Research Article

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“Click” assembly of novel dual inhibitors of AChE and MAO-B from pyridoxine derivatives for the treatment of Alzheimer’s disease

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Abstract: This study fast synthesizes numerous functionalized pyridoxines using click chemistry and assayed *in vitro* as inhibitors of the acetylcholinesterase (AChE), butyrylcholinesterase, and two monoamine oxidase (MAO) isoforms, MAO-A and MAO-B. Most of the obtained compounds demonstrate good AChE and selective MAO-B inhibitory activities in the micromolar range, especially one compound, called **4k5**, exhibits excellent inhibitory performance against AChE ($IC_{50} = 0.0816 \pm 0.075 \mu M$) and MAO-B ($IC_{50} = 0.039 \pm 0.003 \mu M$). Finally, a docking study is carried out, demonstrating potential binding orientations and interactions of the compounds in terms of the AChE and MAO-B active sites.

Keywords: click chemistry, pyridoxine derivatives, dual inhibitors, Alzheimer’s disease, acetylcholinesterase, monoamine oxidase

1 Introduction

Alzheimer’s disease (AD) is a chronic neurodegenerative disorder with multifaceted pathogenesis [1]. More than 50

million people are suffering from dementia worldwide, and AD is the most common cause of dementia, which is the leading cause of 60–70% of cases. By 2030, the total number of dementia patients is evaluated to reach 82 million, and by 2050, 152 million people will be affected [2].

Although the etiology has not been elucidated, the abnormal deposition and accumulation of amyloid- β , tau phosphorylation, oxidative stress, imbalance of biometallic homeostasis, and acetylcholine (ACh) deficiency play an important role in the pathophysiology of AD [3]. Monoamine oxidase B (MAO-B) activity is also increased in association with gliosis, resulting in higher levels of H_2O_2 and oxidative free radicals, which are a possible source of oxidative stress for vulnerable neurons affected by AD [4]. Because of the complexity of AD, a single drug acting on a single pathway or target is not enough as a treatment. As a result, it is now widely accepted that a more effective treatment will have compounds capable of acting on multiple targets [5].

ACh is the neurotransmitter existing in all nerve-muscle junctions and many other nervous system sites [6]. Critical cholinergic pathway deterioration in the central nervous system has been associated with the onset of AD [7]. Acetylcholinesterase (AChE) is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter ACh in numerous cholinergic pathways in the central and peripheral nervous systems [8]. Inhibition of AChE is considered to be a key target for effective treatment of AD because it increases the availability of synaptic ACh in brain regions [9].

In recent years, in addition to AChE, MAOs has attracted attention due to its role in the treatment of AD [10]. MAOs are flavin adenine dinucleotide (FAD)-containing enzymes localized in the outer mitochondrial membranes of neuronal, glial, and other cells [11]. The following two MAO isozymes, MAO-A and MAO-B, have been distinguished by substrate specificity [12]. MAO-A preferentially oxidizes 5-hydroxytryptamine, epinephrine, and norepinephrine, whereas MAO-B preferentially deaminates

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β -phenylethylamine and benzylamine [13]. High levels of MAO-B in neurons may lead to an increase of H_2O_2 and oxidative free radicals, which eventually leads to the cause of AD [14].

Pyridoxine is a 4-methanol in the essence of vitamin B6, an important water-soluble vitamin naturally present in many foods [15]. Pyridoxine and Vitamin B6 are essential nutrients required for the normal functionality of many biological systems of the body. Vitamin B6, principally in its biologically active coenzyme form pyridoxal 5'-phosphate, is involved in a wide range of biochemical reactions [16]. Recent studies focus attention on their anti-oxidative and enzymatic inhibition properties. Numerous functionalized pyridoxine have been presented as potent MAO and/or AChE inhibitors, and some of them have been proposed for treating AD [17].

Therefore, pyridoxine derivatives were an ideal starting compound to design multifunctional drugs for AD treatment. Huisgen Cu(I)-catalyzed alkyne-azide cycloaddition reaction has been widely used in drug designs due to its high modularity and high efficiency [18]. Click chemistry is based on a simple reaction, carried out under mild conditions, producing few by-products and providing an easy separation and purification of the product [19].

