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In situ glucosylceramide synthesis and its pharmacological inhibition analysed in cells by $^{13}C_5$-sphingosine precursor feeding and mass spectrometry

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Glycosphingolipids (GSLs) fulfil diverse functions in cells. Abnormalities in their metabolism are associated with specific pathologies and, consequently, the pharmacological modulation of GSLs is considered a therapeutic avenue. The accurate measurement of *in situ* metabolism of GSLs and the modulatory impact of drugs is warranted. Employing synthesised sphingosine and sphinganine containing ^{13}C atoms, we developed a method to monitor the *de novo* synthesis of glucosylceramide, the precursor of complex GSLs, by the enzyme glucosylceramide synthase (GCS). We show that feeding cells with isotope-labelled precursor combined with liquid chromatography–mass spectrometry (MS)/MS analysis allows accurate determination of the IC_{50} values of therapeutically considered inhibitors (iminosugars and ceramide mimics) of GCS in cultured cells. Acquired data were comparable to those obtained with an earlier method using artificial fluorescently labelled ceramide to feed cells.

Keywords: ^{13}C-labelled lipids; glucosylceramide synthase; glycosphingolipid metabolism; mass spectrometry

Sphingolipids are structural components of the mammalian cell membrane. Glycosphingolipids (GSLs) are particularly present in the outer leaflet of the cell membrane and are unevenly distributed, generating lipid rafts (semi-ordered microdomains) together with cholesterol molecules [1]. Within these lipid rafts reside specific proteins involved in the interaction of cells with a variety of agents ranging from hormones to pathogens. The GSL composition of lipid rafts has been observed in various processes, a topic recently reviewed in

[2]. The metabolism of GSLs is complex [3–6]. The initial step in the formation of the majority of GSLs is the synthesis of glucosylceramide (GlcCer) by the enzyme glucosylceramide synthase (GCS) [7]. Using UDP-glucose as a sugar donor, GCS transfers a glucose moiety to cytosolic ceramide to generate GlcCer. The ceramide is generated in two ways. Firstly, it may be derived from newly formed sphinganine generated from serine and palmitoyl-CoA [4]. Secondly, cytosolic ceramide is provided by the so-called salvage pathway in

Abbreviations

AMP-DNM, *N*-adamantanemethyloxypentyl-1-deoxynojirimycin; CBE, conduritol B epoxide; Eliglustat, *N*-{(1R,2R)-1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(pyrrolidin-1-yl)propan-2-yl}octanamide; Gb3, globotriosylceramide; GBA1, glucocerebrosidase; GBA2, non-lysosomal glucosylceramidase; GCS, glucosylceramide synthase; GD, Gaucher disease; GlcCer, glucosylceramide; GSLs, glycosphingolipids; Ibiglustat, [(3S)-1-azabicyclo[2.2.2]octan-3-yl] *N*-[2-[2-(4-fluorophenyl)-1,3-thiazol-4-yl]propan-2-yl]carbamate; IC$_{50}$, half-maximal inhibitory concentration; LacCer, lactosylceramide; Miglustat, *N*-butyl-deoxynojirimycin; NBD-ceramide, *N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-D-erythro-sphingosine; NHD fibroblasts, normal human dermal fibroblasts; SRT, substrate reduction therapy.

which sphingosine is formed by lysosomal degradation of ceramide, and upon release into the cytosol is employed for re-synthesis of ceramide [8,9]. Thus, as shown in Fig. 1A, both sphinganine (*de novo* synthesis) and sphingosine (salvage pathway) can act as precursors in the formation of ceramide and subsequent GlcCer.

