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Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's β-amyloid

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Abstract **β-Amyloid peptide (Aβ) is the major constituent of senile plaques, the key pathological feature of Alzheimer's disease. Aβ is physiologically produced as a soluble form, but aggregation of Aβ monomers into oligomers/fibrils causes neurotoxic change of the peptide. In nature, many microorganisms accumulate small molecule chaperones (SMCs) under stressful conditions to prevent the misfolding/denaturation of proteins and to maintain their stability. Hence, it is conceivable that SMCs such as ectoine and hydroxyectoine could be potential inhibitors against the aggregate formation of Alzheimer's Aβ, which has not been studied to date. The current work shows the effectiveness of ectoine and hydroxyectoine on the inhibition of Aβ42 aggregation and toxicity to human neuroblastoma cells. The characterization tools used for this study include thioflavin-T induced fluorescence, atomic force microscopy and cell viability assay. Considering that ectoine and hydroxyectoine are not toxic to cellular environment even at concentrations as high as 100 mM, the results may suggest a basis for the development of ectoines as potential inhibitors associated with neurodegenerative diseases.**

Keywords: Alzheimer's disease; β-Amyloid peptide; Ectoine; Hydroxyectoine; Small molecule chaperones

1. Introduction

Alzheimer's disease (AD), currently affecting about 10% of the population age 65 and over, is characterized by the presence of extracellular amyloid plaques and neurofibrilliary tangles in the brain tissue [1,2]. The major component of the amyloid plaques is a 39–42 residues peptide termed β-amyloid peptide (Aβ), which is produced in soluble forms by proteolysis of β-amyloid precursor protein [3]. Although several segments of Aβ fragments are generated during this proteolysis process, the 42-amino acids version (Aβ42) has been shown to have a high propensity to aggregate and was found to be the most predominant in amyloid plaques [3,4]. Environmental stresses are known to trigger the formation of amyloid aggregates having cross β-sheet secondary structure [5–9]. While there are no effective treatments for AD at the present time, substances that can efficiently inhibit the amyloid formation by interfering with the process have been sought as drug candidates for treating AD [10–15].

Ectoines are common solutes of aerobic heterotrophic bacteria and constitute a class of small molecule chaperones (SMCs). SMCs accumulate to high intracellular concentrations, preventing the misfolding/denaturation of proteins and other labile macromolecular structures from environmental stresses [16–19]. The presence of SMCs has already proven highly effective in preserving enzymatic activities against heating, freezing and drying [19,20]. Recently, we reported that SMCs like ectoine, betaine, trehalose, citrulline could successfully inhibit insulin amyloid formation in vitro [21]. Those findings suggest the efficacy of SMCs against amyloid formation, which may make them viable drug candidates for treating neurodegenerative diseases in the future.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is one of the most abundant osmolytes in nature [22]. It has been reported that ectoine protects proteins and enzymes from thermal stress, proteolysis, change of the pH or the salt concentration; their ability to stabilize different proteins, nucleic acids, membranes and whole cells is well studied [19,20,23–26]. Hydroxyectoine is a derivative of ectoine differing in chemical structure by a hydroxyl group (Fig. 1). It is produced by halophilic bacteria under elevated temperature conditions and acts as general dessication protectant for bacterial cells [27]. In the present work, we report that ectoine and hydroxyectoine strongly inhibit the Aβ42 amyloid formation in vitro and reduce the toxicity to human neuroblastoma cells. This finding suggests that ectoine and hydroxyectoine could be potential candidates of anti-amyloid therapeutics for treating AD.

2. Materials and methods

2.1. In vitro Aβ42 amyloid formation

Human Aβ42 was purchased from rPeptide Co. (Athens, GA, USA). Ectoines were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA) and Bitop AG (Witten, Germany). All other chemicals and reagents were purchased from Sigma–Aldrich Co. Aβ42 was dissolved in 100% hexafluoroisopropanol and was sonicated in water bath for 5 min. The dissolved peptide was aliquoted in microcentrifuge tubes, left at room temperature for half an hour, then dried with nitrogen gas and stored at −20 °C. Aβ42 aggregates were formed in 10 mM HCl with 2% DMSO at a concentration of 25 μM at 37 °C for three days according to [28]. Incubation took place in sealed glass vials to prevent any possible evaporation. Ectoines were prepared as stock solutions of 1 M in deionized water before use. For observing the effect on SH-SY5Y cell viability, Aβ42 seed samples were prepared by incubating 25 or 100 μM solution (10 mM HCl, 2% DMSO) at 37 °C for 24 h.

