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Combination of NSAIDs with donepezil as multi-target directed ligands for the treatment of Alzheimer's disease

Lei Fang [a,b,*], Shiyu Shen [a], Qiao Liu [a], Zhikun Liu [a], Jian Zhao [a,b]

[a] Jiangsu Province Hi-Tech Key Laboratory for Bio-medical Research, School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China
[b] State Key Laboratory for Chemistry and Molecular Engineering of Medicinal Resources, Guangxi Normal University, Guilin 541004, China

A B S T R A C T

To search for multi-target directed ligands for the treatment of Alzheimer's disease (AD), eight hybrid compounds from the combination of non-steroidal anti-inflammatory drugs with donepezil were designed and synthesized. The enzyme test revealed that the synthesized compounds had remarkable inhibitory activity towards both AChE and BChE. The IC_{50} values of the most active compound **3a** reached 0.015 and 0.80 μM for AChE and BChE, respectively, much lower than that of donepezil. Besides, the anti-inflammatory assays showed that the target compounds could effectively inhibit COX-1 and COX-2, and prevent the secretion of proinflammatory cytokines (TNF-α and IL-1β) induced by LPS. Moreover, the target compound could also protect the neuron cells from the damage caused by Aβ42 in vitro. All the results suggest that the hybrid compounds, in particular compound **3a**, can be considered as potential candidates for the treatment of AD.

Alzheimer's disease (AD) is a typical multifactorial disease. Ample evidence has revealed that many biological and physiological steps are involved in the eventual pathological condition of AD.[1] The known pathogenic factors of AD at least include the deficiency in cholinergic neurotransmission, the neuroinflammation, the oxidative stress, β-amyloid (Aβ) deposits, the hyperphosphorylation of tau protein, and so on.[2] Currently, four acetylcholinesterase inhibitors (AChEIs) and one *N*-methyl-*D*-aspartic acid receptor antagonist memantine are clinically available; however, these therapeutic agents show only limited effectiveness in ameliorating the symptoms of AD. Thus, developing more effective agents to combat AD is still in great need.

Due to the multifactorial characteristics of AD, multi-target directed ligands, which could simultaneously act on more than one target of AD, have attracted more and more attentions in the past decades.[3,4] For example, many of multifunctional tacrine hybrids were reported to exhibit excellent anti-AD activities.[5] Some galantamine derivatives or analogues were also developed and showed pronounced therapeutic results.[6] Surprisingly, donepezil, one of the most used AChEIs in clinic, is seldom employed for the construction of multi-target directed ligands, which may be probably due to the lack of the functional group (e.g. OH, NH_2 or COOH group) for the structural modification. Considering the well-proven efficacy of donepezil in clinic as well as the success of the former work on the other AChEIs, we believe that it will be really interesting to construct donepezil derivatives as multi-target directed ligands.

From the chemistry point as view, no highly reactive functional group such as OH, NH_2 or COOH group was found in the structure of donepezil. Interestingly, donepezil contains a ketone carbonyl group. Analyzing the interaction mode of donepezil with AChE as revealed by Cheung et al. [7] we found that the ketone carbonyl group did not expose to the solvent, but could interact with Phe295 residue as an H-bond acceptor (Figure 1). So it may be hypothesized that conversion of the carbonyl group to other H-bond acceptor group, e.g. hydrazone group (compound 2), might remain the inhibitory activity of donepezil. In order to predict whether such hypothesis is rational, we performed a docking study. The results turned out that compound **2** could enter the pocket of AChE (PDB code: 7E3H) and have a number of critical interactions with the key residues, including the π-π interaction between the aromatic ring and Trp 86, and the H-bonds formed between the NH_2 group and Tyr 341 and Tyr 337. With this result we may believe compound **2** would possess comparable AChE inhibition activity to donepezil (Figure 1). Thus, it gives a possibility to preform structural modification of donepezil to generate hybrid compounds.