Pharmacological modulation of GSLs proves to be feasible and has been applied to treat disease conditions. Firstly, the pharmacological reduction of GSLs by GCS inhibition is used to treat Gaucher disease (GD) in which GlcCer accumulates due to a deficiency in its degradative lysosomal enzyme named glucocerebrosidase (GBA1) [10,11]. Registered substrate reduction therapy (SRT) drugs for the treatment of type 1 GD are *N*-butyl-deoxynojirimycin (Miglustat) [12,13] and *N*-{(1R,2R)-1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(pyrrolidin-1-yl)propan-2-yl}octanamide (Eliglustat) [14,15]. However, the use of SRT is not advised for some patients and mixed results have been obtained in treating the neuronopathic form of GD with Miglustat [16–20]. The compound [(3S)-1-azabicyclo[2.2.2]octan-3-yl]*N*-[2-[2-(4-fluorophenyl)-1,3-thiazol-4-yl]propan-2-yl]carbamate (Ibiglustat, Venglustat) has been shown to be able to cross the blood–brain barrier in mouse models of GD, and improve the levels of stored GlcCer in both visceral and central nervous system tissues [21]. SRT targeting GCS is intensively further studied in academia and the pharmaceutical industry, resulting in several compounds being investigated and developed, a selection of which is shown in Fig. 1B. Examples are iminosugars (e.g., Lucerastat, Sinbaglustat [22,23]), Eliglustat-based compounds [24], and other structures identified in high-throughput screening [25]. Furthermore, excessive GSLs are associated with obesity

and accompanying insulin resistance. Reduction of GSLs with *N*-adamantanemethyloxypentyl-1-deoxynojirimycin (AMP-DNM) is found to exert beneficial responses in obese rodents regarding various symptoms of the metabolic syndrome, including improved insulin sensitivity [26–29]. In addition, modulation of cell surface GSLs is considered as (preventive) therapy for infectious agents [2].

To identify effective GCS inhibitors determination of the half-maximal inhibitory concentration (IC_{50}) values for GCS in intact cells is warranted. This is currently assessed by feeding cells, in the absence and presence of potential inhibitors, with *N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine (NBD-ceramide) followed by detection of glucosylated NBD-ceramide by high-performance liquid chromatography [30]. This method is laborious and has intrinsic drawbacks. The presence of a fluorophore moiety in NBD-ceramide significantly alters the molecule and might alter metabolism compared to natural ceramide. We envisioned an alternative procedure: the use of natural, but ^{13}C isotope-encoded sphinganine or sphingosine to monitor GSL metabolism, in particular, the *de novo* formation of GlcCer by GCS. We previously reported the synthesis of ^{13}C-encoded lipids, including sphinganine and sphingosine [31–33]. Using natural precursors to assess *in situ* sphingolipid metabolism should render physiological data. Previous research has shown that sphingosine and sphinganine added to the cell medium are easily taken up by cells and enter sphingolipid metabolism [34]. Here, we demonstrate the use of ^{13}C-isotope encoded precursor lipids (^{13}C$_5$-sphingosine and ^{13}C$_5$-sphinganine) to study *in situ* GCS activity in living cells.
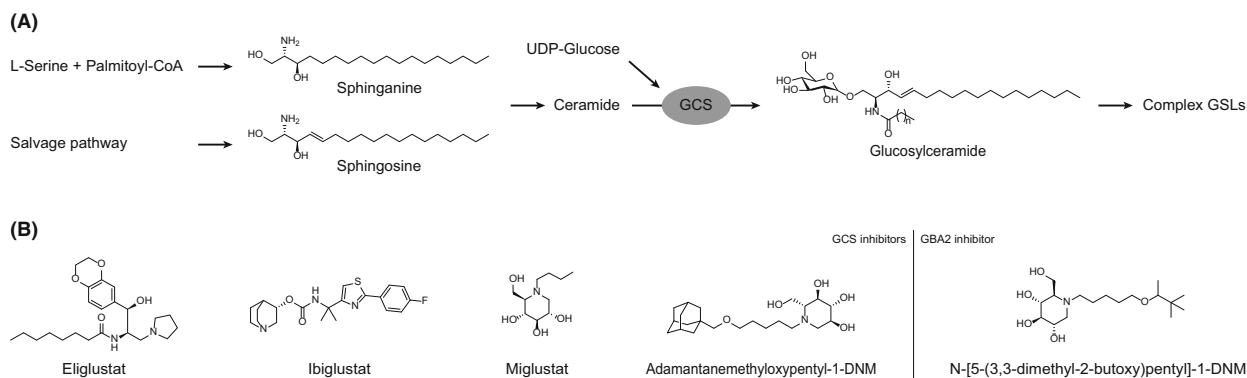

Fig. 1. Overview of (A) ceramide synthesis from the two precursors sphingosine and sphinganine and (B) the inhibitors used in this study. DNM, deoxynojirimycin.

