Doctoral Dissertation Evaluation Review

Degree requested Doctor of Life Science Applicant's name Mariam Elseman Ibrahim Abdelrasoul

Examiner :

Chief examiner	Professor	Kenji Monde
Associate examiner	Professor	Hiroshi Hinou
Associate examiner	Professor	Toyoyuki Ose
Associate examiner	Visiting Associate Professor (Faculty of Advanced Life Science)	Kohei Yuyama
Associate examiner	Associate Professor (Research Faculty of Agriculture)	Yuta Murai

Title of Doctoral Dissertation

Design and evaluation of novel biomimetic molecules with potential pharmaceutical applications
(医薬品応用を指向した新規バイオミメティック分子の設計とその評価)

Results of Evaluation of the Doctoral Dissertation (Report)

Alzheimer's Disease (AD), the most common form of dementia, is clinically characterized by progressive impairment of cognition and memory loss, affecting mainly older people. About 50 million people suffer from AD worldwide, and the number is expected to triple by 2050 if no preventive measures are taken. Pathologically, AD is characterized by the accumulation of Amyloid-beta (Aβ) in brains. Aβ accumulation is caused by impaired clearance of Aβ in the sporadic form of AD and from increased production due to genetic mutations of amyloid precursor protein (APP) or Aβ processing enzymes in the less-common familial form of AD. Hence, decreasing Aβ formation or inducing their clearance can be considered one of the main AD treatment strategies. Previously, the novel exosome-focused method of Aβ clearance has been proposed. Exosomes are nano-scaled extracellular vesicles that carry a variety of cargos including proteins, nucleic acids, and bioactive lipids, and are known for their important role in cell-to-cell communication. Aβ is believed as one of the cargos carried by exosomes derived from neuronal cells of AD patients. In fact, linking the increase of exosome production and the decrease of Aβ accumulation in neurons has been demonstrated. On the other hand, Ceramide, a group of bioactive sphingolipids, has recently been known as a crucial role in exosome production. Oral administration of plant-type ceramide, mainly composed of sphingadienine, promotes neuronal exosome production and alleviates Aβ accumulation in a human amyloid precursor protein (APP) transgenic mouse. Ceramide induces exosome production in a way that is independent of endosomal sorting complex (ESCRT) machinery. Binding of animal-type and plant-type ceramide to lysosome-associated protein transmembrane 4B (LAPTM4B) through its sphingolipid-binding motif was reported to be one of the mechanisms for ceramide-induced exosome production. As the mechanism of ceramide-induced exosome production is still not completely understood, studying the relation between ceramide structure and exosome production activity is necessary. Ceramide, the sole exosome-increasing substance in neurons, has two asymmetric carbons. Therefore, theoretically, there could be four stereoisomers, but the natural form is believed to be of the D-erythro type. Stereochemistry has been involved in the process of drug design and development as a major detrimental factor for drug pharmacodynamic and pharmacokinetic behavior. Studies comparing the different stereoisomers' drug action have been essential for many drugs. Sphingolipid stereoisomers have been reported to show variable responses with different biological targets. For instance, the threo-type isomers were found to have higher activity in the induction of apoptosis of U937 cancer cells, inhibition of mitochondrial ceramidase, inhibition of sphingosine-kinase and inhibition of glucocerebroside

synthetase. Nevertheless, the erythro-type isomers showed higher potency in sphingosine-induced phosphorylation in Jurkat T cells and in the disruption of Golgi complex associated with several cellular events. Herein, this study is the first report on the effect of the stereochemistry of ceramide on exosome production from neuronal cells.

Sphingosine has two asymmetric stereocenters on carbons C2 and C3. So, there are an erythro enantiomeric pair (D-erythro sphingosine: DE and L-erythro sphingosine: LE) and a threo enantiomeric pair (D-threo sphingosine: DT and L-threo sphingosine: LT). The four diastereoisomers of sphingosine with four different tails were synthesized according to the previously established protocol by mainly our group with a few variations. The procedure starts with methyl esterification of D- and L-serine, followed by Boc protection of the amino group and acetal protection of the primary hydroxyl and secondary amino group to give a fully protected D- and L-serine. Conversion of the methyl ester group to phosphonate was required for the formation of the trans double bond on C4 through the Horner-Wadsworth-Emmons (HWE) reaction to give a pair of enantiomeric protected β-ketosphingosines. Erythro-type sphingosines were obtained by the stereoselective reduction of the corresponding β-ketosphingosine with zinc borohydride followed by acid hydrolysis. For the synthesis of the threo-type sphingosines, L-selectride was used instead of the zinc borohydride. Finally, the desired ceramides were obtained by acylation of the amino group of sphingosines with the appropriate acid. The synthesized compounds were characterized by mass spectroscopy and ^{1}H-NMR.

SH-SY5Y cells were treated with each ceramide isomer of 10 μM concentration for 24 h, and exosome levels were measured in the conditioned medium. First, to survey the ceramide effects expeditiously, the conditioned medium was concentrated using centrifugal filtration devices. And then, the exosomes in the concentrated medium were measured by the exosome sandwich enzyme-linked immunosorbent assay (ELISA) system with T-cell immunoglobulin and mucin domain-containing protein 4 (TIM4), phosphatidylserine (PS)-binding protein, and antibody against CD63, an exosome marker protein. The results indicate that exosome production is dependent on the *R* configuration of C3, and to a much lesser extent on the *S* configuration of C2. Furthermore, C16 and C18 fatty acid tails are optimal for activity, which comes in agreement with our previous report. The 24h-treatment with Ceramide isomers tested in this study did not show cell toxicity. The measurements of the exosomes collected by the ultracentrifugation method also showed the effects of DE and DT Ceramide to induce exosome production. Additionally, consistent with the ELISA results, an analysis with a nanoparticle analyzer also revealed that DE Ceramide and DT ceramide with C16 and C18 tails increased exosome particles. Ceramide treatment did not alter the size of exosome particles; the diameter remained at 40–160 nm, with a peak at ~100 nm. It has been reported that ceramide is endocytosed into cells and interacts with LAPTM4B within lysosomes, leading to exosome production. Future studies are needed to analyze the efficiencies of the Ceramide isomers for internalization into the cells or the binding activities on LAPTM4.

Next, a transwell culture system was used to determine whether the increase in exosomes, induced by the treatment with the four C18 Ceramide, promotes Aβ clearance. The exosomes and Aβ are secreted from Aβ-overexpressing SH-SY5Y cells placed on upper inserts that can flow through the membrane into bottom wells with microglial BV-2 cells placed. Under this experimental setting, the ceramide isomers were added to the cultures at 10 μM, and after 24 h of co-incubation, the levels of Aβ in the medium were determined by Aβ ELISA. Both Aβ40 and Aβ42 in the culture media decreased following incubation with DE-18 and DT-18, but not LE-18 or LT-18. In addition, the concentrations of Aβ40 were significantly lower after treating LE-18 than LT-18, showing that LE-18 has a higher potency of exosome-dependent clearance of extracellular Aβ.

In conclusion, the author has new findings on the crucial role of stereochemistry in ceramide-induced exosome production from neurons and decreasing extracellular Aβ levels. DE and DT isomers with C16 and C18 tails showed the best activity without a significant change in the particle size of the released exosomes, implying the essential *R* configuration of C3. Therefore, these findings will contribute to developing natural product-derived ceramide as potential functional food material and natural medicine for exosome-related AD prevention.

Therefore, we acknowledge that the author is qualified to be granted a Doctorate of Life Science from Hokkaido University.