

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

Galina I. Lepesheva, Laura Friggeri and Michael R. Waterman

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA

## Review

**Cite this article:** Lepesheva GI, Friggeri L, Waterman MR (2018). CYP51 as drug targets for fungi and protozoan parasites: past, present and future. *Parasitology* **145**, 1820–1836. <https://doi.org/10.1017/S0031182018000562>

Received: 11 January 2018

Revised: 1 March 2018

Accepted: 5 March 2018

First published online: 12 April 2018

### Key words:

Amoeba; antifungal azoles; crystal structure; CYP51; inhibition; *Leishmania*; sterol 14 $\alpha$ -demethylase; sterol biosynthesis; *Trypanosoma brucei*; *Trypanosoma cruzi*

### Author for correspondence:

Galina I. Lepesheva, E-mail: [galina.i.lepesheva@vanderbilt.edu](mailto:galina.i.lepesheva@vanderbilt.edu)

## Abstract

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 14 $\alpha$ -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 the biosynthesis of ergosterol in fungi at the stage of 14 $\alpha$ -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.

## Introduction

Sterol 14 $\alpha$ -demethylases (CYP51s) are the most conserved cytochrome P450 enzymes (Lepesheva and Waterman, 2004) across phylogeny that catalyse one, essentially the same stereoselective three-step reaction of the oxidative removal of the 14 $\alpha$ -methyl group from one or more of the five naturally occurring cyclized sterol precursors, lanosterol, 24,25-dihydrolanosterol, eburicol, obtusifolol 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 humans, ergosterol in fungi) and also as precursors for regulatory molecules that modulate growth, division, differentiation and development processes (sparkling sterols) (Lepesheva and 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.

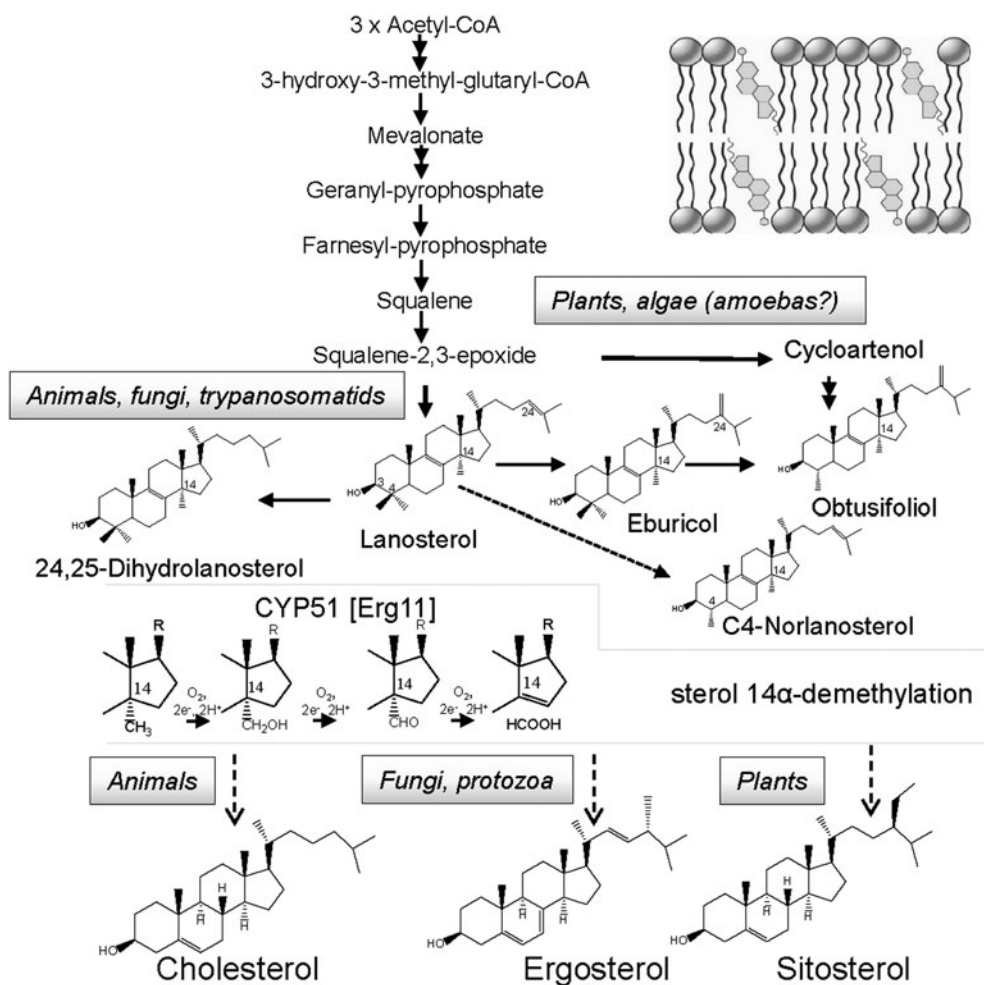
Although sterol biosynthesis involves multiple steps, so far only two of them have become the major targets for systemic clinical drugs. Statins (cholesterol-lowering agents), which act upstream of the pathway, at the step of mevalonate production, are presently the most frequently prescribed medications (Superko *et al.* 2012), while azoles, inhibitors of CYP51, serve as the most widely used antifungals (Lass-Flörl, 2011; Denning and Bromley, 2015) and are under investigation to be repurposed for treatment of human infections with protozoan parasites (Buckner and 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] (Sheehan *et al.* 1999; Heeres *et al.* 2010)]. Initially, the azole drugs were mostly applied topically (Sawyer PR, 1975a, b), with ketoconazole – 1977 [Janssen Pharmaceutica] becoming the first oral systemic antifungal (Graybill and 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 14 $\alpha$ -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 14 $\alpha$ -demethylation occurs in the microsomal fraction, requires NADPH and molecular oxygen and is inhibited by CO suggested that ‘the process must be



**Fig. 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  $14\alpha$ -methyl group of the substrate (lanosterol, 24,25-dihydrolanosterol, eburicol, obtusifolol 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  $\Delta 14$ – $15$  double bond into the sterol core. The CYP51 reaction includes three consecutive cytochrome P450 catalytic cycles, consuming three molecules of oxygen, six electrons and six protons. A detailed description of other reactions of the pathway can be found in Nes (2011). *Inset:* Sterol molecules are incorporated into membranes with the  $3\beta$ -OH facing the water interface and the side chain extending into the hydrophobic core to interact with fatty acyl chains of phospholipids and proteins.

cytochrome P450 dependent' (Mitropoulos *et al.* 1976; Ohba *et al.* 1978). This was proven experimentally, when a specific cytochrome P450 enzyme (then named P450<sub>14DM</sub>) was purified from *Saccharomyces cerevisiae* (Yoshida and Aoyama, 1984) and shown to catalyse all the three steps of the reaction: the  $14\alpha$ -methyl group hydroxylation, the oxidation of the  $14\alpha$ -methylalcohol to the  $14\alpha$ -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<sub>14DM</sub> is 100% inhibited by ketoconazole at a concentration equal to that of the enzyme' (Yoshida and Aoyama, 1987), a feature quite unusual for competitive inhibition.

Later, the orthologous enzyme catalysing the same reaction was purified from rat hepatic microsomes (Trzaskos *et al.* 1986). For some time mammalian P450<sub>14DM</sub> was considered as a potential target for cholesterol-lowering drugs (Frye and 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 (Vanden Bossche, 1985; Vanden Bossche *et al.* 1987; Van den Bossche, 1988).

### Why the enzyme is called CYP51

Because the first CYP51 protein (Aoyama *et al.* 1984) and the first CYP51 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 (Mellado *et al.* 2001; Hargrove *et al.* 2015)] or in *Trypanosoma cruzi* strains (Cherkesova *et al.* 2014), or multiple CYP51 genes in some plants (Lepesheva and 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 CYP51 (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 and 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 (Sheehan et al. 1999; Patel and Dhillon, 2013). 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 transplantation), the incidence of systemic (invasive) fungal infections in the past decades has been increasing sharply (Kauffman et al. 2007; Gullo, 2009; De Pascale and Tumbarello, 2015), resulting in global mortality from fungal infections of 1.5–2 million people per year, far more than are killed by malaria or tuberculosis (Denning and 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 the 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 200–400 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 (Trachtenberg et al. 1983; Trachtenberg and Pont, 1984; Lopez-Barcons et al. 2017) or therapy of organ transplant recipients receiving cyclosporine immunosuppression

(Chapman et al. 1996; Carbajal et al. 2004). 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  $\mu\text{M}$ ) was reached in 1 h, yet in 12 h only 0.1  $\mu\text{M}$  (<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  $\text{kg}^{-1}$  produced maximal plasma concentration of 25  $\mu\text{M}$  1 h after administration, the drug being cleared from the circulation within 8 h (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 analogue of ketoconazole. The drug was also developed at Janssen Pharmaceutica and became the first triazole-based oral systemic antifungal. The daily dosage is 200–400 mg, duration of therapy up to 12 months. Compared with ketoconazole, itraconazole has a wider spectrum of antifungal activity, e.g. it is more efficient in the treatment of aspergillosis (Jennings and 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 h ( $\sim 0.4 \mu\text{M}$ ), the concentration is low, but steady, the clearance time exceeding 20–30 h [0.01  $\mu\text{M}$  was reported detectable on day 3 (Hardin et al. 1988)]. The corresponding PK values in mice are a single oral dose of 20 mg  $\text{kg}^{-1}$  produced maximal plasma concentration of 3  $\mu\text{M}$  1 h after administration and the drug was still detectable after 8 h (0.08  $\mu\text{M}$ ) (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  $\mu\text{M}$ , although high range of variability between individual samples was observed (Denning et al. 1989; Buchkowsky et al. 2005). Similar to ketoconazole, itraconazole is extensively metabolized in the liver, predominantly by CYP3A4,

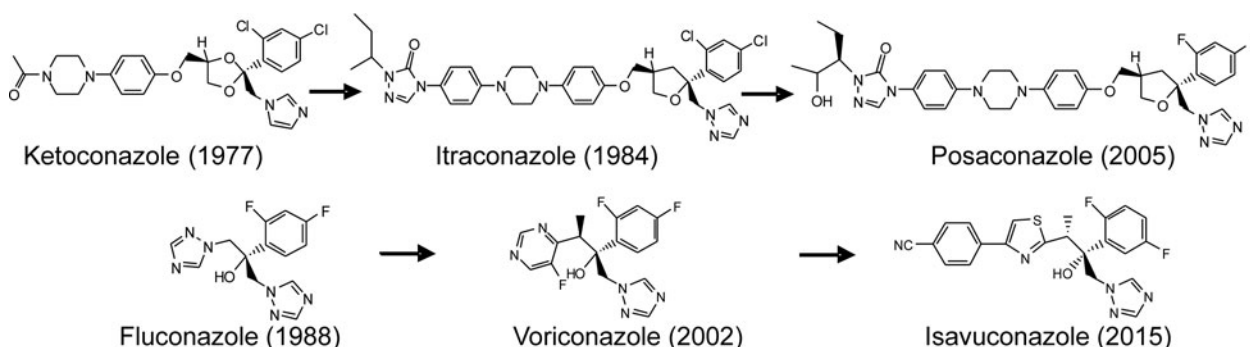


Fig. 2. Clinical antifungal azoles used for the treatment of systemic human infections. The whole set is represented by six derivatives of two basic scaffolds, fluconazole and ketoconazole. Ketoconazole is an imidazole, the others are 1,2,4-triazoles.

