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Approaches for probing and evaluating mammalian sphingolipid metabolism

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Abstract

Sphingolipid metabolism plays a critical role in regulating processes that control cellular fate. This dynamic pathway can generate and degrade the central players: ceramide, sphingosine and sphingosine-1-phosphate in almost any membrane in the cell, adding an unexpected level of complexity in deciphering signaling events. While in vitro assays have been developed for most enzymes in SL metabolism, these assays are setup for optimal activity conditions and can fail to take into account regulatory components such as compartmentalization, substrate limitations, and binding partners that can affect cellular enzymatic activity. Therefore, many in-cell assays have been developed to derive results that are authentic to the cellular situation which may give context to alteration in SL mass. This review will discuss approaches for utilizing probes for mammalian in-cell assays to interrogate most enzymatic steps central to SL metabolism. The use of inhibitors in conjunction with these probes can verify the specificity of cellular assays as well as provide valuable insight into flux in the SL network. The use of inhibitors specific to each of the central sphingolipid enzymes are also discussed to assist researchers in further interrogation of these pathways.

INTRODUCTION.

Sphingolipids and their metabolizing enzymes comprise a dynamic network of critical players in cell signaling and biology. Three central sphingolipids have been extensively studied and implicated in signaling and biology in cells and *in vivo*. Ceramide (Cer), the central lipid in sphingolipid metabolism, has been implicated in cell death, cellular stress responses, senescence and cell cycle arrest (143, 226). Sphingosine (Sph), the immediate metabolite of ceramide breakdown, has been associated with growth arrest and cell death as well (90, 184). On the other hand, sphingosine-1-phosphate (S1P) has roles in cellular

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proliferation, migration, invasion, and inflammation, thereby often representing an opposing signal to Sph and Cer.

Eukaryotic cells regulate this network at both the enzyme and lipid levels throughout multiple sub cellular organelles. Consequently, regulation of the 40 plus enzymes central to metabolism of bioactive sphingolipids, along with hundreds (and possibly thousands) of distinct sphingolipid metabolic products, plays a large role in maintaining membrane composition as well as coordinating lipid signaling events (72). The amphipathic sphingolipids contain a hydrophobic fatty acyl chain attached to another hydrophobic sphingoid backbone, forming ceramide that can be derivatized with the addition of multiple polar head groups. Given the diversity of sphingolipids, both at the molecular and signaling level, there are numerous experimental approaches taking advantage of the various biochemical properties to determine regulation and signaling potential of these potent bioactive lipids.

Within the cell, the hydrophobic nature of sphingolipids, imparted by the two hydrocarbon chains, restricts the movement of Cer and complex sphingolipids to vesicular trafficking or protein-mediated transport, adding another potential level of regulation. On the other hand, the less abundant single chained sphingolipids, such as Sph and SIP, can be soluble in the cytosol and move much more freely between membranes, potentially confounding efforts to identify the compartment of origin of these potent signaling molecules (Fig. 1). To add even more intricacy, sphingolipid pathways are often subject to substrate availability, interconnecting metabolic pathways that utilize the same substrate. Consequently, when assessing sphingolipid metabolism, all of these considerations add levels of complexity to an already multifaceted system.

Thus, complementary approaches to measurements of sphingolipids have been designed and refined to gain the most accurate picture of how sphingolipid metabolism is regulated and which component is modulated under different experimental conditions and in response to various stimuli. For instance, the analysis of ceramide mass via the decades old diglyceride kinase detection assay is still an effective means for this purpose, although this has been largely superseded by LC/MS technology. Though, to further this method, the use of a tracer, such as 17 carbon dihydrosphingosine, can be utilized to distinguish between *de novo* and hydrolytically generated Cer. In this review, we provide an overview of commonly utilized techniques for the analysis of sphingolipid metabolism, along with parameters on the use of labels and inhibitors to probe sphingolipid homeostasis in mammalian cell lines.

1. Sphingolipid metabolism

1.1 Synthesis and Degradation

Sphingolipids represent the third most abundant lipid class found in mammalian cells, after glycerophospholipids and sterols, and are easily the most structurally diverse. They are typically distinguished by their 18-carbon amino alcohol backbone. This “sphingoid” backbone is synthesized in the ER through a condensation of serine and palmitoyl-CoA and eventually *N*-acylated with one of dozens of different fatty acids resulting in the formation of a family of distinct Cers (Fig. 1). This basic sphingolipid is modified at the 1-hydroxyl

group to create a vast subset of sphingolipid subclasses. Thus, addition of phosphate, phosphocholine, or sugar groups creates ceramide 1-phosphate (C1P), sphingomyelin (SM), or glycosphingolipids, respectively. While synthesis and degradation of sphingolipids is almost always compartment-dependent, movement of lipids between membranes makes tracking of biologically relevant accumulations more difficult.

An important aspect of sphingolipid enzyme regulation is localization of the enzymes to different organelles in the cell, as presented in Figure 1. *De novo* synthesis occurs in the ER, where serine palmitoyltransferase (SPT), ceramide synthases (CerS), and ceramide desaturase (DeS) are localized. Transfer of Cer to the Golgi either through the ceramide transporter (CERT) or through vesicular pathways leads to the production of either SM or glucosylceramide (GluCer), respectively. Through vesicular transport, SM can travel to the plasma membrane (PM) whereas GluCer is utilized in the synthesis of more complex glycosphingolipids in additional Golgi sub-compartments prior to transport. At the PM, both SM and glycosphingolipids become part of a dynamic relationship between the catabolizing enzymes, sphingomyelinases (SMases), cerebrosidases, ceramidases (CDase), and others to influence membrane dynamics or be utilized in signaling events (87, 240). Endolysosomal action can begin the processes of clearance and recycling of sphingolipids. As the pH of lysosomes is acidic, another category of sphingolipid enzymes with acidic pH optima resides here. These enzymes help to coordinate the degradation of SM and hexosylCer (HexCer) (denoting GluCer and/or GalCer), and in turn generated from degradation of more complex glycosphingolipids) into Cer and eventually into Sph by the actions of acid CDase (aCDase), acid SMase (aSMase), and glucosyl-ceramidase (GBA). Sph, a soluble metabolite in cells, can be acted on by sphingosine kinases (SK1 or SK2) to generate S1P, which can be irreversibly degraded by S1P lyase (SPL), representing the only known clearance for metabolites from the sphingolipid network. Alternatively, Sph can be acylated to Cer, and S1P can be dephosphorylated by S1P phosphatases (SPP) and then re-acylated in the ER to re-enter the sphingolipid pathway.

1.2 Complexity of Sphingolipids

Sphingolipids can be metabolized and catabolized in specific cellular compartments, potentially resulting in different signaling and biologies. This complexity is underscored by the presence of at least 5 distinct ceramidases and 5 distinct sphingomyelinases (products of distinct genes) that show selective subcellular localization.

Cer can be generated *de novo* or via re-acylation in the ER, or by degradation of SM at the PM (38), in the nucleus (185), in lysosomes (88) or in mitochondria (165). Ceramide can then mediate specific cellular responses through activation of one or more serine/threonine phosphatases (35) as well as possibly other targets (cathepsins (79), PKCz(105)). Similarly, Cer can be degraded to Sph in almost any cellular membrane by the action of compartment-specific CDases (39). Sph can then be phosphorylated to S1P either in the nucleus (85, 176), at the PM (199), or possibly additional compartments by the actions of SKs. S1P can then be transported outside the cell to act on one of five G-protein coupled receptors (S1PRs1–5) generating pro-survival and inflammatory biologies (194, 207). S1P can also act on intracellular targets (200). The metabolic interconnections of bioactive sphingolipids also

add to the complexity of their study. For example, actions attributed to acid sphingomyelinase cannot be *a priori* assigned to ceramide as the direct product. This was recently demonstrated in the case of the function of acid sphingomyelinase in regulating production of cytokines and chemokines where this was found to be dependent on further metabolism of ceramide to ceramide 1-phosphate (139). Given this interconnectivity of sphingolipid metabolism, methods for simultaneously monitoring the mass of multiple sphingolipid species in conjunction with their flux and intracellular transport/location are needed to allow more complete insight into the dynamic origins of sphingolipid signaling in cellular fate.

2. Determining Sphingolipid Mass

Detection of sphingolipid metabolism often begins with the analysis of cellular sphingolipid mass. This was classically accomplished by thin layer chromatography (TLC), including high performance TLC (HP-TLC) and the diacylglycerol kinase assay for ceramide (153), but in the last 15–20 years mass detection and quantitation have moved into the realm of mass spectrometry (MS). This field is now rapidly expanding with the development of new instrumentation and improved sensitivity. However, much of the current methods used to examine sphingolipids are still prone to the major drawback of analyzing total cellular sphingolipids as a snapshot of this dynamic network.

