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CYP51 as drug targets for fungi and protozoan parasites: past, present and future

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Summary

The efficiency of treatment of human infections with the unicellular eukaryotic pathogens such as fungi and protozoa remains deeply unsatisfactory. For example, the mortality rates from nosocomial fungemia in critically ill, immunosuppressed or post cancer patients often exceed 50%. A set of six systemic clinical azoles (sterol 14α-demethylase (CYP51) inhibitors) represents the first-line antifungal treatment. All these drugs were discovered empirically, by monitoring their effects on fungal cell growth, though it had been proven that they kill fungal cells by blocking biosynthesis of ergosterol in fungi at the stage of 14α-demethylation of the sterol nucleus. This review briefs the history of antifungal azoles, outlines the situation with the current clinical azole-based drugs, describes the attempts of their repurposing for treatment of human infections with the protozoan parasites that, similar to fungi, also produce endogenous sterols, and discusses the most recently acquired knowledge on the CYP51 structure/function and inhibition. It is our belief that this information should be helpful in shifting from the traditional phenotypic screening to the actual target-driven drug discovery paradigm, which will rationalize and substantially accelerate the development of new, more efficient and pathogen-oriented CYP51 inhibitors.

Keywords

Trypanosoma cruzi; *Trypanosoma brucei*; *Leishmania*; amoeba; sterol biosynthesis; antifungal azoles; sterol 14α-demethylase; CYP51; inhibition; crystal structure

INTRODUCTION

CYP51 (sterol 14α-demethylases) are the most conserved cytochrome P450 enzymes (Lepesheva & Waterman, 2004) across phylogeny that catalyze one, essentially the same stereoselective three step reaction of the oxidative removal of the 14α-methyl group from one or more of the five naturally occurring cyclized sterol precursors, lanosterol, 24,25-dihydrolanosterol, eburicol, obtusifoliol, and C4-norlanosterol (Fig. 1). In eukaryotes, the reaction occurs in the endoplasmic reticulum and is required for biosynthesis of sterols, which serve as essential components of plasma membranes (bulky sterols, cholesterol in

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CONFLICT OF INTEREST

None

humans, ergosterol in fungi) and also as precursors for regulatory molecules that modulate growth, division, differentiation, and development processes (sparking sterols) (Lepesheva & Waterman, 2007; Nes, 2011). Fast sterol production is most crucial for rapidly multiplying cells. It is also important to bear in mind that while humans can consume cholesterol from the diet, blocking of ergosterol production in unicellular human pathogens is lethal.

Although sterol biosynthesis involves multiple steps, so far only two of them have become the major targets for systemic clinical drugs. Statins (cholesterol lowering agents), which act upstream of the pathway, at the step of mevalonate production, are presently the most frequently prescribed medications (Superko *et al.*, 2012), while azoles, inhibitors of sterol 14α-demethylase, serve as the most widely used antifungals (Denning & Bromley, 2015; Lass-Flörl, 2011) and are under investigation to be repurposed for treatment of human infections with protozoan parasites (Buckner & Urbina, 2012).

A BRIEFING ON THE HISTORY

Chronologically, the use of antifungal azoles was begun long before the mechanism of their action was elucidated. All the drugs were discovered via phenotypic screening, the first of them entering the market more than half a century ago (chlormidazole - 1954; clotrimazole −1958; miconazole −1971 [Janssen Pharmaceutica] (Heeres *et al.*, 2010; Sheehan *et al.*, 1999)). Initially, the azole drugs were mostly applied topically (Sawyer PR, 1975b; Sawyer PR, 1975a), with ketoconazole - 1977 [Janssen Pharmaceutica] becoming the first oral systemic antifungal (Graybill & Craven, 1983).

In 1978, it was reported that azoles kill fungal cells because of the damage to the fungal cell membranes that occurs “as a result of depletion of the major fungal sterol ergosterol” (Van den Bossche *et al.*, 1978). In 1980, it was shown that ergosterol depletion “coincides with the increase of the content of C14-methylated sterol precursors and has to be attributed to an interference with one of the reactions involved in the removal of the 14α-methyl group of lanosterol” (Van den Bossche *et al*, 1980). By that time it was already observed that in artificial membranes incorporation of lanosterol instead of cholesterol substantially affects the membrane fluidity and permeability (Yeagle *et al.*, 1977).

The fact that lanosterol 14α-demethylation occurs in the microsomal fraction, requires NADPH and molecular oxygen, and is inhibited by CO suggested that “the process must be cytochrome P450 dependent” (Mitropoulos *et al.*, 1976 ; Ohba *et al.*, 1978). This was proven experimentally, when a specific cytochrome P450 enzyme (then named $P450_{14DM}$) was purified from *Saccharomyces cerevisiae* (Yoshida & Aoyama, 1984) and shown to catalyze all the three steps of the reaction: the 14α-methyl group hydroxylation, the oxidation of the 14α-methylalcohol to the 14α-methylaldehyde and then elimination of the aldehyde as formic acid with the introduction of the C_{14}-C_{15} double bond into the sterol core (Aoyama *et al.*, 1984). Moreover, it was noticed that “the activity of yeast $P450_{14DM}$ is 100% inhibited by ketoconazole at a concentration equal to that of the enzyme” (Yoshida & Aoyama, 1987), a feature quite unusual for competitive inhibition.

Later, the orthologous enzyme catalyzing the same reaction was purified from rat hepatic microsomes (Trzaskos *et al.*, 1986). For some time mammalian $P450_{14DM}$ was considered as a potential target for cholesterol-lowering drugs (Frye & Leonard, 1999), but strong inhibitors have never been found. Moreover, it was observed that mammalian cells are much less sensitive to azoles than fungal cells (Van den Bossche, 1988 ; Vanden Bossche, 1985; Vanden Bossche *et al.*, 1987).

WHY THE ENZYME IS CALLED CYP51

Because the first sterol 14α-demethylase protein (Aoyama *et al.*, 1984) and the first sterol 14α-demethylase encoding gene (Kalb *et al.*, 1986) were isolated from yeast, all the catalytically orthologous P450s discovered subsequently were placed into one family and named CYP51, the number that in the updated cytochrome P450 (CYP) nomenclature was initially reserved for fungal sequences (Nelson *et al.*, 1993). The CYP51 family now joins members from all biological kingdoms, including >400 bacteria (actinobacteria and proteobacteria), and this enzyme is regarded as a possible evolutionarily ancestor for all other currently existing cytochromes P450 (Nelson, 1999; Yoshida *et al.*, 2000). Since the number of the CYP51 family members is indefinitely large and there are organisms that have more than one *CYP51* gene (*e.g. CYP51A* and *CYP51B* in some fungal species (Hargrove *et al.*, 2015; Mellado *et al.*, 2001)) or in *T. cruzi* strains (Cherkesova *et al.*, 2014), or multiple CYP51 genes in some plants (Lepesheva & Waterman, 2007), attempts to add more labels to the CYP51 name appear to be unreasonable and confusing (*e.g.* sometimes human CYP51 is called “CYP51A1”, although in fact it is the B-type sterol 14α-demethylase (Hargrove *et al.*, 2016)).

CYP51–TARGETING ANTIFUNGALS

CYP51 inhibitors are broadly used as fungicides in agriculture to protect crops. Amongst them are imidazoles (*e.g.* imazalil, since 1973 (Janssen)), pyrimidines (fenarimol, since 1971 (Eli Lilly)) and triazoles (triadimefon, since 1973 (Bayer)) (Morton & Staub, 2008). Topical antifungal drugs that are applied in medicine and veterinary to treat localized infections of the skin, hair, or nails (*e.g.* athlete’s foot, jock itch, ringworm) are predominantly imidazoles (miconazole, clotrimazole, tioconazole, econazole, butaconazole, etc.), with terconazole (1983) and efinaconazole (2013) representing two triazole-based topical antifungals (Patel & Dhillon, 2013; Sheehan *et al.*, 1999). On the contrary, the clinical antifungal azoles that are available for systemic use, except for ketoconazole, are all triazoles (Fig. 2) as it has been proposed that the triazole ring might be generally more advantageous over the imidazole ring of ketoconazole, both in terms of metabolic stability and weaker inhibition of other human cytochromes P450 (Dalvie *et al.*, 2002).

CLINICAL AZOLE ANTIFUNGALS OF SYSTEMIC USE

Due to some advanced life-saving medical techniques and the increase in the number of immunocompromised patients (both HIV-infected and those who are on steroids, recovering after anticancer chemotherapy or undergoing solid organ and bone marrow transportation), the incidence of systemic (invasive) fungal infections in the past decades has been increasing

sharply (De Pascale & Tumbarello, 2015; Gullo, 2009; Kauffman *et al.*, 2007), resulting in global mortality from fungal infections of 1.5 to 2 million people per year, far more than are killed by malaria or tuberculosis (Denning & Bromley, 2015). Yet the number of clinical antifungals available for systemic use remains astonishingly limited. Of the three types of drugs, amphotericin B, a macrolide that removes ergosterol from fungal membrane, is highly toxic, and echinocandins that damage the fungal cell wall have a more limited spectrum of activity, both of them lacking oral formulations (Andes, 2013; Pappas *et al.*, 2016). Therefore, the six clinical azoles presented in Fig. 2 remain the most widely used antifungal medicines.

Ketoconazole (1977)

Ketoconazole (Fig. 2, MW 531, log P 3.54) was developed at Janssen Pharmaceutica as a derivative of miconazole and has served as an oral systemic drug for 40 years (Borelli *et al.*, 1979; Heel *et al.*, 1982; Heeres *et al.*, 2010). The daily dosage is 200 to 400 mg. Ketoconazole displays a broad spectrum of antifungal activity, good oral bioavailability, and is in general well tolerated. Moreover, over the years of use it was reported to be beneficial in anticancer chemotherapy (Lopez-Barcons *et al.*, 2017; Trachtenberg *et al.*, 1983; Trachtenberg & Pont, 1984) or therapy of organ transplant recipients receiving cyclosporine immunosuppression (Carbajal *et al.*, 2004; Chapman *et al.*, 1996). The downside of ketoconazole is a short lifetime in human blood, because the drug is rapidly metabolized in the liver (Dalvie *et al.*, 2002), where it interacts with many drug-metabolizing cytochromes P450, CYP3A4 in particular (Mosca *et al.*, 1985; Zhang *et al.*, 2002). Thus, it was reported that at a single oral dose of 200 mg maximal plasma concentration of ketoconazole (12 μM) was reached in 1 hour, yet in 12 hours only 0.1 μM (less than 1%) of the drug was detectable in the circulation (Huang *et al.*, 1986). The corresponding pharmacokinetic (PK) values reported in mice are: a single oral dose of 40 mg/kg produced maximal plasma concentration of 25 μM 1 hour after administration, the drug being cleared from the circulation within 8 hours (Borelli *et al.*, 1979). Because of the danger of drug-drug interactions, serious hepatotoxicity and the risk of adrenal dysfunction ketoconazole is no longer used for long-term therapy, its systemic use being limited to the cases when patients do not respond to or do not tolerate alternative medications https://www.uptodate.com/contents/pharmacology-of-azoles.

Itraconazole (1984)

Itraconazole (Fig. 2, MW 704, log P 6.77) is the 1,2,4-triazole analog of ketoconazole. The drug was also developed at Janssen Pharmaceutica and became the first triazole-based oral systemic antifungal. The daily dosage is 200 to 400 mg, duration of therapy up to 12 months. Compared to ketoconazole, itraconazole has a wider spectrum of antifungal activity, *e.g.* it is more efficient in treatment of aspergillosis (Jennings & Hardin, 1993; Tracy *et al.*, 2016). It displays a higher lipophilicity and a lack of endocrine-related side effects (weaker inhibition of human steroidogenic P450s) (Hardin *et al.*, 1988). As a result of its lipophilicity itraconazole has a high affinity to tissues, where its concentration can be a few times higher than in plasma (Heykants *et al.*, 1990; Sheehan *et al.*, 1999), including the brain, though its penetration into the cerebrospinal fluid is very limited (Felton *et al.*, 2014). After a single oral dose of 200 mg peak plasma levels of itraconazole in humans are reached in 3 hours

(~0.4 μM), the concentration is low, but steady, the clearance time exceeding 20–30 hours (0.01 μM was reported detectable on day 3 (Hardin *et al.*, 1988)). The corresponding PK values in mice are: a single oral dose of 20 mg/kg produced maximal plasma concentration of 3 μM 1 hour after administration and the drug was still detectable after 8 hours (0.08 μM) (Ishibashi *et al.*, 2007). Absorption of itraconazole can be enhanced by intake of food. It has been reported that after two weeks of therapy combined with the high-fat diet, itraconazole exhibits steady-state kinetics, the average concentration in human blood reaching up to 8 μM, although high range of variability between individual samples was observed (Buchkowsky *et al.*, 2005; Denning *et al.*, 1989). Similar to ketoconazole, itraconazole is extensively metabolized in the liver, predominantly by CYP3A4, and metabolites are excreted into the urine and bile (Bruggemann *et al.*, 2009). Itraconazole is considered safer than ketoconazole, but still can cause different adverse reactions including, gastrointestinal (>10% patients), cardiovascular, dermatologic, hepatic, central nervous system (1–10%) (Tucker *et al.*, 1990). In general, however, the symptoms are mild and can be readily assessed by monitoring the patient clinically (Buchkowsky *et al.*, 2005).