Materials and methods

Materials

Liquid chromatography–mass spectrometry (LC–MS) grade methanol, chloroform, water, butanol, and formic acid were purchased from Biosolve (Valkenswaard, the Netherlands). C_{17}-sphinganine was from Avanti Polar Lipids (Alabaster, AL, USA). Ammonium formate, NaOH, and HCl were purchased from Sigma Aldrich (St. Louis, MO, USA). Conduritol B epoxide (CBE) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). C_{17}-dhCeramide, the $^{13}C_5$ lipids [31], and the GCS [35] and non-lysosomal glucosylceramidase (GBA2) inhibitors [36] were synthesised by the department of Bio-organic Synthesis (Leiden University, the Netherlands).

Cell culture media were also purchased from Sigma Aldrich. Media were supplemented with 10% (v/v) FBS (Bodinco, Alkmaar, the Netherlands), 1% (w/v) penicillin/ streptomycin (P/S; Life Technologies, Carlsbad, CA, USA), and/or 1% (w/v) GlutaMAX™ (Thermo Fisher Scientific, Waltham, MA, USA). Normal Human Dermal (NHD) Fibroblasts, RAW 264.9 and murine fibroblasts NIH/3T3 were purchased from the American Type Culture Collection (Manassas, VA, USA).

Methods

Cell culture

Murine fibroblasts NIH/3T3 were cultured in DMEM high glucose supplemented with 10% (v/v) FBS, 1% (w/v) GlutaMAX™, and 1% (w/v) P/S. Primary human dermal fibroblasts were cultured in DMEM/F12 growth medium, supplemented with 10% (v/v) FBS and 1% (w/v) P/S. RAW 264.7 were cultured in RPMI growth medium, supplemented with 10% (v/v) FBS, 1% (w/v) GlutaMAX™ and 1% (w/v) P/S; an additional 25 mM HEPES was added during incubations. All cell lines were incubated at 37 °C and 5% CO_2. Cells were seeded during passage and the incubations were started when cells were 80–100% confluent. In assays using CBE or the GBA2 inhibitor shown in Fig. 1, cells were pre-incubated with 300 μM CBE or 0.2 μM GBA2 inhibitor per well for 1 h prior to $^{13}C_5$-lipid incubation. Cells were incubated with 0.5 nmol $^{13}C_5$-sphingosine or $^{13}C_5$-sphinganine per well. After optimisation with $^{13}C_5$-sphingosine, where incubation times of 0–24 h were used, incubation times of 1 h for RAW cells and 3 h for fibroblasts were used for inhibitor assessment. After incubation, cells were washed five times with PBS and frozen in 150 μL H_2O per well until lipid extraction.

All cell experiments were performed at least twice, with samples in duplicate or triplicate.

Zebrafish

AB/TL zebrafish were kept in accordance with the directives of the local animal welfare committee (Instantie voor Dierwelzijn, IvD, Leiden, the Netherlands) and guidelines specified by the EU animal Protection Directive 2010/63/ EU. Embryos, derived from natural spawning, were kept in egg water (60 mg·L^{-1} sea salt, 0.01 mg·L^{-1} methylene blue) supplemented with 0.5% DMSO and 20 pmol $^{13}C_5$-sphingosine per fish for up to 5 days post-fertilisation (before the free-feeding stage).

Lipid extraction

Lipid extraction was performed through modified Bligh and Dyer extraction [37]. In short, after the addition of 500 pmol of both C_{17}-sphinganine and C_{17}-dihydroceramide as internal standard, lipids were separated into the lysolipids (upper phase) and neutral GSLs (lower phase), and calibration curve lipids were added to the appropriate samples. The lower phase lipids were deacylated through microwave-assisted hydrolysis [38] to yield their lysolipid counterparts. Samples were further cleaned through butanol extraction and, after drying, reconstituted in MS grade MeOH.

