Membrane Therapy for Chagas' Disease

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Summary

Currently, benznidazole and nifurtimox are the only drugs available for the specific treatment of Chagas' disease. Both are limited by low efficacy in the chronic stage of the disease and considerable toxicity, which is why there is an urgent need for drugs that provide safe and efficient treatment for Chagas' disease. *Trypanosoma cruzi*, the causative agent of Chagas' disease, requires specific endogenous sterols and is therefore very sensitive to sterol biosynthesis inhibitors (SBIs). SBIs are widely used as antifungals and lend themselves to drug repurposing. Sterols are an essential class of lipids in eukaryotes, where they serve as structural components of membranes and play important roles as signaling molecules. The most abundant sterol in vertebrates is cholesterol, whereas fungi synthesize ergosterol, which has a greater degree of unsaturation and an additional methyl group at C_{24}. Like fungi, trypanosomes require the presence of ergosterol and other 24-alkylated sterols; their similar sterol content is the rationale for testing inhibitors of ergosterol synthesis against trypanosomes.
In the framework of this PhD thesis various aspects of sterol anabolism in eukaryotes and its potential exploitation as drug target in parasites were analysed. First, using genome profiling, I did a comparative genomics study of sterol biosynthesis (SB) focusing on eukaryotic parasites. In vitro testing of known SBIs and quantifying the expression levels of SB genes during the different life stages of *T. cruzi* and *Trypanosoma brucei* completed this part of the thesis. Then, I used genetically modified yeast strains as a tool to assess selectivity of SBIs to ergosterol-containing cells. Finally, integrating the results from my work led to a specific proposition how to advance drug development in Chagas' disease.

For the genome profiling an in silico pipeline was developed to globally evaluate sterol metabolism and perform comparative genomics. Hidden Markov model-based profiles for 42 SB enzymes allowed to represent the genomic makeup of a given species as a numerical vector. Hierarchical clustering of these vectors functionally grouped eukaryote proteomes and revealed convergent evolution, in particular metabolic reduction in obligate endoparasites. The only obligate endoparasites found to possess SB genes were the trypanosomatids, *Trypanosoma* spp. and *Leishmania* spp. However,

the origin of SB genes in trypanosomatids remains obscure, as there was no evidence for horizontal transfer.

SBIs are generally thought to act by inhibition of ergosterol anabolism. To investigate this more closely, I developed an assay using genetically modified yeast strains that either synthesize ergosterol or cholesterol. Different efficiencies of a given molecule in inhibiting ergosterol- or cholesterol-producing yeast can thus be attributed to sterol content. Nystatin concentrations required to inhibit growth in the cholesterol-producing yeast strain were 10-fold higher than in the ergosterol-producing strain, demonstrating the validity of the approach. Like amphotericin B, nystatin binds to ergosterol and forms pores in the membrane that lead to death of the target cell. This clear-cut result was only observed for molecules that bind to the finished end product of SB. Inhibitors of enzymes involved in SB did not exclusively inhibit growth of ergosterol-producing yeast strain, showing that the selectivity of SBIs for fungi is not based on differences between cholesterol and ergosterol anabolism. Two possible explanations why SBIs are selective inhibitors of fungal and trypanosomatid growth are brought forward: i) mammalian cells can salvage cholesterol from the environment and thus circumvent inhibition of sterol de novo synthesis whereas trypanosomatids and fungi require the presence of ergosterol and other 24-alkylated sterols, which cannot be replaced by the host's sterols or ii) fungal and protozoan orthologs of SB enzymes are more susceptible to SBIs than the respective mammalian orthologs.

Even though azoles have been used as antifungals for decades, their use against trypanosomatids is still not implemented. Even worse, the most advanced candidate – posaconazole – could not confirm its initial potential in a recent phase II clinical trial for chronic Chagas' disease. Based on my findings and integrating the work of others, posaconazole should not be abandoned but partnered with another drug for combination therapy. In the concluding chapter I elaborate on why a sphingolipid biosynthesis inhibitor is probably the best match.

Abbreviations

24-SMT	Sterol 24-C-methyltransferase
ALP	Alkyl-lysophospholipids
BP	Bisphosphonate
BSF	Bloodstream form
CDC	Centers for Disease Control and Prevention
CL	Cutaneous leishmaniasis
CoA	Coenzyme A
CYP51	Cytochrome P450, Family 51 (Lanosterol 14 alpha-demethylase)
DALY	Disability-adjusted life year
DHCR	Dehydrocholesterol reductase
DMSO	Dimethyl sulfoxide
DNDi	Drugs for neglected diseases initiative
DOXP	Deoxyxylulose 5-phosphate
ELISA	Enzyme-linked immunosorbent assay
FPP	Farnesyl pyrophosphate
FPPS	Farnesyl pyrophosphate synthase
GGPPS	Geranylgeranyl pyrophosphate synthase
GIPLs	Glycosylinositolphospholipids
HAT	Human African Trypanosomiasis
HMG	3-hydroxy-3-methyl-glutaryl
HMM	Hidden Markov model
IPC	Inositolphosphoceramide
MEP	Methylerythritol 4-phosphate
MIPC	Mannosylinositol phosphorylceramide
$M(IP)_2C$	Mannosyl diinositol phosphoryl ceramide
MEV	Mevalonate
NCE	New chemical entity
NECT	Nifurtimox-Eflornitine combination therapy
NTD	Neglected tropical disease

PC	Principal component
PC:PE	Phosphatidylcholine to phosphatidylethanolamine ratio
PCF	Procyclic form
PDP	Product development partnership
R&D	Research and development
SB	Sterol biosynthesis
SBI	Sterol biosynthesis inhibitor
SLT	Spliced-leader trapping
SQS	Squalene synthase
SSG&PM	Sodium stibogluconate & paromomycin combination therapy
Swiss TPH	Swiss Tropical and Public Health Institute
VL	Visceral leishmaniasis

Chapter 1

General Introduction

Sterol Biosynthesis Inhibitors –

From Blockbusters to Neglected Tropical Diseases

General Introduction

1 American Trypanosomiasis

American trypanosomiasis, also known as Chagas' disease, was discovered in 1909 by Carlos Chagas', who characterized the etiologic agent *Trypanosoma cruzi*, its life cycle and vector as well as the transmission process (1). Apart from natural transmission in endemic areas through contact with the faeces of vector insects (triatomine bug) or ingestion of contaminated food, *T. cruzi* infection can also occur through contaminated blood transfusion, organ transplant, congenital transmission from mother to foetus, or laboratory accidents. Infection from mother to child may occur in all endemic countries but is diagnosed only in a few. According to conservative estimates, at least three percent of new-borns from *T. cruzi*-infected mothers acquire the infection through the placenta (2). Debilitating and often fatal, this neglected tropical disease ranks high in terms of disability-adjusted years of life loss in Latin America, where it is a major public health problem and thus also a significant economic burden. Among infectious and parasitic diseases only HIV/AIDS, diarrheal diseases and tuberculosis account for more DALYs lost in this region. In many rural areas Chagas' is by far the most common cause of heart disease.

American Trypanosomiasis is endemic in 21 South American countries but as an enzootic disease, it is more widely distributed than apparent from human infections. It extends approximately from latitude 42° N in northern California and Maryland, to latitude 43° S in southern parts of Argentina and Chile (2). Every year, American Tryponosomiasis causes between 23'000 and 43'000 deaths (2). As a result of population mobility, Chagas' disease now also occurs outside Latin America. Migration, but also travelling to endemic countries, has caused the disease to spread to non-endemic countries. With an estimated 400,000 *T. cruzi* infected individuals outside of Latin America, control of Chagas' disease has become a worldwide challenge (3).

The life cycle of *T. cruzi* consists of three extracellular stages and one intracellular stage which are morphologically and metabolically different from each other: Epimastigotes are found in the gut of the insect vector and they transform into the metacyclic trypomastigotes which are infectious to the mammalian host. Metacyclic

trypomastigotes are transmitted through the faeces of the insect vector upon a blood meal and infect a plethora of mammalian host cells. Inside the cells they transform into amastigotes which multiply through binary fission in the host cell's cytosol. Finally, intracellular amastigotes transform into trypomastigotes which burst out of host cells into the blood stream and are able to infect new cells (4).

2 Human African Trypanosomiasis

Human African Trypanosomiasis (HAT) is caused by two subspecies of *Typanosoma brucei*: *T. b. gambiense*, which causes endemic disease in central and west Africa and *T. b. rhodesiense*, which is prevalent in east and southern Africa and causes acute HAT (5). More than 90% of all reported HAT cases are attributed to *T. b. gambiense* infection. The parasites are transmitted between mammalian hosts by the blood-feeding tsetse flies of the genus *Glossina*. In the fly, the parasites undergo several differentiation steps: In the midgut dividing procyclic trypomastigote forms develop to the epimastigote forms, which migrate to the salivary glands where they develop into the infective metacyclic forms, which are injected with the saliva before the blood meal into the mammalian host. In man there are three distinctive bloodstream forms: dividing slender forms, intermediate forms, and stumpy forms. The latter are non-proliferative and, when ingested by the tsetse fly, transform to diving midgut forms to complete the cycle (6). The disease appears in two stages: the first haemolymphatic stage with non-specific symptoms like headache and fever and the second neurologic stage, after parasites have crossed the blood brain barrier. Invasion of the central nervous system causes the typical symptoms associated with sleeping sickness: serious sleep cycle disruptions, paralysis and progressive mental deterioration. If left untreated, HAT is lethal.

3 Leishmaniasis

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*. Transmission between vertebrate hosts usually occurs by the bite of the blood-sucking female phlebotomine sand fly (7). During a blood meal on an infected host, sand fly vectors take up macrophages infected with amastigotes. In the fly midgut, amastigotes transform into promastigotes, where they divide and then migrate to the proboscis. With the next blood

meal, the infective promastigotes are injected into mammalian skin where they are phagocytized by macrophages. Inside the mononuclear phagocyte the progmastigotes transform into the amastigotes, the proliferative form of the parasite in the mammalian host (8). Many of the more than 20 *Leishmania* species infective to mammals can cause human disease. Parasite species, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Cutaneous leishmaniasis usually presents as ulcers on exposed body parts. Worldwide, the causative agents of most human visceral leishmaniasis (VL) are *Leishmania donovani* and *Leishmania infantum* (9). VL, also known as kala azar, is fatal without treatment.

4 Available Drugs against Trypanosomatids

Currently available drugs to treat *Trypanosoma* and *Leishmania* infections are listed in Table 1 together with their associated key problems and limitations. The first drugs against American Trypanosomiasis were discovered not until 60 years after the initial description of the disease in 1909. Nifurtimox (Lampit®, Bayer) and benznidazole (Rochagan®, Radanil®, Roche) remain to date the only available drugs for the specific treatment of Chagas' disease (10). Both, nifurtimox and benznidazole, are nitroheterocyclic drugs and were originally registered to treat acute *T. cruzi* infections. Benznidazole is generally used as first-line treatment because it has the best safety and efficacy profile (11). Treatment is effective during the acute stage of infection with an estimated parasitological cure of at least 60% (12) but there is insufficient evidence to support the efficacy of nifurtimox or benznidazole in the late chronic stage of Chagas' disease (13-15). Moreover, both drugs display high rates of adverse reactions.

Patients infected with *T. brucei gambiense* are treated with pentamidine if they are in the first stage of the disease; Nifurtimox-Eflornithine Combination Therapy (NECT) is used for second stage HAT. *T. brucei rhodesiense* patients are treated with suramin (first stage) and melarsoprol (second stage). All currently used drugs have their limitations: pentamidine, suramin and melarsoprol show limited to severe toxicity and NECT is not effective against *T. brucei rhodesiense* infections (16).

The recommended treatment against visceral and cutaneous leishmaniasis relies on antimonials. These drugs have been the treatment of choice for many decades and emergence of resistance has been reported and reached epidemic dimensions in Bihar, a

region in India endemic for visceral leishmaniasis (17-19). Alternative drugs to treat leishmaniasis are amphotericin B, pentamidine, and recently also miltefosine. But also against these second-line drugs resistance was observed (20) and side effects as well as high costs of treatment limit their use, especially in low-income countries.

There is an urgent need for safe, efficient and easy-to-use drugs to treat HAT, American Trypanosomiasis and Leishmaniasis. Current drugs are often toxic, not orally available and difficult to administer, too expensive, or becoming impotent due to drug resistance. There are a few drug candidates in development, the most promising and advanced being fexinidazole for HAT and posaconazole for Chagas' disease. But especially for Chagas' disease the situation is not satisfactory as there would be a huge gap in the drug research and development (R&D) pipeline if posaconazole was not able to meet the high expectations. The most promising candidates to date are inhibitors of sterol biosynthesis (SB). Such inhibitors, already widely used as antifungals, are also being developed for Chagas' disease because trypanosomatid and fungal sterol anabolism show striking similarities.

Table 1. Currently available drugs to treat human *Trypanosoma* and *Leishmania* infections and their associated key problems[a]

Disease	Drugs/Treatment	Associated Problems/Issues
Chagas' Disease	Nifurtimox & Benznidazole	Low effectiveness in the chronic phase, limited effectiveness in the acute phase, regional variations in efficacy due to tolerant *T. cruzi* strains, high number of side effects, long period of treatment (30-90 days), high rate of patient non-compliance, dose-dependent toxicity, no paediatric strengths, contraindicated during pregnancy, need for monitoring under specialized medical supervision
Stage 1 HAT	Pentamidine	7-10 daily intramuscular injections; only efficacious for stage 1 HAT
	Suramin	Used primarily for stage 1 *T.b. rhodesiense* HAT, toxicity
Stage 2 HAT	Melarsoprol	10 painful daily intravenous injections, highly toxic with ~5% treatment-related mortality, increasing number of treatment failures (up to 30% in some regions)
	Eflornithine	Difficult administration – 4 intravenous infusions per day required for 14 days, primarily used as second line drug for *T.b. gambiense* HAT
	Nifurtimox	Oral drug developed for Chagas' disease, not registered for HAT, sometimes used compassionately after melarsoprol relapse, probably about 70% efficacy
	Nifurtimox-eflornithine[b]	Simplified stage 2 treatment combining 7 days eflornithine (2 infusions/day) and 10 days oral nifurtimox
Visceral Leishmaniasis (VL)	Pentostam & Glucantime	Quality control, availability, length of treatment (20-30 days), painful injection, toxicity, resistance in India
	Amphotericin B	Need for slow intravenous infusion, dose-limiting nephrotoxicity, heat instability
	Liposomal amphotericin B	High cost, need for slow intravenous infusion, heat instability (stored <25° C)
	Miltefosine	High cost, possible teratogenicity, potential for resistance, patient non-compliance
	Paromomycin sulphate	Efficacy variable between and even within regions
Cutaneous Leishmaniasis (CL)	Sodium stibogluconate & meglumine antimonite	Difficult administration in poor rural areas: intramuscular, intravenous, or intralesional injections, serious side effects, long treatment regime, not affordable for most patients
	Liposomal amphotericin-B	See VL, has not been fully tested on CL, high cost
	Miltefosine	See VL, side effects make it unsuitable to treat CL
	Thermotherapy & cryotherapy	High cost

[a] Adapted from www.dndi.org

[b] Included in WHO's List of Essential Medicines (EML) in May 2009

5 Sterol Anabolism in Eukaryotes

Sterols are ubiquitous in eukaryotes but largely absent from archaea and bacteria. Eukaryotic organisms require sterols as essential structural components of membranes and precursors for biologically active molecules that regulate growth and development. The most abundant sterol in vertebrates is cholesterol, where it is an essential component of cell membranes (bulk function) and acts as a precursor to vitamin D and steroid hormones (sparking function). In smaller eukaryotes, these functions are carried out by different kinds of sterols: Fungi synthesize ergosterol, which has a greater degree of unsaturation than cholesterol and an additional methyl group at C_{24}. Similar to fungi, protozoa require the presence of ergosterol and other 24-alkylated sterols, rendering the latter potentially susceptible to antifungals targeting ergosterol synthesis. This group of unicellular organisms shows a high sterol diversity, involving cholesterol and stigmasterol in *Paramecium* (21), ergosterol and stigmasterols in *Acanthamoeba* (22), cycloartenol and cyclolaudenol in *Dicytostelium* (23), and ergosterol in trypanosomatids (24, 25). Plants make a large variety of phytosterols, such as the 24-alkylated campesterol, sitosterol, or stigmasterol whereas invertebrates such as *Caenorhabditis elegans* or *Drosophila melanogaster* are sterol auxotrophs that rely on uptake of exogenous sterols (26-28).

