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(RESEARCH ARTICLE)

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Antioxidant property of alpha lipoic acid retains after conjugation with SynB3 Peptide

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Abstract

Alpha lipoic acid (ALA), an antioxidant reagent (1) is currently being proposed as a potential therapeutic candidate against central nervous system (CNS) diseases (2, 3). Although it can cross blood brain barrier (BBB), its poor bioavailability due to its heat sensitivity and susceptibility toward enzymes render its pharmacokinetics poor enough to use as a drug (2). Several strategies including bioconjugation and encapsulation have been used successfully to improve pharmacokinetics of drug like molecules (4, 5, 6, and 8). Despite having success in many cases, bioconjugation often leads to perturb structure of drugs and hence reduce its activity (7). Therefore, it is important to study the efficacy of drug like compounds after necessary modification. Here we report that antioxidant activity of ALA retains intact after conjugating SynB3 peptide, which is also a BBB active peptide (9, 14). So, new ALA-peptide conjugate may serve as a potential anti-oxidant with improved bioavailability.

Keywords: Alpha-Lipoic Acid (ALA); Blood–Brain Barrier (BBB); Antioxidant Activity; SynB3 Peptide; Bioavailability; Central Nervous System (CNS)

1. Introduction

ALA (1, 2-dithiolane-3-pentanoic acid) is an endogenous organosulfur compound predominantly present in the mitochondria (1, 10), where it functions as a cofactor of enzymes involved in carbohydrate metabolism (10, 11). Aside its metabolic role, ALA exhibits strong antioxidant properties by scavenging reactive oxygen species (ROS) generated during oxidative processes (1). Due to its unique antioxidant property, ALA has potential therapeutic importance in circumstances associated with oxidative stress (19). Consequently, ALA has been widely evaluated for its role in neurodegenerative and central nervous system (CNS) disorders, including Alzheimer's and related brain disorder syndromes (17).

Although ALA has promising biological activity and ability to cross the blood–brain barrier (BBB) (22), the clinical application of ALA remains restricted, due to its very low water solubility and poor bioavailability (15). Its chemical instability arises from hydrolytic, thermal, and proteolytic sensitivity (15, 16). These physicochemical limitations result in reduced absorption and rapid metabolic clearance, thereby reducing its effectiveness in CNS-targeted therapy (18). To combat these challenges, several formulation and molecular modification strategies such as PEGylation, nano-formulation, liposomal encapsulation, molecular conjugation, and many more methods have been explored to enhance the stability and increase the bioavailability of ALA (20). Recently peptide conjugation becomes growingly popular to improve bioavailability in target-based therapeutics (21). As peptide is a moderately big molecule, it is mandatory that

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structural modifications do not compromise the inherent antioxidant capacity of ALA, as this role of ALA is the foundation of its therapeutic usefulness. The present study focuses on a peptide-conjugation approach to improve the bioavailability and delivery of ALA while maintaining its antioxidant function. We explored a SynB3 (**RRLSYSRRRF**) peptide derived from the antimicrobial peptide protegrin 1 (23) which have the ability to translocate cell membranes and have been successfully applied for delivery of therapeutic molecules across the BBB (27). We coupled ALA with SynB3 via the amide linkage formation between COOH of ALA and N-terminal NH_2 of SynB3. Although ALA itself can cross blood brain barrier, the synergistic effect of conjugation of ALA with another BBB active molecule (here SynB3 peptide) may improve BBA activity and bioavailability further. The question remains whether conjugation inhibits ALA's activity as an antioxidant agent. To address this question, we have performed ROS experiment (24). The result shows the ALA-SynB3 a conjugate has comparable antioxidant property as ALA alone, which validates the use of COOH functionality of ALA for conjugation to other molecules (here SynB3 peptide) to improve its bioavailability.

2. Materials and methods

2.1. Materials

All reagents and chemicals were used ACS grade and purchased from Merck unless otherwise mentioned. Fmoc protected amino acids and HATU were purchased from GL Biochem Ltd (China).

2.2. Synthesis of ALA-SynB3 conjugates

Peptide was synthesized by standard Fmoc strategy describes elsewhere (23). Rink amide MBHA resin was used as solid support. HATU was used as coupling reagent and DIPEA as a base activator. After completing synthesize of linear peptide ALH was attached to N-terminal via HATU, DIPEA method. The peptide and peptide conjugated ALA were cleaved from resin by the cocktail of TFA, H_2O and TIS (90%, 5% and 5% respectively) and precipitated by diethyl ether. Crude peptide was dissolved in H_20 and was purified by HPLC method and characterized by ESI mass spectrometry.

