Investigating resistance mutations in the drug target of triazole drugs

Structural and functional characterisation of *Saccharomyces cerevisiae* lanosterol 14α-demethylase mutants as a model to represent resistance-conferring mutations of pathogenic fungi

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Abstract

Fungal infections affect a broad spectrum of the population, including premature babies, the elderly and individuals with a range of disease- or medically-induced co-morbidities. Fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* cause a variety of conditions, from minor infections to life threatening disease. Some fungi, including *C. albicans* and *Candida glabrata* can be commensal living in harmony with the superficial microflora, but these organisms can behave as opportunistic pathogens when individuals become immunodeficient due to co-morbidities or become immunocompromised due to AIDS or through medical intervention. Fungal infections have become recognised in recent years as a growing health burden that currently causes around 1.5 million deaths per year worldwide.

The azole antifungal drugs (imidazoles and triazoles) are used widely to treat fungal infections and as antifungal prophylaxis. The azole drugs target the fungal enzyme lanosterol 14α-demethylase (Erg11p, CYP51). This monospanning bitopic membrane protein belongs to the CYP51 class in the cytochrome P450 superfamily of enzymes and is involved in the rate-limiting step of ergosterol biosynthesis. Ergosterol is the fungal equivalent of cholesterol and is required for fungal cell growth. Fungal pathogens have evolved several mechanisms of resistance that diminish the action of the azole drugs. The emergence of resistant fungal strains due to mutations in CYP51 can limit therapeutic options and make treatment of fungal infections increasingly problematic. The need for better drugs that overcome resistance is becoming increasingly urgent. The present project builds on the research of Monk et al. who successfully crystallised and obtained the first high-resolution X-ray structures of a fungal CYP51. The aim of this project is to investigate the effect of CYP51 mutations on enzyme structure and function, including different types of triazole drug, by using *Saccharomyces cerevisiae* Erg11p as a model for the homologous enzymes in pathogenic fungi.

A *S. cerevisiae* overexpression system was used to hyper-express the wild type and the mutant ScErg11p enzymes in order to obtain sufficient quantities of protein for structure-function studies. The *C. albicans* CYP51 mutations Y132F/H, K143R, G464S and the double mutation Y132F G464S (Y140F/H, K151R, G464S and Y140F
G464S *S. cerevisiae* numbering), as well as the CYP51A G54E/R/W mutations of *A. fumigatus* (G73E/R/W *S. cerevisiae* numbering) have been reproduced in a C-terminal hexahistidine-tagged version of *S. cerevisiae* Erg11p (ScErg11p6×His). In addition, the innate resistance of *A. fumigatus* CYP51A to fluconazole (FLC) was investigated using the *S. cerevisiae* Erg11p T322I mutant. Microdilution assays were used to determine triazole susceptibilities of these strains. ScErg11p6×His mutant and wild type enzymes were purified from crude membranes by solubilisation with the detergent *n*-decyl-β-D-maltoside followed by affinity and size exclusion chromatography. Spectral analysis of the purified protein was used to determine dissociation constants for triazole drugs. Purified preparations of the enzyme were also used to obtain crystals for X-ray crystallographic analysis. High-resolution (1.98 – 2.35 Å) X-ray crystal structures were obtained for mutant enzymes in complex with triazole drugs and without added ligand, as well as for the wild type enzyme in complex with FLC.

Microdilution assays revealed that strains overexpressing ScErg11p6×His Y140F/H or Y140F G464S had reduced susceptibility to the short-tailed triazoles FLC and voriconazole but not the long-tailed triazole itraconazole. Strains overexpressing ScErg11p6×His G464S, T322I and K151R mutants had triazole susceptibility patterns similar to the wild type enzyme overexpressing strain but the G73E/R/W mutants showed increased susceptibility to all triazoles tested. Binding studies revealed that the triazole binding was tight for all the mutant enzymes.

The high-resolution (2.05 Å) structure of wild type ScErg11p6×His in complex with FLC revealed a water-mediated hydrogen bonding network between residue Y140 and the hydroxyl group of the drug. The crystal structures of the ScErg11p6×His Y140F/H mutants showed that these mutations disrupted the key water-mediated hydrogen-bonding network seen in the wild type enzyme complex. The disruption of these interactions is proposed to weaken the interactions between the drug and the mutant enzyme leading to resistance. These observations explain reduced susceptibility to FLC and voriconazole and the retention of susceptibility to itraconazole of these mutants.

The X-ray crystal structures of the ScErg11p6×His G73E/W mutants in complex with itraconazole showed that the drug bound in different conformations compared to the
wild type enzyme structure. The piperazine ring of the itraconazole molecule acts as a hinge, which can adopt different conformations. The crystal structures indicated potential π-anion interactions between the tail of the itraconazole and the E73 residue and π stacking interactions between W73 and the tail of itraconazole. The bending of the drug molecule was found to accommodate each mutation. The conformation of itraconazole bound to the G73W mutant had not been seen previously. These extra interactions between the drug and the site of the mutation in part explain the increased susceptibility of G73E/W mutant strains to itraconazole.

The structure of ScErg11p6×His G464S revealed that the mutated residue had replaced polar interactions between a water molecule and the propionate group of the heme. No obvious tilting of the heme was observed in this mutant. The ScErg11p6×His T322I and G73W mutant structures without added ligand revealed some density in the active site and some movement of the carbonyl group of helix I residue G314, previously seen in the wild type ScErg11p6×His structure in complex with lanosterol. Residue G314 may be involved in catalysis by potentially stabilising oxygen bound heme iron intermediates.

In summary, this work provides insight into the molecular interactions between the triazole drugs and the CYP51 enzyme. The high-resolution structure of the wild type enzyme in complex with FLC has allowed us to identify the potential basis for resistance of the Y140F/H mutants, which we confirmed by recreating those mutations in our S. cerevisiae system. In addition, other mutations reproduced in our system reveal that despite a relatively high sequence similarity amongst fungal CYP51s, our model does not adequately reflect the effect of the same mutations in pathogenic fungi. This knowledge will aid in the structure-directed design of next-generation azole-based antifungal drugs that can be used to overcome antifungal resistance.
I would like to express my deepest gratitude to my supervisors, Assoc Profs Brian Monk and Joel Tyndall and Dr Mikhail Keniya. Their continuous encouragement and support over the past three and a half years have made my time in Dunedin enjoyable and productive. Mikhail has taught me many of the laboratory techniques and experimental planning skills needed to complete my project. To him I owe my gratitude for his patience, insightful teaching and advice. Brian has been an amazing supervisor and mentor to me over the course of my PhD. He taught me a lot about planning experiments, presenting my results and scientific writing, as well as providing guidance to further myself as a scientist. Joel has taught me about structural biology and how to analyse and interpret crystallographic data, and offered a different perspective on things, especially when I couldn’t see the wood for the trees. I am grateful to you all.

I would like to thank the staff of the Molecular Biosciences Laboratory. They have welcomed me into the lab and have always been ready to help and offer advice. I owe my gratitude to Rajni Wilson and Manya Sabherwal for their help with experiments. Our former lab manager Jenine Upritchard has been so helpful with so many things, she always listened to my rants and complaints and I am thankful for that. I thank my convenor Dr Geoffrey Tompkins for taking his time to attend meetings. I would like to thank Dr Sigurd Wilbanks for your expert advice on crystallography matters. I would also like to thank Dr Franzi Huschmann for teaching me the protein purification techniques. I express my gratitude to my fellow PhD students and friends here in Dunedin.

I thank my partner Chris for supporting me through my PhD journey and putting up with my craziness, which escalated somewhat upon the arrival of our son Boston. Becoming a mum has been amazing, however sleep deprivation and finishing a thesis at the same time has been tiresome, I could not have done it without you. And last but not least, I would like to thank my parents. Their guidance, support and understanding have brought me to this point in my life. They have always managed to give me advice and perspective no matter what stage of my life I was at. I am forever grateful.
for everything that you have done for me and I could only hope that I can be as good a parent to Boston as you are to me.
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*I received travel awards from the conference organisers to attend these meetings.

