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3-Phenethyl-2-phenylquinazolin-4(3H)-one isolated from marine-derived *Acremonium* sp. CNQ-049 as a dual- functional inhibitor of monoamine oxidases-B and butyrylcholinesterase

Jong Min Oh[1] · Prima F. Hillman[2] · Sang-Jip Nam[2] · Hoon Kim[1]

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Abstract Isolation of the culture broth of a marine-derived *Acremonium* sp. CNQ-049 guided by HPLC-UV yielded compound **1** (3-phenethyl-2-phenylquinazolin-4(3H)-one), and its inhibitory activities against monoamine oxidases (MAOs), cholinesterases (ChEs), and β-secretase 1 (BACE1) were evaluated. Compound **1** was an effective selective MAO-B inhibitor with an IC_{50} value of 9.39 μM and a selectivity index (SI) value of 4.26 versus MAO-A. In addition, compound **1** showed a potent selective butyrylcholinesterase (BChE) inhibition with an IC_{50} value of 7.99 μM and an SI value of 5.01 versus acetylcholinesterase (AChE). However, compound **1** showed weak inhibitions against MAO-A, AChE, and BACE1. The K_i value of compound **1** for MAO-B was 5.22±1.73 μM with competitive inhibition, and the K_i value of compound **1** for BChE was 3.00±1.81 μM with mixed-type inhibition. Inhibitions of MAO-B and BChE by compound **1** were recovered by dialysis experiments. These results suggest that compound **1** is a dual-functional reversible inhibitor of MAO-B and BChE, that can be used as a treatment agent for neurological disorders.

Keywords *Acremonium* sp. CNQ-049 · Butyrylcholinesterase · Dual-functional reversible inhibitor · Monoamine oxidase · 3-Phenethyl-2-phenylquinazolin-4(3H)-one

Hoon Kim (✉)
E-mail: hoon@sunchon.ac.kr

[1]Department of Pharmacy, and Research Institute of Life Pharmaceutical Sciences, Sunchon National University, Suncheon 57922, Republic of Korea

[2]Department of Chemistry and Nanoscience, Ewha Womans University, Seoul 03760, Republic of Korea

Introduction

Alzheimer's disease (AD) is one of the famous neurodegenerative diseases and is known to cause dementia [1]. The typical symptoms of AD are memory and cognitive declines [2]. The main cause of AD is brain nerve apoptosis caused by accumulation of beta-amyloid (Aβ), and there is also a decrease in the concentration of neurotransmitters such as serotonin and dopamine (DA) [3-5]. In this reason, inhibitors of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) involving in the production of Aβ, and those of monoamine oxidase (MAO) breaking down neurotransmitters are being developed [1,3,4,6,7]. In addition, a reduction of cholinergic receptor has been reported in AD patient brains. Accordingly, cholinesterase (ChE) inhibitors have been developed as AD treatment agents to increase the concentration of choline receptors [8].

MAO exists in two isoforms such as MAO-A and MAO-B, in the mitochondrial outer membrane [9]. It involved in catecholamine and 5-hydroxytryptamine inactivation, and catalyzes oxidative deamination of monoamines [6,10]. Therefore, MAO inhibitors reduce AD symptoms by increasing dopaminergic transmission and neurotransmitter synthesis factors or blocking degradation of the neurotransmitters [7]. Typically, selective MAO inhibitors such as selegiline, rasagiline, pargyline, and clorgyline are used for AD treatment [11,12]. On the other hand, ChE contains two types, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Two types of ChEs have a common ability to hydrolyze AChE, but differ in their selectivity. AChE specifically hydrolyzes acetylcholine (ACh), and BChE non-specifically hydrolyzes ACh and butyrylcholine (BCh) [13]. ACh is an important neurotransmitter in the brain, which functions in central nervous system and the peripheral nervous system, and regulates cognitive functions through neurotransmission, especially memory and learning [14,15]. ACh is synthesized in presynaptic neurons and released into postsynaptic neurons [16]. Choline inhibitors developed for

the treatment of AD include tacrine, donepezil, galantamine, and rivastigmine, all approved by the FDA [17-20]. However, tacrine has been reported to have a severe hepatotoxicity and is not used currently [11]. Nevertheless, as an AD drug, ChEs inhibitors are still valuable.

