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Moracin derivatives from *Morus* Radix as dual BACE1 and cholinesterase inhibitors with antioxidant and anti-glycation capacities

Su Hui Seong[a], Manh Tuan Ha[b], Byung Sun Min[b], Hyun Ah Jung[c,*], Jae Sue Choi[a,*]

[a] Department of Food and Life Science, Pukyong National University, Busan 48513, Republic of Korea
[b] College of Pharmacy, Drug Research and Development Center, Daegu Catholic University, Gyeongbuk 38430, Republic of Korea
[c] Department of Food Science and Human Nutrition, Chonbuk National University, Jeonju 54896, Republic of Korea

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ABSTRACT

Aims: Morus, a member of the family Moraceae and commonly known as the mulberry, comprises a pharmaceutically important plant group whose major constituents are the moracins. Moracin derivatives have received great attention because they exhibit a diverse range of biological functionalities. However, no studies have considered the anti-Alzheimer's disease (AD) and anti-glycation potential of moracin derivatives.

Main methods: We designed the current study to explore the anti-AD activity of moracin derivatives via in vitro inhibition of BACE1 and cholinesterase, their antioxidant activity via scavenging $ONOO^-$ and $ABTS^{\cdot+}$ radicals, and their anti-diabetic activity through inhibition of advanced glycation end-products (AGEs) formation. Moreover, to define the mechanism of action of moracin derivatives in depth, we performed in silico molecular modeling using a computer-assisted drug design and modeling program.

Key findings: Among the four *Morus*-derived moracins tested, moracin S, which has a prenyl moiety in the 2-aryl benzofuran scaffold, possessed the highest BACE1 inhibitory activity. It also, in a dose-dependent fashion, decreased $ONOO^-$-mediated bovine serum albumin (BSA) nitration and formation of AGEs and amyloid cross-β structures in the glycated BSA system, and it showed notable radical scavenging activity. In addition, enzyme kinetic and molecular docking studies demonstrated that moracin S is a potent, competitive BACE1 inhibitor that could interact with key catalytic aspartyl residues.

Significance: The prenyl moiety in the 2-aryl benzofuran structure plays a crucial role in inhibition of BACE1. These in vitro and in silico results provide valuable information for the design of anti-AD drugs.

1. Introduction

Alzheimer's disease (AD) is a serious, age-related neurodegenerative disorder with a multifactorial etiopathology in which environmental, genetic, and endogenous risk factors including protein aggregation, mitochondrial abnormalities, oxidative stress, and neuro-inflammation play important roles [1]. AD patients suffer various symptoms, such as memory loss, severe cognitive decline, and inappropriate emotional behavior. Despite the number of studies of AD, its pathogenetic mechanisms remain unclear. Proposed mechanisms include the cholinergic hypothesis and the β-amyloid cascade hypothesis [2,3]. In recent years, β and γ-secretases have been proposed as novel targets for treatment of AD. β-Site amyloid-beta-protein precursor (APP) cleaving enzyme 1 (BACE1) is the first protease in the pathway for generation of the Aβ peptide [4]. BACE1 accumulation was observed in the brains of AD patients and APP transgenic mice [5]. Interestingly, it was reported that oxidative stress can affect the expression of BACE1 [6]. Oxidative stress is associated with age-related disorders, including AD. 3-Nitrotyrosine is a stable indicator of oxidative damage to cellular proteins, and increased expression of 3-nitrotyrosine has been demonstrated in the brains of AD patients [7,8].

Advanced glycation end-products (AGEs) are glycated proteins or lipids generated by non-enzymatic reactions between monosaccharides (e.g., glucose, fructose, trioses) and the N-terminal amine residues of a protein. The accumulation of AGEs in the brain is a feature of aging and is especially prevalent in AD. AGE formation is irreversible and causes protease-resistant cross-linking of peptides, leading to amyloidosis [9].

White mulberry (*Morus alba* L.), which belongs to the Moraceae family, is widely distributed in many Asian countries, such as Korea, China, Vietnam, and Japan [10]. All the parts of this plant have been used as silkworm feed, foodstuffs, or traditional medicines [11]. Various types of compounds, including terpenoids, alkaloids, chalcones,

* Corresponding authors.
E-mail addresses: jungha@jbnu.ac.kr (H.A. Jung), choijs@pknu.ac.kr (J.S. Choi).

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(prenyl) flavonoids, anthocyanins, (poly)phenolic compounds, stilbenoids, coumarins, and Diels-Alder type adducts have been isolated from *M. alba* [10,12].

Moracins, which possess 2-aryl benzofuran scaffolds, are found in a wide range of plants and are especially common in *Morus* species [13]. Many moracin derivatives (moracin A to Z) have been described together with their biological activities, including anti-inflammatory [14], anti-cancer [15], anti-microbial [16], anti-tyrosinase [17], anti-phosphodiesterase [18], neuroprotective [19], and antioxidant activities [20]. Recently, the cholinesterase (ChE) inhibitory activities of 2-aryl benzofuran derivatives were reported [1,21]. In addition, moracin M isolated from *Smilax china* exhibited BACE1 inhibition in vitro [22]. To date, however, no one has reported BACE1 inhibitory activity of other moracins nor has anyone considered the ChE and AGE inhibitory activity of moracins.

In this study, we investigated the anti-AD activities of moracins isolated from *M. alba* along with their antioxidant and anti-glycation activity. In addition, we conducted structure-activity relationship (SAR) studies to determine the importance of the functional moiety in the 2-aryl benzofuran scaffolds (through a molecular docking simulation) and to predict the pharmacokinetic parameters of moracins.