Besides the acetylcholine pathway, the inflammation in central nervous system is also believed to play a significant role in AD progression. Neuroinflammation correlates closely with amyloid cascade. It could promote the generation of Aβ, and the deposit of Aβ would in turn exacerbate the inflammatory events which finally cause the death of neurons.[8] It is well documented that non-steroidal anti-inflammatory drugs (NSAIDs) could exert protective effect against the inflammatory

* Corresponding author at: Jiangsu Province Hi-Tech Key Laboratory for Bio-medical Research, School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China.
E-mail address: lei.fang@seu.edu.cn (L. Fang).

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damage. There are studies revealing that combination administration of NSAIDs and AChEIs could offer a better therapeutic result.[9] Our group recently reported that tacrine-ibuprofen hybrids significantly improved the anti-AD activity as compared with tacrine or ibuprofen alone.[10] Encouraged by these findings, we here tried to design a series of hybrid compounds via combination of NSAIDs (e.g. ibuprofen, aspirin, indometacin, naproxen) with donepezil derivatives. Our strategy is to convert the ketone carbonyl group of donepezil to hydrazone by coupling to hydrazine, and then NSAIDs were introduced via different linkers, respectively (Figure 2). We hope that the designed compounds could remain the AChE inhibitory activity of donepezil, and at the same time could prevent the neurons from the damage caused by the inflammation. Thus, an improved anti-AD activity can be expected.

The hybrid compounds were synthesized according to the procedures outlined in Scheme 1. In detail, donepezil was used as the starting material and was firstly coupled to hydrazine, giving the key hydrazone intermediate **2**. In order to confirm the configuration of the C═N bond, we have used compound **2** to performed NOESY test. A clear NOE correlation is found between the protons of NH_2 group and the protons of CH_2 in the side chain. No NOE correlation is found between the NH_2 protons and the aromatic protons (see supporting information). Thus, we are sure that the configuration of C═N double bond is E. Utilizing the free amine group, compound **2** was then reacted with different *N*-protected amino acids, yielding the amide compounds **4a-d**. After the removal of the Fmoc group, ibuprofen was then connected to the linker by the reaction of the carboxyl group with the amine group, offering the target compounds **6a-d**, respectively. Besides, compound **2** was also directly reacted with different NSAIDs, respectively, to give the target compounds **3a-d** without linker.

[a]Reagents and conditions. a) $NH_2NH_2{\cdot}H_2O$, CH_3OH, Na_2SO_4, reflux; b) NSAIDs, CH_2Cl_2, DCC, reflux; c) *N*-Fmoc-amino acid, CH_2Cl_2, DCC, reflux; d) Piperidine, DMF, NEt_3; e) ibuprofen, CH_2Cl_2, DCC, reflux.

ACh and other related neurotransmitters play important roles in the process of learning, recognition and memory. The deficiency of synaptic ACh and other related neurotransmitters is believed to closely correlate with the symptoms of AD. Therefore, various AChEIs have been developed, and are acting as the mainstay for the symptomatic treatment of AD. As the hybrid compounds derived from donepezil, we firstly screened whether our target compounds retained the AChE and BChE inhibitory activity or not. Thus, the target compounds were applied for the cholinesterase inhibition test in vitro by Ellman's assay.[11] The inhibitory effect of ibuprofen, aspirin, indometacin, and naproxen at a dose of 10 μM was also tested. The results (Table 1) turned out that all of the synthesized compounds showed moderate to potent inhibitory effect on the ChEs, with IC_{50} values ranging from 0.015 to 22.17 μM. As expected, the hydrazone compound 2 showed potent inhibitory activity against both AChE and BChE, which was quite consistent with the results of the docking study. In particular, compound **3a** was the most active one whose IC_{50} values reached 0.015 μM against AChE and 0.8 μM against BChE, respectively. The inhibition potency to BChE was even over 5-fold higher than that of donepezil. It should be noted that our HPLC assay indicated **3a** was very stable within 48 h when it was cultured in PBS (pH 7.4) solution (results shown in SI). Thus we can conclude that **3a** can inhibit AChE by itself, without the release of donepezil. As expected, NSAIDs were all not active in the test.