Sterol anabolism either starts with acetyl-CoA and follows the mevalonate (MEV) pathway or proceeds from pyruvate via the deoxyxylulose 5-phosphate (DOXP/MEP) pathway to synthesize isopentenyl diphosphate, the building block of isoprenoids. The condensation of two molecules of farnesyl diphosphate to produce squalene is the first committed step of sterol synthesis. From there, the synthesis proceeds via lanosterol and zymosterol to cholesterol and ergosterol derivatives or via cycloartenol to the various kinds of phytosterols. All in all, more than 40 enzymes are involved in terpenoid synthesis and SB. Figure 1 shows the pathway of biosynthesis of cholesterol, ergosterol and plant sterols including the molecular structures of important sterol intermediates.

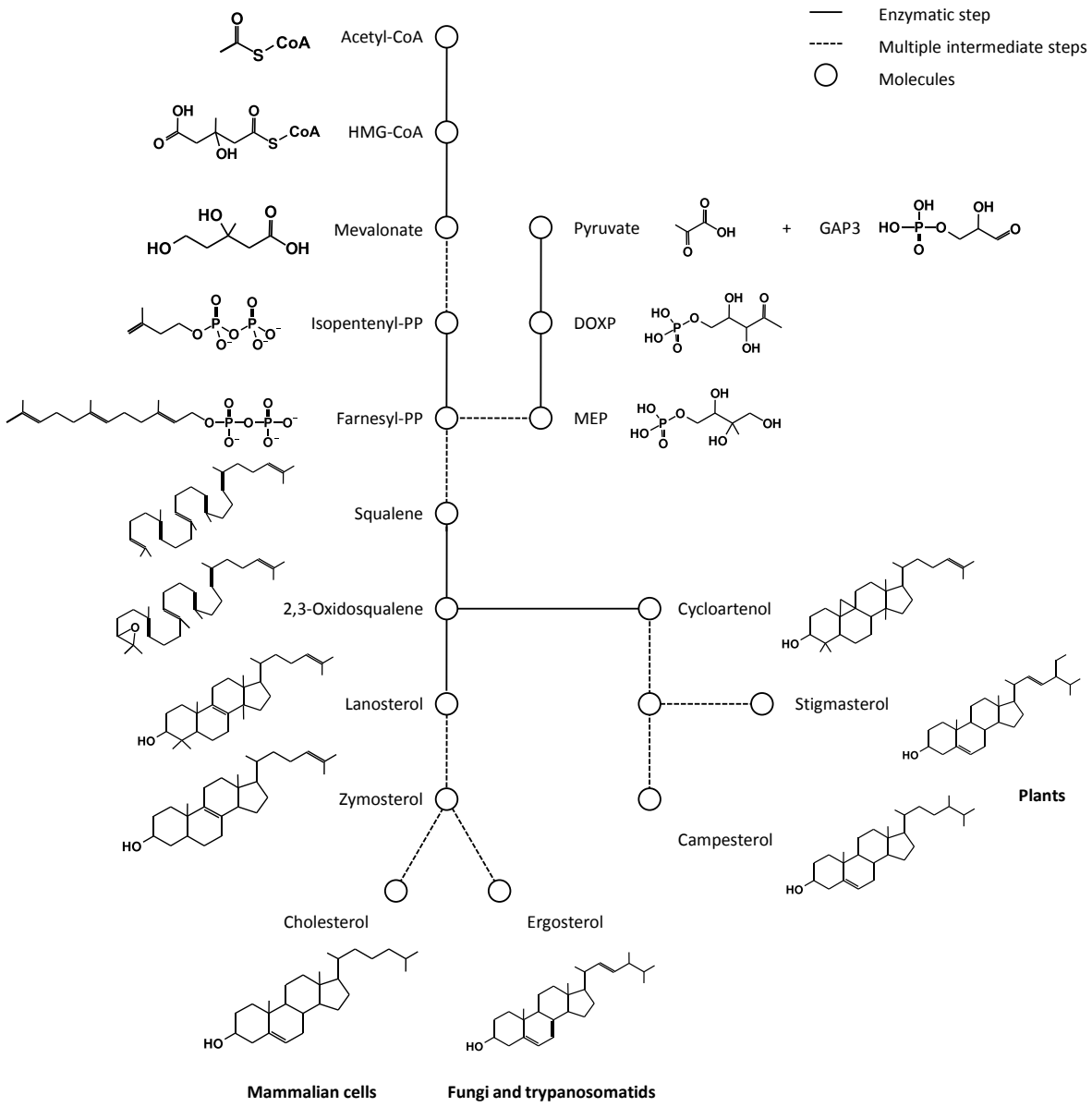


Figure 1. Overview on sterol biosynthesis. Key metabolites are spelled out and their molecular structures are depicted.

6 Sterols in Protozoa

Parasitic protozoa such as *Giardia lamblia* and *Plasmodium spp.* typically do not synthesize their own sterols (29, 30) and predominantly contain host derived cholesterol. However, there are a few exceptions: *Trypanosoma*, *Leishmania* and *Acanthamoeba* are capable of de novo sterol but not cholesterol synthesis (31-33). These protozoa have a sterol content similar to that of fungi. Major sterols found in trypanosomatids are ergosterol and other C_{24}-alkylated $\Delta^{5,7}$-sterols (reviewed in (34)).

Sterol content in any given organism is the result of de novo synthesis as well as uptake and metabolism of exogenous sterols. In the case of parasitic protozoa, the pool of de novo synthesized sterols – if present at all – is always complemented by sterols derived from the host or the culture medium. Thus it is questionable if sterol biosynthesis inhibitors (SBIs) already in use as fungicides can be repurposed as antiparasitic drugs, considering sterol import might circumvent the drugs' effects. However, sterols have dual functions as membrane insert ('bulk function') and signal molecules ('sparking function') and – for the latter – trypanosomatids need specific C_{24}-alkylated sterols such as ergosterol. This two-fold role of sterols explains why trypanosomatids have an essential need for de novo sterol synthesis even though they can import cholesterol from their host.

6.1 Sterols in *Trypanosoma cruzi* and *Leishmania spp.*

In *T. cruzi* the major sterols are ergosterol and other 24-alkylated sterols (35). In addition to the ergostane- (C_{28}) and stigmstane-based (C_{29}) sterols, *T. cruzi* also contains traces of cholesterol (C_{27}) which is derived either from the host or the culture medium (31, 36), as demonstrated for *T. brucei*, imported cholesterol cannot replace endogenous ergosterol (37) and *T. cruzi* requires the specific 24-alkylated sterols for cell viability and proliferation (38, 39). Amastigotes of *T. cruzi* produce no $\Delta^{5,7}$-sterols, indicating the absence of Δ^5-desaturase activity in the intracellular life stage of the parasite (36).
Leishmania species are similar to *T. cruzi* in their sterol content as they mainly contain C_{28}- and C_{29}-sterols with ergostane-based C_{28}-sterols being the most abundant in both amastigotes and promastigotes (40). *Leishmania* can take up and metabolize exogenous sterols (cholesterol) and seem to be rather tolerant of drug-induced alterations in their sterol content (34).

6.2 Sterols in *Trypanosoma brucei*

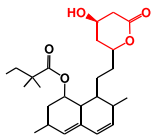
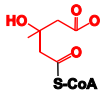
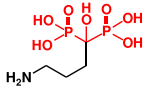
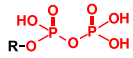
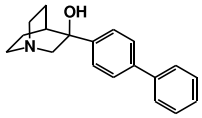
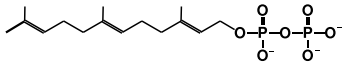
T. brucei procyclic forms (PCF) are capable of de novo SB (41) and 24-alkylated sterols are present, whereas bloodstream forms (BSF) almost exclusively contain cholesterol. Even though *T. brucei* BSF rely on absorption of host cholesterol via a receptor mediated LDL uptake mechanism – a process that was thought to inhibit de novo synthesis of C_{28}

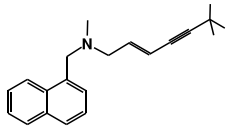
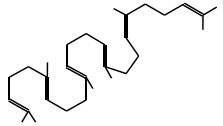
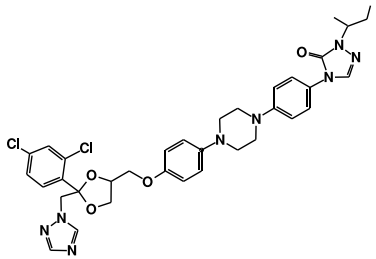
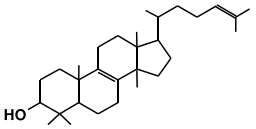
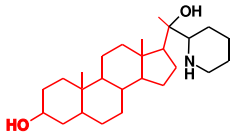
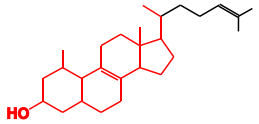
sterols (42), it has recently been shown that endogenous SB does occur in this form of the parasite (43). This finding is also supported by Northern blot analysis showing the presence of sterol 24-C-methyltransferase (24-SMT) mRNA as well as Western blot and activity determinations documenting the expression of the active enzyme in BSF parasites (44).

7 Sterol biosynthesis inhibitors – From Blockbusters to neglected tropical diseases

Sterols are of high pharmacological significance because there are several drug targets in sterol anabolism (Tab. 2): Cholesterol-lowering drugs (statins) are blockbusters in human health; there are a number of statins available on the market, including atorvastatin, which by 2003 became the best-selling pharmaceutical in history. Statins prevent the formation of mevalonate by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) leading to reduced cholesterol anabolism (45). Bisphosphonates inhibit the enzyme farnesyl diphosphate synthase curtailing isoprenoid synthesis and are used for the treatment of osteoporosis and other bone resorption diseases (46). Inhibition of squalene synthase (SQS) with zaragozic acids (47, 48) or quinuclidine-based molecules (49, 50) blocks sterol synthesis without interfering with synthesis of isoprenoids. Intermediate metabolites that accumulate due to SQS inhibition can be metabolized and excreted (51) making SQS an attractive target for cholesterol-lowering drugs.

Table 2. Compound classes of molecules 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. The molecular structure of a representative compound is compared to the substrate of the target enzyme. For structural mimics, similar parts in the molecular structure are highlighted in red.

Class	Target/Mechanism of action	Molecular structure	Target enzyme substrate	Uses	Reference
Statins	Statins are 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.	 Simvastatin	 HMG-CoA	Used as cholesterol-lowering drugs in humans	(45, 52)
Bisphosphonates (BPs)	BPs are 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.	 Alendronate	 Pyrophosphate (Geranyl diphosphate + isopentenyl diphosphate)	Used to treat osteoporosis and other bone resorption diseases	(46, 53, 54)
Quinuclidines/ zaragozic acids	Inhibition of squalene synthase (SQS). Quinuclidines may inhibit SQS by acting as carbocation mimics for farnesyl pyrophosphate (FPP) to squalene conversion. The aryl units may act as isosteres for the isoprenyl subunits in the farnesyl chain.	 3-Biarylquinuclidine	 SQS assembles two molecules of FPP into squalene	Not in use as sterol biosynthesis inhibitor (SBI)	(49)

Allylamines	Allylamines specifically inhibit fungal squalene epoxidase (= squalene monooxygenase).	 Terbinafine	 Squalene	Used for the topical treatment of fungal infections	(55, 56)
Azoles	Azoles bind as the sixth ligand to the Fe^{II} of the heme cofactor in lanosterol 14 α-demethylase (= CYP51) thus occupying the active site and acting as non-competitive inhibitors. Blocking the synthesis of ergosterol leads to the accumulation of methylated sterol precursors.	 Itraconazole	 Lanosterol	Used to treat fungal infections.	(57, 58)
Azasterols	Evidence from yeast shows that azasterols inhibit the enzyme sterol 24-C-methyltransferase (24-SMT).	 22,26-azasterol	 Zymosterol	Not in use as SBI	(59)

The allylamine drugs terbinafine is used for the topical treatment of fungal infections. Terbinafine inhibits fungal squalene epoxidase (= squalene monooxygenase) but not the orthologous enzyme in mammals leading to a depletion of ergosterol in fungi without affecting cholesterol biosynthesis in humans (56). Azoles, inhibitors of sterol 14α-demethylase, are widely used as antifungals – both in human health and crop protection. Finally, azasterols inhibit the ergosterol-synthetic enzyme 24-SMT, which is not involved in cholesterol synthesis and thus not found in mammalian cells. This potentially renders azasterols highly selective for fungi and trypanosomatids.

Given the close resemblance of trypanosomatids and fungi regarding their sterol metabolism, the repurposing of fungicides as anti-parasitic drugs is a promising approach. Indeed, many candidates that are currently investigated as new drugs against *Leishmania* and *Trypanosoma* infections have initially been developed as anti-fungal agents, markedly the azoles, especially posaconazole and ravuconazole (phase II clinical trials for Chagas' disease) or Amphotericin B, which is already in use for CL and VL.

8 Sequence Comparison of Drug Targets in Sterol Biosynthesis

To determine exactly how similar trypanosomatids and fungi are on an enzymatic level, I compared their amino acid sequences of seven SB enzymes that are known drug targets (Fig. 2). I also included sequences from *E. coli*, *P. falciparum*, *A. thaliana* and *H. sapiens*. HMG-CoA reductase, squalene synthase, squalene monooxygenase (squalene epoxidase) and sterol 14-demethylase are absent in bacteria and apicomplexa but present in plants, trypanosomatids, yeast and humans as shown in the respective triangle plots. The DOXP/MEP pathway has been identified in plants (chloroplast), many eubacteria and apicomplexan parasites, whereas the MVA pathway is found in animals, plants (cytosol), fungi, and archaea (60). Consequently, orthologs of DOXP reductoisomerase, which catalyses the reductive isomerization of DOXP to MEP and is the target of the antibiotic fosmidomycin, were only found in *P. falciparum*, *A. thaliana* and *E. coli*.

HMG-CoA-reductase

	E.c.	P.f.*	A.t.	L.m.	T.c.	T.b.	S.c.
P.f.*	0						
A.t.	0	16					
L.m.	0	15	51				
T.c.	0	16	50	65			
T.b.	0	14	53	63	78		
S.c.	0	17	46	50	51	51	
H.s.	0	17	45	50	50	50	40

Squalene monooxygenase

	E.c.*	P.f.*	A.t.	L.m.	T.c.	T.b.	S.c.
P.f.*	18						
A.t.	23	17					
L.m.	21	15	34				
T.c.	21	16	35	58			
T.b.	20	16	34	55	58		
S.c.	21	20	36	32	32	33	
H.s.	21	18	41	33	34	31	37

DOXP reducto-isomerase

	E.c.	P.f.	A.t.	L.m.	T.c.*	T.b.	S.c.*
P.f.	36						
A.t.	44	38					
L.m.	0	0	0				
T.c.*	13	16	15	0			
T.b.	0	0	0	0	0		
S.c.*	18	19	23	0	17	0	
H.s.*	16	14	21	0	21	0	15

Sterol 14-demethylase

	E.c.	P.f.	A.t.	L.m.	T.c.	T.b.	S.c.
P.f.	0						
A.t.	0	0					
L.m.	0	0	31				
T.c.	0	0	30	75			
T.b.	0	0	32	77	83		
S.c.	0	0	31	29	28	28	
H.s.	0	0	38	32	32	32	37

Farnesyl-PP-synthase

	E.c.	P.f.[a]	A.t.[b]	L.m.	T.c.	T.b.	S.c.
P.f.[a]	20						
A.t.[b]	43	22					
L.m.	22	28	22				
T.c.	21	26	19	64			
T.b.	21	29	22	63	70		
S.c.	24	36	21	35	32	33	
H.s.	22	31	19	37	33	38	45

Sterol 24-C-methyltransferase

	E.c.*	P.f.*	A.t.	L.m.	T.c.	T.b.	S.c.
P.f.*	21						
A.t.	18	20					
L.m.	16	19	47				
T.c.	17	21	47	66			
T.b.	18	18	45	63	67		
S.c.	17	21	51	44	43	41	
H.s.*	18	17	21	20	19	19	21

Squalene synthase

	E.c.	P.f.	A.t.	L.m.	T.c.	T.b.	S.c.
P.f.	0						
A.t.	0	0					
L.m.	0	0	43				
T.c.	0	0	43	58			
T.b.	0	0	42	54	62		
S.c.	0	0	40	37	39	35	
H.s.	0	0	42	39	40	39	43

* No true homolog but the best hit that matched the inclusion criteria, even though it is not annotated as the enzyme the profile was built for.

