Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

Solving the enigma: Mass spectrometry and small molecule probes to study sphingolipid function

Alec Millner and G. Ekin Atilla-Gokcumen

Abstract

Sphingolipids are highly bioactive lipids. Sphingolipid metabolism produces key membrane components (e.g. sphingomyelin) and a variety of signaling lipids with different biological functions (e.g. ceramide, sphingosine-1-phosphate). The coordinated activity of tens of different enzymes maintains proper levels and localization of these lipids with key roles in cellular processes. In this review, we highlight the signaling roles of sphingolipids in cell death and survival. We discuss recent findings on the role of specific sphingolipids during these processes, enabled by the use of lipidomics to study compositional and spatial regulation of these lipids and synthetic sphingolipid probes to study subcellular localization and interaction partners of sphingolipids to understand the function of these lipids.

Addresses

Department of Chemistry, University at Buffalo, The State University of New York (SUNY), Buffalo, NY, 14260, USA

Corresponding author: Atilla-Gokcumen, G. Ekin (ekinatil@buffalo.edu)

Current Opinion in Chemical Biology 2021, **65**:49–56

This review comes from a themed issue on **Mechanistic Biology**

Edited by **Jessica Seeliger** and **Jeremy M. Baskin**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.cbpa.2021.05.001

Keywords

Sphingosine, Ceramide, Ceramide-1-phosphate, Sphingosine-1-phosphate, Sphingomyelin, Apoptosis, Inhibitor, Bifunctional, Photoswitchable, Photoactivatable, Deoxyceramides.

The Sphinx of lipids

While analyzing the chemical composition of the brain in the 1870s, Thudichum discovered a new class of lipid. As he struggled with the many structural enigmas that this molecule presented [1], he termed this new molecule 'sphingosin' after the Greek mythological creature, the Sphinx. The members of the sphingolipid family include ceramides, sphingomyelins, glycosphingolipids, and sphingosine-1-phosphate. They play specific signaling and structural roles in numerous cellular processes [2]. In this review, we will mainly focus on signaling roles of sphingolipids in cell death and survival and disease states (i.e. cancer, neurodegenerative diseases, and therapy resistance) associated with these cellular processes. Although many methods exist to study sphingolipid function, we will specifically focus on contributions from lipidomics to study compositional regulation, imaging mass spectrometry to study tissue distribution, and biorthogonal synthetic probes to study subcellular localization and interaction partners of sphingolipids.

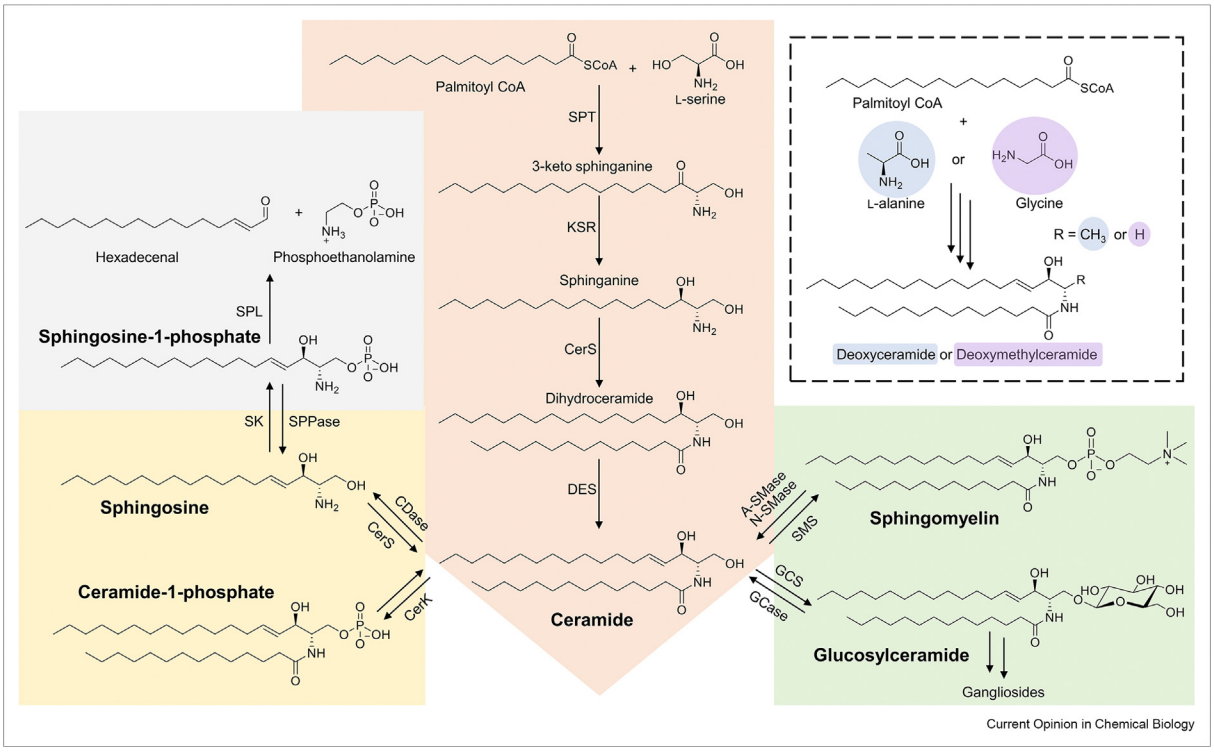
Sphingolipid synthesis involves a network of enzymes that tightly regulate the levels and localization of these lipids. The biosynthesis of sphingolipids begins on the cytosolic leaflet of the endoplasmic reticulum [2]. The first step involves the production of 3-ketosphinganine by serine palmitoyl transferase, which is then reduced to dihydrosphinganine. Dihydrosphinganine is acylated by ceramide synthases (CerSs), producing dihydroceramides. Mammalian cells have six isoforms of CerS (CerS1–6), and different isoforms catalyze the biosynthesis of dihydroceramides with various acyl chain lengths, mainly C14–C26 [2]. Dihydroceramide desaturases form ceramides by inserting a *trans* double bond at C4 of sphinganine (Figure 1, orange box) [2]. Other ceramides can also be produced by the use of L-alanine or glycine by serine palmitoyl transferase, resulting in 1-deoxyceramide or 1-deoxymethyl ceramide, respectively (see inlet in Figure 1) [3].

