Mammalian sphingoid bases: biophysical, physiological and pathological properties

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Abbreviations

3-KTS, 3-keto-sphinganine

BD, Bipolar disorder

CAD, Coronary artery disease

CDases, Ceramidases

CerS, Ceramide synthases

CF, Cystic fibrosis

Chol, Cholesterol

CMC, Critical micelle concentration

COA, Coenzyme A

deoxy(dh)Cer, 1-deoxy(dh)-ceramide

deoxyCer, 1-deoxyceramide

deoxySA, 1-deoxysphinganine

deoxySLs, 1-deoxysphingolipids

deoxySO, 1-deoxysphingosine

DEPE, Dielaidoylphosphatidylethanolamine

DES, Dihydroceramide desaturase

dhCer, Dihydroceramide

DHPC, Diether lipid dihexadecylphosphatidylcholine

dhSM, Dihydrosphingomyelin

DMPC, Dimyristoylphosphatidylcholine

DMPH, N, N-dimethylphytosphingosine

DPPC, Dipalmitoylphosphatidylcholine

DPPS, Dipalmitoylphosphatidylserine

DSB, Deoxy-sphingoid bases

ER, Endoplasmic reticulum

FTY720, Fingolimod

HDAC, histone deacetylases

HSAN1, Hereditary sensory and autonomic neuropathy type 1

LCB, Long chain base

MMPH, N-monomethylphytosphingosine

MS, Multiple sclerosis

NPC, Niemann Pick type C

PA, Phosphatidic acid

PC, Phosphatidylcholine

PE, Phosphatidyethanolamine

PhytoS1P, Phytosphingosine 1-phosphate

PhytoSLs, Phytosphingolipids

PhytoSO, Phytosphingosine

PKC, Protein kinase C

PM, Plasma membrane

POPC, Phosphatidylcholine

PS, Phosphatidylserine

S1P, Sphingosine 1-phosphate

S1PR, Sphingosine 1-phosphate receptors

SA, Sphinganine

SA-1-P, Sphinganine 1-phosphate

SAdiene, sphingadiene/Sphingadienine

SLs, Sphingolipids

SM, Sphingomyelin

SO, Sphingosine

SphK, Sphingosine kinase

SPT, Serine palmitoyltransferase

ssSPT, Small subunit of serine palmitoyltransferase

TPA, 12-O-tetradecanoylphorbol-13-acetate

TRAIL, TNF-related apoptosis-inducing ligand

VPA, Valproate

Abstract

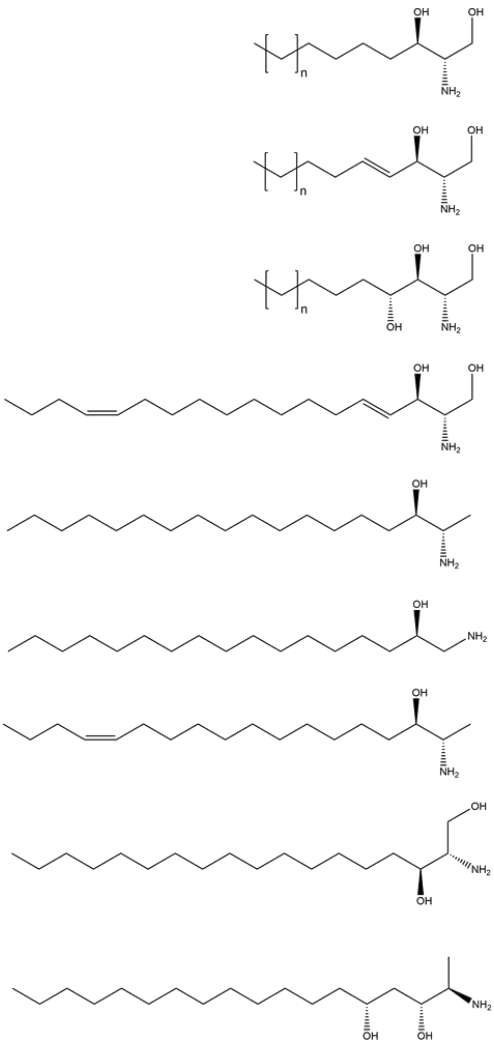
Sphingoid bases encompass a group of long chain amino alcohols which form the essential structure of sphingolipids. Over the last years, these amphiphilic molecules were moving more and more into the focus of biomedical research due to their role as bioactive molecules. In fact, free sphingoid bases interact with specific receptors and target molecules and have been associated with numerous biological and physiological processes. In addition, they can modulate the biophysical properties of biological membranes. Several human diseases are related to pathological changes in the structure and metabolism of sphingoid bases. Yet, the mechanisms underlying their biological and pathophysiological actions remain elusive. Within this review, we aimed to summarize the current knowledge on the biochemical and biophysical properties of the most common sphingoid bases and to discuss their importance in health and disease.

1. Introduction

Sphingolipids (SLs) are essential components of all mammalian tissues, and are highly abundant in the nervous tissue [1]. In blood, SLs constitute a part of the circulating lipoprotein particles (VLDL, LDL, and HDL), are carried by serum albumin, and are also present in blood cells, platelets [2], and exosomes [3–5]. Complex SLs are, together with sterols and glycerophospholipids the major structural components of cellular membranes [6]. SLs, such as sphingomyelin (SM), assemble laterally with cholesterol (Chol) on the outer leaflet of the plasma membrane (PM) thereby forming membrane specialized microdomains, commonly known as lipid rafts [7] that provide conformational support for membrane proteins and for the recruitment of signaling molecules [8]. In addition, certain SL metabolites, such as sphingosine (SO), sphingosine 1-phosphate (S1P), ceramide and ceramide 1-phosphate have well-documented roles as bioactive molecules that act as intra- or inter-cellular messengers. They are involved in a variety of important cellular processes such as proliferation [9], differentiation [10,11], inflammation [12–14], apoptosis [15–17] autophagy [18,19], calcium signaling [20,21] and immune response [13,22].

SLs are derived from a set of aliphatic amino alcohols, referred to as ‘sphingoid’ or long chain bases (LCB). The simplest and most abundant mammalian sphingoid base is the 18-carbon

dihydroxy amino alkane, SO and its unsaturated precursor sphinganine (SA). Although described as early as 1884 and named after the Egyptian Sphinx, the chemical structure of SO was elucidated only in 1947 by Herb Carter, who proposed the designation SLs for lipids derived from this molecule [23,24]. The majority of sphingoid bases are N-acylated to a variable fatty acid, forming ceramides and further substituted with a variety of head group structures forming complex SLs. However, a minor fraction is also present as free LCBs and as such involved in a variety of biological processes [25]. This is particularly true for the phosphorylated forms (S1P, sphingosine 1-phosphate) that has been extensively studied due to its role in cell function and pathogenesis [26,27]. LCBs are found in all eukaryotic organisms including animals, plants, fungi and certain bacterial species (*Sphingomonads* and *Bacteriodes*) encompassing dozens of sphingoid bases and sphingoid base-like compounds, which vary in chain length, number, position, and stereochemistry of double bonds, hydroxyl groups, and other functionalities (Fig. 1) [28,29].



Sphinganine **(1)**

Sphingosine **(2)**

Phytosphingosine **(3)**

Sphingadiene **(4)**

1-deoxysphinganine **(5)**

1-deoxymethylsphinganine **(6)**

1-deoxysphingosine **(7)**

Safingol **(8)**

Enigmol **(9)**

Figure 1 – Schematic representation of some mammalian long chain base structures (1-7) and synthetic derivatives (8,9) (based on [28]); n varies between 14 to 28 [30,31].

The present review will critically discuss the biochemical and biophysical properties of free mammalian sphingoid bases and their implications in pathophysiology, focusing on the most common canonical LCBs - SO and SA. The properties of phytosphingosine (phytoSO) and sphingadienine will also be addressed, and a short overview on the properties of atypical and synthetic LCBs will be presented at the end of the review.

2. Sphingolipid metabolism - an overview

The SL metabolic pathway is complex, involving multiple enzymes and isoenzymes, some of which display tissue and/or subcellular specificity. Such features are thought to be fundamental to the compartmentalization and tight regulation of SL metabolism. The fine balance of SL levels is essential to the preservation of normal cell function. This becomes clear with the increasing number of human diseases linked to genetic mutations causing enzyme dysfunction in the various steps of the SL metabolic pathway (such as, lysosomal and nonlysosomal SL degradation, *de novo* ceramide synthesis, glycosphingolipid synthesis). This issue is discussed in a recent review [32].

A brief description of the SL metabolism is presented below. Further details on the SLs metabolic pathways can be found in recently published reviews [25,33–35].

