Development of a Label-Free LC-MS/MS-Based Glucosylceramide Synthase Assay and Its Application to Inhibitors Screening for Ceramide-Related Diseases

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Abstract
Ceramide metabolism is known to be an essential etiology for various diseases, such as atopic dermatitis and Gaucher disease. Glucosylceramide synthase (GCS) is a key enzyme for the synthesis of glucosylceramide (GlcCer), which is a main ceramide metabolism pathway in mammalian cells. In this article, we developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to determine GCS activity using synthetic non-natural sphingolipid C8-ceramide as a substrate. The reaction products, C8-GlcCer for GCS, could be separated on a C18 column by reverse-phase high-performance liquid chromatography (HPLC). Quantification was conducted using the multiple reaction monitoring (MRM) mode to monitor the precursor-to-product ion transitions of m/z 588.6 → 264.4 for C8-GlcCer at positive ionization mode. The calibration curve was established over the range of 0.625-160 ng/mL, and the correlation coefficient was larger than 0.999. This method was successfully applied to detect GCS in the human hepatocellular carcinoma cell line (HepG2 cells) and mouse peripheral blood mononuclear cells. We also evaluated the inhibition degree of a known GCS inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) on GCS enzymatic activity and proved that this method could be successfully applied to GCS inhibitor screening of preventive and therapeutic drugs for ceramide metabolism diseases, such as atopic dermatitis and Gaucher disease.

Key Words: Ceramide, Chemotherapeutic multi-drug resistance, Gaucher disease, Glucosylceramide, Glucosylerceramide synthase, LC-MS/MS

INTRODUCTION
A deficiency of ordinary ceramides in the corneum is an essential etiologic factor for the dry and barrier-disrupted skin of patients with atopic dermatitis (Choi and Maibach, 2005; Imokawa, 2009; Mizutani et al., 2009). It is known that ceramides are produced from sphingomyelin (SM) and glucosylceramide (GlcCer) as a form of glycosphingolipids (GSL) by enzymatic reactions of sphingomyelinase (Kolesnick, 2002) and GlcCer deacylases (Vielhaber et al., 2001), respectively. Thus, in order to prevent the pathogenesis of atopic dermatitis, many pharmacological approaches have been performed to develop an inhibitor or activator, which could regulate dermal levels of the above lipid mediators (Imokawa, 2009).

GSL are membrane components as a group of membrane lipids in which the lipid portion is embedded in the outer side of the plasma membrane with the sugar chain extending to the extracellular site (Liu et al., 2004; Yoshizaki et al., 2008; Wang et al., 2009; Kartal Yandim et al., 2013). GSL have been known to be involved in many cellular processes, including growth, differentiation, morphogenesis, sensitivity, and response to exogenous compounds (Hakomori, 2008; Kartal Yandim et al., 2013). GlcCer is a crucial GSL metabolic intermediate, which serves as the precursor in the biosynthesis
of a variety of GSL, such as lactosylceramide (LacCer) and the remaining neutral and acidic oligoglycosphingolipids (van Meer and Holthuis, 2000; Duclos, 2001; Degroote et al., 2004; Ekiz and Baran, 2010).

GlcCer is generated by glucosylceramide synthase (GCS) and converted to LacCer by lactosylceramide synthase (LCS). GCS transfers the glucose from UDP-glucose to ceramide, and is degraded both by a glucocerebrosidase in lysosomes and by a non-lysosomal glucocerebrosidase in the cytosol (Maunula et al., 2007; Ekiz and Baran, 2010; Tuuf et al., 2011). GCS is known to be a transmembrane protein localized in the cis/medial Golgi, with an N-terminal signal-anchor sequence and a C-terminal catalytic domain located in the cytoplasm (Abe et al., 1995; Chujor et al., 1998; Miura et al., 1998; Zheng et al., 2006; Wang et al., 2009). This enzyme does not have similarity to other known glycosyltransferases. The structure of the enzyme is quite unique since all other glycosyltransferase involved in GSL synthesis are known to be localized to the lumenal side of the Golgi apparatus or ER (Di Sano et al., 1996; Hayashi et al., 2005). In this study, we report a new LC-MS/MS-based method that uses non-natural sphingolipid C8-ceramide (C8-Cer) and non-radioisotope UDP-glucose as substrates. The product, C8-glucosylceramide (C8-GlcCer), was separated by a C18 column and detected by mass spectrometer in the positive electrospray ion mode (ESI), and the intensity of C8-GlcCer was normalized by the internal standard C12-glucosylceramide (C12-GlcCer). To validate usefulness of this assay, the method was employed to determine the activities of GCS in HepG2 cell and mouse peripheral blood mononuclear cells (PBMC) lysate after incubation with a known GCS inhibitor, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). We successfully evaluated the inhibition degree of PDMP on GCS at different working concentrations to validate this LC-MS/MS-based GCS enzymatic activity assay. Furthermore, we confirmed its additional usefulness by evaluating simultaneously enzymatic activity of LCS, another enzyme involved in a cellular level of GlcCer. This method could be applied to GCS specific inhibitor screening for developing therapeutic drugs against chemotherapeutic multi-drug resistance and Gaucher disease.