"0" indicates that there was no sequence found that matched the inclusion criteria (E value < 10).

Figure 2. Triangle plots showing the percent sequence idendity from a global alignment between representatives of bactaria (*Escherichia coli*, E.c.), apicomplexa (*Plasmodium falciparum*, P.f.), plants (*Arabidopsis thaliana*, A.t.), trypanosomatids (*Leishmania major*, L.m.; *Trypanosoma cruzi*, T.c.; *Trypanosoma brucei*, T.b.), fungi (*Saccharomyces cerevisiae*, S.c.) and human (*Homo sapiens*, H.s.) for n=7 enzymes involved in sterol anabolism. Colour code: <20%, white; between 20% and 80%, gradually from white to black; >80%: black. [a] In *P. falciparum* 3D7 no farnesyl pyrophosphate synthase (FPPS) is annotated, only geranylgeranyl pyrophosphate synthase (GGPPS) which returned the highest score. In *P. cynomolgy* and *P. yoelii* there is a FPPS. [b] In *A. thaliana* FPPS1 is annotated, but GGPP1 returned a higher score.

In humans, trypanosomatids and yeast DOXP reductoisomerase is absent as are all other enzymes that belong to the MEP/DOXP pathway (61). Farnesyl/geranyl diphosphate synthase was found in all genomes analysed including *E. coli*. Finally, sterol 24-methyltransferase orthologs were found in the *A. thaliana*, *L. major*, *T. cruzi*, *T. brucei* and *Saccharomyces cerevisiae* genome, confirming the absence of this protein in humans.

8.1 Sequence Similarity

Not surprisingly, for all enzymes (except DOXP reductoisomerase) the highest sequence similarity observed was between *T. brucei* and *T. cruzi* attaining over 80% in the case of sterol 14-demethylase. Sequences are remarkably conserved even across kingdoms. Generally, trypanosomatids' sequences are – in decreasing order – most similar to *A. thaliana*, human and yeast sequences. Whereas the sequence identity between yeast and trypanosomatid orthologs is not particularly high, it is noteworthy that they show the same genomic makeup of SB enzymes, i.e. the same enzymes are present or absent in both, yeast and trypanosomatids. This is indication of convergent evolution. However, the underlying selective forces remain obscure as the biology of trypanosomatids and fungi is so different. Could it just be a coincidence that both rely on ergosterol as major sterol?

9 Research and development (R&D) project portfolios for neglected tropical diseases

The WHO currently lists 17 diseases as Neglected Tropical Diseases (NTDs) resulting from four different classes of pathogens: viruses, protozoa, helminths and bacteria. Control of these diseases can only be achieved by an integrated approach including i.a. vector control, strengthening of public health systems, improving access to treatment, and drug and vaccine development. For NTDs there is no incentive for the development of new diagnostic tools, drugs and vaccines because these diseases only affect people in low income countries. In the last ten to twenty years Product Development Partnerships (PDPs) have been created that focus on pharmaceutical product development for NTDs. Thanks to PDPs such as Medicines for Malaria Venture, Roll Back Malaria, the Global

Fund and many others there has been considerable progress in the fight against infectious diseases such as AIDS, tuberculosis and malaria. The Drugs for Neglected Diseases Initiative (DNDi) was founded in 2003 and aims to develop new treatments for neglected diseases. DNDi's main focus is on the three protozoan NTDs Chagas' disease, HAT and leishmaniasis. Figure 3 summarizes their portfolio including all current R&D projects that aim to develop and provide access to new drugs for these three diseases.

For HAT, the focus lies on fexinidazole (62, 63) which is currently undergoing two phase II/III clinical trials, one for early second stage and first stage adults and another for children between 6 and 14 years of age. Earlier trials looked very promising and hopes are high that fexinidazole becomes the first new drug to be approved for the treatment of HAT in over thirty years. Since fexinidazole has also shown potent activity against *L. donovani* in vitro and in vivo in a VL mouse model, a phase II proof-of-concept study plans to evaluate fexinidazole for the treatment of primary VL patients in Sudan. Other projects in the HAT portfolio include development of SCYX-7158 (Jacobs et al., 2011) – a molecule belonging to the unique boron-based chemical class of the oxaboroles – or facilitating the implementation of NECT.

The portfolio for leishmaniasis is dominated by development and implementation of new treatments for VL. Large-scale studies are conducted to demonstrate the feasibility of implementing new treatment modalities recommended by the WHO (miltefosine-paromomycin, AmBisome®-miltefosine, AmBisome®-paromomycin, singledose AmBisome®) in primary healthcare settings in Bangladesh and India. In Africa several projects are on-going with the aim to facilitate implementation of and access to sodium stibogluconate & paromomycin combination therapy (SSG&PM) in key endemic areas of East Africa. Safety and effectiveness of SSG&PM is monitored post-implementation in a pharmacovigilance study. Another study evaluates the efficacy of a combination regimen of AmBisome® with miltefosine, and of AmBisome® (at a higher dose) monotherapy in Ethiopian patients co-infected with VL and HIV.

The pipeline for new treatments for Chagas' disease looks less auspicious. Posaconazole and E1224 (a prodrug of ravuconazole) – both triazolic molecules – are the only new candidates that entered clinical trials. K777, which inhibits cruzain, a key protease required for the survival of *T. cruzi*, as well as two interesting candidates from the fenarimol series are only in the pre-clinical phase. A population pharmacokinetic study of the newly registered paediatric dosage form of benznidazole is underway with the

objective to facilitate implementation of and access to this treatment. Posaconazole is thus the most promising new chemical entity (NCE) for the treatment of Chagas' disease and discontinuation of this candidate would seriously aggravate the situation in the already slim Chagas' portfolio.

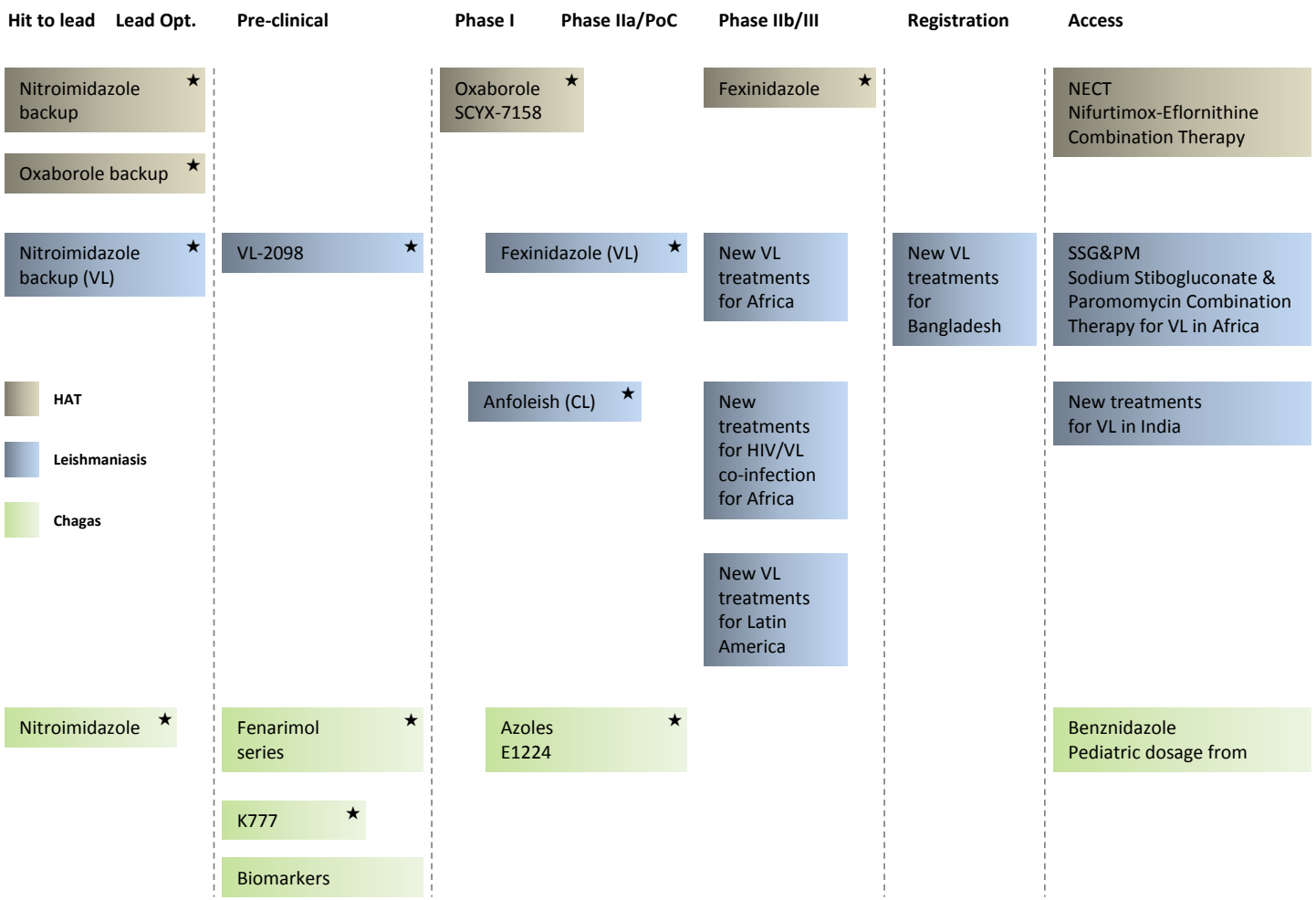


Figure 3. DNDi R&D project portfolio for Human African Trypanosomiasis (HAT), Leishmaniasis (CL, cutaneous leishmaniasis; VL, visceral leishmaniasis) and Chagas' Disease. As of 2013, four new treatments have been made available and eleven new chemical entities (★) were in the pipeline. Adapted from dndi.org.

★ New Chemical Entity (NCE)

Fexinidazole (for HAT and VL) = 1 NCE

10 Bioinformatics

Today, thousands of genome sequences from all domains of life are available. The wealth of genomic data allows the comparison of different genomes covering the whole spectrum from inter-domain to intra-species level. With the human genome published, it was feasible to compare genomes of model organisms to *Homo sapiens* in order to learn about evolution and human diseases. Ever since, researchers have used comparative genomics to aid drug development. In particular, identification of drug targets can be facilitated by identifying similarities and differences between host and pathogen on a genomic level. A promising strategy to pinpoint vulnerable points for chemotherapeutic attack in parasites is to look for essential parasite enzymes that are not present in the host (64). It is important to keep in mind, however, that even though genome analyses can contribute to efficient drug development, they do not replace the wet lab experiment. As will be shown in Chapter 2 in silico results are not always predictive for in vitro or in vivo outcome.

11 Aims and Objectives

The overall aim of this PhD thesis was to scrutinize the potential of sterol anabolism as a target for new drugs against trypanosomatids. I was focussing on Chagas' disease caused by *T. cruzi* because for this disease there is an especially urgent need for new drugs. The thesis' structure reflects the process of progressing differentiation I went through during my work. Chapter 2 presents a holistic view on sterol metabolism in eukaryotes albeit with a clear focus on parasites and trypanosomes in particular. The next chapter describes the probing of different SBIs on yeast strains as well as parasites and mammalian cells in an attempt to shed light on the mechanism of action of these drugs: The specificity of a set of SBIs was assessed using three genetically modified *S. cerevisiae* strains: (i) wild type producing ergosterol, (ii) a cholesterol producing strain and (iii) a strain that produces an intermediate sterol (cholesta-5,7,24-trienol). In the concluding chapter I summarize the lessons learned in this PhD thesis and present the quintessence of the work which ultimately resulted in a proposed way forward for the top-candidate in the drug discovery for Chagas' disease, posaconazole. The following specific objectives were achieved during my PhD thesis:

(i) Development of an in silico pipeline to globally evaluate sterol metabolism and perform comparative genomics (Chapter 2)

(ii) Investigation of ergosterol-specificity in the mechanism of action of SBIs, especially azoles and azasterols (Chapter 3)

(iii) Proposing a combination partner for posaconazole in order to improve efficacy of a new therapy to treat Chagas' disease (Chapter 4)

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Chapter 2

Genome Profiling of Sterol Synthesis Shows Convergent Evolution in Parasites and Guides Chemotherapeutic Attack

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Genome profiling of sterol synthesis shows convergent evolution in parasites and guides chemotherapeutic attack

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Abstract Sterols are an essential class of lipids in eukaryotes, where they serve as structural components of membranes and play important roles as signaling molecules. Sterols are also of high pharmacological significance: cholesterol-lowering drugs are blockbusters in human health, and inhibitors of ergosterol biosynthesis are widely used as antifungals. Inhibitors of ergosterol synthesis are also being developed for Chagas's disease, caused by *Trypanosoma cruzi*. Here we develop an in silico pipeline to globally evaluate sterol metabolism and perform comparative genomics. We generate a library of hidden Markov model-based profiles for 42 sterol biosynthetic enzymes, which allows expressing the genomic makeup of a given species as a numerical vector. Hierarchical clustering of these vectors functionally groups eukaryote proteomes and reveals convergent evolution, in particular metabolic reduction in obligate endoparasites. We experimentally explore sterol metabolism by testing a set of sterol biosynthesis inhibitors against trypanosomatids, *Plasmodium falciparum*, *Giardia*, and mammalian cells, and by quantifying the expression levels of sterol biosynthetic genes during the different life stages of *T. cruzi* and *Trypanosoma brucei*. The phenotypic data correlate with genomic makeup for simvastatin, which showed activity against trypanosomatids. Other findings, such as the activity of terbinafine against *Giardia*, are not in agreement with the genotypic profile.—Fügi, M. A., K. Gunasekera, T. Ochsenreiter, X. Guan, M. R. Wenk, and P. Mäser. **Genome profiling of sterol synthesis shows convergent evolution in parasites and guides chemotherapeutic attack. *J. Lipid Res.* 2014.** 55: **929–938.**

Supplementary key words sterol metabolism • sterol biosynthesis inhibitor • comparative genomics • hidden Markov model • hierarchical clustering • *Trypanosoma* • *Giardia*

Sterols are an intriguing class of metabolites, ubiquitous in eukaryotes but largely absent from archaea and bacteria. Sterols in eukaryotes serve multiple physiological functions associated with two main purposes: as structural components of membranes (bulk function) and as precursors to signaling molecules that regulate growth and development (sparking function). The sterol biosynthetic pathways encompass phylum-specific branches that yield distinctive end products. Cholesterol is the most abundant sterol in vertebrates, where it is an essential component of cell membranes and acts as a precursor to vitamin D and steroid hormones. Invertebrates such as *Caenorhabditis elegans* or *Drosophila melanogaster* are sterol auxotrophs that rely on uptake of exogenous sterols (1–3). Fungi synthesize ergosterol, which has a greater degree of unsaturation than cholesterol and an additional methyl group at C-24. Plants make a large variety of phytosterols, such as the 24-alkylated campesterol, sitosterol, or stigmasterol. Sterol diversity may be equally high in the protozoa, involving cholesterol and stigmasterol in *Paramecium* (4), ergosterol and stigmasterols in *Acanthamoeba* (5), cycloartenol and cyclolaudenol in *Dicytostelium* (6), and ergosterol in trypanosomatids (7, 8).

The sterol biosynthetic pathways involve more than 40 enzymes (**Table 1**). Isopentenyl diphosphate, the building block of isoprenoids, is made either from acetyl-CoA via the MEV pathway or from pyruvate plus glyceraldehyde 3-phosphate via the deoxyxylulose 5-phosphate (DOXP) pathway (**Fig. 1**). The condensation of two molecules of farnesyl diphosphate to produce squalene is the committing step of sterol synthesis. From there, the synthesis proceeds via lanosterol and zymosterol to cholesterol and ergosterol derivatives, or via cycloartenol to the various kinds of phytosterols (Fig. 1). The enzymes that mediate sterol biosynthesis are of dual interest: First, there is a pharmacological interest because sterol biosynthesis

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Abbreviations: DOXP, deoxyxylulose 5-phosphate; MEP, methylerythritol 4-phosphate; MEV, mevalonate; PC, principal component; SBI, sterol biosynthesis inhibitor; SLT, spliced-leader trapping.