Posaconazole (2005)

Posaconazole (Fig. 2, MW 701, log P 5.44) is the itraconazole derivative whose lipophilicity was decreased by the replacement of the two Cl atoms (2,4) in the β-phenyl ring of the molecule with two F atoms. This afforded further elongation of the distal portion of the side chain arm. Posaconazole was synthesized at Schering–Plough (currently Merck). The daily dosage is 800 to 1200 mg, duration of therapy can be more than 1 year (Clark *et al.*, 2015; Keating, 2005). Posaconazole is the broadest spectrum azole antifungal available, with activity against *Aspergillus*, *Candida*, *Cryptococcus*, zygomycota, endemic mycoses, and some agents of hyalohyphomycosis (Kauffman *et al.*, 2007). Yet it is mostly used for prophylaxis in immunosuppressed patients at high risk for invasive fungal infections and for salvage therapy in cancer patients (Clark *et al.*, 2015). Kreiter *et al* report that after a single oral dose of 400 mg a median posaconazole peak plasma concentration of 0.9 μM was achieved in 10 hours, and the drug was still detectable within 183 hours (Krieter *et al.*, 2004). Li *et al* found that after a single dose of 800 mg maximal plasma level of posaconazole was reached at 6 hours, varying from 1.8 μM (high-fat meal) to 0.5 μM (fasting) (Li *et al.*, 2010). Because higher posaconazole concentrations are generally associated with improved treatment response [for example, patients with a mean plasma concentration of 1.25 μg/mL had a 75% response rate, whereas patients with mean concentrations of 0.41 to 0.72 μg/mL and 0.13 μg/mL had response rates of 53 and 24%, respectively], a plasma concentration greater than 1.25 μg/ml (1.8 μM) has been suggested as a conservative target (Clark *et al.*, 2015; Walsh *et al.*, 2007). The PK values reported in mice also vary. A single dose of 20 mg/kg produced maximal plasma concentration of 6 μM 8 hours after administration, the drug concentration at 24 hours was 3 μM (Nomeir *et al.*, 2008). A single dose of 40 mg/kg produced maximal plasma concentration of 6 μM 3 hour after administration, the drug concentration at 24 hours decreased to 1.1 μM (Rodriguez *et al.*, 2009). This supports the notion that posaconazole must display saturable oral absorption (Ezzet *et al.*, 2005). Regardless of its relatively low oral bioavailability, posaconazole has broad tissue distribution (Clark *et al.*, 2015), penetrates the blood-brain barrier (Calvo *et al.*, 2010), and in blood cells its concentration can be >10 fold higher than in plasma (Farowski

et al., 2010). Given the unpredictable plasma concentrations, and wide interpatient variability associated with the use of oral posaconazole, many experts recommend therapeutic drug monitoring (Clark *et al.*, 2015), although now, when the intravenous formulation of posaconazole has become available (Jeong *et al.*, 2016) it could be helpful in resolving the problem. Unlike ketoconazole or itraconazole, posaconazole undergoes limited hepatic metabolism (primarily via glucuronidation) and is mostly eliminated unchanged via feces (Heeres *et al.*, 2010). It has been reported that posaconazole does not affect human liver P450s such as CYP1A2, 2C8/9, 2D6, 2C19 or 2E1 but still inhibits CYP3A4 (Lass-Flörl, 2011; Wexler *et al.*, 2004). Posaconazole is generally well tolerated, and serious side effects are rare. The most commonly reported adverse reactions is gastrointestinal distress including nausea (7–41%), vomiting (2–6%), abdominal pain (1–5%), and elevated transaminases (2%). Longer duration of treatment did not result in incremental or new side effects, which are generally reversible upon treatment discontinuation (Clark *et al.*, 2015).

Fluconazole (1988)

Fluconazole (Fig. 2, MW 306, log P 0.99) is a water soluble triazole derivative of miconazole, developed at Pfizer (Heeres *et al.*, 2010). Both oral and intravenous formulations are available. The daily dosage varies between 150 – 800 mg (maximum 1600 mg), depending on location and severity of infection; the treatment duration may be > 1 year (Debruyne, 1997; Lass-Flörl, 2011; Pappas *et al.*, 2016). Fluconazole displays excellent oral bioavailability (>90%) and achieves high levels in the cerebrospinal fluid. During the past 30 years it has become and remains the first-line agent for treatment and prophylaxis of all types of invasive candidiasis (Kullberg & Arendrup, 2015; Pappas *et al.*, 2016). Fluconazole is also used to treat blastomycosis (Brick & Agger, 2012) and cryptococcal meningoencephalitis (Perfect *et al.*, 2010). Its spectrum of antifungal activity, however, is mainly limited to yeast (Lass-Flörl, 2011). After a single oral dose of 150 mg the peak plasma concentration of 21 μM was reached in 2 hours; 24 hours after administration it remained 8 μM (Debruyne, 1997). The PK values reported in mice are: a single oral dose of 1 mg/kg resulted in maximal plasma concentration of 2.3 μM 1 hour after administration (Fromtling, 1988), while a 5 mg/kg dose led to the concentration of 23 μM (Louie *et al.*, 1998). Fluconazole is a moderate inhibitor of CYP3A4, CYP2C8/9 and CYP2C19, and interacts with several drugs metabolized by these enzymes [https://www.uptodate.com/contents/pharmacology-of-azoles]. The drug is minimally metabolized, about 80% being excreted unchanged in urine. Because the elimination depends on renal function, dose reduction is mandatory in renal failure (Lass-Flörl, 2011). Overall, fluconazole revealed a good long-term safety (Lass-Flörl, 2011), the side effects are mild and might include headache (2 to 13%), dizziness (1%), skin rash (2%), nausea (2 to 7%), abdominal pain (2 to 6%), vomiting (2 to 5%), diarrhea (2 to 3%), and increased liver enzymes though serious hepatotoxicity is rare. However, the long-term use of fluconazole as a clinical drug has led to the development of resistance in many fungal strains (Morschhauser, 2016).

Voriconazole (2002)

Voriconazole (Fig. 2, MW 349, log P 2.28) is a fluconazole analog, where one of the two triazole rings is replaced with the 5-fluoropyrimidine ring. Although it took Pfizer 14 years, which involved synthesis and testing over 1200 fluconazole analogs before voriconazole was

selected (Denning & Bromley, 2015), this modification markedly improved the spectrum of antifungal activity, so that voriconazole not only treats different forms of yeast infections, but is also the agent of choice for invasive aspergillosis (Aperis & Mylonakis, 2006; Denning & Bromley, 2015; Lass-Flörl, 2011): 52% remission, 72% survival (Heeres *et al.*, 2010). Voriconazole is available in both oral and intravenous forms (Heeres *et al.*, 2010). The oral dosing regimen includes a loading dose of 400 mg twice daily for 1 day, followed by 200 mg twice daily, therapy duration is often > 3 months (Elewa *et al.*, 2015; Pappas *et al.*, 2016). The bioavailability after oral administration of voriconazole is > 90% on an empty stomach but it is negatively affected by the presence of food (Heeres *et al.*, 2010), the CSF and CNS penetration is similar to fluconazole (Lass-Flörl, 2011). After a single oral dose of 200 mg the peak plasma concentration of 1.6 μM was reached within 2.4 hours; 24 hours later it decreased to 0.3 μM (Peng & Lien, 2005). After multiple oral doses of 400 mg twice a day the steady state plasma concentrations of voriconazole are reached after 5–7 days of treatment (Bruggemann *et al.*, 2009) and are mainly within the range of 8–17 μM, although high inter- and intrapatient variability is observed (Aperis & Mylonakis, 2006; Elewa *et al.*, 2015), suggesting that therapeutic drug monitoring may be beneficial for optimizing both efficacy and safety (Elewa *et al.*, 2015; Pasqualotto *et al.*, 2010). The PK values in mice are: a single oral dose of 40 mg/kg produced maximal plasma concentration of 31 μM 1 hour after administration, about 15 μM was detectable in 12 hours (Mavridou *et al.*, 2010). Voriconazole is extensively (>98%) metabolized in the liver by N-oxidation, primarily by CYP2C19 and to a lesser extent by CYP3A4 and CYP2C9, its metabolites have little clinical effect and their excretion in urine does not depend on renal function (Aperis & Mylonakis, 2006; Lass-Flörl, 2011). For voriconazole, the main safety issues are the numerous drug interactions and side effects, which include visual disturbances (30%), liver enzyme elevation (13%) skin rush (7%) fever, nausea, vomiting, diarrhea, headache, abdominal pain, and respiratory disorders (<5%) (Elewa *et al.*, 2015; Heeres *et al.*, 2010; Lass-Flörl, 2011).

Isavuconazole (2015)

Isavuconazole (Fig. 2, MW 437, log P 5.24) is produced by Astellas as a water soluble prodrug isavucozonium sulphate (Maertens *et al.*, 2016; Pettit & Carver, 2015). It is the newest antifungal azole approved for clinical systemic use in the USA and Europe. The compound is a derivative of voriconazole and an isomer of ravuconazole (2,5-difluorophenyl *vs* 2,4-difluorophenyl ring), the drug candidate that has been in clinical trials for a long time, but has not passed them yet, mostly due to the problems with its pharmacokinetics. Isavuconazole has been approved as a broad-spectrum triazole for treatment of invasive aspergillosis and invasive mucormycosis. Both oral and intravenous forms are available and can be switched if necessary without losing bioavailability (Cornely, 2017). After a single oral dose of 200 mg the peak plasma concentration of 6 μM is reached within 3.5 hours; 24 hours after administration the concentration decreases to 0.3 μM, elimination time is extended to 76–104 hours. The standard oral dosing regimen includes a loading dose of 200 mg, followed by maintenance doses of 50 or 100 mg daily, so that the steady state concentration of the drug in plasma remains 6 μM (Pettit & Carver, 2015). The median length of therapy is 180 days (Wilby, 2017). The PK values in mice were reported for subcutaneous administration: a single dose of 13 mg/kg produced maximal plasma

concentration of 10 μM in 0.5 hours, the concentration after 8 hours was dropping to 0.5 μM (Warn *et al.*, 2009). Isavuconazole appears to have fewer drug interactions than voriconazole (Pettit & Carver, 2015) and is predominantly metabolized in the liver by CYP3A4 and CYP3A5 followed by UGT glucuronidation, the metabolites are excreted in feces and bile (Rybak *et al.*, 2015). Isavuconazole displays more predictable plasma concentrations and is believed to have improved tolerability (Wilby, 2017). Treatment-emergent adverse events are mild and most commonly included gastrointestinal disorders, pyrexia, hypokalemia, headache, constipation, and cough (Rybak *et al.*, 2015). When compared in the same clinical trials, isavuconazole was shown to be non-inferior to voriconazole, both in terms of efficiency and safety: drug-related treatment-emergent adverse effects were reported in 42% patients receiving isavuconazole and 60% receiving voriconazole (Maertens et al., 2016).

To summarize, though more efficient drugs are clearly needed, so far the 6 clinical azoles represent the safest and most widely used systemic antifungals, the cornerstone of antifungal therapy (Wilby, 2017).

REPURPOSING OF SYSTEMIC ANTIFUNGALS FORHUMAN INFECTIONS WITH PROTOZOA

While many protozoan pathogens (such as *Plasmodium*, *Toxoplasma* (Alveolata), or *Trichomonas* (Excavata)) do not synthesize endogenous sterols, scavenging cholesterol from their hosts, the parasites from the family *Trypanosomatidae* (order Kinetoplastida) and pathogenic free-living amoebae (*e.g.* *Acanthamoeba* (order Centramoebida)) do. These specific endogenous sterols are essential and their functions cannot be fulfilled by human cholesterol.

