Everybody needs sphingolipids, right! Mining for new drug targets in protozoan sphingolipid biosynthesis

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SUMMARY

Sphingolipids (SLs) are an integral part of all eukaryotic cellular membranes. In addition, they have indispensable functions as signalling molecules controlling a myriad of cellular events. Disruption of either the *de novo* synthesis or the degradation pathways has been shown to have detrimental effects. The earlier identification of selective inhibitors of fungal SL biosynthesis promised potent broad-spectrum anti-fungal agents, which later encouraged testing some of those agents against protozoan parasites. In this review we focus on the key enzymes of the SL *de novo* biosynthetic pathway in protozoan parasites of the Apicomplexa and Kinetoplastidae, outlining the divergence and interconnection between host and pathogen metabolism. The druggability of the SL biosynthesis is considered, alongside recent technology advances that will enable the dissection and analyses of this pathway in the parasitic protozoa. The future impact of these advances for the development of new therapeutics for both globally threatening and neglected infectious diseases is potentially profound.

Key words: sphingolipids, ceramide, drug targets, protozoan parasites, apicomplexa, kinetoplastidae.

INTRODUCTION

Protozoan parasites and the global burden of their diseases

Protozoa (kingdom Protista) are single-cell organisms that can be free-living or parasitic in nature (Baron, 1996). Out of more than 50 000 protozoan species that have been described to-date, relatively few have been identified as major contributors to the global burden of human diseases (Kuris, 2012) and animal agriculture (Dubey, 1977). The protozoa represent 19% of all human parasites (83 out of 437 species to-date) and are associated with 30% of parasite-induced human morbidity-mortality (Kuris, 2012).

Of the four groups of infectious protozoa (CDC, 2017), the Mastigophora (flagellates) and Sporozoa contain the Kinetoplastidae and Apicomplexa, respectively. It is to these two phyla that belong many of the causative agents of disease: Mastigophora – the insect vector-borne kinetoplastids *Trypanosoma brucei* (Human African Trypanosomiasis, HAT), *Leishmania* spp. (leishmaniasis, cutaneous and visceral) and *Trypanosoma cruzi* (American trypanosomiasis, Chagas' disease); Sporozoa – the apicomplexan *Toxoplasma gondii* (toxoplasmosis), *Cryptosporidium* spp. (cryptosporidiosis) and *Eimeria* spp. (coccidiosis in poultry and cattle), *Theileria* spp. (East Coast Fever in cattle) and *Plasmodium* spp., including *Plasmodium falciparum* the causative agent of severe malaria and one of the 'Big Three' global infectious diseases alongside HIV and tuberculosis (Torgerson & Macpherson, 2011).

Historically, the diseases caused by some of these parasites have been classified as Neglected Tropical Diseases (NTDs) or Neglected Zoonotic Diseases (King, 2011) and were associated with the classical model of the 'poverty trap' covering tropical and sub-tropical regions in Africa, Latin America and the Indian subcontinent (Kuris, 2012). However, with global changes in climate and human demographics and associated practices, the classical models do not promise safe boundaries that might contain and/or stop the further global spread of many of these parasitic diseases (Colwell *et al*. 2011). The problems associated with these pathogens are further aggravated by the lack of effective vaccines (Dumonteil, 2007; Innes *et al*. 2011; McAllister, 2014; Black & Mansfield, 2016) and the paucity of reliable drugs (Zofou *et al*. 2014), in addition to the difficulties of vector or reservoir control (Colwell *et al*. 2011). Therefore, there is a recognized need to find new therapeutic targets in these causative agents in order to develop effective treatment regimens to avoid potentially catastrophic outbreaks, both in terms of human health and economic impact.

This review presents sphingolipid (SL) biosynthesis and ceramide (CER) homoeostasis as a potential gold mine of tractable drug targets for these protozoan parasites.

State-of-the-art treatment of apicomplexan and kinetoplastid diseases

In general, available treatments for the diseases caused by the Kinetoplastidae and Apicomplexa

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are outdated (if not historic), with relatively few examples that were introduced recently, toxic and require a long treatment regimen, and therefore close monitoring of patients.

The kinetoplastid pathogens in focus here all cause NTDs and as such there are significant problems with the available drug regimens:

Leishmania *spp*. The treatment of leishmaniasis often requires a long course of intravenous pentavalent antimony drugs (e.g. Glucantime and Pentostam), aminosidine (paromomycin) or liposomal amphotericin B (Croft & Coombs, 2003; Center for Food Security and Public Health, 2004; WHO, 2004; Kedzierski *et al*. 2009). The most recent addition was the orally available miltefosine (Sunder *et al*. 2002; Verma & Dey, 2004), originally developed as anti-neoplastic agent. Despite its teratogenic effects (Sunder *et al*. 2002), due to the lack of other effective medications, it has been registered and is now used in India, Colombia, Guatemala and Germany (Soto & Berman, 2006). Other regimens of treatment include Pentamidine (Bray *et al*. 2003), allopurinol, dapsone, fluconazole, itraconazole and ketoconazole. However, to-date all available chemotherapeutic agents suffer from being toxic (Chappuis *et al*. 2007) or inaccessible, both geographically and financially, in endemic areas where public health is under-resourced, poor and underdeveloped. Additionally, the lack of effective vaccines (de Oliveira *et al*. 2009) and the alarming emergence of resistance to these drugs (Croft *et al*. 2006), combined with the short-lived prevention resulting from applying measures such as vector and reservoir host control (WHO, 2004; Figueiredo *et al*. 2012), demand an intensive search for alternative anti-leishmanials to enable effective treatment and control.

Trypanosoma brucei. Another compelling example of the shortcomings of available treatments is HAT (Mina *et al*. 2009; Buckner *et al*. 2012), where there is a lack of effective vaccines (Black & Mansfield, 2016) and treatment depends on the stage of the disease. Whilst in the first stage, the drugs used are less toxic, easier to administer and more effective, treatment in the second stage requires drugs that can cross the blood-brain barrier, specifically the arsenates (Gibaud & Jaouen, 2010), making them considerably more toxic and complex to administer (Babokhov *et al*. 2013). Currently, four drugs are registered for HAT treatment and are provided free of charge to endemic countries through a WHO private partnership with Sanofi-Aventis (Pentamidine, melarsoprol and eflornithine) and Bayer AG (suramin) (Schmidt *et al*. 2012). Unfortunately, all of them exhibit a broad range of adverse effects. Moreover, treatment regimens are usually highly restrictive, particularly in the second stage of the disease, requiring hospital-based I.V. treatment with continuous monitoring.

