In vitro acetylcholinesterase inhibition by psoralen using molecular docking and enzymatic studies

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Received : 24-03-14
Review completed : 16-06-14
Accepted : 06-07-14

ABSTRACT

Introduction: Alzheimer's disease (AD) has increased at an alarming rate and is now a worldwide health problem. Inhibitors of acetylcholinesterase (AChE) leading to inhibition of acetylcholine breakdown constitute the main therapeutic strategy for AD. Psoralen was investigated as inhibitor of AChE enzyme in an attempt to explore its potential for the management of AD. **Materials and Methods:** Psoralen was isolated from powdered *Psoralea corylifolia* fruits. AChE enzyme inhibitory activity of different concentrations of psoralen was investigated by use of *in vitro* enzymatic and molecular docking studies. Further, the enzyme kinetics were studied using Lineweaver-Burk plot. **Results:** Psoralen was found to inhibit AChE enzyme activity in a concentration-dependent manner. Kinetic studies showed psoralen inhibits AChE in a competitive manner. Molecular docking study revealed that psoralen binds well within the binding site of the enzyme showing interactions such as π-π stacking and hydrogen bonding with residues present therein. **Conclusion:** The result of AChE enzyme inhibitory activity of the psoralen in this study is promising. It could be further explored as a potential candidate for further development of new drugs against AD.

KEY WORDS: Acetylcholinesterase enzyme, docking, psoralen

Alzheimer's disease (AD) is a progressive neurodegenerativedisorder, characterized initially by selective loss of cholinergic neurons in the basal forebrain, followed by cognitive and behavioral impairments that progressively disrupt activities of daily living, resulting in impaired memory and behavior, as well as loss of intellectual, social abilities, and eventually death.[1] As of 2010, there were an estimated 35.6 million people with dementia worldwide. According to AD international report, this number will nearly double every 20 years to an estimated 65.7 million in 2030 and 115.4 million in 2050.[2] As of 2011, the prevalence of the disease in India was said to be one in 20 for people over 60 years, and one in five for people over 80 years.[3] There are several strategies to ameliorate AD, although the one that has been most successful so far is the "cholinergic hypothesis". The drugs approved for the AD therapy act by counteracting the acetylcholine deficit, that is, they try to enhance the acetylcholine level in the brain. Inhibition of acetylcholinesterase (AChE) plays a key role not only in enhancing cholinergic transmission in the brain, but also

Access this article online

Quick Response Code:

Website: www.jpbsonline.org

DOI: 10.4103/0975-7406.148775

How to cite this article: Somani G, Kulkarni C, Shinde P, Shelke R, Laddha K, Sathaye S. *In vitro* acetylcholinesterase inhibition by psoralen using molecular docking and enzymatic studies. J Pharm Bioall Sci 2015;7:32-6.

in reducing the aggregation of amyloid-beta (Aβ) peptide and the formation of the neurotoxic fibrils in AD. Currently available AChE inhibitors such as tacrine, donepezil, rivastigmine, and galantamine, are found to be effective to treat mild to moderate AD only. Although around 40-70% patients benefit from AChE inhibitors,[4] the nonselectivity of these drugs, and their limited efficacy, poor bioavailability, adverse cholinergic side effects in the periphery, narrow therapeutic ranges, and hepatotoxicity are some of the severe limitations to their therapeutic success.

Naturally occurring as well as the chemically synthesized coumarin analogs exhibit potent AChE inhibitory activity.[5] Recent reports indicated that coumarins of Psoraleae Fructus including psoralen and isopsoralen alleviate scopolamine-induced amnesia in rats, but exact mechanism of action is unknown.[6] Thus, the present study was carried out in order to find exact mechanism of psoralen so that it could become a new candidate with cholinesterase inhibitor activity in the treatment of AD.