1. Trypanosomatidae parasites

In *Trypanosomatidae*, the sterol biosynthesis pathway resembles that in fungi (Fig. 1): squalene-2,3-epoxide is cyclized directly into lanosterol, and the major products are ergosterol and its C24-alkylated analogs. Sequencing of their genomes confirmed the presence of all the required enzymes of the pathway (El-Sayed *et al.*, 2005). There are three major human diseases due to *Trypanosomatidae*: *Trypanosoma cruzi* causes Chagas disease (American trypanosomiasis), *Trypanosoma brucei* causes sleeping sickness (African trypanosomiasis), and *Leishmania* causes leishmaniasis. Most of the attempts to repurpose antifungal azoles were undertaken on *Trypanosoma cruzi* and *Leishmania*.

Trypanosoma cruzi—*Trypanosoma cruzi* along with its hematophagous triatomine insect vector, also known as kissing bug, were first identified as the origin of human infection by a Brazilian doctor Carlos Chagas in 1908, yet the pathogen has been infecting humans on the American continent for at least 9 000 years (Aufderheide *et al.*, 2004). Chagas disease is a major health problem that is endemic in 21 Latin American countries, with over 25 million people at risk of contracting the disease and more than 10 000 deaths per year [http://www.who.int/chagas/epidemiology]. Furthermore, due to human migration and broadening of the insect vector distribution area, it is now becoming a global health issue (Bern *et al.*, 2011; Perez-Molina & Molina, 2017).

Chagas disease is actually an anthropozoonosis, with >150 mammalian species forming the infection reservoir. *T. cruzi* is transmitted to humans not only by kissing bugs, but also via blood transfusion, organ transplantation, contaminated food and drinks, breastfeeding, and from mother to child. In mammals, *T. cruzi* resides both extra- and intracellularly, as non-multiplying bloodstream trypomastigotes that carry the infection throughout the body and multiplying amastigotes, respectively. *T. cruzi* infects numerous organs and tissues, though damages predominantly the heart, gastrointestinal tract and nervous system. The disease occurs in two phases. The acute phase often manifests with the non-specific symptoms of general inflammation and therefore can pass undiagnosed. Years or sometimes decades later, up to 40% of patients who survive the acute phase develop irreversible cardiomyopathy, arrhythmias, megaviscera, and more rarely, polyneuropathy and stroke, and these frequently lead to death (Bern *et al.*, 2011; Lepesheva, 2013; Perez-Molina & Molina, 2017).

By now it is quite clear that the term *Trypanosoma cruzi* applies to a genetically highly variable population that represents a pool of more than 70 so called strains, which must be in fact different species (Cherkesova *et al.*, 2014). The strains are now often referred to as belonging to one of the six Distinct Typing Units (DTUs) called TcI-TcVI. Colombiana, TcI (Colombia), Y, TcII (Brazil), Tulahuen, TcVI (Chile) are examples of most relevant strains that cause highly virulent infections (Zingales *et al.*, 2009). Depending on the strain, the disease varies in its progression, mortality rates, the severity of the acute *vs* chronic stages, tissue tropism, abundance of dormant forms of the parasite, and susceptibility to the treatment (Filardi & Brener, 1987; Martinez-Diaz *et al.*, 2001), which is still limited to two drugs, benznidazole and nifurtimox. Both of them (nifurtimox in particular) are highly toxic, and have low efficacy, especially in the chronic stage (Perez-Molina & Molina, 2017).

The first ever report on the use of antifungal azoles for *T. cruzi* was on miconazole and econazole, both drugs were shown to inhibit cellular growth of Tulahuen *T. cruzi* at concentrations of about 20 μM (Docampo *et al.*, 1981). In 1983, it was demonstrated that oral ketoconazole (30 mg/kg) is active in a murine model of Chagas disease, protecting mice from death caused by the Y strain *T. cruzi* (McCabe *et al.*, 1983). Ketoconazole was also found efficient in preventing death in mice infection with CL, MR, and Tulahuen *T. cruzi* (60 mg/kg) (McCabe *et al.*, 1984). Similar to fungi, inhibition of *T. cruzi* multiplication by ketoconazole was accompanied by altered composition of the sterols in the parasite cells: sharp (34-fold) accumulation of 24-methylenedihydrolanosterol (eburicol in Fig. 1) and depletion of ergosterol-like products was observed (Beach *et al.*, 1986). Itraconazole was found even more active, preventing death in mice infected with Y, CL, and Tulahuen *T. cruzi* at the dosage of 15 mg/kg and apparently leading to the parasitological cure if mice were treated with 150 mg/kg for about 60 days (McCabe *et al.*, 1986). Moreover, in 2013 the outcome of a 20 years of follow-up of treatment of 46 human patients with itraconazole (Apt *et al.*, 1998) was published, concluding that itraconazole prevented the development of ECG abnormalities and cured 33% of patients (Apt *et al.*, 2013). The efficacy of fluconazole, however, was found to be low (Campos *et al.*, 1992), and correlated well with its weak influence on the *T. cruzi* growth and sterol composition (Goad *et al.*, 1989). Treatment of mice infected with Tulahuen *T. cruzi* with voriconazole, 40 mg/kg for 30 days, was also not particularly satisfactory, as at the end of the trial it resulted in only 75% survival rate (10% survival rate was observed at these conditions for non-treated animals), with high percentage

of mice still having the parasite nests in the myocardium and skeletal muscle (Gulin *et al.*, 2013).

The most promising results were produced by posaconazole. The first study was performed using Y and Bertoldo strains (Urbina *et al.*, 1998) and involved both the acute (Y) and chronic (Bertoldo) murine models of Chagas. In the acute model, all the untreated animals died, while 43 doses of posaconazole (25 mg/kg/day) led to a 100% parasitological cure and 100% survival. In the chronic model, the treatment with posaconazole was started 45 to 60 days postinfection (in the Bertoldo strain infection most of the mice survive the acute stage but then deteriorate because of the cardiac conditions), and after 43 doses (15 mg/kg/day) provided 85% protection from death and 75% parasitological cure. The following study involved both immunocompetent and immunocompromised animals. The acute infection was with benznidazole-susceptible CL, partially resistant Y, and highly resistant Colombiana, SC-28, and VL-10 *T. cruzi*. In the model of chronic infection, the CL, Y, and Colombiana strains were used. The mice were treated with posaconazole (20 mg/kg/day) or benznidazole (100 mg/kg/day). In both cases posaconazole demonstrated better results than benznidazole, especially in immunocompromised hosts (Molina *et al.*, 2000). Posaconazole was also investigated in a murine model of Y *T. cruzi* infection in combination with the anti-arrhythmic drug, amiodarone and the synergetic effect (100% survival, 80% parasitological cure) was observed (Benaim *et al.*, 2006). In 2010 a successful treatment of chronic Chagas in an immunosuppressed human patient has been reported (Pinazo MJ *et al.*, 2010), and this prompted entering of posaconazole into clinical trials for chronic Chagas disease (Leslie, 2011). The trials named CHAGASAZOL were performed in Spain and included three groups of chronic chagasic patients that were receiving low-dose posaconazole (100 mg twice a day), high-dose posaconazole (400 mg twice a day) or benznidazole (150 mg twice a day) for 60 days (Molina *et al.*, 2014). The results were quite disappointing because, although none of the posaconazole patients had to stop treatment due to side effects (versus 15% of the patients treated with benznidazole, or 32% when the dosage of 200 mg was used (Morillo *et al.*, 2017)), the follow-up rtPCR test demonstrated only 10 and 20 % success rates in the low-dose and high-dose groups, respectively. Later, the same group of authors reported that based on the fact that cure ratio was clearly related to the posaconazole dose, higher doses of the drug and longer treatment time should have produced better results (Molina *et al.*, 2015). In our opinion, this is a very reasonable assumption, because (by analogy with fungal infections) effects of inhibitors of sterol biosynthesis are always slower and strongly depend on the drug exposure, as the endogenous sterols in the parasite cells (including its metabolically quiescent forms) must be exhausted before the drug becomes "cidal" *vs* "static". Of special interest is the observation that posaconazole exposure in humans was 5 to 10-fold lower than in animal models (Molina *et al.*, 2015). A prodrug of ravuconazole (E1224, Eisai) was another azole that entered clinical trials for Chagas disease. The trials had a low success rate, and this might well be because of the issues with the compound poor pharmacokinetics, the reason why ravuconazole has never passed clinical trials as antifungal drug. The data on its isomer isavuconazole are not yet available.

In all cases, when the *T. cruzi* cellular sterols were analyzed after the use of antifungal azoles, accumulation of eburicol (Fig. 1) prevailed, implying that this sterol is the most likely substrate of CYP51 in *T. cruzi*, and also that the C24 methylation (catalyzed by 24-

sterol methyl transferase) should be the first reaction after the squalene-2,3-epoxide cyclization. Some traces of obtusifoliol suggested that even when the 14α-methyl group is still present, partial removal of one of the two methyl groups at the C4-position can occur (see Fig. 1), *e.g.* (Beach *et al.*, 1986; Urbina *et al.*, 1998).

Leishmania—More than 20 species of trypanosomatids of the genus *Leishmania* infect humans causing leishmaniasis, a vector-borne disease that is transmitted by sandflies. Sandflies also transmit the parasites to many mammals, such as dogs, rodents, primates, marsupials, bats, etc. (Roque & Jansen, 2014). The disease is spread all over the world, though is more prevalent in the countries with warm climate. The parasite is not found only in Australia and Antarctica. Leishmaniasis is considered endemic in ~90 countries, with 0.7 – 1 million new cases and 20,000 to 30,000 deaths per year [www.who.int/mediacentre/factsheets/fs375/en/].

In the sandfly, *Leishmania* exists in the form of extracellular promastigote. When the infected sandfly bites humans, promastigotes that reach the puncture wound are phagocytized by macrophages and transform into the obligate intracellular amastigotes that multiply and grow, ultimately rupturing the host cell and infecting new mononuclear phagocytes, including those which circulate in the blood.

Different species of *Leishmania* are morphologically indistinguishable but cause different types of the disease and therefore are joined into two major groups, one causing cutaneous and mucocutaneous leishmaniasis (*e.g. L. major*, *L. mexicana, L. braziliensis, L. panamensis*) and the other causing visceral leishmaniasis also known as kala-azar, or black fever (*L. donovani*, *L. infantum*, *L. chagasi*) (Berman, 1997). Cutaneous leishmaniasis manifests as skin lesions on exposed areas of the body that may start as papules, nodules and end up as ulcers. The ulcers can be dry or wet, localized or disseminated (particularly in immunocompromised patients). Frequently, the lesions are self-healing, though without treatment the process may take more than a year. Mucocutaneous leishmaniasis affects the skin and mucous membranes, causing severe destruction of skin and tissues of the mouth and nasal cavity, complications can irreversibly mutilate the face. Visceral leishmaniasis is the most severe form of the disease, the parasite infects mononuclear phagocyte systems of internal organs, such as spleen, liver, bone marrow and blood. The symptoms include high fever, weight loss, enlargement of the spleen and liver, and anemia. If left untreated, visceral leishmaniasis is fatal in over 95% of cases. Post-Kala-Azar-Dermal-Leishmaniasis (PKDL) develops in some patients alongside but more commonly after apparent cure from visceral leishmaniasis (50% and 5–10% of cases in Sudan and India, respectively) (Zijlstra *et al.*, 2003).

The recommended treatment depends on the type and severity of the disease and includes pentavalent antimonials, pentamidine, miltefosine, amphotericin B and (for cutaneous leishmaniasis) antifungal azoles: ketoconazole, itraconazole, and fluconazole [https://www.cdc.gov/parasites/leishmaniasis/health_professionals/index.html]. Interestingly, the attempts to use antifungal azoles for treatment of leishmaniasis were undertaken even before the information about their effects on *Leishmania* sterols or animal models of infection became available. This was probably connected with the successful use of amphotericin B

(since 1959), which is also the antifungal drug (since 1955) acting through removal of ergosterol from the fungal membranes.

The first case of clinical treatment of leishmaniasis with ketoconazole was reported in 1982. Ketoconazole was administered to six patients (three with cutaneous and three with mucocutaneous) at 200 mg twice a day for three months. The cutaneous lesions disappeared after two weeks, while healing of mucocutaneous infections required 12 weeks. No relapses were seen 3 months after the treatment was completed (Urcuyo & Zaias, 1982). Other examples of successful use of ketoconazole (Jolliffe, 1986; Navin *et al.*, 1992; Saenz *et al.*, 1990), itraconazole (Borelli, 1987; Dogra & Saxena, 1996; Momeni *et al.*, 1996; White *et al.*, 2006), posaconazole (Paniz Mondolfi *et al.*, 2011), and even fluconazole (Alrajhi *et al.*, 2002; Daly *et al.*, 2014; Sousa *et al.*, 2011; Toubiana *et al.*, 2006) for treatment of human cutaneous leishmaniasis are known. The antifungal azoles clearly accelerate the healing process and are tolerated much better than the regular medicine (Raether & Seidenath, 1984). But the question, why they have low efficiency, particularly against visceral leishmaniasis, both in humans (Momeni *et al.*, 1996; Sundar *et al.*, 1990) and in animal models (Al-Abdely *et al.*, 1999), remains unresolved.