Sphingolipid *de novo* synthesis starts at the cytosolic surface of the endoplasmic reticulum (ER), where also the synthetic enzymes are located [33]. The first and rate-limiting reaction is catalyzed by the enzyme serine palmitoyltransferase (SPT), which is typically the conjugation of L-serine and palmitoyl-Coenzyme A (CoA) to form 3-keto-sphinganine (3-KTS). Besides this canonical activity, SPT has a variable affinity for other fatty acyl-CoAs, forming sphingoid bases with variable carbon chain length, and an alternative activity towards other amino acid substrates in particular for L-alanine and glycine. This forms a category of atypical 1-deoxysphingolipids (deoxySLs) (Fig. 2) that lack the C1-OH group of canonical sphingoid bases [36]. 3-KTS is instable and rapidly converted to SA by the 3-keto-sphinganine reductase. SA can be phosphorylated at C1 by sphingosine kinase (SphK) to form SA-1-phosphate [33], whereas C4-hydroxylation of SA leads to the formation of phytoSO [37,38]. However, the major synthetic pathway involves the *N*-acylation of SA to form dihydroceramides (dhCer), in a reaction catalyzed by one of the six ceramide synthases (CerS) [39]. Next, dhCer is converted to ceramides

through the Δ4-dihydroceramide desaturase (DES) introducing a ΔC_4-C_5 *trans*-double bond into the SA backbone [40,41]. Ceramides are the central metabolites in SLs synthesis and represent the pivotal branching point for the degradation and salvage pathways [42,43]. In some cell types, ceramide can be converted to galactosylceramide on the lumenal side of the ER [44]. Ceramide is also the precursor of SM and complex glycosphingolipids, which are synthetized in the Golgi [45]. Therefore, ceramides have to be transported from the ER to the Golgi either through vesicular or non-vesicular transport [46,47]. On the catabolic side, ceramides are hydrolyzed by ceramidases (CDases) to SO, which is either reacylated by CerS to form ceramides, or phosphorylated at the C1-hydroxyl by SphK to form S1P [33]. S1P can be de-phosphorylated by S1P phosphatase to form SO again or irreversibly degraded to hexadecenal and phosphoethanolamine by the S1P lyase [33]. These final breakdown products are then further metabolized to Phosphatidyethanolamine (PE) or other phosphoglycerides. A simplified representation of the SL metabolism is presented in Fig. 2.

It is worth mentioning that complex SLs can also be deacylated forming LCB derivatives, with variable headgroups. Well known examples are the sphingosylphosphorylcholine (lyso-sphingomyelin), galactosylsphingosine (psychosine) and glucosylsphingosine, which consist on a SO LCB linked to phosphorylcholine, galactose and glucose, respectively. Different studies suggest that these LCB derivatives have important roles in cell function [48–52] and might contribute to different pathologies, including cancer [53,54] and lysosomal storage diseases [55–58].

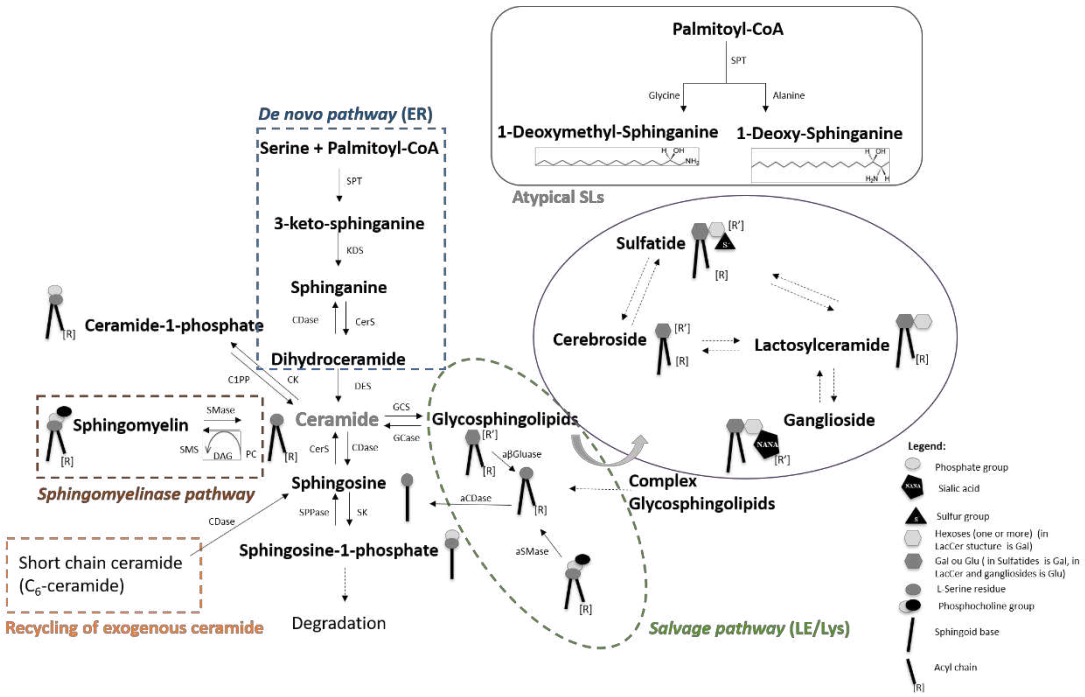


Figure 2: Schematic representation of the SL metabolism. Ceramide synthesis can be achieved through the de novo pathway, sphingomyelinase pathway, salvage pathway and the exogenous recycling of ceramide. Ceramide is a central molecule in the SL metabolism and used as a base for more complex SLs (SM and glycosphingolipids) or degraded to SO. The saccharide headgroup of glycosphingolipids can contain a single sugar unit (cerebrosides) and form glucosylceramide (GlcCer) or galactosylceramide (GalCer) when a glucose or a galactose residue is attached to the ceramide backbone, respectively. The addition of galactose to a GlcCer gives origin to lactosylceramide, the structural unit of gangliosides and globosides. Sulfatides are derived from GalCer via esterification of a sulfate group to the 3-hydroxyl of the galactose moiety. Atypical sphingoid bases such as 1-deoxy-sphinganine and 1-deoxymethyl-sphinganine, are formed by the conjugation of palmitoyl-CoA with L-alanine and glycine, respectively. Abbreviations: aβGluase, acid β-glucosidase; aCDase, acid ceramidase; aSMase, acid sphingomyelinase; C1PP, ceramide-1-phosphate phosphatase; CDase, ceramidase; CerS, ceramide synthase; CK, ceramide kinase; DAG – Diacylglycerol; DES, ceramide desaturase; ER, endoplasmic reticulum; GCase, glucosylceramidase; GCS, glucosylceramide synthase; KDS, ketosphinganine reductase; LE, Late endosomes; Lys, Lysosomes; PC, Phosphatidylcholine; SK, sphingosine kinase; SMase, sphingomyelinase; SMS, sphingomyelin synthase; SPPase, sphingosine phosphate phosphatase; SPT, serine palmitoyltransferase

3. Sphingosine

The most abundant LCB in mammals is C18 (d18:1) SO (Fig. 1, Structure 2). This LCB was first described by J. L. W. Thudichum in 1884 [59], and is chemically characterized as 2S,3R,4E-2-aminooctadec-4-ene-1,3-diol [23]. It is formed by the deacylation of ceramides through the action of 5 known pH-dependent CDases with varying subcellular localization [33,60]. SO can be recycled in the salvage pathway by a set of complex reactions involving activities of both SL synthesizing and hydrolytic enzymes [33]. This pathway is fundamental for the regulation and formation of ceramide [61]. On the catabolic side, SO is also the precursor of S1P, an important signaling molecule that is involved in multiple cellular events with outcomes often opposite to those provoked by SO and ceramides [62–64].

3.1. Biochemical and Biological Properties

SO is a bioactive lipid that acts as a second messenger in cell membranes, by interacting with cellular targets, inhibiting platelet aggregation and growth factor action [61,65–67]. One of the first identified targets of SO is Protein kinase C (PKC), a protein kinase which is widely implicated in the control of cell growth [68]. However, the mechanisms underlying SO-mediated PKC inhibition are not completely resolved. It is suggested that SO inhibits PKC by interfering with the binding of phorbol esters and other PKC activators [66]. Further evidence supporting

this hypothesis was obtained from studies performed on Niemann Pick type C (NPC) fibroblasts, where accumulation of endogenous SO seems to be directly related to the inhibition of PKC. Indeed, the specific binding of [20-^{3}H] phorbol 12,13-dibutyrate to the regulatory domain of the enzyme was significantly reduced in fibroblasts from NPC patients compared to control fibroblasts [69]. Also, stimulating PKC can rescue disease phenotypes, confirming the PKC inhibition by SO as a contributor to disease pathogenesis [70]. The mechanisms underlying SO action seem to be dependent on SO concentration. It is suggested that at higher concentrations (> 5-fold increase) SO directly inhibits PKC, while at lower levels, SO might inhibit the release of PKC activators, such as Ca^{2+} [69] or interact with other membrane lipids such as the negatively charged phosphatidylserine (PS). The later hypotheses was supported by studies on the inhibition of PKC by SO, that suggest that negatively charged PS is important for the electrostatic neutralization and required for activation of the enzyme [71]. Other studies showed that SO interferes with the cellular calcium homeostasis, through the inhibition of Ca^{2+} channels and pumps, particularly the PM Ca^{2+}-ATPase [72], and by decreasing the amount of Ca^{2+} stored in the cellular acidic compartments, contributing to disease states, such as NPC1 (Fig. 3) [73,74]. Additionally, it was reported that SO is involved in the regulation of PKC-dependent phosphorylation in the nucleus, by modulating the association of membrane phospholipids to PKC or its substrates [75].

