Chapter 2

Assaying Ceramide Synthase Activity In Vitro and in Living Cells Using Liquid Chromatography-Mass Spectrometry

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Abstract

Sphingolipids are one the major lipid families in eukaryotes, incorporating a diverse array of structural and signaling lipids such as sphingomyelin and gangliosides. The core lipid component for all complex sphingolipids is ceramide, a diacyl lipid consisting of a variable length fatty acid linked through an amide bond to a long chain base such as sphingosine or dihydrosphingosine. This reaction is catalyzed by a family of six ceramide synthases (CERS1-6), each of which preferentially catalyzes the synthesis of ceramides with different fatty acid chain lengths. Ceramides are themselves potent cellular and physiological signaling molecules heavily implicated in diabetes and neurodegenerative diseases, making it important for researchers to have access to sensitive and accurate assays for ceramide synthase activity. This chapter describes methods for assaying ceramide synthase activity in cell or tissue lysates, or in cultured cells (in situ), using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the readout. LC-MS/MS is a very sensitive and accurate means for assaying ceramide synthase reaction products.

Key words Ceramide, Ceramide synthase, Assay, Mass spectrometry, LC-MS, Liquid chromatography, CERS

1 Introduction

The importance of ceramides as mediators of physiological and pathological processes is now well established and recent advances in mass spectrometry have enabled researchers to delve into the roles for specific forms of ceramide as mediators of different functions and pathologies [1, 2]. Ceramides appear to be particularly important as mediators of metabolic distress and insulin resistance, and neurodegenerative conditions [3–5]. At the cellular level, ceramides are an integral component of the cell death signaling machinery and play an essential role in cell differentiation [6, 2].

Ceramides are synthesized through the addition of a variable length fatty acid to the amine group of a sphingoid base. In mammalian cells, the sphingoid base used for de novo ceramide synthesis is usually the C18:0 lipid dihydrosphingosine (Fig. 1), formed

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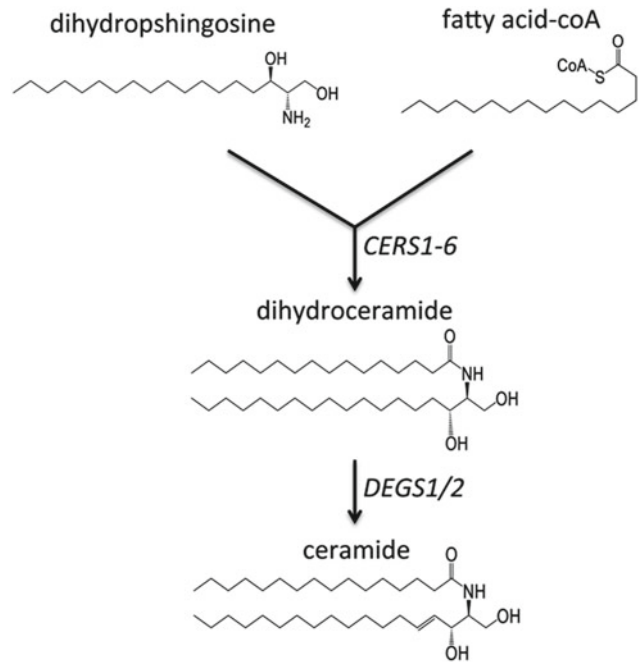

Fig. 1 Diagrammatic illustration of ceramide synthase activity. CERS enzymes catalyze the transfer of a variable length fatty acid, linked to coenzyme A (C16:0 fatty acid is shown), to dihydrosphingosine. In living cells, the dihydroceramides formed are rapidly desaturated by ceramide desaturases (DEGS1/2), forming ceramides. Note that CERS1-6 can use sphingosine as a substrate in place of dihydrosphingosine, directly forming ceramides rather than dihydroceramides

from the condensation of serine and palmitoyl-coenzyme A (C16:0-CoA). C16 or C20 sphingoid bases occur at much lower abundance [7, 8]. Ceramide synthesis is catalyzed by a family of six ceramide synthases (CERS1-6), each of which preferentially transfers fatty acids of different lengths to the amine group of dihydrosphingosine. Thus, CERS1 preferentially catalyzes the transfer of a C18:0 fatty acid, forming C18:0 dihydroceramide (d18:0/18:0 ceramide); CERS2 preferentially transfers very long chain fatty acids (C22–C26); CERS3 transfers even longer chain fatty acids (C26, C28), forming highly hydrophobic ceramides that are a very important part of the water barrier function of skin; CERS4 transfers C18 and C20 fatty acids; CERS5 transfers C16 fatty acids, and potentially also C14 and C18 fatty acids; and CERS6 transfers C14 and C16 fatty acids [1, 9, 10].

