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Synthesis and biological evaluation of 4-arylcoumarins as potential anti-Alzheimer's disease agents

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ABSTRACT:

Alzheimer's disease (AD) is a progressive neurological degenerative disease that has complex pathogenesis. A variety of studies in humans indicate that several enzymes inhibitors can be useful in the treatment of AD, including acetylcholinesterase (AchE), butyrylcholinesterase (BuChE) and monoamine oxidase (MAO). Various substituted 4-arylcoumarin derivatives were synthesised, and their activity *in vitro* were investigated, including AChE/BuChE inhibitory activity, MAO inhibitory activity, and antioxidant activity. Most of the compounds were found to exhibit high inhibitory activity, and individual compounds have extremely excellent activities. Therefore 4-arylcoumarins provides an idea for drugs design for the development of therapeutic or preventive agents for AD.

Keywords: 4-Arylcoumarins; Alzheimer's disease; Cholinesterase inhibitors; Monoamine oxidase; Antioxidant activity

One of the most feared and devastating aspects of aging is the deterioration of memory and other psychological processes that occur frequently with age[1]. Alzheimer's disease (AD) has developed into one of the major diseases that jeopardize the health of elder people in modern society. AD is a progressive neurological degenerative disease that influences up to 5% of people over the age of 65, and this proportion increases to 20% in people over 80 years of age[2]. The most common early symptom is that it is difficult to remember what happened recently. Typical symptoms of AD patients are progressive memory impairment and damage of cognitive function, and often accompanied by behavioral disorders such as aggression, depression and paralysis[3]. AD gradually weakens cognition, emotions, and physical ability, while at the same time disrupting the lives of family caregivers who become their "second

victims"[4].

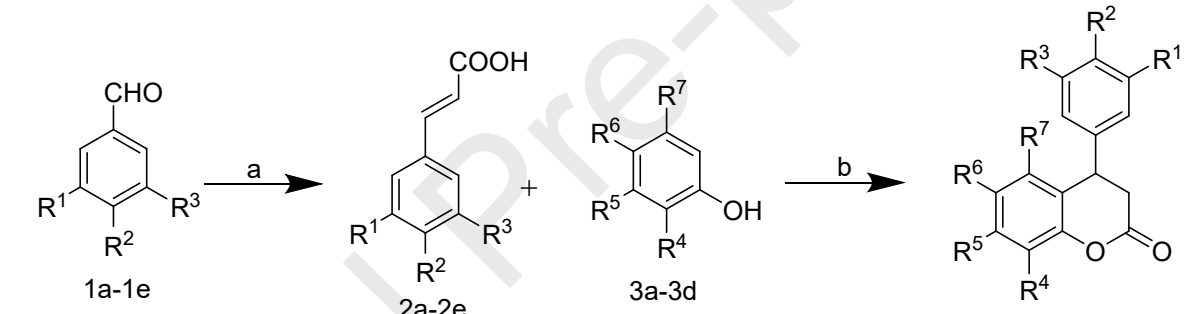
A variety of studies in humans indicate that the dysfunction of acetylcholine (Ach) neurons in the brain has a major impact on cognitive decline in those with advanced age and AD patients. The premise has become the basis for most of AD's treatment strategies and drug development methods to date[5]. With regard to "cholinergic hypothesis", one of the rational and effective methods for treating AD symptoms is increasing the amount of Ach by inhibiting acetylcholinesterase (AchE), which causes Ach to degrade in the presynaptic region. AChE inhibitors are currently recognized drugs with significant therapeutic effects in the world. Based on these studies, several AChE inhibitors containing different chemical backbones were synthesized, there are mainly tacrine[6], donepezil[7], rivastigmine[8], galantamine[9] and so on. It is used clinically to prevent and treat the development of early AD. While the AChE activity of AD patients decreases progressively, butyrylcholinesterase (BuChE) activity shows a certain increase[10]. It has been reported that the differences in enzyme kinetics between AChE and BuChE result in a change in the efficiency of ACh hydrolysis depending on substrate concentration, and BuChE plays a key role in Ach hydrolysis[11]. BuChE inhibition and AChE inhibition are viable therapeutic strategies for cognitive dysfunction in AD[12]. In fact, in the damaged brain, it is critical to preserve the proportion of AChE/BuChE activity for successful treatment of AD. Monoamine oxidase (MAO) inhibitors were first used as antidepressants and have long been used. It now seems that some MAO inhibitors are useful in the treatment of several neurodegenerative diseases, including Parkinson's disease and AD[13].

4-Arylcoumarin refers to a class of compounds having a coumarin skeleton and having an aryl structure at the 4-position[14]. Studies have shown that 4-arylcoumarin compounds are rich in biological activity, such as anti-diabetic[15], anti-oxidant[16], anti-tumour[17], anti-bacterial[18], inhibition of extracellular protein[19], anti-fungal[20], anti-inflammatory[21], anti-viral[22], anti-plasmodium and others[23]. The etiology of AD is complex, and there are currently five drugs used to treat AD cognitive problems. Treatment with a single target is difficult for AD. Multi-target drugs may be an important direction for future AD drug development. Coumarins are a large class of natural and synthetic sources of compounds with a variety of pharmacological activities, including MAO inhibition[24]. Edmondson and co-workers[25] have determined that coumarin derivatives competitively inhibit MAO-B. Bernard and co-workers[26] have reported that 17 coumarins and 2 chromone derivatives having

known inhibitory activities against MAO-A and MAO-B can also be used as AChE inhibitors. These findings encourage us to further explore the potential of 4-arylcoumarins and its derivatives to be candidates for the treatment of AD. The experiments we have performed provide an idea for the development of drug designs for therapeutic or prophylactic agents for AD.