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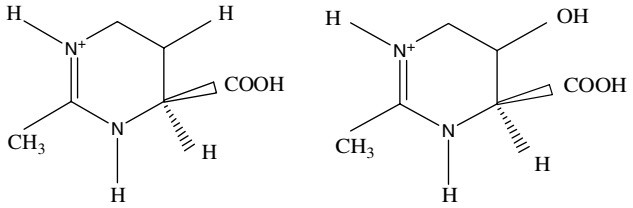

Fig. 1. Chemical structures of ectoine and hydroxyectoine.

2.2. Thioflavin T (ThT) fluorescence measurement

Thioflavin T (ThT)-induced fluorescence changes were measured by spectrofluorophotometer (Model RF5301, Shimadzu Co., Japan) according to [29]. The sample (5 μl) was mixed with 1.5 ml of 50 μM ThT solution in Tris–HCl buffer (pH 8, 20 mM) in a quartz cuvette. Ectoine or hydroxyectoine itself did not interfere with ThT-induced fluorescence. The fluorescence intensity was monitored at an excitation wavelength of 450 nm and an emission wavelength of 482 nm with excitation and emission slit widths at 5 nm each. Readings were done in triplicate and the average was taken as the final value. These average readings were then subtracted from the reading of the ThT-only solution. The results were then normalized against the control Aβ42 sample.

2.3. MTT assay

Human neuroblastoma cells (SH-SY5Y) were maintained in medium with 40% minimal essential medium (MEM), 40% Ham's modification of F-12, 18% fetal bovine serum (FBS), 1% L-glutamine (3.6 mM), and 1% penicillin/streptomycin antibiotics in 5% CO_2 at 37 °C. FBS, penicillin/streptomycin antibiotics and L-glutamine were purchased from Invitrogen (Carlsbad, CA, USA). MEM and Ham's F-12 were purchased from Irvine Scientific (Santa Ana, CA, USA). Cells were harvested from flasks and plated at a concentration of 100 cells/μl medium per well in a 96-well polystyrene plate. Cells were incubated at 37 °C for 24 h to allow the cells to attach to the flask. Aβ42 (25 or 100 μM) seed samples with/without ectoine or hydroxyectoine were preincubated for one day, followed by dilution to two different sets of concentrations (2.5 and 10 μM) with Tris–HCl buffer buffer (pH 7.4, 20 mM). Each sample (10 μl) was then added to the wells containing the cell culture (90 μl), resulting in final concentrations of the samples of either 0.25 or 1 μM. For testing the effect of ectoine or hydroxyectoine alone on the cell viability, ectoines were prepared at concentrations of 250 μM, 10 mM, 100 mM and 1 M in Tris–HCl buffer (pH 7.4, 20 mM) and were diluted 10 times by their application to the cell culture. The treated cells were incubated for an additional 48 h at 37 °C. For the cell viability assay, 10 μl of 5 mg/ml MTT [(3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] in deionized water were added to each well, and cell survival was determined according to [30]. The medium was aspirated from the centrifuged plates after 3 h incubation at 37 °C and the solubilization solution 100 μl of 2-propanol containing 0.1 N HCl was added to the wells. The absorbance of each sample was measured at 560 nm using a multi-well assay plate reader (Victor Wallac, Gaithersburg, MD). Four replicate wells were used for each sample and control. Each experiment was repeated twice, and the average value was calculated. Cell viability was determined by dividing the sample absorbance values by the control sample absorbance value.

2.4. Atomic force microscopy (AFM)

Samples were imaged with Nanoprobe III scanning probe workstation (Digital Instruments Inc., CA, USA) according to [31]. Samples were prepared by placing 5 μl of solution on freshly cleaved mica (Ted Pella Inc., Redding, CA) at room temperature for 60 s; they were then washed twice with 50 μl of deionized water and dried with nitrogen gas. Image data was obtained in tapping mode under ambient conditions at a scan frequency of 1–2 Hz with AFM tips (Veeco Metrology, LLC, Santa Barbara, CA) operating at a resonant frequency of 306–444 kHz. The spring constant of the cantilever having the AFM tip was 103 N/m. The length and the width of the cantilever were 140–180 and 48–52 μm, respectively. The range of oscillation amplitudes and set points used here was 40–100 mV and 0.8–1.0 V, respectively. A scan area of 5 × 5 μm was imaged for two different samples in each case. All images were flattened and presented in height mode by using nanoscope software (Digital Instruments Inc.).

