Match-making for posaconazole through systems thinking

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Currently available drugs for Chagas' disease are limited by toxicity and low efficacy in the chronic stage. Posaconazole, the most advanced new anti-chagasic drug candidate, did not fully confirm its initial potential in a Phase II clinical trial for chronic Chagas' disease. Given that posaconazole is highly active against *Trypanosoma cruzi in vitro*, and was very well tolerated in clinical trials, it should not be abandoned. Rather, a combination therapy may provide a highly promising outlook. Systems-scale approaches facilitate the hunt for a combination partner for posaconazole, which acts by blocking sterol biosynthesis. Mounting evidence suggests the functional interactions between sterols and sphingolipids *in vivo*. Here, we propose combining sterol and sphingolipid biosynthesis inhibitors to advance drug development in Chagas' disease.

Chagas' disease: a global burden and the unmet need for new drugs

Worldwide, an estimated 7 million–8 million people are infected with the protozoan parasite *Trypanosoma cruzi* [1]. Chagas' disease is endemic in 21 South American countries but, as a result of population mobility, also occurs outside the continent [2]. Chagas' disease poses a global challenge due to the lack of safe and effective treatment. Efforts towards the urgently needed new drugs have culminated in the clinical development of triazolic antifungals for Chagas' disease. The most advanced drug candidates were posaconazole and E1224, a prodrug of ravuconazole. Unfortunately, although very well tolerated, both compounds failed to meet the high expectations in recent clinical Phase II trials: they did not cure chronic Chagas' disease as indicated by the high relapse rates observed during follow-up. Considering that posaconazole and E1224 are highly active against *T. cruzi* and well tolerated in clinical trials, we propose not to abandon the triazoles but to find a suitable partner for combination therapy. Combination therapy is an attractive approach because it may improve treatment efficacy while decreasing the likelihood of resistance development [3]. Systems biology aims at revealing interconnections of biological networks and these works serve as useful resources for rational identification of potential interacting partners for chemotherapy. Based on genetic, physical, and functional interactions between sterols and sphingolipids [4] and due to the synthetic lethality of *Saccharomyces cerevisiae* double mutants of sterol and sphingolipid anabolism [5], our opinion is that inhibitors of sphingolipid biosynthesis are promising combination partners for posaconazole or ravuconazole.

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Current drugs for the treatment of Chagas' disease

Chagas' disease remained without an effective treatment for several decades after its original description in 1909 [6]. Nifurtimox and benznidazole, discovered over 40 years ago and still the only available drugs for the specific treatment of Chagas' disease, are limited by toxicity and low efficacy in the established chronic form of the disease [7]. These major drawbacks, along with upcoming reports of resistant *T. cruzi* [8] and the spread of the disease to nonendemic countries [2], spurred renewed drug research and development (R&D) for Chagas' disease. The triazoles posaconazole and E1224 were the only candidates to pass the preclinical phase and enter clinical proof-of-concept trials. However, the results in Phase II clinical trials were disappointing. While the parasitemia dropped below detection limit after treatment, 10 months later, most patients again tested positive for *T. cruzi* [9]. Either candidate was less efficacious than benznidazole. This outcome is arguably attributable to limited systemic exposure resulting from the liquid suspension of the drug and suboptimal treatment duration [10]. Even so, these results have aggravated the situation in the already slim Chagas' portfolio, where the most advanced alternatives to the triazoles have not yet reached clinical Phase I.

Quo vadis posaconazole?

In 1995, V.M. Girijavallabhan described posaconazole (SCH 56592) as a novel, orally active, broad-spectrum antifungal agent [11]. Posaconazole (Nofaxil) was developed by Schering-Plough and was approved by the US Food and Drug Administration (FDA) for the treatment of invasive fungal infection in humans in 2006 [12]. Similar to other triazoles, posaconazole is a potent inhibitor of the