Ceramides are central molecules in the sphingolipid pathway as they are the building blocks for other sphingolipids and have proapoptotic roles [2]. It is now well established that apoptosis-inducing agents promote ceramide production [4] and exogenous addition of a short- and long-chain ceramide can directly induce cell death [5,6]. From a more mechanistic perspective, elevated ceramide levels lead to mitochondrial outer membrane permeabilization by activation of a B-cell lymphoma 2 (Bcl-2) protein family through phosphoinositide-3-kinase and Akt/PBK signaling [7]. Short-chain ceramides, on the other hand, can activate protein phosphatase 2A, which in turn leads to mitochondrial permeabilization through BCL-2 inactivation [8,9]. Other studies have demonstrated that ceramides can directly interact with mitochondria and induce outer membrane permeabilization [10]. Recently, it was shown that the interaction between mitochondrial

voltage-dependent anion channel 2 and ceramides plays a role in mitochondrial membrane permeabilization in apoptosis, providing a new perspective for the ceramides' proapoptotic role [11]. Furthermore, *in vitro* experiments suggested that ceramides can form channels on membranes, and this has been proposed to be linked to its proapoptotic activity [12].

Ceramides form complex sphingolipids (reviewed in [2]). For such metabolic processing, ceramides are transported to the Golgi apparatus by the ceramide transport protein or vesicular transport [13]. Sphingomyelin synthase, primarily found in the luminal trans Golgi and plasma membrane, uses ceramides and forms sphingomyelins, important structural lipids [2,14]. Similarly, ceramides can be glycosylated to form glucosyl and highly glycosylated ceramides, gangliosides, lipids that are involved in cell-cell recognition and signal transduction (Figure 1, green box) [15,16]. Sphingomyelins and glycosphingolipids are trafficked to the plasma membrane [3], where they can participate in the formation of tightly packed domains, called lipid rafts [17]. These domains recruit specific proteins and can directly affect protein function through localization and conformation, and cellular signaling [17]. Ceramide can be regenerated from sphingomyelin and glycosphingolipids via sphingomyelinases and glucocerebrosidase, respectively [2]. It is important to note that there are multiple distinct sphingomyelinases (acid sphingomyelinase and neutral sphingomyelinase), each with their own substrate specificity and subcellular localization [2], which

Figure 1



A simplified diagram of sphingolipid biosynthesis. Only C14 fatty acid–containing species are shown as an example. Sphingolipids that are discussed in this review are shown in bold. Highlighted in orange is the de novo sphingolipid synthesis. Condensation of serine and palmitoyl-CoA by serine palmitoyl transferase produces 3-ketosphinganine. 3-ketosphinganine is reduced by 3-ketosphinganine reductase to produce sphinganine, which is then acylated by ceramide synthases, resulting in dihydroceramide. A trans double bond is introduced on the fourth carbon by dihydroceramide desaturase to yield ceramide. Ceramide can be incorporated into more complex sphingolipids such as glucosylceramide and sphingomyelin, highlighted in green, by glucosylceramide synthase and sphingomyelin synthase, respectively. Glucosylceramides can be further modified to yield gangliosides. Ceramide can also be phosphorylated by ceramide kinase or hydrolyzed by ceramidase to produce ceramide-1-phosphate or sphingosine, respectively, highlighted in yellow. Sphingosine can be phosphorylated by sphingosine kinase, resulting in sphingosine-1-phosphate, which can be broken down to hexadecenal and phosphoethanolamine by sphingosine-1-phosphate lyase, highlighted in gray. Ceramide can be regenerated by the breakdown of glucosylceramide and sphingomyelin by glucocerebrosidase and sphingomyelinase, respectively. Sphingosine can be used as a substrate by ceramide synthase to regenerate ceramide. Alternatively, serine palmitoyl transferase can incorporate alanine or glycine to ultimately lead to the production of deoxyceramide or deoxymethyl ceramide, respectively. SPT, serine palmitoyl transferase; KSR, 3-keto-sphinganine reductase; DES, dihydroceramide desaturase; CDase, ceramidase; GCS, glucosylceramide synthase; GCase, glucocerebrosidase; CerK, ceramide kinase; SMS, sphingomyelin synthase; N-SMase, neutral sphingomyelinase; A-SMase, acid sphingomyelinase; SK, sphingosine kinase; SPPase, sphingosine phosphate phosphatase; SPL, sphingosine-1-phosphate lyase.

allows cells to maintain levels of specific sphingolipids in distinct membranes.

Sphingolipid breakdown is also mediated through ceramide via ceramidases, which forms sphingosine. Much like sphingomyelinases, there are multiple ceramidases, each with their own localization and substrate specificity [2]. Sphingosine can be phosphorylated to sphingosine-1-phosphate (Figure 1, yellow box) [2]. Unlike ceramides, sphingosine-1-phosphate is a key pro survival sphingolipid [18]. It can be released to the extracellular matrix and participate in cell-cell communication and signaling via interaction with sphingosine-1-phosphate receptors [19]. Sphingosine-1-phosphate can also be processed by sphingosine-1-phosphate lyase, which breaks down the phosphorylated sphingoid bases to hexadecenal and phosphoethanolamine, sphingolipid degradation products (Figure 1, gray box) [16]. Overall, the coordinated activity of a number of enzymes regulates the cellular levels of sphingolipids with different biological activities, and dysregulation of these enzymes is involved in numerous diseases.

How can we study sphingolipids?