Sterol analysis, however, indicates that although azoles inhibit *Leishmania* cell growth and impair sterol biosynthesis (Berman *et al.*, 1984), they do not stop the pathway at the step of lanosterol, eburicol or even obtusifoliol (Fig. 1). Instead, the C4,C14-methylated zymosterol (C4-norlanosterol), C14-methylated zymosterol (C4-desmethyllanosterol), and C14-methylated fecosterol (C4-desmethylobtusifoliol) accumulate (Beach *et al.*, 1988; Berman *et al.*, 1984; Goad *et al.*, 1985; Lepesheva *et al.*, 2015), indicating that even though the final structure of the ergosterol nucleus cannot be formed (which must still be crucial for sparking sterol functions), the C4-desmethylated precursors are more likely to be at least partially suitable to serve as membrane components, the role which host cholesterol in *Leishmania* cannot play (Beach *et al.*, 1988). If so, much more efficient azole-based drugs or rather drug combination would be required to produce prompt cytocidal effect in *Leishmania* (Lepesheva *et al.*, 2015). Promising results of combination therapy using allopurinol and ketoconazole (Halim *et al.*, 1993) or amphotericin B and fluconazole (Horber *et al.*, 1993) (visceral leishmaniasis in human) as well as 81% reduction of the parasite burden in *L. donovani* infected mice treated with combination of posaconazole and arylimidamide DB766 (Joice *et al.*, 2017) support this notion.

Trypanosoma brucei—Two subspecies of *Trypanosoma brucei*, *T. b. gambiense* and *T. b. rhodesiense*, cause sleeping sickness (human African trypanosomiasis), while the subspecies *T. b. brucei* causes nagana in cattle but does not infect humans and therefore is often used as a model organism in animal studies. The *T. brucei* species are transmitted by tsetse fly bites. Wild and domestic animals can host these parasites and thus represent an important reservoir of infection. Sleeping sickness occurs in 36 sub-Saharan Africa countries. Based on the WHO report, approximately 70 million people who live/travel in the endemic area are still at different levels of risk, but, due to increased control, the number of new cases reported in 2014 dropped below 4,000, and the estimated number of actual cases was only about 15,000 [http://www.who.int/trypanosomiasis_african/country/en].

In tsetse fly, *T. brucei* multiplies as procyclic trypomastigote in the midgut and then as epimastigote in the salivary gland, where it transforms into metacyclic trypomastigote that infects humans. In humans, it transforms into bloodstream trypomastigote, which then invades blood and body fluids, eventually crossing the blood-brain barrier. At all life stages, the parasite remains extracellular. It evades the host immune system via rapid changes of its VSG coat (Pinger *et al.*, 2017).

T. b. gambiense is responsible for 97% of reported cases of sleeping sickness. It is found in western and central Africa and causes a chronic infection: the symptoms of the disease may emerge months or even years after infection, when the central nervous system is affected. *T. b. rhodesiense* is found in eastern and southern Africa, it causes a rapidly developing acute infection: first symptoms are observed within 1–2 weeks, and death ensues usually within months. The symptoms begin with fever, headache, muscle and joint aches, enlarged lymph nodes and then progress to sleep disturbances, progressive confusion and mental deterioration. Amongst the four drugs that are used to treat sleeping sickness, suramin and pentamidine do not cross the blood-brain barrier and so are only effective at the acute stage of infection. Eflornithine does not work against *T. b. rhodesiense*, and melarsoprol is extremely toxic causing death in >5% of patients [https://www.cdc.gov/parasites/sleepingsickness/treatment.html]. Untreated, sleeping sickness is usually fatal.

Apparently, amphotericin B is not active against *T. brucei*, because this parasite can use host cholesterol as a structural component for the membranes. Based on this fact, at some point it was postulated that "bloodstream forms of *T. brucei* do not synthesize sterols *de novo*" (Coppens & Courtoy, 2000). As a result, the information on the effects of antifungal azoles on *T. brucei*/sleeping sickness is very scarce.

Nevertheless, we found that ketoconazole inhibits growth of both procyclic and bloodstream cells of *T. brucei* in a dose-dependent manner with the EC_{50} of about 15 μM (Lepesheva *et al.*, 2007). Most recently, the corresponding value of EC_{50}=8.5 μM has been reported for posaconazole (Dauchy *et al.*, 2016), and some inhibitory effect was also produced by itraconazole (Haubrich *et al.*, 2015). Moreover, using highly specific anti-*T. brucei* CYP51 antibodies for western blot immunoanalysis, we confirmed that, although at a level lower than in procyclic cells, the *CYP51* gene is expressed in bloodstream *T. brucei* (Lepesheva *et al.*, 2010b). These finding were recently reproduced by another research team (Dauchy *et al.*, 2016), which in addition used RNAi to demonstrate the *CYP51* gene essentiality in bloodstream *T. brucei*. Finally, a clear dose-dependent suppression of *T. brucei* infection in mice was observed after oral administration of an experimental *T. brucei* CYP51 inhibitor VNI or clotrimazole (Lepesheva *et al.*, 2010b), and posaconazole-eflornithine combination showed substantial improvement in mice survival in the infections with different *T. brucei* subspecies (Dauchy *et al.*, 2016). These results imply that even if azole-based drugs alone may not be sufficient to cure sleeping sickness, their ability to cause the parasite growth retardation *in vivo* together with much better safety profile makes this kind of medicine quite attractive for combination therapy.

2. Opportunistic human parasites amongst free-living amoeba

Some details of the sterol biosynthesis pathway in the pathogenic free-living amoebas remain elusive. In 1980s it was reported that *Acanthamoeba polyphaga* (Raederstorff & Rohmer, 1985) and two species of amoeba of the genus Naegleria (Raederstorff & Rohmer, 1987) synthesize sterols *de novo* using the pathway that is more typical for photosynthetic organisms so that squalene-2,3-epoxide is cyclized into cycloartenol, which is then converted into 24-methylenecycloartanol and then forms the CYP51 substrate obtusifoliol. Yet in 2017 another team of authors, when analyzing the sterols of *Acanthamoeba castellanii* failed to identify cycloartenol in this organism and suggested lanosterol as a potential CYP51 substrate (Thomson *et al.*, 2017). Regardless of these discrepancies (or perhaps species-related differences (Thomson *et al.*, 2017)), the products of the pathway appear to be mostly ergosterol-like (see Fig. 1), similar to those in *Trypanosomatidae*.

Humans can be infected by free-living amoeba species from four genera. *Acanthamoeba* (*e.g.*, *A. polyphaga, A. keratitis*, *A. culbertsoni, A. hatchetti, A. castellanii*) and *Balamuthia* (*B. mandrillaris*) belong to the order Centramoebida, *Naegleria* (*N. fowleri*) belongs to the order Schizopyrenida, and *Sappinia* (*S. diploidea*) belongs to the order Euamoebida. These opportunistic pathogens live in water, soil, and air and cause a variety of severe health complications, particularly in immunocompromised patients and contact lenses wearers. Thus, *Acanthamoeba* species are causative agents of blinding keratitis, sinus and lung infections, and granulomatous encephalitis. *B. mandrillaris* may form a skin lesion, or may migrate to the brain and cause granulomatous encephalitis. N. *fowleri*, or brain-eating amoeba, causes non-opportunistic primary amoebic meningoencephalitis, which is acute, fulminant, and rapidly fatal (Trabelsi *et al.*, 2012), and one case of encephalitis due to *S. diploidea* has been described (Gelman *et al.*, 2001). The life-cycle of free-living amoeba has two stages: a motile active trophozoite stage and a dormant stress resistant cyst stage with minimal metabolic activity; both of them are infectious. *Naegleria* also has the third, flagellate stage that can exist in the cerebrospinal fluid. Because the amebic infections are emerging diseases, they are difficult to diagnose clinically, leading to delay in treatment and resulting in a high mortality rate. The treatment usually includes combinations of different drugs, amongst them CDC lists amphotericin B, pentamidine, rifampicin, miltefosine and several antifungal azoles, both systemic (ketoconazole, itraconazole, fluconazole, voriconazole) and topical (miconazole and clotrimazole, for keratitis). Nevertheless, most cases of brain and spinal cord infection with amoeba remain fatal [https://www.cdc.gov/dpdx/freeLivingAmebic/tx.html].

The first clinical use of antifungal azoles for amoeba infection was reported in 1984, when recurrence of the *Acanthamoeba keratitis* in the second eye transplant was cured with systemic ketoconazole and topical miconazole (Hirst *et al.*, 1984). In 1990, the combination of oral itraconazole and eye drops of miconazole was reported to cure three patients with *Acanthamoeba keratitis* (Ishibashi *et al.*, 1990). In 1999, a lung transplant patient who had disseminated acanthamoebiasis was successfully cured with a drug combination that included oral itraconazole and ketoconazole cream (Oliva *et al.*, 1999). Combination of corneal cryosurgery with oral fluconazole was found effective in treatment of *Acanthamoeba keratitis* (Amoils & Heney, 1999). A patient with AIDS diagnosed with granulomatous

amebic encephalitis was treated with fluconazole and sulfadiazine, and the single lesion in the brain was surgically excised. No disease relapse was observed (Seijo Martinez *et al.*, 2000). Voriconazole was first used for treatment of disseminated *Acanthamoeba* infection in in a lung transplant recipient, where it was administered in combination with amphotericin B (Walia *et al.*, 2007). Subsequently, other cases of successful treatment of amoebic keratitis (Arnalich-Montiel *et al.*, 2012; Bang *et al.*, 2010; Tu *et al.*, 2010) and granulomatous amoebic encephalitis (Webster *et al.*, 2012) using voriconazole monotherapy or voriconazole-containing drug combinations were reported.

Most recently, direct inhibition of the *Acanthamoeba castellanii* CYP51 activity with voriconazole, itraconazole and fluconazole was studied *in vitro*, showing that, unlike fluconazole, voriconazole and itraconazole are highly potent inhibitors that could not be displaced by the enzyme substrate during the reaction. In cellular experiments, however, the potency of voriconazole was found more than 10-fold higher in comparison with itraconazole, which the authors suggest might be due to the poor uptake of the bulky itraconazole molecule into the cells. In this study obtusifoliol was experimentally confirmed as the preferred amoeba CYP51 substrate (Lamb *et al.*, 2015). This is in full agreement with the amoeba CYP51 sequences (see Fig. 3, the signature residue is marked with black circle).

CYP51 STRUCTURE/FUNCTION AND INHIBITION

In the absence of structure/functional information on the target CYP51 enzymes, the use of phenotypic screening remained the only option, and the development of new drugs was slow and had low efficiency. With the advances in the molecular biology techniques, sequencings of the genomes of many human pathogens have been performed finally enabling inclusion of the CYP51 enzymes into the drug discovery process.

CYP51 sequences

The CYP51 enzymes from different phyla have very low amino acid sequence identity (Fig. 3). For example, the average sequence identity between trypanosomatid and fungal CYP51s is around 25%, the identity between the amoeba and trypanosomatid CYP51s is 33%, and even the identity between the *C. albicans* and *A. fumigatus* CYP51 sequences is only 46%. The average length of a eukaryotic CYP51 polypeptide chain is about 500 residues, and yet only less than 40 of them are completely conserved across the phyla (Lepesheva & Waterman, 2007). Because of this low sequence identity, attempts to repurpose the antifungal azoles for treatment of protozoan infections may not be the best solution, especially since the set of systemic antifungal azoles is rather limited and the efficacy of treatment of systemic fungal infections is quite unsatisfactory (Denning & Bromley, 2015). New, better CYP51 inhibitors are needed.

The CYP51 genes from the species of interest can now be cloned, the proteins can be heterologously expressed in bacteria (*E. coli*), purified and characterized. The fact that the enzyme is in its functionally active state can be verified by the CO-spectrum, because as a Cys–coordinated hemoprotein, upon the iron reduction CYP51 produce a characteristic absorbance maximum around 450 nm after binding of carbon monoxide (Omura & Sato, 1964).