To assay CERS activity using LC-MS/MS, crude extracts containing cell membranes are firstly prepared from tissues or cultured cells. These extracts are used as the enzyme source for reactions containing deuterated dihydrosphingosine (or sphingosine) and a fatty acid substrate linked to CoA. The precise product formed is

naturally dependent on the substrates used. For example, C24:1 dihydroceramide (d18:0/24:1 ceramide) is formed in a reaction using dihydrosphingosine (C18:0) and C24:1 fatty acid-coenzyme A (nervonoyl-CoA), whilst C24:0 ceramide (d18:1/24:0 ceramide) is formed from sphingosine (C18:1) and C24:0-CoA (lignoceroyl-CoA). Reactions are stopped with the addition of four volumes of methanol and, after clearing insoluble material, reaction products are quantified directly from this reaction mixture using LC-MS/MS. A standard curve may be prepared from commercially available dihydroceramide standards for absolute quantification.

The method we describe is intended for a triple quadrupole MS, as these instruments are very common and exhibit the greatest dynamic range. Users with different instrumentation would need to adapt the instrument set-up and product detection conditions to their particular MS, but the HPLC conditions should remain constant. Although dihydroceramide reaction products could be detected following direct infusion into the MS electrospray source, the use of an HPLC column provides improved sensitivity, greater confidence in identification of the product, and more accurate quantification based on HPLC peak areas. As shown in Fig. 2, the limit of sensitivity using LC-MS/MS on a Thermo Fisher Scientific Quantum Access triple quadrupole mass spectrometer is <40 fmoles on column, permitting the detection of as little as 0.5 pmoles product formed in a standard 50 μL reaction with 500 pmoles substrate.

Compared to traditional TLC methods for product quantification, LC-MS/MS permits the separation and quantification of closely related but structurally distinct ceramide species. For example, C24:1 dihydroceramide and C24:0 dihydroceramide are easily

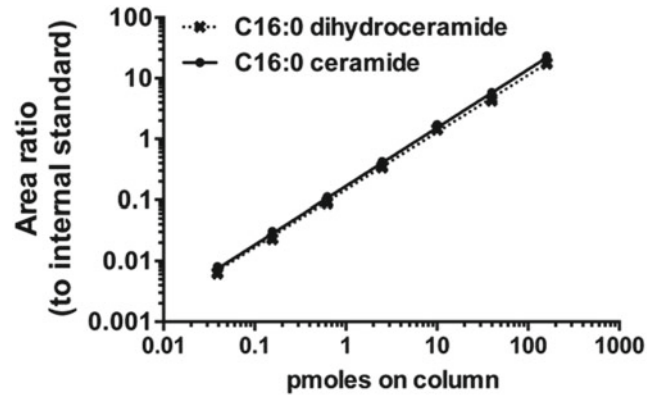

Fig. 2 Linearity of peak areas as a function of pmoles loaded for C16:0 ceramide and C16:0 dihydroceramide. Peak areas are expressed as ratios to 50 pmoles C17:0 ceramide internal standard. The amounts shown (*x*-axis) are pmoles on column (i.e. pmoles/20 μL)

distinguished on the basis of both mass and HPLC elution time. Even ceramide variants with identical mass, such as C24:1 dihydroceramide (d18:0/24:1) and C24:0 ceramide (d18:1/24:0), can be distinguished on the basis of HPLC elution time and differing fragment ions produced under interrogation with tandem mass spectrometry: although both molecules are detected as an ion with mass/charge ratio (m/z) of 650.64, ceramides produce an m/z 264.1 product ion following collision-induced dissociation, whilst the equivalent fragment ion produced by dihydroceramides has m/z 266.1. LC-MS/MS is therefore a very sensitive and accurate approach for quantifying the products of ceramide synthase reactions. Researchers without ready access to LC-MS instrumentation may consider fluorescence-based assays described in Chapter 3, or traditional radioactive methods [11, 12].