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TABLE 1. Sterol biosynthetic enzymes

#	EC	KEGG	Enzyme Name
1	2.3.1.9	K00626	Acetyl-CoA C-acetyltransferase
2	2.3.3.10	K01641	Hydroxymethylglutaryl-CoA synthase
3	1.1.1.34	K00021	Hydroxymethylglutaryl-CoA reductase (NADPH)
4	1.1.1.88	K00054	Hydroxymethylglutaryl-CoA reductase
5	2.7.1.36	K00869	Mevalonate (MEV) kinase
6	2.7.4.2	K00938	Phospho-MEV kinase
7	4.1.1.33	K01597	Diphospho-MEV decarboxylase
8	2.2.1.7	K01662	1-Deoxy-D-xylulose-5-phosphate synthase
9	1.1.1.267	K00099	1-Deoxy-D-xylulose-5-phosphate reductoisomerase
10	2.7.7.60	K00991	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
11	2.7.1.148	K00919	4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase
12	4.6.1.12	K01770	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
13	1.17.7.1	K03526	(*E*)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase
14	1.17.1.2	K03527	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase
15	5.3.3.2	K01823	Isopentenyl-diphosphate delta-isomerase
16	4.2.3.27	K12742	Isoprene synthase
17	2.5.1.1	K00787	Geranyl diphosphate synthase
18	2.5.1.68	K12503	Short-chain *Z*-isoprenyl diphosphate synthase
19	2.5.1.86	K14215	*Trans*,poly*cis*-decaprenyl diphosphate synthase
20	2.5.1.10	K00787	Farnesyl diphosphate synthase
21	2.5.1.21	K00801	Farnesyl-diphosphate farnesyltransferase
22	1.14.13.132	K00511	Squalene monooxygenase
23	5.4.99.7	K01852	Lanosterol synthase
24	1.14.13.70	K05917	Cytochrome P450, family 51 (CYP51; sterol 14-demethylase)
25	1.3.1.70	K00222	Delta14-sterol reductase
26	1.14.13.72	K07750	Methylsterol monooxygenase
27	1.1.1.170	K07748	Sterol-4α-carboxylate 3-dehydrogenase (decarboxylating)
28	1.1.1.270	K09827	3-Keto steroid reductase
29	5.3.3.5	K01824	Cholestenol delta-isomerase
30	1.3.1.72	K09828	Delta24-sterol reductase
31	1.14.21.6	K00227	Lathosterol oxidase
32	1.3.1.21	K00213	7-Dehydrocholesterol reductase
33	3.1.1.13	K01052	Lysosomal acid lipase/cholesteryl ester hydrolase
34	2.3.1.26	K00637	Sterol *O*-acyltransferase
35	1.14.13.13	K07438	Calcidiol 1-monooxygenase
36	1.14.13.126	K07436	Vitamin D3 24-hydroxylase
37	2.1.1.41	K00559	Sterol 24-C-methyltransferase
38	5.-.-.-	K09829	C-8 sterol isomerase
39	1.3.1.71	K00223	Delta24[24 (1)]-sterol reductase
40	5.4.99.8	K01853	Cycloartenol synthase
41	2.1.1.143	K08242	24-Methylenesterol C-methyltransferase
42	n.a.	K09832	Cytochrome P450, family 710, subfamily A

EC, Enzyme Commission number; KEGG, Kyoto Encyclopedia of Genes and Genomes identifier.

inhibitors (SBIs) are widely deployed as chemotherapeutics. As indicated in Fig. 1, human HMG-CoA reductase serves as the target of cholesterol-lowering statins such as simvastatin (9). Human farnesyl diphosphate synthase is the target of bisphosphonates (e.g., tiludronate), used against osteoporosis (10). Squalene epoxidase and sterol 24-methyltransferase are antifungal targets, inhibited by allylamines (e.g., terbinafine) and azasterols, respectively (11–14). A particularly promising target is sterol 14-demethylase (CYP51). Azole inhibitors of CYP51 (e.g., ketoconazole) are widely used for fungal infections and, because trypanosomatid parasites also make ergosterol (15–17), lend themselves for a piggyback approach toward the urgently required new drugs for Chagas's disease. The latest antifungal approved by the U.S. Food and Drug Administration, posaconazole, was shown to be highly active against *Trypanosoma cruzi* in culture and in vivo (18–20), and it is currently undergoing phase 2 clinical trials for the treatment of Chagas's disease. Second, sterol biosynthetic enzymes are of phylogenetic importance. Sterols were proposed to hold a key to understanding eukaryote phylogeny because the evolution of eukaryotes is thought to be interlinked with that of the sterols (21).

Here we present an in silico pipeline for comparative genomics of eukaryotes based on prediction of sterol biosynthetic enzymes. Our aim is to shed light on the evolutionary relationships of these enzymes and to identify new antiparasitic drug target candidates. Focusing on trypanosomatids, we further compare the stage-specificity of expression of the sterol biosynthetic enzymes, and we probe the potential of selected SBIs against a panel of parasites.

MATERIALS AND METHODS

Sequences

Proteome files were downloaded from Uniprot (22), EuPathDB (23), and Integr8 (24). The predicted proteomes were tested for completeness against the 100 most conserved proteins of the Core Eukaryotic Genes Mapping Approach database (25), which we had determined based on HMMer 3.0 profile (26, 27) searches of eukaryote reference proteomes (*C. elegans*,

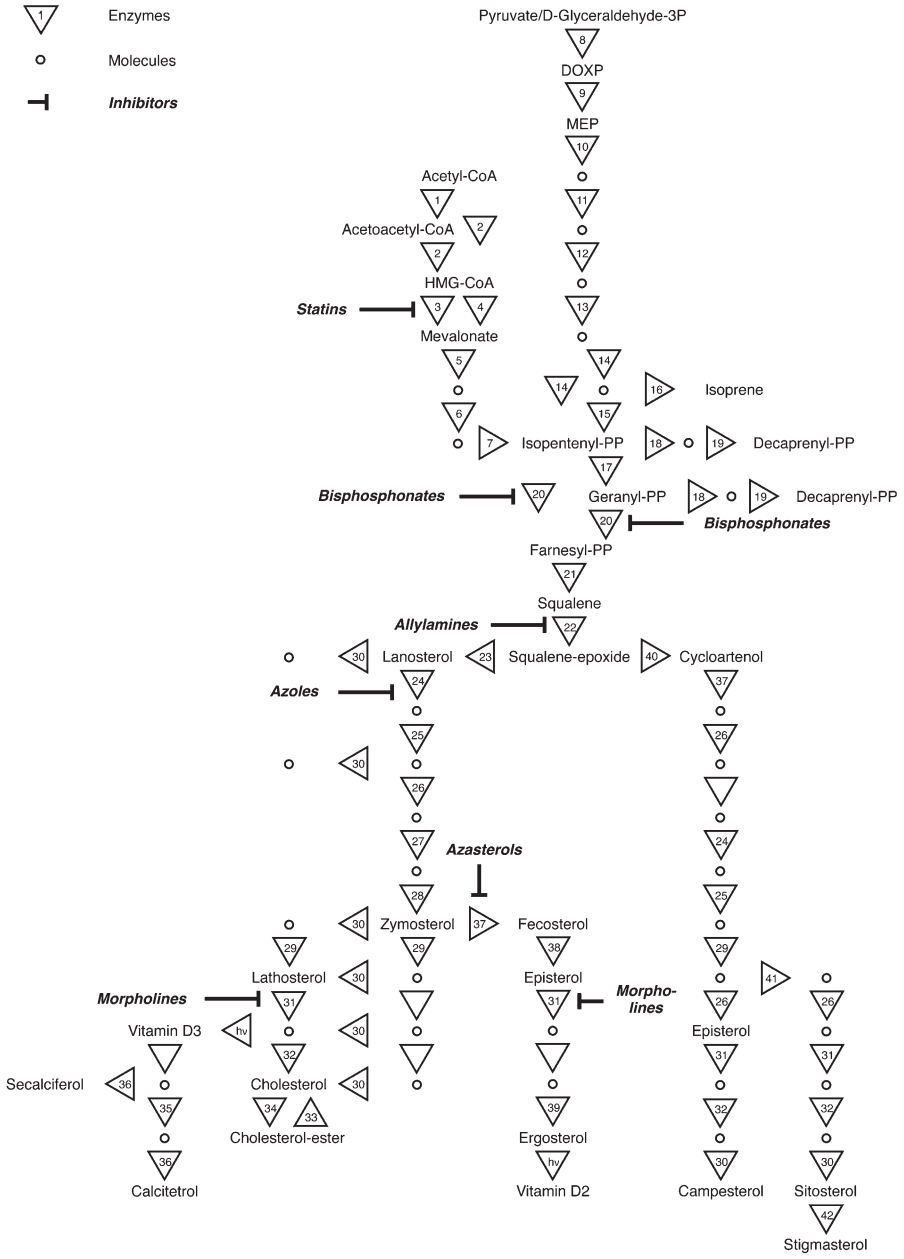

Fig. 1. Overview on sterol biosynthesis. Enzymes are represented by triangles, metabolites by circles. Inhibitors are indicated in bold. Key metabolites are spelled out. See Table 1 for enzyme name EC classifiers.

Chlamydomonas reinhardtii, *D. discoideum*, *D. melanogaster*, *D. rerio*, *E. cuniculi*, *Entamoeba histolytica*, *G. lamblia*, *Homo sapiens*, *K. lactis*, *L. major*, *M. musculus*, *Plasmodium falciparum*, *S. cerevisiae*, *S. pombe*, *Trypanosoma brucei*, *T. cruzi*, *T. parva*, and *T. vaginalis*). Additional proteomes were included only if *hmmscan* of HMMer 3.0 returned a hit for at least 99 profiles with an expectancy (*E*) value $\leq 10^{-30}$. Redundancy-reduced sets of reference sequences for the enzymes of interest (Table 1) were downloaded from UniRef90 (28).

Chemicals

Simvastatin (S6196-25MG), tiludronate disodium salt hydrate (T4580-10MG), terbinafine hydrochloride (T8826-100MG), and ketoconazole (K1003-100MG) were purchased from Sigma-Aldrich. Fenpropimorph was kindly offered by M. Witschel from BASF. The test compounds were dissolved in dimethyl sulfoxide at 10 mg/ml and stored at −20°C. Resazurin sodium salt (Alamar Blue) was purchased from Sigma-Aldrich (R7017-1G), and Chlorophenol red galactopyranoside (CPRG) from Roche (10884308001).

Cell lines

The cell lines used for in vitro drug sensitivity determination were rat L6 myoblasts, *T. brucei rhodesiense* STIB 900, *T. cruzi* Tulahuen C2C4 (expressing *Escherichia coli* β-galactosidase), *Leishmania donovani* MHOM-ET-67/L82 axenic amastigotes, *Giardia intestinalis* strain G1, and *P. falciparum* strain K1.

Bioinformatics

Multiple alignments were performed with ClustalW 2.0.10 (29). All profile constructions and searches were carried out with HMMer 3.0 (26). The heat map was produced with the R library *gplots* (30). Hierarchical clustering was performed with the R library *Pvclust*, using Canberra distance and the McQuitty algorithm (31). Pvclust assesses uncertainty in hierarchical cluster

analysis by implementing multiscale bootstrap resampling (n = 10,000) to estimate "approximately unbiased" (*au*) errors, where $P = (100 - au)/100$. For principal component (PC) analysis we used basic R functions and *ggplot2*. The analyses were automated with Unix shell scripts and with Perl scripts. The phylogenetic trees were constructed from amino acid sequences with Mega 5, using the neighbor-joining algorithm and Jones-Taylor-Thornton substitution model. The number of bootstrap replications was 1,000.

Gene expression

Tag counts of the sterol biosynthesis enzymes were extracted from three published *T. brucei* short-read libraries (long slender bloodstream forms, short stumpy bloodstream forms, and procyclic tsetse fly midgut forms) and from four *T. cruzi* short-read libraries (intracellular amastigotes, trypomastigotes, epimastigotes, and metacyclics). The libraries had been produced by Illumina sequencing using the spliced-leader trapping (SLT) protocol (32). Numbers of reads were normalized by using the DESeq (33) bioconductor package. The gene expression data were modeled with *prcomp* as a numerical matrix of *M* genes times *N* libraries. Eigenvalues and orthogonal eigenvectors were computed based on the square-symmetrical correlation matrix.

In vitro drug sensitivity

In vitro drug sensitivity assays were performed as described (34–37). The tests were done over 72 h of incubation, except for the *T. cruzi* assay, which lasted 96 h. For L6 cells, *L. donovani*, *T. brucei*, and *G. intestinalis*, the redox-sensitive dye resazurin (Alamar Blue) served as an indicator of cell viability. For *P. falciparum*, incorporation of ^{3}H-hypoxanthine was used. For *T. cruzi*, β-galactosidase activity was quantified with the substrate CPRG/Nonidet. IC_{50} values were estimated by linear interpolation based on the semilogarithmic dose-response curves.

RESULTS AND DISCUSSION

Sterol metabolic profiling of eukaryote genomes

Aiming for a broad overview on sterol metabolism, we assembled a list of 42 relevant enzymes ranging from terpenoid backbone synthesis over squalene synthase to the formation of the different sterols and vitamin D derivatives as outlined in Fig. 1. For each enzyme, all the amino acid sequences that had been annotated in the manually curated section of UniProt with the corresponding EC number were retrieved (Table 1). Each of these sequence sets was redundancy reduced to 90%. Then a ClustalW multiple alignment was performed and converted to a position-dependent scoring matrix with *hmmbuild* of HMMer 3.0. The resultant 42 profiles were concatenated to a hidden Markov model (HMM) library for terpenoid backbone and sterol biosynthetic enzymes. This library was used for an in silico screen of eukaryote proteomes. For each proteome and each enzyme, we retrieved the profile-alignment score of the protein that had returned the lowest expectancy (*E*) value. This approach allowed organizing the data in an unbiased, quantitative way, by plotting the obtained high scores as a two-dimensional heat map where the horizontal cross-sections represent the "sterol biosynthetic profile" of a given organism (**Fig. 2**).

The known differences among the analyzed eukaryotes were obvious. The absence of the MEV pathway (enzyme nos. 2 to 7 of Table 1) and the presence of the MEP/DOXP pathway (enzyme nos. 8 to 14) were apparent in most apicomplexan species, with the exception of *Cryptosporidium parvum*, which lacked either pathway (Fig. 2, top). This is in agreement with *C. parvum* having lost the plastid genome in the course of evolution (38). It was interesting to note that *E. histolytica* was also deficient in either pathway, a finding that, to our knowledge, had not been reported before (39). *Entamoeba* was reported to be capable of limited cholesterol synthesis (40). Our data, however, support earlier reports suggesting an absolute requirement for cholesterol by *Entamoeba* (41). The vascular plants possessed both pathways, but the green algae *C. reinhardtii* and *Ostreococcus tauri* only had the MEP/DOXP arm, a fact that had been elegantly demonstrated for *C. reinhardtii* and other green algae by feeding experiments with radiolabeled glucose (42).

As expected, the sterol 24-C-methyltransferases SMT1 and SMT2 (enzyme nos. 37 and 41), characteristic of ergosterol synthesis, were absent in all vertebrates. The genome-wide screen also confirmed the presence of a set of sterol synthetic enzymes, including 24-C-methyltransferases, in trypanosomatids. Surprisingly, though, the trypanosomatids lacked sterol *O*-acyltransferase as well as cholesteryl ester hydrolase (enzyme nos. 34 and 33, respectively), and yet, *T. brucei* had been shown to be capable of sterol esterification (43), suggesting that trypanosomatids might use unusual enzymes for ester synthesis and hydrolysis.

Only 2 of the 42 sterol metabolic enzymes were present in all the analyzed proteomes: geranyl/farnesyl diphosphate synthase (enzyme nos. 17 and 20, respectively). Thus, our proteome-wide profiling approach indicates that the synthesis of farnesylpyrophosphate is essential to all eukaryotes and that farnesylpyrophosphate is a metabolite of central importance to organisms, whether they use the MEV or the MEP/DOXP pathway. A possible explanation is that farnesylation and geranylation of proteins is essential to eukaryotes. This hypothesis is in agreement with the fact that farnesyltransferases (EC 2.5.1.29, 2.5.1.58, and 2.5.1.60) occur in all the analyzed eukaryotes as determined with a profile search analogous to those for sterol biosynthetic enzymes (data not shown).