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<th>Full Form</th>
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<tr>
<td>5-FC</td>
<td>Flucytosine</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>AMB</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy terminal</td>
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<tr>
<td><em>C. albicans</em></td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td><em>Candida glabrata</em></td>
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<tr>
<td><em>C. neoformans</em></td>
<td><em>Cryptococcus neoformans</em></td>
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<tr>
<td>cmc</td>
<td>Critical micelle concentration</td>
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<tr>
<td>CYP450/CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYP51</td>
<td>Sterol 14α-demethylase</td>
</tr>
<tr>
<td>DM</td>
<td>Decyl-β-D-maltoside</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EB</td>
<td>Elution buffer</td>
</tr>
<tr>
<td>Erg11p</td>
<td>Lanosterol 14α-demethylase</td>
</tr>
<tr>
<td>FLC</td>
<td>Fluconazole</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>FSL</td>
<td>Fungus-specific loop</td>
</tr>
<tr>
<td>Fur1p</td>
<td>Uracil phosphoribosyl transferase</td>
</tr>
<tr>
<td>GTED</td>
<td>Glycerol tris EDTA</td>
</tr>
<tr>
<td><em>H. capsulatum</em></td>
<td><em>Histoplasma capsulatum</em></td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIS1</td>
<td>ATP phosphoribosyltransferase gene</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>ISA</td>
<td>Isavuconazole</td>
</tr>
<tr>
<td>ITC</td>
<td>Itraconazole</td>
</tr>
<tr>
<td>KTC</td>
<td>Ketoconazole</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MCZ</td>
<td>Miconazole</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethansulfonic acid</td>
</tr>
<tr>
<td>MFS</td>
<td>Major Facilitator Superfamily</td>
</tr>
<tr>
<td>MH1</td>
<td>Amphipathic helix 1</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;80&lt;/sub&gt;</td>
<td>Minimum inhibitory concentrations at 80% growth inhibition</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600nm&lt;/sub&gt;</td>
<td>Optical density at a wavelength of 600 nm</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<td>PAGE</td>
<td>Poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCZ</td>
<td>Posaconazole</td>
</tr>
<tr>
<td>PDB ID</td>
<td>Protein Data Bank Identity Code</td>
</tr>
<tr>
<td>PDR</td>
<td>Pleiotropic drug resistance</td>
</tr>
<tr>
<td>PDR5</td>
<td>Pleiotropic drug resistance 5</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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x
PGK 3-phosphoglycerate kinase
PMSF Phenylmethylsulfonyl fluoride
rpm Revolutions per minute
S. cerevisiae Saccharomyces cerevisiae
SD Synthetic defined
SDS Sodium dodecyl sulphate
SEC Size exclusion chromatography
SEM Standard error of the mean
T. brucei Trypanosoma brucei
T. cruzi Trypanosoma cruzi
TE buffer Tris-HCl EDTA contained buffer
TEMED Tetramethylethylenediamine
TMH1 Transmembrane helix 1
TR Tandem repeat
URA3 Orotidine-5'-phosphate (OMP) decarboxylase gene
VCZ Voriconazole
YPAD Yeast extract-peptone-dextrose with adenine sulfate
YPD Yeast extract-peptone-dextrose
Z. tritici Zymoseptoria tritici
Preface

This doctoral thesis has been written as a hybrid of thesis chapters and a published article in lieu of Chapter Three, as well as Chapter Four being a manuscript draft at a pre-publication stage. Consequently, there is a certain amount of repetition throughout the thesis especially in the introduction sections for some Chapters.

Chapter One is a general literature review that provides background information and describes the aims of the thesis.

Chapter Two covers the materials and methods utilised in this study.

Chapter Three is a modified version of published article.

“Structural insights into binding of the antifungal drug fluconazole to *Saccharomyces cerevisiae* lanosterol 14alpha-demethylase.”


My contribution to this article was carrying out the majority of experimental work, data analysis and preparing the manuscript. My supervisors, Associate Profs Brian Monk and Joel Tyndall and Dr Mikhail Kenya have helped with experimental design, data interpretation and have edited the manuscript. Dr Rajni Wilson has helped with experimental work. The PDF version of the printed article is also included in Appendix G.

Chapter Four has been written as a manuscript and will be lodged for peer-review after the submission of this thesis.

Chapter Five has been written as a thesis chapter.

Chapter Six is a conclusions chapter, which summarises implications of the work carried out in this thesis.

The X-ray crystal structure published with Chapter Three has been deposited in the Protein Data Bank (PDB ID: 4WMZ). The rest of the protein structures described in this thesis have been deposited in the Protein Data Bank but not released. The coordinate files for the unreleased structures are included on a CD in Appendix G.
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Chapter 1

General Introduction
1.1 The role of fungi in the biosphere

The fungal kingdom is thought to consist of at least 1.5 million species (Hawksworth, 1991, Hawksworth, 2001) that diverged from a common ancestor about 1.5 billion years ago (Hedges et al., 2004). More recent estimates, based on molecular tools and the ratios of fungi to plant species suggest there are as many as 5.1 million fungal species in 10 phyla (Blackwell, 2011). Fungi are eukaryotic organisms that contribute to the biosphere in ways that are essential to many forms of life on Earth. For example, saprophytic fungi recycle organic material, mainly plant detritus, and many fungi have developed symbiotic relationships with the roots of plants. Without decomposition of plant material, the accumulation of plant debris would prevent the growth of new plants and important nutrients would not be recycled. Given the longevity of the fungal kingdom, the presence of fungi in a wide range of ecosystems and their ability to adapt using a variety of interactions and reproductive strategies, it is hardly surprising that the members of this kingdom show extensive genetic, phenotypic and morphological diversity.

Fungi also make major contributions to human activities including industry, medicine and biological research. The food industry benefits from the fermentation processes carried out by yeast to make bread, alcoholic beverages such as beer and wine and products such as soy sauce. Mushrooms are fungal fruiting bodies that have been used as sources of foods, poisons and hallucinogens for millennia. The introduction of antibiotics is regarded as a major turning point in medicine. Penicillin, the first of the modern antibiotics used in the clinic, was prepared on an industrial scale from the fungus *Penicillium notatum* (Elander, 2003). Since then yeast and filamentous fungi have been used by the pharmaceutical industry to produce a variety of antibiotics. *Saccharomyces cerevisiae* or Baker’s yeast is a well-established eukaryotic model organism. *S. cerevisiae* provides a powerful research tool due to its relatively well understood biochemistry, physiology and genetics, as well as several other traits discussed in Section 1.13.

Unfortunately, not all fungi are good for humankind. Around 300 fungal species are known to infect humans (Taylor et al., 2001) while many more species affect the plants and animals that we husband. Among the pathogens of humans, a small group can be regarded as pathogens that cause common diseases while the rest
cause rare mycoses. Members of the fungal genera Candida, Cryptococcus, Pneumocystis and Aspergillus cause invasive fungal infections responsible for about 90% of all reported fungal-related deaths (Denning and Hope, 2010). Fungal pathogens also affect major crops and can significantly affect crop production (Oerke, 2006). For example rice blast disease is caused by Magnaporthe oryzae and wheat rusts are caused by Puccinia spp. Other crop fungal diseases include grey mould which can cause spoilage of ~200 plant species. Prominent examples include powdery mildew of grasses such as barley, leaf scald in cereals and wheat headblight (Dean et al., 2012). Some fungi produce toxic secondary metabolites called mycotoxins (Logrieco and Visconti, 2013). Highly carcinogenic aflatoxins are produced by the phytopathogens Aspergillus flavus and Aspergillus parasiticus. These fungal species usually reside in soil and decompose plant matter but during favourable conditions they can contaminate crops.