2.1 Mass Spectrometry

MS analysis is now recognized as the most sensitive and selective means of determining cellular concentrations of sphingolipids (63). As MS identifies molecules based on mass to charge ratio (m/z), initial studies were quick to identify the 246 m/z “sphingoid backbone” fragment ion that is characteristic of collision-induced dissociation (CID) for most sphingolipid species (63). Though for SM, the 184.1 m/z phosphocholine product ion was found to be the most intense product ion and thus is utilized for its identification (94). Interestingly, these initial studies were able to achieve sub-picomolar sensitivity (63), which, even with large technical advances, has only increased modestly over the past few years. The major advantage of MS over other techniques mentioned in the following sections is the added ability to quantify molecular species characterized by the presence of a specific fatty acid linked to a specific sphingoid backbone (ie. C16, C18, C24-Cer) for each sphingolipid class (i.e. Cer, SM, HexCer), allowing for a more precise and global approach for profiling the sphingolipid network. This aspect acquires particular relevance as it is becoming evident that individual species may exert specific biological functions (73).

High resolution instrumentation has led to an increase in selectivity that has allowed for the deconvolution of complex samples with multiple nearly isobaric (having identical mass) lipid species. Numerous robust and reliable methodologies exist (23, 186) that utilize HPLC MS/MS as a workhorse for the analysis of mammalian sphingolipids, including sphingoid bases, sphingoid base phosphates, Cer, HexCer, SM, C1P, and alpha-hydroxy ceramides (α -HO-Cer). In addition, methodologies for SM often require a hydrolysis step in order to remove contaminating isotopic peaks produced by the more abundant phosphatidylcholine. These contaminating peaks can have overlapping mass transitions generating the common

184.1m/z phosphocholine product ion. As demonstrated by Peng et al,(150) mass differences in lipids can be utilized with high mass accuracy MS to distinguish nearly isobaric sphingolipids. Indeed, these authors were able to separate an odd chain C23:0-Cer (635.6216 m/z) from the hydroxylated C22:1-HO-Cer (635.5852m/z) utilizing the less than 0.2 dalton mass difference generated via the extra oxygen (150). These developing methodologies also have the added benefit of increased functional sensitivity, owed to significantly reduced background when compared to standard low-resolution MS techniques (25, 150).

Though MS analysis of sphingolipids can be utilized without chromatographic separation, termed shotgun lipidomics (62, 67, 174), these methods are susceptible to isobaric interference, as well as loss in sensitivity due to ion suppression. Thus, the addition of the HPLC step, which takes advantage of the amphipathic nature of sphingolipids, represents an orthogonal separation to mass. Nevertheless, there are many considerations that must be taken into account when utilizing MS detections coupled with HPLC separation, especially for quantitative purposes. Primarily, the proper standards must be utilized for both method development and as internal standards to account for extraction efficiencies, ionization effects, and other differences that may arise from specific biochemical properties of the analyte. Ideally, internal standard mixes for simple sphingolipid subspecies would contain a stable isotope label for each molecular species that would generate odd massed parent and product ions. Accordingly, internal standard mass transitions would fall between the even numbered mass transitions that are produced by most endogenous sphingolipids, readily allowing for MS separation of internal standard masses from analytes of interest. Unfortunately, when considering the myriad of sphingolipid molecular species, only a small fraction is currently available as isotopically labeled standards. Therefore, many investigators utilize an array of representative odd chain or stable isotope sphingolipids as internal standards to account for the different chemical and biophysical properties of each sphingolipid subspecies (24, 127). The reverse phase chromatographic conditions typically used to separate sphingolipids prior to MS are also a major consideration. Elution of isobars or isomers, such as GluCer and galactosylceramide (GalCer), require separation prior to MS detection (127, 230). It should also be noted that if separation cannot be achieved under typical HPLC/MS conditions, the analytes are commonly reported as a grouped subspecies if detection is possible; for example, HexCer is used to denote any hexose-modified ceramide primarily including GluCer and GalCer. This is still useful in this case because most non-neuronal tissues contain much higher levels of GluCer than GalCer. In comparison to shotgun lipidomics, chromatographic separation substantially reduces ion suppression in the MS source by reducing the sample complexity at any given point during a gradient. Finally, extraction conditions should be considered carefully based on the sphingolipid species to be analyzed. Sphingolipids have been extracted from cell lysate utilizing a plethora of extraction techniques [reviewed in (36)], but major considerations include the disparity of hydrophobicity between sphingolipid molecular species and the sizable differences in polarity in sphingolipid sub species generated via the derivatized head group. When all of these considerations are accounted for, MS can be utilized as a powerful tool for the quantitative analysis of multiple classes of sphingolipids across many biological matrixes.

2.2 Fluorescence and Ultra Violet detection

Profiling sphingolipids has primarily been achieved via chromatography though the use of traditional HPLC detectors has been hampered as sphingolipids do not contain natural fluorophores or chromophores. Sample derivatization has played a key role in the early understanding of alterations in total cellular sphingolipid content. Iwamori et al. were among the first to derivatize Cer utilizing benzoyl chloride to generate UV absorbent Cer molecules which were detectable in the 230–280 nm range with a detection limit of 10 nmoles (86). Interestingly, these N-acyl benzoylated Cer derivatives were separated via normal phase chromatography on silica columns utilizing a mobile phase containing hexane/ethyl acetate. The major drawback with the derivatization technique is that it is highly sensitive to water, and the chemicals are highly toxic. By comparison, other derivatization techniques with fluorescent molecules yielded an order of magnitude more sensitivity and added selectivity as reverse phase HPLC began to be utilized for sphingolipids (161), thus allowing for the separation of Cer species based on their N-acyl chain length and degrees of desaturation. Indeed, Yano et al. utilized acetonitrile/methanol/ethyl acetate mobile phases in conjunction with a C18 stationary phase and derivatization with the fluorescent reagent anthrolyl-cyanide to separate and detect over 17 distinct Cer and dihydroceramide (dhCer) molecular species (242). Chromatographic separations such as these began to allow for clarification of the role and metabolism of sphingolipids as well as the molecular species contained in each group. The major drawback of these derivatization procedures comes from the inherent instability introduced with the derivatization products. Thus, samples typically cannot be stored for long periods of time and must be analyzed quickly following the reaction (202). Also, unlike MS analysis of the directly extracted lipids, derivatization reactions may introduce more variability, consequently reducing the recovery of sphingolipids or affecting the reproducibility of a sphingolipid analysis.

2.3 Thin Layer Chromatography Detection

Thin layer chromatography (TLC), is an indispensable tool for the analysis of both sphingolipid flux and mass. This relatively rapid method offers many advantages over other techniques noted in this section, as it is robust, simple, and economical. This decades-old system is still utilized by investigators desiring a broad overview of sphingolipid classes, typically by normal phase separation (49). The conditions commonly utilized are a solvent mixture of chloroform/methanol/aqueous solution on silica gel, alumina, or kieselguhr stationary phase (49, 108). After separation on the TLC plate, based on sphingolipid biophysical properties, analytes are visualized and quantified by densitometry after identification and comparison with standards. To increase the sensitivity of TLC analysis, cells are often labeled with radioactive or fluorescent probes and/or precursors, thereby greatly reducing background. Recently, high performance TLC has offered greater reproducibility and fidelity combined with a decrease in the necessary sample volume (49, 108, 205). These advances in chromatography have been utilized by Torretta et al. in conjunction with MALDI MS detection to analyze sphingolipid molecules that can be difficult to define by standard means (217). Thus, although TLC is an older method, it is still used extensively and effectively for measuring sphingolipids.

2.4 Diglyceride Kinase Assay

Another historically important method for the analysis of total cellular ceramide content is the diglyceride kinase (DGK) assay. The assay was developed to monitor diacylglycerol (DAG) content in cells through mass conversion to phosphatidic acid (160), but it was quickly realized that bacterial DGK will also phosphorylate the 1' hydroxyl group on Cer to form C1P, thus allowing for radiometric quantitation of Cer in a given cell lysate as long as *quantitative* (full) conversion of ceramide is achieved (153). This is a critical consideration. Additional special consideration should be given to the form of the *E. coli* DGK enzyme, as it has been noted that lyophilized enzyme preparations may be prone to stimulation by endogenous lipids, potentially skewing Cer readings (153), a shortcoming that is obviated if DGK activity is in large excess and therefore not susceptible to activators or inhibitors. Labeled C1P product identification and quantitation is based on TLC plate mobility when compared to appropriate standards. This assay was also adapted for the analysis of free sphingoid bases by Van Veldhoven et al.; following chemical N-acylation with hexanoic anhydride resulting in the formation of C6-ceramide which was then used as a substrate for *E. coli* DGK and quantified (225). This method has been instrumental in the analysis of Cer and its roles in biology but has been mostly replaced by MS technology.