Materials and Methods

Chemicals and reagents

Sodium hydrogen phosphate, sodium dihydrogen phosphate, and dimethyl sulfoxide (DMSO) were purchased from the S.D., Fine Chemicals Ltd., Mumbai. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) reagent was purchased from Sigma, India. Acetylthiocholine iodide was purchased from Hi-Media, Mumbai. All the chemicals used for this study were of AR grade.

Animals

Adult male Wistar rats, weighing 180-250 g were used. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC/2012/ICT/P80) of Institute of Chemical Technology (ICT), Mumbai, Maharashtra, India. The animals were maintained in standard laboratory conditions with food and water *ad libitum*, under 12 h light/12 h dark cycle.

Isolation of psoralen from *Psoralea corylifolia* fruits

Psoralea fruit powder was soaked insufficient quantity of petroleum ether for 2 days. Petroleum ether fraction was filtered and discarded. Marc, remaining after petroleum ether extract was dried and extracted using chloroform as solvent. Chloroform extract obtained was concentrated and subjected to column chromatography. Silica gel column was used, and chloroform used as mobile phase. Fraction 10-40 contains psoralen. Structure was confirmed by mass and nuclear magnetic resonance (NMR) spectroscopy.

Purification of psoralen with high-performance liquid chromatography

High-performance liquid chromatography (HPLC) analysis was performed with a Jasco (Hachioji, Tokyo, Japan) system consisting of an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), manual sample injection valve (Rheodyne 7725i) equipped with a 20-μL loop, and ultraviolet (UV)-visible (VIS) detector (UV-1575). Compounds were separated on a 250 mm × 4.6 mm i.d., 5-μm particle, Hibar Li Chrocart Purospher Star RP-18 end capped column (Merck, Darmstadt, Germany) with 65:35 (%, v/v) acetonitrile: Water as isocratic mobile phase at a flow rate of 1.0 mL/min. Psoralen was dissolved in methanol. The injection volume was 20 μL, and the detection wavelength was 300 nm (appropriate to the UV absorption maxima of the compounds determined). HPLC was performed at ambient temperature and data were analyzed on a computer equipped with Jasco Borwin software version 1.5.

Experimental design

Preparation of the crude enzyme

All procedures for preparation of crude rat brain AChE enzyme were performed at 4°C. AChE enzyme was isolated as reported earlier. Briefly rat brain was homogenized in phosphate buffer pH 7.2 and then centrifuged at 10,000 rpm for 20 min. The supernatant was subjected to 75% w/v saturated ammonium sulfate solution precipitation. The precipitate was collected after 10,000 rpm centrifugation for 20 min and dissolved in phosphate buffer to obtain crude AChE enzyme. This crude enzyme was used in the study to observe inhibition by different concentrations of psoralen.

In vitro acetylcholinesterase inhibitory assay

The assay for AChE activity was performed using the colorimetric method of Ellman *et al*., with slight modifications utilizing acetylthiocholine iodide (ATCI) as a substrate and crude AChE from rat brain.[7] Briefly, in 3 mL reaction mixture, 2.6 mL of phosphate buffer, pH 7.2, 100 μL of the test samples (psoralen 25-400 μg/mL dissolved in DMSO) or rivastigmine tartarate (1.25-40 μg/mL), 100 μL of 75 mM ATCI (dissolved in buffer), and 100 μL of 10 mM 5,5'-DTNB. The reaction was then initiated by the addition of ATCI. The concentration of DMSO in final reaction mixture was <1%. The hydrolysis of ATCI was monitored by the formation of yellow 2-nitro-5-sulfidobenzene-carboxylate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine for 3 min, at a wavelength of 412 nm using a Shimadzu UV-VIS spectrophotometer. The percentage inhibition of cholinesterase activity was calculated using the following formula:

% Inhibition = (Δ Absorbance control) − (Δ Absorbance drug) / (Δ Absorbance control) * 100

Kinetic assay

The enzyme kinetics on inhibition of AChE activity by psoralen (100 and 200 μg/mL) was studied using increasing concentrations of substrate ATCI (6.25, 12.5, 25, 50,

and 75 mM). The mode of inhibition was determined by Lineweaver-Burk (1/S vs. 1/*v*) plot and kinetic parameters K_m and V_{max} were obtained by curve fitting according to the classical Michaelis-Menten equation.

