A Novel Phytosphingosine Based 1-*O*-Acylceramide: Synthesis, Physicochemical Characterization, and Role in The Lipid Lamellar Organization

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Abstract

Background: Sphingosine-based 1-*O*-acylceramides were first identified in 2013 and speculated to contribute to the stability of lipid lamella phase organization in the human stratum corneum (SC). A novel phytosphingosine-based 1-*O*-acylceramide was synthesized and characterized via physicochemical analyses, molecular dynamics (MD) simulations, and *in vivo* human study.

Methods: Optimizing of a selective 1-*O*-acylation condition, LC/MS, and ^{1}H NMR analyses for structural identification, DSC, XRD analyses, and MD simulation were performed. *In vivo*, a human study for the proof of concept was performed.

Results: 1-*O*-stearoyl-ceramide NP (CerENP) was produced by optimizing the selective 1-*O*-acylation process. LC/MS and ^{1}H NMR analyses confirmed its correct 1-*O*-acyl conformation. DSC analysis revealed that CerENP increased the transition temperature of the 2D multi-layered lamellar membranes while lowering the total heat capacity. An SC mimetic-nanovesicle (SCNV), a 3D multi-lamellar vesicle, showed significantly improved long-term stability in a dose-dependent manner. These findings indicate that CerENP is essential for stabilizing the structure of SCNVs containing SC lipids. MD simulation in conjunction with radial distribution function (RDF) analysis further supports that CerENP can enhance the permeability barrier function of SC lamellar organization via a bidirectional anchoring mode of action. A human study demonstrated that CerENP synergistically enhances skin barrier function in combination with ceramide NP.

Conclusion: CerENP was proved to have a novel function in stabilizing the SC lamellar organization. A proposed bidirectional anchoring model for this new class of ceramide was further supported by a human study.

Keywords: 1-*O*-stearoyl ceramide NP; Lamellar organization; Molecular dynamic simulation; Bidirectional anchoring model; Human study.

Introduction

The intercellular lipid matrix in the human stratum corneum (SC) is a unique lamellar arrangement in which ceramide, free fatty acid, and cholesterol are organized as the main lipids [1]. Lipid composition and organization in the SC play an essential role in the characteristic properties of the SC lipid matrix correlate to a skin barrier function [2]. There are at least 14 classes of ceramide in the human stratum corneum depending on sphingoids and fatty acids binding to the amino group of sphingoids to form an appropriate lipid lamella organization [3,4]. The numbers of ceramides identified in the mammalian stratum corneum have been increasing with the advent of analytical technology for lipids, largely thanks to the LC/MS/MS skills [2]. Given that there are hundreds of ceramide species in human skin, it is challenging to functional characterization and mass production of all the ceramide species for their application in clinics and cosmetics. Numerous studies on physicochemical properties of ceramides having different sphingoid types and alkyl chain lengths have been conducted. Molecular dynamics (MD) simulation is a powerful tool to investigate the structures and interactions among lipid components at the atomistic level and has been used in numerous studies of the SC [5,6]. 1-*O*-acylceramides as a new class of epidermal ceramide were first identified in humans and mice [7]. 1-*O*-acylceramides contain a different acyl chain at the 1-*O*-position. Based on their chemical structure and hydrophobicity, 1-*O*-acylceramides have been speculated to play an essential role in the stabilizing lipid lamellar structure and maintaining permeability barrier despite their relatively low abundance (2~3% of all ceramides) in the skin barrier. However, the role of this new ceramide class in skin barrier function has not been fully elucidated. No reports on phytosphingosine-based 1-*O*-acylceramide and functional characterization of this new class of ceramide have been known. In this study, we synthesized phytosphingosine-based 1-*O*-acylceramides, 1-*O*-stearoyl ceramide NP (CerENP), and proposed a 'bidirectional anchoring model' for the mode of action of 1-O-acylceramide. We performed physicochemical analyses and molecular dynamics (MD) simulations to verify the proposed model's possible role of 1-*O*-acylceramides in SC lipid lamellar organization. Finally, a vehicle-controlled human study was conducted to confirm this notion.

