced toxicity in neuroblastoma cells (Fig. 4).

Thus, compound **9a** that is able to block or hamper $A\beta_{42}$ and H_2O_2 toxic insults may be considered neuroprotector.

Figure 4.

3. CONCLUSION

A series of bifunctional compounds based on the tacrine hybridization with cinnamic acid and cinnamylidene acetic acid derivatives have been developed and biologically evaluated aimed at gathering information about their potential interest as anti-

Alzheimer´s agents. Some compounds showed high inhibitory activity even outperforming the tacrine drug, apparently due to bimodal interaction. The compounds with free cathechol moieties showed very high antioxidant activity, eventually due their iron-chelating contribution. The cellular study performed with neuroblastoma cells treated with $A\beta_{42}$ peptide, a reliable cellular model of AD neurodegeneration, provided substantial evidences supporting that compounds **5c** and **9a** show protective effects on $A\beta_{42}$-induced cell toxicity. Additionally, compounds **9(a,b)**, **10b** and **11a** afforded good neuroprotection against H_2O_2 toxic insult.

Collectively, biological and physicochemical data obtained from this study, allow us to propose the development of new tacrine-cinnamate derivatives as valuable multipotent drugs to arrest AD progression.

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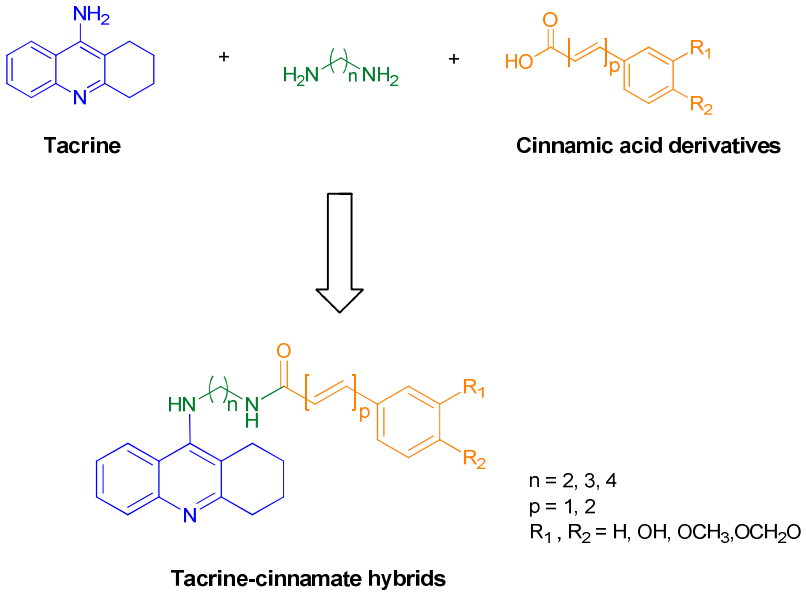


Figure. 1. Design strategy for the synthesis of the tacrine-cinnamate hybrids.

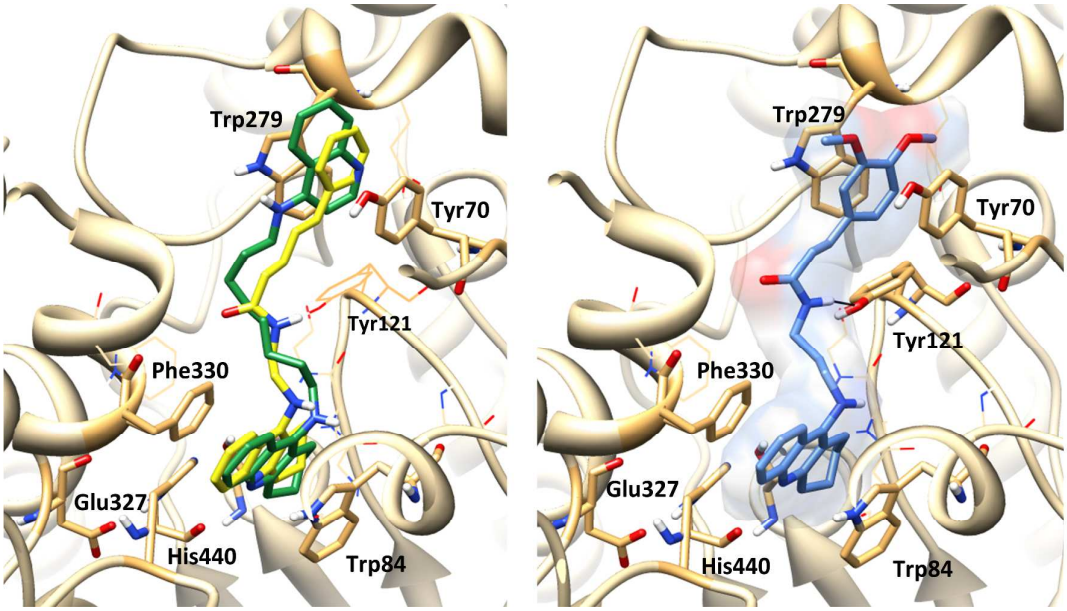


Figure 2. Docking results for the tacrine-cinnamic hybrids with AChE: (a) superimposition of **6a** (yellow) with original ligand (green, PDB code: 1ODC[24]) and (b) **9b** (blue). H-bonds are represented as solid black lines.