1.2 Individuals at risk of fungal infections

Healthy individuals commonly carry fungi as commensals that are kept in check by physical barriers such as the skin and by the innate immune system operating at mucus membranes (Underhill and Iliev, 2014). For example ~40% of healthy individuals carry Candida albicans in their oral cavities. Overgrowth of fungi can cause superficial fungal infections of the skin and mucous membranes even in healthy individuals. Fungal infections of the skin, eyes, nails and hair occur in about 25% of the world’s population (Havlickova et al., 2008). Infections caused by dermatophytes include athlete’s foot (Tinea pedis), ringworm of the groin (Tinea cruris) and nail infections (Tinea unguium). These infections can be transmitted through physical contact with contaminated material. Superficial yeast infections are also common. For example, when the normal microbial flora is disrupted due to antibiotic use, C. albicans can overgrow causing oral and vaginal thrush (Underhill and Iliev, 2014). It is estimated that 3 out of 4 women will experience vaginal thrush in their reproductive years. If physical barriers are breached, for example due to large area skin scrapes or burns, opportunistic fungal pathogens can enter the body more easily, thus putting the affected individual at a greater risk of invasive fungal infection.
Many individuals in modern populations are immune deficient due to underdeveloped, failing or redirected immune systems (Reust, 2013). This situation is exacerbated when medicine applies therapies that compromise patients’ immune systems (Richardson, 2005). Deficient or compromised immune systems render individuals susceptible to various microbial infections, including fungal infections. Immunodeficient groups include HIV patients, premature babies, females of reproductive age, the elderly and the infirm. Immunocompromised patients include cancer patients undergoing chemotherapy or radiotherapy, transplant patients undergoing immune suppressive therapies and AIDS patients (Segal et al., 2006).

The fungal infections of immunodeficient individuals are usually superficial (Havlickova et al., 2008). While such infections are likely to be more extensive than in healthy individuals, and although quality of life can be significantly affected, these infections are usually readily treated. Oral and vaginal thrush can be effectively treated with Canesten®, which contains clotrimazole as the active ingredient. There are a number of different topical agents to treat athlete’s foot such as terbinafine (Lamisil® cream), nystatin (Nilstat® cream) or miconazole (Daktarin® cream). Immunocompromised individuals are not only more susceptible to superficial infections but also to invasive infections that can be fatal because they damage key organs such as the kidney and brain (Richardson, 2005).

1.3 The threat of fungal infections

Among microbial infections, the infections caused by fungi are readily overlooked. This situation is changing due to several factors that have increased the apparent prevalence of fungal infections. These include improved diagnostic techniques, greater numbers of premature babies, co-morbidity especially in an aging population and the use of immunosuppressive agents (Brown et al., 2012). Another factor has been the application of antifungal prophylaxis in susceptible patients, which has led to the emergence of clinical isolates that are resistant to the current treatments. Fungal infections have been estimated to cause about 1.5 million deaths per year worldwide (Global Action Fund for Antifungal Infections, 2015). An even greater segment of the human population is probably adversely affected by the impact of fungal infections on food supplies (Fisher et al., 2012). The Irish potato famine is just one historic example of this problem. Key crops such as wheat,
Chapter 1: General Introduction

soybean, apples, bananas and some grasses would not be sustainable without antifungal intervention.

1.3.1 Candida species

C. albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis and Candida kruzei are reported to cause 95% of all candidiasis cases (Pfaller et al., 2012a). In the United States Candida species are the fourth most common cause of hospital-acquired bloodstream infections. They account for ~10% of all nosocomial infections and have an attributed mortality rate of about 40% (Pfaller and Diekema, 2007).

Infection by C. albicans is more common in Asia-Pacific, European, African and Middle Eastern regions, accounting for ~66% of all Candida infections, than in North and South Americas where it accounts for about 50% of reported infections (Pfaller et al., 2010). In recent years C. glabrata has become much more common in North America (21%) than in South America (7.4%; Pfaller et al., 2010). Treatment of C. glabrata is becoming increasingly problematic due to the emergence of clinical isolates that are innately resistant to echinocandins and fluconazole (FLC), drugs commonly used to treat mycoses caused by Candida species (Pfaller et al., 2012b, Chapeland-Leclerc et al., 2010). Findings from the global antifungal surveillance program ARTEMIS on 256,882 Candida isolates from 41 countries revealed that 15.7% of C. glabrata isolates were FLC resistant compared with only 1.4% of C. albicans isolates (Pfaller et al., 2010).

C. parapsilosis is another emerging fungal pathogen of considerable regional significance. C. parapsilosis is more prevalent in North America (13.6%) and South America (10.3%) than in Europe (4.2%; Pfaller et al., 2010). This pathogen has a tendency to persist in hospitals and rest homes. It is known to cause biofilms in catheters and other prosthetic devices (Trofa et al., 2008). Unlike other fungal pathogens, it is frequently found on the hands of healthcare workers and is thus readily transmitted to patients and from device to device.
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1.3.2 Aspergillus species

Aspergillus species are ubiquitous in the biosphere with their conidia found in the air and soil (Kwon-Chung and Sugui, 2013). Infections related to Aspergillus include allergic bronchopulmonary aspergillosis, severe asthma with fungal sensitisation, chronic pulmonary aspergillosis and invasive aspergillosis (Global Action Fund for Antifungal Infections, 2015). Aspergillus conidia enter the body through the lungs. If they are not cleared by the immune system the patient may be at risk of invasive aspergillosis. Aspergillus fumigatus is the most frequent cause of invasive aspergillosis, and although less common infections by Aspergillus flavus, Aspergillus terreus and Aspergillus niger remain important (Perfect et al., 2001). The individuals most likely to acquire invasive aspergillosis include cancer patients, especially those suffering leukaemia, as well as transplant and lung disease patients. Invasive aspergillosis is less common than candidiasis but is fatal in at least 50% of cases (Lin et al., 2001).

1.3.3 Cryptococcus species

The majority of cryptococcal infections are caused by Cryptococcus neoformans and Cryptococcus gattii, with meningitis likely to result from invasive infections (Lin and Heitman, 2006). C. gattii causes infections in immunocompetent individuals, especially in tropical and subtropical regions, while C. neoformans is an opportunistic pathogen of immunocompromised patients. C. neoformans has been found on eucalyptus trees common in California (Pfeiffer and Ellis, 1991). Prior to the introduction of high activity antiretroviral therapy (HAART) cryptococcal meningitis was responsible for the deaths of many AIDS patients in that region. In Sub-Saharan Africa, where AIDS, tuberculosis and malaria are endemic, cryptococcal infections kill over 400,000 people per year (Park et al., 2009). Solid organ transplant patients have a 0.26 - 5% chance of cryptococcal infection and this has a 50% mortality rate (Wu et al., 2002).

1.3.4 Pneumocystis species

Pneumocystis jirovecii, previously known as Pneumocystis carinii, is a fungal pathogen that causes pneumocystis pneumonia. This disease was predominantly
associated with HIV and AIDS patients, but is now recognised more frequently in other immunosuppressed patients (Calderon et al., 2010). In England, from 2000-2010 there was an average increase of 7% per year in pneumonia cases caused by *P. jirovecii* (Maini et al., 2013). The mortality rates in hospitals range from 7-11% and, among the critically ill, from 26-62% (Morris and Norris, 2012).

### 1.4 Diagnosing fungal infections

The progression of fungal disease can be rapid. Patients can die within 3-4 days of displaying symptoms of infection such as mild fever. Given the variability of responses of and within the fungal species to antifungals in current use, it is now regarded as critical to rapidly identify infective fungi at the phenotypic and genotypic levels to ensure effective treatment can be implemented (Richardson and Warnock, 2012). For example *A. fumigatus* is intrinsically resistant to theazole drugs FLC and ketoconazole (KTC), with voriconazole (VCZ), itraconazole (ITC) and posaconazole (PCZ) being significantly more effective against the pathogen. This is discussed in more detail in Section 5.1.2. The resistance of *A. terreus* to amphoteri cin B (AmB) but not the triazole drugs (Walsh et al., 2003) is discussed in Section 1.7.2.