Trypanosoma cruzi. Despite their toxic side-effects, nifurtimox and benznidazole are the only licenced drugs available for treatment of Chagas' disease (Carabarin-Lima *et al*. 2013; Bermudez *et al*. 2016), with the latter being the first choice due to its lower side effects. Also, benznidazole has been implemented in the treatment of women before pregnancy in order to prevent/reduce vertical transmission (Carabarin-Lima *et al*. 2013; Murcia *et al*. 2013). Due to the lack alternatives, efforts have been directed towards implementing different treatment regimens in order to reduce toxicity, e.g. intermittent administration schedules, combination therapy and re-purposing of commercial drugs (Bermudez *et al*. 2016).

Management of apicomplexan infections is also challenging and faces many of the same shortcomings encountered in the treatment of kinetoplastid infections.

Toxoplasma gondii. Treatment regimens for toxoplasmosis patients have essentially remained the same since the 1950s (Eyles & Coleman, 1953). They largely depend on the repurposing of antibacterials (sulfadiazine, spiramycin and clindamycin) and antimalarials (pyrimethamine and atovaquone) (Opremcak *et al*. 1992; Andrews *et al*. 2014; Antczak *et al*. 2016) in combination, therapies that target parasite folic acid synthesis, protein synthesis or oxidative phosphorylation (Greif *et al*. 2001; Antczak *et al*. 2016). Most of these chemotherapeutics are not readily bioavailable at the site of infection (e.g. unable to cross the blood-brain barrier); cannot be administered by patients with hypersensitivity to sulphonamides; have suspected teratogenic properties (Montoya & Remington, 2008; Paquet & Yudin, 2013); are threatened by the emergence of resistance (Sims, 2009); or require adjuvant therapies (folinic acid supplement) to minimize toxic side effects (for a detailed review see Antczak *et al*. 2016).

Toxoplasmosis is a representative of the urgent need for new antiprotozoal targets. In addition to the fact that *T. gondii* is estimated to infect 2–3 billion people worldwide (Welti *et al*. 2007), its treatment is complicated due to two main factors: (a) the parasite undergoes a complex life cycle with two predominant forms in the human host, namely, tachyzoites (proliferative form) and bradyzoites (encysted form, chronic toxoplasmosis); (b) bradyzoite burden is widespread but usually asymptomatic, although it has been associated with psychiatric disorders (Webster *et al*. 2013). However, in immunocompromised individuals encysted *T. gondii* transform into proliferative tachyzoite forms causing symptomatic disease, toxoplasmic encephalitis. As such

T. gondii is an opportunistic parasite. Notably, all the above-mentioned drugs act only against the tachyzoite stage with no notable effect against encysted bradyzoites (Antczak *et al*. 2016). Recent data from our laboratory (Alqaisi *et al*. 2017) and others (Sonda *et al*. 2005) have shown that the Aureobasidin A and analogous depsipeptides, known to target yeast SL biosynthesis (Wuts *et al*. 2015), exhibit activity against bradyzoite *T. gondii*. This class of compounds may offer a potential treatment for chronic toxoplasmosis and, perhaps, some psychiatric disorders; although the mechanism of action is not via inhibition of parasite SL biosynthesis and is yet to be elucidated (Alqaisi *et al*. 2017).

Plasmodium falciparum. Falciparum malaria remains one of the 'Big Three', most prevalent and deadly infectious diseases across tropical and subtropical regions, with an estimated 154–289 million cases in 2010 (212 million cases in 2015), and 660 000 (429 000 in 2015) associated deaths; although the actual numbers might be even higher (Biamonte *et al*. 2013; WHO, 2016).

Similar to *T. gondii*, *Plasmodium* parasite undergoes a complex life cycle with different stages in different organs of the host, rendering treatment challenging: sporozoites and schizonts in the liver, and merozoites, trophozoites and gametocytes in the blood (Dechy-Cabaret & Benoit-Vical, 2012). Artemisinin-based combination therapies (ACTs) are the standard for treating malaria cases with typical partner drugs including lumefantrine and piperaquine, e.g. Coartem™ (Novartis) and Eurartesim™ (Sigma-Tau) (Biamonte *et al*. 2013). Other regimens include the use of parenteral artesunate (severe malaria) (Dondorp *et al*. 2010*a*), primaquine (liver and transmission, gametocyte, stages) (Dondorp, 2013), mefloquine and sulfadoxine/pyrimethamine in combination (effective as single dose antimalarial drug) (Biamonte *et al*. 2013) and atovaquone/proguanil, Malarone™ (GlaxoSmith Kline), as a prophylactic treatment.

However, although combination therapies have now been adopted, resistance against many existing antimalarials has been observed since the 1950s (Bishop, 1951; Hallinan, 1953; Sandosham *et al*. 1964) and remains a severe threat (Rieckmann & Cheng, 2002; Chinappi *et al*. 2010; Dondorp *et al*. 2010*b*; Newton *et al*. 2016; Parija, 2016; Menard & Dondorp, 2017; Zhou *et al*. 2017). This bleak view of the future of available anti-malarial chemotherapeutics makes it imperative to invest more efforts in identifying new potent chemotypes that will offer both efficacy and safety.

