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Title: Synthesis and pharmacological evaluation of multifunctional tacrine derivatives against several disease pathways of AD

Article Type: Full Length Article

Keywords: Tacrine; Alzheimer's disease; BACE1; Amyloid protein; copper; Multi-targeted ligands; caffeic acid

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Abstract: A novel series of tacrine derivatives were designed and synthesized by combining caffeic acid (CA), ferulic acid (FA) and lipoic acid (LA) with tacrine. The antioxidant study revealed that all the hybrids have much more antioxidant capacities compared to CA. Among these compounds, 1b possessed a good ability to inhibit the β-amyloid protein (Aβ) self-aggregation, sub-micromole acetylcholinesterase (AChE)/butyrylcholinesterase (BuChE) inhibitory, modest BACE1 inhibitory. Moreover, compound 1b also was a DPPH radical scavenger and copper chelatory as well as had potent neuroprotective effects against glutamate-induced cell death with low toxicity in HT22 cells. Our findings suggest that the compound 1b might be a promising lead multi-targeted ligand and worthy of further developing for the therapy of Alzheimer's disease.

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To the Editor of

Bioorganic & Medicinal Chemistry Letter

Pisa, 23 December 2014

Dear Editor,

we submit the revision of our paper " *Synthesis and pharmacological evaluation of multifunctional tacrine derivatives against several disease pathways of AD.*", by M. Digiacomo, Z. Chen, S. Wang, A. Lapucci, M. Macchia, X. Yang, J. Chu, Y. Han, R. Pi[b], and S. Rapposelli. We hope that this revised version will be now suitable for consideration for publication in *Bioorganic & Medicinal Chemistry Letter.*

The manuscript has been revised, following the referee's indications, as summarized in the following point-to-point response. A marked manuscript, highlighting the main changes, is attached as supporting information.
We have performed other experiments and we hope the paper will match all the requirements.

Many thanks for your wonderful jobs on our manuscript.

Sincerely yours,
Simona Rapposelli

The corresponding author
Dr. Simona Rapposelli

***Response to Reviewers**

Dear Editor,

The manuscript has been revised, following the referee indication, as summarized in the following point-to-point response. A marked manuscript, highlighting the main changes, is attached as supporting information.

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Editor's Comments:

Please ensure that your revised manuscript follows the journal-specific guidelines listed below:

1. Address: If there is more than one affiliation, designate authors to institutions by using superscript 'a', 'b', 'c', etc.

Answer: **Done, as suggested**

2. Headings: Please do not use common headings, such as 'Introduction', 'Chemistry', or 'Conclusion', other than 'Acknowledgments' and 'References and Notes'.

Answer: **Done, as suggested**

3. R1, R2: Use a superscript numeral for R1, R2 , etc. (not a subscript R1, R2) to designate substituents in graphic structures, tables, and text.

Answer: **Done, as suggested**

4. Reference Style: For proper reference style, please consult the References and Notes section of the Journal's Guide for Authors found at www.ees.elsevier.com/bmcl. Note that the Journal uses a start page number only for journal articles. For journal title abbreviations, please consult the list provided: Click on Guide for Authors; then click on Journal Title Abbreviation List.

Answer: **The style of references has been corrected**

5. X-ray coordinates: X-ray coordinates must be deposited with the RCSB Protein Data Bank (PDB) database (or Cambridge Crystallographic Data Centre for small molecules) and the PDB (or CCDC) deposition number must be placed in the manuscript.

6. Experimental Information: All experimental information should be placed in the References and notes section as an endnote or placed in Supporting Information.

Answer: **All the experimental information has been placed on supporting information attached to the manuscript**

Reviewers' comments:

Reviewer #2:

The paper by Digiacomo et al. is of the type publishable in BMCL. It describes the synthesis and the pharmacological evaluation of multifuntional derivatives of Tacrine. It is the continuation of a project research of some of the authors, the results of which has been already published and quoted in this paper. The results although not striking it is of certain interest and deserve to be disseminated among the scientific community.

My recommendations are;

- Figure 1, part B is no necessary and can be eliminated. Quoting the appropriate reference is sufficient; The substructure 1,3-diamino-2-proanol is well evidentiated in the compounds reported in part A;

Answer: As suggested by the referee, the part B of Figure 1 has been deleted and the appropriate reference is correctly cited

- A reference compound in table 2 and 4 would offer the reader a better and faster comprehension;

Answer: Table 2 and 4 have been joined in one, large table as requested by the Reviewer #5. The reference compound has not been added in table because of different experimental conditions used in the performed experiments (data collected were not comparable). Anyway a discussion about the different activity and selectivity has been done (see below and the new MS).