Hierarchical clustering reveals convergent evolution

To detect less obvious and possibly new relationships regarding sterol metabolism of eukaryotes, we subjected the sterol metabolic profiles (Fig. 2) to hierarchical clustering. Every analyzed species was represented by a 42-tuple vector consisting of the best scores of the respective proteome to each profile. Hierarchical clustering of these vectors produced the "sterol biosynthesis tree" shown in **Fig. 3**, which basically subdivided the eukaryotes into species that make their own sterols (sterol prototrophs, left side) and species that do not (sterol auxotrophs, right side). The tree locally mirrored the phylogeny of the analyzed eukaryotes. The green plants

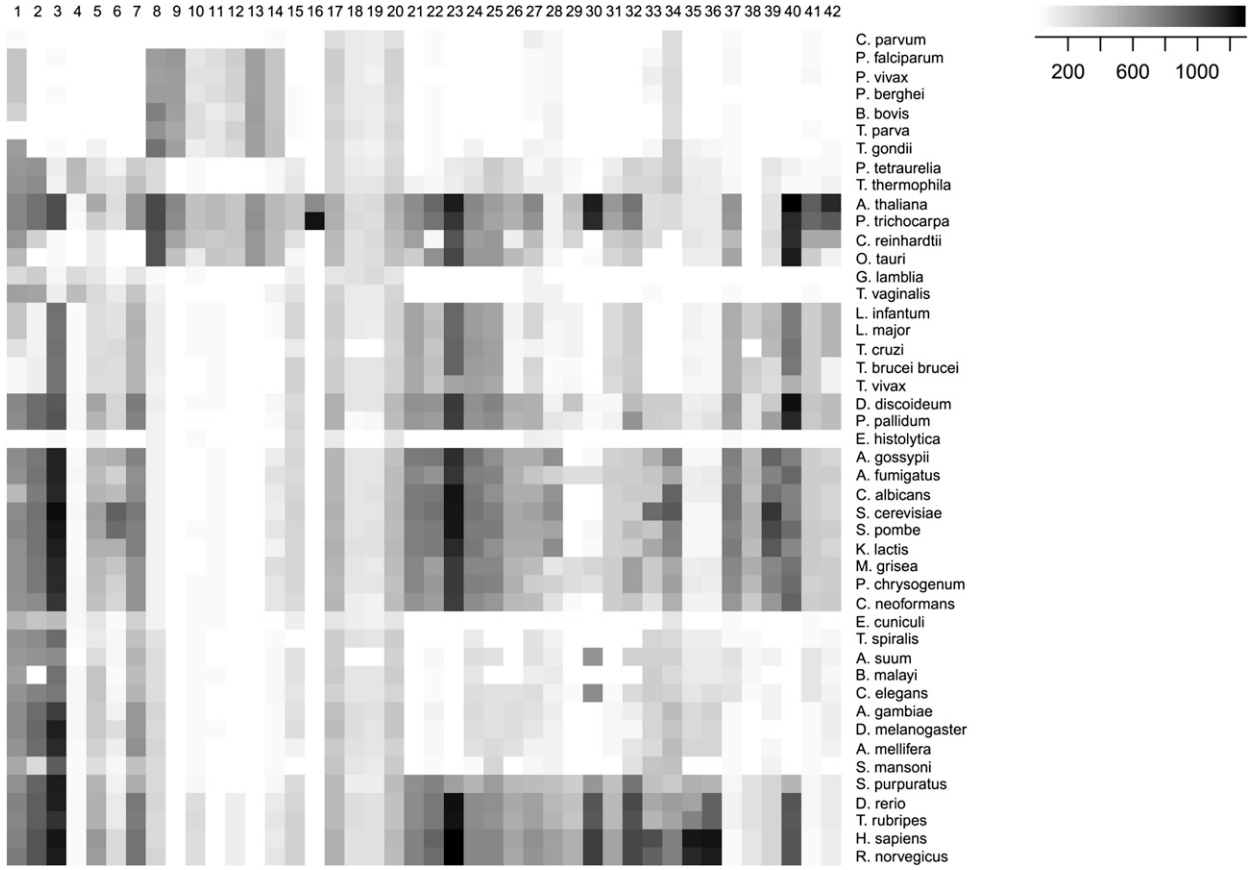

Fig. 2. Sterol biosynthetic profiles. The heat map denotes the best scores achieved by each proteome (rows, n = 46) against each profile for a sterol biosynthetic enzyme (columns, n = 42). See Table 1 for enzyme name and Fig. 1 for its position in the pathway.

cosegregated, as did the trypanosomatids, the protostomes, and the deuterostomes where the sea urchin *Strongylocentrotus purpuratus* clearly clustered with the chordates. The fungi formed a cluster except for *E. cuniculi*, and the apicomplexans formed a cluster except for *C. parvum*. These two outliers segregated with *G. lamblia* and *E. histolytica* in a phylogenetically diverse branch of amitochondriates (Fig. 3). The analyzed ciliates clustered with the insects; however, this association was not significant, and clearly, a larger number of ciliate genomes would be desirable to better resolve their position in the sterol biosynthesis landscape.

We interpret cases where clustering based on sterol biosynthesis enzymes does not coincide with eukaryote phylogeny as indicative of convergent evolution. Thus, the amoebazoon *Entamoeba* and the fungus *Encephalitozoon*, both obligate endoparasites, clustered together on the sterol-auxotrophic branch of the tree, whereas the free-living amoebazoa *D. discoideum* and *P. palladium* segregate on the sterol-prototrophic branch together with the free-living, or facultative parasitic, fungi (Fig. 3). A likely explanation of this clustering is that the eukaryote progenitor synthesized sterols de novo, whereas the obligate endoparasites independently lost the corresponding genes in adaptation to a parasitic lifestyle. The trypanosomatids are a notable exception: of all the included obligate endoparasites, they were the only species located on the sterol prototrophic arm of the tree (Fig. 3).

What is the origin of trypanosomatid ergosterol synthesis?

The sterol biosynthesis tree (Fig. 3) is based on functional predictions and cannot serve for phylogenetic models. To investigate the evolutionary origin of the sterol biosynthetic genes in trypanosomatids, we constructed phylogenetic trees for two key enzymes of ergosterol synthesis: lanosterol 14α-demethylase (enzyme no. 24 in Table 1), the target of azoles, and sterol 24-C-methyltransferase (enzyme no. 37), the dedicative enzyme for ergosterol synthesis. Both trees had a similar topology (except that sterol 24-C-methyltransferase does not occur in animals) with distinct branches for the major groups of eukaryotes and a highly significant separate branch for the included trypanosomatid sequences (Fig. 4). It was impossible to root the trees because there are no suitable outgroups for the two enzymes such as orthologs from bacteria. Acquisition of foreign genes by trypanosomes from plants has been suggested for glycosomal enzymes of *T. brucei* (44, 45). The phylogenetic trees of lanosterol 14α-demethylase and sterol 24-C-methyltransferase clearly do not support such a scenario for the sterol biosynthetic enzymes. Based on this bioinformatic analysis, one would exclude horizontal transfer as the evolutionary origin of trypanosomatid ergosterol synthesis.

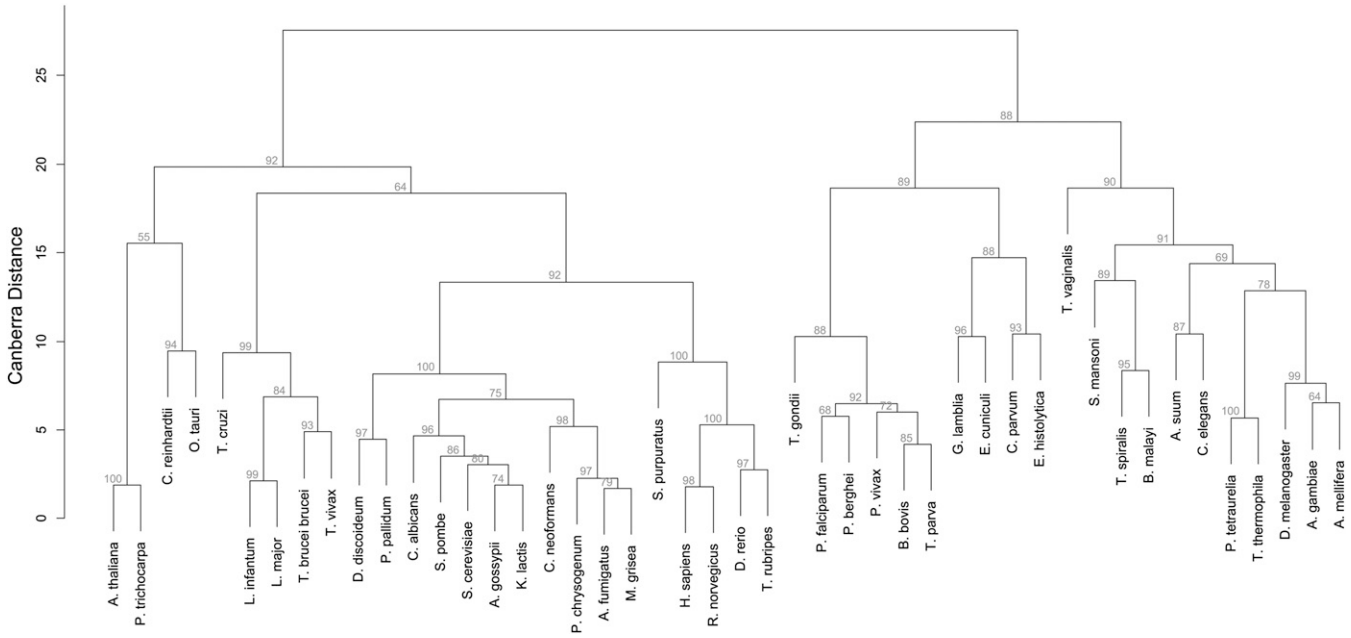

Fig. 3. Sterol biosynthetic tree of eukaryotes. The numerical vectors (rows of Fig. 2) were clustered based on Canberra distance and the McQuitty algorithm. Gray numbers are "approximately unbiased" errors (*au*), where $P = (100 - au)/100$.

Susceptibility of parasites to SBIs

Given the antichagasic potential of azoles, we tested a panel of further known inhibitors of sterol biosynthesis, as indicated in Fig. 1, against parasites (*P. falciparum*, *L. donovani*, *T. cruzi*, *T. brucei*, and *G. lamblia*) and mammalian cells (rat L6 myoblasts). IC_{50} values were determined in vitro (**Table 2**). As expected, the mammalian cells were rather tolerant to most of the tested drugs, except for simvastatin, which had an IC_{50} of 0.70 μM against the L6 cells. This result was surprising because simvastatin is widely used as a cholesterol-lowering agent. We also tested mouse embryonic fibroblast and also against lovastatin, with the same result: IC_{50} values clearly below 1 μM. Apparently, cholesterol synthesis was essential under our test conditions. Tiludronate was inactive against all the tested cells. The most potent compound against *T. cruzi* was ketoconazole, followed by simvastatin, which also showed a moderate activity against *T. brucei* (IC_{50} = 4.6 μM) and *L. donovani* (IC_{50} = 4.7 μM). The activity against *T. cruzi* was not conclusive due to the toxicity of simvastatin to L6 host cells (hence the parentheses in Table 2). Terbinafine, ketoconazole, and fenpropimorph were moderately active

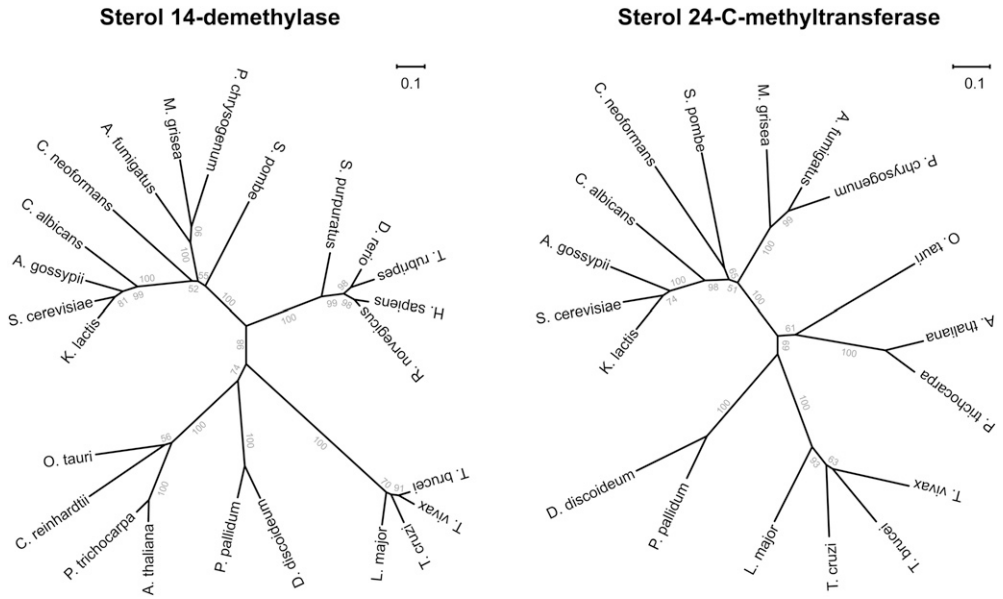

Fig. 4. Phylogenetic trees of key enzymes. Neighbor-joining trees of sterol 14-demethylase (enzyme no. 24), the target of azoles (A), and sterol 24-C-methyltransferase (enzyme no. 37), the dedicative enzyme of ergosterol synthesis (B). The scale bars indicate number of substitutions per site.

TABLE 2. Sensitivity[a] of mammalian cells[b] and parasites[c] to sterol antimetabolites

	L6	Pfa	Ldo	Tcr	Tbr	Gla
Simvastatin	0.70	28.43	4.66	(1.78)	4.58	45.20
Tiludronate	204.00	21.04	119.07	169.82	120.66	64.22
Terbinafine	40.59	6.07	15.95	21.32	88.45	0.62
Ketoconazole	22.25	4.01	28.93	0.01	26.28	10.67
Fenpropimorph	33.59	3.22	35.60	8.95	25.18	4.88

[a] IC_{50} in μM.
[b] L6 rat skeletal muscle cells.
[c] Gla, *G. lamblia*, Ldo, *L. donovani*; Pfa, *P. falciparum*; Tbr, *T. brucei*; Tcr, *T. cruzi*.

against *P. falciparum* with IC_{50} below 10 μM. Terbinafine was the only tested compound that showed a considerable effect on *Giardia* (IC_{50} = 0.62 μM). However, terbinafine inhibits squalene monooxygenase (enzyme no. 22), an enzyme that is absent in *Giardia* (Fig. 2). Comparing the in silico data of Fig. 2 with the in vitro data of Table 2, we would expect a negative correlation between the score of the profile search for a given enzyme and species and the IC_{50} of a known inhibitor of the same enzyme. As shown in **Fig. 5**, this was the case with simvastatin (Spearman rank order correlation coefficient $r_S = -0.89$, $P < 0.05$). For other compound-target pairs, there was either no correlation (ketoconazole and sterol 14-demethylase, $r_S = 0.06$) or a positive, albeit nonsignificant, correlation (tiludronate and farnesyl diphosphate synthase, $r_S = 0.31$; fenpropimorph and lathosterol oxidase, $r_S = 0.64$). For terbinafine and its presumed target squalene epoxidase (enzyme no. 22), there was even a significant positive correlation between profile score and IC_{50} ($r_S = 0.89$, $P < 0.05$; Fig. 5).

Stage-specific regulation of sterol biosynthetic enzymes in trypanosomes

A possible reason for the lack of correlation between genomic makeup and drug susceptibility is that a particular target enzyme may be differently expressed across the life-cycle stages of a parasite. We investigated the expression of sterol biosynthetic enzymes at the mRNA level in the trypanosomatids *T. brucei* and *T. cruzi* using previously published SLT data. SLT takes advantage of the conserved miniexon that is spliced in *trans* to all trypanosomal mRNA (46). We analyzed data from different stages of *T. brucei* (slender bloodstream form, stumpy bloodstream form, and procyclic tsetse-midgut form) and *T. cruzi* (intracellular amastigote form, trypomastigote form, and epimastigote triatomine-midgut form) (32, 47). For both species, we found marked differences between the life-cycle stages regarding the steady-state expression levels of sterol biosynthetic genes. Generally, we detected higher mRNA levels of genes involved in sterol biosynthesis in the insect stages than in the mammalian stages of the organisms. This is in good agreement with the availability of sterols for the parasite in a mammalian host. PC analysis was performed for genes with orthologs in both species (n = 31). Plotting the first two PCs revealed the proliferating insect stages of both *T. brucei* and *T. cruzi* to more closely align with PC-2 than with PC-1, and to positively correlate with either PC (**Fig. 6**). In contrast, the proliferating mammalian stages, as well as all the nonproliferating stages, more closely aligned with PC-1 and oppositely correlated with PC-2 (Fig. 6). Taken together, PC analysis of the steady-state expression levels of sterol biosynthetic genes singled out the insect stages of *T. brucei* as well as *T. cruzi* (Fig. 6), demonstrating parallel metabolic adaptations in African and South American trypanosomes.