Recently, dual-target inhibitors have been developed to increase the efficacy of AD treatment [21], including homoisoflavonoid derivatives [22], donepezil-butylated hydroxytoluene hybrids [23], coumarin-dithiocarbamate hybrids [24], alcohol-bearing dual inhibitors [25], and chalcone oxime ethers [26]. Natural MAO and ChE inhibitors from microbial sources have been isolated and investigated such as 5-hydroxy-2-methyl-chroman-4-one (HMC) from an endogenous lichen fungus (ELF) *Daldinia fissa* [27], alternariol, 5'-hydroxy-alternariol, and mycoepoxydiene from an ELF *Diaporthe mahothocarpus* [28], (*S*)-5-methylmellein (5MM) from an ELF *Rosellinia corticium* [29], chromenone derivatives from *Streptomyces* sp. [30], aplysinopsins from *Aplysinopsis* sp. [31], piloquinones from *Streptomyces* sp. [32], and anithiactins from *Streptomyces* sp. [33]. Especially, we have focused on marine natural inhibitors [34]. In this study, we isolated and identified one compound from a marine-derived *Acremonium* sp. CNQ-049, and investigated its MAOs, ChEs, and BACE1 inhibitory activities, including evaluation of its dual-functional inhibition.

Materials and Methods

General experimental

Low-resolution LC/MS measurements were performed using the Agilent Technologies 1260 quadrupole (Agilent Technologies, Santa Clara, CA, USA) and Waters Micromass-ZQ 2000 MS system (Waters Corp, Milford, MA, USA) using a reversed-phase column (Phenomenex Luna C-18 (2), 50×4.6 mm, 5 μm, 100Å) at a flow rate of 1.0 mL/min at the National Research Facilities and Equipment Center (NanoBioEnergy Materials Center) at Ewha Womans University. ^{1}H and 2D NMR spectra were recorded at 500 MHz in CD_3OD using a solvent signal as an internal standard on Varian Inova spectrometers (Bruker, Billerica, MA, USA). ^{13}C NMR spectra were acquired at 125 MHz on the Varian Inova spectrometer. Medium-pressure liquid chromatography (MPLC) was performed using a Biotage Isolera One System (SE-751 03 Uppsala, Sweden) equipped with a Biotage SNAP KP-Sil column, by a step gradient solvent of dichloromethane (DCM) and methanol (MeOH). The fractions obtained from MPLC were subsequently purified by high-performance liquid chromatography (HPLC) using a reversed-phase Phenomenex Luna column (C-18 (2), 250×10 mm, 5 μm, 100Å).

Fermentation, extraction, and isolation

The strain CNQ-049 was cultured in 80 L of 2.5 L Ultra Yield Flasks, with each flask containing 1 L of SYP SW medium (10 g/L of soluble starch, 2 g/L of yeast extract, 4 g/L of peptone, and 139 g/L of sea salt in 1 L of distilled water) at 27 ºC with shaking at 120 rpm for 7 days. The culture medium was extracted with ethyl acetate (EtOAc), yielding a total of 80 L of extract, which was concentrated in a rotary vacuum evaporator to yield 5 g of crude extract. The crude extract was subjected to separation on a silica gel MPLC column (Biotage® SNAP Cartridge, KP-SIL) using a step gradient of 0 to 100% MeOH in DCM, resulting in the isolation of ten fractions. The second fraction, Q049-2 (1.8 g), was re-separated into six subfractions by C-18 reversed-phase column chromatography using 60% aqueous acetonitrile (CH_3CN). The fifth subfraction, Q049-2-E (179 mg), was further purified by reversed-phase HPLC (Phenomenex Luna C-18 (2), 250×100 mm, 2.0 mL/min, 5 μm, 100Å, UV=210 nm) using 75% aqueous CH_3CN, yielding 1.5 mg of compound **1**.

Compound **1**: ^{1}H (500 MHz, CD_3OD); d_H 8.33 (dd, J =8.1, 1.8 Hz, 1H), 7.86 (m, 1H), 7.68 (m, 1H), 7.62-7.53 (m, 4H), 7.42-7.40 (m, 2H), 7.17-7.15 (m, 3H), 6.84-6.82 (m, 2H), 4.20 (t, J =8.0 Hz, 2H) and 2.91 (t, J = 8.4 Hz, 2H), ^{13}C NMR (125 MHz, CD_3OD); d_C 161.8, 157.0, 146.6, 137.6, 134.51, 134.50, 129.7, 128.3, 128.2, 127.7, 127.0, 126.2, 126.1, 120.4, 47.3, and 33.7, LR-ESI-MS m/z =327.0 $[M+H]^+$.