- In table 1 the selectivity AchEI/BuChEI is calculated, which fine. However, the importance of this parameter, also from a therapeutic point of view should be discussed, also in comparison to the tacrine which is not selective and is one of the drug currently used for the management of Alzheimer's disease.

Answer: Thanks for your kindly suggestions. We have added some discussion in our revised manuscript (See page 7 line 1-3 from the bottom and page 8 line 3-7 from the bottom). In fact, the goal of finding high selective inhibitors of AChE is to minus the peripheral cholinergic effects. But now, increasing evidences demonstrated that long-term inhibition of BuChE might be especially important when exploring any disease-modifying effects of cholinesterase inhibitors. (Anand P, Singh B. A review on cholinesterase inhibitors for Alzheimer's disease. Arch Pharm Res. 2013 Apr;36(4):375-99.-2; Shanks M, Kivipelto M, Bullock R, Lane R. Cholinesterase inhibition: is there evidence for disease-modifying effects? Curr Med Res Opin. 2009 Oct;25(10):2439-46). Both references have been added in the new version of the manuscript

Reviewer #5: *The manuscript "Synthesis and pharmacological evaluation of multifunctional tacrine derivatives against Alzheimer's disease" decscribes the synthesis and biological evaluation of a series of tacrine hybridized to caffeic acid, ferrulic acid and lipoicacidusing a 1,3-diaminopropan-2-ol linker. This work builds on earlier studies by both groups (published in the 2012 BMCL) and a PloS One of one of the groups. The progress when compared to the BMCL is the introduction of another linker, while in the earlier study, a series of diamino alkanes have been used, this study introduces a slightly more polar linker. Interestingly, the authors did not define which compounds 1 or 2 contain the caffeic or ferrulic acid units (no description in scheme 1 or in the text).*

Answer: **Sorry, we added the omitted description both in the scheme and in the text**

The caffeic acid derivative is really similar to the one reported in the 2012 BMCL paper by the same authors (see there compounds 5b and 5e) , the only difference is the introduction of the OH group on the linker. Interestingly, the compound reported in this paper displays lower activity, e.g. in the BuChe assay. Therefore, it would be interesting to compare this new compound to the ones reported in the 2012 BMCL.

Answer: **Yes, in current work, we just simply introduced OH group into the linker and found that 1b have mild BCAE1 inhibitory under 10 microM while the one (T3CA) reported in 2012 BMCL has not (data not shown). This is why we carry out the project. We briefly explained in the first two sentences of sixth paragraph. You know, it is not easy to get the high activities for all targets at one molecule.**

The manuscript can be considered for publication after a number of issues have been addressed:

1. The biological data should be compiled in one, large table (like the presentation in the 2012 BMCL by the same authors)

Answer: **Done, as suggested**

2. For compounds 1 and 2, R' must be defined.
Answer: **Done, as suggested**

3. The title is misleading, as the compounds were not evaluated against AD. It should be rephrased to "Synthesis and pharmacological evaluation of multifunctional tacrine derivatives against several disease pathways of AD"

Answer: **We agreed with the referee and the title has been changed as suggested**

4. In the Cu binding assay, one data point (at 40 compound 1b and 100 uM Cu) is not enough to support the claim. More data points, and the presence of isosbesitc points, have to be presented in order to substantiate this claim. This has been done by the authors in their 2012 study, where the Cu binding was sufficiently characterized by different concentrations.

Answer: **Actually, we have done the experiments as described in the previous study (Chao X, He X, Yang Y, Zhou X, Jin M, Liu S, Cheng Z, Liu P, Wang Y, Yu J, Tan Y, Huang Y, Qin J, Rapposelli S, and Pi R.Design, synthesis and pharmacological evaluation of novel tacrine-caffeic acid hybrids as multi-targeted compounds against Alzheimer's disease. *Bioorganic & medicinal chemistry letters* 22: 6498-6502, 2012.). The maximum absorption suffered a bathochromic shift upon the addition different concentration of CuCl2. We speculated that this effect may be related to the two hydroxyl on the benzene ring. Now, we add the experimental results with different concentration ratio of copper to our compound. (see Fig. 3).**

We hope that the paper is now in a form acceptable for publication in the Bioorganic Medicinal Chemistry Letter.