LC–MS/MS measurements were performed as described previously [33]. Compounds were separated using a BEH C18 column followed by detection on a Waters TQS micro with electrospray ionisation in positive mode (Waters, Milford, MA, USA). The isotope labelled lipids were identified through their distinct parent and daughter ions as follows: $^{13}C_5$-sphinganine 307.3 → 289.3, $^{13}C_5$-sphingosine 305.3 → 287.3, $^{13}C_5$-glucosylsphingosine 467.3 → 287.3, $^{13}C_5$-lactosylsphingosine 629.4 → 287.3, and $^{13}C_5$-lysoGb3 791.4 → 287.3. The endogenous (non-labelled) lipids were identified through their distinct parent and daughter ions as follows: sphinganine 302.3 → 284.3, sphingosine 300.3 → 282.3, glucosylsphingosine 462.3 → 282.3, lactosylsphingosine 624.4 → 282.3, and lysoGb3 786.4 → 282.3. Peak areas were determined using TargetLynx (Waters, Milford). After peak integration, results for both optimisation and determination of inhibitor IC_{50} values were processed using GRAPHPAD PRISM version 8 (GraphPad Software Inc., San Diego, SA, USA). IC_{50} values were estimated using the non-linear regression log(inhibitor) versus normalised response curve fit.

Results

Employing isotope labelled lipid feeding to monitor *in situ* glucosylceramide formation in cultured cells

We first comparatively studied the suitability of $^{13}C_5$-sphinganine and $^{13}C_5$-sphingosine feeding to monitor

GCS-driven GlcCer formation. Sphinganine feeding allows the monitoring of the *de novo* synthesis pathway, whilst sphingosine feeding allows the monitoring of the salvage pathway. The feeding of $^{13}C_5$-sphinganine led to the formation of $^{13}C_5$-dihydroceramide which was converted to $^{13}C_5$-ceramide by dihydroceramide desaturase. In contrast, fed $^{13}C_5$-sphingosine was directly converted to $^{13}C_5$-ceramide by ceramide synthase. At longer incubation time $^{13}C_5$-encoded GlcCer, lactosylceramide (LacCer), and globotriaosylceramide (Gb3) were increasingly detected in cells fed either with $^{13}C_5$-sphinganine or $^{13}C_5$-sphingosine. Examples of the resulting LC–MS/MS chromatograms can be found in Fig. S1. A direct comparison of feeding with both precursors can be found in Fig. S2. Since feeding with $^{13}C_5$-sphingosine led to the comparable generation of GSLs as feeding with $^{13}C_5$-sphinganine, in subsequent experiments we made use of $^{13}C_5$-sphingosine feeding.

To determine the optimal concentration of feeding, we incubated fibroblasts with a range of concentrations of $^{13}C_5$-sphingosine for 4 h. With increasing concentrations of fed $^{13}C_5$-sphingosine, as shown in Fig. 2, the intracellular labelled sphingosine and ceramide also increased. Labelled GlcCer and Gb3 both showed the most incorporation at 500 pmol $^{13}C_5$-sphingosine feeding. At 250 pmol or lower GlcCer and Gb3 are difficult to accurately quantify. Therefore, subsequent experiments were performed with 500 pmol, or 1 μM, $^{13}C_5$-sphingosine feeding per well.

To examine more closely the intracellular flux of the $^{13}C_5$-lipids we incubated the fibroblasts with 0.5 nmol $^{13}C_5$-sphingosine for a wider range of time points (Fig. 3). Cellular $^{13}C_5$-sphingosine was maximal after 1 h of incubation (Fig. 3A), and generation of $^{13}C_5$-ceramide occurred readily (Fig. 3B). The concentrations of $^{13}C_5$-ceramide reached a peak after 2 h of incubation. Figure 3C shows the formation of well-measurable quantities of $^{13}C_5$-GlcCer from $^{13}C_5$-ceramide. Concentrations of $^{13}C_5$-GlcCer increased over time of incubation. Further metabolism to $^{13}C_5$-LacCer and $^{13}C_5$-Gb3 was observed (Figs 3D,E, respectively). Interestingly, the concentration of $^{13}C_5$-Gb3 was higher than that of $^{13}C_5$-LacCer. The same trend is also observed in endogenous lipids. This observation is in accordance with earlier research showing higher concentrations of Gb3 than LacCer in cultured human fibroblasts [39]. After overnight incubation, intra-cellular $^{13}C_5$-sphingosine, $^{13}C_5$-ceramide, and $^{13}C_5$-GlcCer are each in equilibrium. We expect that at longer incubation times $^{13}C_5$-LacCer and $^{13}C_5$-Gb3 will also reach equilibrium. The purpose of the present investigation was the *in situ* measurement of *de novo* GlcCer formation by GCS. We therefore used a $^{13}C_5$-sphingosine incubation of cells for 3 h which allows optimal detection of the enzyme activity.