Chemicals

AChE from *Electrophorus* (electric eel), acetylthiocholine iodide (ATCI), benzylamine, BChE from equine serum, butyrylthiocholine iodide (BTCI), BACE1 inhibitor IV, BACE1 activity detection kit (fluorescent), clorgyline, dimethyl sulfoxide (DMSO), donepezil, kynuramine, pargyline, quercetin, recombinant human MAO-A and MAO-B, safinamide, toloxatone, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphates (mono- and di-basic anhydrous) were purchased from Daejung (Siheung, Korea). DiaEasy™ dialyzer (6-8 kDa) was obtained from BioVision (St. Grove, MA, USA).

Inhibition studies of MAO-A and MAO-B

The MAO activities were determined using 0.06 mM kynuramine for MAO-A and 0.3 mM benzylamine for MAO-B as substrates [35]. The ChE activities were determined using 0.5 mM substrates (ATCI for AChE and BTCI for BChE), and 0.5 mM DTNB as a color reagent [36]. Sample absorbance was measured by continuous assay method [35,37] with slightly modification [38,39]. Inhibitions of compound were compared to the reference inhibitors of MAOs (toloxatone and clorgyline for MAO-A, safinamide and pargyline for MAO-B), ChEs (donepezil), and BACE1 (quercetin and BACE1 inhibitor IV) [35-37,40].

Enzyme kinetics

After first determination at 10 μM, the IC_{50} values of the compounds were calculated by using GraphPad Prism software 5 [41]. The selectivity index (SI) values of compounds were

calculated by (IC_{50} of MAO-A or AChE)/(IC_{50} of MAO-B or BChE) [42]. The inhibition types of compound **1** for MAO-B and BChE were determined at five different substrate concentrations [35,43], and three inhibitor concentrations of ~0.5, 1.0, and 2.0 times of its IC_{50} values [41]. The inhibition patterns and K_i values were determined by comparing the Lineweaver-Burk plots and their secondary plots, respectively [38].

Reversibility studies

Compound **1** was incubated with MAO-B or BChE at a concentration of 2.0 times IC_{50} value for 30 min before the measurement and the reversibilities were evaluated and patterns were determined by comparing undialyzed (A_U) and dialyzed (A_D) values, as previously described [35,43]. Restored enzyme activities of compounds were compared to those of the reference compounds, such as safinamide, pargyline (reversible and irreversible inhibitor of MAO-B, respectively), and donepezil (reversible inhibitor of BChE).

Results and Discussion

Identification of compound 1

Compound **1** was isolated as a white powder with a pseudo-molecular ion peak at the *m/z* =327.0 $[M+H]^+$ in LRMS spectroscopic data. The ^1H NMR spectrum of compound **1** displayed fourteen aromatic protons at d_H 8.33 (dd, J = 8.1, 1.8 Hz, 1H), 7.86 (m, 1H), 7.68 (m, 1H), 7.62-7.53 (m, 4H), 7.42-7.40 (m, 2H), 7.17-7.15 (m, 3H), and 6.84-6.82 (m, 2H), and two methylene groups at d_H 4.20 (t, J = 8.0 Hz, 2H) and 2.91 (t, J = 8.4 Hz, 2H). Moreover, the ^{13}C NMR spectrum of **1** displayed sixteen carbon signals at d_C 161.8, 157.0, 146.6, 137.6, 134.51, 134.50, 129.7, 128.3, 128.2, 127.7, 127.0, 126.2, 126.1, 120.4, 47.3, and 33.7. The spectra were provided in Supplementary (Fig. S1~S6). Finally, compound **1** was identified as 3-phenethyl-2-phenyl-quinazolin-4(3H)-one based on a comparison of its NMR data to the literature [44], as shown in Fig. 1. Compound **1** was a quinazoline derivative and various biological activities of diverse quinazoline compounds have been reported including anti-cancer, anti-inflammatory, anti-viral, and anti-bacterial activities [45]. However, little is known about their potential as Alzheimer's disease (AD) therapeutics. Thus, the bioactivity of compound **1** was investigated on the molecular targets of the neurodegenerative diseases such as MAOs, ChEs, and BACE1.