Docking acetylcholinesterase

Docking study was carried out using standard glide molecular docking protocol implemented within the Maestro molecular modeling suit by Schrodinger, LLC, New York, 2008 installed on Intel Pentium 4 computer (Glide, version 5.0, Schrödinger, LLC, New York, NY, USA, 2008).

The protein structure protein data bank (PDB) code 1EVE, which is three-dimensional (3D) structure of the anti-Alzheimer drug, e2020 (aricept), complexed with its target AChE with a resolution of 2.50 Å was retrieved from Brookhaven PDB and used for the validation of the docking algorithm.[8] All water molecules were deleted, bond orders, and charges of donepezil were assigned properly in the protein preparation step. A grid which is the representation of shape and properties of the receptor using several different sets of fields was generated. The docking study was carried out using extra precision mode.

Structure of psoralen was constructed using build option within Maestro using standard geometries and standard bond lengths. With the help of Ligprep facility, appropriate hydrogens were added, and a single, low-energy, 3D conformation was generated by energy minimization using MMFFs (Schrödinger, LLC, New York, NY, USA, 2008) with a dielectric constant of 1.0. All two-dimensional images of ligand-protein interactions were generated using LIGPLOT version 4.5.3.[9]

Statistical analysis

All results are expressed as the mean ± SD. Statistical evaluation of the data was performed using ANOVA followed by Tukey's multiple comparison test for significant differences using GraphPad Prism software version 5. The minimum level of significance considered was $P < 0.05$.

Results and Discussion

Psoralen, furocoumarins isolated from the plant *Psoralea corylifolia* L. (Leguminosae), is an active component, which is used traditionally to treat symptoms of ageing. Furocoumarins were also reported to possess antiproliferative activity due to their ability to bind DNA upon ultraviolet A (UVA) light irradiation.[10] In fact, photochemotherapy with psoralen and UVA has been successfully employed in the treatment of psoriasis and other dermatological diseases.[11]

Psoralen was isolated from *P. corylifolia* fruits and showed colorless crystal having melting point in the range 165°C. Infrared spectrum of isolated compound showed peaks at 1718/cm (C = O), 1602/cm (C = C), and 1363/cm (carbonyl lactone). Mass spectra showed peak mass spectrometry *m/z* 187.8 (M^+), UV-VIS absorption in methanol was found at 302 and 248 nm. The ^{1}H NMR (300 MHz) δ 5.6 corresponding to benzofuran and 7.6 corresponding to coumarin moiety is present. Based on above chemical and spectral analysis, isolated compound was confirmed as psoralen. Percentage purity of the compound found to be 98% [Figure 1a and b]. On further investigation, psoralen displayed a significant concentration-dependent inhibition of AChE using ATCI as a substrate as shown in Figure 2. For AChE inhibition, psoralen at 400 µg/mL concentration showed almost 53% inhibitory activity with inhibitory concentration $(IC)_{50}$ value of 370 µg/mL, while standard rivastigmine at 40 µg/mL showed almost 66% inhibitory activity with IC_{50} value of 32 µg/mL [Figure 2a and b]. Psoralen activity was moderate and on a molar basis, it is approximately 25 times less potent than rivastigmine tartrate. Despite their moderate activity, these compounds could serve as leads for synthesis of potential analogs with improved inhibitory activity. Encouraging results have been obtained in the analysis of imperatorin and methoxsalen; compounds structurally similar to psoralen, for their activity against neurotoxicity and memory impairment suggest the possible investigation of psoralen as a nootropic.[12,13] Furthermore, functionalization of the aromatic center of coumarins could lead to the development of novel analogs capable of inhibiting Aβ peptide aggregation.

