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Human serum albumin reduces the potency of acetylcholinesterase inhibitor based drugs for Alzheimer's disease

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ABSTRACT

Human serum albumin (HSA) induced modulation of acetylcholinesterase (AChE) inhibition activity of four well-known cholinergic inhibitors like tacrine hydrochloride (TAC), donepezil hydrochloride monohydrate (DON), (−) Huperzine A (HuPA), eserine (ESE) was monitored quantitatively by Ellman's method. Kinetic analysis of enzyme hydrolysis reaction revealed that while the mechanism of inhibition does not change significantly, the inhibition efficiency changes drastically in presence of HSA, particularly for DON and TAC. However, interestingly, no notable difference was observed in the cases of HuPA and/or ESE. For example, the IC_{50} value of AChE inhibition increases by almost 135% in presence of ~250 μM HSA ($IC_{50} = 159 \pm 8$ nM) while comparing with aqueous buffer solution of pH 8.0 ($IC_{50} = 68 \pm 4$ nM) in DON. On the other hand, the change is almost insignificant (<10%) in case of HuPA under the similar condition. The experimentally observed difference in the extent of modulatory effect was correlated with the sequestration ability of HSA towards different drugs predicted from molecular docking calculations. The result in this study demonstrates the importance to consider the plasma protein binding tendency of a newly synthesized AD drug before claiming its potency over the existing one. Further, development of new and intelligent delivery medium that shields the administered drugs from serum adsorption may reduce the optimal drug dose requirement.

1. Introduction

Acetylcholine (ACh) is a neurotransmitter that is primarily released by the neuron in the neuromuscular synaptic junction. The released ACh binds the ACh-receptor located on the muscle and helps in contraction/movement of the muscle through signal transduction pathway. Since excess ACh might induce too much contraction, only a fraction of the released ACh is taken up by the post-synaptic neuron/muscle and the remaining ACh is degraded by the enzyme called acetylcholinesterase (AChE) at the synaptic cleft so that the next nerve impulse is transmitted [1]. Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disorder. The symptoms include confusion, irritability and aggression, mood swings, language breakdown, long-term memory loss, and the general withdrawal of the sufferer as their senses decline. The AD patients suffer from insufficient amount of ACh released by the neuron, mostly due to loss of cholinergic neuron in the hippocampus and in cortex. This situation is further aggravated by the presence of AChE in the synapse that degrades ACh into choline and acetate [2,3]. To ameliorate the problem in AD, cholinergic inhibitors are being used clinically in moderate amount [4,5]. These are essentially a class of chemicals which can take the place of ACh at the active site of AChE, significantly reducing the neurotransmitter's ability to bind with AChE and therefore, limiting the rate at which ACh can be broken down. The result is the presence of sustained amount of ACh at post-synaptic receptor molecules and, subsequently, a continuous firing of action potentials.

A series of chemical compounds are available as potent AChE inhibitors (AChEIs). Some of the early AChEIs include tacrine, metrifonate, galantamine, rivastigmine, (−) HuperzineA, Physostigmine (Eserine) and donepezil to name a few [6]. Active research in this field led to the discovery of many new (philserine, tolserine, esolerine etc.), hybrid (tacrine/8-hydroxy quinolone, donepezil/tacrine etc.) and naturally occurring (Huperzine A & B, *nelumbo nucifera* and *Himatanthus lancifolius* etc.) variants of AChEIs. The list

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is growing continuously over the years and, even some of the potentially toxic DNA intercalators like ethidium bromide (EB) and/or propidium iodide (PI) are also reported to have AChE inhibition activity [7–10]. The most common practice in the development of AChEIs is to test the inhibition of AChE activity in presence of the potent drug (inhibitor) by standard Ellman's method [11]. The medium is often a buffer of pH 8.0 to carry out the enzymatic hydrolysis. However, a buffer solution is considered as an extremely simplistic model to mimic the body fluid and measurement of AChE inhibition activity under this condition can apparently shed a wrong notion both towards the potency as well as efficacy of an inhibitor over the existing drugs. Human serum albumin (HSA), a highly soluble negatively charged protein, present in blood plasma in a high concentration (~0.6 mM) and bind a diverse range of exogenous and endogenous compounds [12–14]. While binding of the drug molecules to HSA is widely used in clinical settings as a drug delivery system due to its potential for improving targeting as well as decreasing the side effects [15], it can significantly impact on the efficacy and delivery of the drug, which in turn alters the pharmacokinetic and pharmacodynamic properties.