Impact of catabolic enzymes on inhibition of GCS

In the past, a variety of cell lines from various species have been used to determine *in situ* IC_{50} values (concentration resulting in a 50% reduction of activity). To assess the importance of cell type, we compared

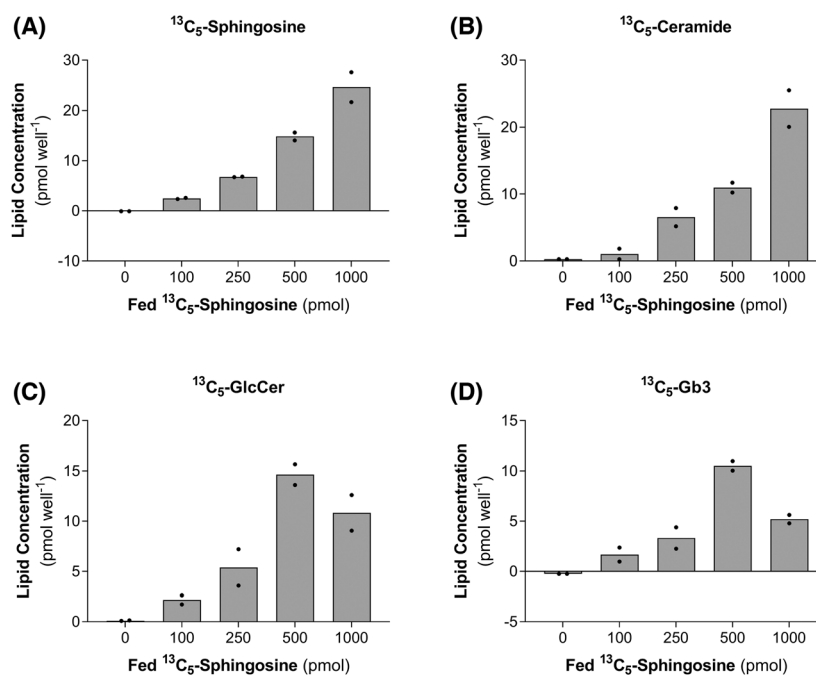**Fig. 2.** The lipid concentrations per well of fibroblasts after 4 h incubation with varying concentrations of $^{13}C_5$-sphingosine. Lipids shown are $^{13}C_5$- (A) sphingosine, (B) ceramide, (C) glucosylceramide, and (D) globotriaosylceramide.

