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REVIEW

Resistance to antifungals that target CYP51

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Abstract Fungal diseases are an increasing global burden. Fungi are now recognised to kill more people annually than malaria, whilst in agriculture, fungi threaten crop yields and food security. Azole resistance, mediated by several mechanisms including point mutations in the target enzyme (CYP51), is increasing through selection pressure as a result of widespread use of triazole fungicides in agriculture and triazole antifungal drugs in the clinic. Mutations similar to those seen in clinical isolates as long ago as the 1990s in *Candida albicans* and later in *Aspergillus fumigatus* have been identified in agriculturally important fungal species and also wider combinations of point mutations. Recently, evidence that mutations originate in the field and now appear in clinical infections has been suggested. This situation is likely to increase in prevalence as triazole fungicide use continues to rise. Here, we review the progress made in understanding azole resistance found amongst clinically and agriculturally important fungal species focussing on resistance mechanisms associated with CYP51. Biochemical characterisation of wild-type and mutant CYP51 enzymes through ligand binding studies and azole IC_{50} determinations is an important tool for understanding azole susceptibility and can be used in conjunction with microbiological methods (MIC_{50} values), molecular biological studies (site-directed mutagenesis) and protein modelling studies to inform future antifungal development with increased specificity for the target enzyme over the host homologue.

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Introduction

Most fungi are saprophytic; however, there are several fungal genera including species that can cause disease in plants, animals and man. Some are primary pathogens, capable of causing disease in a healthy, uncompromised host, whilst others are opportunistic (or secondary) pathogens. These generally only cause disease when host defences are impaired, e.g. when a patient is immunocompromised or immunosuppressed. Many emerging fungal pathogens have appeared and their increase is of concern. Opportunistic fungal pathogens now account for around 10–15 % of nosocomial infections [1].

Worldwide, over 300 million individuals each year contract a serious fungal infection [2]. These infections range from the relatively minor such as a single episode of vulvovaginal thrush to life-threatening conditions such as chronic pulmonary aspergillosis. Global mortality from fungal infection is over 1.35 million people per annum [2], far more than are killed by malaria (~0.63 million) [3]. In addition, fungal infections of hair, nails and skin affect approximately 25 % of the world's population [4].

Oral candidosis affects an estimated 9.5 million people per annum [5], and *Candida* infection of the oesophagus affects a further two million people [6]. Recurrent bouts of vulvovaginal candidosis (thrush) affect at least 75 million women annually [7], and the annual incidence of candidaemia has been estimated at 300,000 cases worldwide with a mortality rate of 30–55 % [8].

Meanwhile, over ten million patients are at risk of invasive aspergillosis (IA) each year through corticosteroid and anticancer therapies with over 200,000 patients developing IA

annually [9, 10] and mortality rates of over 50 % even with treatment. Approximately 4 million of the 193 million asthmatics worldwide develop allergic bronchopulmonary aspergillosis (ABPA), and a further 3 to 13 million adults suffer from severe asthma with fungal sensitisation (SAFS) [11]. In addition, chronic pulmonary aspergillosis (CPA) is estimated to affect more than three million people, with 1.2 million cases following tuberculosis [11–13].

Over one million people, mainly HIV-AIDS patients, develop cryptococcal meningitis each year, caused by the basidiomycete yeast *Cryptococcus neoformans*, which gives rise to a high mortality rate of over 60 % [14]. In addition, around 100,000 to 300,000 cases of coccidioidomycosis occur each year in the USA, mainly amongst immunocompetent individuals [15] with additional outbreaks reported in Central and South America.

Filamentous fungi such as *Aspergillus* sp. and *Fusarium* sp. are also responsible for over one million eye infections in Asia and Africa annually accounting for approximately 10 % of the 284 million visually impaired people worldwide [16]. The main etiological agents of fungal infections of the hair, nails and skin are the basidiomycete *Malassezia* yeasts *Malassezia globosa* and *Malassezia restricta* associated with dandruff, seborrheic dermatitis, pityriasis versicolor and malassezia folliculitis (and may also exacerbate atopic dermatitis and psoriasis) [17–19] and the dermatophytes such as *Trichophyton rubrum*, an important etiological agent in athlete's foot (tinea pedis), ringworm (tinea), nail infections (onychomycosis) and groin infections (tinea cruris) [20, 21]. Azole antifungal agents are an important treatment therapy against all these fungal infections, although there are alternative therapies with the squalene epoxidase inhibitor terbinafine important in treating athlete's foot.

Numerous fungal species are responsible for diseases affecting arable crops. The causative agents of septoria wheat blotch, wheat headblight, eyespot disease in wheat and barley, powdery mildew in wheat, barley and grapes, brown rust in wheat, black sigatoka in bananas and plantain, leaf scald in cereals, leaf spot in sugar beet, apple scab and the spoilage of citrus fruit threaten crops and must be controlled to protect yields. In addition, some phytopathogenic fungi including *Aspergillus*, predominantly *Aspergillus flavus*, and some *Fusarium* and *Penicillium* species also synthesise mycotoxins, e.g. aflatoxins, and must be controlled in agriculture to ensure food safety.

Control of both plant and animal fungal pathogens is clearly desirable, and for several decades, the use of azole antifungal drugs and fungicides has been extensively used to maintain the health and food security of the world [22]. Should azole antifungal agents lose their effectiveness, arable crop yields will fall [23] and mortality rates amongst patients with fungal infections will rise [22]. For example, if the use of azole fungicides ceased, there would be a fall in European wheat production of ~7 % (9.8 million tons) in the short term and ~12 % (18.6 million tons) by 2020 [23]. This decreased productivity would represent a financial loss of 2.4 billion euros in the short term and 4.6 billion euros by 2020. It would also affect the ability of Europe to be self-sufficient in terms of wheat production [23]. Azole use in agriculture extends to animal husbandry; both domestic and agricultural animals have implications for the food chain beyond their use to control fungal infection of food crops [24]. Furthermore, azoles are used to stop fungal growth on materials, e.g. they are included in wood preservatives such as 'Copper azole type B' (96 % copper, 4 % tebuconazole) [25], antimold additives to paints [26] and as copper corrosion inhibitors (tolyltriazole and benzotriazole) added to aqueous coolant systems [27]. It is clear that development of resistance to azole antifungals has the potential to affect health, food supply and quality of life.

Azoles inhibit the synthesis of ergosterol in fungi through direct binding to sterol 14α-demethylase (CYP51). CYP51 is a member of the superfamily of haem-containing enzymes, cytochrome P450. CYP51 is located in the outer membrane of the endoplasmic reticulum and catalyses the removal of the methyl group at carbon 14 [28]. A lone pair of electrons on the nitrogen of the azole ring binds to the iron atom in the haem prosthetic group of CYP51, located in the active site of the enzyme, whilst the N-1 substituent group interacts with amino acids that line the active site pocket [29]. Specificity of azole compounds depends upon the interaction between side groups of the azole compound and the CYP. Azole binding to CYP51 is non-competitive and results in a depletion of the final fungal sterol (usually ergosterol) and a concomitant accumulation of 14-methylated sterols which inhibit fungal growth by disrupting the cell membrane [30]. In some species, including *Candida albicans*, 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol is accumulated, which is known to disrupt the cell membrane [30, 31] whilst other species, such as *C. neoformans*, accumulate 14α-methyl products and the 3-ketosteroid obtusifolione [32].

Clinical azole fungicides

The antifungal action of azole compounds was first reported in 1944 [33, 34]. Various compounds have been introduced over the years; firstly, there were imidazoles (two nitrogen azole ring) which were followed by triazoles (three nitrogen azole ring, see Fig. 1). In 1958, chlormidazole became the first azole antifungal available for topical use in the clinic. Later, in 1969, other imidazole-based azole antifungals, clotrimazole and miconazole, were introduced for topical use followed by econazole in 1974 [35]. The first oral treatment for systemic fungal infections was ketoconazole [36] though its use was limited by its toxicity [37–40]. During the 1990s, the first systemic triazoles, firstly fluconazole and later itraconazole, were introduced in the USA. Both fluconazole and