CONCLUSION

Starting from the assumption that sterol synthesis is intrinsically linked to the evolution of eukaryotes, we performed comparative genomics based on the profiling of sterol biosynthetic enzymes (Fig. 1). The aim was to investigate convergent evolution in eukaryotes and possibly link this to chemotherapeutic strategies against parasites. We refrained from functionally annotating the analyzed

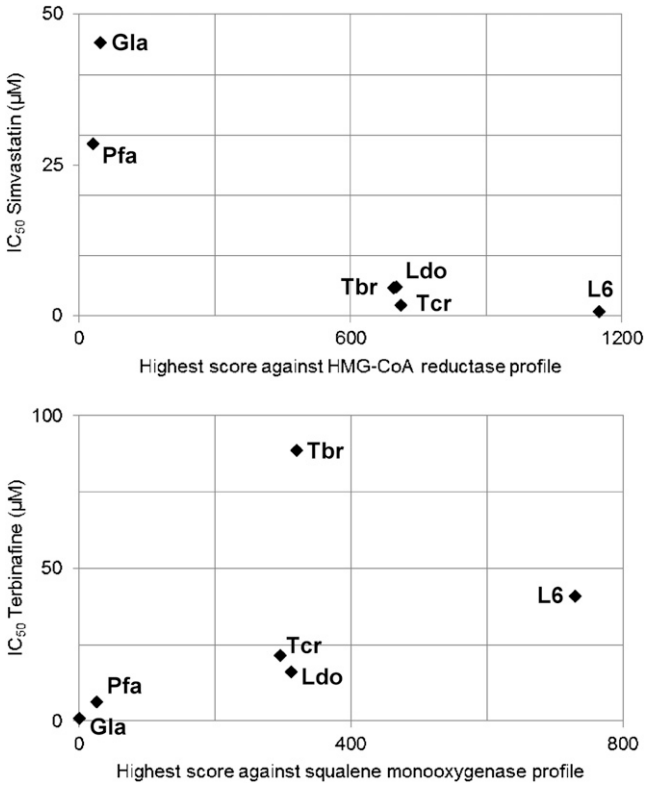

Fig. 5. Correlation of genotype and phenotype. For each species, the in vitro IC_{50} (in μM) of the drugs simvastatin (A) and terbinafine (B) is plotted versus the highest score attained of the species' proteome against the HMM profile of the presumed target of that drug. Gla, *G. lamblia*; L6, rat L6 myoblasts; Ldo, *L. donovani*; Pfa, *P. falciparum*; Tbr, *T. brucei*; Tcr, *T. cruzi*.

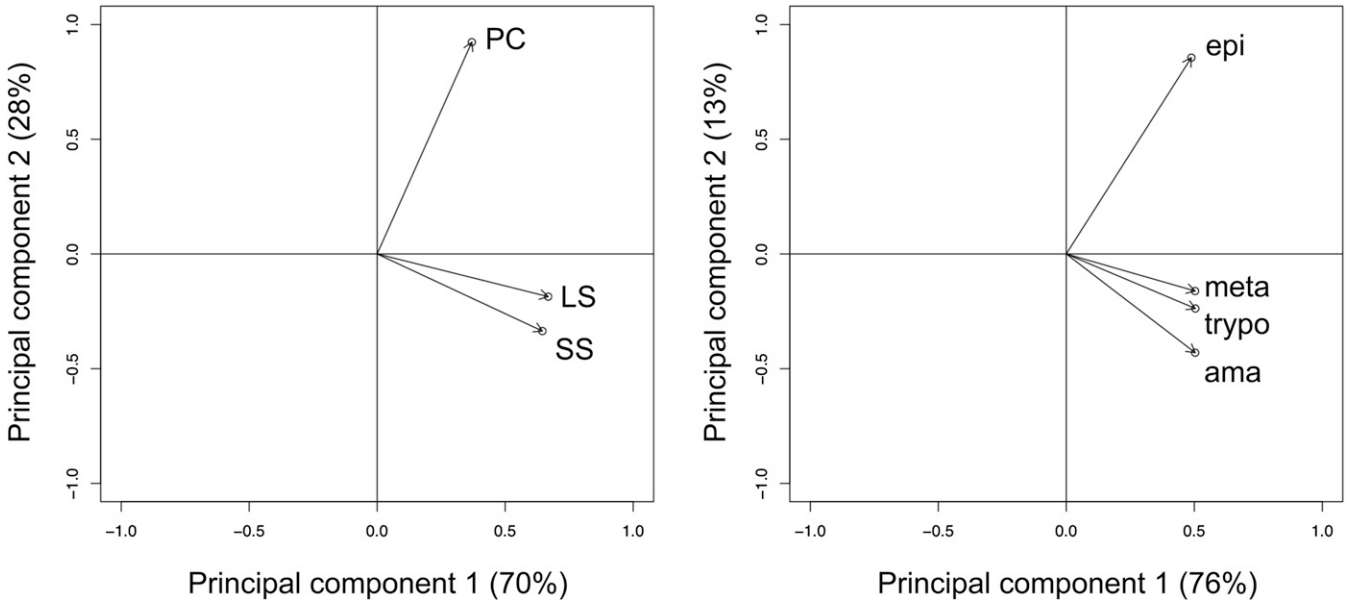

Fig. 6. Expression profiles in *Trypanosoma* spp. Loading plots based on PC-1 and PC-2 of the steady-state mRNA levels of sterol biosynthetic genes expressed in count per million. A: *T. brucei* procyclic forms (PC) and long slender (LS) and short stumpy (SS) bloodstream forms. B: *T. cruzi* epimastigotes (epi), metacyclics (meta), trypomastigotes (trypo), and amastigotes (ama).

proteomes because our in silico pipeline did not furnish proof of function. It provided quantitative scores for all enzymes and proteomes (Fig. 2), which lent itself for clustering. The resulting tree's principal subdivision was between the sterol-prototrophs and the sterol-auxotrophs (Fig. 3). The latter included all of the analyzed protostomes, the former the deuterostomes (i.e., the vertebrates plus the sea urchin *S. purpuratus*).

The interesting branches of the sterol metabolic tree were those where the grouping deviated from evolutionary descent. This was observed for unicellular obligate endoparasites, which appeared to have independently lost genes for sterol biosynthetic enzymes, presumably in adaptation to a parasitic lifestyle. Thus, the microsporidian *Encephalitozoon* did not group with the free-living and facultative parasitic fungi but with parasitic protozoa such as *Giardia*. The same was observed for *Entamoeba*, which did not group with the free-living amoebozoa but with the apicomplexan *Cryptosporidium*. In fact, both *Entamoeba* and *Cryptosporidium* exhibited extreme cases of metabolic reduction, lacking the sterol biosynthetic enzymes as well as either pathway, MEV or non-MEV, for isoprenoid synthesis. The only enzymes present in all the analyzed proteomes were the farnesyl/geranyl diphosphate synthases and the protein farnesyl transferase complex, indicating that protein prenylation is indispensable to all eukaryotes.

The only obligate endoparasites that possessed sterol biosynthetic genes were the trypanosomatids, *Trypanosoma* spp. and *Leishmania* spp. (Fig. 2). Ergosterol has long been known to occur in trypanosomatids (48), and all the analyzed species scored positive for the key enzyme in ergosterol synthesis, sterol 24-C-methyltransferase (enzyme no. 37 in Figs. 1 and 2 and Table 1). In the sterol metabolic tree of Fig. 3, all the trypanosomatids grouped together, sister to the slime molds *Dictyostelium* and *Polysphondylium*, which belong to the amoebozoa but have been proposed to have algal ancestry based on their capability to synthesize cycloartenol (6). An unrooted phylogenetic tree of sterol 24-C-methyltransferase (**Fig. 4**) did not shed light on the ancestry of the enzyme (it was not possible to root the tree due to the lack of an ortholog from prokaryotes to use as an outgroup). The main conclusion from the phylogenetic analysis is that there is no evidence for horizontal transfer as the origin of sterol biosynthetic genes in trypanosomatids.

Apart from their enigmatic history, a major question brought forward by the sterol biosynthetic enzymes of trypanosomatids is to what extent they are exploitable for chemotherapy. Azoles are in development as antichagasic agents because they exhibit selective activity against *T. cruzi*. Their target is sterol 14-demethylase (enzyme no. 24), and in a phylogenetic tree of the enzyme, the trypanosomatid orthologs form a clearly distinct branch (Fig. 4). We tested other known inhibitors of sterol biosynthesis against trypanosomatids and other parasites (Table 2). In general, the in vitro activity of the inhibitors did not correlate with the presence of their presumed target enzyme (Fig. 5). Although the activity of ketoconazole against *T. cruzi* was unmatched, simvastatin also showed activity against trypanosomatids. However, the results were not conclusive because, surprisingly, simvastatin was toxic to mammalian cell lines even though it is widely used as a cholesterol-lowering drug. Furthermore, simvastatin was successfully used for the treatment of mouse (49) and dog (50) models of Chagas's disease.

Comparing the stage-specificity of expression of the sterol biosynthetic enzymes in *T. brucei* and *T. cruzi* revealed interesting parallels. The sterol metabolic enzymes were differentially regulated across the different life stages, and the expression patterns were similar for both species. The

bloodstream forms correlated positively with each other and negatively with the insect forms (Fig. 6). The expression levels of the sterol biosynthetic genes were generally higher in the insect stages than in the mammalian stages of both *T. cruzi* and *T. brucei*. Sterol 24-C-methyltransferase (enzyme no. 37) might be a good drug target because it is highly expressed and thus probably essential in all the life-cycle stages. Finally, the trypanosomatids lacked the bona fide genes for sterol esterification (Fig. 2, enzyme nos. 33 and 34), and yet they had been shown to build sterol esters (43). Thus, we hypothesize that trypanosomatids possess atypical sterol ester synthase and esterase, which represent another possible point for chemotherapeutic intervention. In summary, we conclude that sterol metabolism offers further potential drug targets for selective inhibition of trypanosomes.

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Chapter 3

Lessons from Yeast in Targeting Trypanosomatid Sterol Metabolism

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Working manuscript

Abstract

Sterols are an essential class of lipids in eukaryotes, where they serve as structural components of membranes and play important roles as signaling molecules. Sterols are also of high pharmacological significance: cholesterol-lowering drugs are blockbusters in human health, inhibitors of ergosterol biosynthesis are widely used as antifungals. Similar to fungi – and in contrast to mammals, where the most abundant sterol is cholesterol –, trypanosomatids require the presence of ergosterol and other 24-alkylated sterols, which cannot be replaced by the vertebrate or plant host's sterols. Spearheaded by azoles, different inhibitors of ergosterol synthesis are being developed for Chagas' disease, caused by *Trypanosoma cruzi*. Azoles are very potent inhibitors of lanosterol 14 α-demethylase (CYP51) in fungi and trypanosomes. However, as CYP51 is also present in humans, the question of how azoles exhibit their selectivity is raised. Here, we use genetically modified *Saccharomyces cerevisiae* yeast strains as a tool to asses ergosterol-specific action of two different classes of sterol biosynthesis inhibitors (SBIs) – azoles and azasterols. Integrating the results with in vitro drug sensitivity tests on trypanosomatids and mammalian cells suggests an ergosterol-independent mechanism of action for these SBIs.

Introduction

The azoles are an important class of aromatic molecules that feature a five-membered nitrogen heterocyclic ring stemming from pyrrole. Azoles are widely used as antifungal agents, e.g. the diazoles ketoconazole and miconazole, or the even more effective triazoles such as fluconazole, posaconazole, and ravuconazole. These drugs inhibit the synthesis of ergosterol, an essential component of fungal cell membranes. Like fungi, trypanosomatids also make ergosterol (reviewed in (1)). Given the close resemblance of trypanosomatids and fungi regarding their sterol metabolism, fungicides have been successfully repurposed as antitrypanosomal drug candidates. *Trypanosoma cruzi* and *T. brucei* are very sensitive to ergosterol biosynthesis inhibitors (2, 3). Posaconazole and ravuconazole can cure mice infected with *T. cruzi* (4, 5). Posaconazole and E1224, a prodrug of ravuconazole, have entered clinical trials where they were shown to be very well tolerated and active, but not curative for chronic Chagas' disease (6).

Azoles inhibit lanosterol 14 α-demethylase (CYP51) (7), a cytochrome P450 enzyme which catalyzes the removal of the C_{14} α-methyl group from lanosterol. The interaction between azoles and CYP51 has been extensively studied in fungi (8, 9) and *T. cruzi* (10). The correlation between ergosterol anabolism and susceptibility to azoles strongly suggests a functional link, i.e. that *T. cruzi* and fungal pathogens are both hypersensitive to azoles because they make ergosterol. However, CYP51 orthologs are present in all sterol-prototrophic eukaryotes, including mammals, which make cholesterol and not ergosterol. CYP51 lies upstream of the branching point of cholesterol and ergosterol in the sterol biosynthetic pathway (Figure 1). Why then, are azoles selective for fungi and trypanosomes over mammalian cells? Structural differences between fungal CYP51 (11-13) and trypanosomal CYP51 (14-16) on the one hand, vs. human CYP51 on the other hand, favor binding of azoles to the former and may account for the therapeutic window. In this case, the hypersensitivity of fungi and trypanosomes towards azoles would be coincidental because there is no particular evolutionary relationship between fungal and trypanosomatid CYP51 orthologs (17). However, the selective toxicity of azoles could also be linked to sterol salvage. Blocking sterol synthesis might thus not be lethal to mammalian cells, which can still import cholesterol from their environment, whereas fungal and trypanosomal pathogens die because there is no ergosterol available inside their hosts.

Here, we use genetically modified *Saccharomyces cerevisiae* yeast strains as a tool to interrogate the ergosterol-dependence of sterol biosynthesis inhibitors (SBIs). The yeast strains used in this study are listed in Table 1. RH6825 (*erg5Δ*/*erg6Δ*) lacks C_{22} sterol desaturase and sterol 24-C-methyltransferase (24-SMT), both enzymes specific to the ergosterol branch in the sterol biosynthetic pathway (Figure 1). *S. cerevisiae* RH6825 accumulates an intermediate sterol, cholesta-5,7,24-trienol (Figure 2). *S. cerevisiae* RH6829 has been transfected with 24-dehydrocholesterol reductase (*DHCR24*) and *DHCR7* initially obtained by reverse transcription from *Danio rerio* mRNA, encoding 24- and 7-dehydrocholesterol reductase, respectively. These enzymes are specific to the cholesterol branch of sterol synthesis (Figure 1). Thus RH6829 is a stable yeast strain that efficiently produces cholesterol instead of ergosterol (18). These yeast mutants allowed to correlate sensitivity to selected SBIs with sterol content of the target cell. We have integrated the results with *in vitro* drug sensitivity tests on trypanosomatids and mammalian cells to better understand the reasons for selectivity of SBIs.

Materials and Methods

Chemicals

Tiludronate disodium salt hydrate (T4580-10MG), Terbinafine hydrochloride (T8826-100MG), Ketoconazole (K1003-100MG), Nystatin (N6261-500KU) and (±)-Miconazole nitrate salt (M3512-1G) were purchased from Sigma-Aldrich. Azasterols were kindly offered by BASF. The test compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml (except for miconazole, of which a stock solution of 250 KU was prepared) and stored at -20°C. Resazurin sodium salt (Alamar Blue) was purchased from Sigma-Aldrich (R7017-1G), and chlorophenol red galactopyranoside (CPRG) from Roche (10884308001). Yeast Nitrogen Base was purchased from Sigma-Aldrich (Y0626).