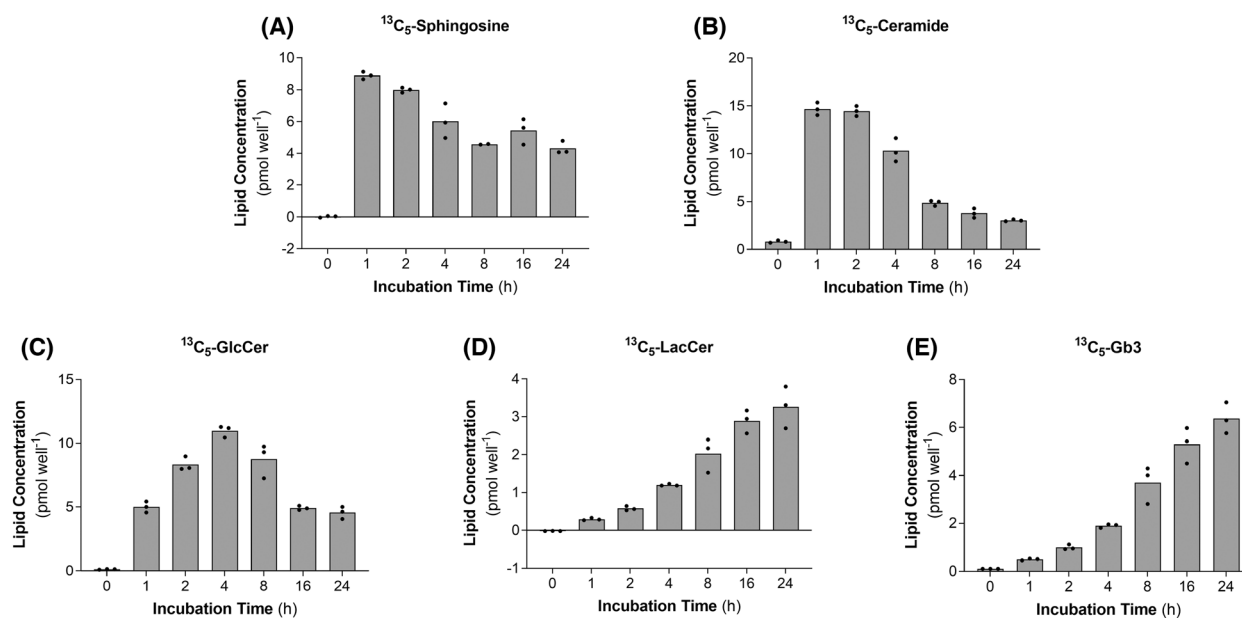**Fig. 3.** The lipid concentrations per well of fibroblasts after varying incubation times with 500 pmol $^{13}C_5$-sphingosine. Lipids shown are $^{13}C_5$-(A) sphingosine, (B) ceramide, (C) glucosylceramide, (D) lactosylceramide, and (E) globotriaosylceramide. Representative results are shown.

the effect of Eliglustat in murine RAW 264.7 cells, NHD fibroblasts, and the murine fibroblast cell line NIH/3T3. When measuring GCS activity by monitoring the formation of $^{13}C_5$-GlcCer, interference by other enzymes metabolising GlcCer needs to be ruled out or prevented. Such interfering enzymes are the lysosomal GBA1 and the cytosol-facing GBA2. For GBA1 and GBA2 specific cell-permeable inhibitors are available, respectively CBE and *N*-[5-(3,3-dimethyl-2-butoxy)pentyl]-1-deoxynojirimycin (GBA2 inhibitor; Fig. 1). It is known that fibroblasts hardly express GBA2 and therefore measurement of GCS activity only requires inhibition of possibly interfering GBA1. We incubated cells with or without the various inhibitors prior to feeding with $^{13}C_5$-sphingosine in the presence of a variable concentration of the GCS inhibitor Eliglustat. The presence of the GBA1 inhibitor CBE, and that of the GBA2 inhibitor in the case of RAW 264.7, did not significantly impact on the observed $^{13}C_5$-GlcCer formation (see Table 1). Of note, quite similar results were obtained with the various cells tested, suggesting broad applicability. Furthermore, at the used experimental conditions (3 h of 500 pmol $^{13}C_5$-sphingosine feeding) when using fibroblasts or RAW 264.7 cells, the presence of GBA1 and GBA2 inhibitors is not required and therefore their addition was therefore omitted in subsequent experiments with these cells.

Inhibitor assessment using NBD-ceramide and $^{13}C_5$-sphingosine feeding of cultured cells

Previously GCS activity in intact cells was studied by the feeding of NBD-ceramide and analysis of its conversion to NBD-GlcCer. This NBD-ceramide substrate assay has been applied in combination with GCS inhibitors and thus has generated IC_{50} values for these compounds. We determined the IC_{50} values of several inhibitors of GCS using the established $^{13}C_5$-sphingosine method and compared these to values previously obtained using NBD-labelled ceramide as substrate. As a comparison, we examined established GCS inhibitors: Eliglustat, Miglustat, Ibiglustat, and the iminosugar AMP-DNM (Table 2).