This study introduces pharmacophores to pyridoxine by the click chemistry. A series of pyridoxine derivatives are designed (Figure 1), synthesized, and the biological activities of the derivatives, including the inhibition of cholinesterase and MAO, are evaluated.

2 Results and discussion

2.1 Chemistry

Figure 2a presents synthesis steps of azide-substituted pyridoxine (4) from commercially available pyridoxine hydrochloride. Alkynes (**k1–k19**) are obtained from commercially available or simple synthesis processes (Figure 2b). The reaction condition is optimized to obtain the triazole products when the reaction is carried out in a *t*-BuOH:H₂O (1:1) solvent mixture with CuSO₄·5H₂O/ascorbate acid as catalysts. Ultimately, pyridoxine derivatives (**4k1–19**) are synthesized through click chemistry (Figure 2c).

2.2 *In vitro* inhibition of AChE and butyrylcholinesterase (BChE)

Using donepezil as a reference drug, the anti-AChE and BChE activities of these new molecules *in vitro* is determined by using modified Ellman's method. The IC₅₀ values for the inhibition of AChE and the inhibition ratios for BChE inhibition are summarized in Table 1.

Table 1 demonstrates that most of the compounds are effective AChE inhibitors, and the IC₅₀ value is in the micromolar range. From these data, we could get some interesting structure-activity relationships. The compounds can be divided into three classes: Class I contains aromatic ring

Figure 1: Design strategy for the pyridoxine derivatives.

Figure 2: The structures of azide and alkyne building blocks and target compounds: (a) azide library, (b) alkyne library, and (c) target library.

Table 1: Inhibition of cholinesterase and MAO activity by pyridoxine derivatives and reference compounds

Inhibitors	AChE ^a IC ₅₀ (μM)	BChE ^a IC ₅₀ (μM)	^d SI	MAO-A ^a IC ₅₀ (μM)	MAO-B ^a IC ₅₀ (μM)	^d SI
4k1	^b n.a.	^b n.a.		14.94 ± 0.02	0.088 ± 0.005	169.770
4k2	0.8011 ± 0.021	20.08 ± 0.011	25.065	^b n.a.	0.086 ± 0.004	
4k3	^b n.a.	10.023 ± 0.054		^b n.a.	0.080 ± 0.003	
4k4	^b n.a.	15.035 ± 0.036		^b n.a.	0.052 ± 0.005	
4k5	0.0816 ± 0.005	^b n.a.		^b n.a.	0.039 ± 0.003	
4k6	0.205 ± 0.004	^b n.a.		33.008 ± 1.004	0.097 ± 0.002	340.280
4k7	0.265 ± 0.024	^b n.a.		23.024 ± 1.003	0.183 ± 0.004	22.960
4k8	2.102 ± 0.014	^b n.a.		^b n.a.	0.345 ± 0.002	
4k9	0.823 ± 0.014	^b n.a.		^b n.a.	0.436 ± 0.003	
4k10	5.674 ± 0.036	^b n.a.		25.304 ± 1.003	28.051 ± 1.204	0.900
4k11	0.321 ± 0.012	^b n.a.		^b n.a.	0.125 ± 0.001	
4k12	24.000 ± 0.022	20.000 ± 0.010	0.830	16.006 ± 1.000	0.268 ± 0.032	59.720
4k13	0.117 ± 0.006	36.897 ± 1.010	315.360	^b n.a.	^b n.a.	
4k14	2.553 ± 0.062	4.605 ± 0.013	1.800	1.904 ± 0.053	4.608 ± 0.135	2.420
4k15	^b n.a.	^b n.a.		^b n.a.	^b n.a.	
4k16	0.632 ± 0.081	32.364 ± 0.014	51.209	^b n.a.	0.135 ± 0.007	
4k17	0.733 ± 0.065	19.472 ± 0.023	26.565	^b n.a.	0.071 ± 0.003	
4k18	15.86 ± 0.031	17.400 ± 0.033	1.090	25.34 ± 0.05	0.053 ± 0.004	478.110
4k19	^b n.a.	^b n.a.		^b n.a.	^b n.a.	^c N.T.
Donepezil	0.013 ± 0.001	23.402 ± 0.003	1800.154	^c N.T.	^c N.T.	^c N.T.
Clorgyline	^c N.T.	^c N.T.		0.004 ± 0.001	^c N.T.	^c N.T.
L-Deprenyl	^c N.T.	^c N.T.		^c N.T.	0.013 ± 0.004	^c N.T.