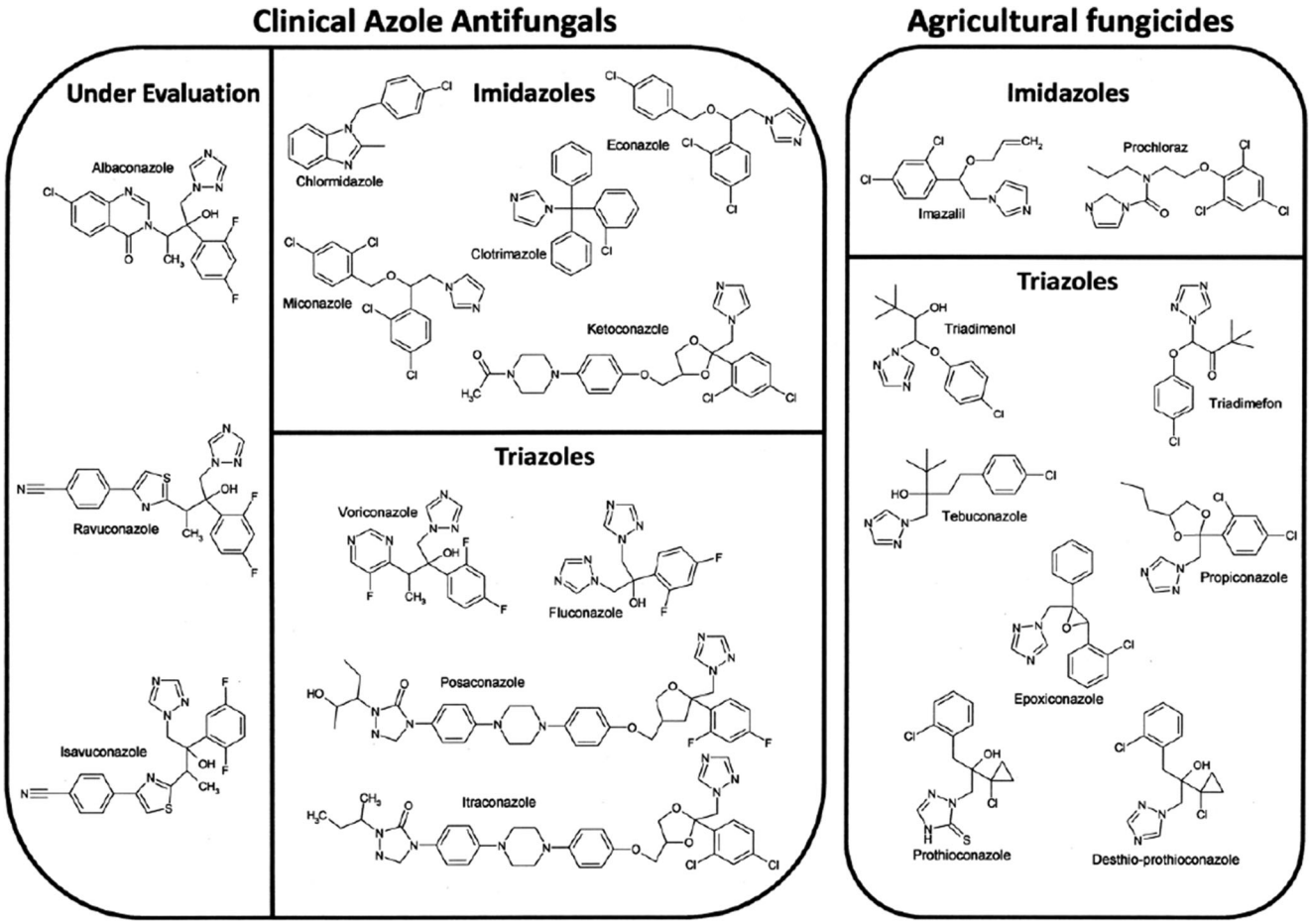

Fig. 1 Structures of the clinical and agricultural azole compounds discussed in this review

itraconazole have good antifungal activity and are less toxic than ketoconazole [41]. In the late 1990s and 2000s, second-generation triazoles were introduced, including voriconazole and posaconazole. They are now used to combat fluconazole-resistant strains and species [42] and have proved especially to be effective against *Aspergillus fumigatus* [43, 44]. Triazole antifungal agents currently undergoing clinical evaluation include albaconazole [45–47], ravuconazole [48, 49], isavuconazole [50, 51] and pramiconazole [52–55].

Agricultural azole fungicides

The first azole fungicides introduced for agricultural use were both imidazole and triazole compounds. The first imidazole fungicides included imazalil (1973) and prochloraz (1977) with imazalil used as a seed treatment and post-harvest treatment, whilst prochloraz was used on cereals. The first triazole fungicides included triadimefon (1973), triadimenol (1977) and propiconazole (1979) [33, 34]. Triazoles have become the most widely used class of fungicide accounting for over 20 % market share with other azole fungicides accounting for a further 5 % [56]. Numerous other triazoles have subsequently been released including tebuconazole (1986), epoxiconazole (1990) and prothioconazole (2002). The three most commonly used fungicides in the UK, the Netherlands and Denmark in 2008 were prothioconazole, epoxiconazole and tebuconazole [57]. Azole fungicides have persisted through the decades because they are high-efficiency broad-spectrum fungicides where resistance has occurred slowly over time to a specific fungicide. Progressive introduction of newer triazoles that are intrinsically more active assists in maintaining the effectiveness (ED_{50} values) of azole fungicides.

Resistance mechanisms

The increasing emergence of azole resistance in yeast and fungi can be attributed to the prophylactic use of azole drugs and prolonged treatment regimens in the clinic and usage of agricultural azole fungicides in crop protection [58–63]. Resistance to azoles can arise through four main mechanisms (see Fig. 2). The affinity for the target enzyme (CYP51) may be reduced through point mutations. The amount of target enzyme present may be increased due to upregulation of the

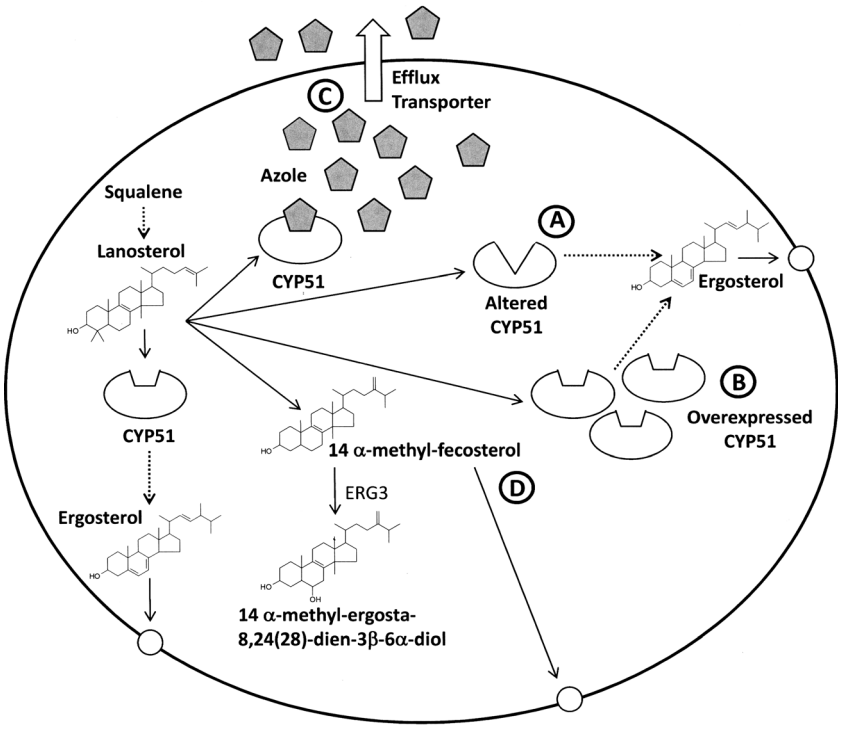

Fig. 2 Azole resistance mechanisms in *C. albicans*. CYP51 is an essential step in the biosynthesis of ergosterol, which is required for membrane stability and functionality. Azoles inhibit CYP51 causing the accumulation of 14 α-methyl-ergosta-8,24(28)-dien-3β-6α-diol. Resistance to azoles can occur through **a** an altered CYP51 (point mutations), **b** overexpression of CYP51, **c** overexpression of efflux transporters and **d** null mutation of ERG3 which blocks the synthesis of 14 α-methyl-ergosta-8,24(28)-dien-3β-6α-diol, resulting in the accumulation of 14 α-methyl-fecosterol which is capable of supporting membrane function

gene, and the efflux of azole from the cell may be increased due to the overexpression of transporters. In addition, it is possible for secondary mutations to occur which confer resistance. This has been observed in *Candida* spp. where an *ERG3* null mutant is resistant to azoles. A lack of ERG3 activity results in the production of 14-methyl fecosterol when treated which is capable of supporting membrane function; thus, the fungistatic effect of the accumulation of other 14-methylated sterols is circumvented [31, 64–66].

The complex nature of resistance mechanisms in fungal species is still not fully understood. In some species, there are multiple copies of CYP51 (e.g. *Aspergillus* and *Fusarium* spp.). In addition, some resistant strains appear to contain adaptations to more than one resistance mechanism. However, mutations that affect the binding of azoles to CYP51 are commonly found in azole-resistant fungal strains. CYP51 mutations have been extensively studied and often correlate directly with observed azole resistance. Understanding the effects mutations have on the interaction between CYP51 and azoles and the inhibition of CYP51 activity is necessary in order to design new improved drugs that combat present and future azole resistance.