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  $\beta$ -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 800–1200 mg, duration of therapy can be more than 1 year (Keating, 2005; Clark *et al.* 2015). Posaconazole is the broadest spectrumazole 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  $\mu\text{M}$  was achieved in 10 h, and the drug was still detectable within 183 h (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 h, varying from 1.8  $\mu\text{M}$  (high-fat meal) to 0.5  $\mu\text{M}$  (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  $\mu\text{g mL}^{-1}$  had a 75% response rate, whereas patients with mean concentrations of 0.41–0.72 and 0.13  $\mu\text{g mL}^{-1}$  had response rates of 53 and 24%, respectively), a plasma concentration >1.25  $\mu\text{g mL}^{-1}$  (1.8  $\mu\text{M}$ ) has been suggested as a conservative target (Walsh *et al.* 2007; Clark *et al.* 2015). The PK values reported in mice also vary. A single dose of 20 mg  $\text{kg}^{-1}$  produced maximal plasma concentration of 6  $\mu\text{M}$  8 h after administration, the drug concentration at 24 h was 3  $\mu\text{M}$  (Nomeir *et al.* 2008). A single dose of 40 mg  $\text{kg}^{-1}$  produced maximal plasma concentration of 6  $\mu\text{M}$  3 h after administration, the drug concentration at 24 h decreased to 1.1  $\mu\text{M}$  (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 (Wexler *et al.* 2004; Lass-Flörl, 2011). Posaconazole is generally well tolerated, and serious side-effects are rare. The most commonly reported adverse reactions are 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 the 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 and 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 and Arendrup, 2015; Pappas *et al.* 2016). Fluconazole is also used to treat blastomycosis (Brick and 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  $\mu\text{M}$  was reached in 2 h; 24 h after administration it remained 8  $\mu\text{M}$  (Debruyne, 1997). The PK values reported in mice are: a single oral dose of 1 mg  $\text{kg}^{-1}$  resulted in maximal plasma concentration of 2.3  $\mu\text{M}$  1 h after administration (Fromtling, 1988), while a 5 mg  $\text{kg}^{-1}$  dose led to the concentration of 23  $\mu\text{M}$  (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 (2–13%), dizziness (1%), skin rash (2%), nausea (2–7%), abdominal pain (2–6%), vomiting (2–5%), diarrhoea (2–3%) 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 analogue, 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 analogues before voriconazole was selected (Denning and 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 and Mylonakis, 2006; Lass-Flörl, 2011; Denning and Bromley, 2015): 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  $\mu\text{M}$  was reached within 2.4 h; 24 h later it decreased to 0.3  $\mu\text{M}$  (Peng and 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  $\mu\text{M}$ , although high inter- and inpatient variability is observed (Aperis and Mylonakis, 2006; Elewa *et al.* 2015),

suggesting that therapeutic drug monitoring may be beneficial for optimizing both efficacy and safety (Pasqualotto *et al.* 2010; Elewa *et al.* 2015). The PK values in mice are a single oral dose of 40 mg kg<sup>-1</sup> produced maximal plasma concentration of 31 µM 1 h after administration, about 15 µM was detectable in 12 h (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 a little clinical effect and their excretion in urine does not depend on renal function (Aperis and 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 rash (7%) fever, nausea, vomiting, diarrhoea, headache, abdominal pain and respiratory disorders (<5%) (Heeres *et al.* 2010; Lass-Flörl, 2011; Elewa *et al.* 2015).

### Isavuconazole (2015)

Isavuconazole (Fig. 2, MW 437, log P 5.24) is produced by Astellas as a water-soluble prodrug isavucononium sulphate (Pettit and Carver, 2015; Maertens *et al.* 2016). 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 PKs. 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 h; 24 h after administration the concentration decreases to 0.3 µM, elimination time is extended to 76–104 h. 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 and 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<sup>-1</sup> produced maximal plasma concentration of 10 µM in 0.5 h, the concentration after 8 h was dropping to 0.5 µM (Warn *et al.* 2009). Isavuconazole appears to have fewer drug interactions than voriconazole (Pettit and 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, hypokalaemia, 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 six clinical azoles represent the safest and most widely used systemic antifungals, the cornerstone of antifungal therapy (Wilby, 2017).

### Repurposing of systemic antifungals for human 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.

### *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 analogues. 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 *T. cruzi* and *Leishmania*.

*Trypanosoma cruzi*. *T. cruzi* along with its haematophagous 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 9000 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 and Molina, 2017).

Chagas disease is actually an anthroponosis, with >150 mammalian species forming the infection reservoir. *Trypanosoma 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. *Trypanosoma 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 and Molina, 2017).

By now it is quite clear that the term *T. 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 and 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 and 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 the cellular growth of Tulahuen *T. cruzi* at concentrations of about 20  $\mu\text{M}$  (Docampo *et al.* 1981). In 1983, it was demonstrated that oral ketoconazole (30 mg  $\text{kg}^{-1}$ ) 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  $\text{kg}^{-1}$ ) (McCabe *et al.* 1984). Similar to fungi, inhibition of *T. cruzi* multiplication by ketoconazole was accompanied by the 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  $\text{kg}^{-1}$  and apparently leading to the parasitological cure if mice were treated with 150 mg  $\text{kg}^{-1}$  for about 60 days (McCabe *et al.* 1986). Moreover, in 2013 the outcome of 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 (Goat *et al.* 1989). Treatment of mice infected with Tulahuen *T. cruzi* with voriconazole, 40 mg  $\text{kg}^{-1}$  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  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) led to a 100% parasitological cure and 100% survival. In the chronic model, the treatment with posaconazole was started 45–60 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  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) 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  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) or benznidazole (100 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ). 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 synergistic 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 *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

[vs 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- 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 5–10-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 PKs, the reason why ravuconazole has never passed clinical trials as an antifungal drug. The data on its isomer isavuconazole are not yet available.

In all cases, when the *T. cruzi* cellular sterols were analysed 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 (catalysed 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 14 $\alpha$ -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 and Jansen, 2014). The disease is spread all over the world, though is more prevalent in the countries with a 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 20 000–30 000 deaths per year ([www.who.int/mediacentre/factsheets/fs375/en/](http://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 anaemia. 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](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 the 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 3 months. The cutaneous lesions disappeared after 2 weeks, while healing of mucocutaneous infections required 12 weeks. No relapses were seen 3 months after the treatment was completed (Urcuyo and Zaias, 1982). Other examples of successful use of ketoconazole (Jolliffe, 1986; Saenz et al. 1990; Navin et al. 1992), itraconazole (Borelli, 1987; Dogra and Saxena, 1996; Momeni et al. 1996; White et al. 2006), posaconazole (Paniz Mondolfi et al. 2011) and even fluconazole (Alrajhi et al. 2002; Toubiana et al. 2006; Sousa et al. 2011; Daly et al. 2014) 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 and Seidenath, 1984). But the question, why they have low efficiency, particularly against visceral leishmaniasis, both in humans (Sundar et al. 1990; Momeni et al. 1996) 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-desmethyl lanosterol) and C14-methylated fecosterol (C4-desmethyl obtusifoliol) accumulate (Berman et al. 1984; Goad et al. 1985; Beach et al. 1988; 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 a prompt cytotoxic 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 *T. 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 4000, and the estimated number of actual cases was only about 15 000 ([http://www.who.int/trypanosomiasis\\_african/country/en](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).

*Trypanosoma 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. *Trypanosoma 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/sleeping-sickness/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 and 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 the growth of both procyclic and bloodstream cells of *T. brucei* in a dose-dependent manner with the EC<sub>50</sub> of about 15 μM (Lepesheva et al. 2007). Most recently, the corresponding value of EC<sub>50</sub> = 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 findings 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.