As exemplified previously, sphingolipids are structurally and functionally very diverse, which calls for the use of different sets of tools to study them in cells depending on the biological question of interest. Many analytical (reviewed in [20]) and imaging-based techniques that rely on the use of sphingolipid sensors [21,22] exist to study sphingolipid function. In this review, we will primarily focus on tools that enable precise, structure-specific measurements mediated by mass spectrometry, chemical and genetic perturbation-based approaches, facilitated mainly by the inactivation of enzymes that produce these lipids, and biorthogonal lipid probes that allow lipid visualization and the temporal control of lipid activation. We discuss recent efforts on elucidating sphingolipid function, mainly of ceramides, sphingosine, and sphingosine-1-phosphate, in cell death and survival in the following sections. These studies benefitted from mass spectrometry to study the composition and spatial regulation of sphingolipids and small molecule probes to study the interactome and achieve temporal activation of these lipids.

Sphingolipidomics

LC-MS–based sphingolipidomics

Sphingolipids exhibit a wide range of concentrations [2,23]. Changes in sphingolipid levels have been associated with numerous pathologies, including neurodegenerative diseases and cancer formation and progression [2] and development of drug resistance and the prosurvival signaling pathway in cancer cells [4]. Liquid chromatography–mass spectrometry (LC-MS, reviewed in [24]) has been the 'go-to' bioanalytical tool to detect, identify, and quantify sphingolipids in biological samples and elucidated many exciting biological questions in sphingolipid flux [23] in cells and their involvement in cancer formation and progression. In this section, we highlight some of the recent studies that used LC-MS to elucidate the role of ceramides, sphingomyelins, sphingosine, and sphingosine-1-phosphate in important cellular processes, including cell death and survival, and briefly discuss the role of these sphingolipids in diseases that are linked to these fundamental cellular processes.

Mitochondrial localization of ceramides has been of high interest because of their proapoptotic activities, as their interactions with certain proteins can lead to membrane permeabilization [7–9]. Through a combination of LC-MS and other bioanalytical techniques, Oleinik et al. [25] identified a new protein, PERMIT (Protein that mediates endoplasmic reticulum-mitochondria trafficking), that traffics CerS1 from the ER to the outer mitochondrial membrane, which, in turn, increases mitochondrial ceramide levels. This study is particularly important because it suggests that the mitochondrial localization of ceramides can be due to their de novo synthesis at the mitochondria in addition to their trafficking to the mitochondria.

Many chemotherapeutics modulate sphingolipid levels [4], and strong correlations between alterations in sphingolipid landscape and therapy resistance have been reported [26–28]. A recent study investigated the effects of sublethal doses of chemotherapeutics on cellular lipids and reported that the increased ceramide pools and localization of these species, specifically at the plasma membrane, were mediated by the conversion of sphingomyelin to ceramide via neutral sphingomyelinase 2 [29]. Subsequent LC-MS analysis showed that plasma membrane–localized ceramides regulate cell adhesion and migration, different than previously established proapoptotic activity of these lipids, which is associated with their mitochondrial interactions. These results demonstrate that cellular localization of ceramides might affect their cellular function [29].

DNA damage–induced apoptosis is a common chemotherapeutic strategy. DNA damage, however, can instead induce senescence, resulting in cell cycle arrest and secretion of inflammatory factors, which reduces the effectiveness of cancer therapy [30]. To investigate potential lipid-related targets in therapy-induced senescence, we used LC-MS–based untargeted lipidomics to elucidate changes in sphingolipid metabolism during therapy-induced senescence in colon cancer cells and showed the depletion of 1-deoxyceramides in senescent cells [26]. Chemical perturbations to increase the levels of 1-deoxyceramides desensitize cells to cellular senescence, suggesting a functional role of 1-deoxyceramides during this process [26].

Sphingosine kinase 1 expression has recently been linked to oncogene-induced senescence. Trayssac et al. [27] found that senescent cells had increased expression of sphingosine kinase 1 and its inhibition enhanced the senescent phenotype by oncogenic K-Ras activation and led to the increase in long- and very long-chain ceramides (C18-26), identified by LC-MS. They also showed that these lipid changes were mediated by CerS2, specifically linking CerS2-derived ceramides as potential regulators of senescence.

Hormone therapy resistance in breast cancer presents a major problem for therapeutics. Sphingosine kinase 1 is commonly upregulated in breast cancer and is linked to more aggressive cancer progression [31]. Maczis et al. [28] found that tamoxifen resistance correlated with an increased expression of estrogen receptor α36, which in turn activated sphingosine kinase 1, leading to higher levels of cellular and secreted sphingosine-1-phosphate. Inactivation of sphingosine kinase 1 reduced the levels of sphingosine-1-phosphate and sensitized tamoxifen-resistant breast cancer to tamoxifen [28], suggesting a new role of sphingosine-1-phosphate signaling in hormone therapy resistance.

LC-MS–based analysis can also pave the way to establish novel correlations between changes in sphingolipid levels and certain oncogenic transformations. Bhadwal et al. [31] used multivariate analysis to demonstrate the elevation of ceramide-1-phosphate and sphingosine-1-phosphate in tumors isolated from patients with breast cancer as compared with healthy tissues. They also established significant upregulations in the expression of ceramide kinase and sphingosine kinase, providing insight into the underlying mechanisms and role of sphingolipids in breast cancer formation and progression.

Overall, LC-MS–based approaches are irreplaceable tools for compositional analysis of sphingolipids and usually the first step for elucidating new roles for these lipids. Untargeted detection methods find their unique applications to obtain a global overview of the changes in lipid composition in an unbiased manner. Targeted LC-MS–based lipid measurements, on the other hand, can provide quantitative information on the changes in lipid composition. Herein, we mainly focused on the analysis of major sphingolipids derived from de novo biosynthesis, which exhibit wide tissue distribution using targeted and untargeted LC-MS. However, it is important to note that certain tissues contain distinct sphingolipids and require the use of specialized targeted LC-MS methods [32].