Current methods for the identification of infective fungi include culture-based approaches, direct microscopy and molecular-based approaches (Richardson and Warnock, 2012). Direct microscopy of a fungal specimen relies heavily on the quality of the tissue sample and requires a skilled mycologist to correctly identify the infective species. For example, *P. jirovecii* has very poor growth *in vitro* but methods for culturing this pathogen are being developed with promising results (Schildgen et al., 2014). Currently, direct microscopic analysis of respiratory samples is the main diagnostic tool for its identification. Polymerase chain reaction (PCR)-based techniques have been shown to be sensitive enough for *P. jirovecii* identification from brochoalveolar lavage fluid but they still strongly depend on clinical validation for diagnosis (Fan et al., 2013).

*Aspergillus* species are difficult to culture from the blood of infected patients so non-culture methods have been applied. One of the techniques used is detection of galactomannan, a component of the cell wall of the *Aspergillus* species, using an
enzyme-linked immunosorbent assay (ELISA) or a more sensitive double-sandwich ELISA (Singh and Paterson, 2005). PCR-based methods have high accuracy in the identification of the *Aspergillus* species but false positives can arise as *Aspergillus* DNA can be found in the lungs of healthy individuals. The PCR-based methods are being explored but have yet to be implemented in diagnostic laboratories (Alanio and Bretagne, 2014).

The identification of *Candida* species can be carried out using culture and non-culture based methods. Chromogenic media-based systems such as CHROMagar *Candida* differentiate amongst *C. albicans*, *C. tropicalis* and *C. kruzei* exploit the cleavage of chromogenic substrates by species-specific enzymes to yield coloured colonies (Horvath et al., 2003). This method requires a 24 - 48 hour culture step and is unable to distinguish closely related species such as *C. dubliniensis* that is frequently co-isolated with *C. albicans* (Campanha et al., 2005). Commercially available biochemical kits vary in price, accuracy and require culture times ranging from 4 – 72 hours. Molecular techniques such as PCR-based methods and mass spectrometry have been shown to effectively identify *Candida* species (Spanu et al., 2012, Kanbe et al., 2002, Lau et al., 2008). They are fast and reliable but most diagnostic laboratories are not equipped to carry out such tests.

### 1.5 Antimicrobial resistance

The discovery of antibiotics has been a major step in the history of science and medicine. The first widely used antimicrobials, the sulphonamides, were introduced in 1937. Resistant microorganisms were detected not long after. Alexander Fleming discovered penicillin in 1928 and resistance caused by the enzyme penicillinase was identified in bacteria before the drug was introduced to the clinic (Abraham and Chain, 1940). Antibiotics are now so widely available that their use is often misdirected. This is a major problem as the incidence of antibiotic resistant bacterial infections is growing and there is a threat of returning to the pre-antibiotic era.

Fungal pathogens also develop resistance and the repertoire of drugs available for treatment of fungal infections is more limited than the range of antibiotics available to treat bacterial infections. The development of broad-spectrum antifungals has
proven difficult. Fungi are eukaryotes, which significantly limits the features that can be used to distinguish between the pathogen and the host. A study by Liu et al. identified 240 essential genes conserved in 10 fungal genomes including \textit{S. cerevisiae}, \textit{C. albicans}, \textit{C. glabrata}, \textit{C. neoformans} and \textit{A. fumigatus}, and only 20\% of those genes have structurally dissimilar homologues (less than 40\% identity) in the human genome (Liu et al., 2006). The same study showed that 85-93\% of essential genes in individual fungal genomes had homologues with >40\% similarity in the human genome i.e. the bulk of genes with highly conserved homologues among fungal species have similar homologues in humans. This limits the number of essential enzymes as potential targets for broad-spectrum antifungals to less than 50.

1.6 Multidrug resistance in fungi

The resistance of an organism to a broad spectrum of structurally and functionally unrelated compounds is termed multidrug resistance (MDR), reviewed by Gulshan and Moye-Rowley (Gulshan and Moye-Rowley, 2007). One of the principal mechanisms by which MDR is achieved in fungi is through the overexpression of efflux pumps. In yeast two major classes of efflux pumps are involved in xenobiotic transport: the ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) transporters. In \textit{C. albicans} Cdr1p and Cdr2p are examples of ABC transporters (Prasad et al., 1995, Sanglard et al., 1997) and Mdr1p is an MFS transporter (Goldway et al., 1995). ABC efflux pumps are primary transporters that couple drug efflux to ATP hydrolysis while MFS transporters are secondary transporters that couple efflux to electrochemical gradients. Drug tolerance is a process that allows for the development of MDR. It is a rapid, xenobiotic-induced response which, when followed by genetic modification, can allow the protective response of MDR to be maintained (Cannon et al., 2007). The tolerance phenotype can be due to the induction of drug efflux pumps with broad-range specificity for xenobiotics, including the imidazole and triazole antifungals. The efflux activity reduces the intracellular concentration of the drugs to levels at which they are no longer effective. The tolerance and subsequent MDR can be life threatening because many of the available antifungal drugs are rendered ineffective. The increased frequency of MDR strains detected in the clinic, and the increased
difficulty associated with treating such strains, including their ability to limit therapeutic options, leads to a need for novel therapies.

1.7 Current antifungal treatments and associated resistance mechanisms

There are five main classes of antifungals currently used in the clinic or as over-the-counter medications. All apart from flucytosine ultimately target the fungal cell wall and membrane structures. Ergosterol and its biosynthesis provides important primary targets because this sterol is required for growth of most fungi and is found in the cell membranes of fungi but not mammals. The ergosterol biosynthesis pathway is described in detail in Section 1.8. The five major classes of antifungal drugs used to treat fungal infections are summarised in the following sections.

1.7.1 Flucytosine

Flucytosine (5-FC) is a base analogue that acts by inhibiting DNA synthesis and aborting RNA translation (Figure 1.1). 5-FC is internalised by cytosine permease and converted to 5-fluorouridine monophosphate by the action of enzymes cytosine deaminase and uracil phosphoribosyl transferase. The 5-fluorouridine blocks fungal DNA synthesis by inhibiting thymidylate synthase and affects fungal RNA biosynthesis by causing premature chain termination (Polak and Scholer, 1975). Resistance to 5-FC occurs mainly by mutations in enzymes associated with its metabolism, in particular cytosine deaminase and uracil phosphoribosyl transferase (Hope et al., 2004). Lowered activity of those enzymes prevents the conversion of 5-FC to 5-fluorouridine. In *C. albicans*, a single nucleotide change that results in an R101C mutation in uracil phosphoribosyl transferase confers resistance to 5-FC (Dodgson et al., 2004).
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Figure 1.1 Antifungals: flucytocine, amphotericin B (polyene), terbinafine (allylamine) and micofungin (echinocandin).

1.7.2 Polyenes

AmB and nystatin are amphipathic molecules that belong to a class called polyene antibiotics (Figure 1.1). The polyenes are thought to self-assemble into ionic transmembrane pores in eukaryotic lipid bilayers that cause the lethal leakage from the cytosol of cations, including mainly K⁺ (Ermishkin et al., 1976). A recent study suggests that the binding to ergosterol alone is sufficient for their antifungal activity while pore formation may be a secondary mechanism of action (Gray et al., 2012). The limited selectivity of polyenes for ergosterol over cholesterol leads to toxicity in mammals (Medoff et al., 1983). As a result AmB has multiple side effects including nephrotoxicity, anemia, fever and nausea and infusion-related problems, causing the drug to acquire the nickname “Amphoterrible”. AmB is usually delivered intravenously, solubilized in sodium deoxycholate (Laniado-Laborin and Cabrales-Vargas, 2009). Incorporating the drug into more expensive liposomal and other lipid formulations has enabled less toxic delivery systems (Walsh et al., 1998). A recent study has found some AmB derivatives to be less toxic than AmB in a mouse model of systemic candidiasis (Davis et al., 2015). These derivatives are
promising as they show greater selectivity for ergosterol than cholesterol compared to AmB.