Analyzing the structure–activity relationship of the target compounds we found that the structure of the linker had an important influence on the activity. When NSAIDs were directly connected to the donepezil part without the application of linker, the resulted compounds (e.g. **3a-3d**) showed generally higher activity than the other compounds. When large-size linker such as valine or β-aminopropionic acid was used, the resulted compounds showed decreased activity. For example, compound **6b** which employed β-aminopropionic acid as the linker showed only a moderate inhibitory activity towards both AChE and BChE. Its IC_{50} value to AChE was 100-fold higher than that of **3a**. Furthermore, the structure of NSAIDs seemed to also have influence on the activity. The ibuprofen hybrid compound showed higher inhibitory activity than the hybrid compounds from the other NSAIDs, especially for the activity towards AChE. It may be attributed to relatively smaller structure of ibuprofen than the other NSAIDs. As the former study revealed, the smaller structure allowed the aromatic ring of the ligand to enter the bottom of enzyme pocket and consequently act on the key residues.[12].

The NSAIDs possess remarkable anti-inflammatory activity via the nonselective inhibition of COX-1 and COX-2, which endows them with the ability to protect the neurons from the damage caused by the neuroinflammation. Since compounds **3a** showed excellent enzyme inhibitory activity, we further examined their COX inhibition activity using an enzyme immunoassay (EIA). Ibuprofen was used as the positive control. As shown in Figure 3, compound **3a** inhibited both COX-1 and COX-2 in a concentration-dependent manner, and the capacity was comparable to the corresponding NSAID ibuprofen. Moreover, similar to ibuprofen, no obvious difference between the inhibition against COX-1 and COX-2 was found for the target compound, indicating **3a** was also a nonselective inhibitor of COX-1 and COX-2.

In order to further confirm the anti-inflammatory effect of the target compound, we selected compound **3a** to perform a qRT-PCR assay, investigating whether the target compound could prevent the secretion

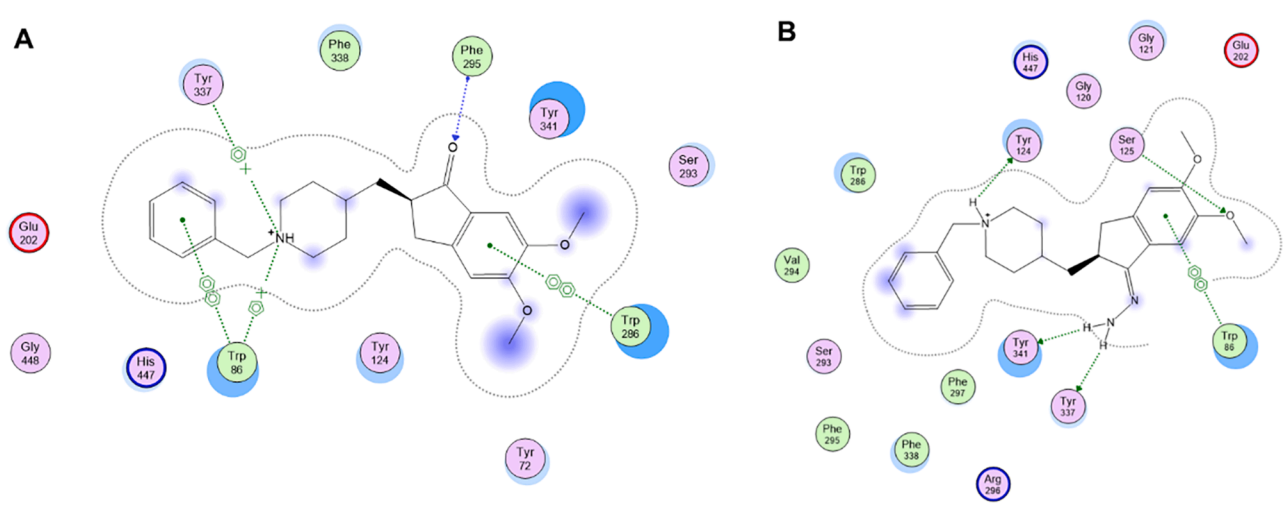


Figure 1. Molecular modeling study of donepezil (A) and Compound 2 (B) with *h*AChE (PDB code: 7E3H). The interaction mode was simulated and analyzed by MOE using the docking function.