Accordingly, in this work we described the biological evaluation of 4-arylcoumarins (synthesised by our research group) as selective ChE/MAO-B inhibitors. The pharmacological profiles of these new compounds including AChE and BuChE inhibition, MAO-B inhibition, the kinetics of enzyme inhibition, and antioxidant.

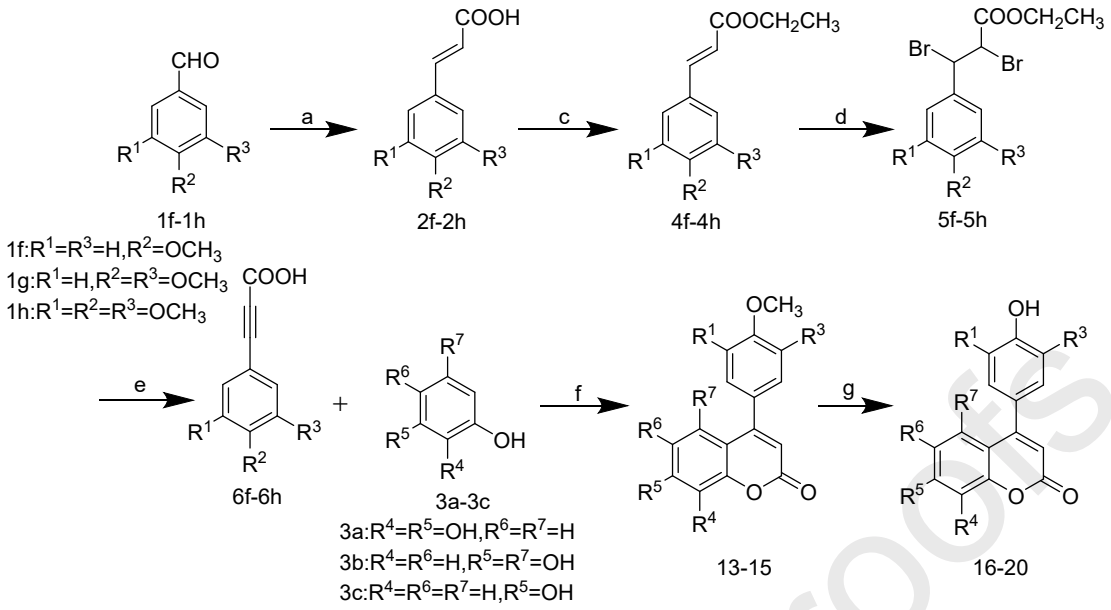
The synthetic methods and chemicals previously reported by our research group were used[27]. Details on the synthetic routes (Scheme 1 and Scheme 2), chemical (Table 1) and spectroscopic characterisations of compounds were described in the references.



1a:R^1=R^3=H,R^2=OCH_3
1b:R^1=OCH_3,R^2=OH,R^3=H
1c:R^1=R^2=OH,R^3=H
1d:R^1=R^2=OCH_3,R^3=H
1e:R^1=R^3=H.R^2=OH

3a:R^4=R^6=R^7=H,R^5=OH
3b:R^4=R^6=OH,R^5=R^7=H
3c:R^4=R^6=H,R^5=R^7=OH
3d:R^4=R^6=OH,R^5=R^7=H

Scheme 1. General synthetic route to 4-aryl-3,4-dihydrocoumarin, and conditions: (a) malonic acid, piperidine, pyridine, 90°C; (b) nitrobenzene or acetic acid, montmorillonite K-10, 100°C.



Scheme 2. General synthetic route to 4-aryl coumarin, and conditions: (a) malonic acid, piperidine, pyridine, 90℃; (c) EtOH, $SOCl_2$, reflux, 2h; (d) Br_2, CH_2Cl_2, 0℃, 20 min; (e) KOH, EtOH, reflux, 6h; (f) nitrobenzene, montmorillonite K-10, 100℃; (g) I_2, Al, acetonitrile, reflux, 5h.

Table 1. Compounds 1-20.