2.5 Antibodies

The subcellular site of sphingolipid generation is potentially of equal importance to changes in their mass since most of the bioactive sphingolipids demonstrate compartment specificity. Spatial context can be given to sphingolipid levels with the use of anti-sphingolipid antibodies (ASABs). Perhaps qualitative at best, ASABs also allow for the study of protein-lipid interactions through pulldown experiments. Therefore, while other methods in this section focus on evaluating sphingolipid mass, these antibodies begin to interrogate compartment-specific signaling events (92). Currently, ASABs are available for determining cellular pools of Cer, Sph, and S1P (172, 175). ASABs have been utilized with flow cytometry, immunohistochemistry and western blotting, as well as for lipid-protein immunoprecipitation. Moreover, ASABs have the potential to be utilized therapeutically. The Cer antibody has been utilized as a countermeasure against the harmful side effects of Cer-induced apoptosis in radiation (172). It should be noted, however, that the lipid specificity of many of these antibodies (especially anti-ceramide antibodies) has not been well established *in vitro* and even less so in cells (43, 92, 102).

3. Evaluating sphingolipid biosynthetic flux; use of probes and inhibitors

Levels of sphingolipid species in the cell can be regulated either by changes in the expression and/or activity of the enzymes that act on them, as well as by substrate availability/load. Additionally, localization of specific sphingolipid enzymes often dictates the metabolic fate of sphingolipids. This flux in the sphingolipid network both in localization and levels of metabolites has been shown to have biological implications. For example, roles have been established for mitochondrial (193) and nuclear Cer production in apoptosis (235), while Cer generated at the PM by nSMase2 is an essential part of breast cancer cells cytostatic responses to certain chemotherapeutics (185). Better understanding of

the effects of Cer generation and metabolic flux will provide significant and more specific insight into Cer-mediated-biological processes and possibly chemotherapeutics.

Thus, analysis of flux in this dynamic network can provide important context to sphingolipid mass levels, be it enzymatic or spatial. Throughout the years, many of the aforementioned techniques for monitoring sphingolipid mass have been adapted into pulse/chase type experiments, often used in conjunction with inhibitors to validate flux at key points in sphingolipid metabolism (182). Many enzymes have a vast array of probes or inhibitors (Figures 2 and 3), and there are many reviews on this topic that comprehensively cover sphingolipid metabolism discussing the importance of functional groups and the classes of inhibitors available (44, 121, 126, 182). This section will highlight some of the more useful probes and the more potent or relevant inhibitors that lend themselves to the analysis of flux *in situ* for mammalian cells. Of note, many of the *in situ* labels can be used to monitor flux through multiple enzymes, briefly reviewed in the section on probing *de novo* flux.

Many of the probes mentioned in the following sections share similar structures with endogenous sphingolipids, and therefore biochemical properties, leading to important considerations in their handling and use. Storing solutions of sphingolipid probes at -80°C can cause them to come out of solution, but most lipids are fairly stable and can be stored at -20°C . If a solution containing a probe appears to contain precipitant or is hazy, sonication and/or slight heat ($<45^{\circ}\text{C}$) can be utilized to get the lipid back into solution. A caveat would be fluorescent lipid probes containing NBD or BODIPY fluorescent groups that can have limited photostability, though it should be noted that BODIPY is the more stable of the two probes.

Most sphingolipid probes are available in a variety of N-acyl chain lengths (from short to long) and are soluble in methanol or ethanol. Though most cells can tolerate small amounts of organic solvent in their media (for probe delivery), probes can also be complexed with bovine serum albumin allowing for delivery in aqueous solutions. Finally, it is important to note that sphingolipid probes or their metabolites have the potential to instigate cellular signaling, due to similarities to their endogenous counterparts, and therefore should be used at the lowest possible dose.

3.1. Serine palmitoyltransferase- probes and inhibitors

The first step in *de novo* sphingolipid metabolism occurs in the ER via SPT. The basic SPT heterodimer complex (SPTLC1 and SPTLC2 or SPTLC3) catalyzes a condensation reaction of serine and palmitoyl-CoA. In yeast, SPT is appreciated to be a multicomponent complex, including additional subunits. Both stable isotope and radioactive substrates have been utilized in the literature to probe this rate limiting step, though as noted by Merrill et al. special care must be exercised with the use of labeled palmitate, as even at low concentrations it can induce sphingolipid biologies (77). Isotopically labeled serine on the other hand was able to be utilized over a wide range of concentrations exhibiting only a slight ability to drive sphingolipid synthesis at mM concentrations (up to 30%) (130). Most methods utilized serine-depleted cell culture media that is reconstituted with isotopically labeled serine to increase the potential label uptake (69, 191). Concentrations of the isotopically labeled serine are typically in the mid μM range for standard cell culture media

(Fig 2). It is important to note that serine is not an essential amino acid, and therefore cells can produce it. This becomes an important consideration for longer pulses, and therefore the levels of serine in the media and cells should be monitored at extended time points. Also of note, the signal to noise ratio is reduced for some stable isotopes (namely ^{13}C serine) due to the relatively high natural abundance of ^{13}C in the hydrocarbon chains of lipids producing large amounts of endogenous sphingolipids with identical masses to their labeled counterparts (93). Radioactive ^{14}C serine has also been extensively utilized in both pulse/chase and steady state assays, and, while it is not subject to the same considerations regarding the background as ^{13}C labeling, the method of detection does not lend itself to separation of sphingolipid molecular species. Typically, cells are labeled for minutes to hours in serine pulse/chase experiments as labeled serine reaches steady state in the cell within 10–20 min (130).

Due to utilization of palmitate by two major steps in ceramide synthesis (SPT and CerS), its incorporation can be more difficult to interpret than serine. One solution to evaluate *de novo* synthesis (which incorporates palmitate into both positions) and salvage (which incorporates palmitate only in the acyl position) can be achieved through the use and analysis of isotopologues (molecules with different isotopes at the same structural positions) and isotopomers (molecules with the same isotopes at different structural positions) generated via the incorporation of label into either the sphingoid backbone, N-acyl chain, or both. Indeed Sims et al. used 0.1mM [^{13}C]palmitate to implicate *de novo* synthesis in the initial autophagic response of RAW264.7 cells to KLA (Kdo₂-lipid A) treatment as most of the Cer mass generated in unlabeled cells could be accounted for in the labeled sphingoid backbone upon addition of the probe (190). As labeling to steady state tends to obfuscate flux results, it should be noted that, in the case of palmitate, labeling to steady state was relatively rapid. When HEK293 cells were labeled with 0.1mM of [^{13}C] palmitate for 6 h, the palmitoyl-CoA pools utilized to produce 3-ketosphinganine nearly reached isotopic equilibrium (77). One major limitation in this approach is the biologic effects that higher concentrations of fatty acids (above 0.1mM) can have on ion transport, gene regulation, and even sphingolipid metabolism (77). A second limitation to both palmitate and serine labeling is the fact that both substrates for SPT are major components of cellular metabolism and therefore are subject to utilization throughout the cell producing numerous nonsphingolipid metabolites.

The discovery that other amino acids (besides serine) can be utilized for the generation of sphingolipids (151) presents an exciting opportunity to utilize other stable-isotope amino acids to probe generation of these non-canonical sphingolipid based (249). In this case, use of alanine or glycine by SPT generates deoxy-dihydrosphingosine and 1-deoxymethyl-dihydrosphingosine. Indeed, the use of isotopically labeled alanine and serine was utilized to verify that deoxysphingolipids, thought to initially be the result of action of mutations in the SPTLC1 gene (151), are regularly produced by mammalian cells (249).

The condensation reaction of SPT to form 3-ketosphinganine has multiple inhibitors including myriocin, lipoxamycin and fungins (44). The most potent of these, myriocin, also known as ISP-1, has an established K_i of 0.28 nM in vitro and an IC_{50} of 15 nM in CTLL-2 cells (133). It is commonly used between 0.1 and 5 μM for up to 24 h in mammalian cells (Fig 3) (5, 82, 104, 134), although the rationale for the higher concentrations is not known

and currently should not be encouraged. Myriocin inhibition is likely driven through the similarities it has to a catalytic intermediate of the SPT reaction (68). Not surprisingly, structurally modified amino acids such as cycloserine and β -chloroalanine have also been utilized for the inhibition of SPT. Though as many enzymes utilize serine and alanine, these inhibitors are not very specific (124, 204). Indeed, Hanada et al. demonstrated that in SPT deficient cells, addition of Sph could rescue cell growth from myriocin treatment, but was ineffective against modified amino acids, indicating that the primary effects of cycloserine and β -chloroalanine on growth were not through SPT (70).