This chapter also includes a method for analyzing ceramide synthase activity in cultured cells. In this method, cells are incubated with deuterated (D7) dihydrosphingosine, which is converted in the cells by ceramide synthases to D7-dihydroceramides, and then to D7-ceramides. These cellular lipids are extracted using a method adapted from Bielawski et al. [13], and quantified using LC-MS/MS.

2 Materials

2.1 Cell or Tissue Lysis for In Vitro Assays

1. Phosphate Buffered Saline (PBS). Can be purchased directly from various suppliers.
2. Lysis buffer: 20 mM Hepes, pH 7.4, 10 mM KCl, 1 mM dithiothreitol, complete protease inhibitor cocktail (Roche), and 3 mM β-glycerophosphate (*see* **Note 1**).
3. Total protein assay kit. We use the BCA assay kit.
4. Plastic cell scrapers.

2.2 CERS Reactions

1. Fatty acid-coenzyme A conjugates may be purchased from Sigma-Aldrich or Avanti Polar Lipids. These may be reconstituted at 5 mM with water and stored in aliquots at −20 °C.
2. Deuterated (D7) dihydrosphingosine is purchased from Avanti Polar Lipids, reconstituted at 10 mM in methanol or DMSO, and stored at −20 °C (*see* **Note 2**).
3. C17 ceramide (d18:1/17:0) or C17:0 dihydroceramide (d18:0/17:0) internal standard (available from Avanti Polar Lipids). This is reconstituted at 5 mM in methanol and stored at −20 °C.
4. Reaction buffer: 20 mM Hepes, pH 7.4, 25 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0.1 % (w/v) fatty acid free BSA

(*see* **Note 3**), 10 μM dihydrosphingosine, and 50 μM of the appropriate fatty acid-CoA (e.g., C16:0-CoA or C24:0-CoA).

5. Eppendorf Thermomixer or similar instrument for agitated incubation of 1.5 mL tubes.
6. Glass HPLC vials with fused inserts and caps. We use screw top glass vials with 0.3 mL fused inserts, and matched rubber caps with PTFE septa.

2.3 Quantification of Dihydroceramide Products Using LC-MS

1. Mass Spectrometer (MS) equipped with autosampler and LC system. We describe settings for a Thermo Fisher Scientific Quantum Access triple quadrupole MS coupled to a Thermo Fisher Scientific Accela UPLC.
2. A C8 or C18 reverse phase chromatography column. Column elution times are shorter with a C8 column (Table 1). The protocol described herein uses an Agilent 3×150 mm XDB-C8 column (5 μM pore size) or an Agilent Poroshell 120, 2.1×150 mm SB-C18 column (2.7 μM pore size).
3. Dihydroceramide standards for quantification (d18:0/16:0, d18:0/18:0, d18:0/24:0, d18:0/24:1), purchased from Avanti Polar Lipids. Stored as 5 mM stock solutions in methanol, at −20 °C.
4. HPLC mobile phase: methanol containing 0.2 % formic acid (v/v) and 1 mM ammonium formate.

2.4 Quantification of CeramideSynthase Activity in Living Cells

1. Cell culture plates (6-well) and growth medium suitable for the cells of interest.
2. 75 % isopropanol/25 % deionized water (v/v) for cell extraction.
3. Ethyl acetate for cell extraction.
4. Borosilicate glass centrifuge tubes with screw caps. We use 15 mL (16×125 mm) tubes from Thermo Fisher Scientific, but 10 mL tubes are also suitable.
5. SpeedVac centrifugal vacuum evaporator system (Thermo Fisher Scientific) or similar.
6. 5 mL borosilicate glass vials (75×12 mm) suitable for use in the SpeedVac or similar system.
7. Glass HPLC vials with fused inserts and caps.