3. Results and discussion

We investigated the effect of ectoine and hydroxyectoine on Aβ42 amyloid formation by using ThT-induced fluorescence and AFM. ThT-induced fluorescence change was observed for 72 h time period in the presence of Aβ42 (25 μM), incubated without/with ectoine or hydroxyectoine (25 μM, 1 mM, 10 mM and 100 mM) (Fig. 2). The Aβ42 amyloid formation followed a typical sigmoidal curve with an initial lag phase, followed by phases of elongation and saturation. The lag time for control samples was approximately 12 h, while the samples coincubated with each of the solutes at 100 mM exhibited a lag time of nearly 24 h. Compared to control Aβ42 sample, the samples co-incubated with ectoine or hydroxyectoine showed less fluorescence increase, indicating fewer cross β-sheets formed in the samples. The inhibitory effect of ectoine and hydroxyectoine was found to be concentration-dependent, while ectoine showed higher inhibitory effect than hydroxyectoine. According to AFM images, the control sample contained a high density of typical unbranched, Aβ42 amyloid fibrils (Fig. 3A1 and A2). The ectoine 100 mM sample contained few aggregates while no fibrils could be observed (Fig. 3B1 and B2). In the case of the hydroxyectoine 100 mM sample, more aggregates and a few fibers could be observed compared to ectoine samples (Fig. 3C1 and C2), which

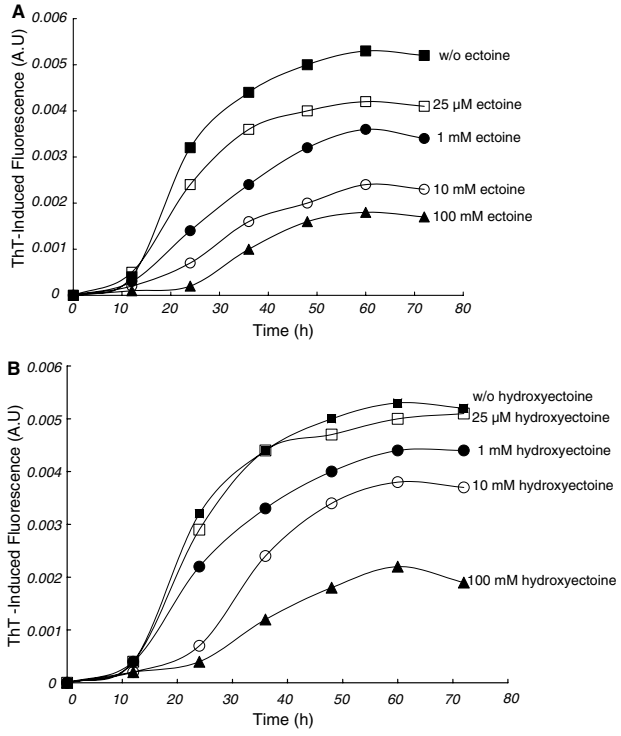

Fig. 2. Effect of: (A) ectoine and (B) hydroxyectoine on Aβ42 amyloid formation. ThT-induced fluorescence changes were measured for Aβ42 (25 μM) solution incubated without/with ectoine or hydroxyectoine at 37 °C for 72 h.

