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Dual inhibition of BACE1 and Aβ aggregation by β-ecdysone: Application of a phytoecdysteroid scaffold in Alzheimer's disease therapeutics

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ABSTRACT

Current medications for the complex neurological disorder, Alzheimer's disease (AD), can neither stop disease progression nor revert back disease pathogenesis. The present study demonstrates the applicability of a phytoecdysteroid, β-ecdysone, as a multi-potent agent in AD therapeutics. β-ecdysone strongly binds to the active site cavity of BACE1 with calculated dissociation constant of 1.75 ± 0.1 μM. Steady-state and time-resolved fluorescence spectroscopy reveal that binding of β-ecdysone induces conformational transition of the protein from open to closed form thereby blocking substrate binding. Even 500 nM of the compound completely blocks the enzyme activity. Furthermore, β-ecdysone strongly inhibits Aβ aggregation, evident from ANS and ThT binding assay. Co-incubation of equimolar peptide and β-ecdysone completely inhibits Aβ fibril formation which is further complemented by the AFM study. Low systemic toxicity of β-ecdysone further extends the applicability of the compound as functional food and dietary supplement for disease management.

1. Introduction

β-ecdysone is the most prevalent ecdysteroid in nature. It is widely distributed in both insects and plant species [1]. In arthropods, it plays dominant roles in controlling crucial functions like metamorphosis, reproduction, molting, and many more [1,2]. Role of ecdysteroid in plant is quite different and is primarily involved in defense against invertebrate predators [2,3]. Interestingly, phytoecdysteroids are present in higher concentrations compared to insects and are commonly found in ferns, gymnosperms, angiosperms and among crop species [1]. They are most abundant in spinach, *Spinacia oleracea* L., and quinoa, *Chenopodium quinoa*. In addition, they are also the main bioactive components of traditional Chinese and Siberian herbs.

Ecdysteroids are widely marketed as dietary supplement for athletes. It is primarily used as body building agents and also as stress busters [1]. Phytoecdysteroids have been shown to elicit several pharmacological effects such as stimulation of growth, enhancement of glucose utilization, reduction of cholesterol biosynthesis and increase in its catabolism [1]. Kizelsztein et al. demonstrated that a dosage of 10 mg/kg body weight of β-ecdysone for 13 weeks to diet-induced obese, hyperglycemic mice increased insulin sensitivity with significant lowering of blood glucose levels and adiposity, with respect to the control group [4]. In addition, β-ecdysone has been shown to stimulate hepatic functions, improve heart and lung functions and also renal functions [1,5]. Phytoecdysteroids can be used as inducers for gene switch systems based on insect ecdysteroid receptor proteins and genes of interest placed under the control of ecdysteroid response elements [1,6–8]. Particularly, due to the property that they do not seem to bind to vertebrate steroid receptors and essentially non-toxic to mammalian cells, they can be applied in gene therapy and functional genomics.

Here, we have evaluated the potential of β-ecdysone in a different perspective, in Alzheimer's disease (AD) therapeutics. Autopsy of the brain of AD patients revealed extensive neuronal loss in the region of the brain that controls memory and cognition [9]. The principal pathological hallmark of the disease is the presence of extracellular proteinaceous aggregates, known as "amyloid plaques" which are primarily composed of a peptide, known as amyloid-β peptide (Aβ) [10]. Aβ is generated due to aberrant pro-

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teolysis of a larger membrane-embedded protein, called amyloid precursor protein (APP) [11]. β-Secretase 1 (BACE1) initially cleaves APP at the N-terminus of the Aβ peptide, which is followed by the second cleavage within the transmembrane domain by γ-secretase, leading to the secretion of Aβ [11]. Aβ peptide is mostly unstructured in solution as evident from several in vitro studies [12,13], however, it's conformation *in vivo* is still unknown. Nevertheless it is known that in the diseased condition Aβ peptide undergoes rapid nucleation dependent dynamic events whereby this irregular conformation aggregates into a highly ordered β-sheet-rich assembly which ultimately develops into amyloid plaques [14]. Aggregated Aβ impairs the function of ion-motive ATPases, glucose and glutamate transporters, and also GTP-binding proteins as a result of covalent modification of the proteins [15,16]. Disruptions of cellular ion homeostasis and energy metabolism make neurons vulnerable to oxidative stress and apoptosis [16].

Acknowledging several established health-promoting and disease-preventing roles such as growth stimulator, enhancer of glucose metabolism as well as inhibitor of cholesterol biosynthesis, played by phytoecdysteroids, we have evaluated the efficacy of β-ecdysone for its ability to act as a BACE1 inhibitor and also as an anti-amyloidogenic agent using steady-state and time-resolved fluorescence spectroscopy, molecular modeling and *in vitro* bioassays.