Recent report showed that the recognition of key structural features within coumarin template has helped in designing and synthesizing new analogs with improved AChE inhibitory activity and additional pharmacological activities including beta-secretase inhibition associated with decreased Aβ peptide deposition and monoamine oxidase (MAO) inhibition.

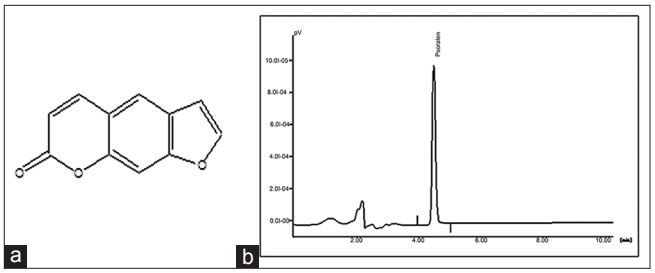

Figure 1: (a) The chemical structure and (b) High-performance liquid chromatography of psoralen

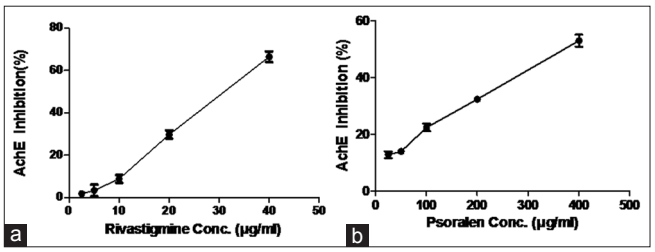

Figure 2: Anticholinesterase activity of (a) Rivastigmine tartarate inhibitory concentration $(IC)_{50}$ = 31.20 µg/mL and (b) Psoralen IC50 = 370 µg/mL. *Values are mean ± standard deviation, *n* = 3

To elucidate the mechanism of AChE inhibition by psoralen, kinetic studies of enzyme activity were performed. Double reciprocal plot of inhibition of ATCI hydrolysis is shown in Figure 3. The relationship between substrate concentration and reaction velocity was in good agreement with Michaelis–Menten equation. The kinetic studies showed that the psoralen showed increased in K_m values without much change in the maximum velocity of enzyme activity or V_{max} [Table 1]. The kinetic results demonstrated that the mechanism of AChE inhibition was of the competitive nature.

The toxicological profiles of psoralen should be considered because existing data on the toxicity of furocoumarins are inconsistent. There are reports indicating that they do not exhibit any observable toxicity upon administration to various experimental animals. On the contrary, other reports have indicated that psoralen (lethal dose 50 = 615 mg/kg), might possess genotoxicty, phototoxicity, or act as ovarian toxicants, or could inactivate cytochrome P-450 2B1 in the liver.[14,15] Furthermore, in-depth studies will be required to find *in vivo* potential of psoralen to become potential therapeutic phytoconstituents in the treatment of cognitive dysfunction. At the same time, efforts should be directed toward reducing toxicity and avoiding photosensitivity of psoralen.[16]

Some recent studies have shown an increased activity of MAO in AD patients which in-turn is correlated with the deposition of Aβ peptide plaque in different areas of the brain that may result in increased production of free radicals to contribute the neuronal damage particularly in the hippocampus. Psoralen is also reported to have *in vitro* inhibitory actions on MAO-A activities in rat brain mitochondria.[17] Whether the alteration of monoaminergic neuronal functions by the potent MAO inhibitory effects of psoralen also participates in the antiamnestic effects, requires more in-depth studies in the future.