3 Methods

3.1 Lysis of Cells or Tissues for In Vitro Assays

1. We recommend lysing 10–20 mg fresh-frozen tissue or a minimum of 10^6 cells in 0.5 mL lysis buffer. This should yield protein concentrations in the range 1–3 mg/mL. Cells should be washed once with PBS and scraped directly into lysis buffer.

Alternatively, cells can be detached with trypsin/EDTA solution, washed with PBS, then pelleted by centrifugation at 200 × *g* for 5 min prior to lysis.

2. Tissue or cells can be lysed using either a glass Dounce homogenizer or a sonicating bath that is suitable for small volumes. We use a Diagenode Bioruptor set to High intensity, with a 30 s on/30 s off cycle. As the sonicating bath heats very rapidly, the Bioruptor is kept at 4 °C and ice is added to prevent sample heating. For solid tissues, a tissue homogenizer should be used, or the tissue should be ground over dry ice or liquid nitrogen prior to lysis, using Eppendorf micropestles.
3. The homogenate is centrifuged for 10 min at 800 × *g* to pellet nuclei and unbroken cells, and the supernatant is transferred to a new tube.
4. The protein concentration is measured using a BCA assay kit (*see* **Note 4**), and the lysate is stored in aliquots at −80 °C.

3.2 CERS Assay

1. Reactions are run in 1.5 mL tubes. The standard reaction volume is 50 μL, using the reaction buffer described above. Reactions are started with the addition of 10–25 μg lysate protein and run at 37 °C on a thermomixer with vigorous shaking (*see* **Note 5**).
2. Reactions are stopped with the addition of four volumes of methanol that includes 50 pmoles C17 ceramide internal standard, and the tubes are vortexed. The tubes are centrifuged for 20 min at 21,800 × *g* to remove any insoluble material, after which the top 0.2 mL of the supernatant is transferred to glass HPLC vials for MS analysis. The vials may be stored at 4 °C (*see* **Note 6**).
3. It is recommended to set up controls in which no enzyme (i.e., lysate) or no fatty acid-coA substrate is added to the reaction.

3.3 Quantifying Ceramide Products on a Triple Quadrupole MS

1. Selected reaction monitoring mode is employed on the mass spectrometer after positive mode electrospray ionization. Precursor and product ion mass-to-charge ratios (m/z) are as indicated in Table 1. Mass to charge ratios for both precursor and product ions of D7-dihydroceramides are increased by 7 mass units compared to the naturally occurring lipids. We strongly recommend that instrument-specific parameters such as most abundant product ion, collision energy, collision gas pressure, electrospray voltage, electrospray source temperature, source gas flow rates, tube lens/S lens voltage, capillary temperature, and skimmer offset be determined empirically in the laboratory in which the assay is being run. This is done by direct infusion of one or more dihydroceramide standards into the MS source, according to the instrument manufacturer's instructions.

Table 1
Exact mass, column elution times, and precursor and product ion masses [M + H] for commonly studied dihydroceramide and ceramide species

Ceramide[a]	Molecular weight	Precursor *m/z* [M + H][c]	Product *m/z* [M + H][c]	Collision energy (eV)	C8 column elution time (min)	C18 column elution time (min)
Dihydroceramides						
d18:0/16:0	539.53	540.5 (547.5)	522.5 (529.5)	17	3.1	3.3
d18:0/18:0	567.55	568.6 (575.6)	550.6 (557.6)	17	3.6	4.3
d18:0/24:0	651.65	652.7 (659.7)	634.7 (641.7)	17	5.8	10.9
d18:0/24:1	649.64	650.6 (657.6)	632.6 (639.6)	17	5.0	8.0
Ceramides						
d18:1/17:0[b]	551.53	552.5	264.1	30	3.2	3.4
d18:1/16:0	537.51	538.5 (545.5)	264.1 (271.1)	30	3.0	3.0
d18:1/18:0	565.54	566.5 (573.5)	264.1 (271.1)	30	3.4	3.9
d18:1/20:0	593.57	594.6 (601.6)	264.1 (271.1)	30	3.9	5.3
d18:1/22:0	621.61	622.6 (629.6)	264.1 (271.1)	30	4.6	7.2
d18:1/24:0	649.64	650.6 (657.6)	264.1 (271.1)	30	5.5	9.9
d18:1/24:1	647.62	648.6 (655.6)	264.1 (271.1)	30	4.7	7.3