^aIC₅₀ value represents the inhibitor concentration required to reduce the enzyme activity by 50%, which is the average value of three independent experiments, and each experiment is conducted three times. The data were expressed as mean ± SD (SD = standard deviation).

^bn.a. = no active. Compounds defined as “no active” are defined as compounds with an inhibition rate of less than 5.0% at a concentration of 100 μM under analytical conditions.

^cN.T. = not tested.

^dSI = [IC₅₀(BChE)]/[IC₅₀(AChE)] and [IC₅₀(MAO-A)]/[IC₅₀(MAO-B)].

(**4k1–4k10**), Class II contains functional groups of AChE inhibitors, such as tacrine and donepezil (**4k11–4k14**), and Class III contains heterocycles amine or bromine (**4k15–4k19**). The results demonstrate that the inhibitory activity of Class I and Class II to AChE is significantly higher than that of Class III derivatives. The results illustrate that the inhibitory activity of AChE is significantly increased by introducing amino groups into aromatic rings (**4k5**, **4k6**, **4k7**). The inhibitory activity of meta substitution was the highest. Through analysis and comparison, it is found that compounds containing fluorine substituent (**4k2**), methyl (**4k3**), ethyl (**4k4**), or the replaced aromatic ring (**4k15–4k19**) had an adverse inhibitory effect on AChE than other compounds.

2.3 In vitro inhibition of MAO

MAO is also an important target for the treatment of AD. Inhibition of MAO-B is beneficial to the treatment of AD. To study the multifunctional biological characteristics

of the designed compounds, the inhibitory activities of MAO-A and MAO-B are evaluated and compared with those of standard Clorgyline and L-Deprenyl. The MAO-A, MAO-B inhibition data, and the selectivity indexes (SIs) of the present compounds are reported in Table 1. It presents that the inhibitory activity of most tested compounds on MAO-B was stronger than that of MAO-A. Surprisingly, compound **4k5** was the best MAO-B inhibitor with an IC₅₀ value of 0.039 μM. It is consistent with the structure–activity relationship of AChE inhibitory activity, suggesting that the introduction of the amino groups has increased both MAO-B and AChE inhibitory activities. This has made it possible to treat AD with multifunctional pyridoxine derivatives.

2.4 Molecular modeling studies

To further study the interaction between the most effective inhibitors and enzymes, we have conducted docking

studies to explore possible binding modes. Using AutoDock, the docking of compound **4k5** (the best inhibitor in this study) to the active center of AChE and MAOs was examined [20].

The docking geometries of **4k5** and donepezil with AChE are shown in Figure 3. There are two functional domains (peripheral anionic site and catalytic anionic site) in the active site of AChE. As with donepezil, the **4k5** is affixed in the active site with pi–pi interactions between Trp86 and Phe295 by the contribution of aromatic rings.

The active site of MAO has three functional domains: the substrate cavity, the entrance cavity, and the “aromatic cage” in the active site of MAO. The substrate cavity is directly in front of the FAD cofactor containing residues 178–221. “Aromatic cage” is consistent with FAD, Tyr407, and Tyr444 in MAO-A and FAD, Tyr435, and Tyr398 in MAO-B. The entrance cavity is situated nearer to the surface of the protein and is composed of residues Phe108-Pro118 (MAOA) or Phe99-Tyr112 (MAO-B) [21].

