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RESEARCH ARTICLE

Acetylcholinesterase and carbonic anhydrase isoenzymes I and II inhibition profiles of taxifolin

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Abstract

Taxifolin, also known as dihydroquercetin, is a flavonoid commonly found in plants. Carbonic anhydrase (CA, EC 4.2.1.1) plays an important role in many critical physiological events including carbon dioxide (CO_2)/bicarbonate (HCO_3^-) respiration and pH regulation. There are 16 known CA isoforms in humans, of which human hCA isoenzymes I and II (hCA I and II) are ubiquitous cytosolic isoforms. In this study, the inhibition properties of taxifolin against the slow cytosolic isoenzyme hCA I, and the ubiquitous and dominant rapid cytosolic isoenzyme hCA II were studied. Taxifolin, as a naturally bioactive flavonoid, has a K_i of 29.2 nM against hCA I, and 24.2 nM against hCA II. For acetylcholinesterase enzyme (AChE) inhibition, K_i parameter of taxifolin was determined to be 16.7 nM. These results clearly show that taxifolin inhibited both CA isoenzymes and AChE at the nM levels.

Keywords

Acetylcholinesterase, carbonic anhydrase, enzyme inhibition, enzyme purification, taxifolin

History

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Introduction

In recent years, natural flavonoids have attracted significant interests in the scientific arena because of their versatility of uses and health-promoting effects. Flavonoids are important compounds that can be found in many plants, including edible fruits and vegetables. Flavonoids act as efficient antioxidants because of their ability to chelate transition metal ions, to scavenge free radicals and to interact with enzymes[1–3]. It was reported that *in vivo* and *in vitro* studies of flavonoids showed that they have a positive impact on many important diseases, such as those affecting cardiovascular system[1,3].

Phenolic compounds contain at last a hydroxyl group (–OH) bonded to aromatic ring and are mildly acidic[4–11]. Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule. This chemical class in terms of biological and pharmaceutical is very reactive[12–24].

Taxifolin (dihydroquercetin) is a representative flavonoid compound. It is present in the plants from the *Pinus genus* and it is also found in citrus fruits and milk thistle seeds[25]. Taxifolin has many a large area of biological effects, including anti-inflammatory, hepatoprotective effects and antitumor effects[26]. More important, taxifolin produces effective antioxidant effects, which contribute to its cardiovascular protective effects. Due to the reducing properties of its hydroxyl groups, taxifolin exhibits antioxidant activity and reacts with free radicals[3].

Researchers have demonstrated that taxifolin decreased the production of lipid radicals in a concentration-dependent manner and reduced the peroxidase activity of the complex of cytochrome c combined with dioleoyl cardiolipin, which is critical for the onset of apoptosis. Taxifolin also ameliorated cerebral ischemia-reperfusion injury by inhibiting oxidative enzymes and reducing the overproduction of ROS[27,28]. Taxifolin inhibited recombinant human aldose reductase and the accumulation of sorbitol in human erythrocyte in diabetes. Taxifolin also retained the clarity of rat lenses incubated with glucose, suggesting that taxifolin might be effective in preventing osmotic stress in hyperglycemia[29]. However, there has been little work on possible beneficial effects of taxifolin for diabetic cardiomyopathy. Caspase enzymes are important factors for modulating the apoptotic cascade. These results have demonstrated that caspase-3 and caspase-9 activities were significantly inhibited by taxifolin[30]. Additionally, it was shown that taxifolin decreases the angiotensin-converting enzyme activity in the aorta of aging rats[31]. Studies have shown the modulatory effects of flavonoids upon multiple enzymes involved in xenobiotic metabolism such as on various cytochrome P450 monooxygenase isoforms and phase II conjugation enzymes and on membrane transport systems including in drug excretion[32–35].