Resistance to polyenes can occur if the ergosterol content in the fungal plasma membrane is lowered (Young et al., 2003). Loss-of-function mutations in C. albicans C-5 sterol desaturase (Erg3p) lower ergosterol levels in the plasma membrane. These mutations also confer cross-resistance to azoles. A. terreus is intrinsically resistant to AmB, however the molecular mechanism responsible for this resistance is poorly understood. It was found that the ergosterol content in A. terreus was more variable than A. fumigatus and that A. terreus isolates with the lowest ergosterol content are least susceptible to AmB (Walsh et al., 2003). In addition, a recent study suggested that A. terreus is more resilient to oxidative damage as it has a higher catalase activity than susceptible strains (Blum et al., 2013), indicating that several factors may contribute to the resistance.

1.7.3 Allylamines

The allylamines, which include terbinafine and naftifine, are antimycotic drugs that inhibit squalene epoxidase (Erg1p; Figure 1.1). Squalene epoxidase catalyses the first step of ergosterol biosynthesis (Section 1.8). Allylamines are fungicidal against filamentous fungi but are fungistatic for most pathogenic yeast, including most Candida species (Kontoyiannis and Lewis, 2002). Mutations in ERG1 and efflux by ABC transporters give rise to allylamine resistance (Leber et al., 2003).

1.7.4 Echinocandins

Despite the discovery of echinocandins in the 1970s, these semisynthetic macrolide derivatives (Figure 1.1) were introduced into the clinic after 2000 and are the newest class of antifungals in clinical use (Richardson and Warnock, 2012). They inhibit the plasma membrane enzyme β-1,3-glucan synthase and thereby interfere with the biosynthesis of the essential cell wall component β-1,3-glucan. Echinocandins are fungicidal against most Candida species, fungistatic against A. fumigatus, but are not effective against C. neoformans (Richardson and Warnock, 2012). Current formulations are expensive and their limited oral bioavailability requires them to be injected. Resistance to echinocandins primarily arises from
mutations in the Fks1p subunit of β-1,3-glucan synthase complex (Park et al., 2005). For the moment there appears to be limited resistance to echinocandins, which may be due to the limited use of these more costly antifungals. More frequent use in the clinic as a prophylactic measure is expected to lead to a greater frequency of mutations that cause echinocandin resistance. Recent reports indicate frequencies of up to 13% echinocandin resistance among clinical isolates of C. glabrata (Alexander et al., 2013). Resistance can arise due to reversible physiological alterations that produce tolerance or via mutations (Walker et al., 2010). MDR does not confer resistance to echinocandins, as caspofungin and micofungin are not substrates of the drug efflux pumps that confer resistance to many azoles i.e. C. albicans Cdr1p, Cdr2p and Mdr1p (Niimi et al., 2006).

### 1.7.5 Azoles

The azoles are the largest class of antifungals used in the clinic as well as over-the-counter medications. They can be divided into two sub-classes: imidazoles and triazoles, based on the heterocycle that coordinates to the heme iron of their target – the cytochrome P450 lanosterol 14α-demethylase. The imidazoles, which contain an imidazole ring as the coordinating moiety, include KTC, miconazole and clotrimazole. The triazoles have a 1,2,4-triazole ring instead. They include FLC and VCZ, which have short tails, as well as PCZ and ITC, which have the long tails (Figure 1.2).
The azole drugs bind to and inhibit the function of the enzyme lanosterol 14α-demethylase (Erg11p) (Yoshida and Aoyama, 1987, Podust et al., 2001), a member of CYP51 family in the cytochrome P450 superfamily. This enzyme is the focus of this dissertation. It is denoted as CYP51 or Erg11p in fungal systems and is involved in the ergosterol biosynthesis pathway (Section 1.8). In mammals, the homologous enzyme is involved in the biosynthesis of cholesterol (Risley, 2002). In many fungal systems, inhibition of the 14α-demethylation of lanosterol by Erg11p depletes cell membranes of ergosterol, thus affecting the fluidity of the lipid bilayer and therefore fungal growth. Inhibition of Erg11p also leads to the accumulation of toxic metabolites of lanosterol e.g. fecosterol (Sanglard and Bille, 2002).

The active site of Erg11p contains an iron protoporphyrin moiety, to which a nitrogen atom in the imidazole or triazole ring can coordinate (Kontoyiannis and
Lewis, 2002). The imidazoles are usually applied as topical agents for mucosal or skin infections while the triazoles are used systemically. Triazoles have better pharmacokinetic properties than imidazoles and interact less with mammalian P450s.

The imidazole and triazole drugs are usually fungistatic rather than fungicidal for pathogenic fungi, including *C. albicans*. This occurs because the drug depletes ergosterol to a level that prevents fungal division and the cells enter a quiescent state (Dahl et al., 1987), with growth resuming once a “sparking” level of ergosterol is synthesised (Pinto and Nes, 1983). The fungistatic effect may also be due to drug tolerance (Sanglard et al., 2003) that results from the azole-induced expression of drug efflux pumps (Monk and Goffeau, 2008). A practical result of such effects is the “trailing growth” found in experiments that measure the minimum inhibitory concentration (MIC) of azole drugs in enriched synthetic medium such as YPD (Manavathu et al., 1998). As a consequence, the impact of azole drugs is normally measured as an MIC$_{80}$ i.e. the concentration of the drug that gives 80% inhibition of cell growth yield compared with the no drug control.

The first generation triazoles FLC and ITC were introduced in the clinic in the 1980s and subsequent generations of triazole drugs including VCZ and PCZ were introduced in the 1990s. Although FLC is not as potent as other azoles it has superior pharmacokinetic properties including a longer half-life and better bioavailability (Table 1.1). While ITC and VCZ are fungicidal against *A. fumigatus*, FLC is ineffective against this important fungal pathogen (Manavathu et al., 1998). The azole drugs were believed to enter fungal cells by passive diffusion but it was recently shown that they are taken up by facilitated diffusion into all four fungal species tested i.e. *S. cerevisiae*, *C. albicans*, *C. neoformans* and *C. kruzei* (Mansfield et al., 2010).

Resistance to azoles can arise in several ways and these mechanisms can occur in different combinations in different fungi. These mechanisms include mutations in Erg11p that reduce the affinity of azole binding, overexpression of Erg11p due to altered regulation or aneuploidy in *ERG11*, efflux via ABC and MFS transporters and tolerance to methylated sterols via mutations in sterol Δ5,6-desaturase (Erg3p; Parker et al., 2014). The loss-of-function mutations in *ERG3* allow for the
conversion of harmful fecosterols to better-tolerated products, as discussed in the next Section 1.8.

Table 1.1 Pharmacokinetic and pharmacodynamic properties of triazole drugs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ISA</th>
<th>FLC</th>
<th>VCZ</th>
<th>ITC</th>
<th>PCZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulations</td>
<td>Oral and IV</td>
<td>Oral and IV</td>
<td>Oral and IV</td>
<td>Oral*</td>
<td>Oral</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>Very high</td>
<td>≥90%</td>
<td>&gt;90%</td>
<td>30% capsules</td>
<td>Dose-dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50% solution</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-life</td>
<td>56 – 104 hours</td>
<td>24 – 30 hours</td>
<td>6 – 12 hours</td>
<td>24 – 30 hours</td>
<td>16 -35 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNS penetration</td>
<td>Low in CSF</td>
<td>High</td>
<td>High</td>
<td>Low (&lt;10%)</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>(&gt;60%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearance (L/hour)</td>
<td>Low (1.9 – 2.8)</td>
<td>Low (1.2)</td>
<td>High (8.4)</td>
<td>Very high (15.9)</td>
<td>Very high (21.7)</td>
</tr>
</tbody>
</table>

This table is modified from Falci and Pasqualotto (Falci and Pasqualotto, 2013). CNS is central nervous system, CSF is cerebrospinal fluid, IV is intravenous. *IV formulations of ITC are no longer widely available.