Materials and Methods.

Synthesis and identification

Optimization of the 1-*O*-acylation process was done to obtain phytosphingosine-based 1-O-stearoyl ceramide NP. LC/MS and ^{1}H NMR analyses were conducted to identify the structure and conformation of the CerENP.

Physicochemical characterization and MD simulation

Multilayered lamellae structures were fabricated through the equimolar molecular assembly of ceramides (CerNP and CerENP), cholesterol, and stearic acid. The lamellar structure and phase properties of the fabricated multilayered lamellae were investigated by using DSC, XRD, and cryo-TEM analyses. MD simulation was performed to investigate the effect of CerENP on the LPP structure and skin barrier characteristics. Two LPP models were constructed, 1) Model A has an equimolar Cer:Chol:FFA ratio with a Cer mixture, CerEOS:CerNS:CerNP ratio of 46:41:13, 2) Model B contains additional CerENP (10% of CerEOS) from Model A. For hydrations, 5 waters per lipid were placed. All simulations were performed using GROMACS[4] with CHARMM36 force field [8]. Steepest-descent energy minimization was performed first to relax the constructed system, which was further equilibrated by performing NVT (constant number of particles, volume, and 305 K temperature) and NPT (constant number of particles, 1 bar pressure, and 305 K temperature) simulations with positional restraints on the heavy atoms. Productions runs were performed in NPT ensemble without any restraints to fully relax the system.

Human study

Skin hydration and SC cohesion were measured by using a Corneometer and Tewameter TM300 (Courage & Khazaka, Cologne, Germany). SC cohesion was expressed by calculating ΔTEWL (ΔTEWL = TEWL immediately after tape stripping – basal TEWL). TEWL was measured after 15 tape-strippings on the forearm of volunteers. Permeation of CerENP into the deep layers of SC was analyzed by TLC, LC/MS from the SC sample collected by tape-stripping.

Results.

1-O-Acylceramide Synthesis and a Proposed Action Model

To establish optimized conditions for 1-*O*-acylceramide, ceramide/acyl donor ratio, reaction time, the volume of solvent, and biocatalysts were considered to reduce side reactions. Optimization of the 1-*O*-acylation process was successfully established to obtain phytosphingosine-based 1-O-stearoyl ceramide NP, CerENP. LC/MS and ^{1}H NMR analyses were conducted to confirm the structure and conformation of the CerENP. The results showed the reaction product had the correct 1-*O*-acyl configuration to be 1-*O*-stearoyl ceramide NP. Based on its characteristic chemical structure showing bidirectionally extended the two acyl chains, it is assumed that 1-*O*-acylceramide might function similarly to an anchor bolt-like action. Therefore, we propose a “Bidirectional Anchoring Model” for a mode of action of CerENP and aimed to prove it in this study (Fig. 1).