Elution times are given for a 3 × 150 mm Agilent XDB-C8 column (5 μm pore size) and an Agilent Poroshell 120, 2.1 × 150 mm SB-C18 column (2.7 μm pore size). Mass/charge (*m/z*) values in parentheses are for D7-labeled compounds formed in the reactions. Note that triple quadrupole mass spectrometers are generally only accurate to unit mass (whole number) (*see* **Note 9**)

[a]d18:0 forms are commonly referred to as dihydroceramides; d18:1 forms are referred to as ceramides

[b]d18:1/17:0 ceramide is the C17:0 ceramide internal standard

[c]Reactions or cell incubations with D7-dihydrosphingosine result in formation of D7-labeled forms of dihydroceramide and ceramide. The *m/z* values for these are given in parentheses

2. The flow rate is 0.5 mL/min using the HPLC mobile phase described in the Materials section.
3. 20 μL of each sample is resolved on the column, with a total run time of 9 min for the C8 column and 15 min for the C18 column. Different dihydroceramide or ceramide products elute at different times from the HPLC column, as listed in Table 1.
4. An external calibration curve spanning the range 1 nM to 2 μM (0.25–500 pmoles in 250 μL HPLC mobile phase) should be constructed for the dihydroceramide or ceramide reaction product that is being measured. For the standard curve, it is ideal to use the naturally occurring equivalent to the form of D7-dihydroceramide that is generated in the reaction. Each standard curve dilution should contain 50 pmoles C17 ceramide internal standard.

5. Data is analyzed using the vendor software (XCalibur for Thermo MS systems). Peak areas are determined for the D7-dihydroceramide reaction product and expressed as a ratio relative to the C17:0 ceramide internal standard for each sample. Similarly, external standard peak areas are expressed as ratios to the internal standard, and the resulting calibration curve (Fig. 2) is used to calculate the pmoles product formed in the reaction. Figure 3 shows chromatograms for an example reaction assaying C24:1 ceramide synthase activity in mouse liver homogenates. As expected, product formation is inhibited with the well characterized ceramide synthase inhibitor fumonisin B1 [14].

3.4 Assaying CERS Activity of Living Cells

1. Cells are seeded on the preceding day at the desired concentration for the assay. We have found 2.5×10^5 cells per well of a 6-well culture plate to be suitable for this assay.