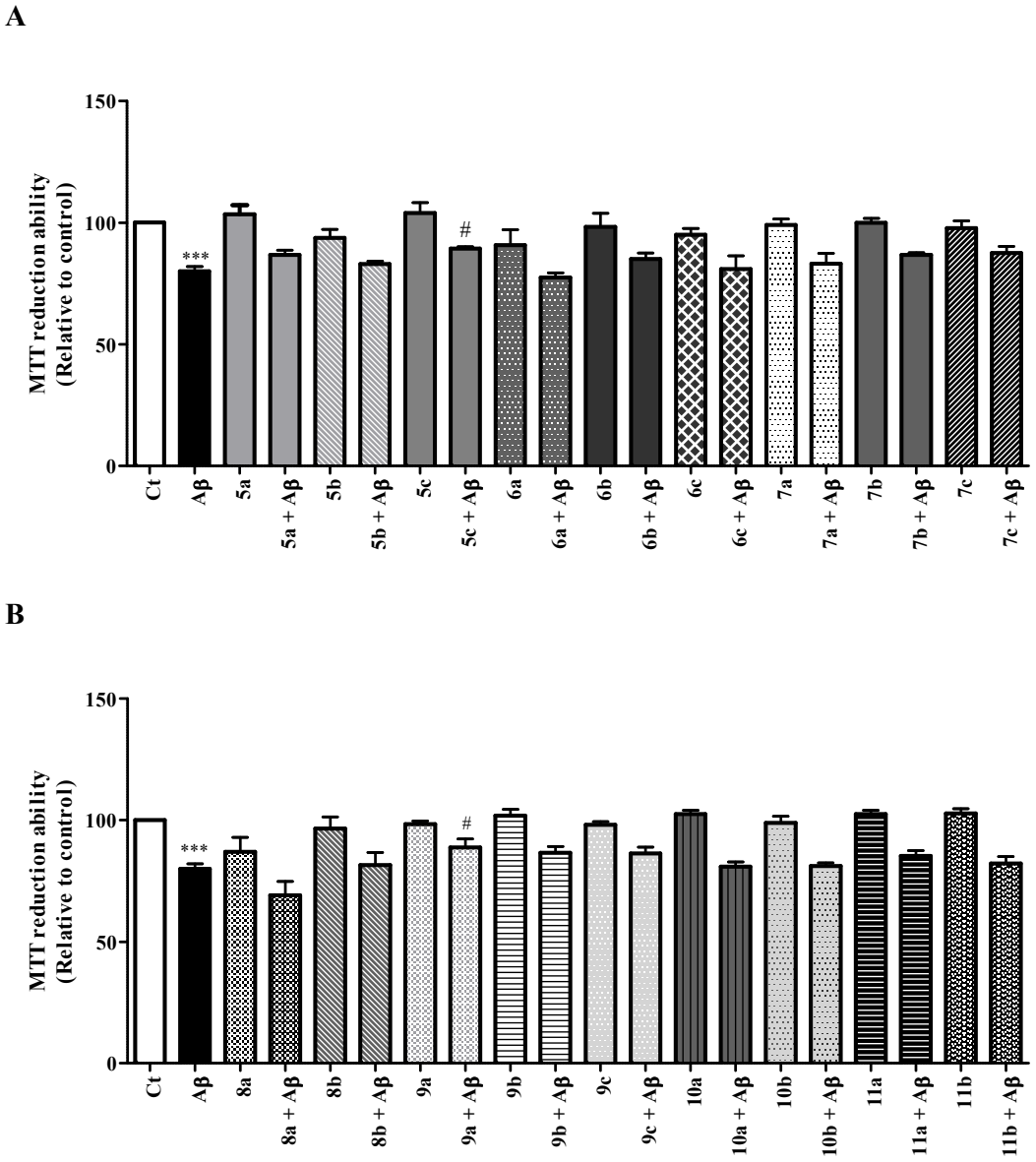


Figure 3. Protective capacity of tacrine-cinnamate derivatives against $A\beta_{42}$-induced toxicity. Neuroblastoma cells were pre-incubated during 1 h with pre-selected compounds concentration and treated with $A\beta_{42}$ (1 μM), for 24 h at 37 ºC. Untreated cells, cells treated only with $A\beta_{42}$ or compounds were used as experimental controls. Compounds protective capacity was evaluated by MTT reduction assay. Results are expressed as percentage relatively to the control (untreated cells), with a mean ± SEM derived from 3-9 different experiments. ***$p < 0.001$, significantly different when compared to the control; #$p < 0.05$, significantly different when compared to $A\beta_{42}$ treated cells.