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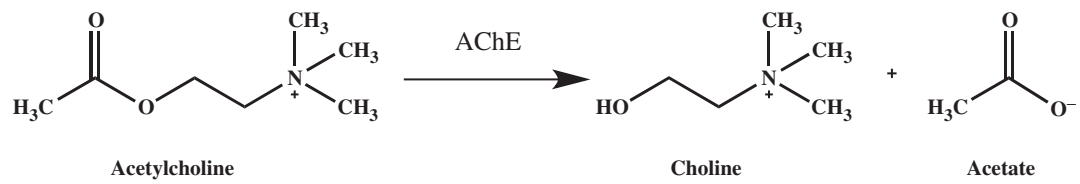
Carbonic anhydrase enzymes (CAs) are ubiquitous in all the living organisms. They have crucial physiological and pathological roles such as in fluid balance, calcification, pH regulation, carboxylation reactions, tumorigenicity, bone resorption, the synthesis of HCO_3^- and in many other pathophysiological processes[36–39]. The CA catalyzes the reversible hydration of carbon dioxide (CO_2) and water (H_2O) to bicarbonate (HCO_3^-) and a proton (H^+)[40–46].

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+$$

An enzyme inhibitor is a molecule that engages to an enzyme and decreases its activity. An inhibitor can prevent a substrate from binding the active site of the enzyme, thus hindering catalysis. It is well known that CA inhibitors (CAIs) bind to a catalytic zinc ion (Zn^{2+}) in the active site of CA isoenzymes and block their activity[47–52]. The clinical use of CAIs had been established as antiglaucoma, and as anticonvulsant agents[48], diuretics[42] and antiobesity drugs[53–55] in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders or osteoporosis[47]. Additionally, CAIs have recently been used as hypoxic tumors management agent[56,57]. The first clinically used heterocyclic and aromatic sulfonamides were clinically used derivatives of acetazolamide (AZA), a known CAI[58,59]. AZA is an inhibitor of CA and used for glaucoma, idiopathic intracranial hypertension, epilepsy and altitude sickness. To regenerate the basic form of CA isoenzyme, a H^+ is transferred from the active site to the solvent. This H^+ transportation may be supported by active site residues or by present buffers in the reaction medium. The fourth position is occupied by H_2O at an acidic pH and is catalytically inactive. At higher pH, a H_2O molecule binds to Zn^{2+} within the CA active site. Then, this proton transfer reaction transfers a H^+ to the solvent, leaving an –OH group[47,49,50,52,57].

Alzheimer's disease (AD) is a neurological disorder in which the patient suffers from memory loss and impaired cognitive abilities. AD is a chronic neurological disorder in which the patient suffers from loss impaired cognitive abilities, deficits in activities of daily living and behavioral disturbances[37,60]. According to the cholinergic hypothesis, memory impairment in patients suffering from AD results from decreased levels of the neurotransmitter acetylcholine (ACh) in the cortex[39]. Acetylcholinesterase (AChE, EC: 3.1.1.7) is a hydrolase that plays a key role in cholinergic transmission through catalyzing the rapid hydrolysis of the neurotransmitter ACh[61]. AChE is a special carboxylic ester hydrolase that hydrolyses the esters of choline[39,60] to produce acetic acid and choline[62].



AChE is found in high concentrations in the brain and in erythrocytes[63]. AChE is a necessary enzyme for the nervous system. AChE inhibitors (AChEI) are used in the treatment of several neuromuscular diseases, and were studied for treatment of AD[39,61,64]. The use of AChEI to block the cholinergic degradation of ACh is therefore considered to be a promising approach for the treatment of AD. Natural products might slow the progression of AD by simultaneously protecting neurons from oxidative stress and by acting as an AChEI[65]. Certain organophosphorus and carbamate derivatives are known to be the best inhibitors of AChE catalytic activity[66]. Carbamate pesticides show their toxicity by irreversibly modifying the catalytic serine residue of AChE, and the inhibiting the AChE[67]. Recently, several studies supported that many active compounds from plant origins, including anthocyanins and terpenoids have AChE inhibition activity[68,69]. Additionally, some studies demonstrated that grape and blueberry anthocyanin[70,71], grape skin anthocyanin[72] lycodine-type alkaloids from *Lycopodiastrum casuarinoides*[73] have neuroprotective roles.

In this study, we identify the potential inhibition profile and associated mechanisms of taxifolin for human CA isoenzymes I and II (hCA I and II) and for AChE which are widely used enzyme in pharmacological industry.