Cryptosporidium *spp*. Like *T. gondii*, *Cryptosporidium parvum* and *Cryptosporidium hominis* usually cause a self-limiting disease in healthy individuals but represent a manifest problem in immunocompromised patients, particularly those with AIDS, where infection leads to acute and protracted life-threatening gastroenteritis (Chen *et al*. 2002). More recent data have led to a radical reassessment of the impact of cryptosporidiosis, with the number of *Cryptosporidium*-attributable diarrhoea episodes estimated at >7·5 million in children aged <24 months in sub-Saharan Africa and South Asia where infection is estimated to contribute to >250 000 infant deaths per year (Sow *et al*. 2016). Current treatment of cryptosporidiosis relies on a single FDA-approved drug, nitazoxanide, which has limited efficacy in those most at risk. More recently, the repurposing of antimalarials, e.g. quinolones and allopurinols, has been proposed (Gamo *et al*. 2010; Chellan *et al*. 2017). The distinctive metabolic features of this parasite from other apicomplexan organisms, e.g. no plastid-derived apicoplast and the absence of the citrate cycle and cytochrome-based respiratory chain (Ryan & Hijjawi, 2015), confer several limitations for the identification of targets necessary for the development of anticryptosporidial drugs. However, the core metabolic pathways, e.g. energy metabolism and lipid synthesis are still present and exhibit high level of divergence from the mammalian host, thus presenting an opportunity to identify new drug targets that promise effective and selective treatment (Chellan *et al*. 2017).

The biological significance of SLs

SLs are a class of lipids that are ubiquitous in eukaryotic cell membranes, particularly the plasma membrane, as well as in some prokaryotic organisms and viruses (Merrill & Sandhoff, 2002). Since their earliest characterization by Thudichum (1884), they have been a subject of controversy. Initially, they had been considered of structural importance only; however, over the last couple of decades, several reports have revealed their indispensability to a plethora of functions including, but not limited to, the formation of structural domains, polarized cellular trafficking, signal transduction, cell growth, differentiation and apoptosis (Huwiler *et al*. 2000; Ohanian & Ohanian, 2001; Cuvillier, 2002; Pettus *et al*. 2002; Buccoliero & Futerman, 2003).

SLs consist structurally of a sphingoid base backbone, e.g. sphingosine (SPH) that can be *N*-acylated to form CER. To the latter, a variety of head groups: charged, neutral, phosphorylated and/or glycosylated can be attached to form complex SLs, e.g. sphingomyelin (SM), as the primary complex mammalian SL; and inositol phosphorylceramide (IPC) in fungi, plants and numerous protozoa (Fig. 1). These molecules have both polar and non-polar regions giving rise to their amphipathic character, which accounts for their tendency to aggregate into membranous structures, yet retaining the interfacial ability to interact with various partners, e.g.

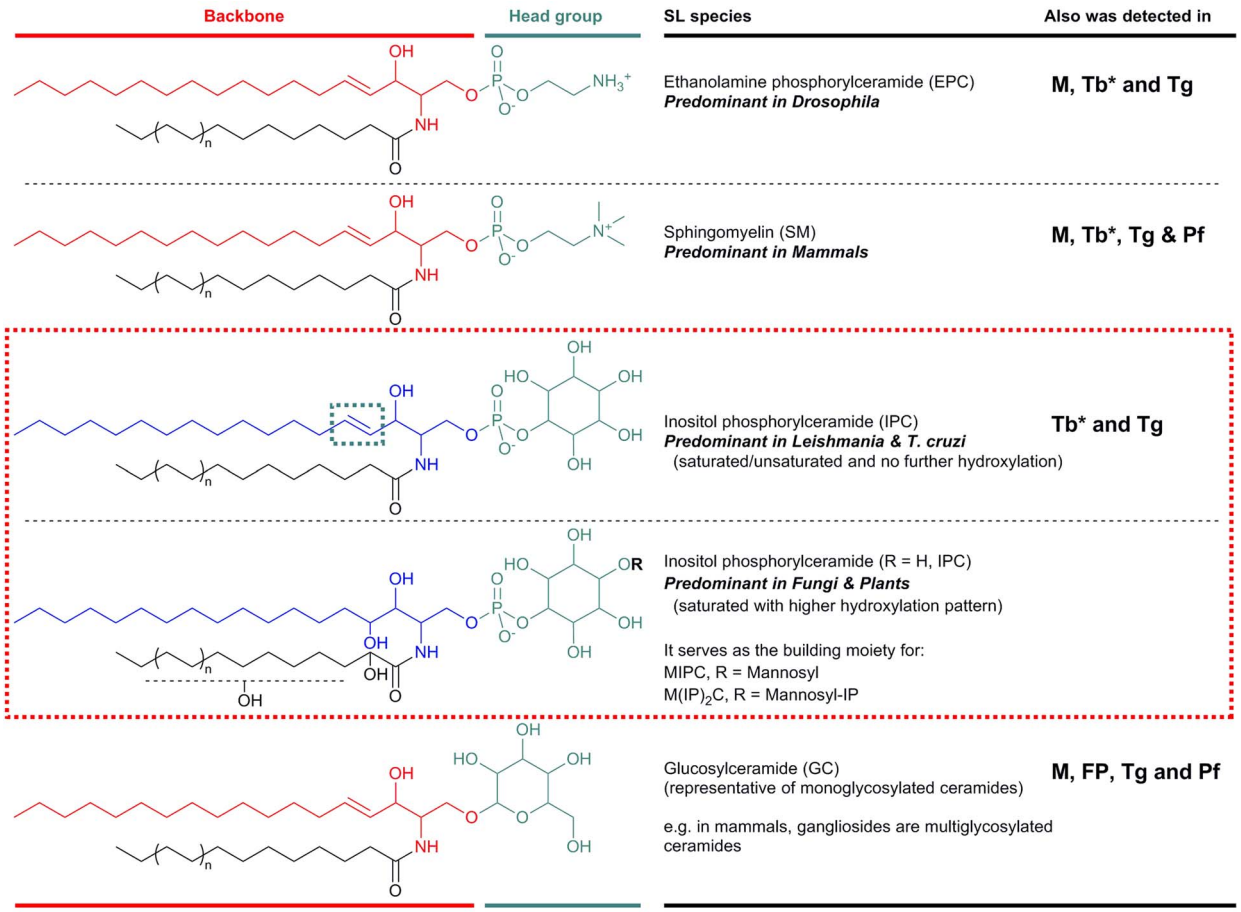

Fig. 1. Illustration of the predominant species of complex sphingolipid in organisms from different evolutionary clades: EPC in Drosophila; SM in mammals; and IPC in Leishmania and *T. cruzi* (as representatives of protozoan parasites) and in fungi and plants. IPC is absent from Mammalian cells but essential for many pathogenic organisms (red box). Glycosylated sphingolipids are also ubiquitous across different species. Backbone chain length is commonly C18 derived from palmitoyl-CoA. Mammals M, Fungi and Plants FP, *Leishmania* spp. L, *Trypanosoma cruzi* Tc, *Trypanosoma brucei* Tb, *Toxoplasma gondii* Tg and *Plasmodium falciparum* Pf. *Denotes developmental regulation. EPC, ethanolamine phosphorylceramide; IPC, inositol phosphorylceramide; SM, sphingomyelin.

involvement of glycosphingolipids (GSLs) in cellular recognition complexes, cell adhesion and the regulation of cell growth (Gurr *et al*. 2002). Furthermore, the diversity of their chemical structures allows for distinctive roles within cellular metabolism, e.g. the signalling functions of SPH and CER *vs* sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) (Merrill & Sandhoff, 2002; Metzler, 2003).