### MATERIALS AND METHODS

**Materials**

HepG2 cells (Hepatocellular carcinoma cell line) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). C57BL/6 mice were purchased from Orient Bio (Gyeonggi, Korea). All animal experiments were performed in accordance with the National Research Council’s Guidelines for Care and Use of Laboratory Animals and the Guidelines for Animal Experiments of Chung-Ang University. C8-Cer, C8-GlcCer, and C12-GlcCer were purchased from Avanti Polar Lipids (AL, USA). Lecithin was obtained from Sigma (MO, USA). All other reagents were obtained at the highest available purity.

**Preparation of lipid standard stock solutions and calibration standards**

Each lipid was dissolved in methanol to prepare the standard stock solution to a final concentration of 1 mg/mL and stored at –80°C. This solution was further diluted in methanol with 0.1% formic acid to obtain standard solutions at several concentrations. Calibration standard samples were prepared by serial dilution with a known amount of each compound. For the internal standard (ISD) solution, C12-GlcCer was dis-
solved in methanol at the concentration of 100 ng/mL. Calibration standard samples were constructed using nine concentration points.

Cell culture
HepG2 cells were cultured at 37°C, 5% CO₂ in MEM supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin in a humidified incubator.

For cell harvest, HepG2 cells were detached from the culture plate using 0.25% trypsin/EDTA and washed thrice with ice-cold PBS.

Mouse PBMC isolation
PBMC were isolated from mouse peripheral blood using the Histopaque-1077 density gradient method. Briefly, whole blood was isolated from pathogen-free male C57BL/6 mice (6-8 weeks) into a 3 mL vacutainer tube coated with EDTA. Subsequently, 1 mL of blood was suspended with 2 mL PBS and carefully layered onto 3 mL Histopaque-1077. Following, the solution was centrifuged at 400×g for 30 min at room temperature. Finally, the PBMC layered in the interface was transferred to a new 15 mL tube and washed thrice with ice-cold PBS.

Preparation of enzyme from HepG2 cells and mouse PBMC
To prepare cell lysates, harvested HepG2 cells and mouse PBMC were suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, containing 1x protease inhibitor and 1 mM EDTA) and lysed by sonication. Following, the cell lysate was centrifuged at 1,000×g, 4°C for 10 min. The supernatants were transferred to a new tube prepared for the GCS activity assay.

Protein assay
The protein concentration of each sample was measured using the bicinchoninic acid protein assay (Pierce, MA, USA), with bovine serum albumin as the standard.