Yeast strains and drug tests

The *Saccharomyces cerevisiae* strains used in this study (Table 1) were kindly provided by Howard Riezman. Cultivation was performed in yeast nitrogen base medium (6.8 g/l) supplemented with glucose (5 g/l), uracil (20 mg/l) and the appropriate amino acid (30 mg/l leucine for all strains, 20 mg/l histidine for RH6825 and RH2881 and 30 mg/l tryptophane for RH2881. Drug sensitivity assays were performed in 96-well plates as follows: Yeast liquid cultures were incubated in the appropriate drop out medium at 30°C over night with shaking at 220 rpm to yield stationary phase cultures (OD_{600} > 1.5). These cultures were diluted 1:10 and incubated for another 4 hours to yield mid-log phase cultures. A further dilution step yielded cultures with an OD_{600} = 0.1. Per well 50 µl of these yeast cultures were added to each drug titrated in 50 µl-duplicates over a 16-fold range. Plates were read in a Sunrise Basic Tecan ELISA reader (measurement λ = 570 nm) at time points t = 0 hours (baseline) and after 24 hours incubation at 30°C. Data from the ELISA reader were collected with Magellan software V5.03. The experimental dimethyl sulfoxide (DMSO) concentration in the assay never exceeded 0.5% (except for the high drug concentration (100 µg/ml) assays) and had no inhibitory effect on cell growth.

Cell lines and drug tests

The cell lines used for in vitro drug sensitivity determination were rat L6 myoblasts, *Trypanosoma brucei rhodesiense* STIB 900, *Trypanosoma cruzi* Tulahuen C2C4 (expressing *Escherichia coli* β-galactosidase) and *Leishmania donovani* MHOM-ET-67/L82 axenic amastigotes. In vitro drug sensitivity assays with parasites and mammalian cells were performed as described (19, 20). The tests were done over 72 h of incubation, except for the *Trypanosoma cruzi* assay, which lasted 96 h. For L6 cells, *Leishmania donovani* and *Trypanosoma brucei* the redox-sensitive dye resazurin (Alamar Blue) served as an indicator of cell viability. For *T. cruzi*, β-galactosidase activity was quantified with the substrate CPRG. IC_{50} values were estimated by linear interpolation based on the semilogarithmic dose-response curves.

Results

We assessed the selectivity of a set of SBIs using genetically modified *Saccharomyces cerevisiae* yeast mutants. Compounds that specifically block ergosterol synthesis, or that rely on the presence of ergosterol in the target cell, will be more active against the parental, 'wildtype' strain RH2881 than against the cholesterol-producing RH6829. The test was validated with nystatin, a natural polyene fungicide produced by *Streptomyces noursei*. Nystatin binds to ergosterol and creates pores in the target cell's membrane (21-23). The growth of RH2881 was completely blocked at nystatin concentrations as low as 15 U/ml whereas RH6829 inhibition required concentrations above 100 U/ml (Figure 2). This is in agreement with previous findings (18). The *erg5*, *erg6* double mutant RH6825 had an intermediate susceptibility to nystatin (Figure 2).

The azoles ketoconazole and miconazole were tested the same way. Neither exhibited selective activity against the ergosterol-producing strain RH2881 (Figure 3). In fact, RH2881 was less sensitive to ketoconazole than the cholesterol-producing strain RH6829. Interestingly, RH6825, which makes neither ergosterol nor cholesterol, was the least sensitive to the azoles, growing at concentrations above 10 μM (Figure 3). This could be due to a compensatory mechanism, possibly involving other classes of lipids (24), that has been selected for in the absence of bulk ergosterol or cholesterol. We also tested the non-azole SBIs terbinafine and tiludronate (not shown), both acting upstream of CYP51 (Figure 1). They had no effect on any of the strains at concentrations up to 30 μM (not shown).

Azasterols were shown to inhibit the enzyme 24-SMT in *S. cerevisiae* (25) and *Candida albicans* (26, 27), but not necessarily in trypanosomatids (28). In *S. cerevisiae*, 24-SMT is encoded by the gene *Erg6*; there is no 24-SMT ortholog in mammalian cells as the methylation at C_{24} is specific to the synthesis of ergosterol (Figure 1). We therefore expected azasterols to be more active against the parental RH2881 than against the *Erg6*-negative strains RH6825 and RH6829. A selection of four azasterols was tested against the three yeast strains, at concentrations >15 μM because none was active at lower concentrations. Surprisingly – and analogous to the azoles – none of the tested azasterols was selective for RH2881 (Figure 4). For two of the azasterols, the ergosterol-producing strain was even the least susceptible (Figures 4A, 4C).

All compounds were also tested against mammalian cells (rat L6 myoblasts) and trypanosomatid parasites: amastigote intracellular *Trypanosoma cruzi*, bloodstream-form *Trypanosoma brucei rhodesiense*, and axenic amastigote *Leishmania donovani*. Table 2 shows the 50% growth inhibitory concentrations (IC_{50}) as determined *in vitro*. Nystatin had a selectivity of about ten-fold towards the extracellular *T. brucei* and axenic *L. donovani* over mammalian cells, but not towards the intracellular *T. cruzi*. As expected, miconazole and ketoconazole were highly active and selective against *T. cruzi*. Tiludronate was inactive against all tested organisms while terbinafine exhibited moderate selectivity towards *L. donovani*. One of the azasterols, LS5761450, had a submicromolar IC_{50} against *T. brucei*. The general trend of LS5761450 and LS5761453 being more active than LS390509 and LS5879325 in yeast, was also seen in trypanosomatids and mammalian cells. Azasterols containing a hydroxyl group in their side chain were most active against *T. brucei* and decreasingly so against L6, *T. cruzi* and *L. donovani*. Remarkably, the molecules that had been inactive in the yeast assay, LS390509 and LS5879325, showed the highest selectivity for *T. cruzi* compared to L6 mammalian cells. These azasterols, in spite of their weak activity as compared to the azoles, nevertheless provide proof-of-concept for selective activity against *T. cruzi* (Table 2).

Discussion

The question whether the selectivity of azoles towards fungi and trypanosomes is (a) coincidental and caused by drugable differences in their CYP51 orthologs as compared to human CYP51, or (b) intrinsically linked to the fact that fungi and trypanosomes both make ergosterol and not cholesterol, is of pharmacological importance because the repurposing of fungicides against *T. cruzi* has been the main avenue towards new drugs for Chagas' disease. There is an urgent need for such new drugs as there is currently no satisfactory treatment for chronic Chagas' disease.

The validity of the used yeast mutants (18) to address ergosterol-dependence of drug action was demonstrated by nystatin, a pore-forming toxin that binds to plasma membrane ergosterol. Nystatin was clearly more toxic to the parental, ergosterol-producing yeast strain than to the ergosterol-deficient mutants (Figure 2). In accordance, nystatin was active against bloodstream-form *T. brucei* and axenic amastigote *L. donovani*, but not against mammalian L6 myoblasts (Table 2). Nystatin was also inactive against the intracellular, amastigote forms of *T. cruzi* (Table 2), which it may be unable to contact. In contrast to nystatin, the tested azoles were not preferrentially toxic to the ergosterol-producing yeast (Figure 3). This disagrees with the model that the selectivity of azoles is intrinsically linked to ergosterol anabolism and is in agreement with the finding, that fungal CYP51 is more sensitive to azoles than human CYP51 (11). Since the amino acid sequence identity between mammalian and *S. cerevisiae* CYP51 is higher than between *S. cerevisiae* and trypanosomatid CYP51, the selectivity of the fungicidal azoles towards *T. cruzi* over L6 cells (Table 2) may in fact be coincidental.

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Tables

Table 1. Strains used in this study.

Strain	Genotype	Major free sterol (18)
RH2881	MATa *ura3 leu2 his3 trp1 bar1*	Ergosta-5,7,22-trienol (ergosterol) (80%)[a]
RH6825	MATa *ura3 leu2 his3 trp1 bar1 erg5Δ::TRP1 erg6Δ::TRP1*	Cholesta-5,7,24-trienol (80%)
RH6829	MATa *ura3 leu2 his3 trp1 bar1 erg5Δ::HIS5-GPD-DHCR24 erg6Δ::TRP1-GPD-DHCR7*	Cholesta-5-enol (cholesterol) (96%)

[a] Determined for strain RH6822 (MATa *ura3 leu2 his3 can1 bar1*)

Table 2. Sensitivity[a] of mammalian cells[b] and parasites[c] to sterol antimetabolites.

	L6	Tcr	Tbr	Ldo
Nystatin	263	272	24	29
Miconazole	25	0.004	40	5.3
Ketoconazole	16	0.002	24	28
Simvastatin	0.92	2.2[d]	3.4	2.0
Tiludronate	>275	>275	217	>275
Terbinafine	41	24	143	9.4
LS5761450	4.4	5.9	0.42	19
LS5761453	3.2	4.5	1.6	35
LS390509	34	3.9	4.7	93
LS5879325	139	19	119	48

[a] 50% inhibitory concentration (IC_{50}) in µM

[b] L6 rat skeletal muscle cells

[c] Ldo, *L. donovani*; Tcr, *T. cruzi*; Tbr, *T. brucei*

[d] The activity of simvastatin against *T. cruzi* was not conclusive due to its toxicity to L6 host cells.

Figures

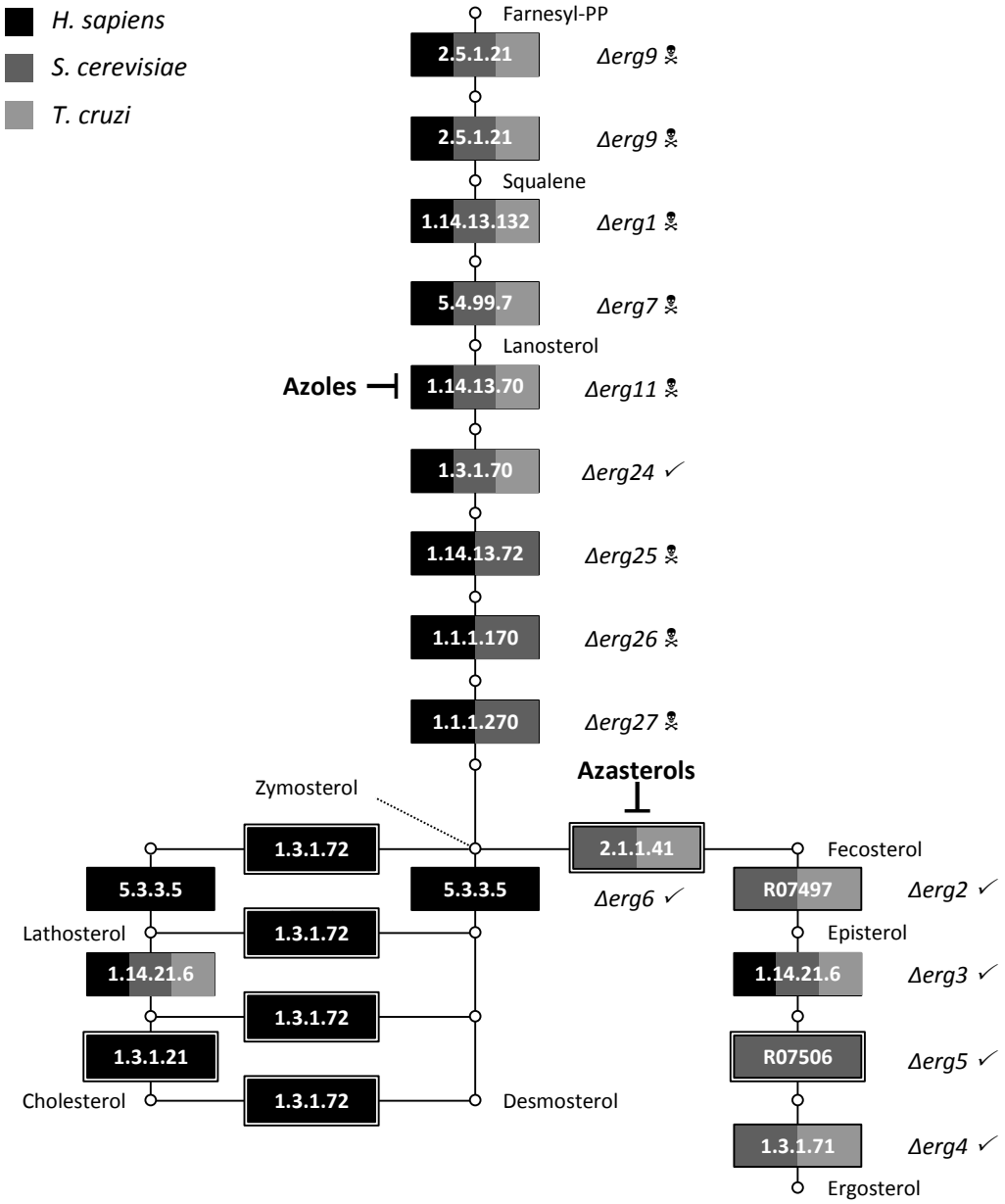

Figure 1. Steroid anabolic pathway. Enzymes are represented by rectangular boxes, metabolites by circles. Boxes are labelled with the EC number of the reaction catalysed by the respective enzyme. The presence of any given enzyme is colour-coded: *H. sapiens* (black), *S. cerevisiae* (dark grey), *T. cruzi* (light gray). Key metabolites are spelled out. Inhibitors are indicated in bold. Enzymes genetically manipulated in RH6825 and RH6829 are double framed. ☠, null mutants were inviable in large scale yeast survey; ✓, null mutants were viable in large scale yeast survey.

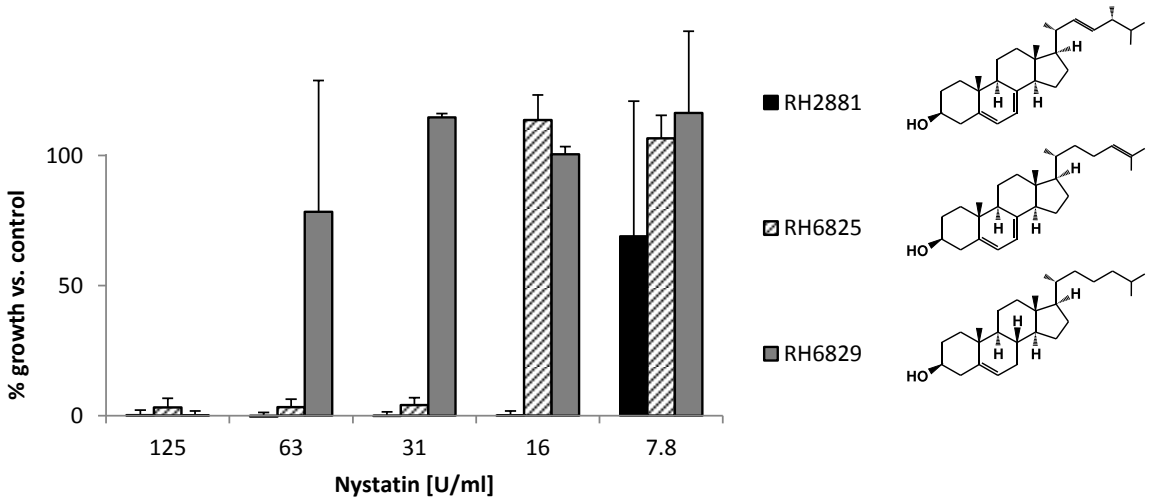

Figure 2. Effect of nystatin on growth of RH2881, RH6825 and RH6829. Bars represent percentage of growth compared to positive control. The values are averages (error bars = SD) form three independent biological replicates. Drug concentrations are indicated in U/ml. The structures of the major free sterol in the three strains are shown: black: RH2881, ergosterol; hatched: RH6825, cholesta-5,7,24-trienol; grey: RH6829, cholesterol.