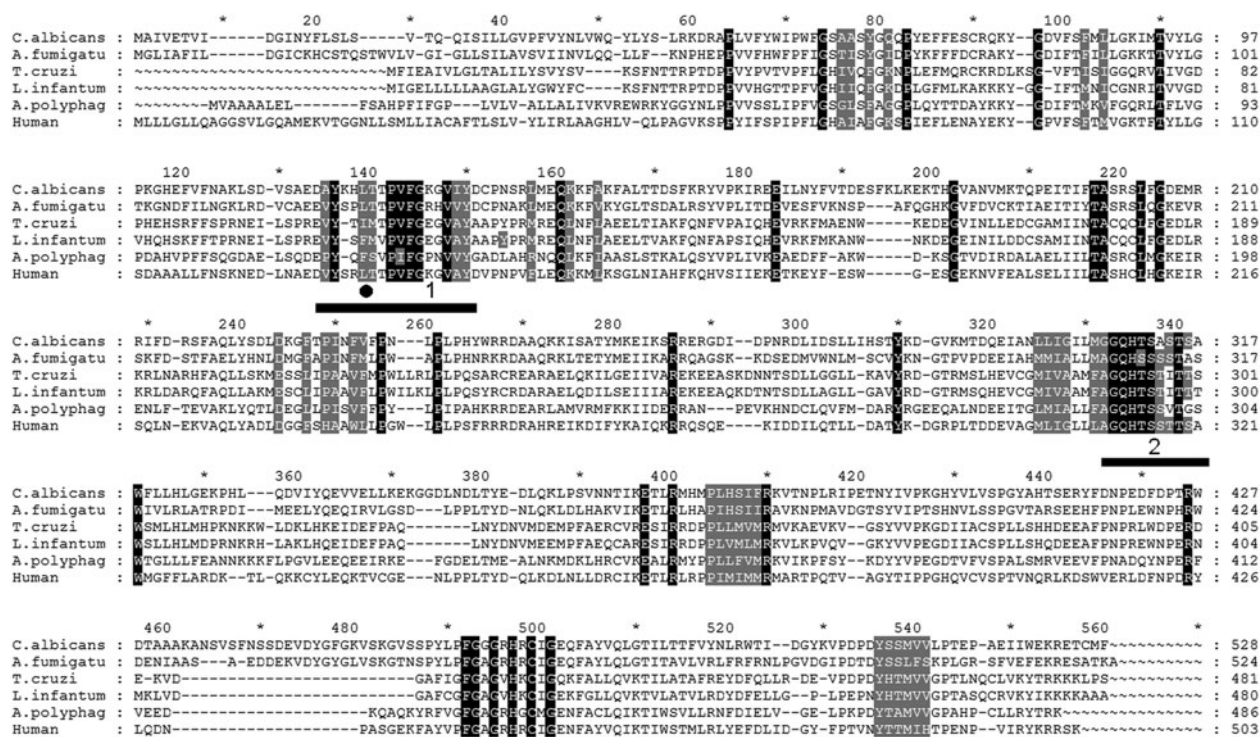
### Opportunistic human parasites amongst free-living amoeba

Some details of the sterol biosynthesis pathway in the pathogenic free-living amoebas remain elusive. In 1980s, it was reported that *Acanthamoeba polyphaga* (Raederstorff and Rohmer, 1985) and two species of amoeba of the genus *Naegleria* (Raederstorff and 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-methylenecycloartenol and then forms the CYP51 substrate obtusifolol. Yet in 2017, another team of authors, when analysing 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. *Balamuthia mandrillaris* may form a skin lesion or may migrate to the brain and cause granulomatous encephalitis. *Naegleria 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 amoebic 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 *A. 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 the treatment of *A. keratitis* (Amoils and Heney, 1999). A patient with AIDS diagnosed with granulomatous amoebic 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 the treatment of disseminated *Acanthamoeba* infection in a lung transplant recipient, where it was administered in combination with amphotericin B (Walia *et al.* 2007). Subsequently, other cases of successful



**Fig. 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 grey. 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 haem-coordinating cysteine, is marked with the dashed line.



treatment of amoebic keratitis (Bang *et al.* 2010; Tu *et al.* 2010; Arnalich-Montiel *et al.* 2012) and granulomatous amoebic encephalitis (Webster *et al.* 2012) using voriconazole monotherapy or voriconazole-containing drug combinations were reported.

Most recently, direct inhibition of the *A. 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, obtusifolium 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 a 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.

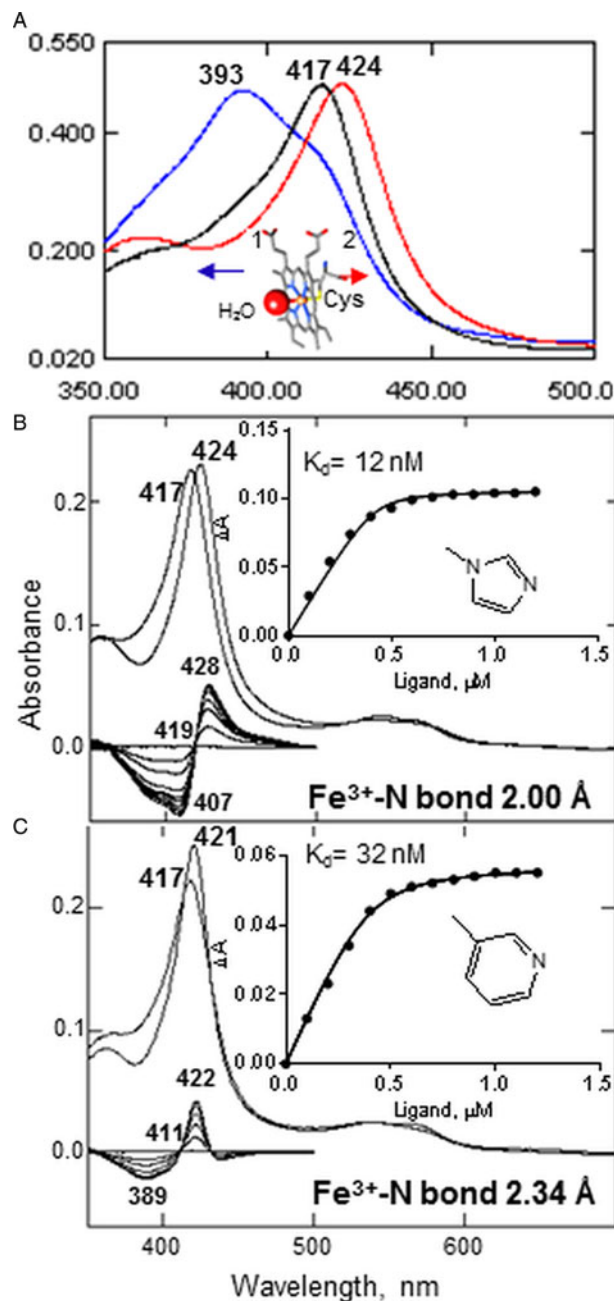
### CYP51 sequences

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 *Candida albicans* and *Aspergillus fumigatus* CYP51 sequences are only 46%. The average length of a eukaryotic CYP51 polypeptide chain is about 500 residues, and yet only <40 of them are completely conserved across the phyla (Lepesheva and 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 and 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 (*Escherichia 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 haemoprotein, upon the iron reduction CYP51 produce a characteristic absorbance maximum around 450 nm after binding of carbon monoxide (Omura and Sato, 1964).

### CYP51 spectral response to ligand binding

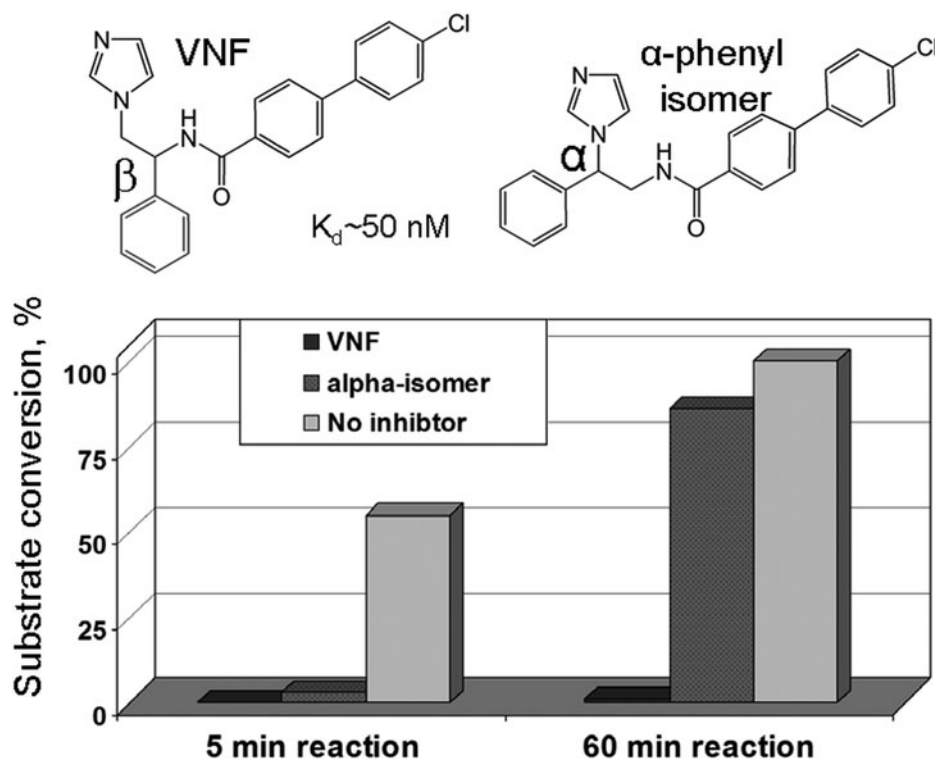
When expressed in bacteria, CYP51 enzymes display the Soret band maximum at 417 nm (Fig. 4A). This means that the purified protein is in the substrate-free form, with the haem iron in the oxidized ( $\text{Fe}^{3+}$ ) low-spin hexa-coordinated state and a water molecule playing the role of the sixth (axial) ligand, bound to the iron on the distal side of the haem plane, the side that forms the catalytic surface of the CYP51 substrate-binding cavity (Hargrove *et al.* 2012a). When the substrate (a substrate analogue) enters the CYP51-binding cavity, it expels the water molecule from the iron coordination sphere. The iron becomes penta-coordinated



**Fig. 4.** CYP51 binding ligands can be identified by spectral titration. (A) Absolute absorbance spectra of water-bound (black), obtusifolium-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 haem 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  $\sim 0.4 \mu\text{M}$ , the optical path length 5 cm. Insets: the titration curves, prepared in Prism.

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 haem 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. (Lepesheva *et al.* 2008; Konkle *et al.* 2009), 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  $K_d$ s do



**Fig. 5.** Low spectral  $K_d$ s do not necessarily mean strong inhibition of the CYP51 activity. While both VNF and its  $\alpha$ -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  $1 \mu\text{M}$  *T. cruzi* CYP51,  $1 \mu\text{M}$  inhibitor and  $50 \mu\text{M}$  substrate.

not necessarily mean strong enzyme inhibition (Fig. 5), and therefore tight binding ligands with the apparent  $K_d$ s <  $1 \mu\text{M}$  (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.* 2007; Lepesheva *et al.* 2008).

### CYP51 inhibition

By definition, antifungal azoles are supposed to work as competitive reversible inhibitors. Indeed, many of them (for example, fluconazole), similar to the  $\alpha$ -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 PK 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 (Yoshida and Aoyama, 1987; Lepesheva *et al.* 2007; Lepesheva *et al.* 2008) (Fig. 6A–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.