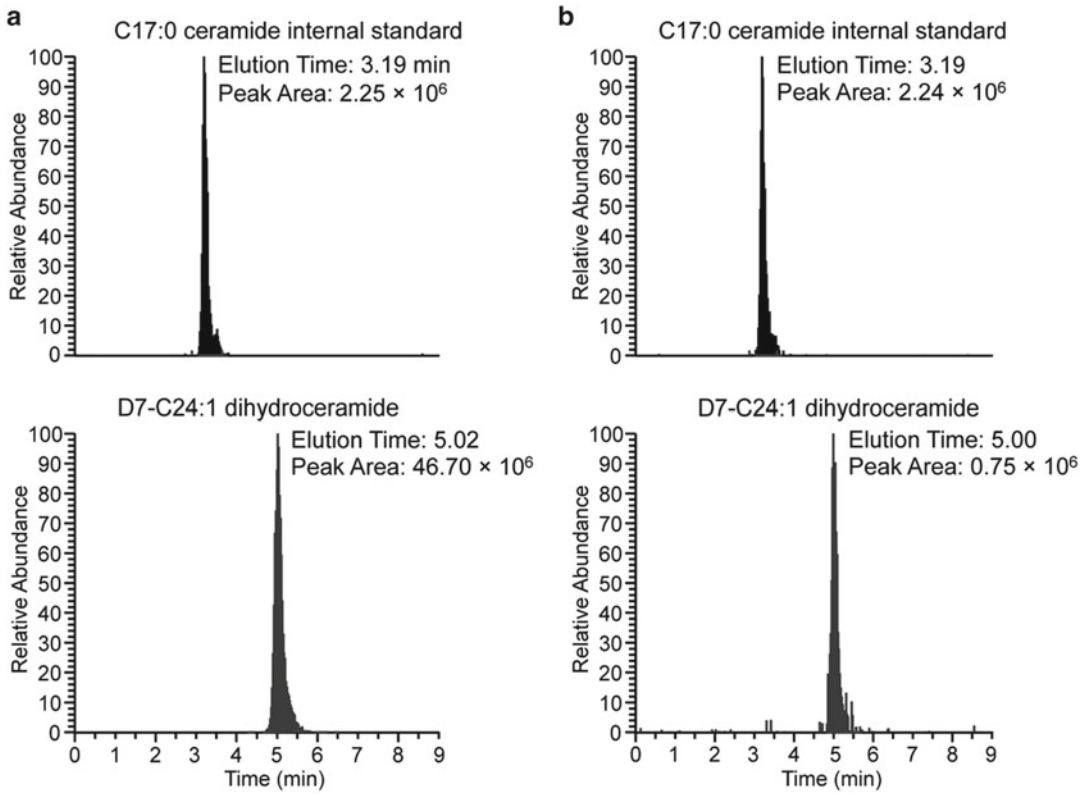

Fig. 3 Chromatograms showing peaks for D7-labeled C24:1 dihydroceramide products. Reactions were set up with 25 μg mouse liver homogenate, and D7-dihydrosphingosine (10 μM) and C24:1-CoA (50 μM) as substrates. Chromatograms shown in (**b**) are for a reaction that included the ceramide synthase inhibitor fumonisin B1 (10 μM), illustrating the inhibition of product (D7-C24:1 dihydroceramide) formation relative to the vehicle control reaction (**a**). Chromatograms for the C17:0 ceramide internal standard are also shown for reference

2. Cells are pretreated with the desired inhibitors or genetic manipulations, then incubated for 1 h with 500 nM D7-dihydrosphingosine.
3. Cells are washed once with PBS to remove serum proteins.
4. The cells are held on ice, and 0.4 mL 75 % isopropanol containing 50 pmoles C17:0 ceramide internal standard is added to each well. A cell scraper is used to collect the cells into the solvent mixture, and the cell extracts are transferred to a 15 mL glass centrifuge tube. The culture wells are then washed with another 0.4 mL 75 % isopropanol, and this extract is combined with the first.
5. Ethyl acetate (1.2 mL) is added to each tube, and the mixture is vortexed, then sonicated for 2 h in a sonicating water bath, with heating to approximately 50 °C (*see* **Note 7**).
6. Cell extracts are centrifuged at 3700 × *g* for 10 min to pellet insoluble debris, and the supernatant is transferred to a 5 mL glass vial.
7. Insoluble debris is re-extracted with another 2 mL ethyl acetate–isopropanol–water (6:3:1, v/v/v) as described above. The supernatants are combined.
8. Cell extracts (i.e., supernatant) are dried down in a SpeedVac or similar vacuum evaporator, or under a stream of nitrogen.
9. Cell extracts are reconstituted with vigorous vortexing in 0.25 mL HPLC mobile phase.
10. Glass tubes are centrifuged at 3700 × *g* for 10 min to pellet any insoluble material, and 0.2 mL of the supernatants are transferred to glass HPLC vials with inserts. Vials are stored at 4 °C pending LC-MS analysis (*see* **Note 6**).
11. Quantify D7-dihydroceramides and D7-ceramides using LC-MS/MS, as described in Subheading 3.3. In cultured cells, the D7-dihydroceramides formed from D7-dihydrosphingosine will be rapidly converted to ceramides by desaturases (*DEGS1* and *DEGS2*). It is therefore important to quantify D7-ceramides as well as D7-dihydroceramides (*see* **Note 9**). Figure 4 shows an example for U251 glioblastoma cells treated with the ceramide synthase inhibitor fumonisin B1.