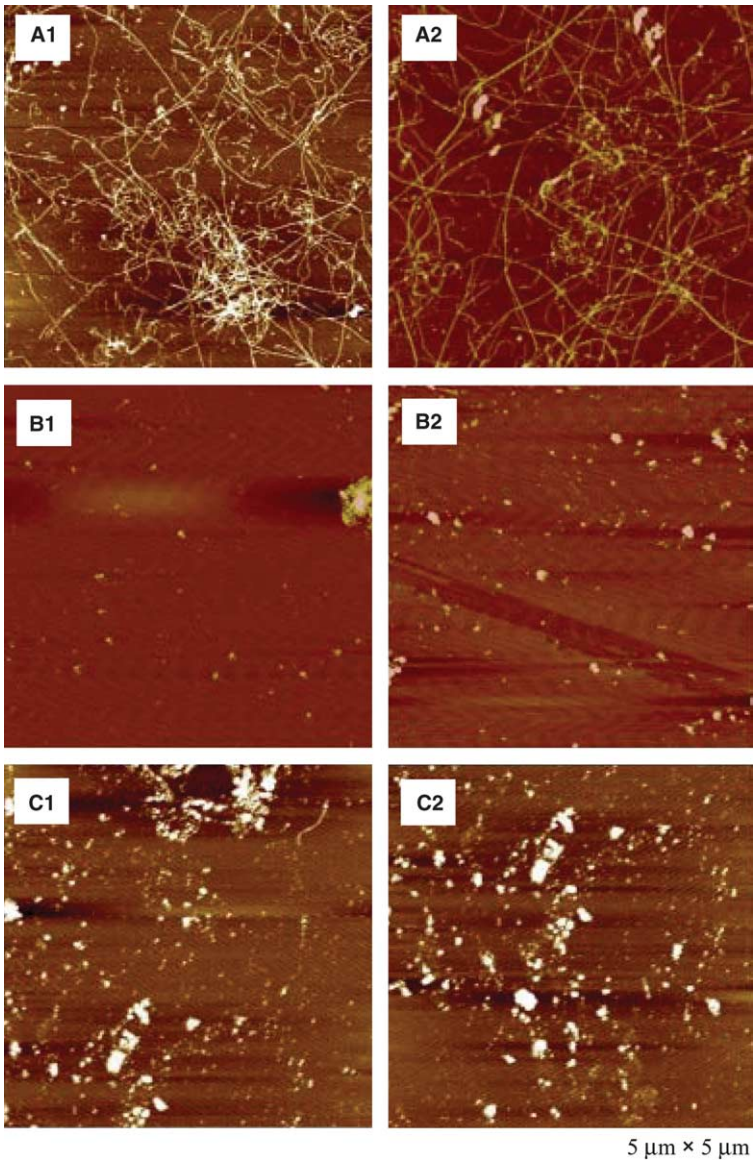

Fig. 3. Representative AFM images of Aβ42 samples incubated for 72 h without any ectoines (A1,A2), with 100 mM ectoine (B1,B2), and with 100 mM hydroxyectoine (C1,C2). Each sample picture represents a scan area of 5 × 5 μm taken twice at different spots.

is in accordance with the ThT data. Neither ectoine nor hydroxyectoine itself affected the binding of amyloid aggregates to AFM mica surface (data not shown). The average height of aggregates in the control, ectoine and hydroxyectoine samples were found to be 6.98, 2.01, and 2.47 nm, respectively.

The effect on cell viability was measured using seed sample of Aβ42 alone (25, 100 μM) and samples co-incubated with ectoine and hydroxyectoine of concentrations 25 μM, 1 mM, 10 mM and 100 mM. As shown in Fig. 4A, the seed sample containing Aβ42 (25 μM) alone caused an approximate 25% decrease in MTT activity compared to the control culture incubated without Aβ42, thereby indicating the toxicity of Aβ42 aggregates. However, the samples co-incubated with both Aβ42 and ectoine showed a decrease in the MTT activity ranging from 3% to 25%, depending on the concentration of ectoine. Hydroxyectoine showed less effect compared to ectoine, causing MTT activity decrease, varying 18–27% from that of the control culture. This result indicates that ectoine could be a potential inhibitor for Aβ42-induced toxicity while hydroxyectoine could provide partial protection. The seed samples with higher concentration of Aβ42 (100 μM) showed more toxicity compared to the 25 μM Aβ42 samples (Fig. 4B). The sample of 100 μM Aβ42 alone showed a 43% decrease in MTT activity while the samples coincubated with ectoine showed 23–40% decrease in MTT activity. Seed samples prepared at pH 7.4 for 10 days were found to exhibit similar effects as Fig. 4 (data not shown). The effect of ectoine or hydroxyectoine alone on cell viability is shown in Fig. 5. According to the result, ectoine/hydroxyectoine neither inhibits nor enhances the cell growth at up to 100 mM concentration. It is noteworthy that ectoines had been reported to be biologically inert, i.e., they do not interfere with most enzymatic and binding reactions [32–36]. According to the reports, ectoines are highly compatible with cell metabolism and tolerated in their producer organisms up to concentrations of 1 M. Many microorganisms in nature, for example, *Escherichia coli*,