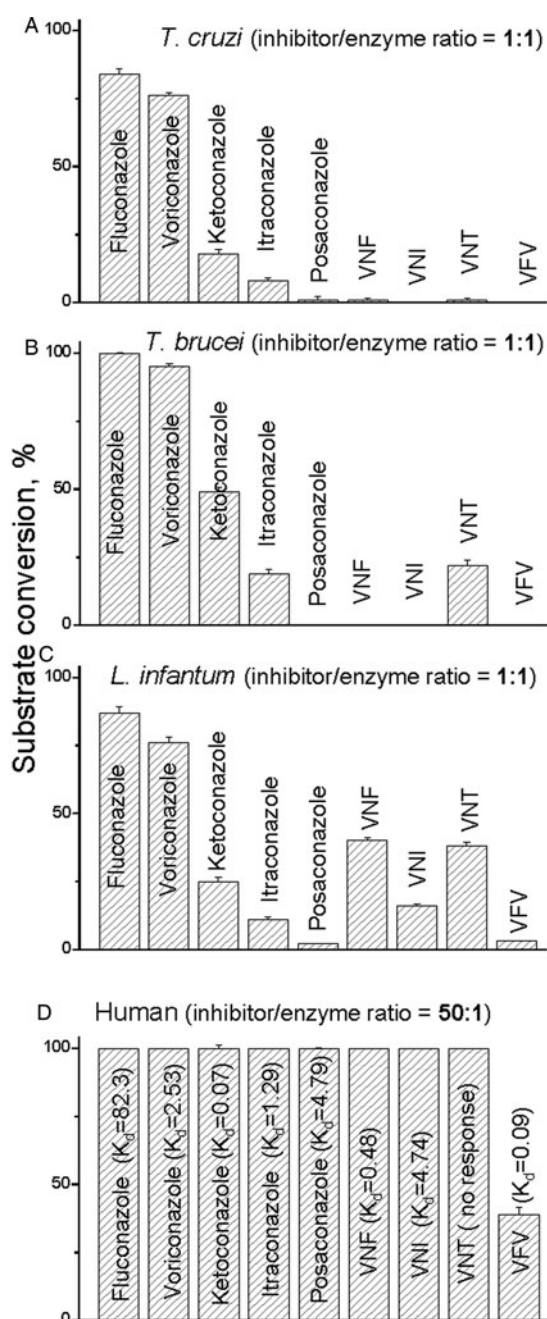
### CYP51 structures and structural basis for the enzyme druggability

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 (Lepesheva *et al.* 2010a; Hargrove *et al.* 2011) and experimental compounds, haem-coordinated

imidazole- (Lepesheva *et al.* 2010a, b; Buckner *et al.* 2012; Andriani *et al.* 2013; Friggeri *et al.* 2014), triazole- (Lepesheva *et al.* 2015), pyridine- (Hargrove *et al.* 2013) and tetrazole-based (Hoekstra *et al.* 2016) as well as the substrate analogue MCP (Hargrove *et al.* 2012b), CYP51s from two fungal human pathogens [*A. fumigatus* (Hargrove *et al.* 2015; Hargrove *et al.* 2017b) 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 and 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 haem-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) (Lepesheva and Waterman, 2011; Hargrove *et al.* 2017a).

All the inhibitors, including the substrate analogue 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.



**Fig. 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  $\pm$  s.e.m. In all experiments the P450 concentration was 0.5  $\mu$ M, the concentration of the sterol substrates [(A) eburicol; (B, C) obtusifoliiol; (D) lanosterol] was 50  $\mu$ M. The values of the apparent spectral  $K_d$ s for human CYP51 are given in  $\mu$ M.

### 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, b). Both of these compounds act as functionally irreversible inhibitors of the *A. fumigatus* and *C. albicans* enzymes (Hargrove et al. 2017a, b). Besides, they have also been found effective *in vivo* against isolates of *Candida glabrata* and *Candida 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 a functionally irreversible inhibitor for all tested CYP51s from human pathogens. In the CYP51 structures, its long arm is exposed above the protein surface (see Fig. 9), forming multiple interactions around the substrate entry (Lepesheva et al. 2010a; Hargrove et al. 2017a). 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

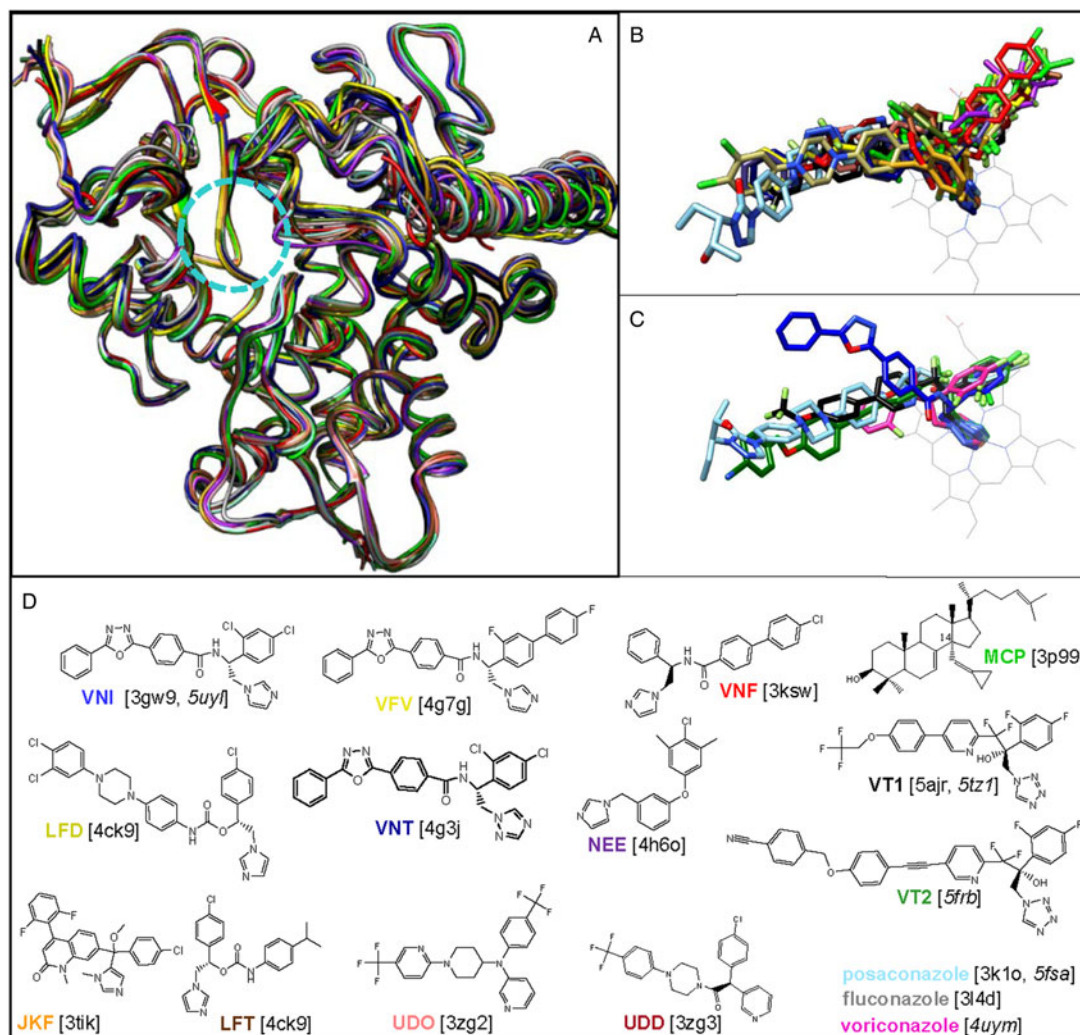
The helices C and I (they contact the biphenyl arm of VFV in Fig. 8) form the deepest portion of the CYP51 substrate-binding cavity, which is unique for CYP51s and is not present in other P450s. This area supports the aliphatic arm of the sterol molecule (Hargrove et al. 2012b), and the compounds that 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 (Lepesheva et al. 2010a; Andriani et al. 2013). Both of these compounds were found to produce a strong anti-parasitic 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



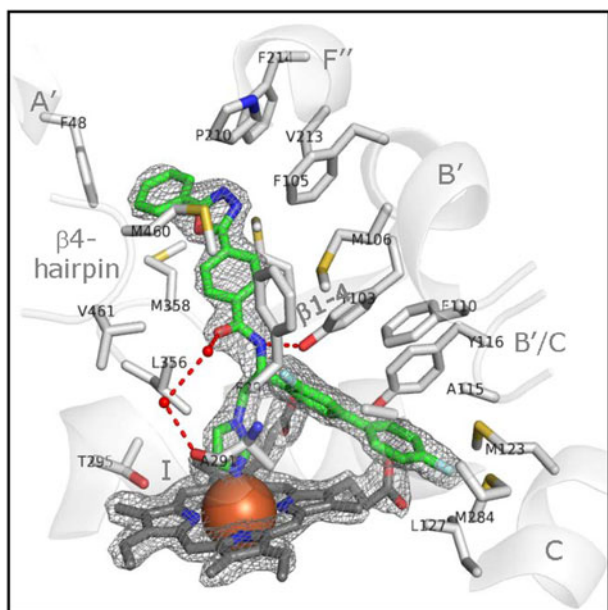
**Fig. 7.** Binding of inhibitors does not cause any large-scale rearrangements in the backbone of the CYP51 molecule. (A, B) Superimposed protozoan CYP51 structures, (A) protein chains of ligand-free *T. brucei* (black) and inhibitor-bound *T. brucei*, *T. cruzi* and *L. infantum* CYP51. The substrate entry is circled. (B) Inhibitors bound in the protozoan CYP51 active site. (C) Inhibitors bound to the fungal CYP51 active site. The haem is depicted in grey. (D) Formulas of the inhibitors (posaconazole, voriconazole and fluconazole are shown in Fig. 2). The colour code of each crystal structure corresponds to the colour 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.

ring may have a positive effect on the compound PKs. 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<sub>50</sub> values within 1 nM (Lepesheva *et al.* 2015). In the same experiments, posaconazole displayed the EC<sub>50</sub> of 5 nM (Villalta *et al.* 2013). Furthermore, both compounds showed the weaker inhibitory effect on CYP3A4 [the IC<sub>50</sub> 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 lifetime 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