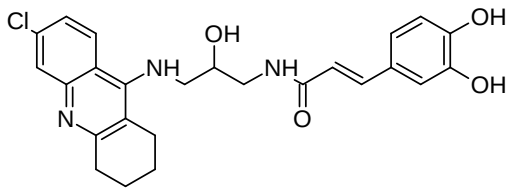
Thank you in advance.

Sincerely,

Simona Rapposelli

*Graphical Abstract (for review)

Graphic abstract



1b

Aβself-aggregation 53% in 50 μM
AChE IC_{50} 0.15 μM
BuChE IC_{50} 0.36 μM
BACE1 13.1% in 10 μM
Copper chelatory
DPPH scavenger 90.36% in 30 μM
Neuroprotection
Low toxicity

Synthesis and pharmacological evaluation of multifunctional tacrine derivatives against several disease pathways of AD

Maria Digiacomo [a,‡], Ziwei Chen [b,c,‡], Shengnan Wang[b,c], Annalina Lapucci [a], Marco Macchia [a], Xiaohong Yang[b,c], Jiaqi Chu[b,c], Yifan Han[c,d],Rongbiao Pi[b,c*], Simona Rapposelli [a*]

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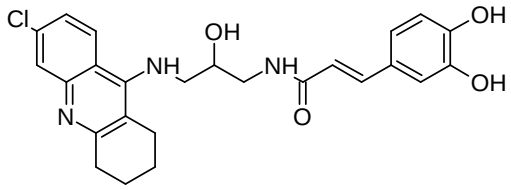
[‡]Contributed equally to this work.

***Corresponding authors:**Rongbiao Pi (pirb@mail.sysu.edu.cn) and Simona Rapposelli (simona.rapposelli@farm.unipi.it)

Graphic abstract



1b

Aβself-aggregation 53% in 50 μM
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Abstract

A novel series of tacrine derivatives were designed and synthesized by combining caffeic acid (CA), ferulic acid (FA) and lipoic acid (LA) with tacrine. The antioxidant study revealed that all the hybrids have much more antioxidant capacities compared to CA. Among these compounds, **1b** possessed a good ability to inhibit theβ-amyloidprotein (Aβ)self-aggregation, sub-micromole acetylcholinesterase (AChE)/butyrylcholinesterase (BuChE) inhibitory, modest BACE1 inhibitory. Moreover, compound **1b** also was a DPPH radical scavenger and copper chelatory as well as had potent neuroprotective effects against glutamate-induced cell death with low toxicity in HT22 cells. Our findings suggest that the compound **1b** might be a promising lead multi-targeted ligand and worthy of further developing for the therapy of Alzheimer's disease.

Keywords: Tacrine; Alzheimer's disease; BACE1; Amyloid protein; copper; Multi-targeted ligands; caffeic acid

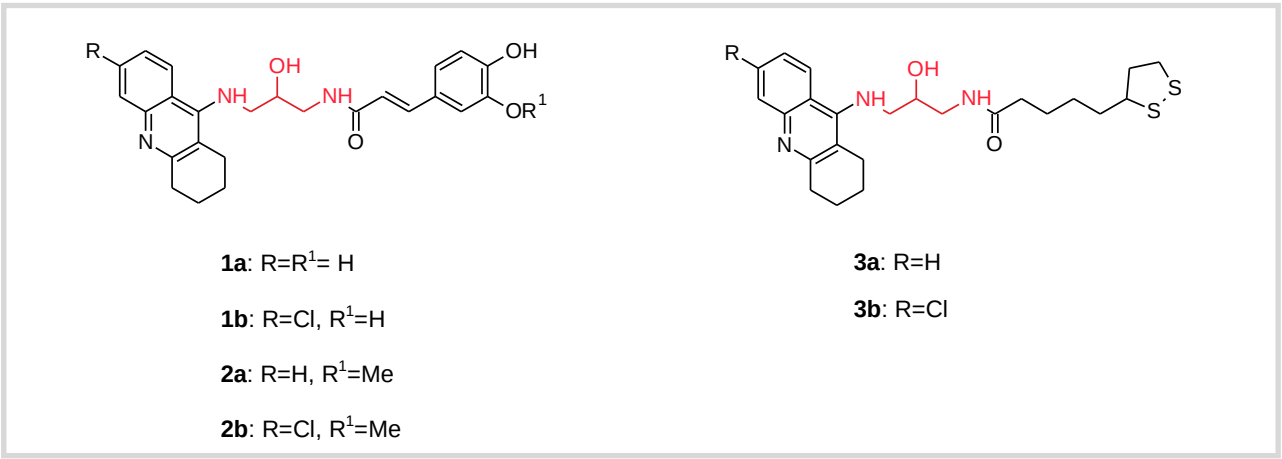
Alzheimer's disease (AD) is the most common form of irreversible dementia in the elderly. It is a multifactorial disorder in which several factors contribute both to its etiology and pathogenesis. In particular, decreased levels of acetylcholine (ACh), the formation of Aß deposits and neurofibrillary tangles, mitochondrial dysfunction and extensive oxidative stress constitute the principle hallmarks of AD.[1] In the last decade, the design of compounds potentially useful for the treatment of AD, focused on the development of new ligands able to interact with different type of targets involved in AD (i.e. BACE inhibitors, γ-secretase modulators),[2,3] but to date, the acetylcholinesterase (AChE) inhibitors, such as tacrine, donepezil, rivastigmine and galantamine, still remain the only drugs currently employed in therapy. The effectiveness of these AChE inhibitors (AChEI) is limited to the reduction of the symptoms of AD. Tacrine (TA), the first AChEI approved by FDA for AD, is the most effective prototype of this class of drugs, nevertheless AChEIs exert limited disease modification and only attenuate the dementia symptom. TA inhibits both AChE and butyrylcholinesterase (BuChE), and induces oxidative stress, due to reactive oxygen species (ROS) production stimulation and glutathione depletion, as demonstrated by *in vitro* studies on human liver cell line HepG2.[4]