Product	R^1	R^2	R^3	R^4	R^5	R^6	R^7
1	H	OCH_3	H	H	OH	H	H
2	OCH_3	OH	H	H	OH	H	H
3	OCH_3	OCH_3	H	H	H	OH	H
4	H	OCH_3	H	OH	OH	H	H
5	OCH_3	OH	H	OH	OH	H	H
6	OH	OH	H	OH	OH	H	H
7	H	OCH_3	H	H	OH	H	OH
8	OCH_3	OCH_3	H	H	OH	H	OH
9	OCH_3	OH	H	H	OH	H	OH
10	H	OH	H	H	OH	H	OH
11	H	OCH_3	H	OH	H	OH	H
12	OCH_3	OCH_3	H	OH	H	OH	H
13	H	OCH_3	OCH_3	OH	OH	H	H
14	OCH_3	OCH_3	OCH_3	OH	OH	H	H
15	H	OCH_3	OCH_3	H	OH	H	OH
16	H	OH	OH	H	OH	H	H
17	H	OH	H	OH	OH	H	H

18	H	OH	OH	OH	OH	H	H
19	OH	OH	OH	OH	OH	H	H
20	H	OH	OH	OH	H	OH	H

In the human brain, AChE is mainly located in cholinergic axons and neuronal cells, while BuChE is mainly distributed in glial cells. These two enzymes are also present in neuritic plaques and tangles in AD patients. The proportion of ChEs present in the human brain changes throughout the AD process[28]. The inhibitory activities of the all compounds on AChE (from electric eel) and BuChE (from equine serum) were tested using the method of Ellman and co-workers[29] with donepezil as reference standard. As shown in Table 2, less than half of the compounds presented inhibitory activity for AChE/BuChE. Only nine compounds (**6**, **13**, **14**, **15**, **16**, **17**, **18**, **19** and **20**) exhibited moderate to excellent AChE inhibitory activity. Notably, compound **19** (IC_{50}=0.025±0.01 μM) had relatively strong activity, which showed a stronger ability than donepezil (IC_{50}=0.029±0.01 μM), and the compound exhibited selective AChE inhibitory activity. Compound **13** (IC_{50}=0.052±0.02 μM) displayed weaker capacity than donepezil but was of the same order of magnitude. The results showed that 4-arylcoumarins with a R^1, R^2, R^3- trihydroxy group has a significantly stronger inhibitory activity than the R^2- monohydroxy or R^2, R^3-dihydroxy 3-arylcoumarin. And R^4, R^5-dihydroxyl is also crucial for inhibiting AChE. However, there is no significant tendency for the inhibitory activity of BuChE.

Table 2. Biological evaluation in vitro.

Product	IC_{50} value (μM)			FRAP value(mmol·g^{-1})
	AChE inhibitory activity	BuChE inhibitory activity	MAO-B inhibitory activity	antioxidant activity
1	>50	>300	>300	0.896±0.02
2	>50	>300	82.507±24.48	9.376±0.07
3	>50	>300	>300	0.922±0.02
4	21.629±0.71	148.655±16.21	2.183±0.03	9.168±0.16
5	10.938±0.47	134.045±9.14	0.257±0.15	10.089±0.01
6	0.283±0.04	72.533±4.14	0.430±0.11	9.603±0.01
7	>50	>300	83.700±3.42	1.061±0.05
8	>50	>300	140.510±22.16	1.499±0.02
9	>50	>300	55.943±10.75	9.014±0.05
10	>50	>300	19.383±1.90	2.259±0.12
11	>50	>300	75.427±3.84	2.846±0.10
12	>50	>300	26.573±7.18	4.759±0.18

13	0.052±0.02	>300	>300	9.895±0.07
14	0.299±0.09	>300	>300	9.648±0.03
15	7.915±0.72	>300	104.053±4.12	1.374±0.02
16	8.857±0.61	>300	>300	9.710±0.08
17	9.454±0.64	>300	293.377±23.29	9.887±0.02
18	7.048±0.29	188.414±17.68	189.080±7.92	10.503±0.01
19	0.025±0.01	106.183±2.05	1.987±0.10	10.101±0.04
20	7.002±0.30	207.984±12.59	22.173±7.45	10.458±0.01
Donepezil	0.029±0.01	2.600±1.49		
Rasagiline			0.230±0.05	
Ascorbic Acid				9.868±0.01

The compound **19** was selected for kinetic measurements because it showed a good inhibitory activity against AChE. The graphical analysis of the steady-state inhibition data of **19** against AChE is shown in Figure 1. The results indicate that both slopes and intercepts increased with increasing inhibitor concentration. This pattern indicates that compound **19** was a mixed-type inhibitor.