CYP51 mediated resistance in the clinic

C. albicans

C. albicans is a dimorphic diploid asexual yeast and the most common fungal opportunistic pathogen of humans [67]. *C. albicans* causes a wide range of clinical infections in humans from mucosal infections such as thrush and oral candidosis to potentially life-threatening systemic candidiasis and candidaemia. *Candida* bloodstream infections cause significant morbidity and mortality, particularly in intensive care patients [68, 69]. The incidence of fungal infections by *C. albicans* and non-albicans *Candida* species has steadily been increasing over the last two decades not only partially as a result of HIV-AIDS but also due to the growing number of patients who are immunodeficient as a result of organ and bone marrow transplants and cancer treatments [70]. Predisposing factors for invasive candidiasis include immunosuppressive and cytotoxic therapies, treatment with broad-spectrum antibiotics, HIV-AIDS or diabetes and in situ central venous catheters and urinary tract catheters [71–73]. Controlling invasive fungal infections amongst haematology, oncology and intensive care patients is now a major challenge [61]. Azole antifungals are relatively safe, easy to administer (often by mouth) and have a high therapeutic index leading to triazole antifungals becoming the standard first-line therapy to combat fungal infections. However, prophylactic use of azole drugs and prolonged treatment regimens in the clinic has led to increasing emergence of azole-resistant yeast and fungal strains. Presently, there are four known mechanisms that confer azole resistance in *C. albicans* [74, 75]. These include mutations that alter the amino acid sequence of CYP51 [76–78], overexpression of efflux transporters (CaCDR1, CaCDR2 and CaMDR1) [79, 80], overexpression of CYP51 and alterations in the ergosterol biosynthetic pathway such as mutations in *ERG3*. These resistance mechanisms are often combined in clinical isolates [66, 81–85].

More than 140 different amino acid substitution mutations have been reported in the CYP51 gene isolated from clinical strains of *C. albicans* [86] (although not all of these mutations confer an azole-resistant phenotype). In addition, the CYP51 genes from both azole-resistant and azole-susceptible clinical *C. albicans* strains may contain several amino acid substitutions [87]. Most of these substitutions cluster into three 'hotspots' [87] located within amino acid residues 105-165, 266-287 and 405-488. Not all amino acid substitutions contribute equally to azole resistance. K143R, S405F, G464S, R467K and I471T have been recovered only from azole-resistant strains, whereas E266D and V488I have been recovered from both azole-resistant and azole-susceptible strains, suggesting that these two latter mutations are probably not responsible for conferring azole resistance [86].

A number of the *C. albicans* CYP51 point mutations have previously been expressed in *Escherichia coli*, purified and biochemically characterised. These include Y132H [88], F145L [88, 89], I471T [90] and S279F [91]. In addition, several mutations were expressed in *Saccharomyces cerevisiae*, and the isolated membrane fractions (G464S [92] and R467K [93]) and purified enzyme (T315A) were used to study azole resistance [94].

A. fumigatus

Aspergillus infections are a problem in both immunocompetent and immunocompromised individuals. Approximately 2 to 10 % of immunosuppressed patients suffer from acute IA annually [95], whilst three million immunocompetent patients worldwide are estimated to have CPA. *Aspergillus* is also responsible for an estimated four million cases of ABPA. Both CPA and ABPA require long-term use of azoles for management of the conditions [96]. Azole resistance is therefore a concern for these patients and also amongst cystic fibrosis patients [97–99]. The mortality rate amongst patients with multiazole-resistant invasive aspergillosis is 88 % [100]. Increasing azole resistance amongst *A. fumigatus* strains will result in increased morbidity and mortality amongst aspergillosis patients as well as placing an extra resource burden (financial and manpower) on hospitals with an increasing reliance on amphotericin B and caspofungin to clear fungal infections which needs to be administered and monitored in a clinical setting. Since 1998, azole resistance in *A. fumigatus* clinical isolates has been increasing around the world. The global ARTEMIS surveillance programme found that 5.8 % of *A. fumigatus* clinical isolates showed elevated minimum inhibitory concentrations (MICs) to one or more triazoles [101], whilst the SCARE programme in Europe found that 3.4 % of *A. fumigatus* clinical isolates were azole-resistant [100]. In the Netherlands, ~10 % of all clinical isolates are now itraconazole-resistant [100] compared to Manchester (UK) where 23 and 31 % of isolates were azole-resistant in 2008 and 2009 [102].

Aspergillus spp. contain two CYP51 isoenzymes, CYP51A and CYP51B. Studies with *A. fumigatus* strains containing gene knock-outs of CYP51A and CYP51B have demonstrated that both isoenzymes can fulfil the role of sterol 14α-demethylase in *A. fumigatus* in vivo, resulting in no phenotypic difference [103–105]. A conditional mutant study indicated that in the absence of both CYP51A and CYP51B, growth was abolished but not when performed individually [106]. The main azole resistance mechanism in *A. fumigatus* is the development of point mutations in CYP51A, and this form was found to have less affinity for azole drugs than CYP51B suggesting a molecular basis for this based on changes in the protein responsible for activity at higher concentrations of drug [107]. Other azole resistance mechanisms such as overexpression of CYP51 isoforms A [96, 108, 109] and B [110], point mutations in CYP51B [111, 112] and the induction and overexpression of the drug transporters atrF, AfuMDR3, AfuMDR4 and Cdr1B [109, 113–115] have been described. Other novel mechanisms that confer azole resistance in *A. fumigatus* strains such as the P88L mutation in transcription factor hapE [116] are still being discovered.

Recently, Lockhart et al. [101] found that 60 % of clinical *A. fumigatus* isolates contained triazole resistance mutations within CYP51A. The five most frequent CYP51A mutations or 'hotspots' reported in *A. fumigatus* are G54, L98, G138, M220 and G448 [96]. These five mutations have been confirmed to confer azole resistance in *A. fumigatus* through gene replacement experiments (though L98H also required a modified promoter sequence) [108, 111, 117–121]. Nearly 40 different CYP51A point mutations in clinical *A. fumigatus* isolates have been reported, although not all mutations confer an azole resistance phenotype [122, 123] and can occur on their own or in combination with other mutations. The CYP51A point mutations G54, G138, M220 and G448 are thought to have arisen during triazole therapy of patients in the clinic, whilst TR34/L98H and TR46/Y121F/T289A (which both contain tandem repeats in the promoter region of the gene) are thought to have arisen in the environment in the Netherlands as a response to the use of agricultural triazole fungicides on crops [118, 124–126]. Interestingly, even at the time of the first report of itraconazole resistance in *A. fumigatus* (1997) [127], the possibility of an environmental origin of the resistance was recognised [128].

C. neoformans

Cryptococcosis is a particularly problematic systemic fungal infection in HIV/AIDS immunocompromised patients from sub-Saharan Africa and is caused by the opportunistic basidiomycete yeast pathogen *C. neoformans* [14] leading to infections of the lungs and brain. Meningoencephalitis is the most lethal manifestation of cryptococcosis with a life expectancy of less than a month if untreated [129]. Pathogenic

Cryptococcus species cause disease in almost one million people annually with over 620,000 deaths, and a third of all HIV/AIDS deaths are attributable to *Cryptococcus* species infection [14]. Current treatment options are limited to only a few drugs, namely initial induction therapy with a combination of amphotericin B and flucytosine followed by a maintenance regime of fluconazole [129]. Even after administering the recommended treatment, 3-month mortality rates of 10 to 20 % are common [130, 131]. In addition, adopting such treatment is costly (with amphotericin B requiring intravenous administration), especially for developing countries where mortality rates can approach 100 % [132, 133]. Most *Cryptococcus* infections of humans and nearly all cryptococcal infections of HIV/AIDS patients are caused by *C. neoformans* var. *grubii*, the most prevalent being the H99 strain, although *Cryptococcus gattii* (found in trees and primarily located in the tropics and sub-tropics) infection is increasing in prevalence amongst immunocompetent individuals, especially in NW America and Africa [134].

Azole resistance associated with enhanced resistance in the CYP51 activity in *C. neoformans* was first observed in isolates from AIDS patients in 1995 [135]. Resistance, especially towards fluconazole, is becoming an increasing problem amongst *Cryptococcus* species in the clinic due to prolonged maintenance treatment regimens [136]. Three main mechanisms for increased azole resistance in *C. neoformans* have been identified. Firstly, point mutations in CYP51 have been identified that confer resistance to azole antifungals. The CYP51 G468S mutation [137] confers resistance to fluconazole, whilst the Y145F mutation (equivalent to Y132F in *C. albicans* and several other species) [138] confers resistance to fluconazole and voriconazole but increases susceptibility to itraconazole and posaconazole. Secondly, *C. neoformans* overcomes stress induced by exposure to azoles, especially fluconazole, by duplication of chromosome 1 (selective disomy) which contains two important genes CYP51, the azole target enzyme, and AFR1, the major transporter of azoles in *C. neoformans*, promoting increased expression levels of these two proteins [139]. Thirdly, general genome plasticity of *C. neoformans* strains post-infection [140] presents the challenge of a constantly moving target in terms of effective drug therapies in combating cryptococcosis. Presently, there are no published studies comparing purified mutant *C. neoformans* CYP51 proteins against wild-type CYP51 protein in terms of substrate and azole binding affinities and inhibition of CYP51 catalysis by azole antifungal agents. Again, such biochemical characterisations of mutant versus wild-type enzymes would provide useful insights into the azole resistance mechanism of this fungal pathogen.