Consequently, the simultaneous administration of free radical scavenger could counteract the tacrine-induced oxidative stress.[4] Moreover, it is well known that also bio-metals such as Cu, Zn and Fe, play a central role in the production of ROS and in the onset of oxidative stress[5] thus contributing to the aggregation of Aβ and progression of AD.

On this basis, new tacrine-like compounds endowed of both AChEI activity and antioxidant properties have been widely investigated.[6-9] This strategy falls into the new approach in medicinal chemistry of multi-target design ligands (MTDL), according to which a single compound able to modulate multiple targets simultaneously, could result a more effective and safety strategy than the "one drug-one target"approach.[10]

In a previous work, we described a series of tacrine-ferulic acid (TAnFA)[11,12] and tacrine-caffeic acid hybrids (TAnCA).[13,14] These hybrids showed a good multiple biological profile, displaying AChE inhibition properties and anti-oxidant activity.

As a further development of our work on the design and synthesis of new TA and antioxidant hybrid compounds with a better pharmacological profile, we here describe a new class of hybrid-molecules (**1a,b-3a,b**)as multi-target derivatives for the treatment of AD. (figure 1)



1a: R=R^1= H

1b: R=Cl, R^1=H

2a: R=H, R^1=Me

2b: R=Cl, R^1=Me

3a: R=H

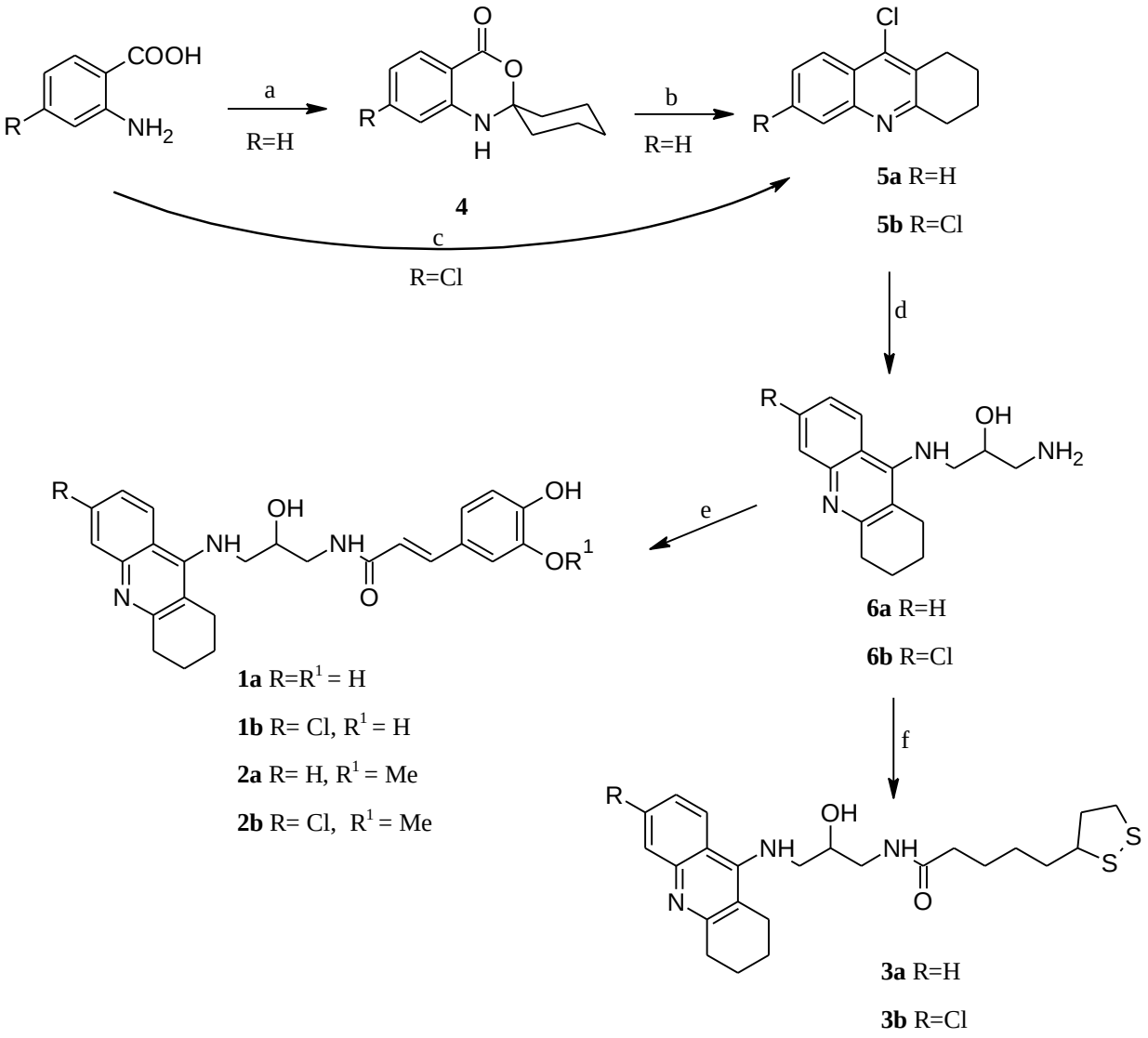
3b: R=Cl

Figure 1. Multifunctional tacrine-derivatives **1a,b-3a,b**.

In particular, in this paper we evaluated the possibility to widen the pharmacological profile of the compounds TAnFA and TAnCA, through the insertion of 1,3-diamino-2-propanol (Figure 1A). Actually this core has been applied successfully to a number of aspartyl proteases, including β-secretase (BACE1).[15-18](Figure 1B) Moreover, the presence of both nitrogen and oxygen atoms within the linker could facilitate the interaction of these ligands with free metals.[19]

The compounds synthesized (**1a,b-3a,b**) were evaluated for their antioxidant and both AChE and BACE1 inhibitor activities. Furthermore the protective effects on glutamate-induced cell death in mouse hippocampal neuronal HT22 cells were investigated. The candidate with the best multi-target profile was also evaluated for its ability to chelate copper.

Final compounds **1a,b-3a,b** were synthesized following the procedures described in Scheme 1.Intermediates **5a,b,** synthesized according to the reported procedure,[20,21]were subjected to a reaction with 1,3-diamino-2-propanol at 160°C. The subsequent condensation of derivatives **6a,b** with the appropriate acids in presence of DCC and DMAP afforded the products **1a,b** and **2a,b**. For the preparation of compound **3a** and **3b,** the lipoic acid was converted to the corresponding acid chloride and treated with the amine-derivative **6a,b**.



Scheme 1. General synthesis of compounds **1a,b-3a-b**. Reagents and conditions: a)cyclohexanone, toluene, reflux, 4 h; (b)$POCl_3$, 120°C, 2h; (c)cyclohexanone, $POCl_3$, reflux, 2h; d) 1,3-diamino-2-propanol, 160°C, 3 h; (e) caffeic acid or ferulic acid,CH_2Cl_2/DMF, DCC, DMAP, rt, 12 h; (f) i) lipoic acid, $SOCl_2$, CH_2Cl_2, 0°C, 4 h; (ii) Et_3N, CH_2Cl_2/DMF, 0°C, 4 h.