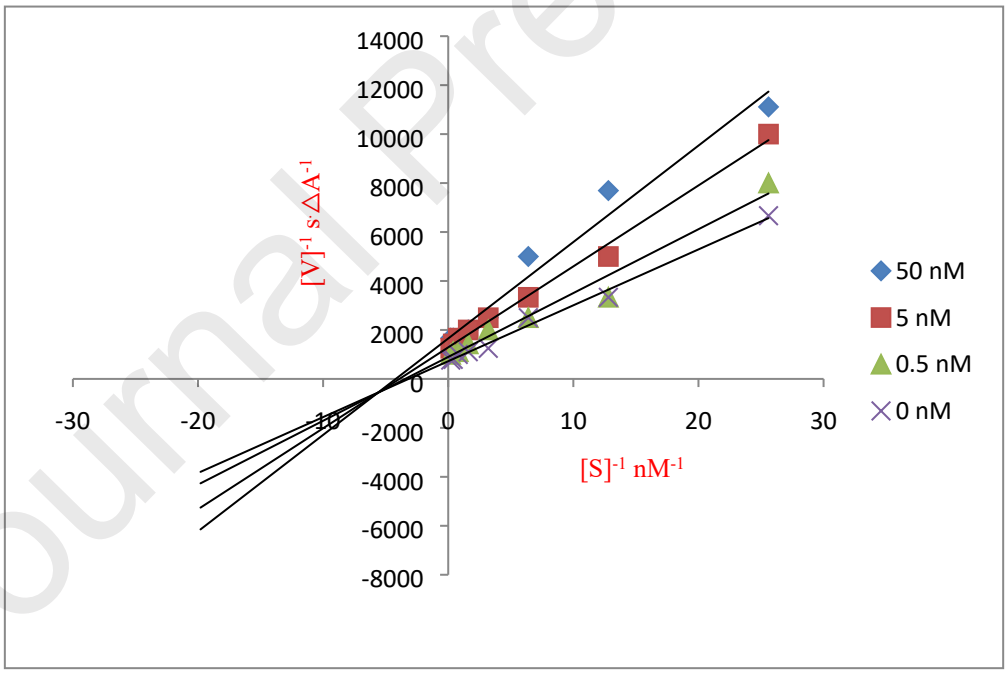


Figure 1. Kinetic study of the mechanism of AChE inhibition by compound **19**.

Three-dimensional (3D) protein structure of AChE (PDB id: 6O4W) was accessed from protein data bank, and for the compounds the 3D-coordinates were generated using ChemDraw software. Molecular docking of the compounds into hAChE protein was performed employing CDOCKER semi-flexible docking algorithm incorporated in the Discovery Studio 2019. Based on the experimental results, we performed docking calculations for the compound **19** (Figure 2). In the

binding pocket, amino acid residues Phe295 and Arg296 (amino acid backbone, non-residue) and a water molecule (797) form hydrogen bonds with the parent carbonyl. In addition, the water molecule 875 forms a hydrogen bond with the ligand B ring phenolic hydroxyl group, and the water molecule 788 forms a hydrogen bond with the ligand A ring phenolic hydroxyl group. Trp286 and Tyr124 and Ligand A ring form a Pi-Pi stack, while Phe338 and Tyr337 have a Pi-donor hydrogen bond with the ligand.

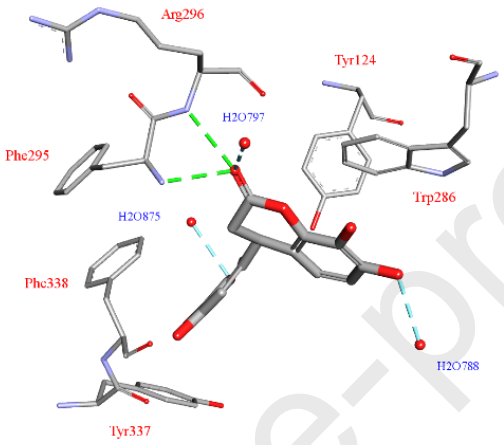


Figure 2. Docking of compound 19 to hAChE protein

It has been reported that MAO is also an important target for the treatment of AD. MAO-B accelerates the oxidative deamination of neurotransmitters, resulting in enhanced production of free radicals thus causing oxidative stress[30]. As a result, selective MAO-B inhibitors are useful in the treatment of AD. The inhibitory activities were evaluated of all compounds against the MAO-B in the way of Holt and co-workers[31] by references. The selective MAO-B inhibitor rasagiline was used as a reference compound in this assay. The MAO used in the experiment was selfmade. The protein content standard curve was drawn according to the absorbance of the protein standards at different concentrations at 595nm, and then the crude enzyme protein content was obtained by a regression equation according to the absorbance of the crude enzyme. The crude enzyme obtained was 3.78mg per mL of protein. It can be seen from Table 2 that most of the compounds showed MAO-B inhibitory activity. Notably, compound **5** (IC_{50}=0.257±0.15 μM) had strongest activity, which displayed weaker capacity than rasagiline (IC_{50}=0.230±0.05 μM). The compound **6** (IC_{50}=0.430±0.11 μM) was slightly weaker than the positive control drug rasagiline. The experimental results show that the 4-aryl-3,4-dihydrocoumarin compounds with R^4, R^5-dihydroxy group have better MAO inhibitory activity, indicating that R^4,

R^5-dihydroxyl is very important for inhibiting MAO.

Compound **5** was used to investigate the mode of MAO-B inhibition because it showed strongest inhibitory activity against MAO-B. The catalytic rates were measured at eight different 4-(trifluoromethyl) benzylamine solution concentrations, and each plot was constructed at four different concentrations of **5**. The graphical analysis of the steady-state inhibition data of **5** against MAO-B is shown in Figure 3. The results show that the plots for different concentrations of **5** were linear and intersected at the x-axis. This pattern indicated that **5** is a noncompetitive MAO-B inhibitor.