CYP51 spectral response to ligand binding

When expressed in bacteria, CYP51 enzymes display the Soret band maximum at 417 nm (Fig. 4A). This means that the purified protein is in the substrate-free form, with the heme iron in the oxidized (Fe^{3+}) low-spin hexa-coordinated state and a water molecule playing the role of the sixth (axial) ligand, bound to the iron on the distal side of the heme plane, the side that forms the catalytic surface of the CYP51 substrate binding cavity (Hargrove *et al.*, 2012a). When the substrate (a substrate analog) enters the CYP51 binding cavity, it expels the water molecule from the iron coordination sphere. The iron becomes penta-coordinated high-spin, and the Soret band maximum shifts to 393 nm, producing the type 1 spectral response. When a ligand stronger than water (*e.g.* a heterocyclic ring nitrogen) coordinates to the CYP51 heme iron, the Soret band maximum shifts to the right, producing the type 2 spectral response, the length of the shift inversely correlating with the length of the Fe-N coordination bond (Hargrove *et al.*, 2013) (Fig. 4B, C).

This feature can and has been used in optical high-throughput screening (HTS) for new CYP51 binding ligands, *e.g.* (Konkle *et al.*, 2009; Lepesheva *et al.*, 2008), and the apparent binding affinities of the HTS hits can be estimated by spectral titration. The results, however, have to be taken with caution because the low K_ds do not necessarily mean strong enzyme inhibition (Fig. 5), and therefore tight binding ligands with the apparent K_ds< 1 μM (close to the average values calculated for the CYP51-substrate complex formation) have to be further tested as inhibitors of the CYP51 activity in the reconstituted enzyme reaction *in vitro* (Lepesheva *et al.*, 2008; Lepesheva *et al.*, 2007).

CYP51 inhibition

By definition, antifungal azoles are supposed to work as competitive reversible inhibitors. Indeed, many of them (for example fluconazole), similar to the α-phenyl isomer of VNF in Fig. 5, strongly affect the initial turnover number but are being replaced by the substrate in the CYP51 active site during the course of the reaction (Lepesheva *et al.*, 2007). These kind of drugs can be useful (provided they have good pharmacokinetic properties), yet their efficacy is always going to be limited, because they cannot completely block sterol biosynthesis in a pathogen, the feature required for the “cidal” *vs* “static” inhibitory effect.

The questions why some of the compounds completely block the CYP51 activity at 1/1 molar ratio inhibitor/enzyme (Lepesheva *et al.*, 2008; Lepesheva *et al.*, 2007; Yoshida & Aoyama, 1987) (Fig. 6A, B, C) and cannot be replaced in the enzyme binding cavity by the substrate, thus acting as functionally irreversible inhibitors, and why this is never the case for human CYP51 (Fig. 6D) had remained enigmatic until the CYP51 crystal structures have been determined.

CYP51 structures and structural basis for the enzyme druggability

We have characterized and solved the structures of CYP51s from three trypanosomatid parasites (*T. brucei* (Lepesheva *et al.*, 2004), *T. cruzi* (Lepesheva *et al.*, 2006) and *L. infantum* (Hargrove *et al.*, 2011)), in the ligand-free form (Lepesheva *et al.*, 2010b) and in complexes with different inhibitors, including clinical azoles fluconazole and posaconazole (Hargrove *et al.*, 2011; Lepesheva *et al.*, 2010a) and experimental compounds, heme-

coordinated imidazole- (Andriani *et al.*, 2013; Buckner, 2012; Friggeri *et al.*, 2014; Lepesheva *et al.*, 2010a; Lepesheva *et al.*, 2010b), triazole- (Lepesheva *et al.*, 2015), pyridine- (Hargrove *et al.*, 2013), and tetrazole-based (Hoekstra *et al.*, 2016) as well as the substrate analog MCP (Hargrove *et al.*, 2012b), CYP51s from two fungal human pathogens (*A. fumigatus* (Hargrove *et al.*, 2017b; Hargrove *et al.*, 2015) and *C. albicans* (Hargrove *et al.*, 2017a)) and the CYP51 counterpart from human (Hargrove *et al.*, 2016). Comparative structural analysis has led us to the following conclusions.

CYP51 enzymes preserve their conserved catalytic function regardless of the extremely low amino acid sequence identity across phylogeny by maintaining strict similarity at the secondary and tertiary structural levels (Lepesheva & Waterman, 2011). For example, nine of the invariant CYP51 family residues (shown in black in Fig. 3) are glycines or prolines that separate the major secondary structural elements, thus maintaining their length and location within the CYP51 molecule. Most of the other invariant family residues either form the heme binding sequence (P450 signature, dashed line in Fig. 3) or are located within the substrate binding cavity, although the surface of the cavity is mostly formed by the residues that are phyla-specific (grey in Fig. 3) (Hargrove *et al.*, 2017a; Lepesheva & Waterman, 2011).

All the inhibitors, including the substrate analog MCP, bind in the CYP51 active site without causing any substantial rearrangements in the protein backbone, as if “freezing” the enzyme in its substrate-free conformation (Fig. 7). The inhibitors simply occupy the available space and acquire the shape of the CYP51 substrate binding cavity. Because large-scale conformational rearrangements in the protein moiety are very likely to be required upon the CYP51 catalysis (binding of the physiological substrate and/or formation of the specific complex with the electron donor protein cytochrome P450 reductase), it appears that the stronger this “freezing” effect is, the stronger the inhibitory potency must be. In addition to the formation of the Fe-N coordination bond (which affects the iron redox potential), this freezing effect is achieved through multiple interactions between the inhibitor molecule and the protein residues. Some of these interactions have a greater impact than others.

Hydrogen bonds—H-bonds are at least one order of magnitude stronger than Van der Waals contacts. For example, we found that the carboxamide fragment of an experimental inhibitors VNI (Lepesheva *et al.*, 2010b) and its derivative VFV (Lepesheva *et al.*, 2015) in the protozoan CYP51 structures forms two H-bonds that connect two functionally essential regions of the CYP51 molecule, the B’ and I helices (Fig. 8A). On the other hand, VNI that acts as a highly potent functionally irreversible inhibitor of protozoan CYP51s is a rather moderate reversible inhibitor of fungal enzymes (Hargrove *et al.*, 2012a). Accordingly, in the structure of *A. fumigatus* CYP51 VNI is oriented differently (see Fig. 7C) and does not form any H-bonds with the protein (Hargrove *et al.*, 2015). Another example, the tetrazole-based investigational antifungal drug candidates from Viamet (VT1 and VT2 in Fig 7D) form the H-bond with the fungal-specific histidine (His374 in *A. fumigatus* and His377 in *C. albicans CYP51*, respectively) (Hargrove *et al.*, 2017a; Hargrove *et al.*, 2017b). Both of these compounds act as functionally irreversible inhibitors of the *A. fumigatus* and *C. albicans* enzymes (Hargrove *et al.*, 2017a; Hargrove *et al.*, 2017b). Besides, they have also been found effective *in vivo* against isolates of *C. glabrata* and *C. krusei*, which were clinically

resistant to other antifungal treatments (Schell *et al.*, 2017). Comparison between fluconazole and voriconazole as inhibitors of *A. fumigatus* CYP51 is probably even more impressive. The two molecules differ only in the composition of a single ring, a smaller triazole ring in fluconazole (weak reversible inhibitor) and a larger 5-fluoropyrimidine ring in voriconazole (see Fig. 2). The pyrimidine ring of voriconazole forms the H-bond with *A. fumigatus* CYP51 Y122, and voriconazole, regardless of its small size, is one of the most potent inhibitors of *A. fumigatus* (Hargrove *et al.*, 2015).

Surface binding subsite around the substrate entrance—Posaconazole represents an example of the compound that acts as functionally irreversible inhibitor for all tested CYP51s from human pathogens. In the CYP51 structures, its long arm is exposed above the protein surface, forming multiple interactions around the substrate entry (Hargrove *et al.*, 2017a; Lepesheva *et al.*, 2010a). It appears that these interactions prevent opening of the substrate access channel. A similar effect was displayed by a potent experimental inhibitor LFD (shown in Fig. 7D), although the compound was only tested as an inhibitor of and co-crystallized with *T. cruzi* CYP51 (Friggeri *et al.*, 2014).

The C/I-helices area—The helices C and I (they contact the biphenyl arm of VFV in Fig. 8) form the deepest portion of the CYP51 substrate binding cavity, which is unique for sterol 14α-demethylases and is not present in other P450s. This area supports the aliphatic arm of the sterol molecule (Hargrove *et al.*, 2012b), and the compounds that bind here (VNF and NEE being two examples, seen in Fig. 7B), regardless of their small size, still cannot be replaced by the substrate in the CYP51 reaction (Andriani *et al.*, 2013; Lepesheva *et al.*, 2010a). Both of these compounds were found to produce strong antiparasitic effect in *T. cruzi* cells, although unfortunately, NEE was not active in mouse models of Chagas disease and VNF has never been tested *in vivo*. Deeper insights into this phenomenon might help select small but potent CYP51 inhibitors with *e.g.* the enhanced ability to penetrate the blood-brain barrier.

CYP51 structure-based drug design

The fact that no conformational changes in the backbone of the CYP51 substrate binding cavity are involved in inhibitor binding makes this enzyme an attractive target for structure-based drug design. Indeed, our first attempts on the VNI scaffold modification (VNT and VFV in Fig. 7) strongly support this notion as all three compounds superimpose perfectly in the protozoan CYP51 active site (Lepesheva *et al.*, 2015).

The reasons for the modifications were as follows. Although we found that VNI is a highly promising molecule (it cured, with 100% efficiency and 100% survival, both the acute and chronic Chagas disease in mice infected with Tulahuen *T. cruzi* (Villalta *et al.*, 2013)), the compound was less potent as inhibitor of CYP51 from *Leishmania infantum* (Fig. 6C) and CYP51A from the Y strain *T. cruzi* (Cherkesova *et al.*, 2014).

VNT was made to test the general belief that replacement of the metabolically more vulnerable imidazole ring with the triazole ring may have a positive effect on the compound pharmacokinetics. VFV was made with the goal to broaden the antiprotozoan spectrum of activity by filling the deepest (CYP51-specific) portion of the substrate binding cavity with

the additional aromatic ring. Both derivatives retained their potency to inhibit the activity of Tulahuen *T. cruzi* CYP51 *in vitro* (Fig. 6A) and were more active than posaconazole in cellular experiments, killing Tulahuen *T. cruzi* amastigotes within cardiomyocytes with the EC_{50} values within 1 nM (Lepesheva *et al.*, 2015). In the same experiments posaconazole displayed the EC_{50} of 5 nM (Villalta *et al.*, 2013). Furthermore, both compounds showed weaker inhibitory effect on CYP3A4 (the IC_{50} 405, 2900 and 3600 nM for VNI, VNT and VFV respectively; the corresponding values for ketoconazole and posaconazole are 8 and 120 nM (Hargrove *et al.*, 2012a)). The life time of VNT in mice plasma was indeed longer, however its concentration did not exceed 5 μM, thus being about 8-fold lower than the maximal plasma concentration of VNI (Fig. 10). Because of this and also since VNT was too selective for Tulahuen *T. cruzi* CYP51 (Fig. 6B, C), it was not tested *in vivo*. VFV, on the other hand, met our expectations. It cured Tulahuen *T. cruzi* infection in mice and was found more potent than VNI in the mouse model of visceral leishmaniasis: 89% *vs* 60% reduction of the parasite burden (Lepesheva *et al.*, 2015). It also showed better results in curing the Y strain *T. cruzi* infection in mice (Guedes-da-Silva *et al.*, 2017). Moreover, after a single oral dose of 25 mg/kg, VFV displayed a longer clearance time in mouse plasma (Fig. 10A) and revealed higher than VNI affinities to tissues (Fig. 10B). Non-toxic, non-mutagenic, VNI and VFV are promising new drug candidates for entering clinical trials.

Human CYP51 is naturally resistant to inhibition

There is still a general concern that inhibitors of pathogenic CYP51s might actually affect the human counterpart. This is, however, a controversial issue, because inhibitors of human CYP51 could potentially serve as cholesterol-lowering agents. Nevertheless, all the attempts to use human CYP51 as a drug target have failed, and none of the known antifungal azoles or experimental inhibitors of CYP51 from human pathogens inhibit the activity of human CYP51 (Fig. 6D) (Hargrove *et al.*, 2016). We believe that this natural resistance of human CYP51 to inhibition results from the higher flexibility of the substrate binging cavity. Unlike the microbial CYP51 structures, human CYP51 displays the loop-like (low-energy) area in the middle portion of the I-helix (the core helix in the P450 structural fold). The structure suggests that this loop-like area is formed because of the long sequence of polar residues (-HTSSTTS-, CYP51 signature 2 area in Fig. 3), which is found in all animal CYP51 sequences but is interrupted by at least one or two hydrophobic residues in the CYP51 enzymes from other phyla. In the human structure, the OH groups of these polar residues disrupt the normal main chain α-helical H-bonding (Hargrove *et al.*, 2016). The notion of higher flexibility of the human CYP51 active site is supported by structural dynamic simulation experiments (Yu *et al.*, 2016), together suggesting that all animal CYP51 enzymes must be naturally resistant to inhibition.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The fact that CYP51 had served blindly but quite successfully as a drug target for more than half a century addresses the question of the enzyme druggability. However, better and more efficient drugs are badly needed. Current progress in understanding the CYP51 structure/function has made it possible to include the target enzyme into the drug discovery process, thus making it quite straightforward. Optical high throughput screening as well as virtual

screening of large libraries of drug-like molecules can be used to identify novel inhibitory scaffolds. The tight-binding ligands can be selected by spectral titration, and those of them that act as functionally irreversible inhibitors in the reconstituted CYP51 reaction should proceed to cellular experiments and/or animal models. The issues of toxicity, bioavailability and pharmacokinetics can be resolved rationally by minor, structure-guided optimization of the molecules. It is our strong belief that a larger set of more efficient, pathogen-oriented CYP51 inhibitors will help to reduce the mortality rates of fungemias, cure Chagas disease and amoebiasis, and must be effective in combination therapy for visceral leishmaniasis and sleeping sickness. Team efforts from academia and pharmaceutical companies are highly advisable to accelerate the process.