After docking into the active site of MAO-A/B, Figure 4 illustrates the most favorable binding mode for **4k5**. At the beginning, the pose of **4k5** shows that the phenyl ring fits into the “aromatic cage” and is sandwiched between Tyr435 and Tyr398 to establish pi–pi stacking interactions. After this, the amino group of the phenyl ring is close to the N5 position of FAD and occupies the active center. Finally, docking results demonstrate that **4k5** is embedded in a large hydrophobic pocket formed by Tyr 69, Ile199, Leu167, Gln206, Ile316, and Leu171. Unexpectedly, in the active site of MAO-A, the phenyl ring is underneath the enzymatic “aromatic cage” formed by Tyr444, Tyr407, and FAD. Moreover, the amino group

of the phenyl ring is far away from the N5 position of FAD and occupies the active center. Thus **4k5** had been failed to form any pi–pi stacking interaction with MAO-A.

3 Experimental

3.1 Materials and methods

3.1.1 Materials

All raw materials, commercial solvents, and reagents are from Innochem Co., Ltd. (Beijing, China). The reaction process was monitored by thin-layer chromatography using silica gel glass plates and observed by UV light (254 nm). ^1H NMR spectra were recorded using a Bruker instrument (500 MHz).

Fourier-transform infrared (FTIR) spectroscopic measurements were performed using KBr pellets on a Perkin Elmer Frontier FTIR spectrometer (USA). The fluorescence measurements were carried out on Tecan Infinite 200 PRO multimode microplate reader (Switzerland). UV-vis spectra were measured on a PERSIE T6 spectrophotometer (Beijing, China). AChE, BChE, MAO-A, and MAO-B were obtained from Sigma-Aldrich (Shanghai, China).

3.1.2 Chemistry

Figure 2 presents the synthetic routes of pyridoxine derivatives. The characterization of all compounds is in supporting information.

Figure 3: 3D docking models of target compounds and AChE: (left) **4k5** with AChE and (right) donepezil with AChE. The enzyme is shown as ribbon. Ligands (yellow) and interacting key residues (gray) are shown in stick models.

Figure 4: 3D docking models of target compounds and MAOs: (left) 4K5 with MAO-A and (right) 4K5 with MAO-B. The enzyme is shown as a ribbon. FAD cofactor (yellow), ligands (blue-green), and interacting key residues (gray) are shown in stick models.

3.1.3 Synthesis of azide-substituted pyridoxines

The azide-substituted pyridoxines were synthesized starting from pyridoxine hydrochloride as outlined in Figure 1. Compounds **1** and **2** were prepared according to the reported methods in the literature [22].

3.1.3.1 Synthesis of 5-azidomethyl-2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridine(**3**)

Compound **2** (1.24 g, 5.79 mmol) was dissolved in dimethylformamide (35 mL), and Et_3N (2 mL, 14.8 mmol) and KI (0.01 g, 0.06 mmol) were added. Afterward, NaN_3 (1.2 g, 18.5 mmol) was added in batches at room temperature (rt), and the reaction mixture was refluxed for 12 h at 80°C , poured into 20 mL water, extracted with Et_2O (3 mL $\times$ 30 mL), and the organic layers were combined and dried over Na_2SO_4 . The residue was purified by silica column chromatography. Elution with petroleum ether/ethyl acetate (1:6) gave pure compound **3** in the form of a yellow oil (yield 85%).

3.1.3.2 Synthesis of 5-azidomethyl-4-hydroxymethyl-2-methyl-pyridin-3-ol(**4**)

The mixture of **3** (1.05 g, 4.5 mmol), acetic acid (10 mL), and H_2O (10 mL) at 60°C was stirred for 12 h. Then, the mixture was neutralized with solid sodium bicarbonate. A large amount of solid precipitates was obtained from the mixture. The precipitates were filtered, washed with *n*-hexane, and dried to obtain compound **4** as a light yellow solid (yield 90%).

3.1.4 Preparation of alkyne blocks

Alkynes (**k1**–**k7**, **k10**, and **k15**–**19**) were available from commercial (Figure 2b). The preparation procedure of alkynes **k8**, **k9**, and **k11**–**k14** may be found in supporting information.