1.7.5.1 New triazoles in clinical trials

There are several triazole drugs that are currently in various stages of clinical evaluation. The ones of special note include VT-1161, isavuconazole (ISA), ravuconazole and albaconazole, all having tails of medium length (Figure 1.3) (Pasqualotto et al., 2010). VT-1161 has a tetrazole ring as a coordinating moiety and it was shown to have superior selectivity for the fungal CYP51 enzyme compared to the human CYP51 (Warrilow et al., 2014). VT-1161 is currently in stage II clinical trials (Hoekstra et al., 2014, Warrilow et al., 2014).

Ravuconazole is very similar to ISA and has the usual 2,4-difluoro phenyl substituent whereas ISA has a 2,5-difluoro phenyl moiety. ISA was approved in the US in March 2015 for the treatment of invasive aspergillosis (McCormack, 2015). This drug is currently in Phase III clinical trials for treatment of candidiasis and invasive fungal infections caused by rare moulds. Isavuconazonium is a water-soluble prodrug of ISA that is administered orally and as an intravenous formulation. Isavuconazonium is rapidly cleaved by plasma esterases yielding its active moiety ISA and an inactive product. ISA has favourable pharmacokinetic
properties compared to triazoles in current use (Table 1.1, Falci and Pasqualotto, 2013, Pettit and Carver, 2015). Given its pharmacokinetic properties and effectiveness against *Aspergillus spp.*, ISA may become the drug of choice for treating invasive aspergillosis.

Figure 1.3 Novel triazole drugs ravuconazole, albaconazole, isavuconazole and VT-1161.
1.8 Ergosterol biosynthesis pathway

Ergosterol, like its mammalian equivalent cholesterol, maintains the rigidity and permeability of the lipid bilayer. The ergosterol biosynthesis pathway (Figure 1.4) is targeted by azole and allylamine antifungals. The presence of ergosterol in yeast membranes instead of cholesterol provides the basis for the differential mechanism of action of the polyene antimycotic drugs.

Lanosterol is synthesised from farnesyl pyrophosphate by enzymes squalene synthase (Erg9p), squalene epoxidase (Erg1p) and lanosterol synthase (Erg7p). The allylamines such as naftifine and terbinafine target squalene epoxidase. Lanosterol 14α-demethylase (Erg11p) is thought to carry out the rate-limiting step in ergosterol biosynthesis and is targeted by theazole drugs. The enzymes sterol C-14 reductase (Erg24p), sterol C-4 methyl oxidase (Erg25p), sterol C-3 dehydrogenase (Erg26p) and sterol C-3 keto reductase (Erg27p) then convert demethylated lanosterol into zymosterol. The recent finding that zymosterol is the main sterol found in purified enzyme preparations of Erg11p (Monk et al., 2014b) may indicate that Erg11p has a central role in a multi-enzyme complex. Erg11p may present its product 4-demethylcholesta-8,14,12-trienol so that subsequent enzymes in the ergosterol biosynthesis pathway can modify it to zymosterol. Erg24p is a membrane–associated protein that reduces the double bond in 4-demethylcholesta-8,14,12-trienol to give 14-dimethyl zymosterol. The occurrence of physical interactions between Erg11p with Erg25p and Erg27p have been demonstrated (Mo and Bard, 2005). Erg25p and Erg26p are known to form a complex that uses Erg27p as a scaffold. The zymosterol is then esterified with unsaturated fatty acid for storage in lipid droplets and transits to the plasma membrane as required (Parks and Casey, 1995).

Sterol C-8 isomerase (Erg2p) converts fecosterol to episterol. C-5 desaturase (Erg3p) then converts episterol to ergostatrienol by means of introducing a C-5-6 double bond. Erg3p normally acts downstream of lanosterol 14α-demethylase (Erg11p) but can also desaturate lanosterol directly in a pathway that leads to toxic fecosterols. The mutations which inactivate Erg3p reduce the buildup of these toxic sterols, with ergosta-7,22-dienol-3β-ol being a main sterol found in resistant strains (Nolte et al., 1997).
Figure 1.4 Ergosterol biosynthesis pathway. The principal sites of inhibition of this pathway by the allylamine andazole drugs are shown.
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1.9 Cytochrome P450 enzymes

The cytochrome P450 (CYP, P450) enzymes catalyse the oxidation of exogenous and endogenous substrates. This heme-thiolate-containing superfamily has diverse roles including drug metabolism and bioactivation, biosynthesis of antibiotics, human steroid metabolism and xenobiotic detoxification. They are found in almost all organisms with some important exceptions, for example, the Archaea kingdom of unicellular organisms and many bacteria including Escherichia coli, Rhodopseudomonas spp. and Chromatium spp. (Volkman, 2003). In humans the P450s are responsible for metabolising about 75% of all marketed drugs with CYP3A4 alone modifying around half of those drugs (Guengerich, 2008). Prokaryotic P450s are soluble cytoplasmic proteins, whereas the eukaryotic enzymes, apart from mitochondrial P450s, have a single transmembrane helix, which tethers it to the lipid bilayer (Johnson and Stout, 2013).

Based on function, the P450s can be broadly divided into two groups. One group is involved in the xenobiotic metabolism and the other takes part in biosynthetic pathways. The first group of P450 families are flexible enzymes, which allow for the binding of various structurally dissimilar substrates. Six substrate recognition sites (SRS) have been identified as parts of the enzyme thought to be involved in binding and recognition of substrates (Gotoh, 1992). The SRSs have considerable sequence variability, which posed difficulties in their original identification. Mutating important residues within the SRS was found to change the substrate specificity of the enzyme (Matsunaga et al., 1990, Lindberg and Negishi, 1989).

The second group of P450s have a much smaller range of substrates and are less able to tolerate mutations. While still maintaining the P450 fold, their substrate channels are able to accommodate particular substrates. Sterol 14α-demethylase (CYP51) is regarded as the most ancient family among the P450s (Aoyama et al., 1996). CYP51s carry out essentially the same role across the kingdoms and have an amino acid sequence identity of at least 28% between the mammalian and fungal enzymes (Lepesheva and Waterman, 2007).
1.9.1 Catalytic cycle of P450s

The cytochrome P450 enzymes contain a protoporphyrin IX ring with iron (III) coordinating to the sulfur atom of a cysteine residue at the active site. The P450s are monooxygenases that use activated molecular oxygen to add one oxygen atom to the substrate and reduce the other to water. This reaction requires two electrons which come from NAD(P)H via its cognate reductase enzyme. Initially this superfamily of enzymes was identified in rat liver microsomes (Klingenberg, 1958) by their unique absorption at 450 nm in a reduced state when CO is bound to the heme iron. However, under the same conditions, an absorbance maximum at 420 nm indicates that the P450 enzyme is no longer active (Omura and Sato, 1967). The CO assay is a useful tool for measuring the quantity of functional enzyme in a preparation (Omura and Sato, 1964b).

In its resting state the heme iron is usually in an oxidised ferric form Fe$^{3+}$ with a water molecule bound as its sixth axial ligand (Figure 1.5, step 1). This results in a low spin state for the iron and gives the heme an absorption peak of around 420 nm. When the catalytic cycle begins the axial water is displaced by a substrate (type I ligand), the heme iron goes into a high spin state but it remains in its ferric oxidation state (Figure 1.5, step 2). It is noteworthy that nitrogen heterocycles such as azoles (type II ligands) replace the water in the active site further stabilising the low spin form of the iron.
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Figure 1.5 The catalytic cycle of cytochrome P450. Figure modified from Ener et al. (Ener et al., 2010). Copyright 2015, Proceedings of the National Academy of Sciences of the United States of America.