2. Materials and methods

2.1. Chemicals and reagents

Electric-eel AChE (EC 3.1.1.7), acetylthiocholine iodide (ATCh), horse-serum BChE (EC 3.1.1.8), butyrylthiocholine chloride (BTCh), 5,5′-dithiobis-(2-nitrobenzoic acid), thioflavin T, bovine serum albumin (BSA), D-(−)-fructose, D-(+)-glucose, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), aminoguanidine hydrochloride, L-penicillamine, trolox, berberine chloride, and quercetin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Peroxynitrite was purchased from Cayman Chemical (Ann Arbor, MI, USA). Monoclonal anti-3-nitrotyrosine antibody (sc-32,757) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (sc-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The BACE1 kit was purchased from PanVera Corp (Madison, WI, USA).

2.2. Isolation of moracins from M. alba

A methanol extract (1.2 kg) of *M. alba* root bark was suspended in distilled water and successively partitioned with *n*-hexane, dichloromethane, and ethyl acetate. The ethyl acetate fraction (304.3 g) was fractionated by open column chromatography (CC) with silica gel eluted by a dichloromethane: methanol solvent system (1:0 to 0:1 gradient) and yielded 20 sub-fractions (E1–E20). Sub-fraction E2 (6.3 g) was separated on silica gel CC eluted with dichloromethane: acetone (15:1, v/v) to yield 11 sub-fractions (E2.1–E2.11). Sub-fraction E2.3 (356.5 mg) was chromatographed on a reversed-phase C_{18} silica gel column using a methanol-water solvent system (3:1, v/v) to yield compound **1** (7.0 mg), compound **2** (5.0 mg), and compound **3** (6.4 mg). In addition, sub-fraction E2.6 (600.5 mg) was chromatographed on a reversed-phase C_{18} silica gel column using a methanol-water solvent system (3:1, v/v) to yield compound **4** (10.5 mg). In comparison with previously reported data, isolated compounds **1–4** were identified as moracin M [23], moracin P, moracin O [24], and moracin S [20], respectively, by spectroscopic analyses, including ^{1}H and ^{13}C NMR. The chemical structures of the moracins are described in Fig. 1.

2.3. In vitro cholinesterase enzyme assay

The inhibitory activities of the four moracin derivatives in the concentration range 4–100 μM were measured against electric-eel AChE and horse-serum BChE using the modified spectrophotometric method developed by Ellman et al. [25]. ATCh and BTCh were used as substrates to assay the inhibition of AChE and BChE, respectively. The 20 μL of moracins or positive control (berberine chloride) were added to 160 μL of 100 mM sodium phosphate buffer (pH 8.0) which contained 0.045 U/mL enzyme. After 15 min at 25 °C, the reactions were initiated by addition of 10 μL of 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 10 μL of 0.6 mM substrate (ATCh or BTCh). After 5 min, the hydrolysis of substrate was monitored by observing the formation of the 5-thio-2-nitrobenzoate anion at 412 nm using a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). The tested concentration range was 5–100 μM for moracins and 0.16–50 μM for berberine chloride, respectively.

2.4. In vitro BACE1 enzyme assay

Assays were conducted using the BACE1 FRET assay kit (Pan Vera Corp.). Equal volume of 50 mM sodium acetate (pH 4.5), 1.0 U/mL enzyme, 750 nM substrate, and various concentrations of samples or positive control (quercetin) were mixed and incubated for 1 h at 25 °C. The reaction was terminated by adding 2.5 M sodium acetate. The fluorescence was read using a GEMINI XPS microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission setting of 545 and 585 nm, respectively. The tested concentration range was 1–50 μM for the four moracin derivatives and 2–10 μM for quercetin.

2.5. Kinetic parameters of BACE1 inhibition

Kinetic studies on the mechanisms of BACE1 inhibition by moracin S were conducted as described by Islam et al. [26]. The test concentrations used for the kinetic study were 0, 5, 10, and 25 μM for moracin M and 0, 1, 2.5, and 5 μM for moracin S. The equation for the Lineweaver-Burk model is as follows:

$$1/V = K_m/V_{max}(1 + [I]/K_i)1/[S] + 1/V_{max}(1 + [I]/\alpha K_i).$$

A secondary plot can be constructed from:

$$K_{m,app}/V_{max,app} = K_m[I]/K_i + K_m$$

$$1/V_{max,app} = 1/V_{max} + [I]/\alpha K_i V_{max}.$$

Here, [S] and [I] denote the concentrations of the Rh-EVNLDAEFK-Quencher and the moracins, respectively. Kinetic plots were drawn using SigmaPlot program (v12.0, Systat Software Inc., San Jose, CA).

2.6. Molecular docking simulations

Molecular docking simulations were carried out using AutoDock 4.2 [27]. X-ray crystallographic structures of closed form (1W51) and apo-form BACE1 (1W50) were obtained from the protein data bank (PDB) at a resolution of 2.55 and 1.50 Å, respectively [28]. Water molecules were removed using Discovery Studio (v16.1, Accelrys, San Diego, CA, USA). The 3D structures of moracin M, moracin S, and 3,5,7,3′,4′-pentamethoxyflavone (PMF) were obtained from the PubChem Compound (NCBI) database, with CIDs of 185848, 42605184, and 97332, respectively. AutoDock 4.2 was used for docking simulations, and grid maps were generated using the Autogrid program. The docking protocol for rigid and flexible ligand docking comprised 10 independent genetic algorithms. The reported allosteric inhibitor PMF was used to compare interaction residues and dispositions [29]. Docking results were visualized and analyzed using PyMOL (v1.7.4, Schrödinger, LLC, Cambridge, MA) and Discovery Studio.