Cyp450-dependent lanosterol 14α-demethylase (Cyp51) in yeasts and molds [13]. Inhibition of Cyp51 blocks the synthesis of ergosterol, which is an essential component in the cell membrane of fungal pathogens. Accumulation of methylated sterol precursors and disruption of the close packing of acyl chains of phospholipids in ergosterol-depleted cell membranes ultimately leads to growth inhibition of the fungi [14]. Similar to fungi, *T. cruzi* synthesizes ergosterol and is sensitive to sterol biosynthesis inhibitors [15]. Posaconazole exhibited excellent *in vitro* and *in vivo* efficacy against both drug-sensitive and -resistant isolates [16,17]. Suitable combination partners for posaconazole might be found in the sterol biosynthesis pathway to enhance the blockade of this highly interconnected metabolic network. The links between distinct steps of the sterol biosynthesis pathway can be exemplified in *S. cerevisiae*: in the presence of *erg6* deletion, the *erg2* gene product works inefficiently [4], resulting in an *erg6* single deletion mutant exhibiting a partial phenotype of an *erg2erg6* double mutant. Furthermore, cells have evolved compensatory mechanisms within metabolic pathways such that accumulating substrates resulting from inhibition of a specific enzymatic step can be alternatively metabolized as a salvage mechanism. Clearly, sterol biosynthesis is of proven druggability and targeting multiple steps in the same pathway can potentiate antiparasitic activity. Lovastatin, a blockbuster used for hypercholesterolemia, enhanced the antiproliferative effects of ketoconazole and terbinafine against *T. cruzi in vitro* and *in vivo* [18]. Evidence from yeast points in the same direction because at least a dozen proteins interacting with Erg11 (Cyp51 ortholog in *S. cerevisiae*) can be found in the sterol metabolic pathway [19]. Druggable pathways that interact with sterol metabolism also represent complementary targets. Glycerophospholipid biosynthesis inhibitors, such as ajoene or alkyl-lysophospholipids (ALP, e.g., miltefosine), have been shown to have antiproliferative effects on *T. cruzi* epimastigotes and amastigotes [20–22]. Growth inhibition correlated with a decrease in the phosphatidylcholine to phosphatidylethanolamine ratio (PC:PE) and, in the case of ALP, also with a marked effect on sterol composition due to inhibition of sterol 22-desaturase (Erg5), a finding that probably explains the antiproliferative synergism of these drugs with the Cyp51 inhibitor ketoconazole against both proliferative stages (epimastigotes and intracellular amastigotes) of the parasite [21,22]. Here, we propose to combine the anti-chagasic triazoles with inhibitors of sphingolipid synthesis, as suggested by systems approaches.

Systems-based matchmaking

Systems approaches were pioneered in model organisms to understand how biological systems act as a whole. Emergence of complex behavior is observed when the biological systems are treated as networks. These can be protein interaction networks, metabolic networks, or genetic networks. All are amenable to large-scale interaction studies, particularly in *S. cerevisiae*, where global approaches such as chemical genetics screens, mutant library screens, protein–protein interactions, and other -omics technologies can be automated. These have led to the availability of databases containing a wealth of information that can be mined to generate new hypotheses of cellular and system functions. It can also guide drug discovery in modern medicine by providing a rational basis to pinpoint interrelated pathways. The interacting partners for *CYP51* in *S. cerevisiae*, for instance, are found on the BioGRID database [23], containing 103 physical and 184 genetic interactions. Candidate pathways can be further narrowed by phenotypes and functionality. Specifically, interactors of Cyp51 that cause synthetic lethality will be appealing.

Capitalizing on sterol–sphingolipid interactions as a combinatorial treatment

Posaconazole blocks sterol biosynthesis; thus, druggable pathways interacting with sterol metabolism and functions represent highly complementary matches for posaconazole. Sterols have been shown to modulate membrane thickness in artificial membranes and this property has been proposed to have a role in membrane protein localisation *in vivo* [24]. It is increasingly known that proteins and lipids do not freely diffuse over the entire surface of the cell and it has been proposed that eukaryotic plasma membranes contain micro- and/or nanodomains (reviewed in [25–27]) that act as platforms creating membrane heterogeneities with many proposed functions. There is clear biophysical evidence that sterols and sphingolipids can segregate from other lipids in simple artificial membrane systems to form liquid ordered domains [28].