Delivery of the first electron from the cognate reductase changes the heme iron from the ferric (Fe$^{3+}$) to the ferrous (Fe$^{2+}$) state (Figure 1.5, step 3). This results in the binding of the molecular oxygen via a coordinate bond to form a ferric superoxide complex (Figure 1.5, step 4). Following the second electron transfer, the distal oxygen is protonated and forms a ferric hydroperoxo complex (Figure 1.5, step 5). A second protonation results in the formation of a water molecule and a very reactive iron (IV)-oxo radical complex. This complex has recently been captured using a CYP119 from Sulfolobus acidocaldarius and characterised spectroscopically and kinetically (Rittle and Green, 2010). This highly reactive species extracts the hydrogen from the substrate (Figure 1.5, step 7) forming a substrate radical, which quickly reacts with the protonated iron (IV) species to generate the ferric enzyme and the hydroxylated product. The findings of Rittle et al. (2010) support this mechanism of action as the kinetic analysis shows that the rate at which the iron (IV) complex oxidizes unactivated hydrocarbons has an incredibly fast second-order rate constant of $k_{app} = 1.1 \times 10^{7}$ M$^{-1}$s$^{-1}$ (Rittle and Green, 2010).

CYP51s convert lanosterol to 4,4-dimethyl-5a-cholesta-8,24-dien-3-β-ol via three consecutive mono-oxygenase reaction cycles as described above. The first step
converts the 14α-methyl group to an alcohol, the second step converts it to an aldehyde and in the final step formic acid is removed (Fischer et al., 1991).

1.9.2 P450 structural fold

The first X-ray structure for a P450 was obtained for camphor hydroxylase, a cytochrome P450\textsubscript{cam} of the CYP101 family that was isolated from the soil bacterium \textit{Pseudomonas putida} (Poulos et al., 1986). Structural fold terminology for P450s originated from the structure of this enzyme (Figure 1.6) (Poulos et al., 1987). Even though the overall structural fold of the P450 catalytic domain is conserved between species, the membrane association of eukaryotic non-mitochondrial P450 proteins has presented a challenge in their purification and structural resolution.

In the P450\textsubscript{cam} structure, the sulfur atom of cysteine 357 is bound to the heme iron. The active site is located on the opposite side of the heme. One edge of the heme is wedged in between helix I and helix L. Residues from helices I, B’, the B’-C loop, β3 sheet and β5 sheet contribute to the substrate channel. Helices F and G together with the F-G loop form a lid that closes off the active site (Figure 1.6).
Figure 1.6 Structure of cytochrome P450$_{\text{cam}}$ (PDB entry: 2CPP). The overall structural fold of cytochrome P450 catalytic domain contains α-helices labelled A-L (for clarity helix J is not labelled - it is located between helix I and helix K) and β-sheets labelled 1-5 according to Poulos (Poulos et al., 1987). The heme moiety borders the active site. The camphor molecule is at the active site in the substrate channel (magenta).

Helix I has a characteristic “kink” in most P450 enzymes. In cytochrome P450$_{\text{cam}}$ of P. putida this “kink” is thought to be due to the disruption of normal α-helix hydrogen bonding pattern in helix I and establishment of a hydrogen bond between the side chain of the highly conserved T252 and the carbonyl group of G248. This arrangement has also been proposed to accommodate the bound dioxygen (Poulos, 2007).

1.9.3 P450s in *Saccharomyces cerevisiae*

There are three cytochrome P450 enzymes in *S. cerevisiae*. Two are involved in ergosterol biosynthesis and the third is cytochrome P450 DIT2, an enzyme that is involved in spore wall development (Briza et al., 1994). Erg11p or CYP51 belongs to a family of CYP51 lanosterol 14α-demethylases and is the main target of the azole drugs. CYP51 knock out strains of the yeast *S. cerevisiae* are lethal. Erg11p
converts lanosterol to its demethylated product as described in Section 1.9.1. C22-desaturase (Erg5p) belongs to a family of CYP61 enzymes and was discovered during the yeast genome project. The ERG5 gene was found to be non-essential in yeast (Skaggs et al., 1996). The azole drugs inhibit Erg5p as well as Erg11p but this C22-desaturase occurs much later in the ergosterol biosynthetic pathway (Figure 1.4; Kelly et al., 1997). With inhibition of Erg11p the subsequent reactions in the pathway are blocked, implying that the effect of azoles on Erg5p is unlikely to be of importance.

1.10 Structural information on fungal CYP51s

In general, the eukaryotic non-mitochondrial CYP51s are thought to have an amphipathic helix (MH1) of variable length followed by a transmembrane helix (TMH1). A series of α-helices (A-L), β-sheets (1-4) and the heme in the active site contribute to the catalytic domain with a common P450 fold. No crystal structures of fungal CYP51s were reported before 2014. Furthermore, the structures of other eukaryotic CYP51s were incomplete because the constructs used were modified, with the transmembrane helix deleted, to assist purification and crystallisation (Chen et al., 2010, Strushkevich et al., 2010). It is of considerable interest to determine whether the truncated enzyme behaves in the same manner as the full-length protein.

1.10.1 In silico models of fungal CYP51s

Some fungal CYP51 structures obtained by homology modelling used published cytochrome P450 X-ray structures as templates. Earlier models are based on P450 structures such as P450_1cam from Pseudomonas putida (Boscott and Grant, 1994, Morris and Richards, 1991) and P450BM3 from Bacillus megaterium (Ji et al., 2000, Holtje and Fattorusso, 1998). The release of M. tuberculosis CYP51 structure allowed for the use of this better suited structure for homology modelling as M. tuberculosis CYP51 has a sequence identity of 26-28% with fungal CYP51s (Podust et al., 2001; Figure 1.7). This led to several models of C. albicans CYP51 (Xiao et al., 2004, Sheng et al., 2004). In silico models for A. fumigatus CYP51A (Xiao et al., 2004, Sheng et al., 2004) and C. neoformans CYP51 (Sheng et al., 2009) were also constructed based on the CYP51 structure of M. tuberculosis. The
structural modelling and docking approaches have been used to investigate the interactions between the enzyme and the azole drugs, with a further aim of designing better antifungals.

Figure 1.7 ClustalW (1.2.1) multiple sequence alignment of the primary sequences of fungal, mycobacterial and human lanosterol 14\(\alpha\)-demethylases. *Saccharomyces cerevisiae* CYP51 (ScErg11), *Candida albicans* CYP51 (CaErg11), *Candida glabrata* CYP51 (CgErg11p), *Aspergillus fumigatus* CYP51A (AfErg11A), *Homo sapiens* CYP51 (HsCYP51) and *Mycobacterium tuberculosis* CYP51 (MtErg11). An asterisk signifies conserved residues, a colon indicates conservation of residues with similar properties and a period indicates conservation of residues with less similar properties. Highlighted residues are frequently found mutated to reduce drug binding in pathogenic fungi. The cysteine residue that coordinates to the heme iron is marked with a triangle. Secondary structure amphipathic helix (MH1), transmembrane helix (TMH1), \(\alpha\)-helices (A-L), most \(\beta\)-sheets and the fungus-specific loop (FSL) are marked. Note: Helix C is shorter in *M. tuberculosis* CYP51. For clarity some \(\beta\)-sheets are not indicated. The figure is on the next page.
Figure legend is on the previous page
1.10.2 X-ray crystal structures of fungal CYP51s

In 2014 a series of X-ray structures of a full-length *S. cerevisiae* CYP51 were published (Monk et al., 2014b). These structures allowed the identification of the orientation of the enzyme in relation to the lipid bilayer. These structures and companion biochemical studies provided evidence that the transmembrane helix helps position the catalytic domain partly in the lipid bilayer (Figure 1.8). The dataset refinement statistics from this study are listed in Appendix G, Table G-2. Two of those structures were released to the Protein Data Bank (PDB) - ScErg11p with ITC in the active site (PDB ID: 4K0F/5EQB) and with lanosterol in the active site (PDB ID 4LXJ). In 2015, an *A. fumigatus* CYP51B structure was published in complex with VCZ (PDB ID: 4UYM) and VNI (PDB ID: 4UYL, VNI has an imidazole group as a coordinating moiety) (Hargrove et al., 2015). The *A. fumigatus* CYP51B structure has the highly conserved CYP450 fold. However, the structure was truncated, with TMH1 and MH1 removed, in order to facilitate purification and crystallisation.