In addition to inhibiting PKC, SO has also been implicated in modulating the activity of other protein kinases [76,77], including calmodulin-dependent kinase, insulin receptor tyrosine kinase [76], the casein kinase Fam20C [78] and SO-dependent protein kinases [79,80], likely due to SO-mediated changes in the electrostatic interactions between the enzymes and membrane lipids [71,76,77]. In addition, an indirect modulation of acid sphingomyelinase phosphorylation within the lysosome has been reported, which is due to SO-mediated PKC inhibition [81].

SO has also been linked to reduced activity of phosphatidate phosphohydrolases [82], and implicated in the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA), suggesting a role of SO in the activation of phospholipase D [83]. Moreover, SO and SphK1 were identified as players in the regulation of endocytic membrane trafficking (Fig. 3) [84], and in the regulation of cell growth arrest, apoptosis and response to chemotherapy [85,86], through multiple mechanisms [87–90]. Additionally, it has been reported that SO might also function as a positive modulator of cell growth, by stimulating DNA synthesis and potentiating the mitogenic response of growth factors [91]. These contradictory effects might somehow be related to the rapid interconversions of ceramide, SO and S1P that often have opposite cellular effects. Among other factors, the cellular response depends on the activity of those enzymes that determine which metabolites are predominantly formed under specific conditions [64].

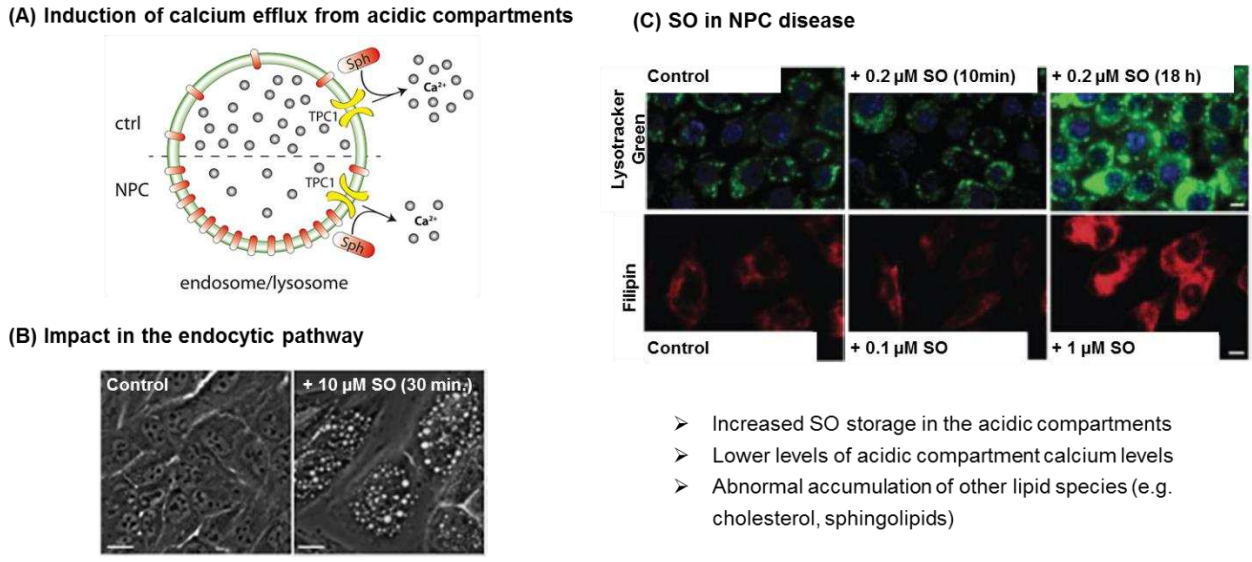


Figure 3 – Examples of Sph biological actions. (A) Sphingosine-induced calcium release from acidic compartments occurs through the action of two pore channel 1 (TPC1). (B) SO induces the formation of dilated intracellular vesicles and has an important role in endocytic membrane trafficking. (C) SO abnormal accumulation in late endosomes/lysosomes is related with calcium depletion in these organelles in NPC cells and is followed by the secondary accumulation of other lipid species. Adapted from [73]; [74]and [84].

3.2. Physico-chemical properties and biophysical implications

It is widely recognized that lipids modulate membrane structure and properties, and that these changes might underlie their biological activity. This is also true for LCBs, which due to their amphiphilic structure, significantly alter the physico-chemical properties of biological membranes.

3.2.1. Physico-chemical characteristics of SO

Differential scanning calorimetry studies revealed that SO displays a single phase transition centered at 39ºC [92], while studies performed using fluorescence spectroscopy showed that SO gel-to-fluid phase transition occurred at ap. 53ºC under neutral conditions (pH 7.4), being reduced to ap. 48°C under acidic conditions (pH 5.0) [93]. These results although distinct, probably due to the different experimental conditions (e.g. techniques, SO concentrations, etc), suggest that SO potentially exists in the gel phase at physiological temperature. Hence, SO is a good candidate to induce changes in membrane fluidity and to participate in the formation of ordered lipid domains. In addition, due to its amphiphilic nature,

SO forms micelles when dispersed in aqueous solutions. However, a large disparity on the critical micelle concentration (CMC) values of SO has been reported, ranging from ~0.99 μM up to 112 μM [92,94,95]. These differences might result from the different techniques used (such as dynamic light scattering [95], fluorescence methodologies [92,94]), and the specific conditions in which the measurements were performed. Particularly, pH and ionic strength may strongly influence ionization state and hydrogen bonding network [95]. In blood, SO concentrations of 0.1 to 1 μM have been reported for both free SO and its phosphorylated form [96]. Considering that free SO is present in the micromolar range in body fluids, it is possible that SO micelles (aggregated SO) exist under physiological conditions. This is of importance, considering that micelles can act as membrane solubilizing agents, thus contributing to membrane structure impairment.

Since the differences in hydration and/or hydrogen bonding affect the pK of the amino group [97], it is not surprising that very different pKa values (ranging from ~ 6 to ~ 9) have been reported for SO in solution, micelles and membranes [66,97,98]. Therefore, it is difficult to predict whether SO is present in uncharged form at the PM [95,97], and becomes charged only within the acidic cell compartments, or if the interaction of SO with other membrane lipid components is sufficient to confer positive charge to the molecule, even at neutral pH [93]. Nonetheless, changes in pH from an acidic to a neutral environment alter the hydrogen-bonding network of SO aggregates, from intramolecular to intermolecular [99], and is therefore expected to influence membrane biophysical properties. Moreover, SO might affect membrane surface charge in a manner dependent on both membrane lipid composition and pH, suggesting that a local enrichment in SO would affect the electrostatic interactions of membrane proteins and consequently protein activity. In the same way, it might drive changes in membrane lipid organization which can impact membrane structure, trafficking and sorting processes.

3.2.2. Effect of SO on the biophysical properties of membranes

Several studies have addressed the effect of SO on bilayer membranes and its ability to change membrane biophysical properties, by promoting changes in fluidity [92–94,97,100–102], domain formation [101,103] or membrane permeability [94,102,104,105] (Fig. 4). The effects of SO on membrane properties are complex and depend on lipid composition and the protonation state of SO. For instance, it is suggested that SO stabilizes dipalmitoylphosphatidylcholine (DPPC) gel domains [92,100] by inducing reorientation of the PC headgroups, which would decrease the lipid cross-sectional area and promote tighter intermolecular packing in the bilayer [92]. This phenomenon might resemble what was already proposed for Chol and for ceramide [106], where a closer interaction with lipids bearing a larger headgroup might help in protecting the

hydrophobic moieties from unfavorable contacts with the aqueous environment. One can therefore hypothesize that in biological membranes SO might be able to intercalate within other membrane lipids with a concomitant increase in membrane packing, thereby enhancing the formation of ordered lipid domains, such as the widely described lipid rafts [8,107]**.** However, it was also described that the effect of SO on membrane fluidity is concentration dependent [100], suggesting that SO might have multiple effects on membrane properties depending on its local concentration. Nonetheless, several studies highlighted that SO induces membrane ordering in a variety of artificial membranes, such as phosphatidylcholine (POPC) [93], dimyristoylphosphatidylcholine (DMPC), diether lipid dihexadecylphosphatidylcholine (DHPC) [92], egg PC [103], DPPC/Chol, SM/Chol and SM/Chol/PE [94], PC/Chol [101] and in POPC/SM/Chol mixtures [93]. Interestingly, the studies performed in membranes containing Chol or Chol/SM showed that SO can form gel domains or become soluble in the l_o phase, depending on the SO-SM and SO-Chol interactions, respectively [93,94,101], a behavior similar to that reported for ceramides [108,109] (Fig. 4). In addition, surface pressure studies revealed a negative deviation from ideal additivity indicating a strong interaction between SO and Chol [110]. The authors suggested that SO and Chol can form pairwise condensed complexes that interact over hydrogen bonds that are formed between the protonated SO amino group and the Chol hydroxyl group. From a structural perspective, this agrees with an opposite cone-shape for these two molecules as sterols have a single hydroxyl group for the polar, but a bulky four-ring structure for the hydrophobic part. Here, the formation of the complexes depends greatly on the cellular location of these lipids, becoming particularly favorable in acidic compartments where SO is likely to exist in its protonated state. This could be particularly relevant in the context of NPC disease, where SO and Chol co-accumulate in lysosomes [73]**.** Interestingly, evidence shows that decreasing the levels of sphingoid bases in NPC models can reduce Chol storage [111], while the reduction of glycolipids levels are not sufficient to revert Chol accumulation [112].