LC-MS/MS-based GCS assay procedures
Acceptor substrate, 1 nmol of C8-Cer, and 130 nmol of lecithin were mixed in 0.2 mL of methanol, followed by evaporation of the solvent. Subsequently, 0.2 mL of water was added, and the mixture was sonicated to form liposomes. For the GCS enzymatic activity assay, 5 µL of 5 mM UDP-Glc, 10 µL of 10 mM EDTA, 10 µL of 25 mM of conduritol B epoxide (CBE), 10 µL of C8-Cer liposome, and 65 µL of enzyme were combined to make a total of 100 µL. Enzyme activity assays were carried out at 37°C for 1 h. For the enzyme activity assay of LCS, 0.1 mM UDP-Gal, 5 mM MgCl₂, 5 mM MnCl₂, 2.5 mM of conduritol B epoxide (CBE), 10 µL of C8-GlcCer liposome, and 65 µL of enzyme were combined. Enzyme activity assays were carried out at 37°C for 1 h. The reaction was stopped by adding 600 µL of chloroform/methanol (1:2, v/v) and 20 µL of ISD (100 ng/mL of C12-GlcCer in methanol) solutions. After vortexing for 1 min, 100 µL of water was added. The mixture was vortexed for 5 min, incubated at room temperature for 10 min, and centrifuged at 5,000×g for 5 min. After centrifugation, the supernatants were taken, and 200 µL of water and 200 µL of methanol were added. The mixture was vortexed for 1 min, centrifuged at 10,000×g for 10 min, and the supernatants were transferred to new tubes. The supernatants were dried and reconstituted with 0.2 mL of methanol. The solution was dried with a nitrogen stream, and the residue was reconstituted with 0.2 mL of methanol. The mixture was vortexed for 1 min, centrifuged at 10,000×g for 10 min, and the supernatants were transferred to new tubes. The supernatants were dried and reconstituted with 0.2 mL of methanol.

Fig. 1. Ceramide metabolic pathway related to glucosylceramide. SMase, sphingomyelinase; GCS, glucosylceramide synthase; GCSase, glucosylceramidase; LCS, lactosylceramide synthase; GalCS, glucosylceramide synthase; GalCase, galactosylceramidase; CERK, ceramide kinase; CPase, Ceramide-1-phosphate phosphatase.
µL of chloroform were added. The samples were vortexed for 5 min and centrifuged at 2,000×g for 5 min to separate the organic phase. The organic phase was evaporated in a speed vacuum dryer. Following, 800 µL of 10 mM NaOMe was added to the dried residue and vortexed well at room temperature for 2 h. The samples were evaporated again and dissolved in 200 µL of 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7, v/v/v).

Liquid chromatography and tandem mass spectrometry
Analyses were determined using LC-ESI/MS/MS, consisting of an HPLC (Agilent 1100, CA, USA) coupled to an API3000 mass spectrometer with an electrospray ion (ESI) source. The analytical column was an X Terra MS C18 (2.0×50 mm, 3 µm, Waters Co., MA, USA). The mass spectrometer was operated in the positive ESI mode using multiple reaction monitoring (MRM). Nitrogen was used as the nebulizer gas and collision gas.

Separation was achieved using 0.1% formic acid added to methanol buffered with 2 mM ammonium formate/tetrahydrofuran (7/3, v/v) as mobile A and 5 mM ammonium formate (pH 4.0)/methanol (9/1, v/v) as mobile B. For HPLC separation, a gradient program was used at a flow rate of 200 µL/min. The initial buffer composition was 50% mobile A and 50% mobile B, then linearly changed to 100% mobile A in 5 min and maintained for 2 min, and immediately returned to initial condition and maintained for 2 min. Each run time was 10 min. Subsequently, 10 µL of sample was injected at a flow rate of 0.2 mL/min.

The optimal mass spectrometry values were obtained by a manual tuning or automatic tuning process with each standard injected by syringe pump. Monitoring ions of each sphingolipid are shown in Table 1. The data acquisition was confirmed by Analyst® 1.6.2 software (AB SCIEX, MA, USA).

The calibration curves for sphingolipid standards were generated by plotting the peak area ratio (analyte/internal standard) versus the concentrations in the calibration standard samples by least-square linear regression.

RESULTS
Ceramide can be synthesized by the condensation of serine with fatty acid acyl-CoA and then converted to other bioactive lipids. Ceramide is converted to ceramide-1-phosphate, GlcCer, sphingosine, etc. by each enzyme, which can be reversibly converted back to ceramide. Among them, GCS catalyzes glucosylation of ceramide, which forms GlcCer. This is the first time to develop a sensitive and selective LC-MS/MS system.
Determination of GCS activities in HepG2 cells and mouse PBMC cells using different protein concentrations and incubation times. GCS activities were determined by LC-ESI-MS/MS using various amounts (0-80 µg proteins) of lysates of HepG2 cells at 37°C for 1 h (A) and using 20 µg of lysate incubated at 37°C for various times from 0.5, 1, 1.5, or 2 h (B). GCS activities were determined by LC-ESI-MS/MS using various amounts (0-80 µg proteins) of lysates of mouse PBMC cells at 37°C for 1 h (C) and using 20 µg of lysate incubated at 37°C for various times from 0.5, 1, 1.5, or 2 h (D). Each point is the average of at least three determinations with a standard deviation. Details were described in the Materials and Methods section.