SLs as indispensable structural components

The unique structural features of SLs (the free 3-hydroxy group, the amide functionality and the C4–C5 *trans* double bond) affect their biophysical properties rendering these molecules different from their glycerolipid counterparts, i.e. SM *vs* phosphatidylcholine (PC) (Boggs, 1980, 1987; Talbott *et al*., 2000; Ramstedt & Slotte, 2002). Such interfacial differences give complex SLs, such as SM, the unique ability to form both *intra*- and *inter*molecular hydrogen bonds (Bruzik, 1988) that are fine-tuned by the *trans* double bond (Ramstedt & Slotte, 2002). This ability is reflected in the tendency of SLs to cluster rather than behave like typical 'fluid' membrane lipids. Naturally occurring SLs undergo the L_β (gel phase) to L_α (lamellar phase) transition near the physiological temperature of 37 °C, in contrast, this transition for naturally occurring glycerolipids is near or below 0 °C. Additionally, the long saturated alkyl chains of SLs allow them to pack tightly with sterols, stabilized by hydrogen bonding (Ramstedt & Slotte, 2002), to form laterally compact hydrophobic micro-domains commonly known as 'lipid rafts' (Futerman & Hannun, 2004). Similar results have been reported with the fungal/plant counterpart of SM, IPC, where it was shown that IPC was able to form sterol containing ordered domains in model systems (Björkbom *et al*. 2010). These membrane micro-domains can readily segregate from the more disordered and expanded domains of unsaturated

acyl chains of glycerolipids (Merrill & Sandhoff, 2002). They have been proposed to function in a diverse array of processes from polarised trafficking of lipid modified proteins (Brown & London, 1998) and the stabilization of other types of biological structures such as lamellar bodies, to the assembly and activation of signal transduction complexes (Brown & London, 2000; Magee *et al*. 2002; Pierce, 2002; Vance & Vance, 2002; Hannun & Obeid, 2008). They have also been involved in the formation of detergent-insoluble gel-phase domains (Ramstedt & Slotte, 2002) via the extensive hydrogen-bonding network in the head groups of GSLs that have been implicated during the formation of 'caveolae' and surface recognition (Merrill & Sandhoff, 2002).

SLs as indispensable signalling agents

SLs can also function as bioactive signalling molecules due to their biophysical properties, e.g. the low pK_a (7–8) of SPH allows it to remain partially uncharged at physiological pH retaining the ability to move across membranes (Merrill & Sandhoff, 2002). Likewise, CER, a neutral species, is able to freely flip flop across membranes (Hannun & Obeid, 2008). Many studies have produced evidence of such signalling functions, e.g. SPH exerts pleiotropic effects on protein kinases; CER mediates many cell-stress responses, including the regulation of apoptosis (Georgopapadakou, 2000); and S1P has crucial roles in cell survival, cell migration and inflammation (Hannun & Obeid, 2008)

SL metabolism and the rationale for druggability

The indispensability of SLs for a myriad of cellular processes and functions, ranging from structural integrity to signalling events, makes it is unsurprising that the SL biosynthesis is highly conserved in all eukaryotes where it is, alongside its proposed regulators (Holthuis *et al*. 2006), an essential pathway (Heung *et al*. 2006; Sutterwala *et al*. 2007). This has lead the pathway to be considered vital for protozoan pathogenesis and, therefore, a drug target; e.g. SM synthase activity in *Plasmodium* (Heung *et al*. 2006). In order to characterise the druggability of protozoan SL biosynthesis, the mammalian pathway, as the most studied system, will be used as the reference model in the following discussions.

SL metabolism constitutes a highly complex network involving critical intersections with various other pathways, particularly glycerolipid biosynthesis (Holthuis & Menon, 2014). CER represents the corner stone for both biosynthesis and catabolism, modulating cell fate (Hannun & Obeid, 2008). Dysregulation of either SL biosynthesis or catabolism could result in cell death, e.g. of protozoan parasites (Yatsu, 1971; Brady, 1978; Chen *et al*. 1999; Merrill & Sandhoff, 2002), however here our focus will be on the former pathway.

Considering the central position of CER, the druggability of SL metabolism revolves around dysregulation of 'Ceramide Homeostasis' (Young *et al*. 2012) which in turn leads to ripple effects perturbing the balance between the pro-apoptotic CER and the mitogenic diacylglycerol (DAG), consequently determining cell fate (Fig. 2) – a mechanism that has been associated with resistance to anti-cancer treatments (Ségui *et al*. 2006) and has been reported in protozoan parasites, e.g. Plasmodium (Pankova-Kholmyansky *et al*. 2003; Labaied *et al*. 2004). The characterisation of several key enzymes involved in SL *de novo* biosynthesis has revealed divergence between mammalian and protozoan species. Thus, attention has been given to the exploitation of the SL biosynthetic pathway (parasite and/or host) for new drug targets or regimens (Sugimoto *et al*. 2004; Zhang *et al*. 2005; Denny *et al*. 2006; Tanaka *et al*. 2007; Pruett *et al*. 2008; Mina *et al*. 2009; Tatematsu *et al*. 2011; Young *et al*. 2012).