Experimental

Purification of CA isoenzymes

Both hCA isoenzymes were purified by sepharose-4B-L-tyrosine-sulphanilamide affinity chromatography[74–77]. For this purpose, the lysate was adjusted with Tris buffer to pH 8.7 and applied to the affinity column. Then, protein content in the eluates was recorded spectrophotometrically at wavelength of 280 nm. SDS–PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was performed after purification of the enzymes. The isoenzymes purities were controlled by SDS–PAGE, and a single band was found for each CA isoenzyme[78]. SDS–PAGE method has been detailed described previously[79] and was performed using acrylamide in the running (10%) and the stacking gel (3%), with 0.1% SDS[80,81].

Determination of CA isoenzymes activities

Both CA isoenzyme activities were performed according to the procedure of Verpoorte et al.[82] and as described previously by our group[81,83]. The absorbance change was spectrophotometrically reordered at 348 nm during 3 min at room temperature (25 °C). One unit of enzyme activity is expressed as 1 mol/L of released *p*-nitrophenol per minute at 25 °C[84]. The protein quantity was spectrophotometrically determined at 595 nm during purification steps by the Bradford method[85]. Bovine serum albumin was used as the standard protein[86,87].

CA isoenzymes inhibition assay

The inhibition property of taxifolin against both CA isoenzymes was determined by hydrolysis of *p*-nitrophenylacetate to *p*-nitrophenol. The later molecule can be monitored spectrophotometrically[49]. The CO_2 hydration reaction catalyzed by CA was first observed in the absence of taxifolin; the resulting rates were measured and used as a control for the CA isoenzymes. Also, the same reaction was measured in the presence of taxifolin. The percent inhibition was determined with $(\%) = [100 - (A_S/A_C) \times 100]$, where A_S is the absorbance of the sample containing taxifolin and A_C is the absorbance of the control sample. The activity (%)–[taxifolin] graphs were drawn and the half maximal inhibitory concentration (IC_{50}) values of taxifolin demonstrated >50% inhibition of CA isoenzymes were calculated after

suitable dilutions. K_i values for taxifolin were determined for both isoenzymes. For this purpose to determine the K_i values, taxifolin was tested at three different concentrations. K_i is the binding affinity constant of the taxifolin to CA isoenzymes. NPA was used as the substrate at five different concentrations, and Lineweaver–Burk curves were drawn[88] in detail as described previously[89–92].

Acetylcholinesterase inhibition assay

Acetylcholinesterase enzyme inhibition assay was determined on commercially available purified AChE (Product no: C3389-Sigma–Aldrich, St. Louis, MO) from electric gel (*Electrophorus electricus*) based on the method of Ellman procedure[93]. Acetyl thiocholine iodide (ATCI) was used as the substrate. Also, 5,5-dithiobis(2-nitrobenzoic) acid (DTNB) was used for the monitoring of AChE activity. Briefly, 150 µl of sodium phosphate buffer (0.1 M, pH 8.0), 10 µl test compound solution and 20 µl of enzyme solution (0.09 units/ml) were mixed and incubated for 15 min at room temperature. Then, 10 µl of DTNB (10 mM) was transferred and the reaction was initiated by the addition of substrate solution (10 µl of ATCI, 14 mM solution). The hydrolysis of the ATCI was measured by the formation of the product, 5-thio-2-nitrobenzoate, which is released by AChE hydrolysis. Absorbance of final solution was spectrophotometrically measured at 412 nm (Beckhman Coulter DU 730) after 10-min incubation. Tacrine, a standard AChE inhibitor, was used as a positive control. The percent of AChE inhibition was calculated as follows:

$$\text{Inhibition } (\%) = 100 - [A_S/A_C] \times 100$$

where A_S is the absorbance of the sample containing taxifolin and A_C is the absorbance of the control sample.