Imaging mass spectrometry

Sphingolipid metabolism is compartmentalized within cells based on the organelle-specific production, breakdown, and transport of these lipids. Similarly, tissue distribution of sphingolipids also varies [33]. LC-MS–based sphingolipidomics provides invaluable information on compositional analysis of sphingolipids and, due to its quantitative nature, can provide absolute concentrations of sphingolipids. However, LC-MS–based sphingolipidomics uses total lipid extracts from cells or tissues, which scrambles lipid species from all cellular compartments, limiting the mechanistic insights that can be obtained. To fill this missing spatial information, it is possible to either biochemically isolate regions of interest and carry out subsequent LC-MS analysis or couple MS-based detections with techniques that provide spatial resolution. Such spatial compositional analysis of lipids can pave the way for further mechanistic investigations on lipid function.

Combining tissue imaging with MS (for review on imaging MS, see Ref. [34]; for a review on imaging MS of sphingolipids, see Ref. [35]) has recently gained traction to analyze the tissue distribution of sphingolipids, including gangliosides, and has been useful in studying neurogenerative disorders and cancer because of the heterogeneous and progressive nature of these diseases. Tobias et al. [36] investigated the localization of different ceramides and gangliosides during cerebral degeneration in a mouse model of Nieman-Pick disease, type 1 and found gangliosides GM2 and GM3 are upregulated in brain tissues of diseased state and localize to certain regions of the brain. They also reported correlations between ceramide and ganglioside levels and redistribution with disease progression, showing key spatial and temporal changes in sphingolipid levels in the disease model.

A recent study characterized the lipid composition of subtypes of breast cancer tissues, including invasive breast cancer, ductal carcinoma, and noncancerous benign tissue. Several ceramide species were upregulated in the invasive breast cancer tissues along with an overall accumulation of sphingolipids in triple-negative and luminal B subtypes, suggesting that these lipid signatures can be useful for molecular subtyping of breast cancer types [37]. MS imaging of metastatic breast and thyroid cancer in human lymph node tissues also showed significant alternations in sphingolipid levels, where ceramides showed significant accumulations in cancerous tissue [38].

A current challenge in adapting MS imaging to study clinical samples is that tissue samples are formalin-fixed for storage, which is not MS friendly. A recent effort on imaging of gangliosides in the fresh frozen and formalin-fixed rat brain led to the development of a method that allows the use of fixed samples of brain tissue to obtain comparable high-resolution information on ganglioside levels with similar spatial distribution [39]. Overall, improvements on sample preparation, data acquisition, and analysis will enable the wide range applications of

imaging MS and the potential integration with LC-MS, providing both spatial and compositional analysis.

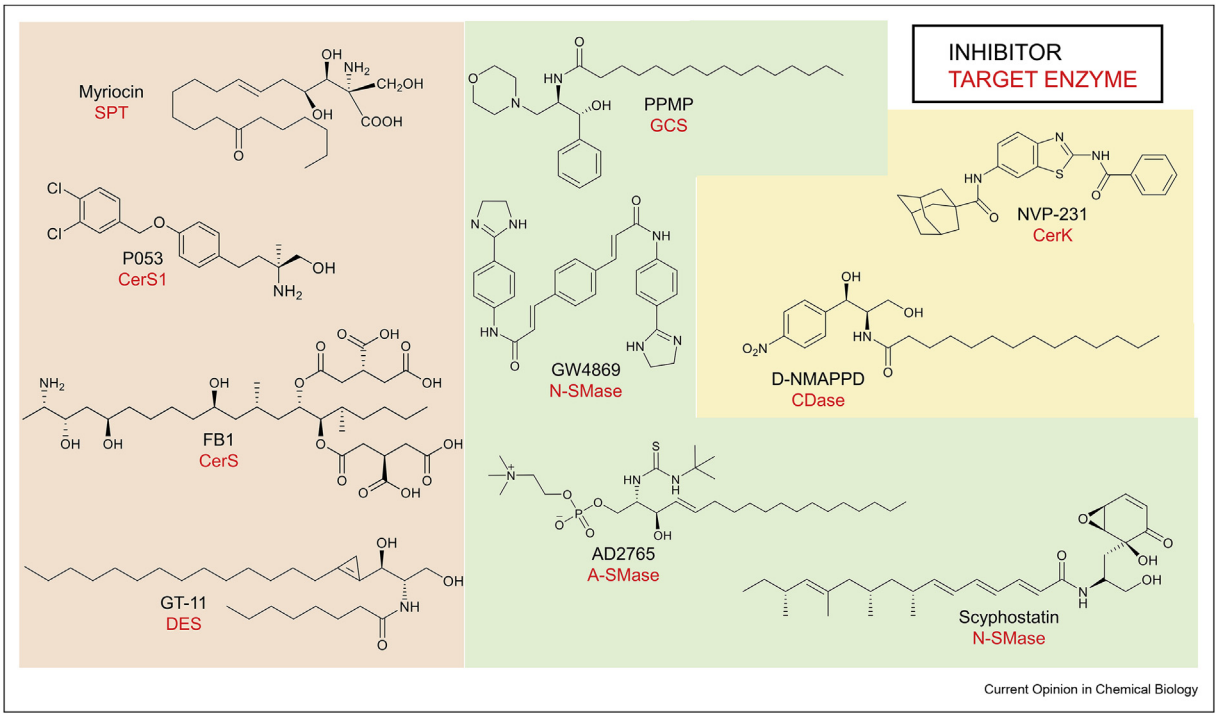
Synthetic sphingolipid probes

Lipids that show different compositional or spatial regulation, identified by MS, as exemplified previously, are ideal candidates for subsequent mechanistic investigations. The use of small molecules, including inhibitors and biorthogonal probes, has advanced our understanding of sphingolipid function. It is possible to alter sphingolipid levels using small molecule inhibitors and investigate the impact of these alterations on phenotypic outcomes. A healthy number and variety of small molecules are available to perturb the sphingolipid pathway (Figure 2, reviewed in [40]). These small molecules target the biosynthesis of ceramides and their downstream complex sphingolipids and breakdown (shown in orange, green, and gray, respectively in Figure 2). Most commonly used are inhibitors of CerSs, including fumonisins, which are effective in reducing overall ceramide levels [21,41]. A recently developed small molecule, P053, that shows specificity toward CerS1, reduces the levels of C18-fatty acid containing sphingolipids [42] (Figure 2). Integration of these chemical perturbations with LC-MS analysis to identify consequent sphingolipid changes can provide invaluable information on how sphingolipid levels affect phenotypes of interest.