Application of the assay of *in situ* GCS activity in intact zebrafish larvae

Genetically modified zebrafish with impaired GSL metabolism have become available in recent years. For example, fish lacking GBA1 have been developed allowing investigations on various aspects of GD [40,41]. Measurement of the metabolic flux of GSLs in full organisms, in addition to the steady-state lipid levels, is of heightened interest. We therefore tested the applicability of $^{13}C_5$-sphingosine feeding, through addition to the egg water, of zebrafish larvae (up to 5 days post-fertilisation, dpf) to measure the *in situ*

Table 1. The IC$_{50}$ of Eliglustat (reported to be 25 nM [14]) was determined in different cell lines using $^{13}C_5$-sphingosine feeding upon the addition of CBE and a GBA2 inhibitor. Mean and standard deviation are shown from two separate experiments.

	GCS IC$_{50}$ (nM)			
GBA1 inhibitor CBE	–	+	–	+
GBA2 inhibitor	–	–	+	+
Murine macrophages RAW 264.7	43.2 ± 0.1	32 ± 11	23 ± 5	33 ± 9
Murine fibroblasts NIH/3T3	9.2 ± 0.01	8.2 ± 0.6	N.A.	N.A.
Human fibroblasts NHDF	6 ± 1	7 ± 2	N.A.	N.A.

Table 2. The IC$_{50}$ values (nM) of GCS inhibitors were determined using both the $^{13}C_5$-sphingsoine feeding methods and a comparison to the published results using the NBD-ceramide substrate method. Mean and standard deviation are shown from two separate experiments.

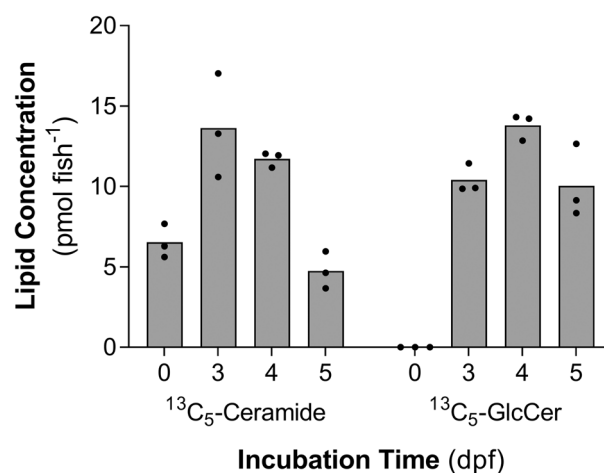|	IC$_{50}$ (nM)	
Inhibitor	NBD-Cer	$^{13}C_5$-sphingosine
Eliglustat	24 [14]	6 ± 1
Miglustat	25 000 [45]	55 000
AMP-DNM	200 [35]	340
Ibiglustat	N.A.	8 ± 1

Fig. 4. Incorporation of $^{13}C_5$-sphingosine into the GSL metabolism of zebrafish larvae up to 5 days post-fertilisation. Three samples of five fish each, fed with 20 pmol $^{13}C_5$-sphingosine per fish, were measured.

metabolism of GSL, in particular the formation of GlcCer. Individual fish were fed at 8 h post-fertilisation with 20 pmol $^{13}C_5$-sphingosine in 100 µL egg water. Three pools of five fish were harvested at daily intervals, lipids were extracted and analysed by LC–MS/MS. As seen in Fig. 4, the $^{13}C_5$-sphingosine is readily taken up by the larvae and metabolised to both ceramide and GlcCer by 5 dpf. Thus, the method is applicable in the fish model and could be used to test the efficacy of drug interventions aiming to reduce GlcCer biosynthesis.

Discussion

When using isotope labelled lipids, both the *de novo* and the salvage pathway of ceramide synthesis can be monitored. Feeding of both $^{13}C_5$-sphingosine and $^{13}C_5$-sphinganine allowed us to track the flux of lipids through the metabolic pathways, and $^{13}C_5$-sphingosine was chosen as the feeding substrate for further work. The $^{13}C_5$-sphingosine is taken up well by the fibroblasts. Levels of $^{13}C_5$-sphingosine increase to a peak after 1 h of incubation. $^{13}C_5$-ceramide shows a peak after 2 h of incubation and, following that, the

glycosylated forms increase. After overnight incubation, levels of $^{13}C_5$-sphingosine, -ceramide, and -GlcCer seem to reach an equilibrium. A further increase in incubation times will likely lead to an equilibrium in the further metabolites as well, in addition to allowing the analysis of the half-life time of the labelled lipids in the fibroblasts.