3.2. 3-Keto-dihydrosphingosine reductase - probes

The 3-keto-dihydrosphingosine reductase (KDSR) is one of the least understood of the enzymes central to sphingolipid metabolism. Only *in vitro* assays have been performed to directly measure KDSR activity. These assays utilize 10–100 μ M of 3-[4,5- 3 H]ketosphinganine for 10 min to 1 h (101, 129, 208) and with the addition of NADPH accounting for the reducing cofactor. The *in vivo* KDSR activity has not been well studied. No labeled 3KDS and DHS were detected in a flux assay where radio-labeled serine (such as [3 H]serine) was added to the live cell culture (128, 133). This led to the notion that the 3KDS is a highly transient sphingolipid intermediate that is converted to downstream metabolites by KDSR at a fast rate, suggesting little/no regulation at the KDSR level. A recent study reported the detection of a stable pool of 3KDS in yeast *S. cerevisiae* at a level comparable to cellular DHS using a HPLC-ESI-MS/MS method (167). We believe the direct detection of both the substrate and product of KDSR makes it possible to develop labeling methods to follow the flux of serine into the sphingolipid pathway through KDSR and thus measure its activity and regulation *in vivo*. Therefore, as there are no known inhibitors for KDSR, methods for probing flux represent the first step to explore the physiological activity of KDSR, a long-ignored enzyme in the sphingolipid biosynthetic pathway.

3.3. Ceramide Synthases - probes and inhibitors

In mammals, CerSs constitute a family of 6 distinct enzymes, products of distinct genes (*S. cerevisiae* has two: Lag1 and Lac1). CerSs1–6 display distinct preference for the fatty acyl CoA substrates, thus generating ceramides, and subsequent complex sphingolipids, with distinct N-linked fatty acids. Probing flux through CerSs presents a challenge in that the CerSs are active in both *de novo* synthesis and salvage pathway; thus, the flow of these two pathways converge in the ER through CerS-mediated N-acylation of both dhSph and Sph, respectively. Regulation and function of these 6 enzymes, which have been implicated in cellular stress responses such as autophagy, apoptosis, and growth arrest, has been thoroughly reviewed by Wenger et al. (236). Thus, many probes have been developed to assess CerS activity *in situ*. The different isozymes of CerS have similar affinity towards the dhSph substrate (K_m between 2 and 5 μ M (103)), and consequently dhCer production appears to be driven by which CerS is present and/or active (135). Hence odd chain sphingoid bases such as d17dhSph and d17Sph are commonly utilized probes. These probes are also easily distinguishable from the 18 carbon backbones of most endogenous sphingolipids and readily allow for the HPLC/MS/MS quantitation of the different CerSs products (183, 196, 197). These d17-sphingoid bases are typically used between 0.5 and 2 μ M from 5 min to 4 hours *in situ* (Fig 2)(134, 183). It is important to avoid higher

concentrations of these probes as concentrations of sphingoid bases in the low μM range can exert significant biological effects (168). Also, when monitoring the product of CerS activity, consideration should be given to the substrate utilized, as dhSph requires a second step (via DeS activity) after CerS activity to generate labeled Cer.

Multiple fluorescent probes that do not require MS for detection have also been developed utilizing Sph analogs, including NBD sphingoid bases (215) and “clickable” derivatives (65). The utilization of a photoactivatable and clickable Sph (PACsph) showed that while the uptake of PACsph was very quick, reaching the ER 5 min after addition to the media, it required 4–6 h for PAC-metabolites to reach the PM (65). This study was not only able to visualize sphingolipid metabolism throughout the cell, but also utilized click chemistry to bind PAC-sphingolipid metabolites to interacting proteins.

[^{13}C] palmitate is another tool used to probe flux through the CerS. As previously mentioned, there are considerations to the use of palmitate as a label (reviewed in (77)), but the incorporation of the [^{13}C]palmitate into the sphingoid base, the N-acyl chain, or both presents an opportunity to deconvolute salvage from *de novo* lipid synthesis. Indeed, in a study of fatty acid effects on insulin resistance utilizing a 3h incubation with ^{13}C palmitate, Hu et al. were able to implicate stimulation of the salvage pathway since most of the N-acyl labeled C16Cer pools (the predominant labeled position) did not contain a labeled sphingoid backbone (81). The ^{14}C radioactive palmitate isotopologue has also been utilized. In a 2005 study, Becker et al. utilized labeled palmitate at 3.0 uCi/ml for 16–24h to validate that phorbol ester treatment was able to increase turnover through hydrolytic and CDase pathways to form free Sph which could be reacylated through salvage to generate bioactive Cer (16). Careful consideration must be given to probing CerS activity as the confluence of salvage and *de novo* pathways makes it one of the most complicated steps in sphingolipid metabolism to interpret.

The judicious use of inhibitors can also be employed to distinguish the salvage and *de novo* pathways. Utilizing inhibitors of SPT in conjunction with the potent and what appears to be highly specific CerS inhibitor fumonisins B1 (FB1), Becker et al. were able to demonstrate activation of the salvage pathway in response to sustained PKC activation. In this study, Cer generation upon PKC activation was sensitive to FB1, but not myriocin, thus metabolic flux through salvage was implicated (16). In mammalian cells, FB1 is typically used at a concentration of 5–100 μM (80, 82, 104, 134, 140, 170), and pretreatment of 1–6 h before further perturbation is common, likely to allow cellular uptake (Fig 3) (146, 239). In renal cells, FB1 has an IC_{50} of 35 μM for inhibition of Cer biosynthesis (141). FB1 contains anionic tricarballic acids and aminopentol backbone that likely drive competition with the sphingoid and acyl-CoA substrates. The inhibitor itself has been linked to renal and liver toxicity, carcinogenesis, neurotoxicity, and pulmonary edema, which limit its use *in vivo*. Interestingly, these toxicities could all potentially be linked to the marked elevation in sphingolipid levels (46). The Sph analog FTY720 has also been utilized to decipher regulation amongst the various CerS isozymes, and while concentrations of FTY720 that could inhibit CerS cannot be reached in humans, it still remains a powerful tool for *in situ* studies. It has been shown to mainly affect long chain Cer synthesis as it predominantly inhibited CerS5 and/or CerS6 (18), though analogues of FTY720 have also been developed

for the preferential inhibition of CerS2 and CerS4 (178). Recently, P053, was shown to specifically inhibit CerS1 activity (IC_{50} of 0.5 μ M), demonstrating that very specific and potent CerS inhibitors can be developed (219).

3.4. Dihydroceramide desaturase- probes and inhibitors

As the last enzyme in the *de novo* generation of Cer, the DeS enzymes catalyze the formation of a double bond between the C4 and C5 position in the sphingoid backbone, although DeS2 also functions as a hydroxylase introducing an OH group on the C4 position of the sphingoid base (effectively making phytoCer). Only relatively recently has dhCer begun to be recognized as a bioactive molecule with implications in autophagy (37), mitochondrial ROS production (55), and growth arrest (123). Therefore, probing the conversion of dhCer to its delta-4 unsaturated metabolite has become an important endeavor. Initial analysis of DeS activity was performed utilizing the fluorescent C6-NBD-dhCer at 0.5 to 10 μ M for up to 24 h *in situ* and *in vitro* (Fig 2)(37, 97, 136). Indeed, C6-NBD-dhCer was instrumental in the 1997 study by Kok et al., which led to the initial observations that desaturation of the sphingolipid backbone occurred during *de novo* synthesis and not later in metabolism via desaturation of complex dihydrosphingolipids (97). In a more selective and sensitive assay for DeS activity, D-erythro-2-N-[12'-(1''-Pyridinium)-dodecanoyl]-4,5-dihydrosphingosine bromide (C12dhCCPS) was utilized *in cellulo* at 0.5 μ M and reached a pseudo steady state at 6 h (time course 15 min to 24 h) in two cancer cell lines (100). It should be noted that utilizing C12dhCCPS as substrate with MS as a detector, the transition of 577.4/79.8m/z/90CE (labeled PhytoCer derivative) can be utilized to also monitor the documented hydroxylase activity of DeS2 (Snider unpublished data), thus allowing for at least partial separation of the isoenzymes activities.

Interestingly, DeS regulation has been shown to occur via several mechanisms in the cell. DeS has been shown to be regulated via transcriptional mechanisms as well as by palmitoylation and translocation in response to fatty acid treatment (15). It has also been shown to be regulated through availability of the NADPH cofactor during oxidative stress conditions (84). Two commonly used inhibitors of DeS in the literature are the sphingolipid analog GT11 and the vitamin A analog Fenretinide. Fenretinide (4-hydroxyphenylretinamide, 4HPR) was reported as a competitive inhibitor with a K_i of 8.28 $\pm$ 1.25 μ M and an IC_{50} of 2.32 μ M for enzyme activity in rat liver microsomes by Rahmaniyan in 2011 (164). 4HPR may also inhibit DeS through ROS generation (84), potentially reducing the cellular pools of the DeS cofactor, NADPH. In turn, these two different regulatory mechanisms of DeS activity may account for the different phenotypes seen in cancer cells at low (cytostatic (100)) and high (cytotoxic (233)) 4HPR doses. Building on previous work (232), Kraveka et al. demonstrated an optimal inhibition of DeS activity at 2 h with 1 μ M 4HPR in human neuroblast cells, however 4HPR for DeS inhibition has been utilized in cells up to and above the cytotoxic dose of 5 μ M for up to 48 h (6, 100, 180). On the other hand, the cyclopropane containing compound GT11 (C8-cyclopropenylceramide, C8CCP) is a sphingolipid analog and exhibited an IC_{50} of 23 nM for the inhibition of formation of Cer by DeS in primary cultured cerebellar neurons (218). GT11 has also been successfully utilized *in vitro* as a competitive inhibitor with a K_i of 13 μ M. When considering the use of GT11, two important issues must be considered: 1) higher

concentrations of GT11 (>5 μM) induce inhibition of SPT via accumulations of sphingoid base phosphates (218), and 2) the cyclopropene structure in GT11 is prone to oxidation via atmospheric oxygen, and stocks must be stored under nitrogen; thus, for extended use, the XM462 analog, which is more chemically stable, may be considered (136). Typically, GT11 concentrations below 2.5 μM are used in cells for 30 min to 2 h pretreatments with a total incubation of up to 24 h (164). Thus, there are multiple very potent and specific options for the inhibition of DeS that are helping to drive forward the understanding of this crucial step in sphingolipid metabolism.