The binding pocket of AChE is a long and narrow region which consists of two separated ligand binding sites: The catalytic (central) site and the peripheral anionic site. The catalytic site is the binding site of classical AChE inhibitors, such as tacrine and huperzine A, which has been studied thoroughly. On the contrary, the function of the peripheral site has not been elucidated clearly as yet. Recent studies have demonstrated that the peripheral site might accelerate the aggregation and deposition of Aβ peptide, which is considered as another cause of AD.[16,18] Therefore, it is supposed that an ideal AChE inhibitor should bind to the catalytic and peripheral sites simultaneously, which could disrupt the interactions between the enzyme and the Aβ peptide, and hence, slow down the progression of the disease.[19]

In order to understand the interaction of psoralen with the catalytic site of the target enzyme, molecular docking study was undertaken. It was revealed that psoralen molecule bind well within the catalytic site of the enzyme showing π–π stacking with Tyr334, hydrogen bonding with Gly119 and Gly118. Glide score of psoralen (-6.844) indicate the stability of the psoralen enzyme complex [Figure 4].

Table 1: Anticholinesterase inhibition with different concentrations of ATCI (6.25-75 mM) was incubated in the absence and presence of psoralen at two different concentrations (100 and 200 μg/mL)

Substrate	Inhibitor	K_m	V_{max}	Type of inhibition
ATCI	DMSO	6.95	0.02	
	Psoralen (100 μg/ml)	12.31	0.02	Competitive type
	Psoralen (200 μg/ml)	13.86	0.02	

ATCI: Acetylthiocholine iodide, DMSO: Dimethyl sulfoxide

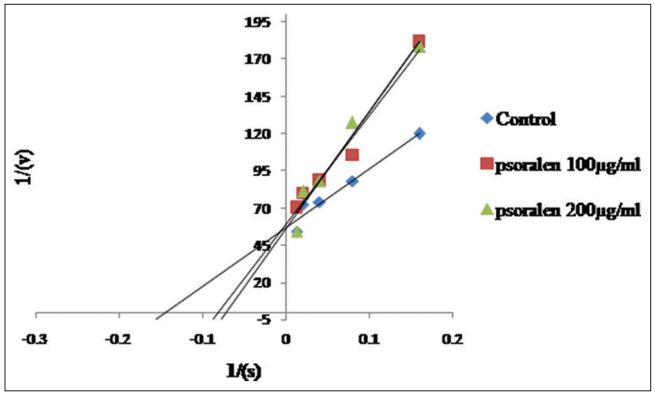

Figure 3: Lineweaver–Burk plot of anticholinesterase inhibition by different concentrations of the psoralen

Conclusion

Psoralen could be a potential candidate for inhibition of AChE. Thus, it could be further explored for its possible application in AD.

Acknowledgment

Author would like to acknowledge financial support from DPST, Mumbai to carry out the research work.

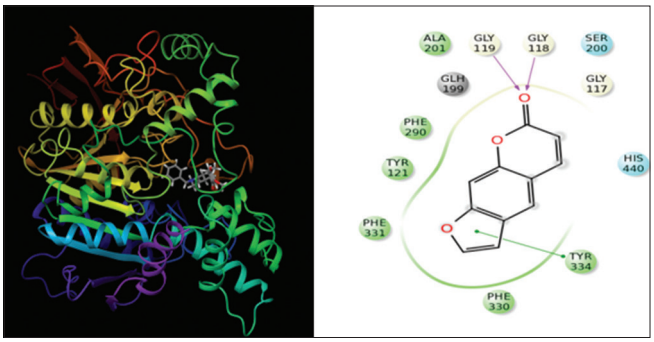

Figure 4: Ribbon structure of acetylcholinesterase (AChE) and ligand interaction architecture of AChE target protein (protein data bank accession no. 1EVE) with the superimposed docked molecule of psoralen