Sterol–sphingolipid interactions have also been demonstrated *in vivo*. Evidence in the budding yeast, *S. cerevisiae*, suggests a genetic interaction between mutants in sterol and sphingolipid biosynthesis [4,5,29,30]. For example, mutants that affect the hydroxylation pattern of sphingolipids display synthetic growth defects with mutations in late-acting ergosterol biosynthetic genes [4]. By contrast, mutations that affect the synthesis of the sphingolipid-specific very-long chain C_{26} fatty acid display strong synthetic lethality with mutations in *ERG6*, a methyltransferase that catalyzes the addition of a fungal-specific methyl group at position C_{24} in the aliphatic side chain or ergosterol [5]. Figure 1 summarizes experimental evidence on genetic interactions between the sterol and sphingolipid synthetic genes. While the bulk of experimental evidence comes from high throughput screens and needs to be treated with caution, there is solid support for synthetic lethality between *ELO3* and *ERG6* [5], and for synthetic growth defects of sterol synthetic genes with *ISC1*, *SUR2*, and *SCS7* [4]. Strikingly, synthetic lethality has been demonstrated between *CYP51* and *SCS7* [31].

In yeast and higher eukaryotes, it has further been shown that sterols and sphingolipids are important for proper trafficking of transporters (amino acids and proton pumps) to the cell surface and their stability at the plasma membrane [32–35]. Adaptation to changes in sterol composition by adjusting sphingolipid levels and variants is not unique to the unicellular eukaryotes but is also present in Metazoa, exemplified by the fruit fly, *Drosophila melanogaster*. As a sterol auxotroph, *D. melanogaster* cannot synthesize sterols but this lipid is required for larval growth and development. A drop in sterol levels caused developmental arrest but cells remain viable, possibly due to a compensatory increase in sphingolipid levels and composition [36].

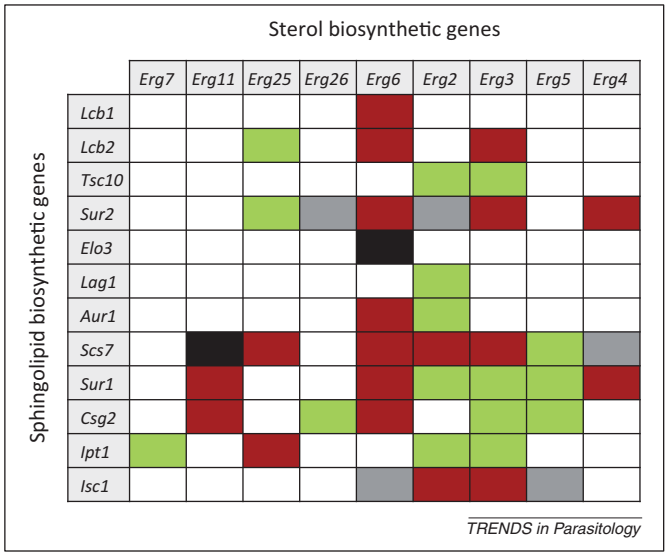

Figure 1. Genetic interactions between sterol and sphingolipid biosynthetic genes in yeast. Only genes for which an interaction has been experimentally documented are shown. Data are from BioGRID v. 3.2.117 [23]. Green, positive genetic interaction; red, negative genetic interaction or synthetic growth defect; black, synthetic lethality; gray, equivocal results from different screens. For gene symbol definitions, please see Figure 2.

Figure 2 shows the structures of mannosyl diinositol phosphoryl ceramide [$M(IP)_2C$] and ergosterol, which is the most abundant sphingolipid and sterol species in yeast. Together with glycerophospholipids, sterols and sphingolipids comprise the major classes of eukaryotic membrane lipids. Many membrane characteristics, such as composition and integrity, turnover or trafficking, and signaling, fulfill the requirements for bona fide drug targets in parasites: they must be (i) essential for parasite survival; (ii) druggable; and (iii) sufficiently different from the host. Indeed, 'membrane-lipid therapy' was coined by Pablo Escribá and is defined as the therapeutic approach based on the regulation of the membrane-lipid composition and structure to modulate cell functions [37]. In a broader sense, we think of membrane therapy as interfering with membranes directly or via curtailing lipid biosynthesis.