3.5. Sphingomyelin synthase - probes and inhibitors

The generation of SM represents a metabolic fork in the road for ER pools of Cer. The transfer of Cer from the ER to the Golgi via CERT leads to the preferential generation of SM through sphingomyelin synthase 1 (SMS1) (212). SMS2 exhibits similar substrate specificity as SMS1, but can be localized at both the PM and Golgi (210). Non-sphingolipid metabolites may also need to be considered when probing SMS biological function since these enzymes also affect proliferative cellular signaling through the metabolism of phosphatidylcholine (the donor of the choline phosphate head group in the SMS reaction) to form the bioactive molecule DAG (71, 228). SMS activity has traditionally been assessed utilizing C6-NBD-Cer in cells at concentrations of 1–5 μM for 1–8 h (Fig 2) (7, 29, 96, 228). An *in vitro* study by the Ye group determined the K_m for C6-NBD-Cer as a substrate for SMS at 7.5 μM . In a 2007 study evaluating the contribution of the two SMS isozymes to cellular SM, Tafesse et al. utilized C6-NBD-Cer in human cervical carcinoma HeLa cells to evaluate both flux and localization of SMNDB following knock down of either enzyme using RNA interference. While an HPLC analysis quantified the reduced incorporation of the label into SM in either SMS1 or SMS2 knockdowns, utilizing fluorescent microscopy indicated abolished Golgi staining in response to SMS1 knockdown. Therefore, the use of a single probe provided complementary information when different detection methods are employed (206).

The toxins lysenin and equinatoxin-II (EqII) bind SM with high affinity and specificity, and both are effective probes for visualizing SM and for identifying relative levels of SM at the PM. Yachi et al. observed that, after permeabilization of cells, these stains localized to different organelles, with EqII staining abolished upon knockdown of SMS1, indicating that these probes actually recognized different pools of SM (241). Finally, radioactive SMS substrate precursors, such as [^3H] choline, are also utilized to monitor perturbations in SMS activity (47, 75, 119, 206) in cells, though newer stable isotope assays present less hazardous substrates. Methyl[^{14}C]choline is typically utilized at 0.5–2 $\mu\text{Ci/ml}$ for 4–5 hours for pulse conditions (206, 221) and takes between 24–60 hours to reach steady state labeling of SM (4, 119).

As there are differences in sub-cellular localization of SMS isoforms and distinct regulation of SM pools, inhibitors that could distinguish these activities would be highly desirable. The initial inhibitor discovered for SMS was the anti-viral reagent D609 (119), that exhibited an IC_{50} of 375 μM towards SMS1 and was less effective at inhibiting SMS2. It has been utilized in multiple cell types at a concentration of 20–25 $\mu\text{g/ml}$ for 2–6 h (Fig 3) (119, 125,

169). However, particular care must be taken with stocks as the xanthate group of D609 can be readily oxidized ($t_{1/2}$ is less than 20 min in saline conditions at 24 °C (11)), causing a loss of biological activity. A more stable prodrug (methylenedioxybutyryl D609)) has been described (11). More potent and specific inhibitors of the isozymes activity have recently been developed, including D-Series inhibitors, SAPAs, and 2-Quinolone derivative (1, 45, 109). Li et al. reported on the 2-(4-(N-phenethylsulfamoyl)phenoxy)acetamides (SAPAs) molecules, one of which had a K_m of 2.1 μ M for SMS1 (109), a 100 fold more potent than D609. Also, greater specificity was achieved by the Kawamoto group who recently developed a 2-Quinolone derivative that has an IC_{50} in the nM range with over 100 fold selectivity for SMS2, potentially providing a much more effective tool for the interrogation of SMS activity (1). However these inhibitors are not suitable for cellular studies.

3.6. Glucosylceramide synthase - probes and inhibitors

While SM synthesis represents an “anabolic dead end”, the generation of GluCer, through glucosylceramide synthase (GCS) activity, is a stepping stone to the vastly complex family of glycosphingolipids. In this complicated arm of sphingolipid metabolism, utilizing molecular probes is the most effective way to track metabolic changes. Typical tracer experiments in cells utilize C6-NBD-Cer as a direct substrate for GCS at 1–100 μ M for 30 min to 3 h and analyze the products using TLC/fluorimetric analysis (Fig 2) (29, 47, 96, 114, 122, 189, 231). Fluorescent molecules have been extensively utilized in the study of vesicular trafficking of newly synthesized complex sphingolipids. In an early study, Babia et al. demonstrated distinct NBD-GluCer or NBD-SM containing vesicles in HT-29 cells labeled with C6-NBD-Cer (9). An important consideration in the analysis of GCS flux is GalCer, an isomer of the GCS product, which can often be difficult to separate with HPLC and fluorescent detection techniques. As tissue distribution between these glycosphingolipid isomers is highly distinct, it is sometimes possible to deduce GCS activity in tissues with very low/absent formation of GalCer. Though to circumvent this issue, radioactive UDP-glucose and UDP-galactose have also been utilized in many studies. Brenkert et al. defined the effects of age on patterns of Cer glycosylation in rat brain lysate utilizing either glucose or galactose tracers to separate the metabolism of Cer into GluCer or GalCer isomers (33). It is also possible to separate these isomers utilizing borate impregnated HPTLC plates, as was demonstrated by Gupta et al. with a 2 h incubation of 500 μ M C6-NBD-Cer in a breast cancer cell line (64).

Traditionally, inhibitors for glycosyltransferases are few, but GCS is an exception, with inhibitors generated from analogs of both substrates, UDPglucose and Cer. 1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP) is a ceramide analog which contains an aromatic ring and tetracyclic system in place of the alkyl chain and sphingoid base, respectively. PDMP is a mixed-type inhibitor that is typically used *in situ* at IC_{10} values (50 μ M) (Fig 3) (14, 29, 56). Improved derivatives of PDMP include P4, a compound with an IC_{50} of 90 nM towards the inhibition of ceramide glycosylation by GCS *in vitro* (106). Recently C10, an analog of the potent drug eliglustat (used in treatment of Gaucher's disease) has been utilized for the inhibition of GCS *in situ* (IC_{50} in low nanomolar range), and of particular importance, appears to have less off target effects than PDMP (unpublished data and (187, 188)). Subathra et al. utilized 0.15 μ M C10 in mesangial cells for 48 h,

decreasing GCS activity by 90% (201). As a counterpart to the ceramide analogs, immunosugar derivatives of UDPGlu have also been utilized to inhibit GCS, in particular deoxynojirimycin (DNJ) analogs, some of which have low μM IC₅₀s (29, 41, 158, 159). There is the possibility of immunosugars having off-target effects throughout the cell and on other steps in glycosphingolipid metabolism, therefore when probing the initial step in this metabolic pathway, Cer analogs may present a better option.

3.6. Ceramide kinase - probes and inhibitors

Ceramide kinase (CERK) has been implicated in several cellular processes, including survival and proliferation (58) as well as apoptosis and inflammatory responses (60). C6-NBDCer has been employed as a substrate for probing CERK activity *in vitro* with a reported K_m of 4.0 μM (224) when utilized with purified enzyme. Pettus et al. utilized 20 μM C6-NBD-Cer for 2 h in A549 lung adenocarcinoma cells to demonstrate cytokine activation of CERK (154). More recently Matsuzaki et al. were able to take advantage of the increased sensitivity afforded by technical advances in chromatography and detectors to create a CERK activity assay utilizing 30 min treatment of 10 μM C6-NBD-Cer in HEPG2 cells (Fig 2) (122). Of note, due to the large range in NBD metabolite concentrations, absolute quantification was difficult, and results were expressed as a percentage of the total cellular concentration of the NBD metabolites. Matsuzaki also noted the additional polarity offered by the phosphorylation to the already less hydrophobic NBD labeled metabolite can affect partitioning during extraction; therefore, single phase extractions (48) or a solid phase extraction (224) should be considered to achieve optimal recovery. Fluorescent C5-BODIPY-Cer has also been utilized to study C1P trafficking, though at lower concentrations than its NBD counterpart (5 μM for 30–45 min in COS-1 cells) (28). Radioactive phosphate tracers ([³²P] orthophosphate) are also popular and typically used at 0.1–10 mCi/ml for 2–15 h in cells (60, 222–224).