SL METABOLISM

The key steps in de novo biosynthesis

SL *de novo* biosynthesis can be simplified into three key steps: a gate-keeper and two cell fate modulator steps. The former comprises the up-stream rate-limiting step of the condensation of acyl-CoA and L-serine, in the endoplasmic reticulum (ER) via serine palmitoyltransferase (SPT), to produce dihydrosphingosine. The latter comprises first the formation of CER in the ER by the action of ceramide synthase (CerS), and then the formation of complex SLs in the Golgi. These products vary depending on the species, and are formed under the catalysis of what could be generically termed SL synthases: SM synthase in mammals and IPC synthase in fungi, plants and protozoa. It is worth mentioning that another Golgi localized metabolic pathway results in the formation of glycosylated CER species, and also contributes to the regulation CER levels (Holthuis & Menon, 2014) (Fig. 2).

Protozoan parasites vs *host: differences & opportunities*

The cross-species differences encountered in the first, SPT-catalysed, step are mostly minor in terms of the chemical structure of the product; mainly due to the chain length of the acyl-CoA utilised in the reaction, e.g. myristoyl-CoA (in *Leishmania* spp. amongst other odd sphingoid base lengths (Hsu *et al*. 2007)) and palmitoyl-CoA, with the latter more predominant across the Eukaryota

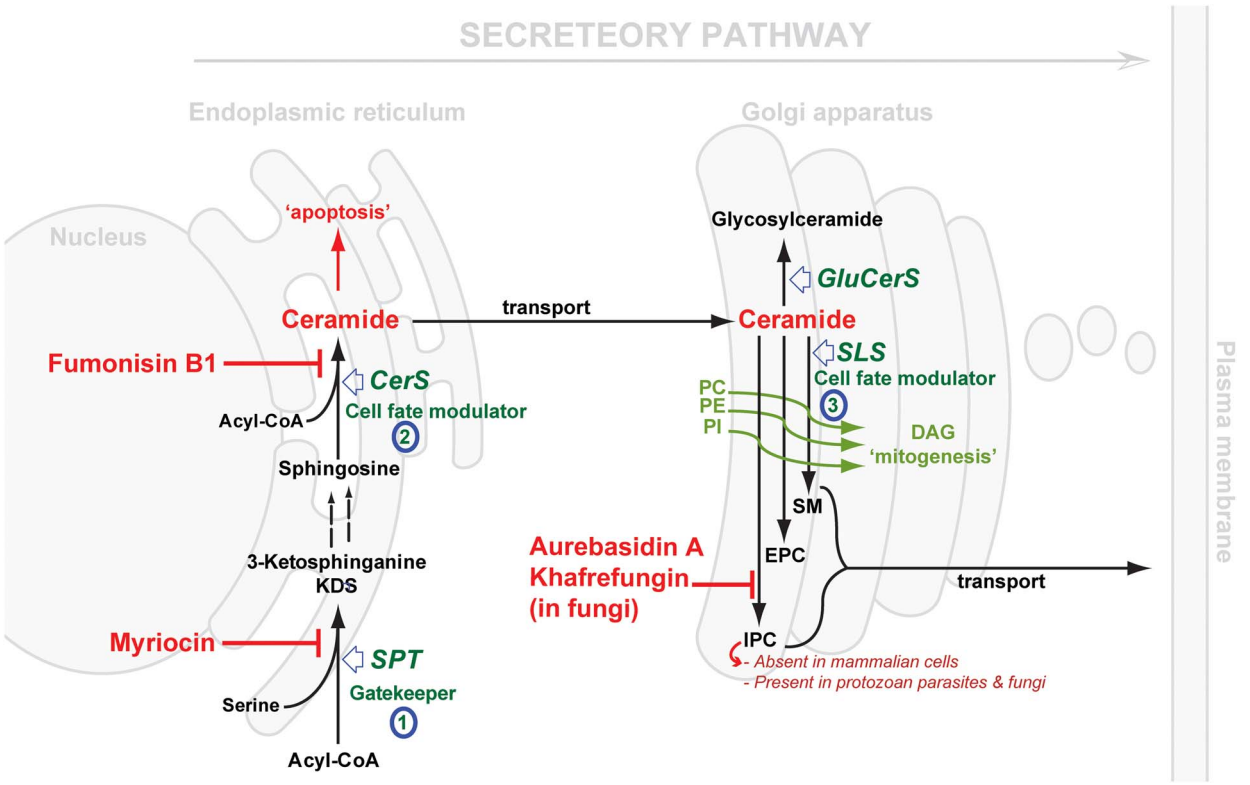

Fig. 2. Schematic representation of *de novo* sphingolipid metabolism. Three key steps are highlighted: (1) SPT, evolutionary divergent in *T. gondii*; (2) CerS, fewer isoforms in protozoan parasite (c.f. 6 isoforms in mammals); SLS, while predominantly synthesising SM in mammals and to a lesser extent EPC, orthologues in protozoan parasites (*Leishmania* spp., *T. brucei*, *T. cruzi* and *T. gondii*) can synthesise IPC, an activity that is absent from mammalian cells and the target of the highly specific fungal inhibitors shown. The scheme also illustrates the differential cellular effects of ceramide *vs* DAG (diacylglycerol). Accumulation of ceramide elicits an apoptotic response while increasing concentrations of DAG promotes cell growth. CerS, ceramide synthase; GluCerS, glucosylceramide synthase; SLS, sphingolipid synthase; SPT, serine palmitoyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; EPC, ethanolamine phosphorylceramide and IPC, inositol phosphorylceramide.

(in mammals, *Plasmodium* and *T. brucei*) (Richmond *et al*. 2010; Botté *et al*. 2013). Further differences may be apparent with respect to the catalysing enzyme, SPT (*vide infra*). However, clear divergence is observed in the second and the third steps, both of which represent a cell-fate modulator process. CerSs exhibit differential preferences for the chain length of the acyl-CoA substrate (Park *et al*. 2014) and its hydroxylation pattern (Layre & Moody, 2013), with 6 isoforms present in humans suggesting a different role for each CER species produced (Levy & Futerman, 2010; Figueiredo *et al*. 2012). To-date, one or, maximum, two genes encoding CerS function have been identified in protozoan parasite species (Koeller & Heise, 2011). However, most interesting is the variation in the complex SL formed in the Golgi, reflecting significant differences in the active site of the SL synthases catalysing the transfer reaction. The divergence of the protozoal complex SL synthases, and the synthetic products, with respect to the mammalian host, may provide opportunities to design selective inhibitors. Previously, this step has been validated as a promising drug target in fungi using aureobasidin A (AbA) (Fig. 2) (Denny *et al*. 2006).