Results and discussion

Carbonic anhydrases catalysis the crucial pathophysiological processes that are connected with CO_2/HCO_3^- transport and homeostasis, biosynthetic reactions including gluconeogenesis, ureagenesis and lipogenesis, respiration, calcification, tumorigenicity, electrolyte secretion in a variety of tissues and organs, and bone resorption[94]. Phenolic compounds are a class of chemicals containing of a –OH bonded directly to an aromatic hydrocarbon group and are categorized either as simple phenols or as polyphenols depending on the number of phenol units in the molecule[24]. Recently, a lot of phenolic acid, phenols and phenolic derivatives were investigated in detail as inhibitors of the Zn^{2+}-containing CA isoenzymes by our group[95–99]. It was reported all CA isoforms are inhibited by three different mechanisms: (i) inhibition by coordination of the inhibitor to the Zn^{2+} located in the active site of CA isoenzymes, thereby replacing the Zn^{2+}-bound H_2O/–OH, which leads to a tetrahedral geometry for Zn^{2+}. This geometry can also arise by the addition of an inhibitor to the metal coordination sphere when the Zn^{2+} has trigonal bipyramidal geometry[1]. (ii) Inhibition by anchoring of the inhibitor to the Zn^{2+}-bound solvent molecule, i.e. an H_2O/–OH. Phenolic compounds and polyamine molecules can bind to CA in this way, as shown schematically for phenol or (iii) inhibition by inhibitor occlusion of the active site or activator-binding site of CA[100–102]. Thus, by binding in a non-classical way to CAs, phenols and their derivatives provide interesting leads for identifying novel types of CAIs. Taxifolin has two classical structural characteristics in one molecule: phenols and ketone.

It has been reported that phenols act as an inhibitors of the Zn^{2+}-containing CA isoenzymes[103]. Phenol binds to CA in a diverse manner when compared to the classic sulfonamide inhibitors. Sulfonamides coordinate to the Zn^{2+} ion in the CA active site by replacing the fourth non-protein ligand, which is typically an H_2O molecule or a –OH ion. By binding in a non-classical way to CAs, phenols and their derivatives constitute interesting leads for identifying novel types of CAIs[94,100,102]. In the present study, we report the inhibition profiles of taxifolin against the slower cytosolic isoform, hCA I and the more rapid isoenzyme hCA II. Taxifolin showed effective inhibition of both isoforms (Table 1).

To describe inhibitory effects, researchers often list an IC_{50} value; however, a more suitable measure is the K_i constant. K_i values were calculated from Lineweaver–Burk graphs (Figures 1, 2 and 3), and both the K_i and IC_{50} parameters of the taxifolin were determined in this study. The Lineweaver–Burk plot is a graphical representation of the Lineweaver–Burk equation of enzyme kinetics[104]. The plot provides a useful graphical method for analysis of the Michaelis–Menten equation:

$$V = V_{max} + \frac{[S]}{K_m + [S]}$$

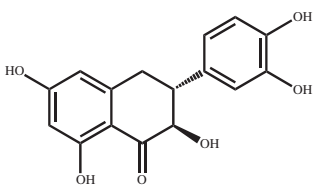
Taking the reciprocal gives

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

where V is the reaction velocity, K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity and [S] is the substrate concentration.

As shown in Table 1 and Figure 1, the IC_{50} and K_i values were found for taxifolin against both CA isoenzymes. For the cytosolic isoenzyme hCA I, taxifolin had an IC_{50} value of 27.72 nM and a K_i value of 29.20 ± 2.85 nM (Table 1). On the other hand, AZA is a hCA I that is used for the medical treatment of glaucoma, altitude sickness, epileptic seizure, idiopathic intracranial hypertension, central sleep apnoea, cystinuria, periodic paralysis and dural ectasia. AZA was extensively used as positive control for both CA isoenzymes. In the present study, AZA demonstrated IC_{50} values of 315.52 and 123.53 nM against hCA I and II,

Table 1. The inhibition profiles of taxifolin on purified hCA I and II from human erythrocytes by sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography and inhibition of AChE purified from electric gel (*Electrophorus electricus*).