While there currently is no evidence in *T. cruzi* on the interactions of sterols and sphingolipids, it is intuitive that the simultaneous inhibition of both sterol and sphingolipid metabolism will have a major impact on membrane homeostasis. Moreover, there are several lines of evidence that these lipids have critical roles in trypanosomatids. Endogenous sterols and sphingolipids are required for proliferation of trypanosomes [38–40]. Interestingly, reduced inositolphosphoceramide (IPC) levels due to inhibition of serine palmitoyltransferase (Spt2) in *T. brucei* have been shown to be compensated for by increased levels of phosphatidylcholine and cholesterol, demonstrating a tight interaction of sterol and sphingolipid homeostasis [41]. As in yeast, IPC rather than glycerophospholipids is utilized as lipid anchor constituent of glycoproteins and free glycosylinositolphospholipids (GIPLs) in *T. cruzi* [42]. Furthermore, inhibition of IPC synthesis impaired *T. cruzi* differentiation [43].

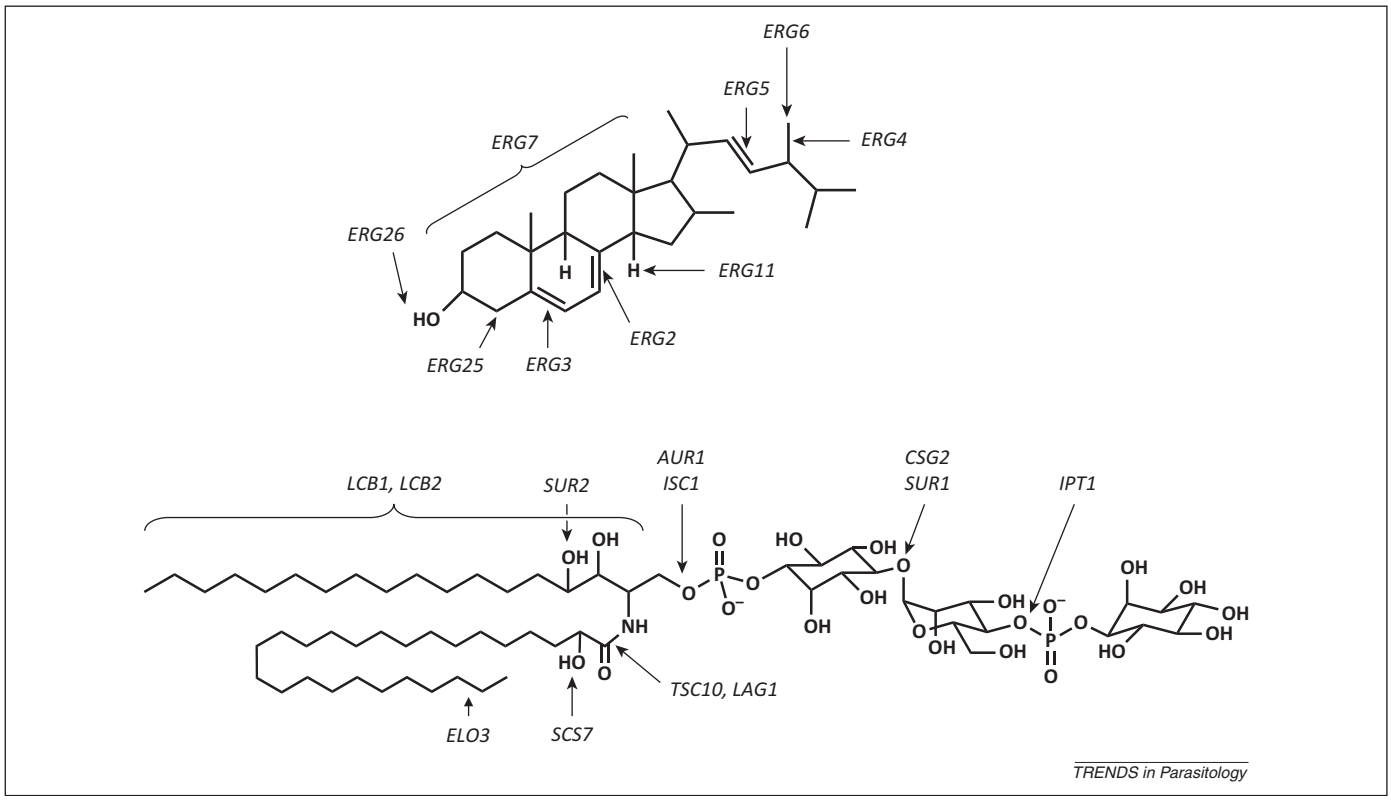

Figure 2. Structures of ergosterol and the sphingolipid, mannosyl diinositol phosphoryl ceramide [$M(IP)_2C$]. Genes shown encode enzymes that catalyze major steps in sterol and sphingolipid metabolism, respectively. The list of genes is not exhaustive but based on sterol/sphingolipid interactions (see also Figure 1). For ergosterol: *ERG7*, 2,3-oxidosqualene cyclase; *ERG11*, C_{14} demethylation; *ERG25*, $C_{4\alpha}$ methyl oxidation; *ERG26*, C_3 decarboxylation; *ERG6*, C_{24} methylation; *ERG2*, C_8 isomerization; *ERG3*, C_5 desaturation; *ERG5*, C_{22} desaturation; *ERG4*, C_{24} reduction. For $M(IP)_2C$: *LCB1*, serine C-palmitoyltransferase; *LCB2*, serine C-palmitoyltransferase; *TSC10*, 3-dehydrosphinganine reductase; *SUR2*, long chain base hydroxylase; *ELO3*, fatty acyl elongase; *LAG1*, sphingosine N-acyltransferase; *AUR1*, phosphatidylinositol:ceramide phosphoinositol transferase; *SCS7*, fatty acyl hydroxylase; *SUR1*, mannosylinositol phosphorylceramide (MIPC) synthase; *CSG2*, MIPC synthase regulatory subunit; *IPT1*, inositolphosphotransferase; *ISC1*, inositol phosphosphingolipid phospholipase C.