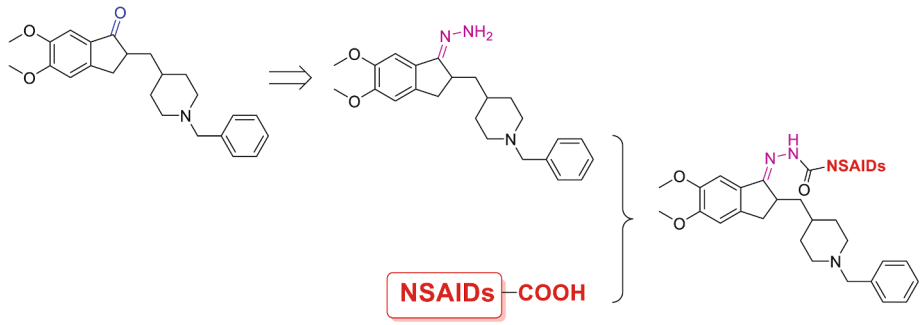
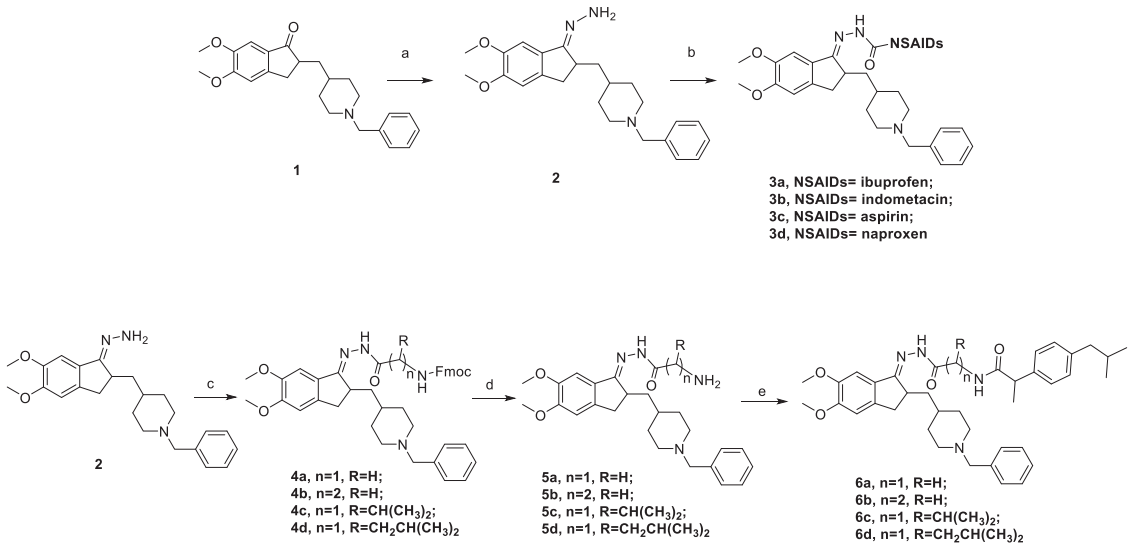


Figure 2. Structures of donepezil, and the structural modification leading to the donepezil-NSAID hybrid compounds.



Scheme 1. Synthetic procedure of the target compounds.[a.]

Table 1
Inhibitory effect of the target compounds and donepezil on AChE and BChE (IC_{50} values).

Compound	IC_{50} (μM) [a]	
	AChE [b]	BChE [b]
Donepezil	0.025	4.53
3a	0.015	0.80
3b	0.510	8.53
3c	0.046	0.80
3d	0.150	0.88
6a	1.190	1.64
6b	4.850	11.6
6c	0.400	3.23
6d	0.350	20.17
2	0.19	2.54
ibuprofen	NA[c]	NA[c]
aspirin	NA[c]	NA[c]
indometacin	NA[c]	NA[c]
naproxen	NA[c]	NA[c]

[a] Data are the mean values of at least three determinations.
[b] AChE from Electric Eel and BChE from equine serum were used.
[c] Not active when tested at a dose of 10 μM.