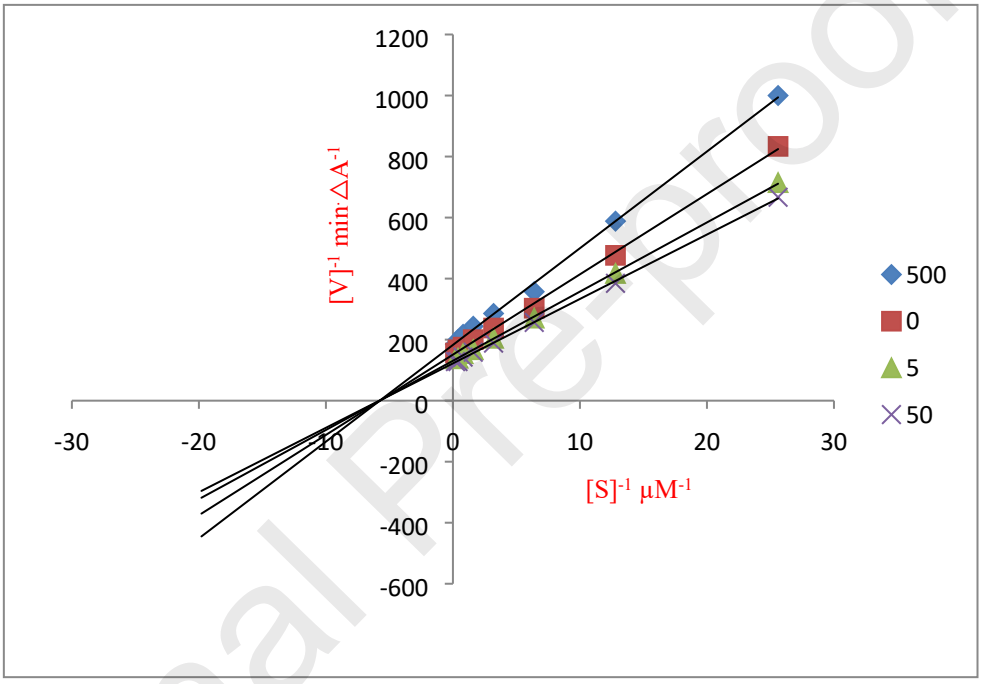


Figure 3. Kinetic study of the mechanism of MAO inhibition by compound **5**.

3D protein structure of MAO-B (PDB id: 4A79) was accessed from protein data bank. Based on the experimental results, we performed docking calculations for the compound **5** (Figure 4). The Tyr435 in the binding pocket and a water molecule (2183) form a hydrogen bond with the carbonyl group of the ligand core. Another water molecule in the pocket (2348) also forms a hydrogen bond with the hydroxyl group at the 8-position of the ligand. In addition, Tyr398 and the ligand A ring, Tyr326 and ligand C ring became Pi-Pi stacking. Leu171 and Ile199 form a hydrophobic interaction with the ligand, Cys172 and the ligand C ring form a Pi-sulfur interaction.

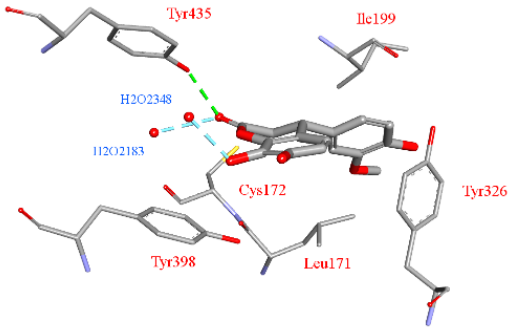


Figure 4. Docking of compound 5 to MAO-B protein

The antioxidant activity of the all compounds were tested using the method of Benzie et al.[32] with vitamin C as reference standard. The standard curve was drawn with $FeSO_4$ as standard material, and the antioxidant capacity of the sample is expressed as FRAP value. It can be seen from Table 2 that more than half of the compounds presented good antioxidant activity. Notably, six compounds (**5**, **13**, **17**, **18**, **19** and **20**) showed a stronger ability than vitamin C (FRAP value=9.868±0.01 $mmol \cdot g^{-1}$). Compound **18** (FRAP value=10.503±0.01 $mmol \cdot g^{-1}$) has the strongest antioxidant capacity. The antioxidant capacity of compound is related to the position and number of hydroxyl substitution, and the experimental results show that the 4-arylcoumarins compounds with R^4, R^5-dihydroxy group have better antioxidant activity.

In conclusion, we study the pharmacological activities against AD of 4-arylcoumarins synthesised by our research group, including cholinesterase inhibitory activity, monoamine oxidase inhibitory activity and antioxidant activity. All the synthesised compounds were evaluated for their antioxidant activities in the way of the Ferric Reducing Ability of Plasma. Most compounds demonstrated moderate to high activity, and the compound **18** with several hydroxyl groups have excellent antioxidant activity. According to ChE inhibitory and MAO inhibitory test results, most of the compounds showed moderate to excellent inhibitory activity. The selective AChE inhibitory compound **19** (IC_{50}=0.025±0.01 μM) showed a stronger ability than positive drug. The same is true for the MAO inhibitory compound **5** (IC_{50}=0.257±0.15 μM). From the perspective of structure, the maximum inhibitory activity was displayed by compounds with R^4, R^5-dihydroxy group, indicating that 4-arylcoumarins with R^4, R^5-dihydroxyl is very important for inhibiting AChE and

MAO. Thus, we selected compound **19** and **5** to study kinetic experiment of enzyme inhibition. Our study illustrates the importance of the number and position of hydroxyl groups on the aromatic ring, and among the compounds tested, two hydroxyl substituents in R^4, R^5 to be the most functional for the AChE and MAO inhibition. In summary, the experiment we did about 4-arylcoumarins provides an idea for drugs design for the development of therapeutic or preventive agents for AD.