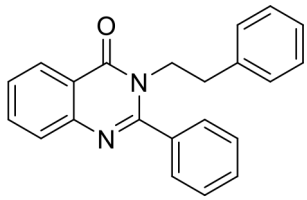

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Fig. 1 Chemical structures of 3-phenethyl-2-phenylquinazolin-4(3H)-one (**1**)

Inhibition studies of MAO-A and MAO-B

Compound **1** was analyzed for inhibitory activities against MAOs, ChEs, and BACE1. Compound **1** effectively inhibited MAO-B and BChE with the residual activities of 47.92% and 39.02%, respectively, at 10 μM. Compound **1** showed MAO-B inhibition with an IC_{50} value of 9.39 μM, and the SI value was >4.26, indicating that compound **1** was a selective MAO-B inhibitor (Table 1). On the other hand, compound **1** showed potent BChE inhibition with an IC_{50} value of 7.99 μM, and SI value was >5.01, indicating compound **1** was a selective BChE inhibitor (Table 1). These results showed that compound **1** was a dual-functional inhibitor against MAO-B and BChE. Dual-functional inhibitor is an inhibitor with two or more therapeutic effects and higher therapeutic ability can be expected than single functional inhibitor [21]. Recently, various dual-functional inhibitors have been developed and reported, such as dual-functional inhibitors for

Table 1 Inhibitions of MAOs, ChEs, and BACE1 by compound **1**[a]

Compound	Residual activity at 10 μM (%)					IC_{50} (μM)		**SI**[b]	IC_{50} (μM)		**SI**[c]
	MAO-A	MAO-B	AChE	BChE	BACE1	MAO-A	MAO-B		AChE	BChE	
1	96.04±1.40	47.92±0.56	106.49±4.59	39.02±2.68	109.36±14.46	>40	9.39±0.19	>4.26	>40	7.99±0.49	>5.01
Toloxatone						1.08±0.025					
Clorgyline						0.007±0.001					
Safinamide							0.105±0.033				
Pargyline							0.140±0.006				
Donepezil									0.001±0.002	0.180±0.004	
Quercetin					13.4±0.035[d]						
BACE1I IV*					0.440±0.064[d]						

[a]Results are the means ± standard errors of duplicate or triplicate experiments
[b]Selectivity index (SI) values are expressed for MAO-B as compared with MAO-A
[c]SI values are expressed for BChE as compared with AChE
[d]These are IC_{50} values of BACE1 reference compounds
*BACE1 inhibitor IV

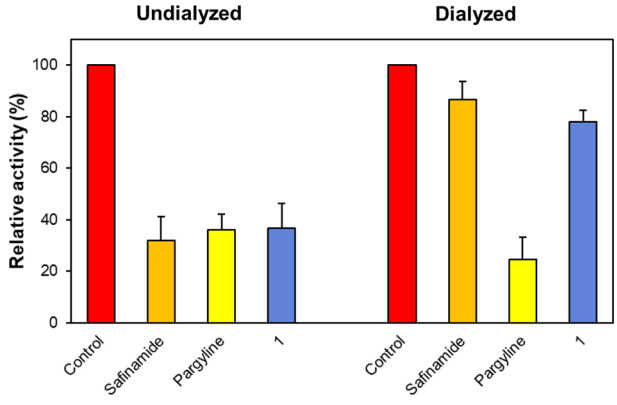

Fig. 2 Recovery of MAO-B inhibition by compound **1** using dialysis experiments. The concentration of inhibitor was used at ~2×IC_{50}. Enzyme was preincubated with inhibitor for 30 min before the measurement and residual activity was measured after dialysis

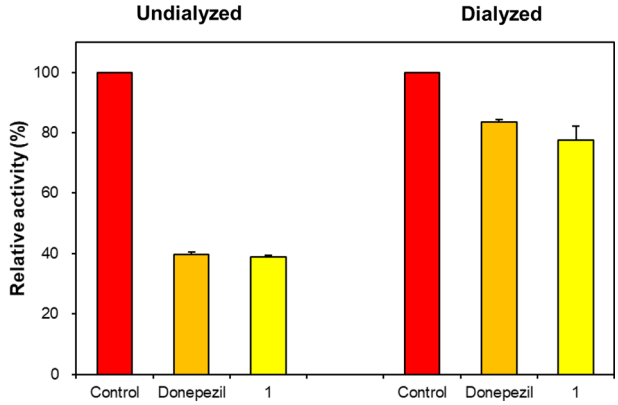

Fig. 3 Recovery of BChE inhibition by compound **1** using dialysis experiments. The experiment was performed as mentioned in Fig. 2, except BChE instead of MAO-B

MAO, ChE, BACE1, antioxidant, and carbonic anhydrase VII [46-51]. However, compound **1** showed weak inhibitory activity against BACE1 as well as MAO-A and AChE. Compared with other natural inhibitors, MAO-B inhibitory activity of compound **1** was similar or higher than 5MM (IC_{50}=9.15 μM) [29], alternariol (AT, IC_{50}=20.7 μM) [28], glycyrol (GC, IC_{50}=29.48 μM) [40] and chromenone derivative **1** (IC_{50}=27.0 μM) [30], but lower than chromenone derivative **2** (IC_{50}=3.42 μM) [30], and liquiritigenin (IC_{50}=0.098 μM) [40]. In addition, BChE inhibitory activity of compound **1** was slightly lower than GC (IC_{50}=7.22 μM) [40].