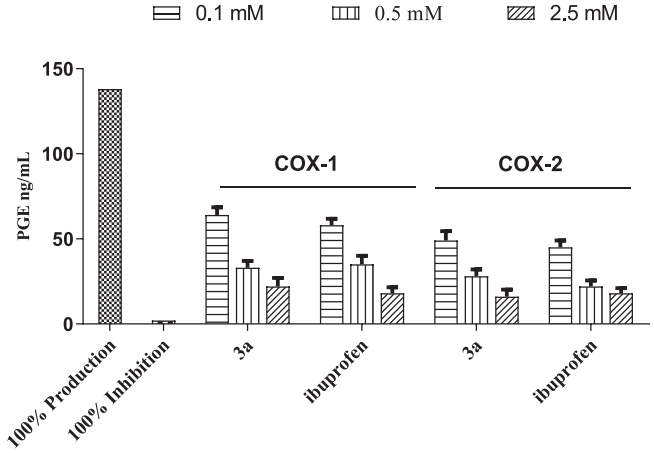


Figure 3. The inhibition effect of **3a** and ibuprofen on COX-1 and COX-2 evaluated by an enzyme immunoassay.

of proinflammatory cytokines such as TNF-α and IL-1β. Generally, BV-2 cells were firstly treated with compound **3a** for 6 h, and then lipopolysaccharide (LPS), a kind of endotoxin that can induce significant inflammatory reactions, was administrated and co-incubated for another 24 h. Thereafter, qRT-PCR assay was performed to determine the total RNA according to the previous reports.[13–14] The results in Figure 4 showed that the stimulation of LPS (1 μg/mL and 3 μg/mL) significantly increased the expression of IL-1β and TNF-α. When treated with 3 μg/mL of LPS, the relative mRNA expression of IL-1β and TNF-α increased to 3.76 and 3.91, respectively. This clearly indicates that LPS is capable of inducing inflammation in BV-2 cells. However, when pretreated with **3a** (5 μM, 10 μM) for 6 h, the relative mRNA expression of IL-1β in BV-2 cells significantly decreased to 1.19 and 0.78 in a concentration-dependent manner, and the expression of TNF-α decreased to 1.51 and

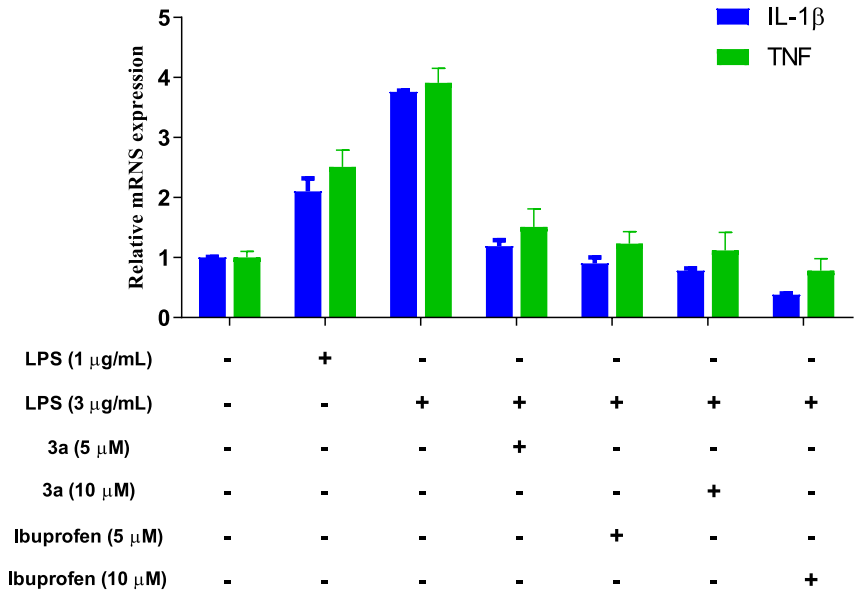


Figure 4. The inhibition effect of **3a** on the secretion of proinflammatory cytokines including TNF-α and IL-1β.