Cholinergic neurotransmission damage is believed to be one of the major causes of memory impairment associated with AD. AChE inhibitor could maintain the Ach in the synaptic cleft by inhibiting acetylcholinesterase (AChE), and this is the only known means of treating AD [22]. The

synthesized compounds (**1a,b-3a,b**) were firstly tested for their inhibitory activity of AChE and BuChE following the experimental protocol previously described.[11]

The IC_{50}s for AChE and BuChE and the ratios of BuChE to AChE, are summarized in Table 1. Tacrine was taken as reference compound. Compounds **2a, 2b** and **3b,**were found to be more active than reference drug in inhibiting AChE. The presence of chlorine atom in 6 position of the tetrahydroacridine fragment (**1b, 2b, 3b**) improves the AChE-inhibitory activity and selectivity toward AChE over BuChE, with respect to the no-substituted derivatives (**1a, 2a, 3a).** It is also reported that BuChE is involved in the metabolic degradation of ACh. BuChE activity increases as AD progresses. Therefore, the concurrent inhibition of both AChE and BuChE should provide additional benefits in the treatment of AD [23,24]. As list in Table 1, compounds **1b**, **2a** have equal inhibitory activity against BuChE. The best results, in terms of affinity and selectivity, were obtained for compounds **2b** and **3b,** that are ferulic and lipoic chlorine-substituted derivatives, respectively.

Table 1.

The inhibition of AChE and BuChE activities ($IC_{50,}$ μM) and DPPH radical scavenging activities (10μM and 30μM) of new compounds and references.

Compounds	**IC_{50} (μM)**		**Ratio of BuChE/AChE IC_{50}**	**DPPH radical scavenging rate (%) (10 μM)**	**DPPH radical scavenging rate (%) (30 μM)**
	AChE	**BuChE**			
1a	0.70	1.01	1.45	41.50 ±3.60	89.6±0.57
1b	0.15	0.36	2.40	60.87±2.7	90.36±1.4
2a	0.03	0.31	10.33	10.30±2.6	25.0±3.1

2b	0.02	0.71	34.53	17.11±1.9	22.68±4.1
3a	0.10	1.18	11.80	4.23±0.96	8.47±2.91
3b	0.04	1.03	25.75	0.98±0.55	1.57±0.79
Tacrine	0.10	0.04	0.40	>1000	>1000
Caffeic acid	na	na	na	44.10 ±4.40	90.27±1.50

na.: not available

Compounds (**1a,b-3a,b**) were also tested for their radical scavenging activities by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. The compounds were tested to 10 μM and 30 μM and the data are summarized in Table 1. The only derivatives endowed of radical scavenging activity, comparable to that of caffeic acid, were **1a** and **1b.**

In order to evaluate the influence of the tested compounds on amyloid fibrils, a Th-T fluorescence assay was performed. The compounds were tested to 50μM and 5 μM and the results are reported in Table 2. Compounds **1a,b-2a,b**, at concentration of 50 μM, induced a slight significant inhibition of A$\beta_{1\text{-}42}$ self-aggregation with percentage values ranging from 46 (**2a**) to 53% (**1b**). On the contrary, the tacrine-lipoic acid hybrids **3a** and **3b** did not affect the A$\beta_{1\text{-}42}$ self-aggregation

Table 2. Inhibition of $A\beta_{1-42}$ aggregation.

Compounds (5μM)	Inhibition of Aβ aggregation (%)	Compounds (50μM)	Inhibition of Aβ aggregation (%)
1a	12.8	***1a***	49.8
1b	9.2	***1b***	53.2
2a	8.4	***2a***	46.3
2b	13.5	***2b***	48.9
3a	na	***3a***	na
3b	na	***3b***	na
Congo red	76.6	Congo red	93.2

na.: not available

Hybrids (**1a,b-3a,b**) were then tested for their BACE1 inhibitory activity by using a commercially available kit (Sigma-Aldrich, MO, USA). As showed in Table 3, lipoic acid derivative **3b** was able to induce a slight significant inhibition of BACE1 at 10 μM with a percentage of about 47% .On the contrary, the compounds **1a,b, 2a,** and **3a** exhibited only a modest BACE1 inhibitory activity.

Table 3.

The BACE1 inhibitory activity of compounds.