There are few inhibitors for CERK activity. It has been noted that some SK inhibitors modestly inhibit CERK activity, though treatments with the SK inhibitors MP-A08 and F-12509A were in the 100's of μM range to achieve only a 50% inhibition of CERK (155, 203). The inhibitor NVP-231 is highly specific and has an IC₅₀ value of 12 nM for CERK activity, with an observed K_i of 7.4 nM (59). NVP-231 provided a 90% inhibition of CERK activity when utilized in cells at a concentration of 100 nM for 6 h (Fig 3), and increasing the concentration to 1 μM or increasing the time to 24 provided no significant improvement in its inhibitory effect (59). While there are few inhibitors for CERK, NVP-231 represents a potent option for the interrogation of CERK signaling potential.

3.7 Probing *de novo* flux

As mentioned above, many of the fluorescent products used to measure enzymatic activity in cells are recognized and further metabolized by sphingolipid enzymes. Therefore, if a comprehensive approach is taken to analyzing *de novo* sphingolipid synthesis, the metabolic fate of probes can provide insight into the activity of multiple enzymes. While exogenous treatments with C6-NBD-Cer have been extensively utilized to probe sphingolipid metabolism mostly at the Golgi (SMS, CERK, GCS) (112), there is still some contention as to the confounding effects that an omega positioned NBD fluorophore and short N-acyl

chain length have on the recognition of this molecule versus endogenous sphingolipids by Golgi enzymes of sphingolipid metabolism. Thus, the utilization of a d17 sphingoid backbone or isotopically labeled precursor may produce the most faithful results to indicate regulation of endogenous sphingolipid metabolic flux. Indeed, a method was recently developed by Snider et al. utilizing mass spectrometry to monitor temporally distinct phases for the incorporation of a d17dhsph pulse into d17dhCer, d17Cer, d17SM, d17HexCer, d17Sph, and d17dhSph 1-phosphate, allowing for the dissection of enzymatic activities for CerS, DeS, SMS, GCS, CDases, and SK1, respectively (195). This method offers a much more comprehensive insight into *de novo* sphingolipid flux than those using fluorescent probes, as mass spectrometry can follow the incorporation of the d17dhsph probe into the myriad of sphingolipid molecular species that can be generated in the ER and Golgi. Additionally, the utilization of a dihydro sphingoid base as a probe allows for accurate monitoring of ceramidase activity, which occurs to a much greater extent on Cer molecules (as opposed to dhcer), thereby producing a different molecule (d17Sph) from the initial probe (d17dhSph) (195). In such a dynamic network, these methods for probing entire sections of sphingolipid metabolism will play a crucial role in moving forward the understanding of sphingolipid metabolism and transport.

4. Sphingolipid Turnover and Clearance

4.1. Sphingomyelinases - probes and inhibitors

SM is the most abundant sphingolipid at the PM, but it is also found in several other subcellular membranes. Therefore, hydrolysis of these SM pools provides an efficient means for cells to generate Cer quickly and in a compartment-specific manner. Consequently, the SMases play an important role in signaling for Cer-driven biologies, including apoptosis (3), growth arrest (185), and autophagy (152). As pH dependent activity has been observed, these enzymes are separated into 3 categories, acid (aSMase), neutral (nSMases), and alkaline. The radioactive substrate [choline-methyl-¹⁴C]-SM and fluorescent probes have been utilized for *in vitro* enzyme activity assays (116), and changing the pH from 4.0 to 7.4 (as well as the addition of Mg²⁺) will change the assay specificity from aSMase to nSMase activity. The C6-NBD-SM substrate is typically utilized in cells through direct addition to media at 2–4 μM for 30–60min to probe SMase activity (Fig 2) (96, 117). PM isolations (96) and back exchange conditions (0–4 degrees with BSA) (246) have been utilized to optimally assess SMase activity on fluorescent probes in a cellular context. Utilizing back exchange conditions, Hao et al. were able to establish two routes for C6-NBD-SM recycling at the PM, one with a label halftime of 1.5 min and one with a slower recycling halftime of 12 min (74). Further, utilizing a lipophilic dye (FM) that cannot flip-flop across the lipid bilayers, they interrogated endosomal fusion and were able to verify the hypothesis that both of the recycling pools were indeed internalized and included passage through early endosomes (74). Altering the temperature in the back exchange protocol was utilized to demonstrate defects in SM degradation in Niemann-pick type-A fibroblasts (NPA) that allowed SM to accumulate in the lysosomes. Liposomes containing 25 μM NBD-SM were initially loaded into the PM of cells at 7 degrees, with similar rates of internalization between normal and NP-A cells, but, as temperature and time were increased, normal cells demonstrated increased fluorescence in the Golgi apparatus while NBD-SM accumulated in the lysosomes

in NP-A cells. Therefore, NBD-SM can be utilized to monitor flux through multiple components of SM degradation based on its transport between intercellular compartments (99).

For evaluation of cellular activity of SMases, steady state labeling of SM substrate can be reached within 16–48 h depending on the cell line and substrate utilized (e.g choline or palmitate)(2, 4, 50). Depletion of labeled SM then provides insight into SMase activity. To distinguish SM pools that were sensitive to hydrolysis in response to TNF α signaling, Levade's group employed [methyl- ^3H]choline at 2 μCi for 48h in conjunction with exogenous SMase treatments(2). Demonstrating that pools of SM generated after TNF α treatment were not accessible to exogenous SMase, highlighting a multipronged approach to interrogate SMase flux. There is a plethora of inhibitors for the SMases that can be employed to evaluate activation and roles of specific SMases. Many of these inhibitors are structurally unrelated to SM, including desipramine, SR33557, NB6, C11AG, and GW4869, though some are rather unselective. Of these, GW4869 is a selective noncompetitive inhibitor with an IC $_{50}$ of 1 μM for nSMase activity, with no effect on aSMase (120). This inhibitor is typically utilized in cells at 10–20 μM (120, 213). Recently, 2,6-Dimethoxy-4-(5-Phenyl-4-Thiophen-2-yl-1H-Imidazol-2-yl)-Phenol (DPTIP), was identified as a potent inhibitor of nSMase2 through a high throughput screen. DPTIP demonstrated better solubility than previous nSMase inhibitors which contributed to its ability to cross the blood-brain barrier (171). aSMase is commonly inhibited by desipramine and related tricyclic compounds by disrupting aSMase binding to lipid bilayers in the lysosome, thereby inducing proteolytic degradation of the enzyme (83, 98). This class of compounds has been utilized in cells, normally at 25 μM for 1–2 h (Fig 3)(83, 89). However, these molecules also inhibit acid CDase (245) and probably have additional effects on lysosomal proteins. It has been demonstrated that both aSMase and nSMase can also be inhibited by cellular metabolites. Testai et al. observed non-competitive inhibition of aSMase with phosphatidyl-myo-inositol 3,4,5-triphosphate [PtdIns (3,4,5)P] (83), with a IC $_{50}$ of 3 μM for Cer generation by SMase activity (214). Scaphostatin, one of the original molecules used to inhibit SMase activity, still remains one of the most potent inhibitors available for nSMase activity with an IC $_{50}$ value of 1 μM for nSMase and 49.7 μM for aSMase (137). At 5mM, glutathione exhibited greater than 95% inhibition of nSMase *in vitro* (due to changes in buffer pH), but also exhibited inhibitory effects at 10mM in human airway epithelial cells and in hepatocytes (107, 113, 173). As these molecules are pivotal players in cellular metabolism, they cannot be utilized as pharmacological inhibitors, but they may constitute a means for cellular regulation of these enzymes.

4.2. Glucosylcerebrosidases - probes and inhibitors

The generation and catabolism of the structurally diverse complex glycosphingolipids is regulated by many enzymes, though the final degradation and clearance of the majority of these species (those based on GluCer) funnels down to the catabolic action of Glucosylcerebrosidases (GCases). The majority of GCases action occurs in the lysosomes through GBA1 activity, though hydrolysis of GluCer at the PM has been documented via the membrane-bound GBA2 (30, 244). Defective lysosomal activity of GBA1 results in toxic accumulations of GluCer and glucosylsphingosine that have been linked to the pathogenesis

of Gaucher's disease, an inherited disorder (32). Multiple NBD derivatives of the substrate have been utilized in the literature, including C6, C12 and C16-NBD-GluCer, indicating that GCase has no specificity for N-acyl chain length. In cells, C6-NBD-GluCer is typically utilized between 5–50 μ M for 0.5–3 h (Fig 2). Non-lysosomal GBA2 activity was initially tested in COS-7 cells using multiple fluorescent probes, including both C6-NBD-GluCer and 4-methylumbelliferyl- β -gluco-side. In combination with GCase and lysosomal inhibitors these experiments were able to take advantage of the different localization of the GCase enzymes to tease their activities apart (30). Most studies of these enzymes focus on GBA1 activity as it pertains to Gaucher's disease. Indeed, both NBD and BODIPY Cer analogs along with 2-Hexadecanoylamino-4-nitrophenyl β -D-glucopyranoside and 1-O-Glucosyl-2-N-(dimethylaminonaphthalene-5-sulfonyl) sphingosine have been utilized as *in vitro* fluorescent substrates to diagnose Gaucher's disease (51, 131). However, a major issue when utilizing these fluorescent molecules in cells, is that flux through GBA1 is confounded by exogenous substrate degradation at the PM through GBA2 activity, as well as apparent activity occurring in the cytosol, perhaps through a neutral GCase (76).