We applied the $^{13}C_5$-sphingosine feeding to evaluate the inhibitory properties of several known GCS inhibitors. To do so, we first examined the influence of catabolic enzymes on the assay results by employing inhibitors of both GBA1 and GBA2. The presence of active GBA1 appeared to have no impact on the found values, for which the short incubation times are likely responsible. For GBA1 to have an effect, the newly synthesised glycolipids need to reach the lysosome. In the short incubation times used here, it is unlikely that the $^{13}C_5$ glycolipids have already been reabsorbed, and an extension of the incubation times will probably show an influence of a GBA1 inhibitor on the results. A far more likely candidate for interference in our values is GBA2, as this is found on the cytosolic leaflet of the plasma membrane. While fibroblasts contain almost no GBA2, RAW 264.7 cells have a much higher expression. Therefore, we studied the effect of the GBA2 inhibitor on apparent IC$_{50}$ values for GCS in RAW 264.7 cells. This analysis revealed that the apparent IC$_{50}$ values of GCS inhibitors are similar with or without GBA2 inhibitors. Thus, under the assay conditions used there is no influence of both GBA1 and GBA2 on the assessed IC$_{50}$ values of compounds for GCS.

We compared the IC_{50} values found using the isotope method to those earlier obtained using NBD-ceramide as substrate. Though the two methods are fundamentally distinct, the established IC_{50} values are pretty similar. Besides the method of lipid measurement as such, the two protocols also differ in other aspects. Firstly, we here use a natural sphingosine precursor allowing the swift formation of ceramide *in vivo*. Secondly, uptake of NBD-ceramide by cells from the medium has a limited capacity and the unnatural chemical structure of NBD-ceramide might impact on its cellular fate.

The novel method appears particularly suited to studying at organismal level GSL metabolism in zebrafish. Pilot experiments have shown that the $^{13}C_5$-sphingosine is taken up and tolerated by fish larvae up to 5 days post-fertilisation. Delivery through both feeding in the egg water and injection into the yolk (data not shown) resulted in the incorporation of $^{13}C_5$-sphingosine into the backbone of higher GSLs.

Another elegant approach to studying *in situ* GSLs metabolism has been developed by Hannun and co-workers [42] They make use of odd-chain lipids to distinguish newly synthesised molecules derived from those. It was demonstrated that the *de novo* GSL metabolism can be examined through feeding cells with C_{17} sphinganine. As C_{17} sphinganine does not naturally occur, the altered length of the chain allows the distinguishing of newly synthesised (glyco)sphingolipids by means of mass spectrometry. While both Snider et al. [42] and our study examined the short-term incorporation of labelled lipids in the GSL metabolism, with longer incubation times, other steps in GSL metabolism downstream from GCS can *a priori* also be monitored. Such future studies can elegantly be assisted by the availability of specific inhibitors of lysosomal glycosidases involved in the degradation of GSLs, for example, GBA1 [43,44].

In conclusion, isotope labelled lipids can be exploited to study *in situ* GSL metabolism in intact cells. The $^{13}C_5$-sphingosine is readily taken up by various cell types, and the generation of GSL metabolites can be detected after 3 h. This can be applied for the determination of the IC_{50} of various inhibitors of GCS. Our novel method rendered similar IC_{50} values of SRT drugs as earlier established with NBD-ceramide as the substrate for GCS. The use of isotope labelled precursors has the potential to find broader applications than that presented in this study. Downstream pathways from GCS could be analysed, and fluxes of GSLs could be measured, even in intact zebrafish. The method may assist in further optimisation of GCS inhibitors for therapeutic purposes.

Data accessibility

The raw data of this study (IC_{50} titrations) are available on request from the authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The LC-MS/MS chromatograms of ^{13}C$_5$ -sphingosine, and deacylated ^{13}C$_5$ - ceramide, glucosylceramide, lactosylceramide, and globotriaosylceramide.

Fig. S2. A comparison of 0.5 nmol ^{13}C$_5$ -sphingosine (light bars) and -sphinganine (dark bars) feeding with 2 hour and 4 hours incubation time.