References

1. Zheng XY, Zhang ZJ, Chou GX, Wu T, Cheng XM, Wang CH, *et al.* Acetylcholinesterase inhibitive activity-guided isolation of two new alkaloids from seeds of *Peganum nigellastrum* Bunge by an *in vitro* TLC-bioautographic assay. Arch Pharm Res 2009;32:1245-51.
2. UK: Alzheimer Disease International: Global Knowledge Research; 2012, http://www.alz.co.uk/. Available from: http://www.alz.co.uk/research/statistics/. [Last accessed on 2013 Jun 09].
3. India: The Hindu: Online Edition of Indian National Newspaper, Alzheimer's will be on the rise in India, 2011 April 21. Available from: http://www.hindu.com/seta/2011/04/21/stories/2011042155291700.htm/. [Last accessed on 2013 Jun 09].
4. Anand P, Singh B, Singh N. A review on coumarins as acetylcholinesterase inhibitors for Alzheimer's disease. Bioorg Med Chem 2012;20:1175-80.
5. Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. Phytomedicine 2007;14:289-300.
6. Wu CR, Chang CL, Hsieh PY, Lin LW, Ching H. Psoralen and isopsoralen, two coumarins of Psoraleae Fructus, can alleviate scopolamine-induced amnesia in rats. Planta Med 2007;73:275-8.
7. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
8. Kryger G, Silman I, Sussman JL. Structure of acetylcholinesterase complexed with E2020 (Aricept): Implications for the design of new anti-Alzheimer drugs. Structure 1999;7:297-307.
9. Fadaeinasab M, Hadi AH, Kia Y, Basiri A, Murugaiyah V. Cholinesterase enzymes inhibitors from the leaves of *Rauvolfia reflexa* and their molecular docking study. Molecules 2013;18:3779-88.
10. Wang Y, Hong C, Zhou C, Xu D, Qu HB. Screening antitumor compounds psoralen and isopsoralen from *Psoralea corylifolia* L. seeds. Evid Based Complement Alternat Med 2011;2011:363052.
11. Stern RS, Nichols KT, Väkevä LH. Malignant melanoma in patients treated for psoriasis with methoxsalen (psoralen) and ultraviolet A radiation (PUVA). The PUVA Follow-Up Study. N Engl J Med 1997;336:1041-5.
12. Budzynska B, Kruk-Slomka M, Skalicka-Wozniak K, Biala G, Glowniak K. The effects of imperatorin on anxiety and memory-related behavior in male Swiss mice. Exp Clin Psychopharmacol 2012;20:325-32.
13. Kim JK, Bae H, Kim MJ, Choi SJ, Cho HY, Hwang HJ, *et al.* Inhibitory effect of *Poncirus trifoliate* on acetylcholinesterase and attenuating activity against trimethyltin-induced learning and memory impairment. Biosci Biotechnol Biochem 2009;73:1105-12.
14. Lagatolla C, Dolzani L, Granzotto M, Monti-Bragadin C. Genotoxic activity of 4,4',5'-trimethylazapsoralen on plasmid DNA. Teratog Carcinog Mutagen 1998;18:239-48.
15. Stern RS, Liebman EJ, Väkevä L. Oral psoralen and ultraviolet-A light (PUVA) treatment of psoriasis and persistent risk of nonmelanoma skin cancer. PUVA Follow-up Study. J Natl Cancer Inst 1998;90:1278-84.
16. Shen LL, Liu GX, Tang Y. Molecular docking and 3D-QSAR studies of 2-substituted 1-indanone derivatives as acetylcholinesterase inhibitors. Acta Pharmacol Sin 2007;28:2053-63.
17. Kong LD, Tan RX, Woo AY, Cheng CH. Inhibition of rat brain monoamine oxidase activities by psoralen and isopsoralen: Implications for the treatment of affective disorders. Pharmacol Toxicol 2001;88:75-80.
18. Inestrosa NC, Alvarez A, Pérez CA, Moreno RD, Vicente M, Linker C, *et al.* Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: Possible role of the peripheral site of the enzyme. Neuron 1996;16:881-91.
19. Bartolini M, Bertucci C, Cavrini V, Andrisano V. Beta-amyloid aggregation induced by human acetylcholinesterase: Inhibition studies. Biochem Pharmacol 2003;65:407-16.

Source of Support: Nil, **Conflict of Interest:** None declared.