Other studies reported that SO abolishes the pre-transition of DMPC and DHPC, suggesting that SO increase the fluidity of the membranes by destabilizing the interdigitated gel phase formed by these lipids [92]. Furthermore, SO might have a detergent-like action, leading to the formation of very small, highly curved vesicles, or even mixed micelles of SO/ dielaidoylphosphatidylethanolamine (DEPE) [100]. Whether such situation also occurs in biological membranes is currently unknown. However, those structural changes would be compatible with cellular events that involve vesicle formation and membrane sorting, such as the endo-lysosomal pathway and exosome formation, placing SO as a good candidate for the

modulation of these cellular events. Indeed, a recent study identified SO as a key player in endocytic membrane trafficking [84].

Protonated SO was also shown to have concentration dependent effects on the thermotropic behavior of acidic phospholipids [92,97]. At low concentrations, SO stabilizes dipalmitoylphosphatidylserine (DPPS) membranes by promoting a tighter packing because of increased hydrogen bonding and/or electrostatic interactions between the anionic phospholipids and the cationic SO. This effect is, however, abolished in mixtures containing high SO concentration [92,97]. Moreover, SO causes deprotonation of DPPS carboxyl group with consequent lowering of its apparent pKa due to electrostatic interactions between the two lipids [113]. These interactions also contribute to an overall electrostatic charge neutralization preventing the binding between the positively charged Ca^{2+} and the negatively charged headgroup [113], suggesting that, in a cellular context, positively charged SO might affect the binding of proteins to membranes. This could also explain why SO interferes with Ca^{2+} homeostasis, as discussed above [73]. Similar changes in the thermotropic behavior were reported for the acidic phospholipids dimyristoylphosphatidic acid and egg PA [92]. It was shown recently, that the interaction between SO and PA leads to transient non-lamellar structures, namely cubic phases [102].

The impact of SO on membrane structure, thermotropic behavior and lipid phase separation, can have consequences on membrane permeability, both in model and cell membranes [94,102,104,105]. Changes in membrane permeability might derive from i) structural defects created by SO as a consequence of lipid phase separation into rigid and fluid lamellar phases [94,105]; ii) the formation of small short-lived channels that allow the passage of small molecules and ions [104]; and iii) non-lamellar structures formed upon interaction of SO with negatively charged lipids [102]. Additionally, it was shown that SO-induced membrane permeability depends on pH and membrane lipid composition: SO promotes a rapid increase in membrane permeability in conditions mimicking the lysosome pH environment, i.e., with internal acidic and external neutral pH. Moreover, high levels of Chol and SM, as observed in NPC1, enhance SO-induced membrane permeability in those lysosomal-mimicking vesicles [105], suggesting that SO might impair membrane structure under pathological conditions (Fig. 4).

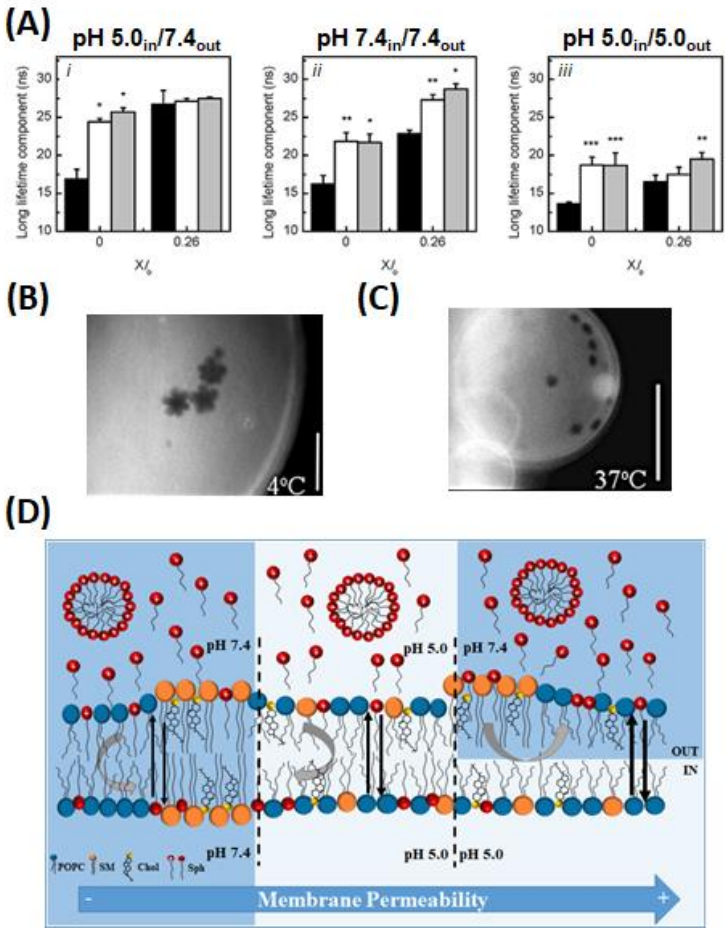


Figure 4 – SO-induced biophysical changes in artificial membranes. (A) SO-induced alterations in the biophysical properties of Lysosome Mimicking Vesicles (LMVs – pH $5.0_{in}/7.4_{out}$) and POPC/SM/Chol vesicles with no pH gradient. Long lifetime component of trans-Parinaric Acid (t-PnA) fluorescence intensity decay in ternary POPC/SM/Chol vesicles without (0) or with 26% of liquid ordered phase fraction (XI_o = 0.26) before (black) and immediately after addition of 5 (white) and 10 (light grey) mol% of SO. The values are the mean ± SD of at least three independent experiments. *p < 0.001 versus 0% SO; **p < 0.01 versus 0% SO; ***p < 0.05 versus 0% SO. Addition of SO to ternary mixtures causes a decrease in membrane fluidity, which is more pronounced in mixtures containing low Chol content (XI_o = 0). SO-induced changes in membrane fluidity are also dependent on pH. For further details see Figure 8 of [105]. (B) SO induces gel /fluid phase separation in ternary mixtures composed by PC/SO/Chol (70/20/10 mol%). (C) SO is also able to induce I_o/I_d phase separation in quaternary mixtures composed by PC/SO/SM/Chol (40/20/20/20 mol%). (D) Schematic representation of the effects of external addition of SO to POPC/SM/Chol vesicles, at different physiological pH conditions. SO cause multiple changes in membrane properties that include, decrease in membrane fluidity, increase in membrane

fluidity, change in membrane surface charge, all of which dependent on pH. For further details see Figure 9 from [105]. Adapted from (B,C) [101] and (A,D) [105].

3.3. SO in disease

SO has been implicated in different pathologies. It has been shown that lysosomal SO storage is an initiating factor in NPC disease [73,114] (Fig. 3). NPC is an inherited atypical neurodegenerative lysosomal storage disorder, characterized by mutations in two genes encoding for the late endosomal/lysosomal transmembrane protein NPC1 and NPC2 [115]. The exact function of these proteins is still unclear, but both have been related to Chol transport in the endo-lysosomal system [116,117]. NPC1 was also pointed as a possible intervenient in the mediation of the lysosomal efflux of SO [73]. Indeed, molecular modelling studies using the solved crystal structure of NPC1 identified a potential binding site for SO inside NPC1, strengthening the hypothesis that NPC1 is a lysosomal SO transporter [118]. This hypothesis is further supported by a study that uses click chemistry-based sphingosine probes to measure the lysosomal release of sphingosine from NPC cells [119]. NPC cells are characterized by an intra-lysosomal accumulation of SO as well as Chol, glycosphingolipids and SM [73]**.** Moreover, NPC cells have a characteristic defect in the transport of lysosomal LDL-derived Chol to the ER for esterification. As described above, SO accumulation might affect the biophysical properties of lysosomal membranes (Fig. 4). Indeed, synthetic membranes enriched in SO have impaired stability under acidic conditions and enhanced propensity to form larger aggregates, likely due to SO-induced changes in membrane surface charge [105]. A defective NPC1 function not only affects the storage of lipids, but also reduces the levels of lysosomal Ca^{2+} and causes unique endocytic transport and fusion defects [73]. This in turn, was shown to cause defects in the endocytic transport leading to a secondary accumulation of Chol, SM and glycosphingolipids in NPC. Furthermore, the exogenous addition of SO to normal cells induced an NPC like phenotype, such as altered calcium homeostasis in acidic compartments, trafficking defects and a secondary accumulation of other lipid species [73]. Reducing SO levels by inhibiting *de novo* synthesis with the SPT inhibitor myriocin, reversed these cellular phenotypes in NPC cells [73]. Considering that SO has been shown to alter membrane permeability [94,102,104,105], it can be hypothesized that, in addition to inhibition of PKC mediated by SO, changes in lysosomal permeability are a possible mechanism for the observed changes in the lysosomal Ca^{2+} stores upon SO accumulation [20,73]. It was also suggested, that an inhibition of PKC by SO could be involved in the Rab9 trafficking defect seen in NPC [120]. A better understanding of the molecular mechanisms underlying NPC disease might help developing therapeutic strategies to treat this