Compounds (10μM)	**Inhibition rate %±SEM**
1a	10.51±2.55
1b	13.07±5.81
2a	14.97±1.25
2b	2.57±7.29
3a	24.77±5.46
3b	46.78±3.12

The new synthesized compounds (**1a,b-3a,b)** were further investigated for their cell toxicity in HT22 cell, at different concentrations (10, 30 and 100μM) (Figure 2A). No cytotoxic effects were observed for caffeic acid-derivative **1a** and the ferulic acid-derivative **2a** at various concentrations whereas the 6-chloro analogues **1b,** and notably **2b,** appeared to be cytotoxic only at the highest concentrations (100μM). Derivatives **3a** and **3b** showed to be cytotoxic both at 30 and 100 μM, and in particular compound **3b** showed an appreciable cytotoxicity already 10 μM.

In order to evaluate the neuroprotective effects in HT22 cell against glutamate-induced neuronal death, the synthesized compounds were tested at different concentrations (0.3, 1, 3 and 10 μM). As reported in Figure 2B, derivatives **1a, 2a** and **2b** didn't show any neuroprotective effect. At 10μM tacrine-caffeic acid hybrid**1b** produced a significant neuroprotection, while compounds **3a** and **3b** displayed an appreciable neuroprotective effect already at 3μM. Unfortunately, both derivatives **3a** and **3b** were toxic even at 10uM, and therefore both compounds cannot be

considered as potential pipelines to select for further studies. Conversely compound **1b** seems to possess the minimum characteristics sought for this type of multifunctional ligand.