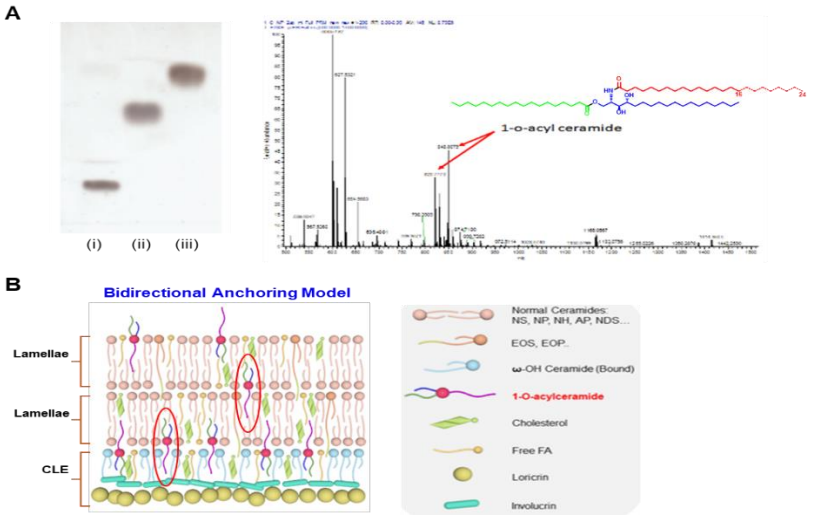

Figure 1. (A) Synthesis of 1-*O*-acylceramide (CerENP) and structural identification: (i) Ceramide NP, (ii) FA (C18:0), and (iii) CerENP. (B) Proposed action model, "Bidirectional Anchoring Model" based on the chemical structure.

Pysicochemical charaterization

To investigate how the incorporation of CerENP affects multilayered lamellar structure in SC model membrane, we produced the 3-D multilamellar vesicles as SC mimetic-nanovesicles (SCNVs) using CerNP/cholesterol/fatty acid (model SC lipids) in combination with CerENP. The average particle size of the nanovesicles was 200～300 nm. Their TEM images showed a typical lamellar structure regardless of the presence of CerENP. In addition, the SCNVs displayed typical Maltese cross patterns under a polarized microscope, indicating a concentric lamellar structure (Fig. 2A). However, it is difficult to maintain the initial structure of spherical multilamellar vesicles consisting of the SC lipids because of the intrinsic property of the SC lipids to assemble into planar membranes. Considering the structure of CerENP with bidirectional acyl chains, we expected that CerENP can be advantageous to make the SCNVs maintain their structure by connecting individual layers in the multilamellae. To verify it, we investigated the change in the size of the SCNVs depending on the incorporated concentration of CerENP. As a result, the SCNVs showed significantly improved long-term stability with the increase in the incorporated CerENP (Fig. 2B). These findings indicate that CerENP is important for stabilizing the structure of SCNVs containing SC lipids.

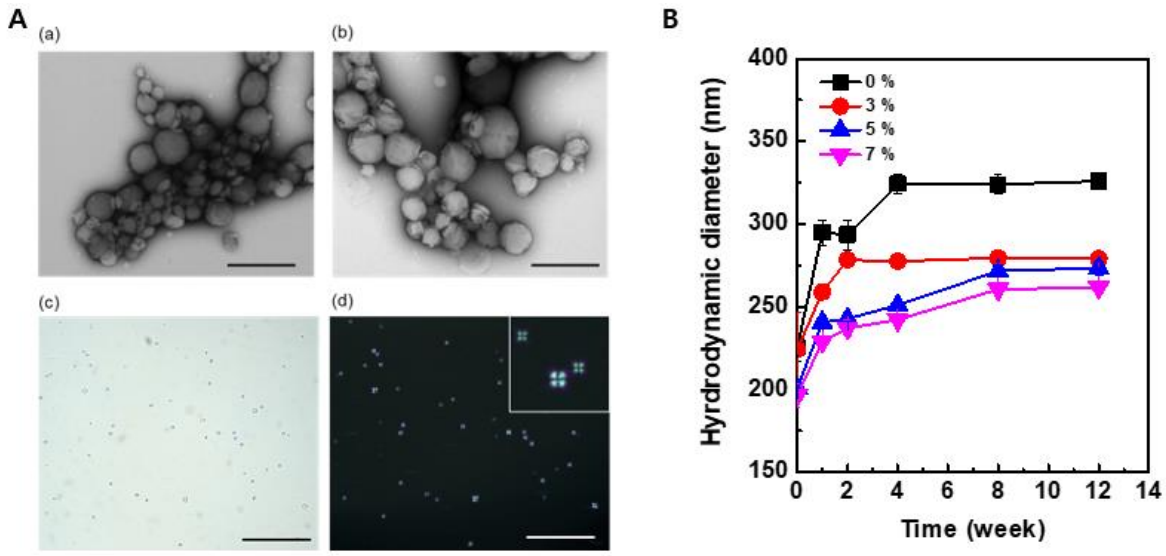

Figure 2. A. TEM images of SCNVs: (a) 0% CerENP, (b) 7% CerENP [Scale bar = 500 nm]. (c) Bright-field microscope and (d) polarized microscope images of SCNV containing 7% CerENP [Scale bar = 50 μm]; **B.** Size change of SCNVs a function of time during storage at 4 ℃ for the different concentrations of CerENP incorporated in the SCNVs.