Table 1. Sphingolipid biosynthesis inhibitors in clinical trials or on the market

Compound name	Clinical phase or drug name (if on the market)	Mechanism of action	Indication	Refs
N-butyldeoxynojirimycin	Miglustat, Zavesca®	Glucosylceramide synthase inhibitor	Gaucher disease	
FTY720	Gilenya	Sphingosine-1-phosphate receptor inhibitor	Multiple sclerosis	[49]
Safingol	Phase I	Sphingosine kinase inhibitor	Cancer	[50]
Phenoxodiol	Phase III	Sphingosine kinase inhibitor	Cancer	[51]
ABC294640	Phase I	Sphingosine kinase inhibitor	Cancer	[52]
Sphingomab	Preclinical	Anti-sphingosine-1-phosphate antibody	Cancer	[53]
Fenretinide	Phase I	Ceramide desaturase inhibitor	Cancer	[54]
Desipramine	Treyzafagit, Norpramin, and Pertofrane	Acid sphingomyelinase inhibitor	Antidepressant	
Imiglucerase	Cerezyme	β-glucocerebrosidase replacement	Gaucher disease	
Amitriptyline	Phase IIb	Acid sphingomyelinase inhibitor	Cystic Fibrosis	[55]
	Elavil, Endep, and Vanatrip		Antidepressant	
			Analgesic	
Fluoxetine	ROzac, PROzac Weekly, Sarafem, Rapiflux, Selfemra, and PROzac Pulvules	Acid sphingomyelinase inhibitor	Antidepressant	
Aureobasidin A[a]	Phase I	Inositol phosphorylceramide synthase inhibitor	Antifungal	[56]

[a]Failed in clinical Phase I.

The relation between sterols and sphingolipids, evident in yeast and the fruit fly, could indicate a potential evolutionarily conserved adaption mechanism for membrane homeostasis. Thus, concomitant perturbation of these two classes of lipids may promote synergistic lethality. Therefore, it will be interesting to test the interactions between posaconazole or ravuconazole and sphingolipid inhibitors on *T. cruzi* focusing on 100% cidality rather than potential synergism.