Other species of fungi have shown some resistance to azole antifungal drugs. The prolonged treatment regimens often required to treat dermatological infections have led to the emergence of azole-resistant *T. rubrum* and *M. globosa* strains, especially against fluconazole [141–143], although no CYP51 point mutations have so far been identified in azole-resistant isolates with the mechanism(s) of azole resistance remaining unclear [144]. However, fluconazole resistance in *Histoplasma capsulatum* in AIDS patients has been correlated with a single mutation (Y136F) in CYP51 [145].

CYP51 mediated azole resistance in agriculture

Azole fungicides are used to treat several economically important arable crops to minimise crop damage caused by plant fungal pathogens. These pathogens include *Oculimacula yallundae* and *Tapesia yallundae* which cause eyespot disease in wheat and barley, *Blumeria graminis* (powdery mildew in wheat and barley), *Puccinia triticina* (brown rust in wheat), *Mycosphaerella fijiensis* (cause of black sigatoka in bananas and plantain), *Erysiphe necator* (powdery mildew in grapes), *Rhynchosporium secalis* (leaf scald in cereals), *Cercospora beticola* (leaf spot in sugar beet), *Venturia inaequalis* (apple scab), *Penicillium digitatum* (mold on citrus fruit), *Zymoseptoria tritici* (formerly called *Mycosphaerella graminicola* and *Septoria tritici* and the cause of septoria leaf blotch in wheat) and *Fusarium* sp. (headblight).

There are presently no reports of mutations in the CYP51 of *O. yallundae*, *T. yallundae*, *P. digitatum*, *R. secalis*, *C. beticola*, *V. inaequalis* or *Fusarium* spp. that correlated with azole resistance. However, CYP51 mutations have been documented in *B. graminis* (Y136F and K147Q), *E. necator* (Y136F) [146, 147], *P. triticina* (Y134F) [148], *M. fijiensis* (Y136F, A313G, Y461D, Y463D, Y463H and Y463N) [149] and *Z. tritici* (over 30 CYP51 mutations) [150]. In addition, constitutive overexpression of CYP51 contributes to azole resistance in field isolates of *Z. tritici* [151], *P. digitatum* [152, 153], *P. triticina* [148] and *V. inaequalis* [154, 155]. Azole resistance in *P. digitatum* [156] and *B. cinerea* [157] has also been found to be mediated by the overexpression of efflux transporters. Resistance to azoles in *R. secalis* has been found to be mediated by selection of strains possessing both CYP51A and CYP51B, where previously the majority of isolates possessed a single CYP51B [158].

Mutations in the sole CYP51 enzyme present in *Z. tritici* (formerly called *M.* graminicola) are well documented [58, 150, 159–164]. Azoles have been used extensively since the early 1980s to combat septoria wheat blotch which has given rise to a large number of CYP51 mutations, with up to eight coding changes in one haplotype, reducing the effectiveness of some compounds. CYP51 alterations are the most common form of azole resistance in *Z. tritici*. Mutations at 14 residues have been reported, which appear in different combinations resulting in over 30 CYP51 variants [150]. However, some mutations are lethal in isolation and may only exist in combination with other mutations enabling CYP51 function to be maintained. Mutations are often found in combinations with

different CYP51 variants leading to resistance to different azole compounds [159, 162–164]. Studies of mutations, including heterologous systems and protein modelling, have provided insight into the development of resistance to different azoles [160, 161] and the differential nature of resistance [159]. The impact of various combinations of mutations in *Z. tritici* is complex and has been comprehensively reviewed in relation to the current status of variants in the field by Cools and Fraaije [150].

Several of the *Z. tritici* CYP51 mutations are equivalent to those seen in other fungal species. Mutations at the equivalent residue to Y137 are the most widely reported azole resistance mutations found in both clinical and agricultural isolates including *E. necator*, *B. graminis*, *P. triticina* and *M. fijiensis* [78, 138, 145, 146, 148, 149, 165–167]. The mutation Y137F was prevalent in the 1990s and later in 2000 in combination with S542T and confers resistance to triadimenol. However, triadimenol is no longer used and Y137F and Y137F-S542T (double mutation) are now rarely seen in the population as newer azoles have been selected for other mutations. Alterations at residues 459-461 in *Z. tritici* confer resistance to many azoles but are also required to accommodate some other mutations (e.g. I381V, V136A) which are lethal on their own [160]. Mutations in this region are also found in combination in *C. albicans* [82, 86, 167], *M. fijiensis* [149] and *A. fumigatus* [59]. The mutation D134G is another mutation found in combination, which has also been identified in the structurally equivalent region in *C. albicans* (G129A) where it has also only been observed in combination with other mutations [78].

The use of many different azoles to combat *Z. tritici* has led to a shift in variants found in the population adapting to the azole challenge. Of particular concern at present are isolates carrying V136A, I381V and D134G which have reduced sensitivity to prochloraz, epoxiconazole and prothioconazole [150]. Another recently emerged mutation, S524T, decreases sensitivity to all azoles, but when combined with L50S, D134G, V136A and Y461S, it is less sensitive to prothioconazole, the most recently introduced fungicide [58]. The majority of *Z. tritici* isolates from the field now contain mutations in CYP51 and demonstrate an extreme change due to selective pressure from azole use. This extensive development of mutations in *Z. tritici* CYP51 compared to the present status of clinical fungal strains may represent an indicator of the mutations that may eventually occur in clinical species given similar selection pressures.

Assessing azole susceptibility of CYP51

DNA sequencing of strains is used to identify point mutations within CYP51 genes. Correlated with MIC data, they indicate those mutations that may confer resistance. Further genetic analysis, including mutating genes in host organisms and heterologous expression of mutated and wild-type CYP51s, can be used to functionally assess the effect such mutations have on azole resistance [105, 108, 111, 117–121, 160]. However, biochemical analysis allows an assessment of the impact CYP51 susceptibility alone has on resistance.

Substrate and azole ligand binding studies using UV–visible spectroscopy under oxidative conditions and determination of the azole concentration required for 50 % inhibition of CYP51 catalysis (IC_{50} value) using purified recombinant CYP51 enzymes are valuable tools in assessing the susceptibility of CYP51 enzymes to particular azole compounds and the impact of point mutations on that susceptibility. These methods are usually performed using truncated (soluble) CYP51 enzymes since eukaryotic CYP51 enzymes generally express poorly in *E. coli* without modification of the N-terminal membrane anchor region. Due to the difficulty in expressing full-length eukaryotic CYP51 proteins, no systematic study has been performed to assess the impact of the N-terminal membrane anchor region on eukaryotic CYP51 catalytic efficiency, azole susceptibilities, ligand binding affinities and structural/spatial conformation. However, we have studied the difference between truncated and full-length forms of *Homo sapiens* CYP51. The removal of the N-terminal membrane anchor of *H. sapiens* CYP51 increased the turnover number with lanosterol by 3.7-fold compared to the full-length enzyme [168], suggesting that the N-terminus impedes optimal catalysis in CYP51 in vitro reconstitution assays. The N-terminal region did not affect the azole or sterol ligand binding properties as both the full-length and truncated *H. sapiens* CYP51 bound pharmaceutical azole antifungal agents and lanosterol with similar affinities [168]. Further studies are required to fully elucidate the effects of the removal of the N-terminal membrane anchor on the catalytic properties of CYP51 enzymes both in free solution and in lipid bilayers/membranes. However, comparison of wild-type CYP51s against those that contain point mutations makes it possible to biochemically determine whether a compound is likely to be effective against strains harbouring point mutations in CYP51 and whether azoles are likely to be effective against a species. Furthermore, these studies in conjunction with in silico protein modelling enable a better understanding of azole–CYP51 interactions including which structural elements of azole antifungals confer increased selectivity for a specific CYP51 enzyme and will assist in the design of the next generation of azole antifungals both for agriculture and the clinic.

Ligand binding studies

Progressive titration of CYP51 enzymes with their sterol substrates (lanosterol for mammals and yeasts, lanosterol/eburicol for higher fungi and obtusifoliol for plants) give type I difference spectra (Fig. 3a) with an absorbance maximum at 380–390 nm and an absorbance minimum at 415–420 nm.