Ethical statement

All experiments involving living animals and their care were performed in strict accordance with the National Care and Use of Laboratory Animals by the National Animal Research Authority (China) and guidelines of Animal Care and Use issued by the University of Jinan Institutional Animal Care and Use Committee. The experiments were approved by the Institutional Animal Care and Use Committee of the School of Medicine and Life Sciences, University of Jinan. All efforts were made to minimise animal's suffering and to reduce the number of animals used.

Acknowledgments

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References and notes

1. Coyle, J.T., D.L. Price, and M.R.J.S. Delong, *Alzheimer's disease: a disorder of cortical cholinergic innervation.* 1983. **219**(4589): p. 1184-1190.
2. Camps, P., et al., *New tacrine− huperzine A hybrids (huprines): highly potent tight-binding acetylcholinesterase inhibitors of interest for the treatment of Alzheimer's disease.* 2000. **43**(24): p. 4657-4666.
3. Francis, P.T., et al., *The cholinergic hypothesis of Alzheimer's disease: a review of progress.* 1999. **66**(2): p. 137-147.
4. Filley, C.M.J.A.j.o.o. and gynecology, *Alzheimer's disease in women.* 1997. **176**(1): p. 1-7.
5. Terry, A.V., J.J.J.J.o.P. Buccafusco, and E. Therapeutics, *The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development.* 2003. **306**(3): p. 821-827.
6. Tumiatti, V., et al., *Tacrine derivatives and Alzheimer's disease.* 2010. **17**(17): p. 1825-1838.
7. Sugimoto, H., et al., *Donepezil hydrochloride (E2020) and other acetylcholinesterase inhibitors.* 2000. **7**(3): p. 303-339.
8. Birks, J.S., L.Y. Chong, and J. Grimley Evans, *Rivastigmine for Alzheimer's disease.* Cochrane Database Syst Rev, 2015. **9**: p. CD001191.
9. Kavanagh, S., et al., *Galantamine and behavior in Alzheimer disease: analysis of four trials.*

Acta Neurol Scand, 2011. **124**(5): p. 302-8.

10. Giacobini, E., *Cholinesterase inhibitors: new roles and therapeutic alternatives.* Pharmacol Res, 2004. **50**(4): p. 433-40.
11. Giacobini, E., et al., *Inhibition of acetyl-and butyryl-cholinesterase in the cerebrospinal fluid of patients with Alzheimer's disease by rivastigmine: correlation with cognitive benefit.* 2002. **109**(7-8): p. 1053-1065.
12. Furukawa-Hibi, Y., et al., *Butyrylcholinesterase inhibitors ameliorate cognitive dysfunction induced by amyloid-beta peptide in mice.* Behav Brain Res, 2011. **225**(1): p. 222-9.
13. Youdim, M.B., D. Edmondson, and K.F. Tipton, *The therapeutic potential of monoamine oxidase inhibitors.* Nat Rev Neurosci, 2006. **7**(4): p. 295-309.
14. Garazd, M., Y.L. Garazd, and V.J.C.o.n.c. Khilya, *Neoflavones. 1. Natural distribution and spectral and biological properties.* 2003. **39**(1): p. 54-121.
15. Korec, R., et al., *Antidiabetic effect of neoflavonoid coutareagenin in STZ diabetic rats and diabetic menopausal women.* 2000. **50**: p. 42.
16. Zhang, K., et al., *Antioxidant and antitumor activities of 4-arylcoumarins and 4-aryl-3,4-dihydrocoumarins.* Biochimie, 2014. **107 Pt B**: p. 203-10.
17. Chen, H., et al., *Design, synthesis, and anti-tumor activities of novel triphenylethylene-coumarin hybrids, and their interactions with Ct-DNA.* Bioorg Med Chem Lett, 2013. **23**(17): p. 4785-9.
18. Canning, C., et al., *Antibacterial and cytotoxic activity of isoprenylated coumarin mammea A/AA isolated from Mammea africana.* J Ethnopharmacol, 2013. **147**(1): p. 259-62.
19. Combes, S., et al., *Synthesis and biological evaluation of 4-arylcoumarin analogues of combretastatins. Part 2.* J Med Chem, 2011. **54**(9): p. 3153-62.
20. Taechowisan, T., et al., *Secondary metabolites from endophytic Streptomyces aureofaciens CMUAc130 and their antifungal activity (Retracted Article. See vol 153, pg 915, 2007).* 2005.
21. Ducharme, Y., et al., *The discovery of setileuton, a potent and selective 5-lipoxygenase inhibitor.* ACS Med Chem Lett, 2010. **1**(4): p. 170-4.
22. Nichols, D.B., et al., *Evaluation of coumarin and neoflavone derivatives as HCV NS5B polymerase inhibitors.* Chem Biol Drug Des, 2013. **81**(5): p. 607-14.
23. Pierson, J.T., et al., *Synthesis and antiprotozoal activity of 4-arylcoumarins.* Eur J Med Chem, 2010. **45**(3): p. 864-9.
24. Patil, P.O., et al., *A comprehensive review on synthesis and designing aspects of coumarin derivatives as monoamine oxidase inhibitors for depression and Alzheimer's disease.* Bioorg Med Chem, 2013. **21**(9): p. 2434-50.
25. Binda, C., et al., *Structures of human monoamine oxidase B complexes with selective noncovalent inhibitors: safinamide and coumarin analogs.* 2007. **50**(23): p. 5848-5852.
26. Brühlmann, C., et al., *Coumarins derivatives as dual inhibitors of acetylcholinesterase and monoamine oxidase.* 2001. **44**(19): p. 3195-3198.
27. Wang, B., et al., *Synthesis and biological evaluation of novel neoflavonoid derivatives as potential antidiabetic agents.* RSC Advances, 2017. **7**(55): p. 34448-34460.
28. Savelev, S.U., E.J. Okello, and E.K. Perry, *Butyryl- and acetyl-cholinesterase inhibitory activities in essential oils of Salvia species and their constituents.* Phytother Res, 2004. **18**(4): p. 315-24.
29. Ellman, G.L., et al., *A new and rapid colorimetric determination of acetylcholinesterase*