Bifunctional probes

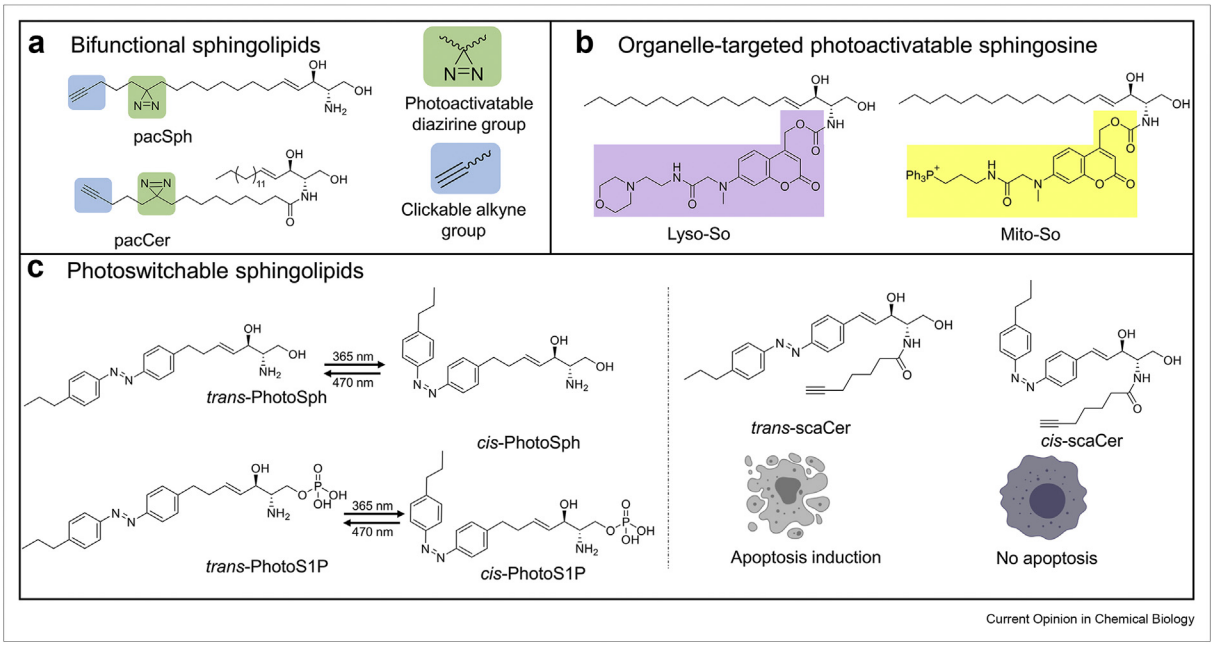
Biorthogonal sphingolipid probes with multiple functionalities (i.e. clickable, crosslinkable, photoactivatable, and photoswitchable) have been instrumental in identifying proteins that interact with these lipids and their subcellular localization and also allow temporal activation. A group of these probes, known as bifunctional analogs, contain terminal alkyne and diazirine moieties. The photoactivatable diazirine ring reacts with neighboring biomolecules (i.e. proteins that the probes interact with) and results in covalent labeling of the probe upon irradiation. The alkyne is used to install a reporter molecule, mainly either an azido fluorophore to study cellular localization or an azido biotin for affinity-based enrichment and subsequent analysis of bound proteins (Figure 3a). Haberkant et al. [43] developed a photoactivatable and clickable sphingosine (pacSph, Figure 3a) analog to study the cellular metabolism of the lipid probe to ceramides, sphingomyelins, and glycosylated sphingolipids and identified hundreds of novel sphingolipid-binding proteins. A similar bifunctional analog of ceramide (pacCer) led to the identification of new ceramide-binding

Figure 2



Small molecule inhibitors of sphingolipid synthesis and their targets. Small molecule inhibitors and their structure are provided along with their targeted enzyme indicated in red. Inhibitors are highlighted corresponding to the location of the pathway they target; colors correspond to de novo (orange), glucosylceramide/sphingomyelin (green), and sphingosine/ceramide-1-phosphate (yellow) synthesis. FB1, Fumonisin B1; PPMP, D-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol; SPT, serine palmitoyl transferase; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; N-SMase, neutral sphingomyelinase; A-SMase, acid sphingomyelinase; CerK, ceramide kinase; CDase, ceramidase.

Figure 3



Small molecule probes for temporal control of sphingolipids. (**a**). Bifunctional sphingolipid analogs contain photoactivatable diazirine and clickable alkyne groups. The diazirine ring can be activated to form a covalent bond with a neighboring protein, and the alkyne group can be used to install a reporter molecule. pacSph (photoactivatable and clickable sphingosine) and pacCer (photoactivatable and clickable ceramide) are shown. (**b**) Sphingosine analogs are modified to localize to specific organelles. Uncaging these targeted probes releases sphingosine in the specified organelles. Lyso-So (Lyso-caged sphingosine) and Mito-So (mito-caged sphingosine) can be targeted to subcellular structures. (**c**) Azobenzene-containing, photoswitchable sphingolipid probes. On radiation, these probes are converted from their inactive *cis* to an active *trans* isomer, which mimics endogenous sphingolipids. PhotoSph, photoswitchable sphingosine; PhotoS1P, photoswitchable sphingosine-1-phosphate; scaCer, short-chain, clickable, and photoswitchable ceramide.

proteins, ceramide transport protein–related steroidogenic acute regulatory protein D7 [44], and voltage-dependent anion channel 2 [11] and provided insight into the mechanism framework by which ceramides induce mitochondria-mediated toxicity. It is important to note that these lipid probes are commercially available, allowing researchers to access and apply them to new studies.