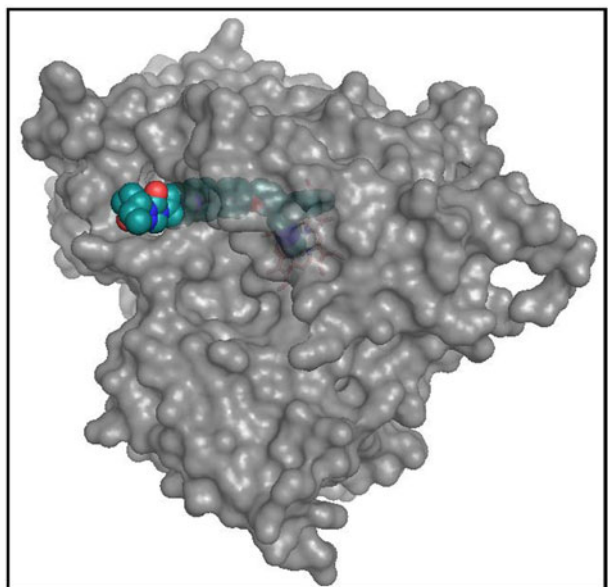
also showed better results in curing the Y strain *T. cruzi* infection in mice (Guedes-da-Silva *et al.* 2017). Moreover, after a single oral dose of 25 mg kg<sup>-1</sup>, VFV displayed a longer clearance time in mouse plasma (Fig. 10A) and revealed higher than VNI affinities to tissues (Fig. 10B). 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-binding 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

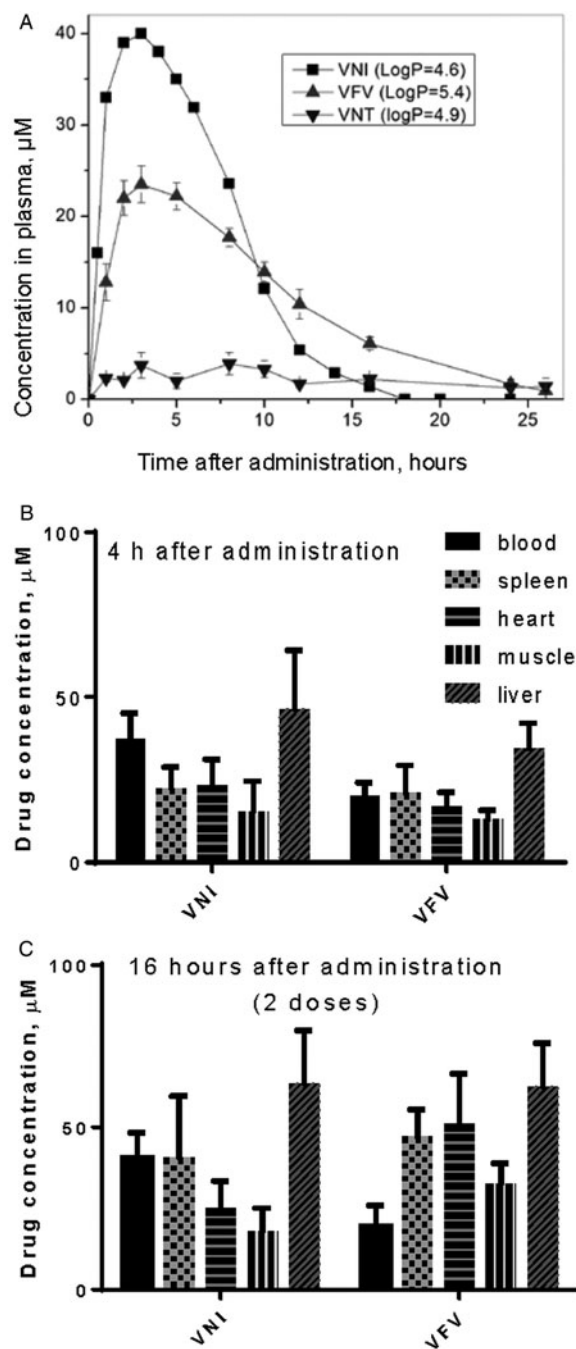


**Fig. 8.** VFV bound in the CYP51 active site. The  $2F_o - F_c$  electron density map around the haem and the inhibitor is contoured at  $2.0 \sigma$  and shown as a grey mesh. The carbon atoms of VFV are green, the carbon atoms of 22 CYP51 residues that form Van der Waals contacts with the inhibitor are light grey, and the carbon atoms of the haem are dark grey. The atoms of oxygen, nitrogen, and sulphur are read, blue, and yellow, respectively. The haem iron is presented as an orange sphere. The active site-defining secondary structural elements (semitransparent cartoon) are labelled for clarity. The H-bonds between the enzyme and inhibitor are displayed as red dashes.



**Fig. 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.

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  $\alpha$ -helical H-bonding (Hargrove *et al.* 2016). The notion of the higher flexibility of the human CYP51 active site is supported by structural dynamic simulation experiments (Yu *et al.*



**Fig. 10.** Pharmacokinetics of VNI and derivatives. (A) Plasma concentration curves after a single oral dose of  $25 \text{ mg kg}^{-1}$ . (B) VNI and VFV tissue distribution 4 h after administration (single dose) and 16 h after administration (two doses).

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 HTS, as well as a 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 PKs 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.

**Financial support.** Financial support was provided by the National Institutes of Health Grant R01 GM067871 (to G.I.L.).

**Conflict of interest.** None.

**Ethical standards.** Not applicable.

## References

- Al-Abdely HM, *et al.* (1999) Efficacy of the triazole SCH 56592 against *Leishmania amazonensis* and *Leishmania donovani* in experimental murine cutaneous and visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy* **43**, 2910–2914.
- Alrajhi AA, *et al.* (2002) Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. *New England Journal of Medicine* **346**, 891–895.
- Amoils SP and Heney C (1999) *Acanthamoeba keratitis* with live isolates treated with cryosurgery and fluconazole. *American Journal of Ophthalmology* **127**, 718–720.
- Andes D (2013) Optimizing antifungal choice and administration. *Current Medical Research and Opinions* **29**(Suppl. 4), 13–18.
- Andriani G, *et al.* (2013) Antitrypanosomal lead discovery: identification of a ligand-efficient inhibitor of *Trypanosoma cruzi* CYP51 and parasite growth. *Journal of Medicinal Chemistry* **56**, 2556–2567.
- Aoyama Y, Yoshida Y and Sato R (1984) Yeast cytochrome P-450 catalyzing lanosterol 14 alpha-demethylation. II. Lanosterol metabolism by purified P-450(14)DM and by intact microsomes. *Journal of Biological Chemistry* **259**, 1661–1666.
- Aperis G and Mylonakis E (2006) Newer triazole antifungal agents: pharmacology, spectrum, clinical efficacy and limitations. *Expert Opinion on Investigational Drugs* **15**, 579–602.
- Apt W, *et al.* (1998) Treatment of chronic Chagas' disease with itraconazole and allopurinol. *American Journal of Tropical Medicine and Hygiene* **59**, 133–138.
- Apt W, *et al.* (2013) Treatment of Chagas' disease with itraconazole: electrocardiographic and parasitological conditions after 20 years of follow-up. *Journal of Antimicrobial Chemotherapy* **68**, 2164–2169.
- Arnalich-Montiel F, *et al.* (2012) Successful monitoring and treatment of intraocular dissemination of *acanthamoeba*. *Archives of Ophthalmology* **130**, 1474–1475.
- Aufderheide AC, *et al.* (2004) A 9,000-year record of Chagas' disease. *Proceedings of National Academy of Sciences of the United States of America* **101**, 2034–2039.
- Bang S, *et al.* (2010) Treatment with voriconazole in 3 eyes with resistant *Acanthamoeba keratitis*. *American Journal of Ophthalmology* **149**, 66–69.
- Beach DH, Goad LJ and Holz GG (1986) Effects of ketoconazole on sterol biosynthesis by *Trypanosoma cruzi* epimastigotes. *Biochemical and Biophysical Research Communications* **136**, 851–856.
- Beach DH, Goad LJ and Holz Jr. GG (1988) Effects of antimycotic azoles on growth and sterol biosynthesis of *Leishmania* promastigotes. *Molecular and Biochemical Parasitology* **31**, 149–162.
- Benaim G, *et al.* (2006) Amiodarone has intrinsic anti-*Trypanosoma cruzi* activity and acts synergistically with posaconazole. *Journal of Medicinal Chemistry* **49**, 892–899.
- Berman JD (1997) Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clinical Infectious Diseases* **24**, 684–703.
- Berman JD, Holz Jr. GG and Beach DH (1984) Effects of ketoconazole on growth and sterol biosynthesis of *Leishmania mexicana* promastigotes in culture. *Molecular and Biochemical Parasitology* **12**, 1–13.
- Bern C, *et al.* (2011) *Trypanosoma cruzi* and Chagas' disease in the United States. *Clinical Microbiology Reviews* **24**, 655–681.
- Borelli D (1987) A clinical trial of itraconazole in the treatment of deep mycoses and leishmaniasis. *Reviews of Infectious Diseases* **9**(Suppl. 1), S57–S63.
- Borelli D, *et al.* (1979) Ketoconazole, an oral antifungal: laboratory and clinical assessment of imidazole drugs. *Postgraduate Medical Journal* **55**, 657–661.
- Brick KE and Agger WA (2012) Successful treatment of brainstem blastomycosis with fluconazole. *Clinical Medicine and Research* **10**, 72–74.
- Bruggemann RJ, *et al.* (2009) Clinical relevance of the pharmacokinetic interactions of azole antifungal drugs with other coadministered agents. *Clinical Infectious Diseases* **48**, 1441–1458.
- Buchkowsky SS, Partovi N and Ensom MH (2005) Clinical pharmacokinetic monitoring of itraconazole is warranted in only a subset of patients. *Therapeutic Drug Monitoring* **27**, 322–333.
- Buckner FS and Urbina JA (2012) Recent developments in sterol 14-demethylase inhibitors for Chagas disease. *International Journal for Parasitology, Drugs and Drug Resistance* **2**, 236–242.
- Buckner F, *et al.* (2012) Pharmacological characterization, structural studies, and in vivo activity of anti-Chagas disease lead compounds derived from tipifarnib. *Antimicrobial Agents and Chemotherapy* **56**, 4914–4921.
- Calvo E, *et al.* (2010) Murine model of a disseminated infection by the novel fungus *Fonsecaea monophora* and successful treatment with posaconazole. *Antimicrobial Agents and Chemotherapy* **54**, 919–923.
- Campos R, *et al.* (1992) Evaluation of the therapeutic activity of fluconazole in acute experimental infection caused by *Trypanosoma cruzi*. *Revista do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* **47**, 174–175.
- Carbajal H, *et al.* (2004) Cyclosporine and low-dose ketoconazole in renal transplant recipients: a single-center experience. *Transplantation* **77**, 1038–1040.
- Chapman SA, *et al.* (1996) Considerations for using ketoconazole in solid organ transplant recipients receiving cyclosporine immunosuppression. *Journal of Transplant Coordination* **6**, 148–154.
- Cherkesova TS, *et al.* (2014) Sequence variation in CYP51A from the Y strain of *Trypanosoma cruzi* alters its sensitivity to inhibition. *FEBS Letters* **588**, 3878–3885.
- Clark NM, Grim SA and Lynch 3rd JP (2015) Posaconazole: use in the prophylaxis and treatment of fungal infections. *Seminars in Respiratory and Critical Care Medicine* **36**, 767–785.
- Coppens I and Courtroy PJ (2000) The adaptive mechanisms of *Trypanosoma brucei* for sterol homeostasis in its different life-cycle environments. *Annual Review of Microbiology* **54**, 129–156.
- Cornely OA (2017) Isavuconazole: is there a need for a new antifungal? *Journal of Antimicrobial Chemotherapy* **72**, i2–i4.
- Dalvie D, *et al.* (2002) Biotransformation reactions of five-membered aromatic heterocyclic rings. *Chemical Research in Toxicology* **15**, 269–299.
- Daly K, *et al.* (2014) Intermediate cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis* successfully treated with fluconazole. *Clinical and Experimental Dermatology* **39**, 708–712.
- Dauchy FA, *et al.* (2016) *Trypanosoma brucei* CYP51: essentiality and targeting therapy in an experimental model. *PLoS Neglected Tropical Diseases* **10**, e0005125.
- Debruyne D (1997) Clinical pharmacokinetics of fluconazole in superficial and systemic mycoses. *Clinical Pharmacokinetics* **33**, 52–77.
- Denning DW and Bromley MJ (2015) How to bolster the antifungal pipeline. *Science* **347**, 1414–1416.
- Denning DW, *et al.* (1989) Treatment of invasive aspergillosis with itraconazole. *American Journal of Medicine* **86**, 791–800.
- De Pascale G and Tumbarello M (2015) Fungal infections in the ICU: advances in treatment and diagnosis. *Current Opinion in Critical Care* **21**, 421–429.
- Docampo R, *et al.* (1981) Biochemical and ultrastructural alterations produced by miconazole and econazole in *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology*, **3**, 169–180.
- Dogra J and Saxena VN (1996) Itraconazole and leishmaniasis: a randomised double-blind trial in cutaneous disease. *International Journal of Parasitology* **26**, 1413–1415.