To disclose the detection limits of C8-GlcCer by LC-MS/MS method based on the reaction product determination to assess GCS enzymatic activity from the cell lysate. Due to the interference of endogenous sphingolipids, an excess C8-Cer was added as a substrate, and the generated C8-GlcCer was quantitatively determined (Fig. 1). In this method, C8-GlcCer and C12-GlcCer were separated by a reverse-phase LC method and quantified by ESI-MS/MS. Fig. 2 shows the precursor and product ion fragment peak. The MS parameter for each sphingolipid is shown in Table 1.

In our previous studies, we successfully separated sphingolipids at the start ratio of 80:20 (mobile A: mobile B); while in this study, we used C8-Cer as the GCS substrate. Both the substrate C8-Cer and the product C8-GlcCer have a shorter fatty acid chain, which means that they are less hydrophobic than the endogenous sphingolipids. Therefore, we reduced the hydrophobic phase at the start ratio to 50:50 (mobile A: mobile B), and both of the C8-GlcCer and internal standard were separated successfully. The retention time of each sphingolipid is shown in Fig. 2C.

To disclose the detection limits of C8-GlcCer by LC-MS/MS assay, the relationship between the amount of C8-GlcCer and peak areas were examined according to Agilent technical note, ‘signal, noise, and detection limits in mass spectrometry’. It was shown less than 0.625 ng/mL of reaction product, C8-GlcCer, could be detected quantitatively by the assay using reverse-phase HPLC-ESI-MS/MS and the respective mobile phase described above. The standard curve was linear from 0.625 ng/mL to 160 ng/mL of C8-GlcCer (Fig. 2D), providing both a sensitive and wide ranging assay for GCS.

Moreover, this method was used to evaluate the inhibition degree of GCS by PDMP, which is a known GCS inhibitor. PDMP was treated at 5, 10, or 50 µM in HepG2 cell and mouse PBMC lysates and incubated with the substrate mix for 1 h. The results are shown in Fig. 4. PDMP inhibited GCS in a dose-dependent manner, nearly 90% of GCS activity was inhibited when PBMD was treated at 50 µM. These results show that the inhibition degree of GCS enzymatic activity by PDMP depends on the target enzyme. Further, this method could be possibly applied to screening of specific GCS inhibitors for development of therapeutic drugs towards chemotherapeutic multi-drug resistance and Gaucher disease.

Finally, we analyzed LCS activity, an enzyme that converts GlcCer to LacCer, in HepG2 cells (Fig. 5). The precursor and product ion fragment peak is shown in Fig. 5A and 5B. A linear relationship between the protein amount of cell lysate added and C8-LacCer generation was observed at up to 80 µg of total protein for HepG2 cells and 40 µg of total protein for mouse PBMC. This was in the reaction mixture for the assay when the incubation was performed at 37°C for 1 h under the study conditions. The linearity lasted at least 2 h for HepG2 cells and 1.5 h for mouse PBMC when 20 µg of total protein was incubated with 1 µM substrate (Fig. 3B, 3D).

To disclose the detection limits of C8-GlcCer by LC-MS/MS method, LC-MS/MS was determined by LC-ESI-MS/MS using various amounts (0-80 µg proteins) of lysates of HepG2 cells at 37°C for 1 h (A) and using 20 µg of lysate incubated at 37°C for various times from 0.5, 1, 1.5, or 2 h (B). GCS activities were determined by LC-ESI-MS/MS using various amounts (0-80 µg proteins) of lysates of mouse PBMC cells at 37°C for 1 h (C) and using 20 µg of lysate incubated at 37°C for various times from 0.5, 1, 1.5, or 2 h (D). Each point is the average of at least three determinations with a standard deviation. Details were described in the Materials and Methods section.