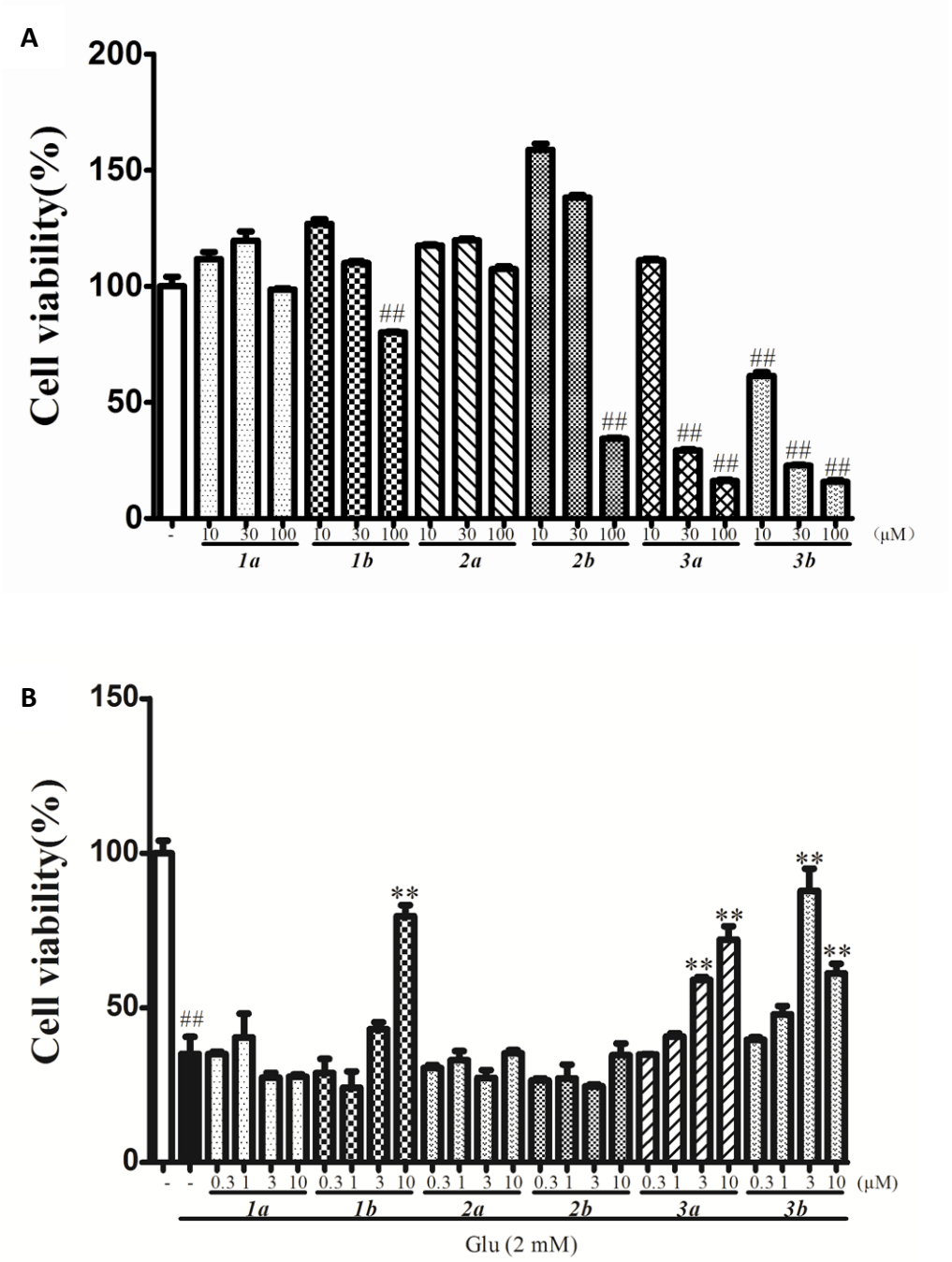


Figure2.The effects of compounds on cell viability in HT22 with or without glutamate.(A) Cell toxicity of test compounds on HT22 cells. Cell viability was determined using the MTT assay (n = 6). Cells were

treated with indicated concentration of test compounds for 24h. ** P < 0.01 versus control. (B) Compounds protect HT22 cells against glutamate-induced cytotoxicity. Cells were pretreated with different concentration of compounds for 30 min and then incubated with / without 2mM glutamate for 24 h. Cell viability was determined using the MTT assay (n = 6). ## P < 0.01 versus control; **P < 0.01 versus glutamate-treated group.

On the basis of the results obtained, compound **1b** was subjected to an additional assay, in order to evaluate its capacity to chelate copper. It is, now, established that metal ions, such as Cu^{2+}, contribute to development of AD, throughout the production of ROS and its interaction with Aβ peptides with consequently aggregation.

The complexation ability of **1b** toward Cu^{2+} was studied by UV–vis spectrometry and the results were illustrated in Figure 3. The bathochromic shift upon the addition of $CuCl_2$ of the maximum absorption induced by **1b** suggests the formation of a complex with Cu^{2+}.

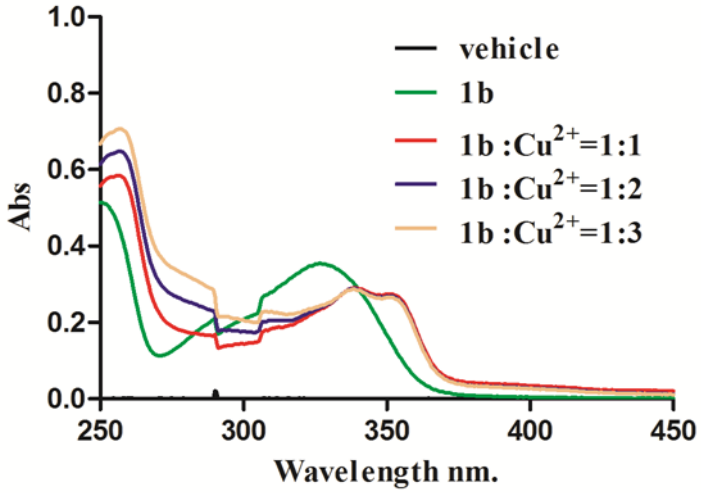


Figure 3.UV–vis (250–500 nm) absorption spectra of **1*b*** (40 μM) with 40, 80 or 120 μM $CuCl_2$ in ethanol.

In conclusion, this study focused on the identification of new ligands endowed of a multi-target pharmacological profile for the treatment of AD. In particular we evaluated the AChE, BuChE and BACE1 inhibitory activities and the ability of the compounds to inhibit the Aβ aggregation. Moreover a determination of the antioxidant, neuroprotective and chelating properties has also been carried out.

All together these results led us to designate **1b** as a lead multi-target ligand to further investigate in AD models. **1b** possessed a good ability to inhibit the Aβ aggregation, even if it displayed modest BACE1 and AChE inhibitor activities. Moreover, it possessed antioxidant activity and proved to chelate copper ions and thus delineate a multi-functional profile. Our findings suggest that the compound **1b** might be a promising lead multi-targeted ligand worthy of further developing for the therapy of AD. Effectively, even if the high in vitro potency is considered as a necessary requirement to carry on with further studies in the "one-drug-one-target" approach, in the multi-targeted ones a low potency towards one target does not necessarily mean a loss of therapeutic efficacy. Rather a weaker but more cooperative interaction with different targets involved in the pathology could be positive in terms of pharmacokinetic and pharmacodynamic of a multi-targeted compound, when compared with a specific highly selective single-target molecule.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version

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