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ETHICAL STANDARDS

Not applicable

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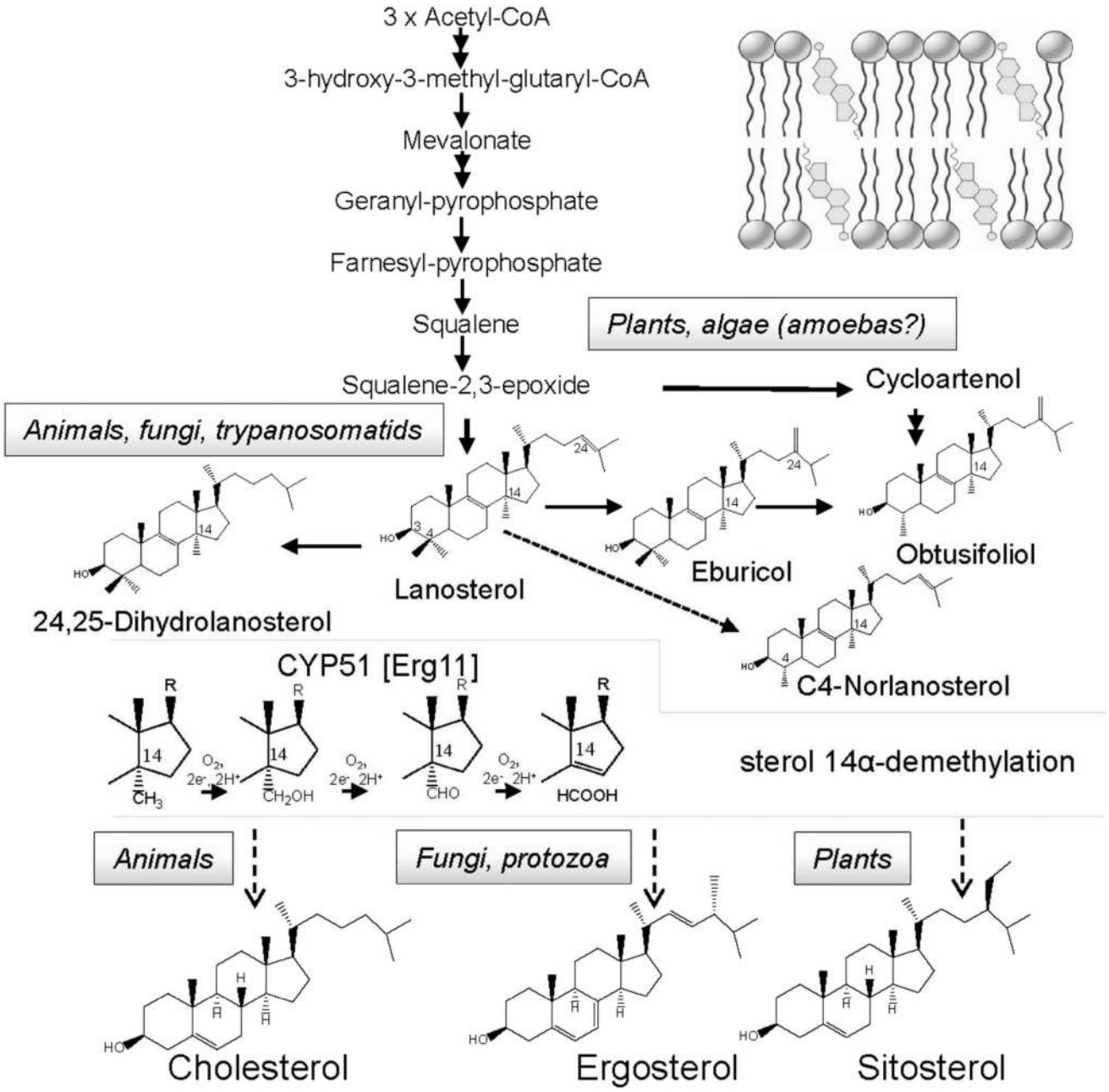

Figure 1. CYP51 reaction is an essential step upon sterol biosynthesis

The pathway involves multiple (>30) steps, beginning with the condensation of acetyl-CoA molecules that serve as initial building blocks, and proceeds to squalene, which then forms epoxide and cyclizes into the triterpene sterol skeleton (cycloartenol or lanosterol). These precursors are further modified to produce cholesterol, ergosterol, or sitosterol, which are the major membrane sterols in humans, fungi/protozoa, and plants, respectively. The CYP51 reaction occurs either immediately or soon after squalene cyclization. The 14α-methyl group of the substrate (lanosterol, 24,25-dihydrolanosterol, eburicol, obtusifoliol, and/or C4-norlanosterol) is converted into the alcohol, then into the aldehyde derivative and finally is removed as formic acid with the introduction of the Δ14–15 double bond into the sterol core.

The CYP51 reaction includes three consecutive cytochrome P450 catalytic cycles, consuming three molecules of oxygen, 6 electrons and 6 protons. Detailed description of other reactions of the pathway can be found in Nes, 2011. ***Inset***: Sterol molecules are incorporated into membranes with the 3β-OH facing the water interface and the side chain extending into the hydrophobic core to interact with fatty acyl chains of phospholipids and proteins.

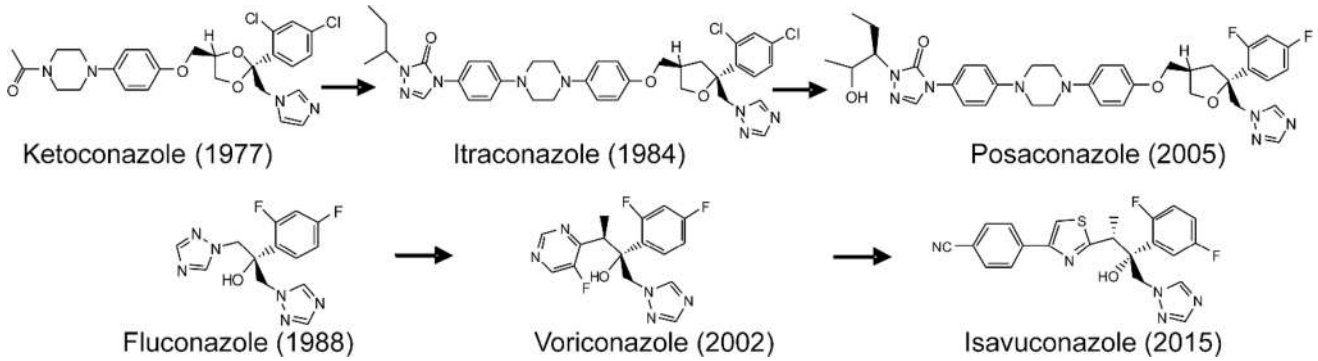

Figure 2. Clinical antifungal azoles used for treatment of systemic human infections

The whole set is represented by six derivatives of two basic scaffolds, fluconazole and ketoconazole. Ketoconazole is an imidazole, the others are 1,2,4-triazoles.

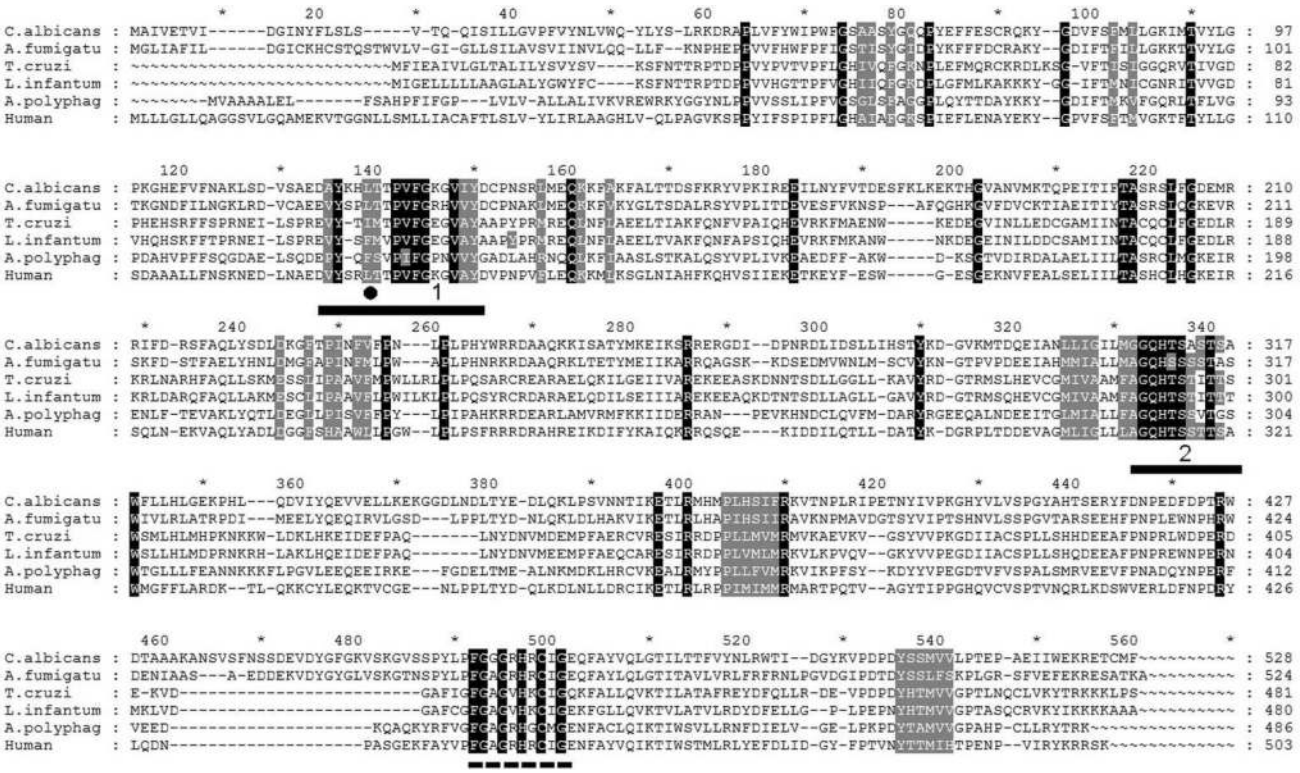

Figure 3. Amino acid sequence alignment of eukaryotic CYP51

The alignment was performed using >200 proteins. The sequences of two fungal (*C. albicans* and *A. fumigatus*), three protozoan: two Trypanosomatidae (*T. cruzi* and *L. infantum*) and amoeba (*A. polyphaga*), and human CYP51s are displayed as examples. The residues conserved in >99% CYP51 family members are in black, the phyla-specific residues that form the surface of the substrate binding cavity are in gray. The residue that defines the CYP51 substrate preferences is marked with black circle (●): F- C4-monomethylated sterols, L/I – C4-dimethylated sterols). Two CYP51 family signatures are underlined, the P450 signature, involving the heme-coordinating cysteine, is marked with the dashed line.