**DISCUSSION**

Ceramide, central to sphingolipid metabolism, regulates the activity of various molecular and biochemical targets involved in anti-proliferative and cellular responses (Kartal Yandim...
Determination of LCS enzymatic activity of HepG2 cells. C8-LacCer (A) and C12-LacCer (B) were detected using an API3000 mass spectrometer with an Electrospray Ion (ESI) Source (AB SCIEX) at positive ion mode. Both C8-LacCer and C12-LacCer were eluted in 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7) at 10 ng/mL and separated on a reverse-phase C18 column using an HPLC (Agilent 1100) system with mobile A (5 mM ammonium formate (pH 4.0)/methanol, 95/5) and mobile B (2 mM ammonium formate in methanol with 0.1% formic acid/tetrahydrofuran, 7/3). (C) LCS activity was determined by LC-MS/MS using various protein amounts of lysates of HepG2 cells (0-80 µg proteins). The enzyme reaction was carried out at 37°C for 1 h. Each point is the average of four determinations. Details were described in the Materials and Methods section.

Fig. 5. Determination of LCS enzymatic activity of HepG2 cells. C8-LacCer (A) and C12-LacCer (B) were detected using an API3000 mass spectrometer with an Electrospray Ion (ESI) Source (AB SCIEX) at positive ion mode. Both C8-LacCer and C12-LacCer were eluted in 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7) at 10 ng/mL and separated on a reverse-phase C18 column using an HPLC (Agilent 1100) system with mobile A (5 mM ammonium formate (pH 4.0)/methanol, 95/5) and mobile B (2 mM ammonium formate in methanol with 0.1% formic acid/tetrahydrofuran, 7/3). (C) LCS activity was determined by LC-MS/MS using various protein amounts of lysates of HepG2 cells (0-80 µg proteins). The enzyme reaction was carried out at 37°C for 1 h. Each point is the average of four determinations. Details were described in the Materials and Methods section.
known to be an important sphingolipid, which plays a crucial role in the potency and effectiveness of chemotherapeutic drugs, such as doxorubicin-induced cell apoptosis. Therefore, GCS activity could determine the working concentrations of chemotherapeutic drugs to be administrated. Considering the possible toxic side effect of chemotherapeutic drugs at the desired concentrations, it is very important to reduce the dosage of these drugs. Currently, several inhibitors have been developed to inhibit GCS activity, including PDMP, 1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) and, which are known to be potent competitive inhibitors of GCS as structural analogues of its natural sphingolipid substrate (Abe et al., 1992, Senchenkov et al., 2001). PDMP and its analogue PPPP at concentrations that maximally inhibit GlcCer synthesis have no effect on cell viability, but they sensitize cells to doxorubicin (Senchenkov et al., 2001). PDMP is firstly known as a GCS inhibitor, which has been reported to suppress GCS activity and to potentiate the apoptotic effect of C6- ceramide (Vunnam and Radin, 1980; Senchenkov et al., 2001). Several drugs have been used to treat Gaucher disease. For instance, macrophage-targeted enzyme replacement therapy (ERT) has long been the accepted form of treatment for Gaucher disease, but there are still several limitations in treating all aspects of this disease (Limgala et al., 2016). To complement the disadvantages of ERT, oral substrate replacement therapy (SORT) was developed using GCS inhibitors. SORT minimized the accumulation of GlcCer within cells by inhibiting GCS (Limgala et al., 2016; Shawky and Elsayed, 2016). Recently, some GCS inhibitors were also developed to treat Gaucher disease at a high cost (Bennett and Mohan, 2013; Nagral, 2014). In the present study, we developed a sensitive and specific LC/MS/MS-based GCS enzymatic activity assay method and validated by using PDMP as the best known GCS inhibitor (Radin, 1996). Further, this method successfully evaluated the inhibition degree of PDMP on GCS at different working concentrations, showing that this assay could be applied to a GCS-specific inhibitor screening for developing therapeutic drugs against chemotherapeutic multi-drug resistance and Gaucher disease.

**CONFLICT OF INTEREST**

The authors declare no conflicts of interest.

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