activity. 1961. **7**(2): p. 88-95.

30. Lopez, N., et al., *Oxidative stress in Alzheimer's disease and mild cognitive impairment with high sensitivity and specificity.* J Alzheimers Dis, 2013. **33**(3): p. 823-9.

31. Holt, A., et al., *A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates.* 1997. **244**(2): p. 384-392.

32. Benzie, I.F. and J.J.J.A.b. Strain, *The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay.* 1996. **239**(1): p. 70-76.

The AChE/BuChE inhibitory activity of the 4-arylcoumarin compounds was determined by the method of Ellman and co-workers with slight modifications. To a 96-well plate, 120 μL of phosphate buffer solution (0.1 M, pH=8.0), 20 μL of 5,5-dithiobis-2-nitrobenzoic acid (3.3 mM in 0.1 M phosphate buffer solution, pH=8.0) were added sequentially, 20 μL AChE solution (0.2 $U \cdot mL^{-1}$ in 0.1 M phosphate buffer solution, pH=8.0), 20 μL of different concentrations of the sample solution (10.0, 100.0, 500.0, and 1000.0 $\mu g \cdot mL^{-1}$), shaken well, and incubated at 37℃ for 5min. Then 20 μL of substrate (5 mM in distilled water) were added, shaken well, and incubated at 37 ℃ for 20 min. The absorbance at 412 nm of the samples was measured using a microplate reader, and the inhibition rate of cholinesterase and the IC_{50} value of each sample were calculated according to the formula. Determination of the inhibitory activity of BuChE is similar. The sample blank group replaced the substrate with distilled water, and the blank group replaced the sample solution with a solvent. The sample solution was set to four concentration gradients and the experiment was repeated three times. Donepezil was used as a positive control.

Cholinesterase inhibitory effect (%) = $[A_0 - (A_1 - A_2)]/A_0 \times 100\%$

where A_0 is the absorbance of blank group; A_1 is the absorbance of sample group; A_2 is the absorbance of sample blank group.

The compound that showed better inhibitory activity against AChE/BuChE was selected for kinetic measurements. To obtain the mechanism of action of compound, reciprocal plots of $[velocity]^{-1}$ versus $[substrate]^{-1}$ were constructed at different substrate solution concentrations by using Ellman and co-workers method. The experiment were measured at eight different substrate solution concentrations (0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 mM) and four different concentrations of target compound (0, 0.5, 5 and 50 nM). To a 96-well plate, phosphate buffer solution, 5,5-dithiobis-2-nitrobenzoic acid, AChE solution and target compound were added sequentially, shaken well, and incubated at 37 ℃ for 5 min. Then substrate were added, shaken well, and incubated at 37 ℃ for 20 min. The absorbance at 412 nm of

the samples was measured using a microplate reader, and the experiment was repeated three times. Slopes of these reciprocal plots were then plotted against the concentration of compound in a weighted analysis and Ki was determined as the intercept on the negative x-axis.

The MAO inhibitory activity of the 4-arylcoumarin compounds was determined by the method of Holt and co-workers with slight modifications. The crude enzyme was extracted from the liver of Wistar rats. Wistar rat, weight 200–250 g, were obtained from Jinan Peng Yue Experimental Animal Co. (License number: SCXK (Lu) 2014–0007), Ltd. The animals were housed under standard laboratory conditions and maintained on a standard pellet diet and water ad libitum. After extracting the crude enzyme, the crude enzyme protein content was determined by the Bradford/method using a Bradford Protein Assay Kit (Beyotime). 40 μL of enzyme solution and 40 μL of sample solution (125.0, 250.0, 500.0, and 1000.0 $\mu g \cdot mL^{-1}$) were added to a 96-well plate. The solution was then incubated at 37 ℃ for 20 min. 120 μL of the 4-(trifluoromethyl) benzylamine solution and 40 μL of the chromogenic agent (1 mM vanillic acid, 0.5 mM 4-aminoantipyrine, 4 $U \cdot mL^{-1}$ horseradish peroxidase, 0.2 M pH=7.6 phosphate buffer solution constant volume) were added subsequently, and incubated at 37 ℃ for 90 min. The absorbance was measured at 490 nm using a microplate reader, and the inhibition rate of MAO and the IC_{50} value of each sample were calculated according to the formula. The control group replaced the sample solution with a solvent, the positive control replaced the sample with the positive drug, and the blank group replaced the substrate with PBS, and each group was measured three times in parallel to average.