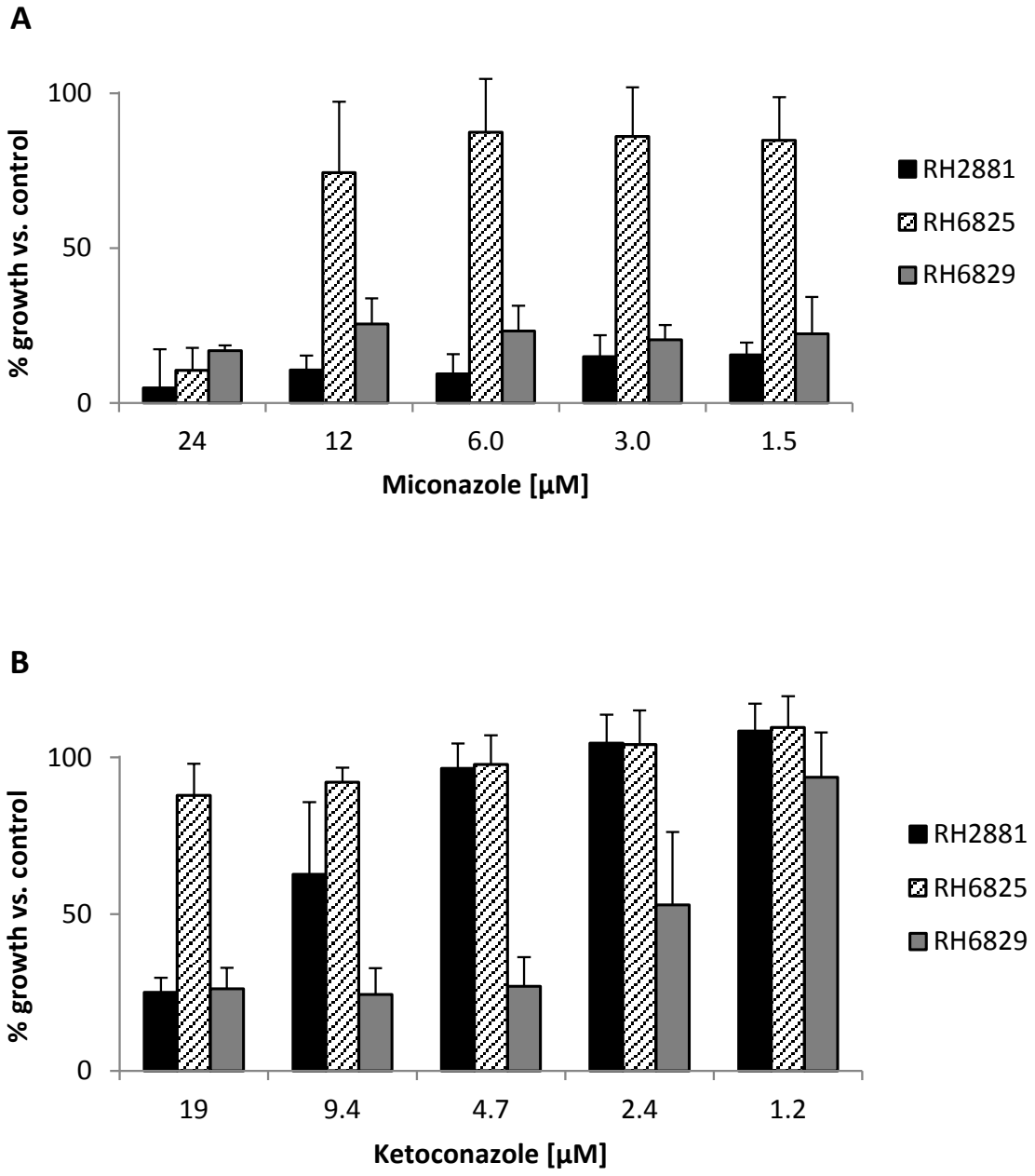

Figure 3. Effect of the imidazoles miconazole (A) and ketoconazole (B) on growth of RH2881, RH6825 and RH6829. Bars represent percentage of growth compared to positive control. The values are averages (error bars = SD) form three independent biological replicates. Drug concentrations are indicated in µM.

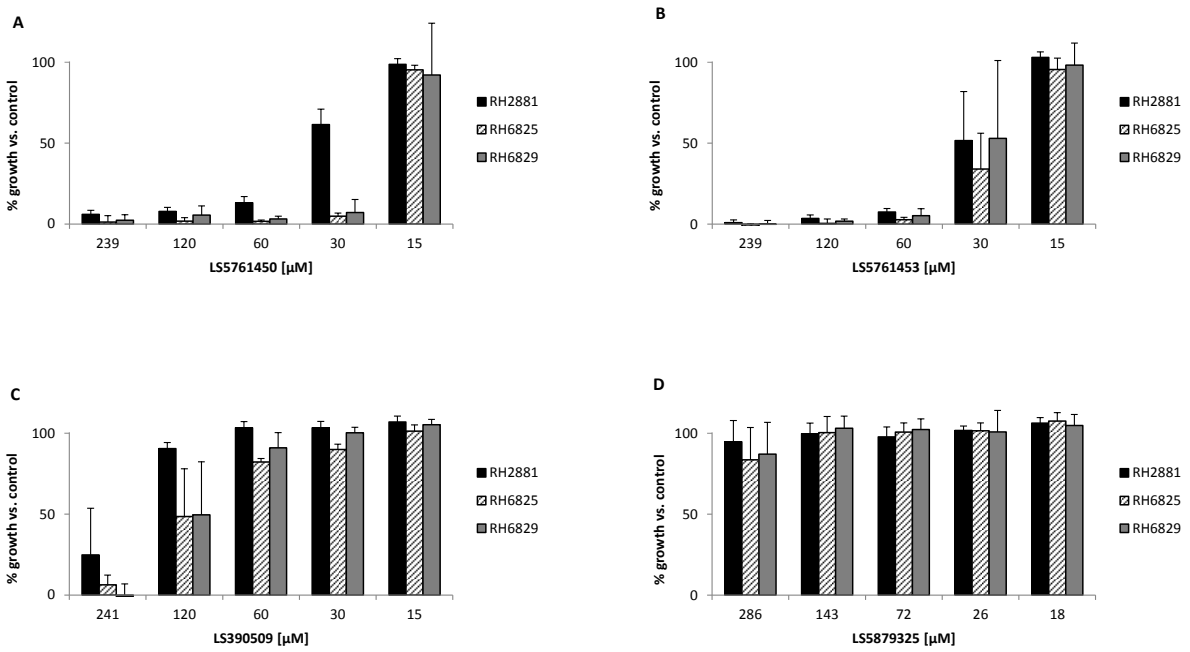

Figure 4. Effect of azasterols on growth of RH2881, RH6825 and RH6829. Bars represent percentage of growth compared to positive control. The values are averages (error bars = SD) form three independent biological replicates. Drug concentrations are indicated in µM. A, LS5761450; B, LS5761453; C, LS390509; D, LS5879325.

Chapter 4

Match-making for Posaconazole through Systems Thinking

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Trends in Parasitology

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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.

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

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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].

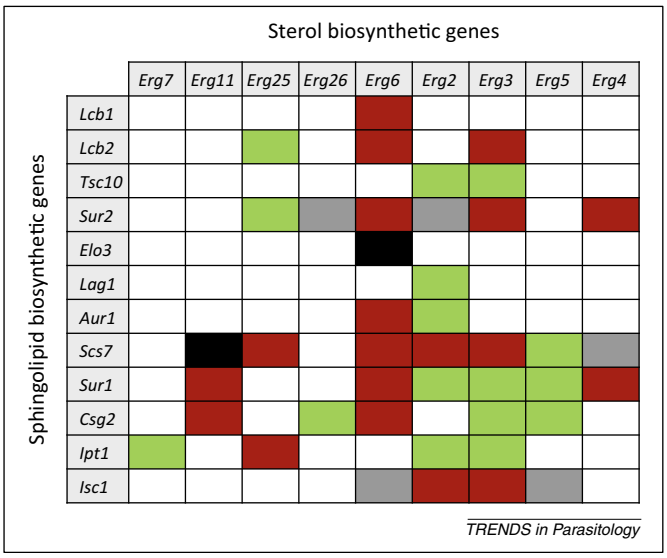

Figure 1. Genetic interactions between sterol and sphingolipid biosynthetic genes in yeast. Only genes for which an interaction has been experimentally documented are shown. Data are from BioGRID v. 3.2.117 [23]. Green, positive genetic interaction; red, negative genetic interaction or synthetic growth 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].

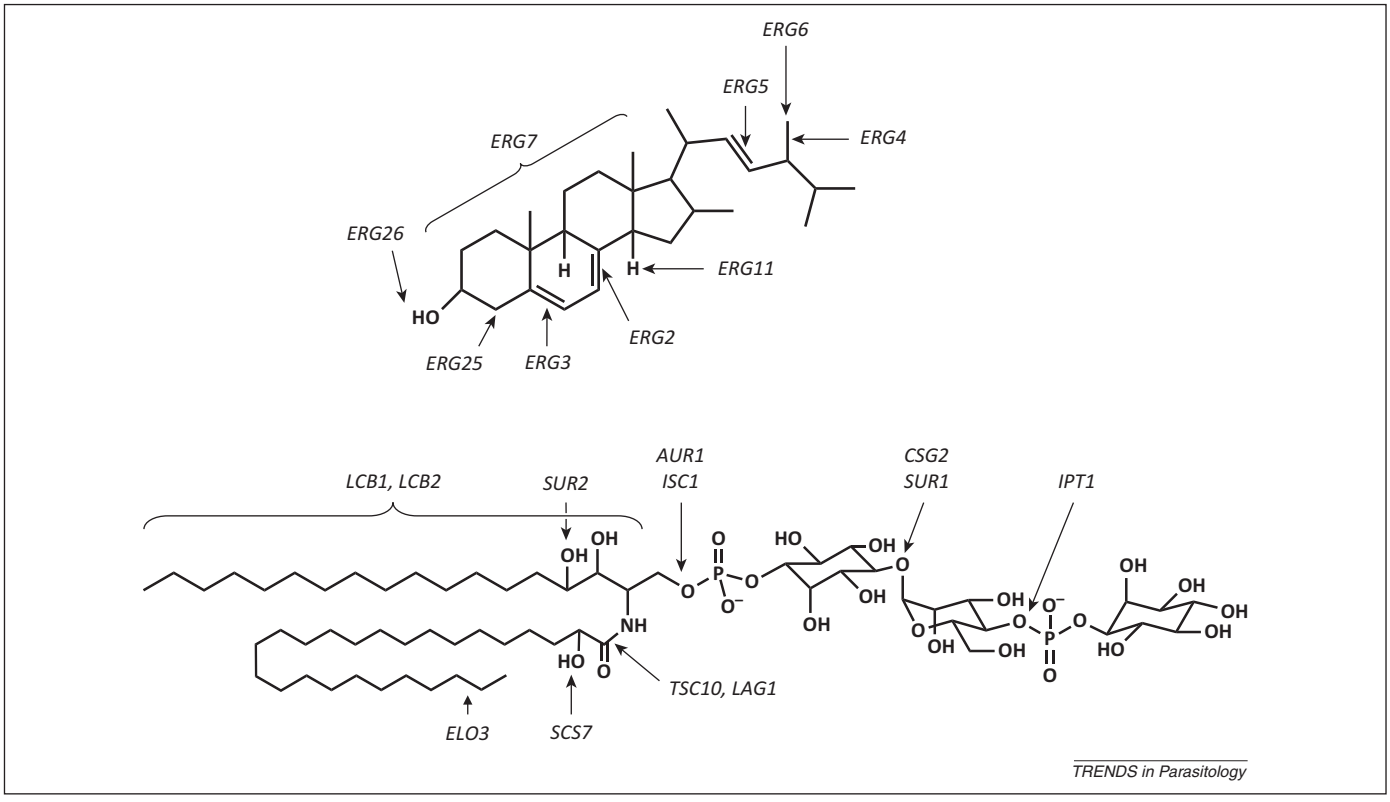

Figure 2. Structures of ergosterol and the sphingolipid, mannosyl diinositol phosphoryl ceramide [$M(IP)_2C$]. Genes shown encode enzymes that catalyze major steps in sterol and sphingolipid metabolism, respectively. The list of genes is not exhaustive but based on sterol/sphingolipid interactions (see 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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Chapter 5

Conclusion and Outlook

Discussion and Conclusion

Sterols are an essential class of lipids in eukaryotes, where they serve as structural components of membranes and play important roles as signaling molecules. Sterols are also of high pharmacological significance: cholesterol-lowering drugs are blockbusters in human health and inhibitors of ergosterol biosynthesis are widely used as antifungals. Routine screening procedures unearthed many of these antifungals. Their mechanism of action has only subsequently been found to be linked to sterol biosynthesis (SB). Today, the SB pathway is well characterized as drug target in fungi but also in humans (1). In addition to its pharmacological significance, SB is of phylogenetic importance. Despite their ubiquity in eukaryotes, sterols generally do not occur in prokaryotes, raising the question of their evolutionary origin. Finally, sterols and other lipids are important modulators of host-pathogen interactions (2). Despite their high significance in biology, lipids have long been neglected and compared to other "-omics" such as genomics and proteomics, lipidomics has only recently become an emerging field of research (3). With this PhD thesis I contribute to a better understanding of some of the aforementioned aspects of lipid biology focusing on SB in *Trypanosoma cruzi*.

Several lines of evidence show that lipids play critical roles in trypanosomatids. Specifically, in *T. cruzi* the major sterols are ergosterol and other 24-alkylated sterols (4). In addition to the ergostane- (C_{28}) and stigmstane-based (C_{29}) sterols, *T. cruzi* also contains traces of cholesterol (C_{27}) which is derived either from the host or the culture medium (5, 6). However, imported cholesterol cannot replace endogenous ergosterol as *T. cruzi* requires these specific sterols for cell viability and proliferation (7, 8). On the other hand, growth of *Trypanosoma brucei* blood stream from (BSF) disrupted by a specific inhibitor of sterol 24-methylation was not rescued by cholesterol absorption from the host, suggesting an essential role for ergosterol in cell proliferation (9). Thus, trypanosomatids have a sterol content similar to that of fungi (10) which makes repurposing of antifungals a promising approach in parasite chemotherapy.

In this thesis, first, an in silico pipeline was developed that allowed for systematic investigation of SB in eukaryotes (11). The only obligate endoparasites that possessed SB genes were the trypanosomatids, *Trypanosoma spp.* and *Leishmania spp.* However, the origin of SB genes in trypanosomatids remains obscure as the phylogenetic analysis provided no evidence for horizontal gene transfer.

Then, I developed an assay based on genetically modified yeast strains in order to investigate more closely if the activity of sterol biosynthesis inhibitors (SBIs) is linked to ergosterol. The results obtained suggest that the tested azoles and azasterols exhibit an ergosterol-independent mechanism of action.

Together with glycerophospholipids and sterols, sphingolipids represent the major classes of eukaryotic membrane lipids. Integrating several lines of evidence on the importance of sphingolipids in trypanosomatids (12, 13), knowledge gained in the framework of this PhD thesis and learning from yeast where sterol-sphingolipid interactions have been amply demonstrated (14-16), I conclude that combining sterol and sphingolipid biosynthesis inhibitors is a promising approach to advance drug development in Chagas' disease.

Outlook

Taken together, this thesis makes a significant contribution to lipid research and parasite chemotherapy. Nevertheless, outstanding questions are manifold: First, the proposed drug combinations have to be tested in *T. cruzi* to prove the concept. While most sphingolipid and SBIs are experimental compounds, there are a number in clinical use or in clinical trials for a spectrum of human diseases. Therefore drug repurposing is feasible, especially for SBIs where azoles have already reached proof of concept trials for Chagas' disease. I believe it is promising to also consider other combination partners for posaconazole. E.g., the results of pharmacogenomic and pharmacokinetic studies suggest combining azoles with benznidazole (17, 18). Also, simultaneous chemotherapeutic attack at different sites of the same pathway provides a highly potential outlook. As we have shown, sterol metabolism offers further potential drug targets for selective inhibition of trypanosomes (11). In particular, trypanosomatids lack the bona fide genes for sterol esterification and yet they had been shown to build sterol esters (19). Trypanosomatids might possess atypical sterol ester synthase and esterase, which represent a possible point for chemotherapeutic intervention.

Rational match-making in drug development as described in Chapter 4 can be broadly applied, e.g. to develop antimalarias, where combination therapies are paramount. There, data repositories (such as BioGRID) can assist decision-making when drug combinations have to be found for clinical trials. On the one hand, interacting pathways can be found where simultaneous targeting might enhance effectiveness. On the other hand, if selectivity is an issue, because a drug target is also present in humans, partners that do not interact with this specific target might be sought after. Genomic profiling (Chapter 2) is widely applicable as well, as our approach can readily be adapted to different metabolic pathways.

The use of SBIs against *T. cruzi* is a form of membrane therapy. 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 (20). In a broader sense, I think of membrane therapy as interfering with membranes directly or via curtailing synthesis of lipid synthetic enzymes. SBIs have the potential to impair membrane functionality and thus may kill not only proliferating but also dormant parasites. As currently available drugs lack sufficient efficacy in the

chronic stage of for Chagas' disease, targeting lipid metabolism is a highly promising approach to advance drug discovery for Chagas' disease.

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