2. Materials and methods

2.1. Materials

BACE1 protein, BACE1 activity assay kit and Thioflavin T (ThT) were purchased from Sigma–Aldrich while β-ecdysone was purchased from Extrasynthase. $A\beta_{25-35}$ (GSNKGAIIGLM) was purchased from ANASPEC Inc. ANS (8-anilino-1-naphthalene sulfonate) was a kind gift from Dr. Rajat Banerjee (Department of Biotechnology, University of Calcutta). All solvents used in the study were of analytical grade (E. Merck). Deionized water from Milli-Q apparatus (Millipore Corp., Billerica, MA) was used throughout the experiments.

2.2. Interaction of BACE1 with β-ecdysone studied using steady-state and time-resolved fluorescence

For fluorescence experiments, BACE1 (100 nM) was prepared in 0.2 M acetate buffer, pH 4.8, while a concentrated stock solution of β-ecdysone (10 mM) was prepared in DMSO. During fluorescence titration studies, gradual addition of aliquots of the inhibitor from the stock solution was added to the protein solution to attain the desired final concentration of the inhibitor. The final concentration of DMSO was kept <1% (by volume) in all samples. After the addition, each reaction mixture was allowed to equilibrate at ambient temperature for 30 min. Steady state fluorescence spectra were carried out with Parkin-Elmer LS-55 spectrofluorimeter using a 10 mm path-length quartz cuvette. Emission spectra were collected in the region of 300–450 nm using λ_{ex} = 295 nm, whereas excitation spectra were obtained by scanning over the wavelength range 260–310 nm using λ_{em} = 340 nm. All measurements were carried out at room temperature.

Fluorescence lifetime decay measurements of BACE1 in absence and presence of 3.5 μM β-ecdysone were performed on a Horiba Jovin-Yvon time-resolved fluorescence spectrometer operated in the time-correlated-single-photon-counting (TCSPC) mode using a pulsed LED source (pulse width < 1 ns) at 295 nm for selective tryptophan excitation. Fluorescence intensity decay curves were analyzed by fitting using a multi-exponential decay function:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad (1)$$

where I(t) is the fluorescence intensity at time t. α_i represents the fractional contribution to the time-resolved decay of the component with a lifetime of τ_i. χ^2 value and the Durbin–Watson (DW) parameter were used to validate the goodness of fit. The average life-time was calculated using the equation defined by:

$$<\tau> = (\sum_i \alpha_i \cdot \tau_i^2)/(\sum_i \alpha_i \cdot \tau_i) \quad (2)$$

2.3. Analysis of BACE1 inhibitory activity of β-ecdysone

β-secretase activity assay kit was used to evaluate the BACE1 inhibitory activity of β-ecdysone. Experiments were carried out in accordance with the manufacturer's instructions. Fluorescence was measured using a HITACHI F-7000 FL spectrofluorimeter using excitation at 320 nm and emission was monitored at 405 nm.

Percentage of inhibition was calculated using the following equation:

$$\text{Inhibition}(\%) = [1 - (C - S_0)/(S - S_0)] \times 100 \quad (3)$$

where S and S_0 are the fluorescence of the substrate in presence and absence of BACE1, respectively, after 120 min of incubation. C is the fluorescence of the substrate in presence of the inhibitor and the enzyme after 120 min of incubation.

2.4. Molecular modeling studies of BACE1-β-ecdysone complex formation

BACE1 crystal structure (PDB ID: 3TPP) in closed conformation was considered for the docking study. 3D structure of β-ecdysone was generated in SYBYL mol2 format. The active site of BACE1 was then defined by selecting the bound ligand as the reference ligand and choosing a 10 Å cutoff around the reference ligand. FlexX [17] was used to dock β-ecdysone into the BACE1 active site and docked solutions were ranked using an empirical scoring function. Preparation of the receptor, ligand and all the post docking interaction analyses were carried out using LeadIT (http://www.biosolveit.de/LeadIT/).

2.5. Analysis of inhibition of Aβ aggregation by β-ecdysone

2.5.1. Preparation of $A\beta_{25-35}$ and β-ecdysone stock solution

$A\beta_{25-35}$ was dissolved in 1,1,1,3,3,3- hexafluoro-2-propanol (HFIP) at a concentration of 1 mM and it was very gently vortexed to prepare a uniform, non-aggregated Aβ solution and immediately stored at −80 °C. On the day of use, the HFIP-dissolved amyloid samples were lyophilized and dissolved in PBS, pH 7.4, to initiate aggregation. β-ecdysone stock solution was prepared in DMSO at 10 mM concentration and diluted in appropriate concentration using PBS (pH 7.4).

$A\beta_{25-35}$ peptide (100 μM) in absence and presence of different concentrations of β-ecdysone in PBS buffer (pH 7.4) were incubated at 37 °C for 3 days.

2.5.2. ANS binding assay

During the experiment, incubated peptide in presence and absence of β-ecdysone were diluted 5 times with PBS buffer and aliquots of ANS were added from the concentrated stock solution of 1 mM in Tris buffer (pH 8.0) such that the final concentration of ANS was 4 μM. Fluorescence measurements were carried out in a Parkin-Elmer LS-55 fluorescence spectrophotometer using