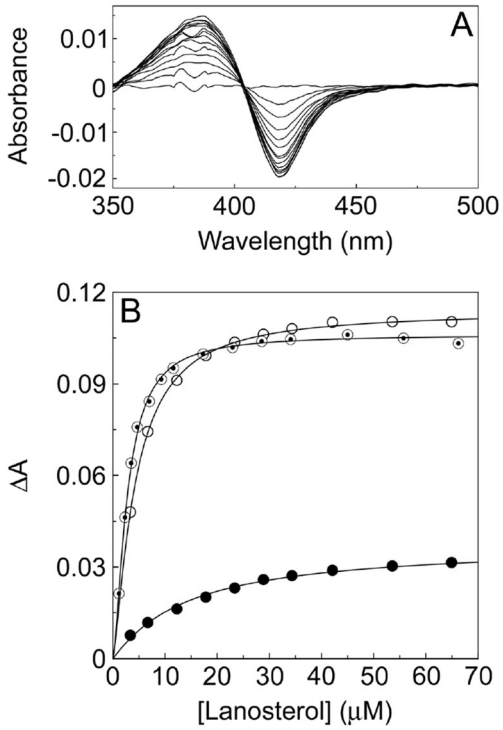

Fig. 3 Lanosterol binding studies with 5 μM wild-type, S279F and I471T *C. albicans* CYP51 proteins. The type I difference spectrum obtained for the wild-type protein is shown **a** along with the lanosterol saturation plots **b** for wild-type (*black circle*), S279F (*white circle*) and I471T (⊙) *C. albicans* CYP51. The Michaelis–Menten equation was used to fit the data and derive K_s values

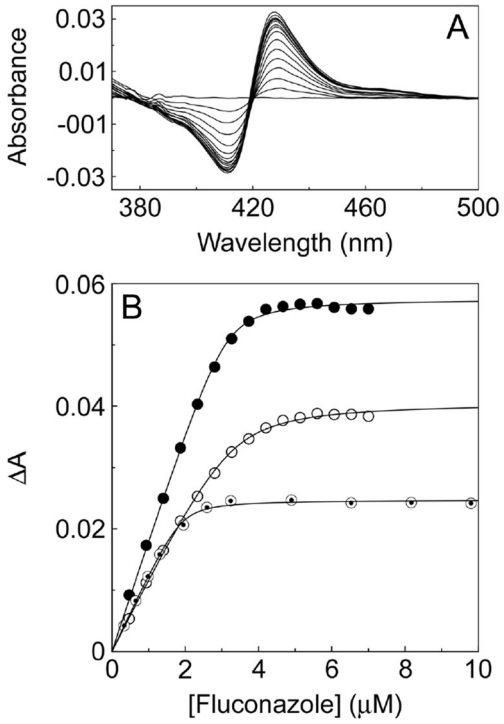

Fig. 4 Fluconazole binding studies with wild-type, S279F and I471T *C. albicans* CYP51 proteins. The type II difference spectrum obtained for the wild-type protein is shown **a** along with the fluconazole saturation plots **b** for wild-type (*black circle*), S279F (*white circle*) and I471T (⊙) *C. albicans* CYP51. A rearrangement of the Morrison equation [172] was used to fit the data and determine K_d values

Type I binding spectra are formed when the substrate (or other molecules) displaces the water molecule coordinated as the sixth ligand to the low-spin hexa-coordinated heme prosthetic group, causing the heme to adopt the high-spin penta-coordinated conformation [169]. These spectra are useful for investigating substrate affinity with the CYP51 protein by determining the dissociation constant for the enzyme–substrate complex (K_s) (Fig. 3b), often an indicator of catalytic efficiency, and will quickly identify whether a CYP51 point mutation alters the binding affinity for substrate. The K_s values for wild-type, S279F and I471T *C. albicans* CYP51 enzymes were 13.5, 7.1 and 4.8 μM, respectively [90, 91]. Therefore, the S279F and I471T mutant proteins had higher apparent affinities for lanosterol than the wild-type enzyme suggesting increased catalytic efficiency was one way these two point mutations conferred a reduction in azole susceptibility. The type I difference spectrum only measures the low- to high-spin state conversion, and studies have shown that the CYP–substrate complexes can exist as an equilibrium of high- and low-spin states [170].

Progressive titration of CYP51 proteins with azole antifungal agents gives type II difference spectra (Fig. 4a) with an absorbance maximum at 425–435 nm and an absorbance minimum at 390–410 nm [169]. Type II difference spectra arise due to the triazole ring N-4 nitrogen or the imidazole ring N-3 nitrogen coordinating as the sixth ligand with the heme iron [171]. Fluconazole saturation curves (Fig. 4b) were best fitted using a rearrangement of the Morrison equation [172], indicating that the observed binding was 'tight'. Tight binding is observed when the dissociation constant for a ligand (K_d) is similar to or lower than the concentration of the enzyme present [173]. The fluconazole K_d values for wild-type, S279F and I471T *C. albicans* CYP51 enzymes were 56±4, 241±18 and 84±17 nM [90, 91]. This suggests the S279F point mutation confers reduced fluconazole susceptibility through weakening fluconazole binding, whereas the I471T mutation binds fluconazole with similar affinity to the wild-type CYP51 enzyme. Azole ligand binding experiments are useful to screen new azole fungicides for potential effectiveness against the target CYP51 and that they should ideally exhibit reduced affinity towards the host CYP51 homologue. Candidate azole fungicides that bind weakly to the target CYP51 are unlikely to be effective inhibitors in situ unless the compound is a pro-fungicide which breaks down over time to release the active component such as prothioconazole [174, 175].

Type I substrate binding studies can also be used to gain insight into how a CYP51 inhibitor disrupts/alters substrate binding. This is achieved by progressively titrating the CYP51 enzyme with substrate in the presence of fixed concentrations

of inhibitor, such as voriconazole, prothioconazole and prothioconazole-desthio. A secondary Lineweaver-Burk plot of 1/ΔA against 1/[substrate] can elucidate the type of inhibition being observed towards lanosterol binding (Fig. 5). Voriconazole and prothioconazole-desthio (Fig. 5a, b) clearly show non-competitive inhibition (converging lines that meet at 1/ΔA=0) as would be expected for an azole antifungal agent. In contrast, prothioconazole inhibition appeared to be competitive (converging lines at 1/[lanosterol]=0) [175] suggesting competition with lanosterol for the same binding site. The primary and strongest interaction between an azole antifungal and a CYP51 enzyme is through the direct coordination of the triazole N-4 nitrogen atom as the sixth ligand of the heme ferric ion. CYP51 substrates do not bind directly through coordination with the heme ferric ion but instead bind through numerous interactions with the amino acid side chain residues that line the substrate access channel and binding pocket in CYP51. As the azole and substrate binding sites are different, non-competitive inhibition would be expected to occur unless the primary binding mechanism of the azole antifungal was no longer through direct coordination with the heme ferric ion but was through interactions of the azole side chain groups with the amino acid residues lining the substrate access channel. In such circumstances, the antifungal agent would be potentially competing for the same sites as lanosterol, which appeared to be the case for the pro-fungicide prothioconazole. Type I substrate binding studies can also be utilised to study the interactions of non-azole CYP51 inhibitors, especially if the inhibitor binds within the CYP51 substrate binding pocket.

CYP51 catalysis studies

CYP51 catalysis studies using the CYP51 reconstitution assay system developed by Lepesheva et al. [176] allow detailed inhibition studies to be performed. The reconstitution assay utilises recombinant CYP51 protein and a partner cytochrome P450 reductase in suspension with substrate (lanosterol) in an artificial lipid bilayer of dilauryl phosphatidylcholine (DLPC) along with a reduced nicotinamide adenine dinucleotide phosphate (NADPH) regeneration system. If the recombinant CYP51 protein has low catalytic activity after purification, up to 50 μl of the membrane fraction (isolated from *E. coli* of the expression clone) can be used instead by omitting the DLPC from the assay. The reaction is initiated with NADPH, shaken at the desired temperature for a predetermined time before termination and extraction with ethyl acetate. Dried reaction metabolites are then derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and tetramethylsilane prior to analysis by gas chromatography–mass spectrometry [175]. Azole IC_{50} determinations can easily be performed by the inclusion of 2.5 μl of ×200 stock azole solutions in

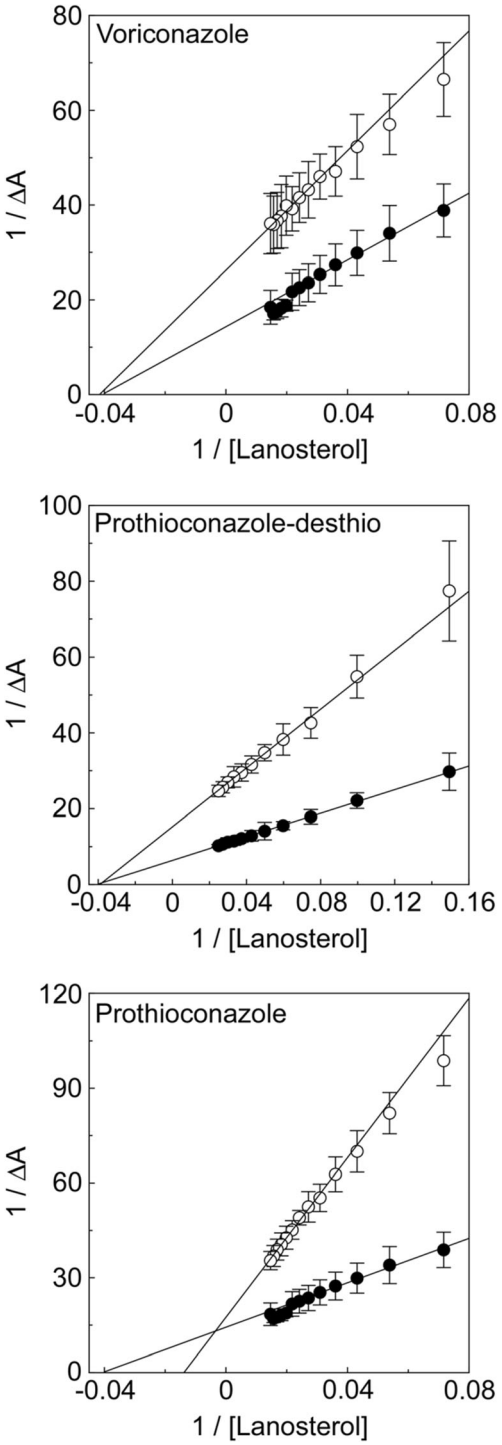

Fig. 5 Inhibition of lanosterol binding to *C. albicans* CYP51 by azole fungicides. Type I difference spectra were measured during the progressive titration of 10 μM CYP51 with lanosterol in the absence and presence of azole fungicides. Lineweaver-Burk plots were constructed from the type I binding spectra obtained in order to compare lanosterol binding in the absence (*black circle*) and presence (*white circle*) of 4 μM voriconazole, 4 μM prothioconazole-desthio and 100 μM prothioconazole. One representative example of each experiment is shown, although all experiments were performed in triplicate

dimethylformamide, followed by preincubation for 10 min prior to commencement of the reaction with NADPH [168].