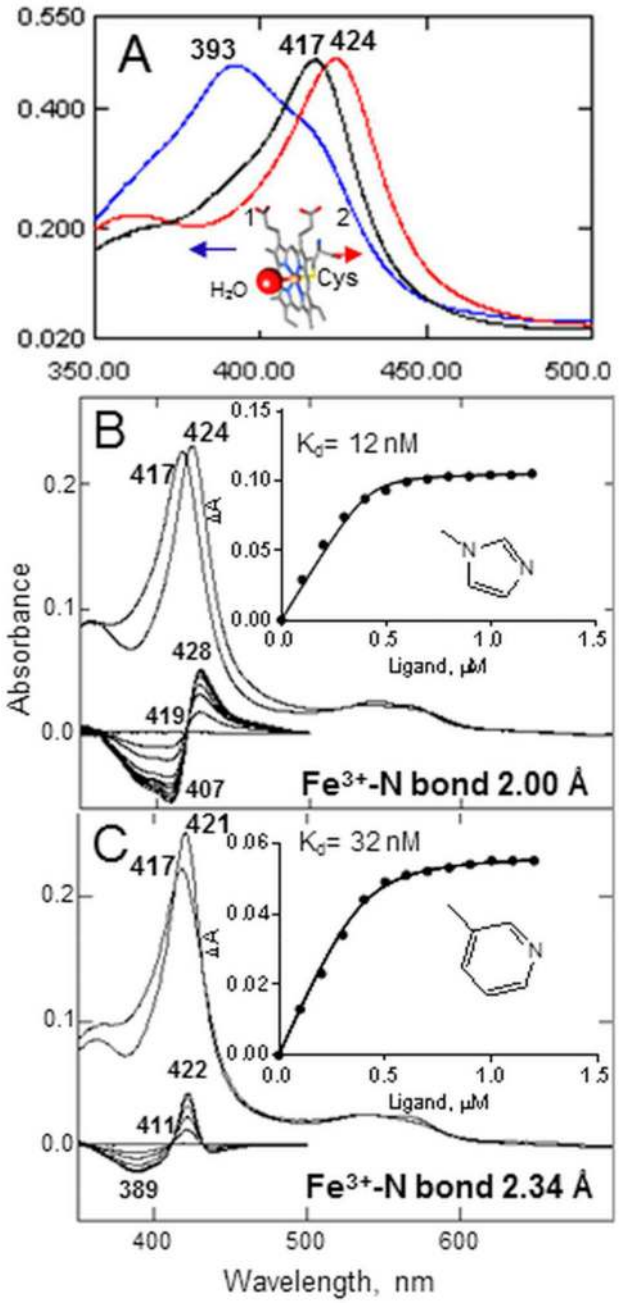

Figure 4. CYP51 binding ligands can be identified by spectral titration

A. Absolute absorbance spectra of water-bound (black), obtusifoliol-bound (blue, type 1 response), and azole-bound (red, type 2 response) *T. brucei* CYP51.The Soret band maxima are marked. Inset: the water-bound heme iron. **B, C**. Type 2 response of *T. cruzi* CYP51 to the binding of imidazole-based VNI [PDB code 3gw9] (**B**) and, pyridine-based UDO [PDB code 3zg3] (**C**). Absolute (top) and difference (bottom) absorption spectra. The P450 concentration ~0.4 μM, the optical path length 5 cm. *Insets*: the titration curves, prepared in Prism.

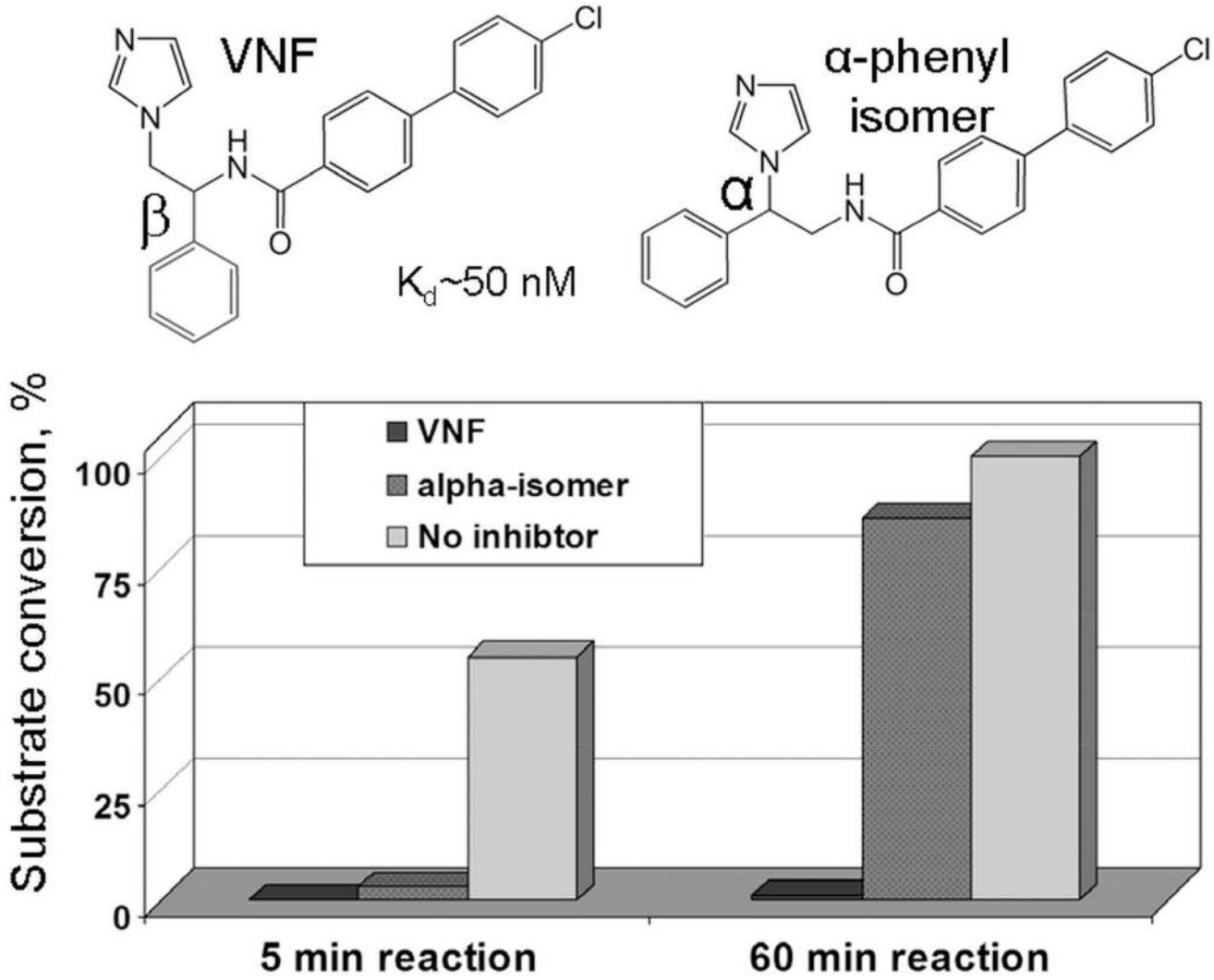

Figure 5. Low spectral K_ds do not necessarily mean strong inhibition of the CYP51 activity

While both VNF and it's α-phenyl isomer display high spectral binding affinity and comparable inhibitory effects on the initial rate of reaction (5 min), VNF is not replaced in the CYP51 active site with the substrate overtime (60 min). The reaction mixture contained 1 μM *T. cruzi* CYP51, 1 μM inhibitor, and 50 μM substrate.

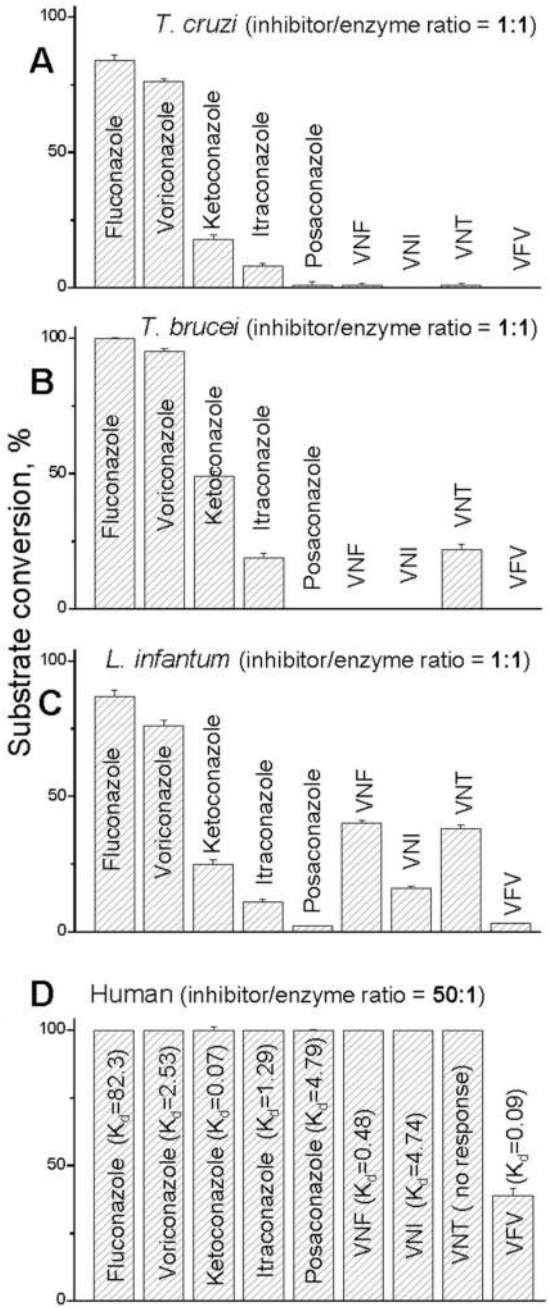

Figure 6.

Inhibitory effects of systemic clinical antifungal azoles and experimental inhibitors on the activity of (A) *T. cruzi, (B) T. brucei*, (C) *L. infantum*, and (D) human CYP51 orthologs; 60 min reaction. The results are presented as means ± SEM. In all experiments the P450 concentration was 0.5 μM, the concentration of the sterol substrates (**A**, eburicol; **B**, **C**, obtusifoliol; **D**, lanosterol) was 50 μM. The values of the apparent spectral K_ds for human CYP51 are given in μM.

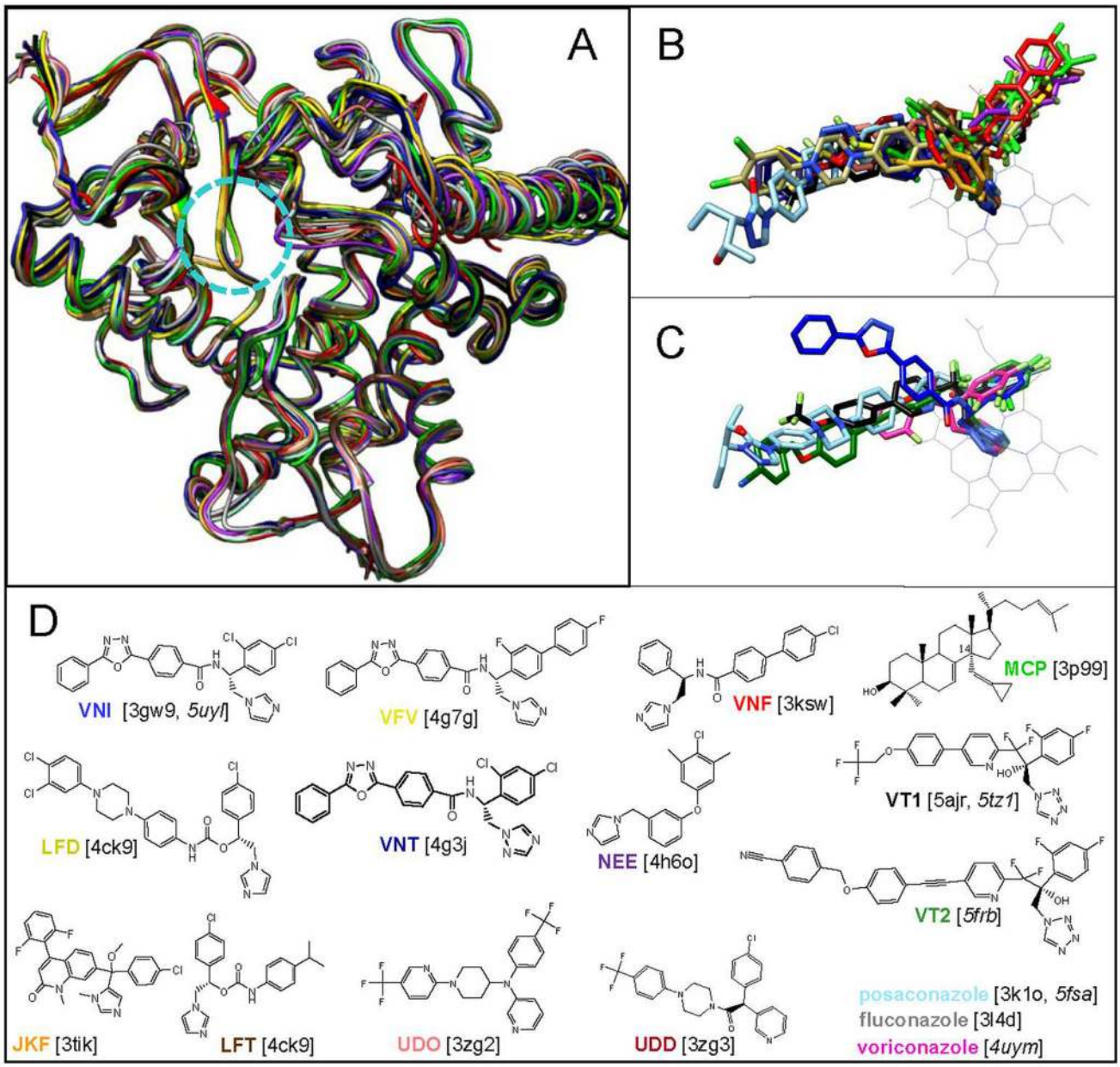

Figure 7. Binding of inhibitors does not cause any large-scale rearrangements in the backbone of the CYP51 molecule

A, B. Superimposed protozoan CYP51 structures, **A**: protein chains of ligand-free *T. brucei* (black) and inhibitor-bound *T. brucei*, *T. cruzi* and *L. infantum* CYP51. The substrate entry is circled. **B**: inhibitors bound in the protozoan CYP51 active site. **C**. Inhibitors bound in the fungal CYP51 active site. The heme is depicted in grey. **D**. Formulas of the inhibitors (posaconazole, voriconazole and fluconazole are shown in Fig. 2). The color code of each crystal structure corresponds to the color of the inhibitor name (PDB ID) beneath the formulas. The correspondent PDB codes of the crystal structures are shown in brackets, the codes of fungal structures are italicized.