Taxifolin	Kinetic parameters	hCA I	hCA II	AChE
	IC_{50} (nM)	27.7	43.3	30.1
	R^2	0.9858	0.9682	0.9723
	K_i (nM)	29.2 ± 2.9	24.2 ± 6.5	16.7 ± 3.9
	Inhibition type	Non-competitive	Non-competitive	Non-competitive

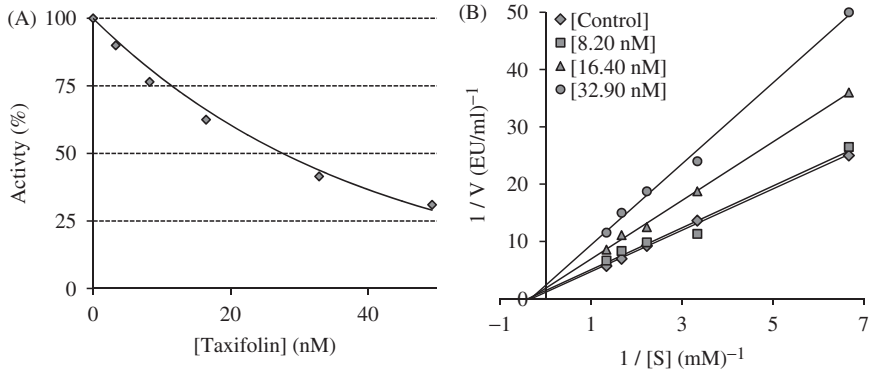


Figure 1. Determination of the half maximal inhibitory concentration (IC_{50}) (A) and inhibition constant (K_i) values (B) of taxifolin for human erythrocyte carbonic anhydrase I isoenzyme (hCA I) by using a Lineweaver–Burk graph.

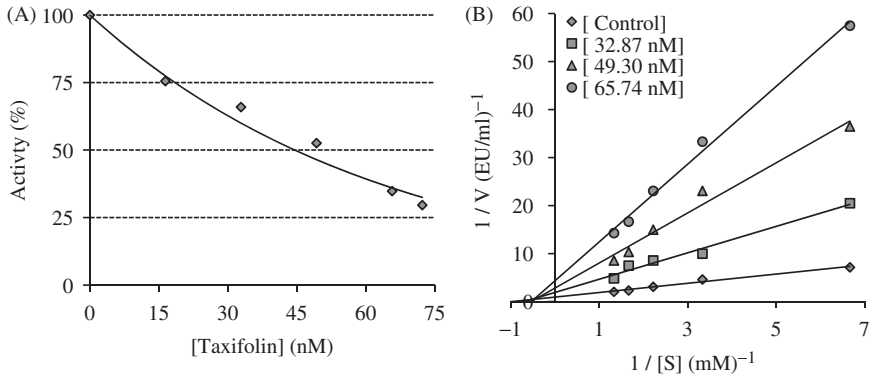


Figure 2. Determination of the half maximal inhibitory concentration (IC_{50}) (A) and inhibition constant (K_i) values (B) of taxifolin for human erythrocyte carbonic anhydrase II isoenzyme (hCA II) by using a Lineweaver–Burk graph.

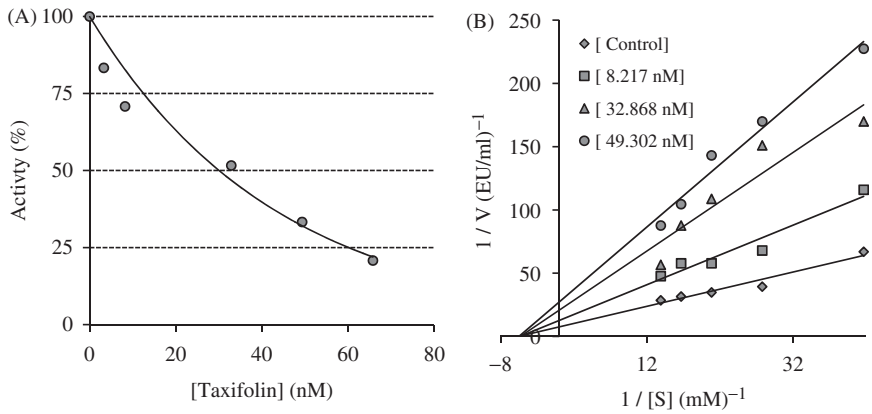


Figure 3. Determination of the half maximal inhibitory concentration (IC_{50}) value (A) and inhibition constant (K_i) value (B) of taxifolin for acetylcholinesterase enzyme (AChE) by using a Lineweaver–Burk graph.

respectively. On the other hand, its K_i values for both isoenzymes were found as 184.34 and 61.12 nM, respectively. These results clearly show that taxifolin had more CA isoenzyme inhibitor effects than that of AZA.