The resultant IC_{50} plot of relative velocity against azole concentration (Fig. 6a) quickly identifies azole drug candidates that strongly inhibit the target CYP51 enzyme and is the enzymological equivalent of the whole organism MIC_{50} determinations often performed to determine the effectiveness of an azole fungicide. The IC_{50} values for fluconazole with wild-type, S279F and I471T *C. albicans* CYP51 (Fig. 6a) were 1.39, 3.28 and 1.28 μM, respectively, confirming the S279F point mutation had reduced the fluconazole susceptibility of the CYP51 enzyme, whilst the I471T point mutation had not affected the observed fluconazole susceptibility; however, I471T had more than 2-fold higher catalytic turnover than the wild-type and S279F enzymes. Even though the K_s value obtained for S279F was half that of the wild-type enzyme, this did not result in an increased enzyme reaction velocity for S279F, confirming that the binding affinity for substrate is not always the rate limiting step. In contrast, I471T had nearly a 3-fold lower K_s value than the wild-type enzyme, and this did result in more than 2-fold increase in reaction velocity [90, 91].

The IC_{50} values for voriconazole, prothioconazole-desthio and prothioconazole with wild-type *C. albicans* CYP51 (Fig. 6b) were 1.6, 1.9 and ~120 μM, respectively. As expected, voriconazole and prothioconazole-desthio strongly inhibited *C. albicans* CYP51 activity with IC_{50} values approximately half the CYP51 concentration indicative of tight binding inhibitors. Prothioconazole, however, weakly inhibited *C. albicans* CYP51 with 55 % residual activity remaining in the presence of 100 μM prothioconazole. IC_{50} determinations can also be performed using non-azole CYP51 inhibitors broadening the range of inhibitors that can be screened and studied. For a more comprehensive picture of azole inhibition of the target CYP51 enzyme, the IC_{50} determinations can be performed at different CYP51 concentrations and different substrate concentrations (resources permitting). However, performing sterol substrate K_m determinations is complicated by the discontinuous nature of the CYP51 assay system making determination of initial reaction velocities difficult.

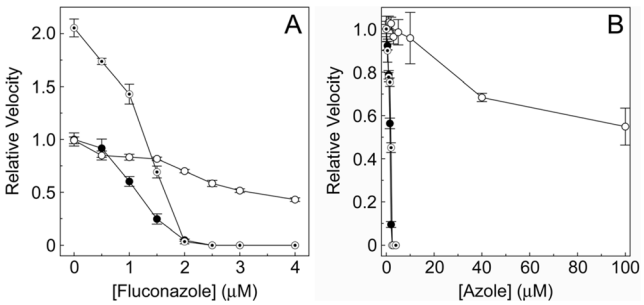

Fig. 6 Azole IC_{50} determinations. **a** The CYP51 activities of 2.5 μM wild-type (*black circle*), S279F (*white circle*) and I471T (⊙) *C. albicans* CYP51 were determined at fluconazole concentrations ranging from 0 to 4 μM. A relative velocity of 1.0 corresponds to the velocity observed with the wild-type enzyme in the absence of fluconazole (0.062 nmol min^{-1}). **b** The CYP51 activities of 2.5 μM wild-type *C. albicans* CYP51 were determined at voriconazole (*black circle*) and prothioconazole-desthio (⊙) concentrations ranged from 0 to 4 μM and prothioconazole (*white circle*) concentrations ranged from 0 to 100 μM. Relative velocities of 1.0 equate to actual velocities of 0.080, 0.098 and 0.087 nmol min^{-1} for the IC_{50} determinations with voriconazole, prothioconazole and prothioconazole-desthio, respectively. Mean values from three replicates are shown along with associated standard error bars

Structural modelling of the wild-type *C. albicans* CYP51 protein indicated S279 was located outside the protein, on an external β turn. With the S279F substitution, F279 became incorporated within a short section of α helix preceding the long I helix, resulting in a large movement of position (Fig. 7a). During fluconazole docking, the oxygen atom of S507 interacts with the second triazole ring of fluconazole in the wild-type *C. albicans* CYP51 protein, assisting in orientating fluconazole so that a more favourable binding conformation to heme is achieved (Fig. 7b). In contrast, for the S279F point mutation, this S507-fluconazole interaction is absent, providing an explanation for the higher K_d value observed with fluconazole [91].

Therefore, studying the biochemical properties of CYP51 enzymes is important to validate the in silico models and that the two techniques can then be used together to further understand the mechanisms of resistance exhibited by CYP51 point mutations, develop appropriate strategies to combat the growing problem of azole resistance in fungal pathogens and screen for new antifungals.

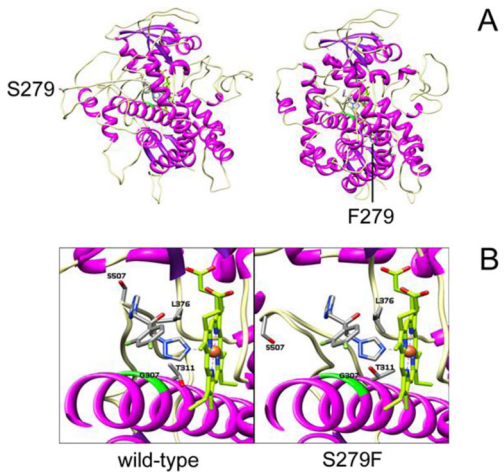

Fig. 7 Structural modelling of wild-type and S279F *C. albicans* CYP51 proteins with and without fluconazole docked. **a** Wild-type protein, with S279 located peripherally on an external β turn. The S279F mutant protein also shown has F279 incorporated within a short section of α helix preceding the long I helix. **b** The wild-type protein shows fluconazole in the centre (*coloured by element*) and the haem group to the right, and residues predicted to be within the range of contact of fluconazole are *labelled*. The S279F mutant protein is also shown with the S279F substitution leading to conformational changes that result in S507 being removed from interaction with fluconazole but still bordering the haem cavity

Specificity of azole fungicides for the target CYP51 enzyme

The ideal new clinical azole antifungal drug/fungicide should have a far greater affinity for the target fungal CYP51 enzyme than the host CYP51 analogue to avoid disruption of the host's sterol biosynthetic pathway. It must also prove to be effective against fungal CYP51 point mutations that confer resistance against previous azole antifungal compounds. In addition, the azole drug/fungicide should bind poorly to other cytochrome P450 enzymes in the host to avoid the disruption of important biosynthetic and regulatory pathways, such as the endocrine system and liver drug/xenobiotic metabolism in mammals. Designing such an ideal azole drug/fungicide is extremely challenging and will inevitably lead to compromises being made. As a consequence, it is important that any new azole compound is fully characterised in terms of selectivity for its drug target over the host homologue and that interactions with other CYPs are investigated. In a recent publication, the selectivity of five therapeutic azole antifungal drugs and seven agricultural azole fungicides for *C. albicans* CYP51 over the human homologue [168] demonstrated that azole binding and IC_{50} studies can be used as a measure of selectivity for an azole compound. Comparison of the K_d values (Fig. 8a) obtained from type II binding spectra indicated that the selectivity of the azole compounds for *C. albicans* CYP51 over the human homologue varied considerably from 0.95-fold for prothioconazole-desthio to 543-fold for fluconazole. Likewise, comparison of the IC_{50} values (Fig. 8b) also showed variable selectivity from 1.3-fold with tebuconazole to ~1,300-fold with fluconazole for *C. albicans* CYP51 over the human homologue. Fold selectivity based on IC_{50} values was significantly greater for itraconazole (175-fold), propiconazole (183-fold) and prothioconazole-desthio (167-fold) than observed with K_d values (4.8-, 8.8- and 0.95-fold) (Fig. 8c). This was primarily due to the high IC_{50} values obtained with human CYP51 for these three azoles even though the K_d values obtained were relatively low. The differences observed between K_d determinations and IC_{50} values may in part be due to the CYP51 enzyme adopting a slightly different structural conformation when inserted into a lipid bilayer along with substrate, cytochrome P450 reductase redox partner and NADPH. Therefore, azole ligand binding studies are useful in excluding new compounds that bind poorly to the target CYP51 enzyme, but IC_{50} studies are required to confirm that high initial binding affinities are translated into effective inhibition of the target CYP51 enzyme. Conventional MIC studies can then be performed using