3.1.5 Click assemble target library

A solution (*t*-BuOH: H_2O = 1:1) of azide **4** (1.0 equiv.) was dispensed into a 25 mL round bottom flask containing a corresponding alkyne (1.0 equiv.) and a catalytic amount of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.04 equiv.) and sodium ascorbate (0.08 equiv.) and the reaction mixture was refluxed for overnight at rt, gave a crystalline precipitate. Pure pyridoxine derivatives (**4k1**–**19**) were separated by filtering as white/yellow solids, and yields ranged from 90 to 94%. The compounds (**4k1**–**19**) were characterized by using IR and proton nuclear magnetic resonance (^1H NMR).

3.1.6 5-[4-(3-Amino-phenyl)-[1,2,3]triazol-1-ylmethyl]-4-hydroxymethyl-2-methyl-pyridin-3-ol(**4k5**)

Reddish brown solid, yield 90% ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.87 (s, 1H), 8.59 (s, 1H), 8.14 (s, 1H), 7.71 (ddd, J = 7.7, 1.9, 1.2 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.08 (t, J = 1.9 Hz, 1H), 6.80 (ddd, J = 7.7, 1.9, 1.2 Hz, 1H), 5.50 (s, 2H), 5.12 – 4.91 (m, 6H), and 2.47 (s, 3H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 152.87, 148.56, 147.16, 143.00, 141.58, 134.93, 130.93, 130.35, 128.40, 119.13, 115.84, 114.02, 108.62,

56.14, 47.45, 18.48. IR (cm^{-1}) 3,392, 2,102, 1,636, 1,418, 1,225, 1,031, 798, and 661.

3.2 Biological activities evaluation

3.2.1 Detection of enzyme inhibition

To evaluate the inhibitory activity of compounds against AChE and BChE, an improved Ellman's method was adopted. The general procedures were described in the literature [23]. For ChE inhibition assays, a mixture (240 μL) containing acetylthiocholine iodide (1.5 mM, 40 μL), phosphate-buffered solution (0.1 mM, pH = 8.0, 140 μL), 0.5 U/mL ChE (40 μL) and different concentrations of test compounds (40 μL) is incubated at 37°C for 30 min. Then 5,5-dithiobis-(2-nitrobenzoic acid) (0.3 mM, 20 μL) is added and incubated at 37°C for 40 min. Changes in absorbance are detected at 412 nm for ChE in a multimode reader. Compounds inhibiting AChE activity reduces color generation. Thus, ChE activity inhibition is expressed by IC_{50} . Donepezil is used as a reference drug. All samples are assayed in triplicate.

MAO inhibitory activity is assayed using the method of Koichi Takao with minor modifications [24]. Briefly, 140 μL of 0.1 M potassium phosphate buffer (pH 7.4), 8 μL of 0.75 mM kynuramine, and 2 μL of inhibitor solution are incubated at 37°C for 10 min. MAO (50 μL) is then added to obtain a final protein concentration of 0.0075 mg/mL (MAO-A) or 0.015 mg/mL (MAO-B) in the assay mixture. Further incubation is carried out at 37°C, and the reaction is stopped after 30 min by the addition of 75 μL of 2 M NaOH. The fluorescence is generated by MAO and measured at Ex 310 nm/Em 400 nm using a microplate reader. Clorgyline and Pargyline are used as reference drug. All samples are assayed in triplicate. The results are expressed by IC_{50} and the SI.

3.2.2 Molecular modeling studies

The MAO-A and MAO-B with inhibitors Clorgyline and Deprenyl enzyme models 2BXR and 2BYB are downloaded from the Protein Data Bank (PDB) site, whereas the X-ray crystallographic structures of AChE (PDB ID: 4EY7) along with donepezil is used for the AChE target. The docking study is conducted using the Autodock 4.2 program. The enzyme code is extracted by using Uedit32 <https://www.ultraedit.com/> software to prepare a protein structure for docking simulation. The simulation results are visualized by using PyMOL software <https://www.pymol.org/>.

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