As seen in Figure 2, for the physiologically predominant CA II, taxifolin had an IC_{50} value of 43.31 nM and a K_i value of 24.15 ± 6.45 nM. Many studies have shown that the inhibition of CA II is brought about by an inhibitor's ability to bind to the catalytic Zn^{2+} in the CA active site and mimic the tetrahedral transition state[47,49,50].

There are important differences in inhibition between the two isoenzymes. The main difference is found in the active site architectures of the two hCA isoenzymes and is due to the presence of more histidine residues in the CA I isoform[47]. In addition to the Zn^{2+} binding ligands (His 94, His 96 and His 119) discussed in the introduction, the His 64 residue of CA I play an important role in catalysis. Another difference between the two isozymes is that CA II contains a histidine cluster consisting of the following residues: His 64, His 4, His 3, His 10, His 15 and His 17 which are absent in CA I. Hence, these two isozymes exhibit different affinities for the inhibitors. In general, CA II has a higher affinity for the inhibitor than CA I[47].

hCA I is highly abundant in red blood cells and is found in many tissues but its precise physiological function is unknown. CA I is associated with cerebral and retinal edema; thus, the inhibition of CA I may be a valuable tool for fighting these conditions. The physiologically predominant cytosolic isoform hCA II is ubiquitous, and it is associated with several

diseases including epilepsy, edema, glaucoma and altitude sickness[56,57,105].

AZA is a well-known example of a clinically established CAI[106,107] and in recent years we have reported its strong inhibition of both human cytosolic CA I and II. CAI effects are also exhibited by a wide spectrum of phenolic compounds including melatonin[40], morphine[41], vitamin E[74], CAPE[84], antioxidant phenols[100], phenolic acids[98], natural product polyphenols and phenolic acids[96], natural phenolic compounds[51,92,95], antioxidant polyphenol products[92,95] (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and its derivatives[52], natural and synthetic bromophenols[57,91,108], novel sulfonamide derivatives of aminoindanes and anilines[44], novel phenolic sulfamides[108], novel phenolic benzylamine derivatives[45], sulfonamide derivatives[60] and novel sulfamide analogues of dopamine-related compounds[38], new benzotropone derivatives[50], guaiacol and catechol derivatives[55], capsaicin[109], hydroquinone[110] and brominated diphenylmethanone and its derivatives[46], novel sulfamides and sulfonamides incorporating a tetralin scaffold[39]. These extensive studies indicate the importance of CA I and II isoenzyme inhibitors.

AChE was very strongly inhibited by taxifolin (Table 1). Taxifolin had an IC_{50} value of 30.1 nM and a K_i value of 16.7 ± 3.9 nM (Figure 3). On the other hand, donepezil hydrochloride, which is used to the treatment of mild-to-moderate AD and various other memory impairments, had been shown to lower AChE inhibition activity (IC_{50}: 55 nM)[106]. Donepezil hydrochloride contains *N*-benzylpiperidine and an indanone moiety that shows longer and more selective action.

Conclusions

Taxifolin demonstrated unique inhibition profiles against both CA isoforms I and II. These results demonstrated that in the light of the high homology between these two CAs, they exhibit similar activity. Taxifolin was first identified as a potent CAI because phenolic compounds with aromatic rings have previously been identified as inhibitors of CA. In this study, nanomolar levels of K_i and IC_{50} values were observed for taxifolin. We show that taxifolin is a selective inhibitor for both cytosolic CA isoenzymes. These results clearly indicate the potential for use of bioactive taxifolin to identifying more CAIs and for eventually targeting additional isoforms. Additionally, taxifolin had effective AChE inhibition properties and it can be a good candidate for the treatment of mild-to-moderate AD and various other memory impairments.

Declaration of interest

The authors declare no conflict of interest. I.G. and S.H.A. would like to extend his sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP-VPP-254.

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