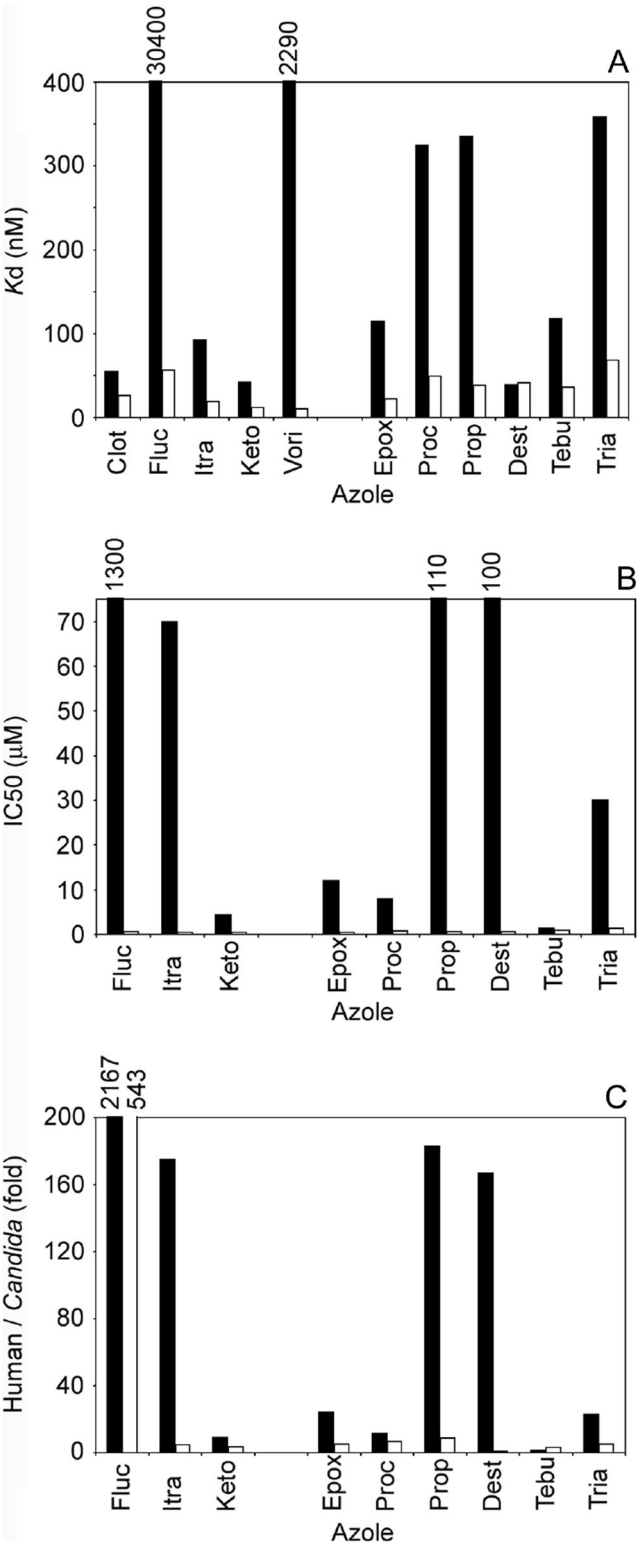

Fig. 8 Comparison of azole binding parameters for *C. albicans* and human CYP51 enzymes. **a** K_d values were determined for azoles binding to human (*black bar*) and *C. albicans* (*white bar*) CYP51 proteins from ligand saturation curves using a rearrangement of the Morrison equation [172]. **b** IC_{50} values for azole antifungal drugs and fungicides. IC_{50} values were determined for azoles binding to human (*black bar*) and *C. albicans* (*white bar*) CYP51 proteins using the CYP51 reconstitution assay system. **c** Fold selectivity for the fungal drug target over the human homologue was calculated by dividing the IC_{50} values (*black bar*) and K_d values (*white bar*) for human CYP51 by those obtained for *C. albicans* CYP51

the target organism to confirm biological effectiveness against clinical and agricultural isolates. Fluconazole had the highest selectivity for the fungal CYP51 enzyme over the human homologue by a significant margin with some of the agricultural fungicides, such as tebuconazole, having the poorest selectivity [168]. Similar studies could be performed using CYP51 enzymes from fungal plant pathogens and the host CYP51 (CYP71) homologues to determine selectivity of the agricultural fungicides.

Prothioconazole is a special case and does not behave like the other azoles when interacting with *C. albicans* and human CYP51 proteins. Ligand binding experiments (Fig. 9) gave weak type I binding spectra typical of substrate binding and not the typical type II interaction observed when azole antifungals coordinate through the triazole N-4 or imidazole N-3 nitrogen atom with the haem ferric ion. In addition, prothioconazole did not appear to inhibit human CYP51 and only very weakly inhibited *C. albicans* CYP51. This alternate behaviour is attributed to the 'bulky' sulphur atom covalently attached adjacent to the triazole N-4 nitrogen atom prevented direct coordination to the haem ferric ion. Further work on prothioconazole has shown that prothioconazole is readily converted to the desthio form which accounts for the antifungal effect [175].

In silico structural modelling of CYP51 enzymes

In silico structural modelling is increasingly being used to investigate CYP51-mediated azole resistance [119, 161, 177–179]. These investigations have primarily focussed on differences in azole ligand docking between the wild-type enzyme and the point mutations found in the CYP51 enzymes present in azole-resistant strains from the clinic or field. Ideally, azole ligand docking studies need to be performed using the template of the species CYP51 of interest. However, this is not possible, as crystallising membrane-bound CYP51 proteins of eukaryotes with the N-terminal membrane anchor intact is difficult. Presently, six CYP51 enzyme structures have been resolved by X-ray crystallography. They are the eukaryote CYP51 enzymes from *Trypanosoma brucei* [180], *Trypanosoma cruzi* [181], *Leishmania infantum* [182], *H. sapiens* [183] and *S. cerevisiae* [184] and the prokaryote CYP51 enzyme from *Mycobacterium tuberculosis* [185]. All of the eukaryotic CYP51 enzymes, with the exception of *S. cerevisiae*, were crystallised in truncated form (N-terminal membrane anchor removed). To date, the only fungal, membrane-bound, non-truncated CYP51 to be crystallised and the structure resolved is that of *S. cerevisiae* CYP51 [184]. This is an important development which may increase the reliability of models in the future with regards to fungal specific regions of CYP51.

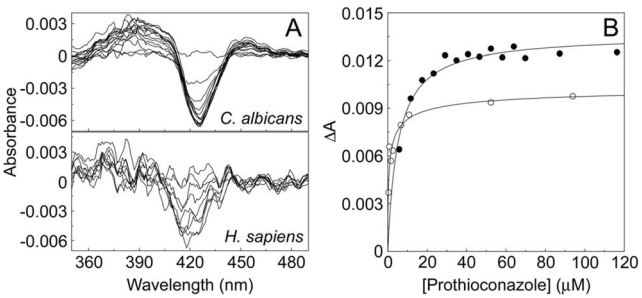

Fig. 9 Prothioconazole binding with *C. albicans* and human CYP51 proteins. **a** Weak type I difference spectra were obtained using 5 μM solutions of the CYP51 proteins during progressive titration with prothioconazole. **b** Ligand saturation curves were constructed from the change in absorbance against prothioconazole concentration using the Michaelis–Menten equation

As more CYP51 crystal structures are published, the more refined these models have become. Most of the CYP51 point mutations observed in azole-resistant clinical *C. albicans* isolates were not predicted by models to interact directly or through hydrophobic interactions with the main azole antifungal drugs, since they are based on homology models to a single bacterial enzyme [177–179]. Recently, modelling techniques based on using multiple cytochrome P450 proteins as templates have been used to describe how point mutations in *C. albicans* and *Z. tritici* CYP51 might exert their effect in conferring azole resistance [58, 91, 161]. For example, the model constructed for *C. albicans* S279F revealed a large change in conformation compared to the wild type which precludes an important interaction with fluconazole, accounting for the resistant phenotype [91]. These scaffolds have proved particularly useful in understanding the importance of a region associated with azole resistance mutations in *Z. tritici* [161], *C. albicans* [82, 86, 167], *A. fumigatus* [59] and *M. fijiensis* [149]. Models of *Z. tritici* CYP51 [58, 125] predicted this fungal-specific region (Y459–Y461) to be integral to the active site and directly impact azole binding, which was consistent with in vivo findings whilst past models had predicted this region to be on the surface of the protein [186]. Models of mutations in this region directly impact azole binding and are consistent with in vivo findings. These multiple scaffold models are also useful in predicting the impact of individual and combinations of mutations [58, 91, 161]. Interactions with docked ligands and substrates can be predicted, explaining the differential azole sensitivities seen in different variants. Models of *Z. tritici* CYP51 have also shown that successive mutations have led to an enlarged active site pocket reducing the interaction of azoles with surrounding amino acids and therefore reducing sensitivity to azoles [58, 161]. In silico models may therefore be a tool to aid the management of fungicides in agriculture in response to existing and developing resistance. These models can also be used without prior biological knowledge of a specific mutation. They can be used to predict how new compounds will bind and how existing compounds will bind to mutants

for which there is no in vitro data. This is the case with azole-resistant mutants of *Z. tritici* CYP51 and *A. fumigatus* CYP51A, which have not yet been expressed and purified, precluding biochemical comparison of the azole and substrate binding properties, CYP51 catalytic efficiency and azole inhibitory properties (IC_{50} values) of these proteins. In the case of *Z. tritici*, there is still a requirement for a functional cytochrome P450 reductase partner in order to be able to obtain catalytic efficiency and IC_{50} data. In the absence of the availability of such biochemical analyses, in silico structural models are of particular importance.