Molecular dynamics modeling

We constructed two lipid matrix models (Fig. 3A), based on the long periodicity phase (LPP) model with ~13 nm repeat distance, the bilayer−slab−bilayer (sandwich) structure [6]. We observed the rigid (ordered) lipid bilayers and the relatively flexible (disordered) slab layer, which is consistent with the previous study of the LPP model. In particular, the high concentration of CerEOS is important for the LPP structure and fluid-like property of the slab layer in which the linoleate tail of the long fatty acid chain of CerEOS apparently plays a role. Cholesterols (Chol) are concentrated within the bilayers while free fatty acids (FFA) are distributed throughout the whole lipid matrix. Water molecules are placed at the interfaces between the bilayer and slab layer with other polar groups of lipid components, such as head groups of lipids and ester groups of CerEOS. Interestingly, the result of over 700 ns MD simulations shows that the inserted CerENP molecules cause a significant change in the surface area (SA) by ~10% (Fig. 3B). The splayed CerENP molecules are likely to induce not only more disorder in the slab layer but also denser bilayers, resulting in a smaller SA. This suggests that the denser lipid matrix by adding CerENP may suppress the permeability of the SC, and thus play a role in preventing water loss from the skin, although further studies and experimental validation should be followed. We believe that this computational approach together with experiments can provide insights into understanding the role of 1-*O*-acylceramides on the SC and developing cosmetic products.

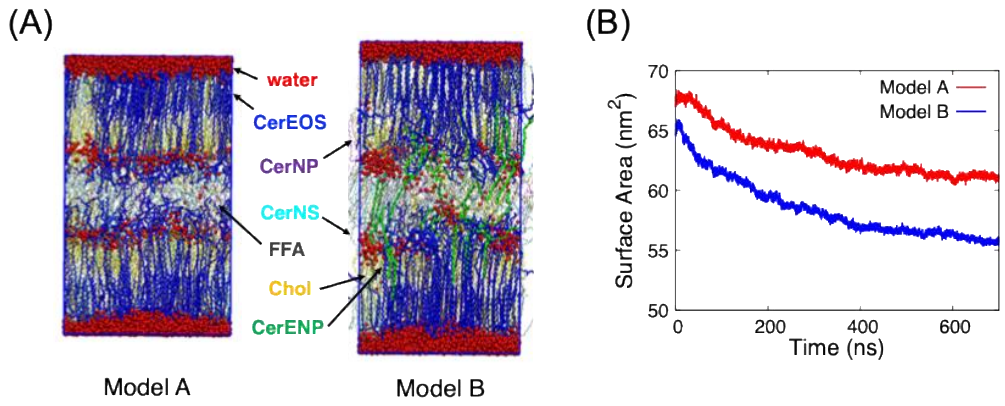

Figure 3. Atomistic model structures of stratum corneum. (A) Two model structures of LPP lipid matrix, where Model B contains additional CerENP, and CerEOS and CerENP are highlighted with thick lines. (B) Comparison of surface area as a function of simulation time for Model A and B.

SC permeation of 1-*O*-acylceramide

A cream containing 0.5% CerENP was applied to the forearms of 5 volunteers. After 30 min., the stratum corneum samples were obtained by fifteen tape strippings using D-squame standard tape. Skin ceramides were extracted using methanol. To analyze ceramides, the extracts were dissolved in chloroform/methanol (1/9, v/v) and diluted 10-fold with methanol. Aliquots were subjected to the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system to analyze CerNP (18:1/18:0) and CerENP (18:0/18:1/18:0) in the samples. As shown in Figure 4, the levels of CerENP (18:0/18:1/18:0) (p=0.006) were significantly increased in the treatment group. The results indicated that 1-O-Acylceramide could quickly and significantly permeate into the intercellular space of the SC.