Reversibility studies

The reversibility tests of compound **1** for MAO-B and BChE were performed by the dialysis method with slight modification [37,52]. In this study, concentrations of compound **1** were ~2.0-times of its IC_{50} concentrations, i.e., 20 μM for MAO-B and 16 μM for BChE. The recovery patterns were compared using the activities of A_U and A_D relative activities. MAO-B inhibition by compound **1** was recovered from 36.79 to 77.93% (Fig. 2). This recovery value of the compound **1** was similar to that of safinamide (from 31.89 to 86.70%), a reversible MAO-B inhibitor, and it can be distinguished from that of pargyline (from 36.11 to 24.42%), an irreversible MAO-B inhibitor. On the other hand, BChE inhibition by compound **1** was recovered from 38.79 to 77.66% (Fig. 3). The recovery value of compound **1** was similar to that of donepezil, a reversible inhibitor of BChE (from 39.72 to 83.51%). These results indicated that compound **1** was a reversible inhibitor of MAO-B and BChE.

Enzyme kinetics

Enzyme kinetics of MAO-B and BChE were analyzed at five substrate concentrations (benzylamine and BTCI, respectively) and at three inhibitor concentrations. In Lineweaver-Burk plot, compound **1** appeared to be a competitive MAO-B inhibitor (Fig. 4A), and secondary plot showed that the K_i value was 5.22 ± 1.73 μM (Fig. 4B). On the other hand, compound **1** showed a mixed-type BChE inhibition (Fig. 5A), and secondary plot showed that the K_i value was 3.00±1.81 μM (Fig. 5B). In previous studies, most of MAO inhibitors were reported as competitive inhibitors [27-30,32,33], and ChE inhibitors were reported as mixed-type inhibitors [22-24]. These results suggested that compound **1** was a competitive MAO-B inhibitor and a mixed-type BChE inhibitor. In this study, compound **1** was isolated from a marine-derived *Acremonium* sp. CNQ-049. Compound **1** showed effective MAO-B and BChE inhibitions with reversible competitive and mix-type

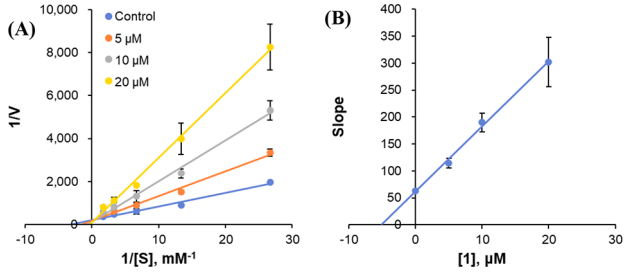

Fig. 4 Lineweaver–Burk plots for MAO-B inhibition by compound **1** (**A**), and their respective secondary plots (**B**) of the slopes vs. inhibitor concentrations

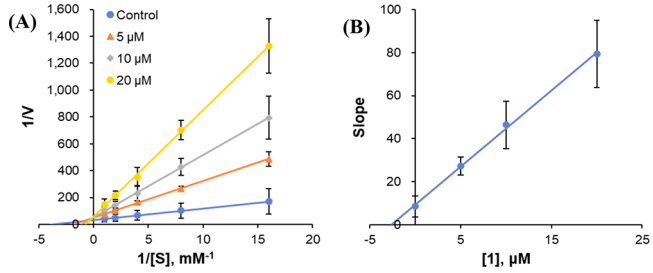

Fig. 5 Lineweaver–Burk plots for BChE inhibition by compound **1** (**A**), and their respective secondary plots (**B**) of the slopes vs. inhibitor concentrations

patterns, respectively. These results suggest that compound **1** is a potential dual-target inhibitor and can be used as a natural candidate for neurodegenerative disease treatment.

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Supplementary material Supplementary Figures S1 to S6: ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, and LRMS spectra of the compound **1**.

Declaration of Competing Interest The authors declare no competing financial interest.

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