2.3. Trypan Blue assay

The viability of SH-SY5Y cells were first checked against ALA and ALA-SynB3 peptide conjugate by simple Trypan blue assay. After having confluent cells, cells were suspended in complete (RPMI, FBS (10%), Penicillin-Streptomycin (1%) media. 10 µl Trypan blue and 10 µl of the cell suspension (1:1 ratio) was mixed and 10 µl of the mixture was loaded in haemocytometer for the cell count. Live and dead cells were differentiated by selective staining of dead cells by Trypan blue.

2.4. MTT assay

The cell viability was further confirmed by MTT assays. In a micro well plate 200 µl of cell (1.5 X 10^5 cells/well) and incubate overnight. ALA and ALA-SynB3were added in different concentration and incubated for 24 hours. MTT stock was prepared in PBS at 5 mg/ml. To each well 10 µl of MTT solution from stock was added and incubate 2 hour at 37oC. Then, the media was removed carefully and 200 µl of DMSO was added to solubilize the purple crystal. The purple colors of live cells were measured at 570 nm in a multiwall spectrophotometer. Cells without treatment of either ALA or ALA-SynB3 were used as control. The cell viability was measured as:

$$Viability\ (\%) = (Mean\ Absorbance\ of\ treated\ cell)/(Mean\ absorbance\ of\ control\ cell)$$

2.5. ROS assay

ROS assay was performed by H_2O_2 and DCFH-DA method. Cells were counted (5X105 cells/ml) and 200 µl confluent SH-SY5Y Cells were plated into 96 wells plate. From top 100 µl media was discard and 100 µl of RPMI media was added. Oxidative stress was generated with H_2O_2 in presence or absence of ALA as well as ALA-SynB3 conjugate. After overnight incubation cells were treated with DCHF-DA and incubate for 1 hour and then, discard the media and finally cells were washed with 100 µl PBS and fluorescence was measured. DCFH-DA, (2', 7', dichlorodihydrofluoresceindiacetate) is a cell-permeable, non-fluorescent probe used to detect and measure intracellular Reactive Oxygen Species (ROS). It reacts with reactive oxygen and emits green fluorescence. The fluorescence intensity was measured using fluorescence micro plate reader at 530 nm. The excitation wavelength was set at 490 nm.

3. Result

3.1. Characterization of ALA-SynB3 conjugates

We conjugated ALA with SynB3 peptide (RRLSYSRRF) via amide bond formation between the carboxylic acid group of ALA and n-terminal amine of the peptide. We added one 6-amino-hexanoic acid followed by a glycine at the N-terminal of SynB3 to introduce a spacer between ALH and SynB3 as illustrated in (figure1).High-Performance Liquid Chromatography (HPLC) was used to separate the conjugate from the impurity. The chromatogram (figure 2) displays a distinct peak at RT = 21.22 minutes corresponds to the ALA-SynB3 conjugate which was further verified by the Electrospray Ionization Mass Spectrometry (ESI-MS). The ESI_MS chromatogram reveals multiple charged ion peaks corresponding to different charge states of the conjugate. The observed peaks at m/z = 400.78 (M+4), 534.12 (M+3), and 800.53 (M+2) correspond to molecular mass 1599 Da which is same as calculated mass of the ALA-SynB3 conjugate validate the synthesis of the conjugate.

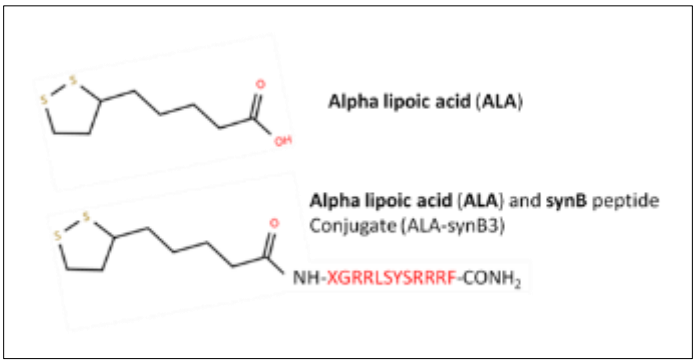

Figure 1 Structure of alpha lipoic acid (ALA) and SynB3 peptide conjugate (ALA-SynB3). X marked in red color represents 6-aminohexanoicacid. Conjugation was formed by the condensation of COOH group of the ALA with terminal NH_2 group of the peptide resulting an amide bond formation