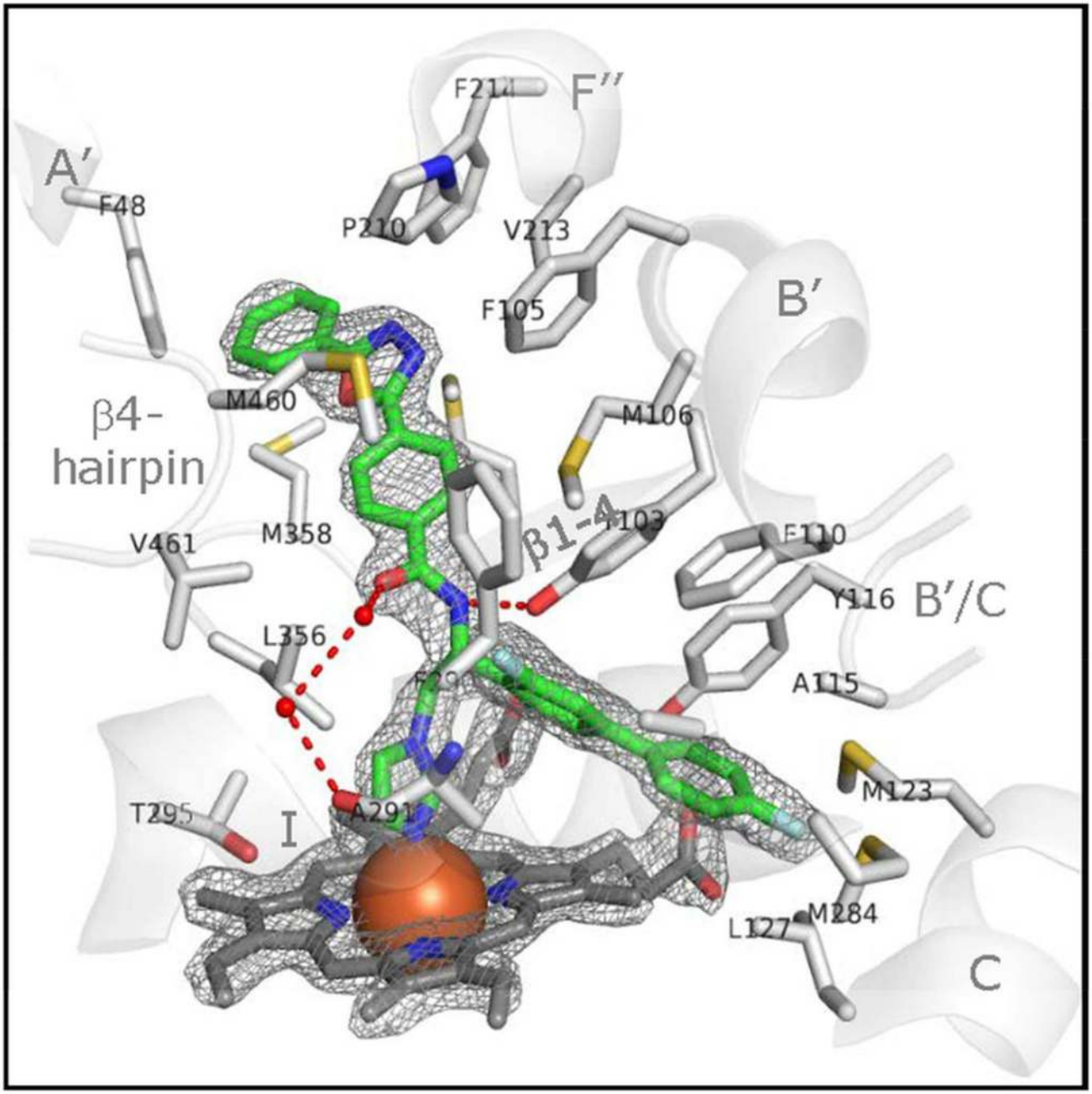

Figure 8. VFV bound in the CYP51 active site

The $2F_o$-F_c electron density map around the heme and the inhibitor is contoured at 2.0 σ and shown as grey mesh. The carbon atoms of VFV are green, the carbon atoms of 22 CYP51 residues that form Van der Waals contacts with the inhibitor are light grey, and the carbon atoms of the heme are dark grey. The atoms of oxygen, nitrogen, and sulfur are read, blue, and yellow, respectively. The heme iron is presented as an orange sphere. The active site-defining secondary structural elements (semitransparent cartoon) are labeled for clarity. The H-bonds between the enzyme and inhibitor are displayed as red dashes.

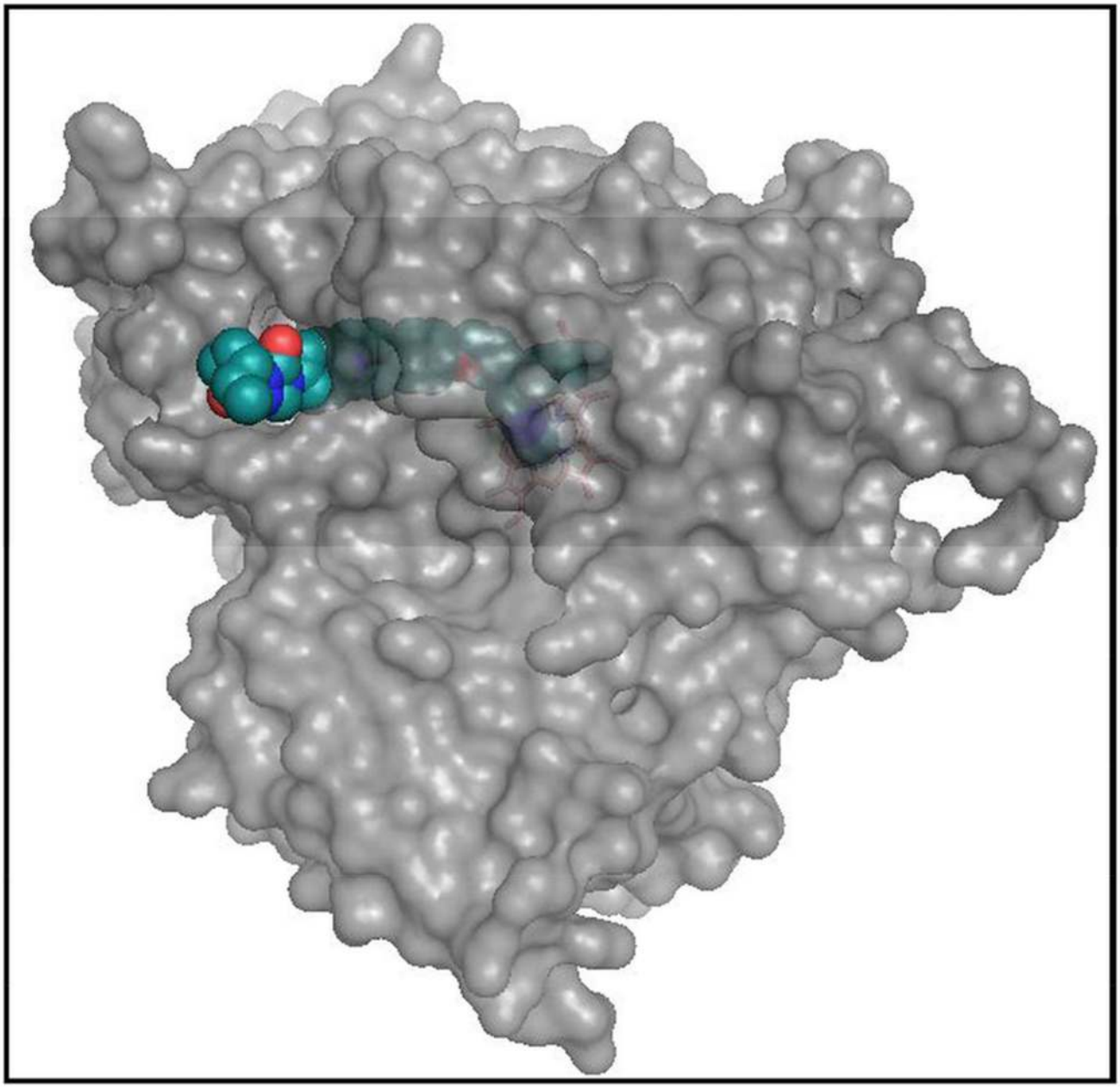

Figure 9. Surface representation of *T. cruzi* CYP51 bound to posaconazole

The long arm of the inhibitor protrudes above the entrance into the substrate access channel.

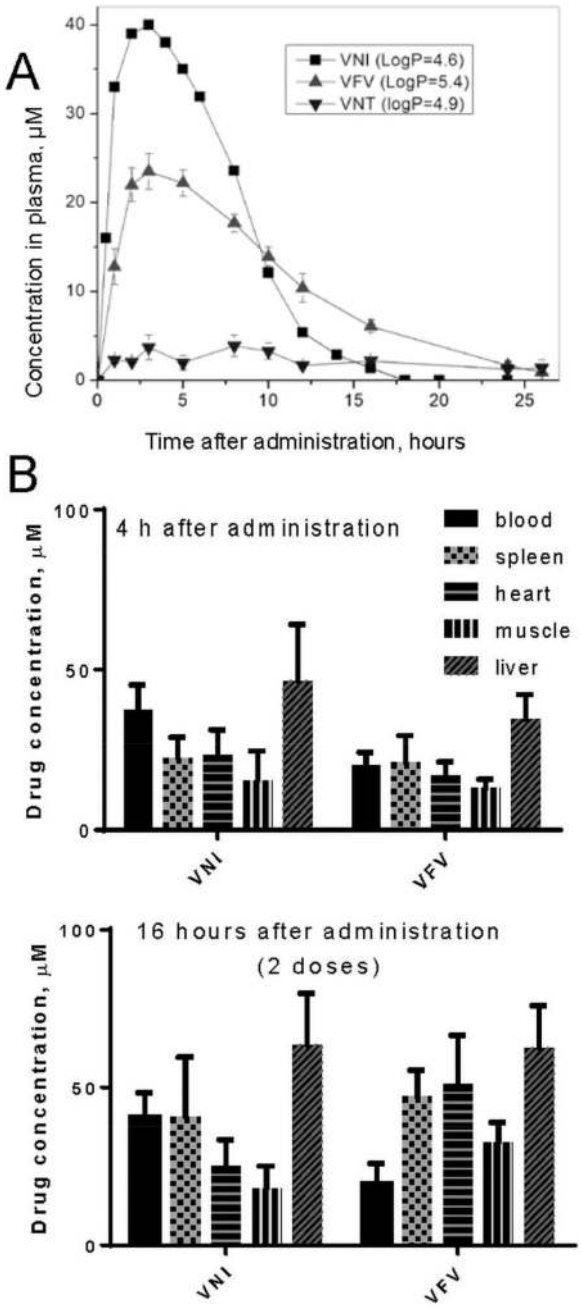

Figure 10. Pharmacokinetics of VNI and derivatives

A. Plasma concentration curves after a single oral dose of 25 mg/kg. **B**. VNI and VFV tissue distribution 4 hours after administration (single dose) and 16 hours after administration (2 doses).