Multiple groups have published crystal structures of GCase with different inhibitors bound to the active site, such as the iminosugars isofagomine and DNJ analogues, which have led to structural modifications of the compounds to increase potency (34, 110). Specifically, modifications to DNJ structure have led to more potent inhibitors, such as the N-(5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM) compound, which is a hydrophobic derivative of DNJ. AMP-DNM also displayed increased selectivity with an IC₅₀ of 1 nM for GBA2 activity, compared to a 200 nM IC₅₀ for GBA1 (238). Of note, this inhibitor can also antagonize GCS activity at an IC₅₀ of 25 nM (238). Towards the inhibition of GBA1, modifications to Isofagomine have led to the development of an N-alkylated group of Isofagomine derivatives, of which the longest hydrocarbon chain (nonyl) was noted to have an IC₅₀ of 0.6 nM for GBA1 activity (248). Therefore, extremely effective inhibitors are available for the distinction of GluCer hydrolysis by GBA1 versus GBA2.

4.3. Ceramidases - probes and inhibitors

As the first step in the clearance of Cer from cellular pools, ceramidases (CDases) represent a crucial regulatory point in sphingolipid metabolism. The five CDase enzymes are characterized by their pH optimum for activity, acid (aCDase), neutral (nCDase) and three alkaline CDases (ACERs), and by their specific subcellular localization and substrate specificity (39). Thus, analyzing these enzymes in cells with a single probe can be difficult. Initial studies utilized radio tracers ([³H] or [¹⁴C] labeled ceramide) to follow production of the radioactive fatty acid from Cer hydrolysis via CDase activity (132, 243). Fluorescent assays have utilized C6-NBD-Cer at 6 μ M for 12 h (140), but it was realized that C12-NBD-Cer was more effectively processed by ACERs & nCDase (211). In a comprehensive *in vitro* study performed by Tani et al. utilizing enzymes from each of the pH groups with C12-NBD-Cer at concentrations between 5 and 50 μ M for 0.5 to 3 h (Fig 2), K_ms of 22, 59, and 111 μ M for nCDase, aCDase, and ACER were measured, respectively (211). In another study by the same group, C12-NBD-Cer and C6-NBD-Cer were utilized in B16 melanoma cells at a concentration of 5 μ M for 1 h to demonstrate CDase preference for the longer chain

length *in situ* (209). It has also been noted that BODIPY derivatives of Cer are more effectively hydrolyzed by aCDase (78). Bhabak et al. developed a FRET compatible Cer analog combining NBD and Nile Red fluorophores into (NBD)₁₂CerC₇(NR). *In vitro*, the probe demonstrated K_m values of 182 μ M and 142 μ M for aCDase and nCDase, respectively (19). After a 60 min incubation in HeLa cells, the probe localized to the Golgi, though no cleavage of the probe was observed (19). Optimization of these FRET compounds is ongoing (20). A high-throughput assay has been developed by Fabrias and coworkers that employs a coumarin dye attached to the Cer substrate. The hydrolytic CDase activity releases the amino diol group, which is subsequently chemically oxidized by periodate, generating the fluorescent molecule umbelliferone (17). Finally, reverse CDase activity, which is FB1 insensitive, has also been evaluated in lymphoid cells after a 2 h treatment utilizing 20 μ M C12-NBD-fatty acid and 10 μ M Sph to produce fluorescent Cer in conditions where CerS activity is inhibited (145).

CDases have been targeted by multiple inhibitors with ongoing development in this area of research. The initial inhibitor of CDase activity was N-Oleylethanolamide (NOE), which took advantage of structural and stereochemical modifications to directly inhibit the CDases, though it suffered from poor selectivity (198) and low efficacy (K_i of 500 μ M) (61). ACERs can be inhibited with the ceramidase inhibitor D-erythro-MAPP ((1S,2R)-N-myristoylaminophenylpropanol-1) which has an IC_{50} of approximately 1–5 μ M *in vitro* and can induce growth arrest in HL-60 cells in a dose and time dependent manner (22). Inhibition of aCDase has also been pursued, and D-e-MAPP served as the basis for subsequent work that developed B-13 (1R,2R)-N-myristoylamino-4'-nitro-phenylpropanediol-1,3) analogs (12, 20). As ceramidases are localized to specific subcellular compartments, inhibition of these enzymes should ideally be achieved by targeting these specific compartments. As an illustration, second and third generation B13 analogs targeting the lysosome were developed. LCL204 inhibited aCDase with IC_{50} ~40 μ M for MCF-7 cell growth (21). More recently LCL521 was developed as a prodrug that is deacylated to B13 in the lysosome (10). This compound exhibited the most potent effects in cells, but because of its design as a prodrug, it showed little effects *in vitro*. The tricyclic anti-depressant desipramine has also been found to inhibit aCDase, likely by the same indirect lysosomal mechanism by which it inhibits aSMase (245). Finally, Carmofur, a 5-fluorouracil-releasing drug, has demonstrated greater potency (IC_{50} of 29 nM) and specificity than other currently available aCDase inhibitors, both *in vitro* and *in vivo* (166).

Recently molecules that are structurally unrelated to Cer have been employed as inhibitors. The most potent and stable of these are a class of benzoxazolone carboxamides that covalently bind to cysteine-143 in the catalytic site of aCDase (157). In particular, Compound 17a demonstrated an IC_{50} of 79 nM *in vitro*, which remained below 1 μ M when examined in both human and mouse cells (156). Importantly, this class of inhibitors is very efficient as they covalently bind to the enzyme, and inhibition occurs in a time dependent manner.

Inhibition of nCDase has recently been achieved with the ceramide analog C₆ ureaceramide. This competitive inhibitor of nCDase was developed using structure-activity analysis (142, 220), and has been shown to maximally inhibit nCDase at 25 μ M in cells with treatments up

to 24 h (Fig 3) (54). This inhibitor did not modulate ceramide levels in epithelial fibroblasts derived from nCDase knock out mice, thus demonstrating its selectivity among the ceramidases (54). Taken together these inhibitors offer a comprehensive array of tools to probe CDase activity throughout the cell.

4.4. Sphingosine Kinases - probes and inhibitors

Two known isoforms of SK, SK1 and SK2, are responsible for the production of S1P from Sph. This bioactive lipid has been implicated in numerous biologies mostly due to its activation of one of five G protein-coupled receptors (S1PRs1–5) (95), but additional intracellular targets have been proposed (200, 216). SK activity and S1P generation have primarily been probed through the use of radioactive substrates, though fluorescent and odd chain sphingoid bases are becoming more popular. Initial studies utilized radiolabeled ATP as a substrate *in vitro* and radioactive phosphate in cells (to label cellular ATP) often between 1–5 uCi for up to 1 h (147, 192). Though these assays are highly accurate, they are also labor intensive and not high throughput. Safer and faster alternatives have come from the use of odd chain sphingoid bases. Similar to the measurement of CerS activity, 17CSph is added to cells to assess SK activity at concentrations between 0.5 and 2 μ M for 15–30 min (Fig 2), as 17CS1P is rapidly generated in response to this exogenous substrate (52, 162). This accurately assesses SK activity and requires MS for detection. However, fluorometric detection of NBD-Sph lends itself to high-throughput assays for the screening of potential SK inhibitors, and indeed this is how it has been employed. Lima et al. developed a 384 well-plate format assay to monitor the phosphorylation of NBD-sphingosine by SK activity, owed to an increase in emission once the probe has been phosphorylated (111).

Numerous inhibitors of SK have been utilized in the literature, and many of them (mostly those mimicking the substrate Sph) also result in proteosomal degradation of the protein in the cell (53). Classically, inhibition of SKs was achieved with dimethylsphingosine (DMS), which acts as a competitive inhibitor of SK1 (K_i =5 μ M) and a non-competitive inhibitor of SK2 (K_i =12 μ M) (163, 227). SKI-I, with a K_i of 10 μ M for SK1 has been used to inhibit growth of leukemia cells (149). SKI-II is perhaps the best characterized, but it is rather non-specific and has a K_i of ~17 μ M (148). Most of these inhibitors suffer from lack of specificity for SK1 vs SK2 and have significant off target effects. In particular, SKI-II has recently been shown to inhibit DeS1, a component of *de novo* sphingolipid synthesis (8, 118, 155). The recent identification of the SK1 structure has aided the development of the potent SK1 specific inhibitor PF543 with a K_i of 3.6 nM (179). Later this molecule was co-crystallized with SK1 and led to insights regarding its 130-fold difference in selectivity for SK1 over SK2 (234). Importantly, many of the studies with PF-543 effects on cell proliferation have utilized much higher concentrations of PF-543 (10 and 25 μ M) which are several fold higher than the K_i (Fig 3) (66, 91). Inhibition of SK1 is a promising therapeutic strategy given the important biologies associated with S1P, but it requires further study and development.