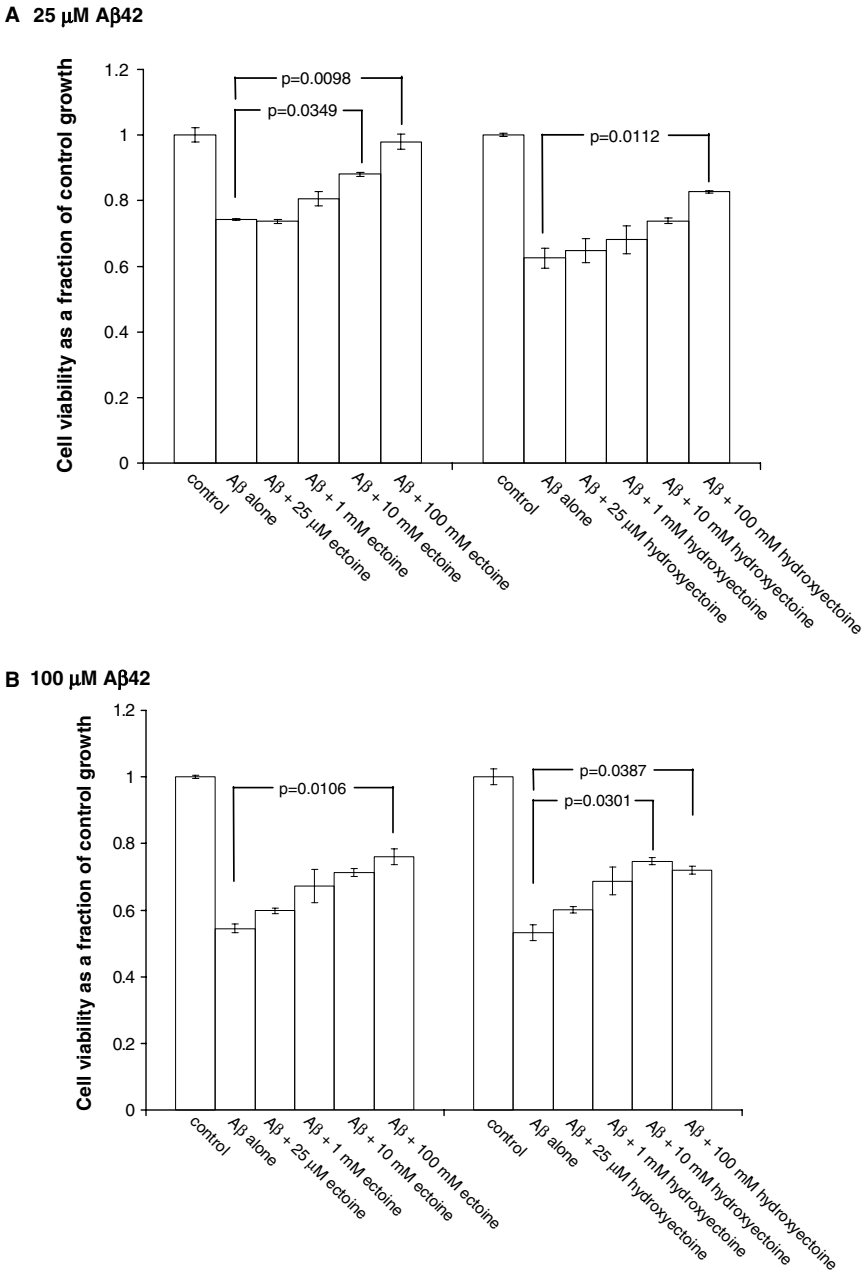

Fig. 4. Dose response of SH-SY5Y apoptosis induced by: (A) Aβ42 (25 μM) seed samples without/with ectoine or hydroxyectoine, (B) Aβ42 (100 μM) seed samples without/with ectoine or hydroxyectoine. Seed samples were 100-times diluted in cell culture. Data are expressed as a fraction of control value from two independent experiments with each experimental value being the average of four trials. The error bars were calculated with a 95% confidence level. Statistical analysis was performed by means of one-way analysis of variance (ANOVA). A *P*-value less than or equal to 0.05 was considered statistically significant.

can take up a large amount of ectoines under stress conditions from their environment to increase their stress resistance.