Serine palmitoyl transferase (SPT)

SPTs are members of the pyridoxal 5′-phosphate (PLP)-dependent (Sandmeier *et al*. 1994) α-oxoamine synthase family and share a conserved motif (T[FL][GTS]**K**[SAG][FLV]G) around the PLP-binding lysine (in bold) (Young *et al*. 2012). SPT catalyses the first rate-limiting step in the *de novo* biosynthesis of SLs (Weiss & Stoffel, 1997; Hojjati *et al*. 2005) (Fig. 2), a reaction involving the decarboxylative Claisen-like condensation of serine and an acyl-CoA (Lowther *et al*. 2012), to yield the sphingoid base backbone, 3-ketodihydrosphingosine (3-KDS) (Hanada, 2003; Raman *et al*. 2009; Lowther *et al*. 2012). Therefore, SPT represents the 'Gatekeeper' of the SL biosynthetic pathway.

All eukaryotic SPTs studied to date are ER-resident and membrane bound with a heterodimeric protein core consisting of two subunits sharing ~20% identity: LCB1 and LCB2, ~53 and ~63

kDa respectively (Hanada, 2003; Denny *et al*. 2004; Han *et al*. 2004; Chen *et al*. 2006). The latter contains the canonical PLP cofactor binding site while the former has been suggested to be important for complex stability (Lowther *et al*. 2012). In contrast, the orthologous SPT from sphingomonad bacteria is a soluble 45 kDa homodimer (Ikushiro *et al*. 2001). SPT activity in apicomplexan parasites has been detected and was proposed as a potential drug target (Gerold & Schwarz, 2001; Bisanz *et al*. 2006; Coppens, 2013), however the enzyme(s) responsible have yet to be further characterized (Mina *et al*. 2017). In contrast, kinetoplastid parasites have been shown to possess a heterodimeric SPT similar to the mammalian orthologue (Denny *et al*. 2004). Inhibiting SPT activity (e.g. using myriocin, Fig. 2) results in various effects in different species. Mammalian cells exhibited a loss of viability, with a partial loss of SPT function resulting in a rare SL metabolic disease, Hereditary Sensory Neuropathy type I (HSN1) (Hanada, 2003). In contrast, *Saccharomyces cerevisiae* were found to be relatively tolerant (Nagiec *et al*. 1994), and *Leishmania major* lacking LCB2 were viable but unable to differentiate into infective metacyclic forms (Zhang *et al*. 2003). However, *T. brucei* procyclic forms in which SPT expression was reduced were non-viable (Fridberg *et al*. 2008).

The SPT catalysed reaction product, 3KDS, is subsequently reduced by 3-ketosphinganine reductase to form sphinganine (dihydrosphingosine). Subsequent minor metabolic differences are encountered across different species; mainly concerning the order of the hydroxylation (in fungi and higher plants) and acylation to produce CERs (Sugimoto *et al*. 2004).

Ceramide synthase

In all eukaryotic systems studied to date, CerSs are ER-resident integral membrane proteins catalysing the *N*-acetylation of dihydrosphingosine to produce dihydroceramide, which is then oxidized to form CER, the simplest SL species and a key bioactive molecule in numerous cellular pathways (Lahari & Futerman, 2007).

Mammalian CerSs are orthologues of longevity-assurance genes, LAG1p and LAC1p identified in yeast (Guillas *et al*. 2001). The eukaryotes studied to date have been found to encode at least two CerSs, with humans expressing six – each generating CER with a defined acyl chain length (C18 to C26) (Pewzner-Jung *et al*. 2006; Levy & Futerman, 2010). Whilst little is known regarding structure-function relationships or regulation of CerS,, the ubiquitous Lag1 motif has been shown to be important for functionality (Spassieva *et al*. 2006), likely forming part of the active site.

Experimental evidence (from our laboratory and others) has previously indicated the presence of CerS activity in *Leishmania* spp (Zhang *et al*., 2003; Denny *et al*., 2004, 2006) and in *T. cruzi* (De Lederkremer *et al*. 2011). More recently LAG1 orthologues have been identified and functionally and molecularly characterized in the latter parasite (Figueiredo *et al*. 2012). Other results indirectly suggest the presence of such activity in *T. brucei* (Patnaik *et al*. 1993; Richmond *et al*. 2010; Smith & Bütikofer, 2010). Similarly, CerS activity in the Apicomplexa has been inferred (Welti *et al*. 2007; Zhang *et al*. 2010; Pratt *et al*. 2013), but remains unexplored.

Once formed in the ER, CER is transported, by CER transfer protein CERT in mammals (Kumagai *et al*. 2005; Kudo *et al*. 2010; Rao *et al*. 2014), to the Golgi apparatus where the synthesis of complex SLs occurs (Ohanian & Ohanian, 2001; Bromley *et al*. 2003; Bartke & Hannun, 2009; Pata *et al*. 2010). ER CER concentration is kept under tight control as accumulation of CER here has been shown to result in induction of the mitochondrial apoptotic pathway (Vacaru *et al*. 2009; Tafesse *et al*. 2014) via an unknown mechanism (Bockelmann *et al*. 2015).

Sphingolipid synthase

In the Golgi, CER can be phosphorylated by CER kinase (Rovina *et al*. 2009), glycosylated by glucosyl or galactosyl CerS (Raas-Rothschild *et al*. 2004), or acquire a variety of neutral or charged head groups under the catalysis of what could be called generically SLSs, to form various complex phosphosphingolipids. Phylogenetic analyses have identified at least 4 clades of SLS (Huitema *et al*. 2004; Denny *et al*. 2006).

In mammals CER is a substrate for the SLS, SM synthase, to produce SM (Huitema *et al*. 2004). Whilst in fungi and higher plants phytoceramide is utilized by a different SLS, IPC synthase, to produce IPC as the principal phosphosphingolipid (Nagiec *et al*. 1997; Wang *et al*. 2008). This landscape is significantly divergent when it comes to protozoa.