Problems encountered with current azole antifungals

Since the haem prosthetic group is not unique to CYP51 but is shared by all cytochrome P450 (CYPs), and all CYP51s are similar, containing structurally conserved regions [187], there may be significant cross-reaction and toxicity problems with azoles. Firstly, azole compounds may inhibit human CYP51, resulting in a reduction in the synthesis of cholesterol and affect the endocrine system [188]. Secondly, CYPs are the most abundant form of drug-metabolising enzymes in humans; therefore, there is the potential for drug–drug interactions due to the inhibition of other human CYPs [189] which may cause adverse effects. There is increasing concern in Europe over the effects of agricultural azoles in mammals [190, 191] and the hepatotoxicity of these fungicides [192]. Hepatotoxicity may be caused by induction of the expression of liver cytochrome P450 enzymes (CYP1, CYP2 and CYP3 families), which in turn increases the abundance of reactive oxygen species in liver cells. This may result in lipid peroxidation and DNA damage [192]. In addition, azoles may also inhibit liver P450 enzymes, interfering in the phase I metabolism of xenobiotics [192–195]. Alterations in cytochrome P450 in humans associated with cholesterol, steroid, vitamin D and eicosanoid synthesis are known to cause diseases [196]. Therefore, interference in these pathways may cause adverse effects. Azoles can disrupt the endocrine system by inhibiting several highly substrate-selective cytochrome P450 enzymes (CYP11A1, CYP11B2, CYP17A1, CYP19A1 and CYP21A2) involved in mammalian steroid hormone biosynthesis. This can lead to impaired reproduction, alterations in sexual differentiation, impaired growth and development and the formation of hormone-dependent cancers [38, 39, 190, 191, 197]. In addition, there is growing evidence that inhibition of CYP46A1 by azoles impairs 24S-hydroxycholesterol synthesis in the brain and can result in visual disturbances [198]. Recent analysis has shown that the azole affinity for CYP46A1 and fungal CYP51 enzymes is similar [199].

Many yeasts and fungi that are causative agents of clinical infections, such as *Candida* species and *Aspergillus* species, are also present in the general environment and are exposed to the selective pressure of agricultural azoles in the field. There are now concerns that azole resistance in clinical infections has emerged through the use of agricultural azoles [200]. *Aspergillus* is a relatively weak phytopathogen, though it is abundant in the environment and therefore exposed to azoles through treatment of other agriculturally important species. Recently, point mutations in *A. fumigatus* CYP51A that confer resistance to azole antifungal agents were attributed to resistance acquired outside the clinic, in the general environment [125, 200–202]. Unlike resistance acquired as a result of clinical azole treatment, wild-type strains were not recovered from patients infected with either of the two resistant strains CYP51ATR34/L98H and TR46/Y121F/T289A [125], isolated from some azole-naïve patients.

Resistance acquired in the environment is a growing concern. It is likely that more azole-resistant *Aspergillus* sp. strains that have evolved in the environment will appear in clinical isolates. It is possible that other opportunistic pathogens, such as *Fusarium* spp. may also emerge as resistant strains in the clinic. *Fusarium* species cause many types of infection in humans, ranging from superficial infections (keratitis and onychomycosis) to systemic infections in immunocompromised patients [203]. Around 50 % of cases are caused by *Fusarium solani*, around 20 % by *Fusarium oxysporum* and around 10 % each by *Fusarium verticilliodis* and *Fusarium moniliforme* [203]. As yet, azole resistance mediated by CYP51 mutations has not been observed either in the field or the clinic. However, Fan et al. have generated Y123H mutations in CYP51B and a mutant overexpressing CYP51A in *F. verticillioides* in the laboratory by subjecting the strain to successive selection with azole [204]. Both strains are prochloraz-resistant and exhibit some cross-resistance to triazoles. This indicates the potential for azole resistance to arise in *Fusarium* spp. and may apply to other opportunistic pathogens, which are also exposed to agricultural azole treatments in the environment.

In addition to agricultural use, many fungicides are used both by industry and domestically to prevent fungal growth and decay. Difenoconazole, tebuconazole and propiconazole are used to treat lawns. Tebuconazole present in 'Copper azole type B' [25] and propiconazole are used to treat wood. Propiconazole is also licenced for use in adhesives, paints, leather, paper and textiles [26] and is the active compound in antimold paints. Also, copper corrosion inhibitors added to aqueous coolant systems also contain azoles (tolyltriazole and benzotriazole) [27]. Therefore, environmentally prevalent fungi will be exposed to these azoles [57]. In addition, azole compounds are used in toiletries and shampoos (climbazole and ketoconazole), and excreted medicines (clotrimazole) will also increase the environmental exposure of fungi to azoles [205].

Summary

CYP51 is an effective drug target that has enabled the introduction of a large number of new antifungal compounds to be developed for use against it over the past four decades. Compounds with other modes of action have also been introduced to target fungal diseases, such as echinocandins and strobilurins; however, resistance to these quickly developed [206–208] and compounds targeting CYP51 are still an essential tool in tackling fungal diseases. There is a necessity to develop new antifungal compounds with increased selectivity for the fungal CYP51 enzyme over the human homologue and other human CYPs and which will also minimise the risk of cross-resistance. Currently, several new azole and non-azole CYP51 inhibitors are being developed for the next generation of antifungal drugs [176, 209–214]. It may also be possible to design new compounds which are pro-fungicides in a manner similar to that of prothioconazole [174, 175].

Understanding which agricultural azoles may drive resistance to clinical azoles (especially in those species such as *Aspergillus* which are already intrinsically resistant to many clinical azoles) is of the upmost importance. It is therefore necessary to be able to determine differences between agriculturally and clinically important strains and their susceptibility to azoles. Traditionally, antifungals used in agriculture may be less specific for fungal CYP51 and may cause cross-resistance problems with human CYPs (as demonstrated with human CYP51 [168]). Azole compounds which are not closely related structurally to medical azoles are best used in agriculture. In addition to binding and inhibition of human CYPs, azole compounds may also induce some human CYPs. Therefore, the potential for drug–drug interactions and toxicity needs to be addressed.

All these factors must be considered when designing new antifungals, and the assessment of CYP51 susceptibility, through the biochemical techniques described above, is central to the design and assessment of such compounds. However, the increasing concern over cross-resistance emanating from environmental use of azoles may need to be addressed through altering agricultural antifungal treatment regimens. Interestingly, triazole fungicides were used on crops nearly two decades before triazole antifungal drugs were routinely used in the clinic to treat systemic infections of humans [215]; therefore, the recent emergence of cross-resistance may be due to a change in use of agricultural antifungals or a change towards azoles which interact with fungal CYP51 enzymes more akin to clinical azoles. Alternative antifungal strategies including mixes of azoles with non-azoles and a rotation in the use of antifungals with different modes of action may aid to stem the increase in cross-resistance.

The continued discovery of inhibitors of CYP51 in fungi as drugs and fungicides shows the success of this approach and the difficulty of discovering other targets to control fungal infection. Management of resistance or tolerance is possible, although even in azole sensitive fungi cure rates in the clinic are not ideal. Molecular modelling may allow the cycling of fungicide use as implied by the emerging pattern of resistance on exposure to CYP51 inhibitors in agriculture, and something similar could arise in the clinic as resistant strains to current therapeutics become sensitive to earlier drugs. As more CYP51 structures from pathogenic fungi are solved, in silico structural modelling will continually improve enabling modelling of azole docking to drug-resistant CYP51 mutants and use as a predictive tool to decide on therapy strategies such as whether cycling of azole drugs would prove beneficial in the clinic. Future work will require the investigation of the enzymology to dissect the roles of mutations in reducing azole affinity, changing enzyme turnover rates and compensating for the role of other mutations on activity and strain fitness. Previous fluconazole IC_{50} studies with *C. albicans* CYP51 have shown that some point mutations (F145L) cause only a modest increase in the IC_{50} value but facilitate low residual CYP51 activity at high azole concentrations [88] compared to the wild-type CYP51 that exhibits no such residual activity. This ‘leaky’ phenotype deserves further investigation to elucidate the molecular mechanism of resistance involved. Long range as well as close proximity amino acid sequence changes relative to the active site can be examined for conferring these effects. Better inhibitors with reduced toxicity that are resistance breakers and less stable compounds that break down in the environment are needed for the longer term.

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