- Elewa H, et al.** (2015) Therapeutic drug monitoring of voriconazole in the management of invasive fungal infections: a critical review. *Clinical Pharmacokinetics* **54**, 1223–1235.
- El-Sayed NM, et al.** (2005) The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* **309**, 409–415.
- Ezzet F, et al.** (2005) Oral bioavailability of posaconazole in fasted healthy subjects: comparison between three regimens and basis for clinical dosage recommendations. *Clinical Pharmacokinetics* **44**, 211–220.
- Farowski F, et al.** (2010) Intracellular concentrations of posaconazole in different compartments of peripheral blood. *Antimicrobial Agents and Chemotherapy* **54**, 2928–2931.
- Felton T, Troke PF and Hope WW** (2014) Tissue penetration of antifungal agents. *Clinical Microbiology Reviews* **27**, 68–88.
- Filardi LS and Brener Z** (1987) Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 755–759.
- Friggeri L, et al.** (2014) Structural basis for rational design of inhibitors targeting *Trypanosoma cruzi* sterol 14 $\alpha$ -demethylase: two regions of the enzyme molecule potentiate its inhibition. *Journal of Medicinal Chemistry* **57**, 6704–6717.
- Fromling RA** (1988) Overview of medically important antifungal azole derivatives. *Clinical Microbiology Reviews* **1**, 187–217.
- Frye LL and Leonard DA** (1999) Lanosterol analogs: dual-action inhibitors of cholesterol biosynthesis. *Critical Reviews in Biochemistry and Molecular Biology* **34**, 123–140.
- Gelman BB, et al.** (2001) Amoebic encephalitis due to *Sappinia diploidea*. *Jama* **285**, 2450–2451.
- Goad LJ, Holz Jr GG and Beach DH** (1985) Sterols of ketoconazole-inhibited *Leishmania mexicana mexicana* promastigotes. *Molecular and Biochemical Parasitology* **15**, 257–279.
- Goad LJ, et al.** Jr (1989) The activity of ketoconazole and other azoles against *Trypanosoma cruzi*: biochemistry and chemotherapeutic action in vitro. *Molecular and Biochemical Parasitology* **32**, 179–189.
- Graybill JR and Craven PC** (1983) Antifungal agents used in systemic mycoses. *Activity and therapeutic use. Drugs* **25**, 41–62.
- Guedes-da-Silva FH, et al.** (2017) Antitrypanosomal activity of sterol 14 $\alpha$ -demethylase (CYP51) inhibitors VNI and VFV in the Swiss mouse models of Chagas disease induced by the *Trypanosoma cruzi* Y strain. *Antimicrobial Agents and Chemotherapy* **61**, pii: e02098-16. doi: 10.1128/aac.02098-16.
- Gulin JEN, et al.** (2013) Efficacy of voriconazole in a murine model of acute *Trypanosoma cruzi* infection. *Journal of Antimicrobial Chemotherapy* **68**, 888–894.
- Gullo A** (2009) Invasive fungal infections. *Drugs* **69**, 65–73.
- Halim MA, et al.** (1993) Successful treatment of visceral leishmaniasis with allopurinol plus ketoconazole in a renal transplant recipient after the occurrence of pancreatitis due to stibogluconate. *Clinical Infectious Diseases* **16**, 397–399.
- Hardin TC, et al.** (1988) Pharmacokinetics of itraconazole following oral administration to normal volunteers. *Antimicrobial Agents and Chemotherapy* **32**, 1310–1313.
- Hargrove TY, et al.** (2011) Substrate preferences and catalytic parameters determined by structural characteristics of sterol 14 $\alpha$ -demethylase (CYP51) from *Leishmania infantum*. *Journal of Biological Chemistry* **286**, 26838–26848.
- Hargrove TY, et al.** (2012a) CYP51 structures and structure-based development of novel, pathogen-specific inhibitory scaffolds. *International Journal of Parasitology. Drugs and Drug Resistance* **2**, 178–186.
- Hargrove TY, et al.** (2012b) Structural complex of sterol 14 $\alpha$ -demethylase (CYP51) with 14 $\alpha$ -methylenecyclopropyl- $\Delta$ 7-24, 25-dihydrolanosterol. *Journal of Lipid Research* **53**, 311–320.
- Hargrove TY, et al.** (2013) Complexes of *Trypanosoma cruzi* sterol 14 $\alpha$ -demethylase (CYP51) with two pyridine-based drug candidates for Chagas disease: structural basis for pathogen selectivity. *Journal of Biological Chemistry* **288**, 31602–31615.
- Hargrove TY, et al.** (2015) Structure-functional characterization of cytochrome P450 sterol 14 $\alpha$ -demethylase (CYP51B) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *Journal of Biological Chemistry* **290**, 23916–23934.
- Hargrove TY, et al.** (2016) Human sterol 14 $\alpha$ -demethylase as a target for anticancer chemotherapy: towards structure-aided drug design. *Journal of Lipid Research* **57**, 1552–1563.
- Hargrove TY, et al.** (2017a) Structural analyses of *Candida albicans* sterol 14 $\alpha$ -demethylase complexed with azole drugs address the molecular basis of azole-mediated inhibition of fungal sterol biosynthesis. *Journal of Biological Chemistry* **292**, 6728–6743.
- Hargrove TY, et al.** (2017b) Crystal structure of the new investigational drug candidate VT-1598 in complex with *Aspergillus fumigatus* sterol 14 $\alpha$ -demethylase provides insights into its broad-spectrum antifungal activity. *Antimicrobial Agents and Chemotherapy* **61**, pii: e00570-17. doi: 10.1128/aac.00570-17.
- Haubrich BA, et al.** (2015) Discovery of an ergosterol-signaling factor that regulates *Trypanosoma brucei* growth. *Journal of Lipid Research* **56**, 331–341.
- Heel RC, et al.** (1982) Ketoconazole: a review of its therapeutic efficacy in superficial and systemic fungal infections. *Drugs* **23**, 1–36.
- Heeres J, Meerpoel L and Lewi P** (2010) Conazoles. *Molecules* **15**, 4129–4188.
- Heykants J, et al.** (1990) Pharmacokinetics of oral antifungals and their clinical implications. *British Journal of Clinical Practice. Supplement* **71**, 50–56.
- Hirst LW, et al.** (1984) Management of *Acanthamoeba keratitis*. A case report and review of the literature. *Ophthalmology* **91**, 1105–1111.
- Hoekstra WJ, et al.** (2016) Clinical candidate VT-1161's antiparasitic effect in vitro, activity in a murine model of Chagas disease, and structural characterization in complex with the target enzyme CYP51 from *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **60**, 1058–1066.
- Horber FF, et al.** (1993) Visceral leishmaniasis after orthotopic liver transplantation: impact of persistent splenomegaly. *Transplant International* **6**, 55–57.
- Huang YC, et al.** (1986) Pharmacokinetics and dose proportionality of ketoconazole in normal volunteers. *Antimicrobial Agents and Chemotherapy* **30**, 206–210.
- Ishibashi Y, et al.** (1990) Oral itraconazole and topical miconazole with debridement for *Acanthamoeba keratitis*. *American Journal of Ophthalmology* **109**, 121–126.
- Ishibashi H, et al.** (2007) Oral administration of itraconazole solution has superior efficacy in experimental oral and oesophageal candidiasis in mice than its intragastric administration. *Journal of Antimicrobial Chemotherapy* **59**, 317–320.
- Jennings TS and Hardin TC** (1993) Treatment of aspergillosis with itraconazole. *Annals of Pharmacotherapy* **27**, 1206–1211.
- Jeong W, et al.** (2016) Safety, clinical effectiveness and trough plasma concentrations of intravenous posaconazole in patients with haematological malignancies and/or undergoing allogeneic haematopoietic stem cell transplantation: off-trial experience. *Journal of Antimicrobial Chemotherapy* **71**, 3540–3547.
- Joice AC, et al.** (2017) Antileishmanial efficacy and pharmacokinetics of DB766-azole combinations. *Antimicrobial Agents and Chemotherapy* **62**, pii: e01129-17. doi: 10.1128/aac.01129-17.
- Jolliffe DS** (1986) Cutaneous leishmaniasis from Belize--treatment with ketoconazole. *Clinical and Experimental Dermatology* **11**, 62–68.
- Kalb VF, et al.** (1986) Isolation of a cytochrome P-450 structural gene from *Saccharomyces cerevisiae*. *Gene* **45**, 237–245.
- Kauffman CA, et al.** (2007) Posaconazole. *Nature Reviews Drug Discovery* **6**, 183–184.
- Keating GM** (2005) Posaconazole. *Drugs* **65**, 1553–1567, discussion 1568–1559.
- Konkle ME, et al.** (2009) Indomethacin amides as a novel molecular scaffold for targeting *Trypanosoma cruzi* sterol 14 $\alpha$ -demethylase. *Journal of Medicinal Chemistry* **52**, 2846–2853.
- Krieter P, et al.** (2004) Disposition of posaconazole following single-dose oral administration in healthy subjects. *Antimicrobial Agents and Chemotherapy* **48**, 3543–3551.
- Kullberg BJ and Arendrup MC** (2015) Invasive candidiasis. *New England Journal of Medicine* **373**, 1445–1456.
- Lamb DC, et al.** (2015) Azole antifungal agents to treat the human pathogens *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* through inhibition of sterol 14 $\alpha$ -demethylase (CYP51). *Antimicrobial Agents and Chemotherapy* **59**, 4707–4713.
- Lass-Flörl C** (2011) Triazole antifungal agents in invasive fungal infections. *Drugs* **71**, 2405–2419.
- Lepesheva GI** (2013) Design or screening of drugs for the treatment of Chagas disease: what shows the most promise? *Expert Opinion on Drug Discovery* **8**, 1479–1489.
- Lepesheva GI and Waterman MR** (2004) CYP51--the omnipotent P450. *Molecular and Cellular Endocrinology* **215**, 165–170.