In the kinetoplastid *Leishmania* spp. and *T. cruzi*, CER acquires a phosphorylinositol head group from phosphatidylinositol (PI) to produce IPC via IPC synthase (Zhang *et al*. 2005; Denny *et al*. 2006; Mina *et al*. 2010), although there are some reports of SM in *T. cruzi* (Quiñones *et al*. 2004) (Fig. 2). Whilst *Leishmania* encodes a single copy IPC synthase, *T. cruzi* has two highly related copies (Denny *et al*. 2006). Further divergence, and possible redundancy, is encountered in *T. Brucei*, which harbours 4 genes that encode SLSs (Denny *et al*. 2006; Sutterwala *et al*. 2008). This enzyme portfolio results in a diverse profile of the complex SL species (SM, IPC and ethanolamine phosophorylceramide [EPC]) which are developmentally

regulated during the life cycle of the parasite (Sutterwala *et al*. 2008).

In apicomplexan parasites, previous reports have indicated the presence of glycosyl-ceramide and SM in *P. falciparum* and *T. gondii*, as summarized in Zhang *et al* (2010). However, other findings reported the presence of EPC in *T. gondii* (Welti *et al*. 2007) and, more recently, IPC (Pratt *et al*. 2013). The latter study also characterized *T. gondii* SLS as demonstrating IPC synthase activity *in vitro* (Pratt *et al*. 2013).

The divergence of SLS function, with respect to the host, seen in both kinetoplastid and apicomplexan protozoan parasites in intriguing and, perhaps, indicated them as a tractable drug target. In support of this hypothesis, ceramide-analogues with anti-*Plasmodium* activity have already been identified (Labaied *et al*. 2004).

In general, SLSs are Golgi-resident transmembrane proteins, presumed to have 6 transmembrane domains with the active site facing the Golgi lumen (Holthuis *et al*. 2006; Sutterwala *et al*. 2008). Those orthologues identified in kinetoplastids demonstrated two conserved regions (CGDX_3SG**H**T & **H**YTX**D**VX_3YX_6FX_2YH) with respect to the animal SM synthases (Huitema *et al*. 2004; Denny *et al*. 2006). These regions contain the so-called the catalytic triad (two Histidines and one Aspartate residues) that mediates a nucleophilic attack on lipid phosphate ester during the transferase/hydrolase activity (Mina *et al*. 2010). Apicomplexan orthologues form a separate evolutionary clade, yet retain the catalytic triad (Denny *et al*. 2006; Pratt *et al*. 2013), as does the fungal orthologue AUR1p (Heidler & Radding, 2000). Further evidence for the essentiality of these residues was provided when mutation of the active histidine of the triad was shown to deactivate fungal IPC synthase and mammalian SM synthase-related activity (Levine *et al*. 2000; Vacaru *et al*. 2009). Furthermore, recently it has been shown that substrate selectivity, and so the diversity of SLS activity, may depend on key residues close to the transferase active residues or on a luminal loop of the protein (Sevova *et al*. 2010; Kol *et al*. 2017).

In the Eukaryota SLS's occupy a central position at the intersection of glycerolipids (PI/PC/PE and DAG) and SLs ([phyto]ceramide and IPC/SM/EPC). Accordingly, these enzymes act as regulators of a delicate balance between pro-apoptotic CER and pro-mitogenic DAG (Holthuis *et al*. 2006).

The most significant previous example of SL biosynthesis inhibition as a drug target was reported in fungi. Aureobasidin A (AbA), a depsipeptide, was first reported by Ikai *et al*. (1991) and soon after its antifungal properties were highlighted (Takesako *et al*. 1993). The target gene was further characterized (Hashidaokado *et al*. 1995) revealing its identity to be the IPC synthase (AUR1p). AbA is a specific and potent (low nanomolar) inhibitor of the fungal IPC synthase. This ushered in a new era in the search for anti-fungal chemotherapeutics, positioning IPC synthase as a promising, broad spectrum, anti-fungal drug target (Sugimoto *et al*. 2004). Other specific inhibitors were later added to the arsenal of fungal IPC synthase inhibitors: khafrefungin (Mandala *et al*. 1997), rustmicin (Harris *et al*. 1998; Mandala *et al*. 1998) and others (Ohnuki *et al*. 2009). Unfortunately, further development of these inhibitors stalled, either due to physical properties, e.g. aureobasidin A is very sparingly soluble in water (Georgopapadakou, 2000; Sugimoto *et al*. 2004), or because their highly complex chemical structures rendered chemical synthesis challenging, with the few synthetic efforts reported resulting in compounds with either reduced or no activity (Sugimoto *et al*. 2004; Aeed *et al*. 2009). However, recent works have highlighted that semi-synthetic strategies may overcome these barriers (Wuts *et al*. 2015).

Perhaps reflecting the evolutionary divergence of these enzymes, the protozoan IPC synthase orthologues, from *Leishmania major* and *T. gondii* are not susceptible to AbA inhibition (Denny *et al*. 2006; Pratt *et al*. 2013). Some studies have reported the inhibitory effects of AbA and analogues against *T. gondii* in culture (Sonda *et al*. 2005; Alqaisi *et al*. 2017), however this is not associated with inhibition of SL biosynthesis. Despite this, the protozoan SLS's remain tractable drug targets with no functional equivalent in mammalian cells. Surprisingly, at least one SLS isoform from *T. brucei* was acutely sensitive to AbA treatment (Mina *et al*. 2009), although these findings stirred some controversy due, in part, to the redundancy of *T. brucei* SLSs (4 isoforms) compared with the single copy found, for example, in *L. major* and *T. gondii* (Sutterwala *et al*. 2008).

THE ENIGMATIC NATURE OF SL DRUGGABILITY

Difficulties in pinpointing SL functionality

Investigation and deciphering of the functions of each specific SL species remains challenging. This is due to the complexity in SL metabolic interconnections, their varied biophysical properties (neutral or charged), chain length variation, the hydrophobic nature of the involved enzymes and the presence of multiple pathways that can operate in parallel (Hannun & Obeid, 2008). The interaction with other cellular metabolic pathways (e.g. glycerolipid metabolism) introduces another layer of complexity.