4.5. Sphingosine-1-Phosphate Lyase - probes and inhibitors

The gatekeeper to clearance of metabolites from sphingolipid metabolism is sphingosine-1-phosphate lyase (SPL). Regulation of SPL activity is partially responsible for flux through S1P and has been correlated with resistance to specific anticancer therapies (40, 138, 229).

The observation of defects in lymphocytic egress from lymph nodes in SPL knockout mice has increased interest in SPL as a druggable target to suppress the immune response (181, 229). Fluorescent probes have been developed that allow rapid assays for S1PL activity. Bandhuyula et al. developed an *in vitro* assay for the analysis of SPL activity utilizing 5 nmol per sample of a NBD-S-1-P molecule for 15 min. A Bodipy probe has also been developed, though with a slightly higher K_m of 35 μ M compared to the NBD derivative (14.6 μ M) (13). Very recently, the fluorescent substrate RBM148 was used and effectively added to intact cells to probe S1P metabolism. While it is an exciting step forward in the analysis of this critical enzyme, the authors did utilize RBM148 at a seemingly high concentration of 580 μ M and had to encapsulate it in anionic liposomes for delivery (177). SPL inhibition has primarily been explored *in vivo*. The SPL inhibitor 2-acetyl-4-tetrahydroxybutyl imidazole (THI) is a component of caramel food coloring and induces lymphopenia by altering the S1P gradient *in vivo* (57). Though there has been controversy over the mechanism of action of THI, Ohtoyo et.al argued that this might be due to the fact that previously attempted *in vitro* SPL assays did not adequately represent SPL activity because they lacked pyridoxal 5'-phosphate, a key factor in SPL activity (144). Another potent SPL inhibitor is the sphingolipid analog 2-vinylsphinganine-1-phosphate (2VS1P) which has an IC_{50} of 2.4 μ M (31). In a comprehensive approach to develop SPL inhibitors, Billich et al screened over 200,000 compounds, which eventually resulted in the development of compounds C and D, with IC_{50} s of 0.21 and 0.024 μ M, respectively (27, 115, 237). Compound C was later co-crystallized with SPL, revealing structural details about fitment in the substrate binding site (42). The pyridoxal 5'-phosphate analog 4'-deoxy pyridoxine (DOP) is a nonspecific inhibitor of SPL. As DOP also modulates the activity of multiple enzymes involved in DNA synthesis, it can be highly toxic over time (26). Endogenous activation of SPL may also prove to be a more useful therapeutic target, instead of inhibition, as activation of this enzyme would clear S1P from the sphingolipid network and could be used to augment apoptosis in response to chemotherapy.

5. Unresolved Questions and General Considerations

In the last two decades, the field of sphingolipidomics has extensively moved forward, with improving detection of lipid species, sensitivity, and methods for probing metabolism. However, there are several aspects of sphingolipid analysis that could still benefit from improvements. The use of NBD probes for fluorescent detection of sphingolipid metabolites can affect the biophysical properties of downstream metabolites, as the label is bulky and typically located on the omega carbon of the N-acyl chain. Indeed, the need to adapt extraction procedures for the analysis of the much less hydrophobic C6-NBD-C1P molecule has been documented (122). This large change in hydrophobicity may also have metabolic consequences, potentially providing inaccurate flux information. While radioactive probes for assessing flux are more hazardous, they do constitute/convert into virtually identical molecules as endogenous sphingolipids, yet TLC separation must be utilized instead of HPLC. All this considered, radioactive assays typically used for the detection of flux in sphingolipid metabolites are being replaced thanks to the increased number and improved detection of stable isotope labeled sphingolipids. However, analysis of stable isotope probes can require extensive bioinformatic strategies due to mass overlap of probes that fall within

the isotopic distribution window of the corresponding sphingolipid (247). Finally, the recent development of probes that utilize click chemistry to visualize sphingolipid flux presents an exciting opportunity to add spatial context to sphingolipid flux; however, quantitation with fluorescent microscopy is still under development (65).

When probing sphingolipid metabolism, it is also important to consider the potential biological effects of the probes even at low concentrations. Many of the probes that enter sphingolipid metabolism after the SPT reaction are bioactive molecules. However, because of detection limits of current technologies, they are rarely used at truly trace levels. This becomes particularly important when probing metabolites with receptors, such as flux in SK activity, where the addition of exogenous substrate provides a large generation of S1P proximal to the S1P receptors at the PM.

6. Conclusion

Advancements in the understanding of the structural specificity involved in sphingolipid metabolism have led to the development of many complimentary molecular tools for the interrogation of sphingolipid flux and mass. Moreover, this has led to the development of inhibitors and molecular probes for nearly every step central to sphingolipid generation and clearance. In the future, labeling techniques that can define regulation of distinct pools of lipids, combined with genetic manipulation of distinctly localized enzymes will hopefully add some much needed clarity to the bioactive roles of the “sphinx” of lipids.

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Highlights:

- Sphingolipids metabolism is complex and thus requires specific labeling and inhibitor strategies to probe
- The combination of inhibitors and molecular probes for in-cell assays allows for the most authentic representation of sphingolipid regulation
- Overview of commonly utilized techniques for the analysis of sphingolipid metabolism, along with parameters on the use of labels and inhibitors to probe sphingolipid homeostasis in mammalian cell lines

Figure 1.

Bioactive sphingolipids can be generated in multiple membranes throughout the cell. Generation of sphingolipids *de novo* begins with the condensation of serine and palmitoyl-CoA via the serine palmitoyltransferase complex (SPT). The 3-ketosphinganine (3-KSph) product is reduced by 3-ketosphinganine reductase (3-KR) to generate dihydrosphingosine (dHSph). Next, ceramide synthases (CerS) derivatize dHSph with one of various fatty acids to form dihydroceramides (dHCer) of various acyl-chain lengths. Dihydroceramide desaturase (DES) then reduces dHCer to form ceramide (Cer), which can be metabolized in the ER forming either ceramide phosphoethanolamine (CPE) or galactosylceramide (GalCer). Ceramide can also be transported to the Golgi via ceramide transport protein (CERT) or through vesicular trafficking to generate sphingomyelin (SM) or glycosphingolipids (GSL), respectively. These complex sphingolipids can then be transported to the plasma membrane (PM) where hydrolytic pathways can degrade SM (SMases) or GSL (Gcase) to produce Cer at the PM or in endolysosomes. The Cer that is produced can be further broken down by ceramidases (CDase) to form sphingosine (Sph). Free Sph can either serve as a substrate for CerS to generate ceramide in a processed termed

“salvage/recycling” or begin clearance from the pathway by phosphorylation and subsequent degradation via sphingosine kinase (SK) and sphingosine lyase (SPL), respectively.

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		Metabolic Labels	Concentration	Duration
SPT	<div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> <div>↓</div> 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Figure 2.

An overview of commonly used probes for sphingolipid metabolism. All labels represent non-radioactive label tracers that can be utilized to probe sphingolipid metabolism in cells. Typical concentrations and duration of labeling to accurately monitor flux are presented.

		Inhibitor	Concentration	Duration
<p>SPT</p> <p>↓</p> <p>CerS</p> <p>↓</p> <p>DeS</p> <p>↓</p> <p>GCS</p> <p>↓</p> <p>SMS</p> <p>↓</p> <p>Gcase</p> <p>↓</p> <p>SMase</p> <p>↓</p> <p>CDase</p> <p>↓</p> <p>SK</p>		<p>Myriocin</p>	0.1 – 5μM	< 24 h
		<p>Fumonisin</p>	5–100μM	1 – 6 h
		<p>Fenretinide GT-11</p>	0.5 μM	0.5 – 2 h
		<p>D609 PDMP NVP-231</p>	<p>SMS 20– 25μg/ml</p> <p>GCS 50μM</p> <p>CERK 100nM</p>	<p>2-6 h</p> <p>24 h</p> <p>6 h</p>
		<p>DNJ</p>	1 – 5μM	24 h
		<p>GW4869</p>	10 – 20μM	24 h
		<p>C6UreaCer</p>	5-25μM	24 h
		<p>PF 543</p>	2.5 – 25μM	24-72 h

Figure 3.

An Overview of commonly used inhibitors of sphingolipid metabolism. Structures of commonly used inhibitors with concentration and duration for typical treatments for cellular use.