- Lepesheva GI and Waterman MR** (2007) Sterol 14 $\alpha$ -demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochimica et Biophysica Acta* **1770**, 467–477.
- Lepesheva GI and Waterman MR** (2011) Structural basis for conservation in the CYP51 family. *Biochimica et Biophysica Acta* **1814**, 88–93.
- Lepesheva GI, et al.** (2004) CYP51 from *Trypanosoma brucei* is obtusifoliol-specific. *Biochemistry* **43**, 10789–10799.
- Lepesheva GI, et al.** (2006) CYP51 from *Trypanosoma cruzi*: a phyla-specific residue in the B' helix defines substrate preferences of sterol 14 $\alpha$ -demethylase. *Journal of Biological Chemistry* **281**, 3577–3585.
- Lepesheva GI, et al.** (2007) Sterol 14  $\alpha$ -demethylase as a potential target for antitrypanosomal therapy: enzyme inhibition and parasite cell growth. *Chemistry Biology* **14**, 1283–1293.
- Lepesheva G, et al.** (2008) CYP51: a major drug target in the cytochrome P450 superfamily. *Lipids* **43**, 1117–1125.
- Lepesheva GI, et al.** (2010a) Structural insights into inhibition of sterol 14  $\alpha$ -demethylase in the human pathogen *Trypanosoma cruzi*. *Journal of Biological Chemistry* **285**, 25582–25590.
- Lepesheva GI, et al.** (2010b) Crystal structures of *Trypanosoma brucei* sterol 14  $\alpha$ -demethylase and implications for selective treatment of human infections. *Journal of Biological Chemistry* **285**, 1773–1780.
- Lepesheva GI, et al.** (2015) VFV as a new effective CYP51 structure-derived drug candidate for Chagas disease and visceral leishmaniasis. *Journal of Infectious Diseases* **212**, 1439–1448.
- Leslie M** (2011) Drug developers finally take aim at a neglected disease. *Science* **333**, 933–935.
- Li Y, et al.** (2010) Pharmacokinetic/pharmacodynamic profile of posaconazole. *Clinical Pharmacokinetics* **49**, 379–396. doi:10.2165/11319340-000000000-000000000.
- Lopez-Barcons L, et al.** (2017) P450 inhibitor ketoconazole increased the intratumor drug levels and antitumor activity of fenretinide in human neuroblastoma xenograft models. *International Journal of Cancer* **141**, 405–413.
- Louie A, et al.** (1998) Pharmacodynamics of fluconazole in a murine model of systemic candidiasis. *Antimicrobial Agents and Chemotherapy* **42**, 1105–1109.
- Maertens JA, et al.** (2016) Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by *Aspergillus* and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. *The Lancet* **387**, 760–769.
- Martinez-Diaz RA, et al.** (2001) Biological characterization of *Trypanosoma cruzi* strains. *Memorias do Instituto Oswaldo Cruz* **96**, 53–59.
- Mavridou E, et al.** (2010) Impact of cyp51A mutations on the pharmacokinetic and pharmacodynamic properties of voriconazole in a murine model of disseminated aspergillosis. *Antimicrobial Agents Chemotherapy* **54**, 4758–4764.
- McCabe RE, Araujo FG and Remington JS** (1983) Ketoconazole protects against infection with *Trypanosoma cruzi* in a murine model. *American Journal of Tropical Medicine and Hygiene* **32**, 960–962.
- McCabe RE, Remington JS and Araujo FG** (1984) Ketoconazole inhibition of intracellular multiplication of *Trypanosoma cruzi* and protection of mice against lethal infection with the organism. *Journal of Infectious Diseases* **150**, 594–601.
- McCabe RE, Remington JS and Araujo FG** (1986) In vitro and in vivo effects of itraconazole against *Trypanosoma cruzi*. *American Journal of Tropical Medicine and Hygiene* **35**, 280–284.
- Mellado E, et al.** (2001) Identification of two different 14- $\alpha$  sterol demethylase-related genes (cyp51A and cyp51B) in *Aspergillus fumigatus* and other *Aspergillus* species. *Journal of Clinical Microbiology* **39**, 2431–2438.
- Mitropoulos K, Gibbons G and BE R** (1976) Lanosterol 14 $\alpha$ -demethylase. Similarity of the enzyme system from yeast and rat liver. *Steroids* **6**, 821–829.
- Molina J, et al.** (2000) Activities of the triazole derivative SCH 56592 (posaconazole) against drug-resistant strains of the protozoan parasite *Trypanosoma (Schizotrypanum) cruzi* in immunocompetent and immunosuppressed murine hosts. *Antimicrobial Agents and Chemotherapy* **44**, 150–155.
- Molina I, et al.** (2014) Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. *New England Journal of Medicine* **370**, 1899–1908.
- Molina I, Salvador F and Sa' nchez-Montalva' A** (2015) The use of posaconazole against Chagas disease. *Current Opinion in Infectious Diseases* **5**, 397–407.
- Momeni AZ, et al.** (1996) Treatment of cutaneous leishmaniasis with itraconazole. Randomized double-blind study. *Archives of Dermatology* **132**, 784–786.
- Morillo CA, et al.** (2017) Benznidazole and posaconazole in eliminating parasites in asymptomatic *T. cruzi* carriers: the STOP-CHAGAS trial. *Journal of American College of Cardiology* **69**, 939–947.
- Morschhauser J** (2016) The development of fluconazole resistance in *Candida albicans* – an example of microevolution of a fungal pathogen. *Journal of Microbiology* **54**, 192–201.
- Morton V and Staub T** (2008) *A short history of fungicides*. doi: 10.1094/APSnetFeature-2008-0308.
- Mosca P, et al.** (1985) In vivo and in vitro inhibition of hepatic microsomal drug metabolism by ketoconazole. *British Journal of Experimental Pathology* **66**, 737–742.
- Navin TR, et al.** (1992) Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *Journal of Infectious Diseases* **165**, 528–534.
- Nelson DR** (1999) Cytochrome P450 and the individuality of species. *Archives of Biochemistry and Biophysics* **369**, 1–10.
- Nelson DR, et al.** (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA and Cell Biology* **12**, 1–51.
- Nes WD** (2011) Biosynthesis of cholesterol and other sterols. *Chemical Reviews* **111**, 6423–6451.
- Nomeir AA, et al.** (2008) Posaconazole (Noxafil, SCH 56592), a new azole antifungal drug, was a discovery based on the isolation and mass spectral characterization of a circulating metabolite of an earlier lead (SCH 51048). *Journal of Mass Spectrometry* **43**, 509–517.
- Ohba M, et al.** (1978) Involvement of cytochrome P-450 and a cyanide-sensitive enzyme in different steps of lanosterol demethylation by yeast microsomes. *Biochemical and Biophysical Research Communications* **85**, 21–27.
- Oliva S, et al.** (1999) Successful treatment of widely disseminated acanthamoebiasis. *Southern Medical Journal* **92**, 55–57.
- Omura T and Sato R** (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry* **239**, 2370–2378.
- Paniz Mondolfi AE, et al.** (2011) Successful treatment of Old world cutaneous leishmaniasis caused by *Leishmania infantum* with posaconazole. *Antimicrobial Agents and Chemotherapy* **55**, 1774–1776.
- Pappas PG, et al.** (2016) Clinical practice guideline for the management of candidiasis: 2016 update by the infectious diseases society of America. *Clinical Infectious Diseases* **62**, e1–e50.
- Pasqualotto AC, et al.** (2010) Voriconazole therapeutic drug monitoring: focus on safety. *Expert Opinion on Drug Safety* **9**, 125–137.
- Patel T and Dhillon S** (2013) Efinaconazole: first global approval. *Drugs* **73**, 1977–1983.
- Peng LW and Lien YH** (2005) Pharmacokinetics of single, oral-dose voriconazole in peritoneal dialysis patients. *American Journal of Kidney Disease* **45**, 162–166.
- Perez-Molina JA and Molina I** (2017) Chagas disease. *Lancet* **391**, 82–94. doi: 10.1016/s0140-6736(17)31612-4.
- Perfect JR, et al.** (2010) Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases* **50**, 291–322.
- Pettit NN and Carver PL** (2015) Isavuconazole: a new option for the management of invasive fungal infections. *Annals of Pharmacotherapy* **49**, 825–842.
- Pinazo MJ, et al.** (2010). Successful treatment with posaconazole of a patient with chronic Chagas disease and systemic lupus erythematosus. *American Journal of Tropical Medicine and Hygiene* **82**, 583–587.
- Pinger J, Chowdhury S and Papavasiliou FN** (2017) Variant surface glycoprotein density defines an immune evasion threshold for African trypanosomes undergoing antigenic variation. *Nature Communications* **8**, 828.
- Raederstorff D and Rohmer M** (1985) Sterol biosynthesis de nova via cycloartenol by the soil amoeba *Acanthamoeba polyphaga*. *Biochemical Journal* **231**, 609–615.
- Raederstorff D and Rohmer M** (1987) Sterol biosynthesis via cycloartenol and other biochemical features related to photosynthetic phyla in the amoeba *Naegleria lovaniensis* and *Naegleria gruberi*. *European Journal of Biochemistry* **164**, 427–434.
- Raether W and Seidenath H** (1984) Ketoconazole and other potent antimycotic azoles exhibit pronounced activity against *Trypanosoma cruzi*, *Plasmodium berghei* and *Entamoeba histolytica* in vivo. *Zeitschrift Fur Parasitenkunde* **70**, 135–138.