Photoactivatable probes

Another group of biorthogonal sphingolipid probes includes the ones that allow spatiotemporal activation, including caged sphingolipids and azobenzene derivatives. Removal of certain chemical moieties upon irradiations (i.e. uncaging) is one of the first strategies that have been implemented to study spatiotemporal activation of sphingolipids. Feng et al. [45] developed lysosome- and mitochondrial-targeted [46] photo-caged sphingosine to study site-specific sphingolipid metabolism. These probes allow sphingosine targeting to the organelle of interest, and the levels of sphingosine and its metabolism can be observed over time which allowed locale-dependent metabolism and functional analysis of these lipids (Figure 3b) [45].

Photoswitchable probes

Trauner and colleagues [47] developed new classes of probes that use light for activation. These probes include clickable, azobenzene-containing sphingosine, sphingosine-1-phosphate [47], ceramides [48], and galactosylceramide [49]. The azobenzene moiety is 'photoswitchable', meaning it can be converted into metabolically active forms upon irradiation. The irradiation induces a reversible isomerization between a *cis* and *trans* isomer of the probe. The *trans* isomer mimics the shape of the endogenous lipid, thus enabling temporal activation (Figure 3c). Photoswitchable sphingosine and sphingosine-1-phosphate show prolonged metabolic stability and can be used to modulate light-dependent activation of lipid signaling and consequent phenotypic alterations both in culture cells and also *in vivo* [47]. Similarly, photoswitchable ceramides can be activated upon irradiation and exhibit properties that are similar to these of endogenous versions, including incorporation to membrane and lateral lipid packing and metabolic processing by sphingolipid-related enzymes to form sphingomyelins and glucosylceramides [48]. The *trans* isomers of the photoswitchable ceramides can also induce apoptosis under the conditions where there

is no appreciable apoptotic activity with the *cis* isomer, allowing time-dependent induction of ceramide-mediated apoptosis [50]. The increased metabolic stability combined with temporal activation of these probes now allows researchers to study sphingosine-1-phosphate— and ceramide-induced phenotypes 'on-demand' upon irradiation and allows to turn on and off these phenotypes. Overall, small molecules are the keys to unlock sphingolipid functions as they allow efficient perturbation at the lipid level and allow the temporal lipid activation and identification of new protein targets.

Conclusions

Since the discovery of key signaling roles of ceramides [5] and sphingosine-1-phosphate [51] around three decades ago, research on sphingolipids gained traction and their many exciting roles have been discovered. These discoveries were enabled by important technological advances in high-resolution LC-MS and imaging MS. In parallel, an increased number of small molecule inhibitors that target sphingolipid biosynthesis and synthetic lipid probes to identify the localization, interaction partners and allow temporal activation have provided invaluable insights into the function of specific sphingolipids. In this review, we just scratched the surface of recent studies and highlighted some that use these methods and shed light on sphingolipid functioning in different cellular processes. The integration of these methods will accelerate the new discoveries and will start bridging the current gap in our knowledge on many exciting roles of these 'enigmatic' lipids.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We acknowledge support from the National Science Foundation (grant MCB1817468 to G.E.A.G.).

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Thudichum JLW: *A treatise on the chemical constitution of the brain*. Archon Books; 1962.

2. Hannun YA, Obeid LM: **Sphingolipids and their metabolism in physiology and disease**. *Nat Rev Mol Cell Biol* 2018, **19**: 175–191.

3. Merrill Jr AH: **Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics**. *Chem Rev* 2011, **111**:6387–6422.

4. Lewis AC, Wallington-Beddoe CT, Powell JA, Pitson SM: **Targeting sphingolipid metabolism as an approach for combination therapies in haematological malignancies**. *Cell Death Discov* 2018, **4**:4.

5. Obeid LM, Linardic CM, Karolak LA, Hannun YA: **Programmed cell death induced by ceramide**. *Science* 1993, **259**: 1769–1771.

6. Law BA, Liao X, Moore KS, Southard A, Roddy P, Ji R, Szulc Z, Bielawska A, Schulze PC, Cowart LA: **Lipotoxic very-long-chain ceramides cause mitochondrial dysfunction, oxidative stress, and cell death in cardiomyocytes**. *Faseb J* 2018, **32**: 1403–1416.

7. Bourbon NA, Sandirasegarane L, Kester M: **Ceramide-induced inhibition of Akt is mediated through protein kinase Cζ: implications for growth arrest**. *J Biol Chem* 2002, **277**:3286–3292.

8. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA: **Ceramide activates heterotrimeric protein phosphatase 2A**. *J Biol Chem* 1993, **268**:15523–15530.

9. Mukhopadhyay A, Saddoughi SA, Song P, Sultan I, Ponnusamy S, Senkal CE, Snook CF, Arnold HK, Sears RC, Hannun YA, Ogretmen B: **Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling**. *Faseb J* 2009, **23**:751–763.

10. Patwardhan GA, Beverly LJ, Siskind LJ: **Sphingolipids and mitochondrial apoptosis**. *J Bioenerg Biomembr* 2016, **48**: 153–168.

11. * Dadsena S, Bockelmann S, Mina JGM, Hassan DG, Korneev S, Razzera G, Jahn H, Niekamp P, Muller D, Schneider M, Tafesse FG, Marrink SJ, Melo MN, Holthuis JCM: **Ceramides bind VDAC2 to trigger mitochondrial apoptosis**. *Nat Commun* 2019, **10**:1832.
This paper used a photactivatable ceramide analog to identify VDAC2 as a ceramide binding protein and showed that loss of VDAC2 resulted in resistance to ceramide-induced apoptosis.

12. Siskind LJ, Kolesnick RN, Colombini M: **Ceramide forms channels in mitochondrial outer membranes at physiologically relevant concentrations**. *Mitochondrion* 2006, **6**:118–125.