disease. To date, there is no cure for NPC patients. The current therapeutic approaches delay the progression of the disease or attenuate its clinical symptoms [121][122]. For instance, Miglustat is used to limit the accumulation of gangliosides and neutral glycosphingolipids, like glucosylceramide and globosides, through the inhibition of glucosylceramide synthase, and is able to delay neurological manifestations of the disease and improve patient survival[122,123]. Recently, Fingolimod (FTY720), was proposed as a new therapeutic agent for NPC disease. It was shown that this S1P analog is able to increase NPC1 and NPC2 expression, contributing for a reduction in Chol and SL accumulation in NPC fibroblasts [124].

SO might also be important in the prevention of disease and/or disease related complications. There is increasing evidence of the protective antibacterial action of sphingoid bases and other lipids in infection [125]. SO was shown to be crucial for host defense against *Pseudomonas aeruginosa* and other lung infections [126,127]. Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the CF Transmembrane conductance Regulator gene [128]. Despite the multisystemic implications of this mutation, the major cause of patient mortality are chronic pulmonary infections. Different microorganisms are involved in the lung infections of CF patients. In particular *Pseudomonas aeruginosa* infections are incident in these patients [129]. Due to the long-term antibiotic treatments the number of resistant *Pseudomonas aeruginosa* strains is increasing, which requires alternative therapeutic options. It was shown that ceramide levels are elevated in the respiratory tract of CF patients [130,131], while decreased SO levels result in a diminished antibacterial response [126]. Increasing SO levels either by the inhalation of SO, the S1P analog FTY720, or by treatment with acid CDase had a positive effect in CF mice [126,127] and might become a preventive/therapeutic measure to reduce bacterial infections in CF patients. The detailed molecular mechanisms related to the protective effect of SO against bacterial infection are yet to be resolved. Nonetheless, it is known that PKC signaling is involved in *Pseudomonas aeruginosa* elastase-induced epithelial barrier disruption [132], indicating that SO-induced protection against infection might be related to PKC inhibition, as previously suggested [133]. Other hypothesis might be related with SO-induced bacterial cell wall damage, for example by affecting bacterial membrane permeability, and defects in adherence, as suggested for SA [125,134].

SO, together with SA have also been related to protection mechanisms against cancer. By inhibiting proliferation and inducing apoptosis in different cancer cell lines, these molecules have a special importance as tumor suppressors [15,135–137]. Multiple molecular mechanisms have been attributed to SO-mediated cell death. For example, elevation in SO levels were shown to induce cell death via either Golgi fragmentation or lysosomal rupturing [138,139]. In U937 human monoblastic leukemia cells, SO was shown to activate JNK and to inhibit ERK leading to

apoptosis [88]. SO has also been implicated in human hepatoma cell apoptosis through activation of caspase-3 [89] and/or caspase-7 and -8 [140]. Whether SO induced apoptosis occurs via a specific mechanism or a combination of multiple mechanisms operating in a concerted manner, as previously suggested [90], remains to be elucidated. The evidence that SO can act as a tumor suppressor has already led to the development of synthetic SO derivatives, such as N,N-dimethylsphingosine and N,N,N-trimethylsphingosine, that showed promising effects in cancer therapy [141–144]

4. Sphinganine

Compared to SO, less is known about the biological, biophysical and pathological effects of SA (Fig. 1, structure 1). Dihydro-sphingolipids were for long considered biologically inactive [145]. However, there is emerging evidence that dihydro-sphingolipid species contribute to various cellular events, such as, autophagy [146], cell proliferation [147], oxidative stress [146,148] and metabolic regulation [149,150], by yet unidentified mechanisms. Nonetheless, most studies are focused on dhCer and little is known about the free base SA. SA has been implicated in T cell death [151], and as a pro-apoptotic molecule in colon cancer cells [15,152,153]. Other study suggests that elevation in SA enhances 4-HPR-induced tumor cell cytotoxicity [154]. In addition, SA and dhCer were identified as mediators of cell death in prostate cancer cells treated with different variants of vitamin E[155,156]. Yet, the mechanistic aspects of SA-mediated cell death have not been resolved. It is suggested that SA cytotoxic action involves caspase activation both in T cells [151] and hepatoma cells [89]. Another study suggests that SA-induced cytotoxicity and growth inhibition of CHO cells occurs via inhibition of PKC [157]. SA mediated inhibition of PKC has also been related to the mechanism by which SA reduces cell proliferation and induces apoptosis in human myeloid leukemia cells [158,159], and prevents monocytic differentiation of HL-60 cells [160]. Indeed, SA and SO have similar abilities to inhibit PKC [66] and to induce apoptosis by indirect and direct mechanisms involving degradation of DNA and the induction of apoptotic cytoarchitectural changes [158]. Elevated levels of SA have also been reported in NPC disease, however SA was shown to have less or no effect in inducing NPC disease phenotype compared to SO [111,161,162].

The biological importance of SA can also be accessed from studies using fumonisin B1 (FB1). Fumonisin is a mycotoxin that inhibits CerS resulting in increased accumulation of SA and SO and a decrease in complex SL in mammalian serum and tissues [163–165]. Fumonisin is associated with kidney and liver toxicity [166–168] and other pathological conditions such as increased oncotic necrosis [164] and induction of neural tube defects [169]. Comprehensive

reviews on this subject were already published [164,170,171]. Of note is the increased accumulation of SA compared to SO and its rapid conversion to SA1P that is considered to be the mediator of fumonisin induced toxic effects [169,172,173]. In LM/Bc mouse embryonic fibroblasts treated with FB1, SA1P was already shown to be able to decrease histone deacetylase activity and increased histone acetylation that could be related with neural tube defects [169]. SA1P was also shown to be related with the kidney and liver toxicity with more severe consequences for the kidney cells [166]. This toxicity could be related with increased calmodulin expression after SA accumulation [165].

Nevertheless, there is a contradictory study on the importance of SA1P for fumonisin toxicity. Fumonisin was shown to decrease the phosphorylation of ERK2. Nevertheless, SPT and glucosylceramide synthase inhibitors caused similar decreases. This indicates that rather the inhibition of the *de novo* SL biosynthesis than the accumulation of SA and SA1P was responsible for the ERK2 status after FB1 treatment [174].

Finally, to our knowledge biophysical studies that address the implications of SA in membrane structure and properties have not yet been performed, except one study that shows that SA, similarly to SO, is able to stabilize Chol-enriched domains [175]. The missing Δ4-5 double bond might contribute for a tighter packing of the membrane. Indeed, studies performed with dihydrosphingomyelin (dhSM) showed more ordered membranes compared to regular SM [176–178] and more tightly packed sphingolipid-cholesterol-enriched domains [179]. However, the absence of an acyl chain and the smaller headgroup in SA compared to dhSM will certainly influence its membrane interactions. It is therefore important to investigate how SA changes the fluidity, surface charge and structure of model and cell of membranes and evaluate if these alterations are implicated in SA biological actions.

5. Phytosphingosine

5.1. Biological and biophysical properties

Phytosphingolipids (PhytoSLs) are a group of functionally related long-chain aliphatic 2-amino-1, 3, 4-triols. Among them, 4-hydroxysphinganine or PhytoSO (t18:0) ((2S,3S,4R)-2-aminooctadecane-1,3,4-triol) (Fig. 1, Structure 3) is the most prevalent [180]. They are formed after the hydroxylation of SO through the action of DES2, and are found abundantly in plants, yeast, fungi, and marine organisms, as well as, in mammalian tissues [35,181,182]. PhytoSO is the structural backbone of PhytoSLs and normally constituted by 18 carbons. PhytoSO with other chain lengths, such as C20, can be detected in minor amounts depending on their origin

[180,183]. Variations in the PhytoSO chain length result in different biological responses. For instance, McCarthy and co-workers showed that the length of the PhytoSO chain, controls the affinity of T-cell receptor binding to α-galactosylceramide–human CD1d–glycolipid complexes and the activation of iNKT cells [184].