- Rodriguez MM, et al. (2009) Correlation of In vitro activity, serum levels, and in vivo efficacy of posaconazole against *Rhizopus microsporus* in a murine disseminated infection. *Antimicrobial Agents and Chemotherapy* **53**, 5022–5025.
- Roque ALR and Jansen AM (2014) Wild and synanthropic reservoirs of *Leishmania* species in the Americas. *International Journal for Parasitology: Parasites and Wildlife* **3**, 251–262.
- Rybak JM, et al. (2015) Isavuconazole: pharmacology, pharmacodynamics, and current clinical experience with a new triazole antifungal agent. *Pharmacotherapy* **35**, 1037–1051.
- Saenz RE, Paz H and Berman JD (1990) Efficacy of ketoconazole against *Leishmania braziliensis panamensis* cutaneous leishmaniasis. *The American Journal of Medicine* **89**, 147–155.
- Sawyer PR, et al. (1975a). Miconazole: a review of its antifungal activity and therapeutic efficacy. *Drugs* **9**, 406–423.
- Sawyer PR, et al. (1975b) Clotrimazole: a review of its antifungal activity and therapeutic efficacy. *Drugs* **9**, 424–447.
- Schell WA, et al. (2017) Fungal CYP51 inhibitors VT-1161 and VT-1129 exhibit strong in vitro activity against *Candida glabrata* and *C. krusei* isolates clinically resistant to azole and echinocandin antifungal compounds. *Antimicrobial Agents and Chemotherapy* **61**, pii: e01817-16. doi: 10.1128/aac.01817-16.
- Seijo Martinez M, et al. (2000) Granulomatous amebic encephalitis in a patient with AIDS: isolation of *acanthamoeba* sp. Group II from brain tissue and successful treatment with sulfadiazine and fluconazole. *Journal of Clinical Microbiology* **38**, 3892–3895.
- Sheehan DJ, Hitchcock CA and Sibley CM (1999) Current and emerging azole antifungal agents. *Clinical Microbiology Reviews* **12**, 40–79.
- Sousa AQ, et al. (2011) High-dose oral fluconazole therapy effective for cutaneous leishmaniasis due to *Leishmania (Vianna) braziliensis*. *Clinical Infectious Diseases* **53**, 693–695.
- Sundar S, Kumar K and Singh VP (1990) Ketoconazole in visceral leishmaniasis. *The Lancet* **336**, 1582–1583.
- Superko HR, Momary KM and Li Y (2012) Statins personalized. *Medical Clinics of North America* **96**, 123–139.
- Thomson S, et al. (2017) Characterisation of sterol biosynthesis and validation of 14 $\alpha$ -demethylase as a drug target in *Acanthamoeba*. *Scientific Reports* **7**, 8247.
- Toubiana J, et al. (2006) Oral fluconazole treatment for extensive cutaneous leishmaniasis in an 11-year-old child. *The Pediatric Infectious Diseases Journal*, **25**, 1083–1084.
- Trabelsi H, et al. (2012) Pathogenic free-living amoebae: epidemiology and clinical review. *Pathologie Biologie (Paris)* **60**, 399–405.
- Trachtenberg J, Halpern N and Pont A (1983) Ketoconazole: a novel and rapid treatment for advanced prostatic cancer. *Journal of Urology* **130**, 152–153.
- Trachtenberg J and Pont A (1984) Ketoconazole therapy for advanced prostate cancer. *Lancet* **2**, 433–435.
- Tracy M, et al. (2016) Allergic bronchopulmonary aspergillosis. *Journal of Fungi* **2**, 17.
- Trzaskos J, Kawata S and Gaylor JL (1986) Microsomal enzymes of cholesterol biosynthesis. Purification of lanosterol 14  $\alpha$ -methyl demethylase cytochrome P-450 from hepatic microsomes. *Journal of Biological Chemistry* **261**, 14651–14657.
- Tu EY, Joslin CE and Shoff ME (2010) Successful treatment of chronic stromal *acanthamoeba keratitis* with oral voriconazole monotherapy. *Cornea* **29**, 1066–1068.
- Tucker RM, et al. (1990) Adverse events associated with itraconazole in 189 patients on chronic therapy. *Journal of Antimicrobial Chemotherapy* **26**, 561–566.
- Urbina JA, et al. (1998) Antiproliferative effects and mechanism of action of SCH 56592 against *Trypanosoma (Schizotrypanum) cruzi*: in vitro and in vivo studies. *Antimicrobial Agents and Chemotherapy* **42**, 1771–1777.
- Urcuyo FG and Zaias N (1982) Oral ketoconazole in the treatment of leishmaniasis. *International Journal of Dermatology* **21**, 414–416.
- Van den Bossche H, Willemsens G, Cools W, Cornelissen F, Lauwers WF and van Cutsem JM (1980) In vitro and in vivo effects of the antimycotic drug ketoconazole on sterol synthesis. *Antimicrob. Agents Chemother.*, **17**, 922–928. doi: 10.1128/aac.17.6.922.
- Vanden Bossche H (1985) Biochemical targets for antifungal azole derivatives: hypothesis on the mode of action. In McGinnis MR (ed.). *Current Topics in Medical Mycology*, vol. 1. New York: Springer-Verlag, pp. 313–351.
- Van den Bossche H (ed.) (1988). *Mode of Action of Pyridine, Pyrimidine and Azole Antifungals*. Chichester: Ellis Horwood, pp. 79–119.
- Van den Bossche H, et al. (1978) Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. *Chemo-Biological Interactions* **21**, 59–78.
- Vanden Bossche H, et al. (1987) Interaction of azole derivatives with cytochrome P-450 isozymes in yeast, fungi, plants and mammalian cells. *Pesticide Science* **21**, 289–306.
- Villalta F, et al. (2013) VNI cures acute and chronic experimental Chagas disease. *Journal of Infectious Diseases* **208**, 504–511.
- Walia R, et al. (2007) A case of successful treatment of cutaneous *Acanthamoeba* infection in a lung transplant recipient. *Transplant Infectious Diseases* **9**, 51–54.
- Walsh TJ, et al. (2007) Treatment of invasive aspergillosis with posaconazole in patients who are refractory to or intolerant of conventional therapy: an externally controlled trial. *Clinical Infectious Diseases* **44**, 2–12.
- Warn PA, et al. (2009) Pharmacokinetics and pharmacodynamics of a novel triazole, isavuconazole: mathematical modeling, importance of tissue concentrations, and impact of immune status on antifungal effect. *Antimicrobial Agents and Chemotherapy* **53**, 3453–3461.
- Webster D, et al. (2012) Treatment of granulomatous amoebic encephalitis with voriconazole and miltefosine in an immunocompetent soldier. *American Journal of Tropical Medicine and Hygiene* **87**, 715–718.
- Wexler D, et al. (2004) Effect of posaconazole on cytochrome P450 enzymes: a randomized, open-label, two-way crossover study. *European Journal of Pharmaceutical Sciences* **21**, 645–653.
- White JM, et al. (2006) Cutaneous leishmaniasis: three children with *Leishmania major* successfully treated with itraconazole. *Pediatric Dermatology* **23**, 78–80.
- Wilby KJ (2017) A review of the clinical pharmacokinetics and pharmacodynamics of isavuconazole. *European Journal of Drug Metabolism and Pharmacokinetics*, PMID:29101732. doi: 10.1007/s13318-017-0445-7.
- Yeagle PL, et al. (1977) Differential effects of cholesterol and lanosterol on artificial membranes. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 4924–4926.
- Yoshida Y and Aoyama Y (1984) Yeast cytochrome P-450 catalyzing lanosterol 14  $\alpha$ -demethylation. I. Purification and spectral properties. *Journal of Biological Chemistry* **259**, 1655–1660.
- Yoshida Y and Aoyama Y (1987) Interaction of azole antifungal agents with cytochrome P-450<sub>14DM</sub> purified from *Saccharomyces cerevisiae* microsomes. *Biochemical Pharmacology* **36**, 229–235.
- Yoshida Y, et al. (2000) Sterol 14-demethylase P450 (CYP51) provides a breakthrough for the discussion on the evolution of cytochrome P450 gene superfamily. *Biochemical and Biophysical Research Communications* **273**, 799–804.
- Yu X, et al. (2016) Ligand tunnels in *T. brucei* and human CYP51: insights for parasite-specific drug design. *Biochimica et Biophysica Acta* **1860**, 67–78.
- Zhang W, et al. (2002) Inhibition of cytochromes P450 by antifungal imidazole derivatives. *Drug Metabolism and Disposition* **30**, 314–318.
- Zijlstra EE, et al. (2003) Post-kala-azar dermal leishmaniasis. *Lancet Infectious Diseases* **3**, 87–98.
- Zingales B, et al. (2009) A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Memorias do Instituto Oswaldo Cruz* **104**, 1051–1054.