13. Hanada K: **Co-evolution of sphingomyelin and the ceramide transport protein CERT**. *Biochim Biophys Acta* 2014, **1841**: 704–719.

14. Slotte JP: **Biological functions of sphingomyelins**. *Prog Lipid Res* 2013, **52**:424–437.

15. Schnaar RL, Kinoshita T: **Glycosphingolipids**. In *Essentials of glycobiology*. Edited by Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH, Prestegard JH, Schnaar RL, Seeberger PH, NY: Cold Spring Harbor; 2015:125–135. rd.

16. Gault CR, Obeid LM, Hannun YA: **An overview of sphingolipid metabolism: from synthesis to breakdown**. *Adv Exp Med Biol* 2010, **688**:1–23.

17. Sezgin E, Levental I, Mayor S, Eggeling C: **The mystery of membrane organization: composition, regulation and roles of lipid rafts**. *Nat Rev Mol Cell Biol* 2017, **18**:361–374.

18. Maceyka M, Spiegel S: **Sphingolipid metabolites in inflammatory disease**. *Nature* 2014, **510**:58–67.

19. Pyne NJ, Pyne S: **Sphingosine 1-phosphate and cancer**. *Nat Rev Cancer* 2010, **10**:489–503.

20. Li M, Yang L, Bai Y, Liu H: **Analytical methods in lipidomics and their applications**. *Anal Chem* 2014, **86**:161–175.

21. Leier HC, Weinstein JB, Kyle JE, Lee JY, Bramer LM, Stratton KG, Kempthorne D, Navratil AR, Tafesse EG, Hornemann T, Messer WB, Dennis EA, Metz TO, Barklis E, Tafesse FG: **A global lipid map defines a network essential for Zika virus replication**. *Nat Commun* 2020, **11**:3652.

22. Kiyokawa E, Baba T, Otsuka N, Makino A, Ohno S, Kobayashi T: **Spatial and functional heterogeneity of sphingolipid-rich membrane domains**. *J Biol Chem* 2005, **280**:24072–24084.

23. ** Snider JM, Snider AJ, Obeid LM, Luberto C, Hannun YA: **Probing de novo sphingolipid metabolism in mammalian cells utilizing mass spectrometry**. *J Lipid Res* 2018, **59**:1046–1057.
This paper demonstrated the use of a non-endogenous 17 carbon dihydrosphingosine to study the activity of different enzymes in the

sphingolipid by measuring the incorporation and metabolism of the labeled precursor

24. Sullards MC, Liu Y, Chen Y, Merrill Jr AH: **Analysis of mammalian sphingolipids by liquid chromatography tandem mass spectrometry (LC-MS/MS) and tissue imaging mass spectrometry (TIMS)**. *Biochim Biophys Acta* 2011, **1811**: 838–853.

25. ** Oleinik N, Kim J, Roth BM, Selvam SP, Gooz M, Johnson RH, Lemasters JJ, Ogretmen B: **Mitochondrial protein import is regulated by p17/PERMIT to mediate lipid metabolism and cellular stress**. *Sci Adv* 2019, **5**, eaax1978.

This paper characterized a protein which is responsible for trafficking of CerS1 to the outer mitochondrial membrane and provides insights into the mechanim of ceramide accumulation in the mitochondria during cell death.

26. * Millner A, Lizardo DY, Atilla-Gokcumen GE: **Untargeted lipidomics highlight the depletion of deoxyceramides during therapy-induced senescence**. *Proteomics* 2020, **20**, e2000013.

This paper used LC-MS-based untargeted sphingolipidomics to show depletion of noncanonical sphingolipid, deoxyceramides in therapy-induced senescence and demostrated that increasing deoxyceramide levels can reduce senescence.

27. Trayssac M, Clarke CJ, Stith JL, Snider JM, Newen N, Gault CR, Hannun YA, Obeid LM: **Targeting sphingosine kinase 1 (SK1) enhances oncogene-induced senescence through ceramide synthase 2 (CerS2)-mediated generation of very-long-chain ceramides**. *Cell Death Dis* 2021, **12**:27.

28. Maczis MA, Maceyka M, Waters MR, Newton J, Singh M, Rigsby MF, Turner TH, Alzubi MA, Harrell JC, Milstien S, Spiegel S: **Sphingosine kinase 1 activation by estrogen receptor alpha36 contributes to tamoxifen resistance in breast cancer**. *J Lipid Res* 2018, **59**:2297–2307.

29. Canals D, Salamone S, Santacreu BJ, Nemeth E, Aguilar D, Hernandez-Corbacho MJ, Adada M, Staquicini DI, Arap W, Pasqualini R, Haley J, Obeid LM, Hannun YA: **Ceramide launches an acute anti-adhesion pro-migration cell signaling program in response to chemotherapy**. *Faseb J* 2020, **34**: 7610–7630.

30. Demaria M, O'Leary MN, Chang J, Shao L, Liu S, Alimirah F, Koenig K, Le C, Mitin N, Deal AM, Alston S, Academia EC, Kilmarx S, Valdovinos A, Wang B, de Bruin A, Kennedy BK, Melov S, Zhou D, Sharpless NE, Muss H, Campisi J: **Cellular senescence promotes adverse effects of chemotherapy and cancer relapse**. *Cancer Discov* 2017, **7**: 165–176.

31. Bhadwal P, Dahiya D, Shinde D, Vaiphei K, Math RGH, Randhawa V, Agnihotri N: **LC-HRMS based approach to identify novel sphingolipid biomarkers in breast cancer patients**. *Sci Rep* 2020, **10**:4668.

32. Kawana M, Miyamoto M, Ohno Y, Kihara A: **Comparative profiling and comprehensive quantification of stratum corneum ceramides in humans and mice by LC/MS/MS**. *J Lipid Res* 2020, **61**:884–895.