Considering that the physiological Aβ concentrations in human cerebral cortex are in the range of 3–8 nM [37], ectoines may be needed in much lower concentrations for therapeutic purpose than those used in this work. According to our results, the inhibitory effect of ectoines was dependent on the ratio between Aβ and ectoines. As shown in Fig. 4, the effect became statistically significant at above around 100-fold higher concentrations of ectoines than Aβ, suggesting that the actual concentration of ectoines needed as drug candidates can be lower than 1 μM. Since ectoines are 'zwitterionic' low-molecular weight molecules at physiological conditions like many amino acids, the penetration of ectoines through the blood brain barrier in brain may not be highly affected. Further experiments on this issue need to be done in order to develop ectoines as drug candidates for treating Alzheimer's disease patients.

Lack of detailed structural information on soluble or aggregated Aβ had made structurebased drug design difficult [10]. The residues 29–42 of Aβ42 contain a large portion of nonpolar amino acids. Recent reports suggest that π-stacking interactions between aromatic residues of Aβ may play a key role in amyloid aggregation [10,38]. It is conceivable that ectoine and hydroxyectoine may interfere with the hydrophobic and/or

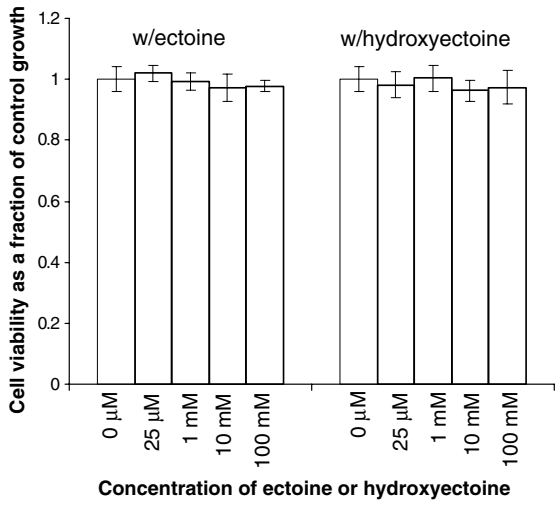

Fig. 5. Effect of ectoine or hydroxyectoine alone on the viability of SH-SY5Y in the absence of Aβ42. Data are expressed as a fraction of control value from two independent experiments with each experimental value being the average of four trials. The error bars were calculated with a 95% confidence level.

π–π interactions and hence prevent, or severely disrupt, the self-association of Aβ42 into aggregates. The higher inhibitory effect of ectoine compared to hydroxyectoine might be due to its higher hydrophobicity caused by the lack of hydroxyl group in the ring structure.

According to our previous work [21], ectoine inhibited insulin amyloid formation in vitro. Unlike Aβ42 that is known to be natively unfolded and random in configuration [2,4,9,39], insulin has a folded globular conformation under ambient conditions [40–43]. Insulin had been reported to form amyloid fibrils via formation of partially unfolded intermediates from the native globular form, triggered by various environmental stresses. Considering those structural aspects, the inhibitory action by ectoine on the insulin amyloid formation seems to occur by stabilizing partially unfolded intermediates, not the compact globular structure. Stabilization effects of SMCs such as ectoine and hydroxyectoine on proteins are not clearly understood yet, but they are believed to be due to preferential hydration and specific solute/protein interactions, resulting in preferential exclusion of SMCs from protein monomers and hydration of protein with solvent molecules [25,44,45]. Thus, it is hypothesized here that the preferential hydration of (partially) unfolded amyloidogenic proteins by ectoine and hydroxyectoine may suppress the formation of amyloid fibrils; this possibility needs future investigation. The stabilization might be further strengthened by specific hydrophobic interaction between the solutes and protein, which can disrupt the hydrogen bond formation between the partially unfolded monomers.

In this work, ectoine and hydroxyectoine were found to inhibit Aβ42 amyloid formation and reduce the toxicity to human neuroblastoma cells. Previously we demonstrated that several SMCs successfully inhibited insulin amyloid formation in vitro [21]. The inhibitory effect of SMCs on amyloid formation is further strengthened by a recent report on the efficacy of trehalose against Huntington disease [46]. Supported by those reports, current work suggests that naturally-occurring, non-toxic SMCs can be potential drug candidates against the amyloid formation associated with neurodegenerative disorders.

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