1.12 (Figure 4). Such expression level of both IL-1β and TNF-α was somehow as same as that of untreated group. It was also found that the potency of compound **3a** was comparable to that of the positive control. This finding definitely revealed that compound **3a** possessed the anti-inflammatory activity by preventing the secretion of cytokines (TNF-α and IL-β).

Numerous reports have revealed NSAIDs could protect neuron from the exogenous toxins.[9,15] Since compound **3a** showed remarkable anti-inflammatory activity in the former studies, the neuroprotective effect may be expected for the target compounds. Thus, Aβ42 was used as the toxins, and the neuroprotective effect of compound **3a** on PC12 cells was determined in vitro by MTT assay.[4,10] For the comparison, ibuprofen and ibuprofen/donepezil mixture (1:1, n/n) were also included in the test as controls. The results were shown in Figure 5. When **3a** (0.1, 1, 10 μM) was administrated alone, almost no change of the cell viability was observed as compared with the vehicle group. However, when the cells were treated with Aβ42 (5 μM), a significant decrease of the cell viability was observed, indicating clear toxicity was caused by Aβ42. Interestingly, when PC12 cells were co-treated with compound **3a** (0.1, 1, 10 μM), the induced toxicity was obviously relieved in a dose-dependent manner, indicating compound **3a** could effectively protect the neurons from the damage of Aβ42. Its potency was slightly higher than ibuprofen. As far as the mixture of donepezil and ibuprofen (molar ratio: 1:1) was concerned, a moderate protective effect against the Aβ42-induced toxicity was observed. It should be noted that at the high concentration (10 μM), the mixture caused a decrease of the cell viability, suggesting side effects induced by the mixture. Given all of the results, we can conclude that combination of donepezil and ibuprofen in one hybrid molecule could effectively protect neurons from the damage of Aβ42, and is safer than a simple mixture of both components at a high dose.

In this work we have designed and synthesized a set of hybrid compounds from non-steroidal anti-inflammatory drugs and donepezil via the structural modification of the ketone carbonyl group of donepezil. The pharmacological studies showed that the target compounds possessed pronounced ChE inhibitory activity. The potency of the most active compound **3a** against both AChE and BChE was over 10-fold higher than that of donepezil. Moreover, the target compounds could inhibit COX-1 and COX-2, and prevent the secretion of proinflammatory cytokines such as TNF-α and IL-β. In the cellular assay, compound **3a** showed remarkable neuroprotective efficacy against the Aβ-induced toxicity. All the results suggest that the hybrid compounds, in particular compound **3a**, can be considered as potential candidates for the treatment of AD.

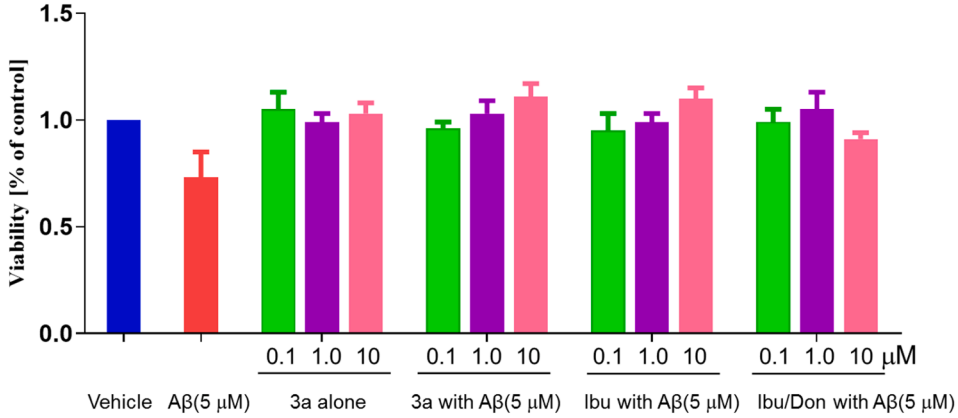


Figure 5. The neuroprotective effect of ibuprofen (Ibu), **3a** and the corresponding ibuprofen/donepezil mixture (ibu/Don, molar ratio: 1:1) against the Aβ42-induced toxicity (n = 3).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2022.128976.

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