33. Jones EE, Dworski S, Canals D, Casas J, Fabrias G, Schoenling D, Levade T, Denlinger C, Hannun YA, Medin JA, Drake RR: **On-tissue localization of ceramides and other sphingolipids by MALDI mass spectrometry imaging**. *Anal Chem* 2014, **86**:8303–8311.

34. Porta Siegel T, Hamm G, Bunch J, Cappell J, Fletcher JS, Schwamborn K: **Mass spectrometry imaging and integration with other imaging modalities for greater molecular understanding of biological tissues**. *Mol Imaging Biol* 2018, **20**: 888–901.

35. Luberto C, Haley JD, Del Poeta M: **Imaging with mass spectrometry, the next frontier in sphingolipid research? A discussion on where we stand and the possibilities ahead**. *Chem Phys Lipids* 2019, **219**:1–14.

36. * Tobias F, Pathmasiri KC, Cologna SM: **Mass spectrometry imaging reveals ganglioside and ceramide localization patterns during cerebellar degeneration in the Npc1(-/-) mouse model**. *Anal Bioanal Chem* 2019, **411**:5659–5668.

This paper utilized imaging mass spectrometry to investigate the localization of sphingolipids in a mouse model of Nieman-Pick Disease, type 1, and found that specific gangliosides were upregulated in specific regions in diseased brain tissue.

37. Santoro AL, Drummond RD, Silva IT, Ferreira SS, Juliano L, Vendramini PH, Lemos M, Eberlin MN, Andrade VP: **In situ DESI-MSI lipidomic profiles of breast cancer molecular subtypes and precursor lesions**. *Cancer Res* 2020, **80**:1246–1257.

38. Zhang J, Feider CL, Nagi C, Yu W, Carter SA, Suliburk J, Cao HST, Eberlin LS: **Detection of metastatic breast and thyroid cancer in lymph nodes by desorption electrospray ionization mass spectrometry imaging**. *J Am Soc Mass Spectrom* 2017, **28**:1166–1174.

39. Harris A, Roseborough A, Mor R, Yeung KK, Whitehead SN: **Ganglioside detection from formalin-fixed human brain tissue utilizing MALDI imaging mass spectrometry**. *J Am Soc Mass Spectrom* 2020, **31**:479–487.

40. Skacel J, Slusher BS, Tsukamoto T: **Small molecule inhibitors targeting biosynthesis of ceramide, the central hub of the sphingolipid network**. *J Med Chem* 2021, **64**:279–297.

41. Riley RT, Merrill Jr AH: **Ceramide synthase inhibition by fumonisins: a perfect storm of perturbed sphingolipid metabolism, signaling, and disease**. *J Lipid Res* 2019, **60**:1183–1189.

42. Turner N, Lim XY, Toop HD, Osborne B, Brandon AE, Taylor EN, Fiveash CE, Govindaraju H, Teo JD, McEwen HP, Couttas TA, Butler SM, Das A, Kowalski GM, Bruce CR, Hoehn KL, Fath T, Schmitz-Peiffer C, Cooney GJ, Montgomery MK, Morris JC, Don AS: **A selective inhibitor of ceramide synthase 1 reveals a novel role in fat metabolism**. *Nat Commun* 2018, **9**:3165.

43. Haberkant P, Stein F, Hoglinger D, Gerl MJ, Brugger B, Van Veldhoven PP, Krijgsveld J, Gavin AC, Schultz C: **Bifunctional sphingosine for cell-based analysis of protein-sphingolipid interactions**. *ACS Chem Biol* 2016, **11**:222–230.

44. Bockelmann S, Mina JGM, Korneev S, Hassan DG, Muller D, Hilderink A, Vlieg HC, Raijmakers R, Heck AJR, Haberkant P, Holthuis JCM: **A search for ceramide binding proteins using bifunctional lipid analogs yields CERT-related protein StarD7**. *J Lipid Res* 2018, **59**:515–530.

45. * Feng S, Harayama T, Chang D, Hannich JT, Winssinger N, Riezman H: **Lysosome-targeted photoactivation reveals local sphingosine metabolism signatures**. *Chem Sci* 2019, **10**: 2253–2258.

This paper used a lysosome targeted photo-caged ceramide analog to provide insigths on lysosomal metabolism of sphingolipids.

46. Feng S, Harayama T, Montessuit S, David FP, Winssinger N, Martinou JC, Riezman H: **Mitochondria-specific photoactivation to monitor local sphingosine metabolism and function**. *Elife* 2018, **7**.

47. Morstein J, Hill RZ, Novak AJE, Feng S, Norman DD, Donthamsetti PC, Frank JA, Harayama T, Williams BM, Parrill AL, Tigyi GJ, Riezman H, Isacoff EY, Bautista DM, Trauner D: **Optical control of sphingosine-1-phosphate formation and function**. *Nat Chem Biol* 2019, **15**:623–631.

48. Kol M, Williams B, Toombs-Ruane H, Franquelim HG, Korneev S, Schroeer C, Schwille P, Trauner D, Holthuis JC, Frank JA: **Optical manipulation of sphingolipid biosynthesis using photoswitchable ceramides**. *Elife* 2019, **8**.

49. Hartrampf N, Seki T, Baumann A, Watson P, Veprek NA, Hetzler BE, Hoffmann-Roder A, Tsuji M, Trauner D: **Optical control of cytokine production using photoswitchable galactosylceramides**. *Chemistry* 2020, **26**:4476–4479.

50. ** Morstein J, Kol M, Novak AJE, Feng S, Khayyo S, Hinnah K, Li-Purcell N, Pan G, Williams BM, Riezman H, Atilla-Gokcumen GE, Holthuis JCM, Trauner D: **Short photoswitchable ceramides enable optical control of apoptosis**. *ACS Chem Biol* 2021, **16**:452–456.

This paper introduces short chain photoswitchable ceramide with improved uptake and metabolism compared to long-chain analogs and allowed optical control of ceramide-induced apoptosis.

51. Zhang H, Desai NN, Olivera A, Seki T, Brooker G, Spiegel S: **Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation**. *J Cell Biol* 1991, **114**:155–167.