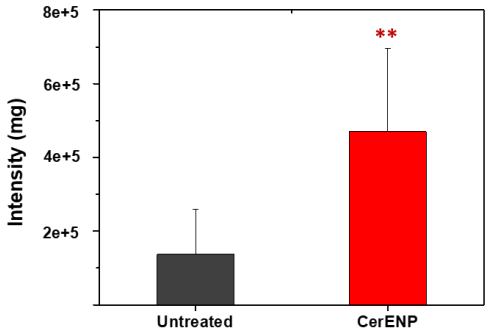

Figure. 4 A significant increase of 1-*O*-stearoyl ceramide NP (CerENP) was observed from the skin applied with CerENP

Human study on skin barrier functions

A vehicle-controlled human study was conducted to find a relative concentration of CerENP to CerNP when both ceramides were formulated together. Twenty volunteers initially participated and 18 of them completed the test. Test creams TC1, TC2, and TC3, which all contained 0.2% of CerNP, were prepared by adding 0%, 0.02%, and 0.05% of CerENP to the control cream, respectively. A base cream containing no CerNP was used as vehicle control. Volunteers were asked to apply test creams twice a day for four weeks. The skin barrier functions, including basal TEWL, SC hydration, and SC integrity, were measured. Skin hydration and basal TEWL were assessed at baseline and every week for four weeks. SC hydration was significantly higher in the site applied with TC2, and TC3 than with the TC1, a control cream that contains only ceramide NP ($p < 0.001$; Fig. 5A). Compared to baseline, both TC2 and TC3 showed an increase of more than 50%. It is noteworthy that the ratio of NP to ENP in the formulation. The ratio of ENP to NP seems to be appropriate at 10 to 25% ,which is similar to the ratio of NPs and ENPs found in human skin. SC integrity, expressed by calculating ΔTEWL (ΔTEWL = TEWL immediately after tape stripping – basal TEWL), was significantly enhanced in the site applied with the TC2 and TC3 ($p = 0.004$, 0.015 respectively; Fig. 5B). This study was approved by the Institutional Review Board of Dongguk University Committee on Human Research (Approval no. DUIRB202205-01). Written consent of participation from each volunteer was documented according to the ethical standards.

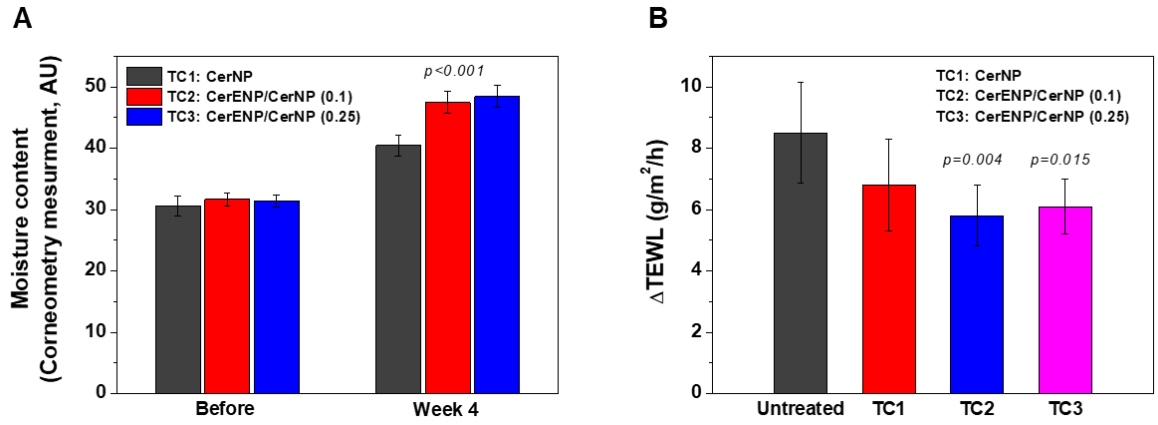

Figure 5. Skin barrier functions after 4-weeks of application of test cream compared to vehicle cream. SC hydration (A), SC integrity (B), Data represent mean ± SEM (n= 18)

Discussion.