Free PhytoSO was shown to have antiproliferative and anti-inflammatory effects, and has major importance for the maintenance of skin homeostasis [185,186]. For example, PhytoSO showed a protective role for the skin of 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated hairless mice. It can block epidermal thickening and edema, and the infiltration of inflammatory cells into the dermis [185]. The anti-inflammatory effects of PhytoSO were promoted through the block of TPA-induced generation of prostaglandin E_2 in peripheral mononuclear leukocytes [185]. PhytoSO is also an effective antimicrobial compound, able to inhibit the growth of Gram-negative and Gram-positive bacteria even at low concentration [125,187]. The underlying mechanism of the antimicrobial activity of PhytoSO (or other sphingoid bases, as discussed above) are not clearly elucidated, but is suggested to be related to the disruption of bacterial cell wall [125], reduction of bacterial adherence [134], and inhibition of bacterial protein kinase [188]. In yeast, both PhytoSO and SA levels are increased during heat stress, suggestive of an important role of these lipids in the regulation of stress responses [189]. These molecules are required to regulate translation of heat shock mRNA [190]. Like SO, PhytoSO has also been associated with apoptotic events activated through different mechanisms. For instance, studies with human T-cell lymphoma Jurkat cells showed that PhytoSO induced chromatin DNA fragmentation, a hallmark of apoptosis, through the activity of caspases-3 and -9 [191]. Other studies showed that PhytoSO can use two distinct MAPK signaling pathways for amplifying apoptotic cascade [192]. Moreover, in radiation-resistant human T-cell lymphoma the combined treatment using PhytoSph and γ-irradiation caused nuclear translocation of apoptosis-induced factor, which in turn is mediated by ROS-dependent Bax relocalization and ROS-independent PARP-1 activation [193]. This finding suggests an alternative therapeutic approach against radiation resistant cancers [194]. It is also suggested that PhytoSO has antifungal properties, inducing apoptosis in filamentous fungi [194].

Contrary to SO and similarly to S1P, the phosphorylated form of PhytoSO (PhytoS1P) is associated with cellular growth and proliferation. PhytoS1P in combination with epidermal growth factor revealed a synergistic anti-aging effect and was able to restore the extracellular matrix in human dermal fibroblasts [195]. Moreover, it was demonstrated that this lipid is involved in the regulation of genes that are required for mitochondrial respiration in yeast [196]. Structurally, PhytoSO differs from SO due to the presence of a hydroxyl group linked to the 4-ene. The presence of this polar group might have a strong influence in the biophysical properties

of membranes, particularly considering that it provides an additional source for H-bond formation. Unfortunately, studies addressing the biophysical properties of PhytoSO as a free molecule have, to our best knowledge, not been performed, and information can only be obtained indirectly from studies performed with PhytoCeramide and PhytoSphingomyelin. However, extrapolation of the biophysical behavior should be made with caution, since the *N*-acyl chain of SLs strongly influences their biophysical behavior. Nonetheless, it should be stressed that the additional OH group present in the sphingoid backbone seems to be responsible for a higher gel-to-fluid transition temperature of these phytoSLs compared to the SO-based SLs, and higher ability to increase membrane order [197–200], likely due to stronger network of hydrogen bonds and/or interactions between the polar head groups [198,201]. It can therefore be hypothesized that these stronger interactions are related to the protective effect that PhytoSO-based SLs have in skin, where they are highly abundant [197]. Also noteworthy, is the altered affinity of sterols for membranes containing phytoSphingomyelin compared to SM [200], suggesting that these lipids might influence the organization and properties of the lipid raft domains, and thus raft-dependent signaling events [107]. For example, PhytoSO/PhytoSL-enriched ordered domains in biological membranes might be involved in the activation of apoptotic signaling cascades through specific membrane death receptors, which could account for the observed apoptotic effects described for PhytoSO in different cell types. It is also tempting to hypothesize that the antimicrobial effects of PhytoSO could be related to its impact on membrane organization. Like SO, PhytoSO might also compromise the permeability and integrity of bacterial cell wall or other microbial membranes, thus preventing microbial infection in humans.

5.2. Role in disease

PhytoSO is recognized as a potential therapeutic agent in the treatment of inflammatory and proliferative cutaneous diseases [185]. In fact, it has been demonstrated that synthetic derivatives of PhytoSO (mYG-II-6 and fYG-II-6) have anti-inflammatory and therapeutic effects in TPA- or cytokine-stimulated keratinocytes, mouse T-cells, and cutaneous inflammatory models, as well as, induced human peripheral blood mononuclear cells [186]. The effect of these compounds was stronger than those of the parent compound, PhytoSO, and their activities were related to the inhibition of NF-kB, JAK/STAT and MAPK signaling, resulting in the induction of apoptosis [186]. Moreover, based on its strong antimicrobial activity and anti-inflammatory effects, PhytoSO can complement existing acne therapies as an active cosmetic component [187].

Other PhytoSO derivatives have been developed based on the inhibition of SphK, which has been implicated in cell growth and inhibition of apoptosis. The cytotoxic effect of N-monomethylphytosphingosine (MMPH) and N, N-dimethylphytosphingosine (DMPH) and the pegylated forms MMPH-PEG and DMPH-PEG was evaluated in human leukemia HL60 cells, and all of them revealed a concentration dependent inhibition of cell growth and viability [202]. Despite this, DMPH was the most effective cytotoxic compound against leukemia. It was able to induce apoptosis via the activation of caspases-3,-8 and -9 [202] and, because of its apoptotic effects, PhytoSO has been suggested as an alternative therapeutic approach against radiation resistant cancers [193]. Additionally, PhytoSO can sensitize cancer cells to TNF-related apoptosis-inducing ligands (TRAIL). This occurs through the synergistic up-regulation of DR4 and DR5 in a NF-kappaB-dependent manner, resulting in caspase-8 activation and subsequent mitochondrial dysfunction [203]. These results suggest that the combination of PhytoSO with TRAIL might be useful in the treatment of cancers that are more resistant to TRAIL.

PhytoSO was also suggested for the treatment and prevention of diabetes and obesity. Murakami and coworkers [204], showed that feeding high fat diet supplemented with PhytoSO to DES2 knockout mice inhibits inflammatory events in adipose tissue, which prevents the development of glucose intolerance and obesity [204]. PhytoSO is synthesized in yeast and fungi, thus being present at relatively high levels in fermented foods. This suggests that its ingestion might help to prevent the development of type II diabetes and obesity [204]. The mechanism by which PhytoSO provides this protection could be related to the transcription of peroxisome-activated receptor γ, involved in lipid and glucose homeostasis [205].

Recently, Jadhav and collaborators have shown that treatment of yeast cells with valproate (VPA), a widely used drugs to treat bipolar disorder (BD), increases the levels of PhytoSO via *de novo* synthesis and at the same time leading to inositol depletion and upregulation of unfolded protein response [206]. They further demonstrated that VPA mediated increase in SL de novo synthesis is due to a downregulation of Orm1/2 expression, and upregulation of expression of fatty acid elongases Sur4 and Fen1, as well as of genes involved in the export and metabolism of PhytoSO [206,207]. Since, VPA induced inositol depletion is also observed in mammalian cell models [208,209] these findings suggest that inositol depletion and the consequent increase in PhytoSO levels might underlie the therapeutic action of VPA [207], which is promising for the development of new therapeutic approaches for BD treatment.

6. Sphingadienine

The dienic sphingoid base, sphingadienine (Fig. 1, Structure 4) also known as sphingadiene (SAdiene) differs from the most common mammalian LCBs in the number of double bonds [210]. Whereas, the common sphingoid base, SO presents a unique trans double bond at the Δ4-5 position, SAdienes have an additional double bond within the hydrocarbon chain [211,212]. In humans this second C-C double bond is located at Δ14-15 [210,211] and in the same position as the double bond in the deoxySO [213]. The enzyme responsible for the introduction of this Δ14-15 double bond has not yet been identified (see also section 8). Nevertheless, studies using *Drosophila melanogaster* [214] and studies with cultured human and murine cells [215] showed that SAdiene is a downstream product of SO, which provide evidence for an interconnection between the metabolism of SAdiene and deoxySO.

Free SAdienes and their synthetic analogues exhibit cytotoxic and anti-proliferative effects in cancer and non-cancer cultured cells in vitro [216–218]. SAdienes were also associated with inhibition of colon tumorigenesis in mouse models by reducing the viability of cancer cells in a dose dependent manner [219–221]. Interestingly, Δ4,8-C18-SAdiene and Δ4,6-C14-SAdiene were shown to have anti-inflammatory [222] and neurotropic effects [223], suggesting their importance in the treatment of neurodegenerative diseases. Mitochondrial and cellular pools of SAdienes-ceramide, but not of canonical SO-based ceramide, are elevated in embryonic fibroblasts derived from double knockout BAX-BAK mice and mouse derived immortalized iBMK cell line [224]. These findings suggest a novel role for BCL2 proteins in regulating SAdiene-ceramide species, and the later might provide a missing connection between apoptosis and SL synthesis.

Even though these species are relatively abundant in plasma (~18%) [225] and have been associated with different pathophysiological events, there is still a lack of information regarding their mechanism of action. In blood, a significant proportion of SAdiene is also found in phosphorylated form as SAdiene-1P.

Likewise, the physico-chemical properties of free SAdiene and its impact in membrane structure and organization is yet to be investigated. Therefore, additional studies aiming at elucidating these aspects are fundamental.