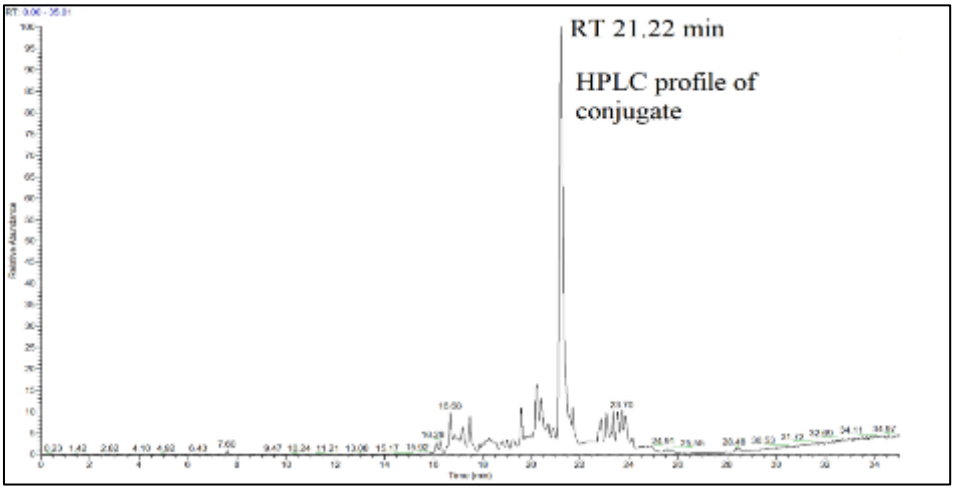

Figure 2 HPLC profile of crude peptide. C18 column was used as a stationary phase. The gradient was used to separate peptide was as 0 to 60 percent acetonitrile for 35 min. Chromatogram was monitor at 280 nm absorbance. The purified product was further characterized by ESI-MS method

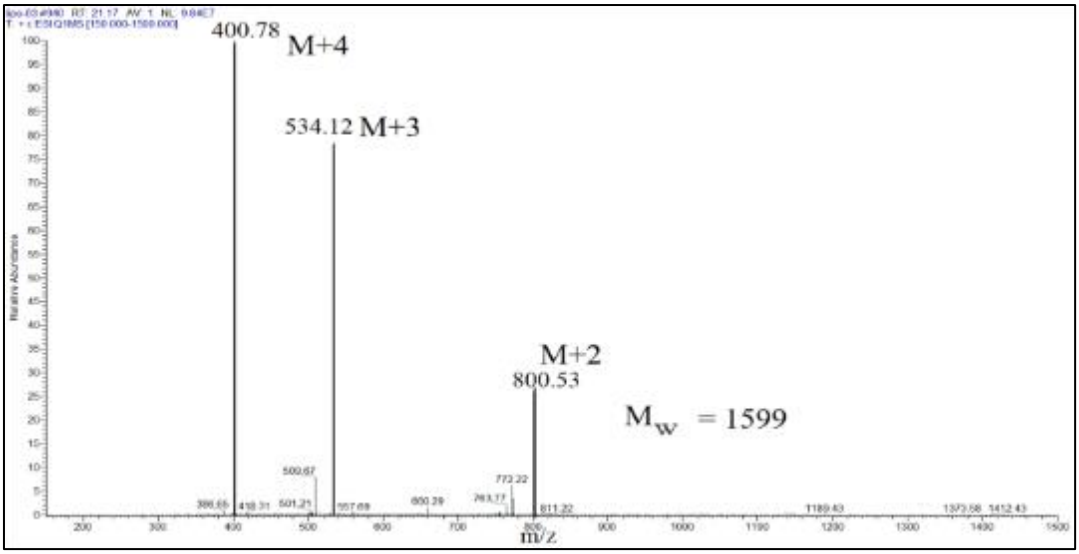

Figure 3 ESI-MS spectra of ALA-SynB3. The observed peaks at m/z = 400.78 (M+4), 534.12 (M+3), and 800.53 (M+2) represent the molecular mass as 1599 which is identical to the calculated mass

3.2. Cell viability (MTT) Assay

Throughout the study we have used the SH-SY5Y cell line which is a human-derived neuroblastoma cell line commonly used in neuroscience research. The figure 4 illustrates the effect of ALA and ALA-SynB3 conjugate on cell viability. Fifty percent cell viability (LD50) in presence of ALA and ALA-SynB3 were found as 1.27 mg/ml and 1.08 mg/ml respectively. These results suggest that although, cells are more viable in presence of ALH, ALA-SynB3 conjugate also be useful up to 1mg/ml concentration.