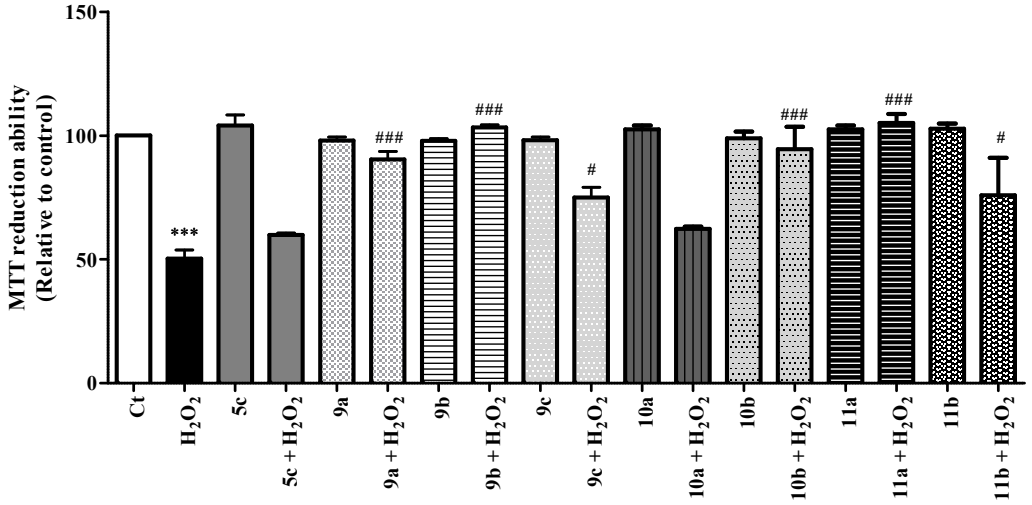
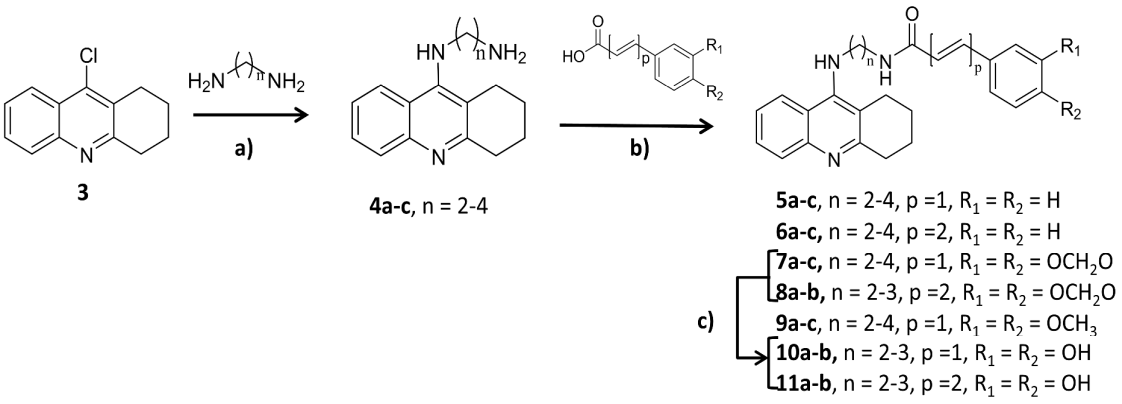


Figure 4. Evaluation of tacrine-cinnamate derivatives protective effect against H_2O_2-induced toxicity. Neuroblastoma cells were pre-incubated during 1 h with **5c**, **9a-c**, **10a-b** and **11a-b** and treated with H_2O_2 (50 μM), for 24 h at 37 ºC. Untreated cells and cells treated only with H_2O_2 were used as experimental controls. Compounds protective capacity was evaluated by MTT reduction assay. Results are expressed as percentage relatively to the control (untreated cells), with a mean ± SEM derived from 3 different experiments. ***$p < 0.001$, significantly different when compared to the control; #$p < 0.05$, ###$p < 0.001$, significantly different when compared to H_2O_2 treated cells.



Scheme 1. *Reagents and conditions*: **a)** Phenol, KI, 4 h, 180 °C; **b)** CH_2Cl_2, T3P, NMM, 4 h, N_2; **c)** BCl_3, *n*-Bu_4NI, CH_2Cl_2, -78 °C, N_2.