Concluding remarks and outstanding questions

Sterile cidality also against nonproliferating trypanosomes is imperative to Chagas' disease chemotherapy. To this aim, three different strategies have been proposed to select a suitable combination partner for azoles. The partner could be a drug such as benznidazole, which is 100% cidal itself and additive in action with posaconazole [44–46]. It could also be a drug that is not 100% cidal itself but shows synergistic interaction with posaconazole, such as amiodarone, amlodipine, or clemastine [46,47]. In addition, aiming to completely block sterol synthesis, the combination partner could be another sterol biosynthesis inhibitor [15,18]. Here, we propose as an additional strategy the partnership between posaconazole and sphingolipid inhibitors. This is based on the hypothesis that such a combination will be most effective in disrupting membrane integrity and functions, which is critical also for quiescent cells. Exploration of the chemotherapeutic potential of this proposed partnership will require: (i) systems knowledge of *T. cruzi* lipid physiology; (ii) sphingolipid biosynthesis inhibitors; and (iii) a test for 100% sterile cidality on the relevant *T. cruzi* stages.

Currently, there is no evidence that sterols and sphingolipids functionally interact in *T. cruzi*. The advancing technologies for system-scale analyses of genes, transcripts, proteins, and metabolites (including lipids) accompanied by high throughput genetic and chemical screening, will revolutionize our understanding of *T. cruzi* biology and identify possible pathways for combination therapies. Concomitant chemotherapeutic attack of sterol

Table 2. Sterol biosynthesis inhibitors[a]

Class	Target and/or mechanism of action	Indication	Refs
Statins	Competitive inhibitors of HMG-CoA reductase, preventing the formation of mevalonate from HMG-CoA; they occupy the HMG-binding pocket and part of the binding surface for CoA	Used as cholesterol-lowering drugs in humans	[57,58]
Bisphosphonates (BPs)	Potent inhibitors of bone resorption. The selective action on bone is based on the binding of the BP moiety to the bone mineral; nitrogen-containing BPs bind to, and inhibit the activity of, farnesyl diphosphate synthase	Used to treat osteoporosis and other bone resorption diseases	[59–61]
Quinuclidines and/or zaragozic acids	Inhibition of squalene synthase (SQS); quinuclidines may inhibit SQS by acting as carbocation mimics for FPP to squalene conversion. The aryl units may act as isosteres for the isoprenyl subunits in the farnesyl chain.	Not in clinical use	[62]
Allylamines	Specific inhibition of fungal squalene mono-oxygenase	Used for topical treatment of fungal infections	[63,64]
Azoles	Bind as the sixth ligand to the haem in lanosterol 14 α-demethylase (= CYP51), thus occupying the active site and acting as noncompetitive inhibitors; blocking the synthesis of ergosterol leads to the accumulation of methylated sterol precursors	Used to treat fungal infections	[65,66]
Azasterols	Evidence from yeast shows that azasterols inhibit the enzyme C24-sterol methyltransferase	Not in clinical use	[67]

[a]Compound classes of molecule known to interfere with sterol metabolism. Target enzymes and mechanisms of action are indicated, as well as clinical indications where molecules are already on the market.

and sphingolipid biosynthesis is facilitated by the availability of sphingolipid inhibitors [48], owing to the interests in their functions in human health. As with every drug candidate, consideration must be given to the potential toxicity of sphingolipid biosynthesis inhibitors. Toxicity is one reason why most of the numerous existing sphingolipid inhibitors remain experimental compounds [48]. Nonetheless, this class of compounds is promising, because there are several in clinical use or in clinical trials for a spectrum of human diseases (Table 1) and, given the role of sphingolipids in many other human diseases, efforts to discover novel compounds are ongoing. The same applies for sterol biosynthesis inhibitors (Table 2). A crucial requirement for R&D of next-generation anti-chagasic agents will be an *in vitro* test that is amenable to medium throughput and that can demonstrate 100% cidality against nonproliferating intracellular amastigote *T. cruzi*. Such an assay must be able to predict the lack of sterile cidality of posaconazole and ravuconazole.

In summary, we argue that the potential of posaconazole must be further explored with a view of rational target identification and achieving combination therapy through systems-scale approaches. Based on evidence in model organisms, particularly the budding yeast, the matching of sphingolipid synthesis inhibitors as partners of triazoles can impair membrane functionality and, thus, may kill proliferating as well as dormant parasites.

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