Medicinal chemists routinely use several techniques like equilibrium dialysis, ultrafiltration or ultracentrifugation to monitor the altered drug absorption, distribution, metabolism and excretion (ADME) pathway of novel pharmaceuticals to determine suitable drug doses. For example, in a comparative study Barré et al. [16] determined the reliability and applicability of the above methods towards binding of an anticonvulsant drug, valproic acid, by plasma proteins. Results by ultracentrifugation differed from those by equilibrium dialysis and ultrafiltration, which agreed reasonably well with each other. Transil (XL) plasma protein binding (PPB) kits are also often used for pre-clinical testing and clinical development of lead candidates [17]. However, a deeper understanding on why and how plasma protein binding impact the bio-chemical pathways is still lacking. In this communication, we report the modulatory effect of HSA, the most abundant plasma protein in vertebrates, on the activity of four well-known reversible AChE inhibition drugs like tacrine hydrochloride (TAC), donepezil hydrochloride monohydrate (DON), (−) HuperzineA (HuPA), eserine (ESE). It is shown that while the inhibition mechanism remains practically unaffected; the inhibition efficiency, characterized by IC_{50} values, shows drastic change in HSA medium in comparison with aqueous buffer environment. The extent of the modulatory behavior of HSA critically depends on its sequestration ability toward the individual drug predicted from molecular docking calculation. This study demonstrates the importance to consider the effect of delivery media in determining the AChE inhibition efficiency while developing new inhibitor drugs toward the treatment of Alzheimer's disease through cholinergic pathway.

2. Experimental

2.1. Chemicals

The type V-S, lyophilized powder form of acetylcholinesterase, activity ≥1000 units/mg protein, from *Electrophorus electricus* (electric eel) was purchased from Sigma Aldrich Chemical Company (cat. No. C2888). The chemicals like tacrine hydrochloride (TAC, purity ≥99%, cat. no. A79922), donepezil hydrochloride monohydrate (DON, purity ≥98% HPLC, cat no. D6821), (−) HuperzineA (HuPA, purity ≥98% TLC cat no. H5902), eserine (ESE, purity ≥99% HPLC cat no. E8375) and acetylthiocholine iodide (purity ≥98% TLC, powder or crystals cat no. A5751) were received from Sigma—Aldrich Chemical Company and used directly as received without any further purification. The extrapure form of dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman reagent), and sodium bicarbonate were procured from Sisco Research Laboratories (SRL), India (product no. 054817, 044883 and 1944142 respectively. Fatty acid and globulin free, >99% (agarose gel electrophoresis), lyophilized powder form of human serum albumin (HSA, USB Corp. USA, cat. no. 10878 and Sigma—Aldrich Chemical Company cat no. A1887) was used as received. The gelatin was received from Qualigens fine chemicals (a division of GlaxoSmithkline Pharmaceuticals Ltd., India). The other reagents such as anhydrous GR form of di-potassium hydrogen phosphate and sodium di-hydrogen phosphate monohydrate (CAS no. 7758-11-4 and 10049-21-5 respectively) were obtained from Merck, whereas extra-pure analytical grade of sodium hydroxide pellets were received from Sisco Research Laboratories (SRL), India. The analytical grade type II water used in this study (resistivity ~10 MΩ cm at room temperature) was obtained from Elix 10 water purification system (Millipore India Pvt. Ltd. The buffer pH was checked with Systronics μ-pH system 361.

2.2. Measurement of AChE enzyme activity

The effect of all the inhibitors towards the AChE enzyme activity in the absence and presence of HSA (concentration fixed at 250 μM) was carried out in synergy H1 hybrid meter plate reader instrument (BioTek) by using 96 well plates following the method of Ellman et al. [11] in 0.1 M phosphate buffer of pH = 8.0 at room temperature (298 K) with slight modification. All the components of the reaction mixture for both enzymatic as well as non-enzymatic studies were incubated for 5 min on ice cold water bath followed by total run time of 50 min inside the plate reader at the experimental temperature to follow the kinetics. In the experiments for measuring the Michaelis–Menten constant, K_m and maximum hydrolysis rate V_{max}, the substrate concentration was varied from 50 μM to 2.0 mM. Two different concentrations of inhibitors were tested to see the effect on the kinetic parameters. To study the effect of HSA on the IC_{50} (concentration of the inhibitor at half maximal inhibition) values of the inhibitor, the substrate concentration was kept fixed at the saturated reaction condition of AChE catalysis reaction (~1.5 mM); whereas, the inhibitors concentrations were varied from very low till the saturation inhibition condition. The corresponding blank for each reaction were taken by adding all the components according to their concentration except enzyme and was treated as non-enzymatic hydrolysis reaction. In both the non-enzymatic as well as enzymatic reaction conditions, the [DTNB] was kept fixed at ~317 μM; whereas, the enzyme concentrations were ~0.079 units/ml in all the enzymatic reactions. All the results are reported by taking the average of three independent run.

2.3. Evaluation of kinetic parameters for the enzyme catalysis and data analysis

The data analysis and calculation of kinetics parameters were done by using the Microcal Origin 6.0 software. For calculating the initial rate V_0 for the substrate hydrolysis, the non-enzymatic hydrolysis curve was subtracted off from the corresponding enzymatic hydrolysis curve. The V_0 for the substrate hydrolysis was calculated from the initial linear portion of the progress curve by using Equation (1)

$$\text{Initial rate, } V_0 \text{ (in moles per liter per seconds)} = \frac{\text{Slope}}{(1.36 \times 10^4) \times 0.442} \quad (1)$$

where, 1.36×10^4 is the molar absorptivity (ε, in M^{-1} cm^{-1}) of the yellow anion [18], 0.442 is the path length (in cm) and the slope is