Monoamineoxidase inhibitory effect (%)= $[(A_1-A_2)-(A_3-A_4)]/(A_1-A_2)\times 100\%$

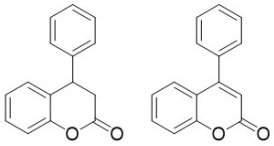
where A_1 is the absorbance of blank group; A_2 is the absorbance of blank background group; A_3 is the absorbance of sample group; A_4 is the absorbance of sample background group.

Data processing method of the kinetic characterization of MAO inhibition is similar to the kinetic measurements of AChE. The experiment were measured at eight different substrate solution concentrations (0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 mM) and four different concentrations of target compound (0, 5, 50 and 500 μM). To a 96-well plate, enzyme solution and target compound were added sequentially. The solution was then incubated at 37 ℃ for 20 min. The 4-(trifluoromethyl) benzylamine solution and the chromogenic agent were added, and incubated at 37 ℃ for 90 min. The absorbance was measured at 490 nm using a

microplate reader, and the experiment was repeated three times.

The total antioxidant capacity of the 4-arylcoumarin compounds was measured by the FRAP (the Ferric Reducing Ability of Plasma) assay of Benzie and co-workers[32] with slight modifications. This method is based on the reduction of colourless Fe(III)-TPTZ(2,4,6-Tris(2-pyridyl)-s-triazine) complex to coloured Fe(II)-TPTZ complex by the compounds. FRAP working solution (300 mM acetate buffer, 10 mM TPTZ, 20 mM $FeCl_3$) was ready to use. 180 μL of FRAP reagent and 5 μL of sample solution were added to a 96-well plate, and then incubated at 37 ℃ for 5 min. The experiments set up 3 groups of parallel groups. The absorbance was measured at 593 nm using a microplate reader. The standard curve was drawn with $FeSO_4$ as standard material, and the regression equation was obtained. With 1.0 mM $FeSO_4$ as standard, the antioxidant activity of the sample is expressed in millimoles of $FeSO_4$ required to achieve the same absorbance.

Computer simulation molecular docking steps are as follows. The ligand is first treated, and the method is Discovery Studio 2019 built-in prepare ligand method. The molecule does not form tautomerism and isomers, the protonation pH is set to 7, and the remaining parameters are default parameters. The energy optimization of the formed ligand is carried out by Discovery Studio 2019 built-in minimization, and the force field used is the MMF force field. After the above steps are completed, the resulting ligand is treated for molecular docking. The protein required for docking was treated by Discovery Studio 2019 built-in prepare protein method, and the main purpose was to hydrogenate the protein. Considering the structure of the docking ligand, we retain the water molecules in the protein binding pocket as well as the endogenous ligand, and other parameters are set to default. Based on the active sites included in the PDB database and the location of the ligands in the original eutectic complex, the coordinates and radius of the active sites are determined. The specific parameters are as follows: hAChE: X=89.214403, Y=85.422867, Z=-5.545143, r=11; MAO-B: X=52.076244, Y=157.208276, Z=30.314829, r=11. Molecular docking of the compounds into protein was performed employing CDOCKER semi-flexible docking algorithm incorporated in the Discovery Studio 2019.

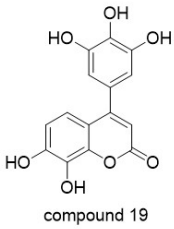


Experimental:

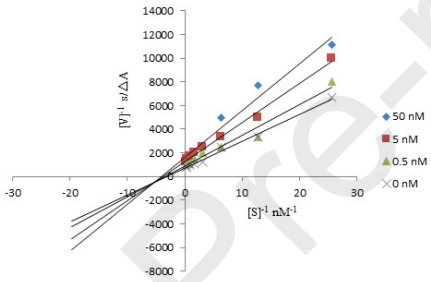
1. *In vitro* cholinesterase inhibitory activity
2. Kinetic characterization of AChE inhibition
3. *In vitro* monoamine oxidase inhibitory activity
4. Kinetic characterization of MAO inhibition
5. *In vitro* antioxidant activity
6. Molecular docking

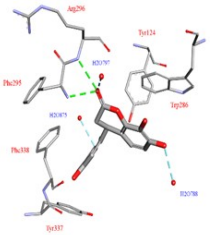
Results and discussion:

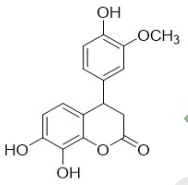
1. AChE inhibition: compound 19 had relatively strong activity.
2. Kinetic study of AChE inhibition: 19 is a mixed-type inhibitor.
3. MAO-B inhibition: compound 5 had strongest activity
4. Kinetic study of MAO inhibition: 5 is a noncompetitive MAO-B inhibitor
5. *In vitro* antioxidant activity: more than half of the compounds presented good antioxidant activity



compound 19







compound 5