7. LCB with atypical carbon chain lengths

A great variety of sphingoid bases with atypical chain lengths (in the range of C16-C20) are present in blood, although the relative proportions of the LCBs differ between mammalian species. These atypical LCBs are formed in a reaction catalyzed by the SPTLC3 subunit of SPT, which metabolizes also other acyl-CoAs in the range of C12 to C18 [30,226,227]. In contrast to

the ubiquitously expressed subunits SPTLC1 and SPTLC2, the expression of the SPTLC3 is restricted to few tissues and cell types [228]. SPTLC3 expression is especially high in trophoblasts and keratinocytes, which indicates a special need of skin and placenta tissue for atypical LCBs structures or ceramides [30,229]. The atypical sphingoid base variants also include odd number of carbons such as linear d17- and d19- SO [230]. Even though, SO (61.5%) and SAdiene (18.65%) are the most abundant LCBs in plasma, yet approximately about 15 % of the LCBs in human plasma are of atypical chain length [225].

Among the atypical LCBs, SO d20:1, which is formed when SPT metabolizes stearoyl-CoA, appears to be of specific interest. A SO d20:1 backbone was reported in gangliosides of rat and human brains [231], in human stomach mucosa [232] and in SM of rat liver [233], and which reflects less than 1% of the total sphingoid bases in human plasma [225]. Studies in yeast suggested the formation of C20 LCBs is dependent on the small subunit of SPT (ssSPTb), which promoted the use of stearoyl-CoA when co-expressed with the SPT subunits [226]. Enhanced formation of C20 LCBs are also found in a mouse model that bears a spontaneous *de novo* mutation in the ssSPTb gene associated with axon degeneration and aberrant accumulation of membrane structures [234]. Interestingly, several SPT mutations, which were reported in the context of a severe form of the inherited neuropathy HSAN1 (Hereditary sensory and autonomic neuropathy type 1) showed increased C20 LCB formation. In contrast to the majority of HSAN1 mutations, which appear to cluster around the active site of the enzyme, these mutations are located on the surface of the SPT protein [235], possibly interfering with the binding to an interaction partner. A recent study found that SO d20:1 is a predictive biomarker for the risk to develop cardiovascular events even after adjusting for traditional risk factors including the use of lipid-lowering drugs and angiographically-determined CAD at baseline [236].

Whether the structural and biophysical properties of LCBs with atypical chain lengths determine these pathophysiological effects, solely or at least in part, is at present unknown. Indeed, very few biophysical studies have been conducted to address this issue, and none has been performed with the LCBs in their free forms. Nonetheless, it is worth mentioning that the longer the LCB of the ceramide molecules, the higher the T_m and the lower the concentration of ceramide needed to induce gel domain formation [237]. However, due to the unique biophysical properties displayed by ceramides [238], it is difficult to predict whether the effects of structurally different unconjugated free LCBs would be comparable to those observed for ceramide bearing different LCBs.

8. Deoxy-sphingoid bases

Apart from its canonical substrate L-serine, SPT also uses L-alanine and glycine as alternative substrates (Fig. 2). This forms a category of atypical deoxySLs, which lack the C1-OH group [230,239]. The conjugation of L-alanine forms 1-deoxysphinganine (deoxySA) (Fig. 1, Structure 5), while the conjugation of glycine forms 1-deoxymethylsphinganine (Fig. 1, Structure 6) [36,239]. Several mutations in the SPT subunits are associated with increased production of deoxy-sphingoid bases (DSB) [36,240,241], which is due to an enhanced activity with L-alanine and glycine without changes in the binding affinities for these amino acids [242]. DeoxySA is converted further to 1-deoxy(dh)-ceramide (deoxy(dh)Cer) and 1-deoxyceramide (deoxyCer). However, while the conversion of canonical dhCer into ceramide occurs by the introduction of a Δ4,5 trans double bond [41,243] the double bond in deoxyCer is Δ14,15cis [213]. This suggests that the metabolism of deoxySLs deviates from that of canonical SLs. Due to the lack of the C1-OH, deoxyCer cannot be further metabolized into more complex SLs. Catabolically, deoxyCer is degraded by CDase to form 1-deoxysphingosine (deoxySO) (Fig. 1, Structure 7). However, deoxySO cannot be phosphorylated to form a catabolic intermediate similar to S1P. This prevents its breakdown by S1P-lyase, meaning that deoxySLs cannot be terminally degraded by the canonical catabolic pathway [239]. A detailed overview on the metabolism and biological aspects of deoxySLs is described in a very recent review [244]. Herein, we briefly describe the biological, biophysical and pathological implications of DSB.

8.1. Biological and biophysical properties of DSB

DSBs, the backbone of all deoxySLs, exist naturally in mammalian cells and in circulation although in residual amounts when comparing to other SLs [245]. However, elevated levels of DSB are directly associated with the development and progression of HSAN1 and metabolic disorders, such as, diabetes [36,246].

The pathological role of DSB might be related to their cytotoxic action [247] that causes multiple cellular effects, including loss of actin stress fibers mediated by the activation of an atypical PKC isoform (PKC ζ) [248,249]. However, such effects are cell type dependent and cells with neuronal origin are significantly more sensitive to DSB action. Particularly, deoxySA causes neurite fragmentation and retraction [36], which might be associated to deoxySA-induced changes in mitochondrial morphology and mitochondrial dysfunction [250], reduced ATP generation [251,252], ER stress and altered Ca^{2+} influx [251] and activation of the N-methyl-D-aspartate receptor [253]. In vivo studies also showed the neurotoxicity of deoxySA [254].

The cytotoxic effect of deoxySA is dose dependent and more significant than canonical SA [36]. It is likely that the structural differences between these LCB might in part contribute to their mechanism of action. From a physico-chemical perspective, the absence of the OH group at the C1-position makes this DSB more hydrophobic and, in addition, will affect the intra- and inter-molecular interactions established by DSB within the membrane, but also with the surrounding aqueous environment. In this regard, the higher hydrophobicity of DSB might increase their propensity to form micelles at lower concentrations, which might also lead to a faster and/or more extensive membrane permeabilization compared to canonical LCBs [255]. Such mechanism could partially explain the higher toxicity of DSB and therefore deserves further investigation. In cells, deoxySA is converted into deoxy(dh)Cer and deoxyCer [252] that are known to be toxic metabolites and to significantly alter the biophysical properties of model membranes [252,256].

Besides the lack of the C1-OH group, the relative position of the double bond also differs between the canonical SO and the deoxySO [213]. While the former displays a trans-unsaturation at Δ4-5, deoxySO has a cis double bond located at the Δ14-15 position (Fig. 5), which might interfere with the packing of the hydrophobic core of the membrane. Thus, while SO decreases the fluidity of the membranes and induce gel-fluid phase separation [93,105], deoxySO might have the opposite effect. To date, no studies have been performed to elucidate the physico-chemical properties of free DSB, nor their effects on membrane structure and organization. Indirect evidence was obtained from studies performed with 1-deoxySL, which showed that the lack of the C1-OH influences their ability to drive gel-fluid phase separation [257]. The emerging importance of these DSB in pathophysiology demands for additional studies that provide mechanistic details regarding their mode of action.