4 Notes

1. We include the β-glycerophosphate as a protein phosphatase inhibitor. A recent publication has demonstrated that ceramide synthase activity in yeast is regulated by phosphorylation [15].
2. Deuterated (D7) dihydrosphingosine is used as the substrate to avoid any baseline level of dihydroceramide products attrib-

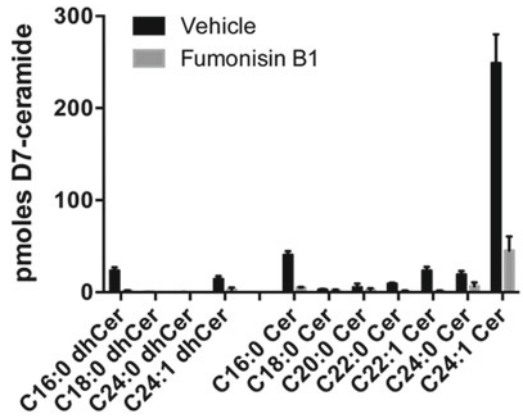

Fig. 4 Formation of D7-ceramides from D7-dihydrosphingosine in cultured cells. U251 glioblastoma cells (2.5×10^6 cells) were pretreated for 1 h with 5 μM fumonisin B1, prior to a 1 h incubation with 500 nM D7-dihydrosphingosine. As expected, the D7-dihydrosphingosine is converted to dihydroceramides and ceramides in the cells, and this is inhibited with fumonisin B1. *Cer* ceramide, *dhCer* dihydroceramide

uted to endogenous dihydroceramides in the cell or tissue extracts. Note that these endogenous dihydroceramides are likely to be low abundance, and it is therefore also feasible to use natural dihydrosphingosine as the substrate in place of the D7 form, provided that control reactions with cell or tissue extract but without dihydrosphingosine substrate are included to assess endogenous levels of dihydroceramides.

3. Fatty acid free BSA, available from a number of common biochemical suppliers, is used as a carrier for the ceramide formed in the reaction. Standard laboratory BSA contains lipids and fatty acids that may interfere with the reaction or quantification of reaction products.
4. Serum proteins will interfere with estimation of protein concentration by BCA or similar protein assay. Protein concentration may also be determined using other common assays.
5. It is recommended for the user to run a test assay confirming that CERS activity is linear with respect to time and/or lysate concentration before picking a single time and lysate protein concentration at which to compare different samples. In our experience, reactions are linear over 30 min, using between 10 and 30 μg lysate protein, but this will obviously depend on the level of CERS activity in the samples of interest.
6. Prolonged storage at 4 °C, or particularly at −20 °C, may result in further precipitate forming. In this case the supernatant should be transferred to new HPLC vials. Care should be taken

to ensure that no precipitate is loaded onto the LC-MS system, as this may clog the injection port or column.

7. It is important not to allow the sonication to cause overheating of the glass tubes, as this will result in solvent spillage into the water bath and a potential inhalation hazard with the ethyl acetate.
8. When considering lipids that are separated in mass by only two mass units, it is important to consider the peaks produced by naturally occurring heavy carbon (^{13}C) isotopes. These represent around 1 % of all carbon atoms. For example, C24:1 ceramide, monitored with m/z 648.6, will also produce a peak with m/z 650.6, albeit with greatly reduced intensity compared to the m/z 648.6 peak. Since m/z 650.6 will also detect C24:1 dihydroceramide and C24:0 ceramide, it is very important in these instances to employ external standards for each lipid of interest, in order to confirm the expected HPLC column elution times. This issue is not particularly problematic when monitoring a single product formed in an in vitro reaction, but is an important consideration when monitoring a complex lipid mixture isolated from cultured cells. We note that the C18 column provides better resolution of very long chain ceramide species (e.g., C24:1 and C24:0 ceramides and dihydroceramides) than does the C8 column.
9. When using a water loss event to monitor for dihydroceramides, as indicated in Table 1 (e.g., C24:1 dihydroceramide precursor ion m/z 650.6; product ion m/z 632.6), it is important to note that this will detect any molecule in the range m/z 650.1–651.1 that loses a water molecule (m/z 18.0) following collision-induced dissociation. This MS event will therefore also detect isobaric ceramide species such as C24:0 ceramide (also detected with m/z 650.6), and heavy carbon forms of C24:1 ceramide as discussed in **Note 8**. In these instances, HPLC column elution time is particularly important for correct lipid identification.

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