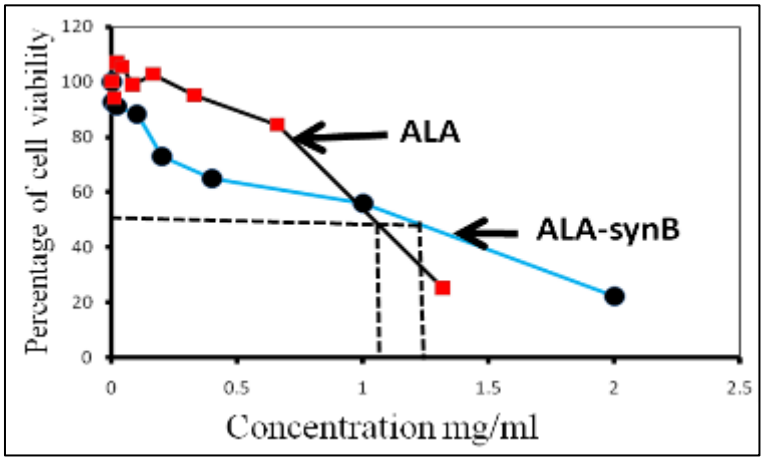

Figure 4 Viability test of ALA and ALA-SynB3. MTT method was used to test viability of SH-SY5Y Cells against different concentration of ALA and ALA-SynB3. LD_{50} of ALA and ALA-synB3 were found as 1.27 mg/ml and 1.08 mg/ml respectively

3.3. ROS measurement

This figure depicts the fluorescence intensity (A.U), which reflects reactive oxygen species (ROS) levels in SH-SY5Y neuroblastoma cells under different treatment conditions. Cells treated with H_2O_2 alone exhibited the highest fluorescence intensity indicating total oxidative stress. Treatment with ALA as well as ALA-SynB3 reduced ROS levels approximately 20 percent. Although, this reduction is quite lower it is enough to say that both ALA and ALA-SynB3 conjugate exhibit similar level of quenching power of free radicals hence, antioxidant property.

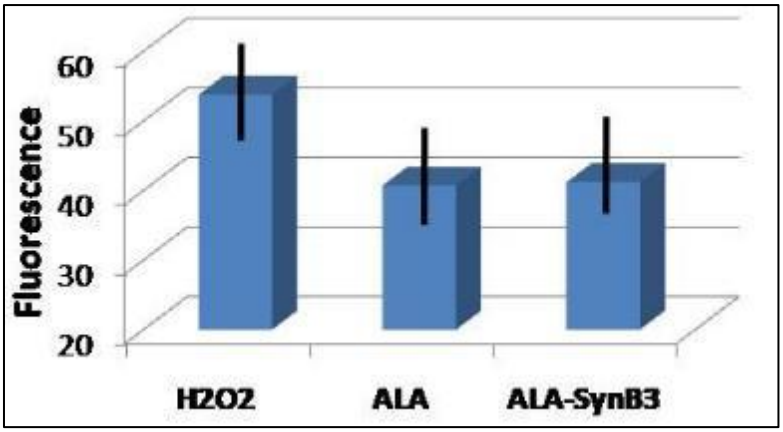

Figure 5 ROS assay- H_2O_2 was used as ROS inducer. The induced ROS was challenged by ALA and ALA-SynB3. ROS was measured by the DCFH-DA. DCFH-DA reacts with ROS and become fluorescent

4. Discussion

In the present study, ALA was conjugated with the SynB3 peptide to improve its solubility, bioavailability, and potential for absorption in brain. SynB3 peptide was selected due to its water-soluble nature and its ability to cross the blood–brain barrier, making it an appropriate carrier for CNS-targeted drug delivery systems (25). The MTT assay is widely applied in cell biology, pharmacology, and toxicology to assess cell viability, proliferation, and cytotoxicity (26). Cell viability and activity can be assayed using the MTT protocol, providing reliable measurements of cellular responses. The antioxidant activity of the ALA–SynB3 conjugate was assessed using reactive oxygen species (ROS) scavenging free radicals and compared with that of free ALA.

This observation indicates that conjugation with the SynB3 peptide did not affect the required antioxidant properties of ALA, which are essential for its biological function. Preservation of antioxidant activity suggests that the molecular stability of the active functional groups of ALAS remained intact following conjugation.

Furthermore, the conjugate exhibited improved aqueous solubility relative to free ALA, which is expected to facilitate better absorption, bio-distribution and systemic availability. The enhanced solubility, combined with the BBB-crossing capability of the SynB3 peptide, could enhance the possibility for efficient delivery of ALA to brain tissues. Although direct in-vivo studies were not conducted at this stage, the observed physicochemical improvements while maintaining intact antioxidant property support the viability of the conjugation strategy. Overall, the results indicate that because of the multi-component activity, ALA-SynB3 conjugate represents an effective approach for improving the drug delivery characteristics of ALA without compromising its antioxidant activity.

5. Conclusion

Poor bioavailability of alpha lipoic acid renders its therapeutic potential as an antioxidant. Conjugation of alpha lipoic acid with BBB permeable molecule could be the possible solution to overcome poor bioavailability and improve distribution of alpha lipoic acid in brain. Our study shows the ALA-SynB3 conjugate has potential antioxidant property; hence the conjugates may be a potential therapeutic candidate against CNS diseases and it need to be tested *in vivo.*

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

Anwesha Banerjee, Soni Kumari and Shrayoshree Putatunda contributed equally and be considered as first author.

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