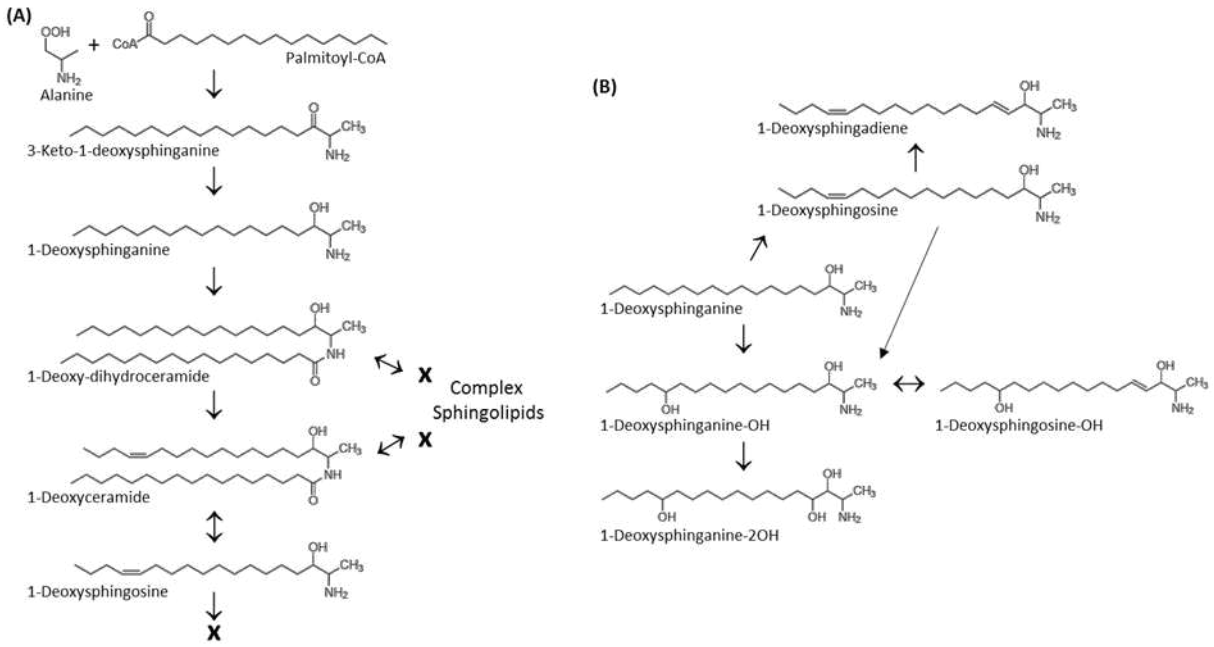


Figure 5 – Metabolism of DSB. **(A)** The reaction between palmitoyl-CoA and L-alanine is catalyzed by SPT forming deoxySA that is acylated to deoxy(dh)Cer, which is further metabolized to deoxyCer. The desaturation of deoxy(dh)Cer introduces a Δ14,15-cis double bond instead of the canonical Δ4,5-trans double bond. The enzyme responsible for this reaction is still not known. Due to the lack of the hydroxyl group in the position C1 they cannot form complex SLs nor be degraded through the canonical pathway of sphingolipid metabolism. **(B)** Metabolites formed downstream of deoxySO constitute three non-canonical metabolic branches. These metabolites are part of a relatively slow cellular detoxification/elimination pathway mediated by CYP4F family of enzymes.

8.2. Role of deoxysphingoid bases in disease

Even though these are relatively newly discovered lipid species, their implication in pathology is extensively known, and accumulating evidence places these lipid species as biomarkers and as important players in pathology development. This issue is thoroughly discussed in a very recent review [244] and therefore will not be subject of analysis herein. Moreover, the pathological effects of these lipids are at least in part caused by deoxySLs and not only by free DSB. Nonetheless, it is important to highlight that mutations in SPT that cause increased DSB formation [235,258] [240,241] were correlated with the severity of HSAN1 - an autosomal dominant inherited axonal neuropathy which affects mainly the peripheral sensory and to a variable extent also the motor and autonomic nerves - suggesting that plasma sphingoid base profile could be used as predictor of the clinical symptoms [235].

Several clinical studies also showed that DSB are elevated in metabolic diseases like the metabolic syndrome and diabetes [246,259]. However, this relation is not caused by SPT mutations as seen for HSAN1 but most likely by a dysregulated carbohydrate and fatty acid metabolism, two pathways that generate substrates for the *de novo* SL synthesis. It was also recently found that deoxySLs are reliable biomarkers for type 2 diabetes mellitus and are thought to be involved in its progression [260]. It should however be stressed that studies showing increased levels of DSB in patients refer to both free and *N*-acylated DSB forms, since lipidomic quantifications are performed after extraction and hydrolysis of the lipid extracts [246,260,261]. Additionally, once within cells, DSBs are immediately converted to deoxySLs and this metabolic conversion is responsible, at least in part, for DSB toxicity [262]. Therefore, it is not yet known if the observed pathological features in diabetes and metabolic syndrome are associated with elevated levels of DSB or of deoxySLs. SL metabolism provides an interconnection between fatty and amino acid metabolism and thereby indirectly of carbohydrate metabolism as well. Thus, a complex regulatory mechanism might exist which dictates SPT activity in response to flux of metabolites within these pathways. Further studies are needed to map these networks and as well to understand the role of free DSB in health and disease.

9. Synthetic sphingoid bases

Even though this review is mostly dedicated to natural mammalian sphingoid bases, a short reference should be made to two important synthetic LCB analogues, Enigmol and Safingol, due to their recognized therapeutic importance in human disease.

9.1. Enigmol

Enigmol (2S,3S,5S-2-amino-3,5-dihydroxyoctadecane) (Fig. 1, Structure 9) is a saturated sphingoid base analogue presenting a hydroxyl group at the carbon 5. The relative position of the OH-group prevents its phosphorylation by SphK [263–265]. Phosphorylated sphingoid bases (such as S1P) are generally associated with cell growth, proliferation and having specific roles in cancer development, Enigmol thus appears as a promising anti-cancer compound. Indeed, Enigmol revealed to be toxic for different human cancer cell lines, and to suppress tumor growth both in Min mouse models of colon and in prostate cancer cell xenografts in BALBc mice [265]. Enigmol cytotoxic effects are restricted to cancer cells: it decreased tumor size in mice models, without affecting body size or having adverse effects on major organs [265]. The mechanisms by which Enigmol exerts its activity are not yet clear, but enhanced caspase activity compared

to SO, and a reduced aberrant nuclear β-catenin staining were reported for HT29 cells [266]. Like SO, Enigmol could act through the regulation of protein kinases, that appears to be deregulated in different types of cancer [267].

Recently, fluorinated analogs of Enigmol were developed (CF2-Enigmol), which showed enhanced anti-tumor potency *in vivo* [268]. Using mouse xenograft models of prostate cancer, it was shown that CF2-Enigmol has an enhanced antitumor activity compared to Enigmol, without associated systemic toxicity, even when the compounds accumulated at higher levels in plasma and tissues [268].

9.2. Safingol

Safingol (Fig.1, Structure 8) is the L-threo enantiomer of SA affecting PKCs and phosphoinositide 3-kinase activity [269,270] but is primarily used as a SphK1 inhibitor [271]. Due to the anti-apoptotic and pro-growth activity of S1P, and its identification as an oncogene, SphK is a therapeutic target in many forms of cancer. In fact, Safingol already entered Phase I clinical trials for solid tumors, in combination with doxorubicin [272] and cisplatin [273], and was shown to significantly decrease S1P levels post treatment [273]. Safingol was shown to exert its effects on solid tumors by triggering autophagy, inhibiting PKCε, phosphoinositide 3-kinase, mTOR and MAPK, but without affecting the levels of other SLs, such as ceramide and dhCer [269].

In spite of its stereochemistry, Safingol was shown to be metabolized into dhCer, dhSM and gangliosides in primary cerebellar neurons and in Swiss 3T3 fibroblasts, although to a lower extent compared to the natural variant, D-erythro-SA [274]. Compared to D-erythro-SA, safingol does not undergo catabolism and most of it is found in the cells in the *N*-acylated form. However, in neuroblastoma B104 cells a significant amount of safingol is channeled towards the catabolic pathway [274]. These differences may point to a tumor specific metabolism of or SLs, likely associated with a different biological role and resistance mechanism. Further studies are thus needed to better understand the role of sphingoid bases on tumor cell development, progression and therapeutic resistance.

10. Concluding remarks

It is established beyond doubt that sphingoid bases and their phosphorylated derivatives are important bioactive lipids. These molecules have been implicated in numerous cellular processes, of which some are crucial in the determination of cell fate. Due to their bioactive role in the cell, they can also be associated with numerous human diseases, and either used as biomarkers for specific disorders or as potential therapeutic targets in various diseases. The

specific targeting of SL metabolism has been extensively explored to develop more efficient therapeutic approaches. Even though therapeutics that target SL metabolism are already being applied as primary lines of treatment (FTY720), there are several obstacles to overcome, that require a deeper understanding on the mechanisms underlying the biological action of these lipids. In particular, special attention must be paid while considering the opposing biological outcomes of some sphingoid bases to avoid adverse effects. Thus, mechanistic studies that provide further details on the biological and biochemical actions of LCBs will certainly contribute to the identification of new therapeutic targets, and to the development of innovative therapeutic strategies against several diseases. Likewise, characterization of the physico-chemical properties of these SLs' backbones and of their effects on the biophysical properties of biological membranes will help to clarify the structural mechanisms underlying the biological function of these bioactive compounds. Indeed, a growing body of evidence suggests that the functional roles of bioactive lipids are not only linked to direct interactions with cellular targets but are in part related to their effects on biological membranes. Therefore, an interdisciplinary research approach that integrates physical, chemical and biological studies of these lipids is required to advance our knowledge further, and to promote a faster route for clinical translation of some of the already identified SL-associated therapeutics. The application of innovative methodologies will also contribute significantly to move this field forward. For instance, the use of click chemistry/caged lipids [67,74,275–277] might be very useful in the study of local lipid metabolism and to unravel the role of signaling lipids that are only transiently produced in order to induce specific cellular responses. The use of coherent anti-Stokes Raman scattering (CARS) microscopy, can also be useful in addressing some aspects related to the subcellular localization of endogenous lipids and how it is changed in pathological conditions. It has the advantage of being a versatile technique with submicron three-dimensional spatial resolution and represents a good alternative to the use of more invasive methodologies that use fluorescently tagged lipid molecules [278,279]. Moreover, advances in mass spectrometry imaging might be a promising strategy to address membrane lipid composition and lipid trafficking in intact cells [280–282]. Progress in super resolution imaging techniques and development of suitable fluorescent probes to address membrane organization and properties will also be fundamental to gain further insight into the mechanisms that link the structural and biological properties of sphingoid bases.

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