Overall, the signalling effect/role of an individual SL could be defined on spatial-temporal basis with at least five parameters: (a) subcellular localisation, (b) regulation (c) chain length specificity, (d) kinetics of trafficking and (e) mechanism of action. For

example, phosphorylation of 1–3% cytosolic SPH may double the levels of S1P that acts on G protein-coupled receptor (GPCR) to elicit a specific response in a particular cellular locality for certain period of time (Hannun & Obeid, 2008). Such signalling events can be described as a function of cytosolic S1P that is regulated by S1P Kinase, with the signal caused through the interaction of S1P with a GPCR. The elucidation of such complex systems remains challenging and a comprehensive discussion of the issue is beyond the scope of this review. However, an additional layer of significant complexity in terms of the pathogenic protozoa arises when considering the SL signalling network in the case of obligate intracellular parasites, where host SL biosynthesis, and its interaction with parasite *de novo* synthesis, must be taken into account.

Parasite–host SL interplay

The intimate parasite–host interaction in terms of SL metabolism has been well documented; *L. major* pathogenic amastigotes isolated from mammalian hosts showed normal IPC levels (Zhang *et al*. 2005) despite lacking LCB2, a functional SPT and the ability to synthesis CER *de novo*. Alterations in host, macrophage, cell SL biosynthesis upon infection may compensate for this deficiency (Ghosh *et al*., 2001, 2002). These studies suggest a complex and multifaceted interplay between host and parasite SL metabolism comprising nutritional factors and signalling pathways that could modulate parasite survival and/or host defence (Zhang *et al*. 2010). Similar observations have been reported in the apicomplexan parasites (Romano *et al*. 2013). This highlights the striking potential of host and parasite SL modulation as an anti-protozoal target, as is similarly proposed for pathogenic fungi (Zhang *et al*. 2010; Ramakrishnan *et al*. 2013).

PERSPECTIVE

Classically dissecting the role and locale of critical enzymatic steps in SL biosynthesis and assessing the effect on the parasite fitness and virulence could turn into an overwhelmingly challenging task aggravated by: the complexity of the metabolic pathway itself; the ability of the parasite to salvage (Coppens, 2013), hijack and remodel host SL; and developmental regulation during the parasitic life cycle, which adds another layer of intricacy rendering the deconvolution of any observed effects difficult to interpret. Fortunately, many of those problems can be now overcome with advances in technology. High resolution localization studies in protozoan parasites can benefit greatly from new microscopic techniques such as Airy-scan (Huff, 2015), super-resolution microscopy (Florentino *et al*. 2014) and upcoming technologies, e.g. phase-modulation nanoscopy (Pal, 2015; Ward & Pal, 2017), which can elucidate spatial arrangement of proteins of interest within the parasite to reveal potential interaction partners and shed light on mechanistic features. Similarly, new advances in chemical probes, and SL analogues in particular, such as bifunctional lipid technology (Haberkant & Holthuis, 2014) coupled with high throughput proteomic (Ramaprasad *et al*. 2015), could identify different interaction partners that would help map the biosynthetic pathway and its critical interactions. The effects of these probes on the parasite (and host) cell can now be comprehensively evaluated by monitoring the transcriptome, proteome, metabolomics (Watson, 2010) and lipidome (Marechal *et al*. 2011). Such studies could reveal multiple windows of opportunity to exploit as potential drug targets. The targets identified in this way can now be rapidly genetically validated in the parasitic protozoa by applying modern gene editing technologies, such as CRISPR/Cas9 (Sugi *et al*. 2016). Compared with the classical methodologies, this tool enables fast and efficient application for single gene (Serpeloni *et al*. 2016), and systematic genome-wide knockout generation (Sidik *et al*. 2016). Additionally, the development of novel orthogonal approach for conditional knockout strategies, e.g. tetracycline-induced gene disruption Tet-system (Meissner *et al*. 2002), rapamycin-induced Cre recombinase-assisted gene excision (Andenmatten *et al*. 2013; Collins *et al*. 2013; Jimenez-Ruiz *et al*. 2014), has allowed testing of essential gene functionality, in *Leishmania* spp. (Duncan *et al*. 2016) and *T. gondii* (Pieperhoff *et al*. 2015).

Aside from the increase ability to robustly validate targets such as SL biosynthesis, global collaboration between academia and pharmaceutical partners is expediting the process of drug discovery of new anti-protozoal drugs. For example, within the sphere of targeting SL biosynthesis in the protozoa, we have managed several projects with industrial partners, MRCT and Tres Cantos Open Lab Foundation (https://www.openlabfoundation.org, an initiative of GlaxoSmithKline), in the pursuit of identifying new compound scaffolds active against the *Leishmania* spp IPC synthase utilising yeast (Norcliffe *et al*. 2014) as a vehicle for drug discovery (Denny & Steel, 2015). The generated results and techniques could readily be translated to other disease targets. Other global initiatives include Open Innovation Drug Discovery, Eli Lilly, which is focused on cancer, cardiovascular disease, endocrine disorders, neuroscience and tuberculosis. The Centers for Therapeutic Innovation, facilitates Pfizer and academic researchers to work together in order to develop new biologics programs and WIPO Re:Search, provide participant researchers with access to patents and expertise related to drug

discovery for 19 NTDs, malaria and tuberculosis (Sheridan, 2011).

Finally, SL biosynthesis represents a gold mine for new drug targets alongside at least two axes, *de novo* synthesis and salvage and remodelling. On one hand, the protozoan *de novo* SL biosynthetic pathway comprises three key steps, and considering their divergence compared with the mammalian host, identifying specific inhibitors for those could open an opportunity for anti-protozoal drugs with synergistic effects and lower incidences of resistance. On the other hand, the nature of obligate intracellular parasites dictates that further efforts should be directed towards the catabolic/salvage pathway where parasite–host dependencies could be exploited in order to identify additional key steps, or host enzymes, where inhibitors would exert further synergism with the *de novo* inhibitors.

To summarize, the landscape of anti-protozoan drug discovery requires immediate attention: with the re-evaluation of knowledge gained, the application of recent technologies; and the support of coordinated global discovery efforts. The multifaceted effects of SLs as a dynamic matrix of interaction (spatial and temporal) and function makes SL biosynthesis highly alluring for drug intervention, after all, everybody needs SLs, right?

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