This work is the first report on the synthesis of a novel 1-*O*-acylceramide, CerENP. All of the 12 significant classes of ceramide, which are created by a combination of 4 sphingoids and 3 different

fatty acids such as NS, NP, NG, AP, EOS, and EOP, have only one acyl chain attached to the amino group. On the other hand, CerENP contains one more acyl chain at the 1-*O*-acyl position. Chemically, CerENP is expected to be more hydrophobic than other ceramides, and structurally, the hydrophilic head group is flanked by two hydrophobic acyl chains. Therefore, we postulated that it might play a critical role in stabilizing the SC multilamellar organization through bidirectional anchoring neighboring lipid lamellas. As expected, in the presence of CerENP, the SCNVs, an SC mimetic model for the multilamellar organization, showed improved long-term stability while maintaining the initial multilamellar structure, providing direct proof of its role in stabilizing the SC multilamellar organization. Our notion for the mode of action was further supported by the data obtained from MD simulation studies. Skin barrier function was A significant improvement of skin barrier functions was observed from the skin site applied with a very low relative concentration of CerENP compared to CerNP. The ratio of CerNP to CerENP of TC2 was 10 to 1, yet more than a 17% increase in hydration was observed compared to TC1, which contains only 0.2% of CerNP in the cream. The result was consistent with the fact that the content of 1-O-acylceramides was estimated at 2~3% of the total content of all ceramides in SC.

Conclusion.

1-*O*-stearoyl ceramide NP, a novel phytosphingosine-based 1-*O*-acylceramide has been demonstrated to play an essential role in human SC. All the results strongly suggest that the mode of action of this new ceramide is very much likely exerted via a “bidirectional anchoring model” to stabilize the lipid lamellar organization as proposed. It is noteworthy that a synergistic effect was clearly demonstrated from the human skin test when it was formulated in combination with ceramide NP.

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Conflict of Interest Statement.

NONE.

References.

1. Bouwstra, J. A.; Ponec, M., *Biochim Biophys Acta 2006, 1758, (12), 2080-95*.

2. van Smeden, J.; Hoppel, L.; van der Heijden, R.; Hankemeier, T.; Vreeken, R. J.; Bouwstra, J. A., *J Lipid Res 2011, 52, (6), 1211-21.*
3. van Smeden, J., Janssens, M., Gooris, G. S. & Bouwstra, J. A. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids 2014, 1841: 295-313.*
4. Moore, T. C., Hartkamp, R., Iacovella, C. R., Bunge, A. L. & McCabe, C., *Lipid Bilayers. Biophys. J. 2018, 114: 113-125.*
5. M. Lundborg, A. Narangifard, C. L. Wennberg, E. Lindahl, B. Daneholt, L. Norlen, *J. Struct. Biol. 2018, 203, 149.*
6. E. Wang, J. B. Klauda, *J. Am. Chem. Soc. 2019, 141, 16930.*
7. M. Rabionet, A. Bayerle, C. Marsching, R. Jennemann, H.-J. Grone, Y. Yildiz, D. Wachten, W. Shaw, J. A. Shayman, R. Sandhoff, *J. Lipid Res. 2013, 54, 3312.*
8. J. B. Klauda, R. M. Venable, J. A. Freites, J. W. O'Connor, C. Mondragon-Ramirez, I. Vorobyov, D. J. Tobias, A. D. MacKerell, R. W. Pastor, *J. Phys. Chem. B 2010, 114, 7830.*