![Figure 1.8](image)

**Figure 1.8** The overall fold of *S. cerevisiae* Erg11p with its proposed orientation in the lipid bilayer (PDB ID: 4ZDY).

To date (September 2015) there are 49 published structures of CYP51s complexed with various ligands lodged to the PDB ranging in resolution from 1.5 Å to 3.12 Å. Of these structures, 19 are of *Trypanosoma cruzi* CYP51, 9 of *Trypanosoma brucei*...
CYP51, 3 of *Homo sapiens* CYP51, 1 of *Leishmania infantum* CYP51, 12 of *M. tuberculosis* CYP51, 2 of *A. fumigatus* CYP51 and 3 of *S. cerevisiae* CYP51 (including 1 from this thesis).

1.11 CYP51s as drug targets

Organisms that rely on sterol production and cannot obtain compatible sterols from the host organism are good candidates for targeting CYP51. The CYP51 family of enzymes are a major drug target in fungi and are also under extensive development as drug targets for tropical diseases. The CYP51 of *T. cruzi* has been shown to be a valid target for treatment of Chagas disease (Lepesheva et al., 2008). Clinical trials revealed that PCZ has antityranosomal activity in the treatment of chronic Chagas disease (Molina et al., 2014). Targeting CYP51 is also effective in some protozoan *Leishmania* species (de Macedo-Silva et al., 2013, Shakya et al., 2011). CYP51 is also a potential target in the disease-causing, free-living amoebae *Acanthamoeba* and *Naegleria* that are also dependent on sterol biosynthesis (Choi et al., 2014). In contrast in *T. brucei*, the cause of African sleeping sickness, CYP51 is a poor target because the pathogen obtains cholesterol from the host by receptor mediated endocytosis.

Sterol 14α-demethylases have five known substrates: lanosterol, 24,25-dihydrolanosterol, 29-norlanosterol, 24-methylene-dihydrolanosterol (eburicol), and 24-methylene-dihydro-29-norlanosterol (obtusifoliol; Figure 1.9). Sterol biosynthesis in fungi and protozoa are strain and species specific, for example fungal CYP51s use lanosterol but *A. fumigatus* and some filamentous phytopathogens catalyse the conversion of eburicol to its demethylated product (van Nistelrooy et al., 1996, Price et al., 2015b). Vertebrates produce cholesterol in a pathway similar to fungi in which CYP51 14-demethylates lanosterol, as discussed in detail by Risley (Risley, 2002). Mammalian and yeast CYP51s demethylate both lanosterol and 24,25-dihydrolanosterol (Lepesheva and Waterman, 2007). Plants produce phytosterols via the isoprenoid pathway. This results in obtusifoliol that is demethylated by the CYP51 enzyme obtusifoliol 14α-demethylase (Piironen et al., 2000).
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Figure 1.9 CYP51 substrates. Lanosterol, 24-methylenedihydrolanosterol (eburicol), 24,25-dihydrolanosterol, 24-methylenedihydro-29-norlanosterol (obtusifoliol) and 29-norlanosterol. * denotes carbon 14 in lanosterol.

1.12 Resistant Erg11p mutants

Fungal infections are becoming a significant burden on healthcare systems worldwide (Diekema et al., 2012, Pfaller and Diekema, 2010, Pfaller et al., 2010). Challenges in the treatment of fungal infections currently lie with the limited classes and numbers of effective drugs and the increasing incidence of resistant isolates. The limited number of effective drugs results from high diversity of fungal organisms that limit the options for broad-spectrum antimycotic drugs as discussed in Section 1.5. Prophylactic use of fungistatic azoles in susceptible patients and prolonged administration of those treatments has contributed to the emergence of resistant fungal pathogens (Perlin, 2009). The use ofazole fungicides in agriculture can also lead to development of cross-resistance to medical triazoles e.g. in A.
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*fumigatus* and *C. glabrata* (Snelders et al., 2012, Faria-Ramos et al., 2014). Both studies were able to induce resistance in susceptible strains using azole-based fungicides in laboratory conditions and confirmed that those strains have cross-resistance to medical triazoles. Moreover, the resistant laboratory strains evolved similar mechanisms of resistance to those commonly found in the environmental and clinical isolates.

Fungal CYP51 is a target for the azole drugs and one of the ways resistance to azoles arises is due to the mutations in CYP51. Many of these mutations reduce the affinity of the drug for its target, rendering the drug less effective. There is a need to design better drugs to combat fungal infections. In order to overcome antifungal resistance, an in depth understanding of how the mutations affect the interactions between CYP51 and the azole drugs is needed. The list of mutations discussed in this thesis are summarised in Table 1.2. A more detailed description of these mutations is covered in the introduction to Chapters 4 and 5.
Table 1.2 S. cerevisiae Erg11p amino acid substitutions equivalent to mutations in clinical isolates of the pathogenic fungi Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus and Histoplasma capsulatum

<table>
<thead>
<tr>
<th>Phenotypes of resistant clinical isolates</th>
<th>Mutations in CYP51 from clinical isolates of resistant strains</th>
<th>Equivalent positions in ScErg11p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 4-fold increase in resistance to fluconazole</td>
<td>CaCYP51 Y132F/H CnCYP51 Y145F HcCYP51 Y136F</td>
<td>ScErg11p Y140F/H</td>
</tr>
<tr>
<td>Increased resistance to itraconazole and posaconazole</td>
<td>AfCYP51A G54E/W/R</td>
<td>ScErg11p G73E/W/R</td>
</tr>
<tr>
<td>Intrinsic resistance to fluconazole</td>
<td>AfCYP51A I302</td>
<td>ScErg11p T322I</td>
</tr>
<tr>
<td>Increased resistance to fluconazole and voriconazole</td>
<td>CaCYP51 K143R</td>
<td>ScErg11p K151R</td>
</tr>
<tr>
<td>Increased resistance to fluconazole and ketoconazole</td>
<td>CaCYP51 G464S AfCYP51A G448S</td>
<td>ScErg11p G464S</td>
</tr>
</tbody>
</table>

CaCYP51 = Candida albicans CYP51; CnCYP51 = Cryptococcus neoformans CYP51; HcCYP51 = Histoplasma capsulatum CYP51; ScErg11p = Saccharomyces cerevisiae CYP51; AfCYP51 = Aspergillus fumigatus CYP51

1.13 S. cerevisiae as a model organism

S. cerevisiae or Baker’s yeast provides a robust eukaryotic model that has been widely used in many scientific fields, including metabolic analysis, drug discovery and biotechnology. The genome sequence of this organism was released in 1996 (Goffeau et al., 1996). The many molecular biology-based applications of S. cerevisiae include gene-cloning, genetic engineering, heterologous protein expression for laboratory and commercial purposes as well as in screens that detect protein-protein interactions. Several factors have encouraged the use of this simple unicellular eukaryote. For example, it is readily grown on a defined media, it has a well-understood life cycle, its genome sequence is known and many of its post-translational modifications are characteristic of eukaryotes.
1.13.1 Homologous recombination

Homologous recombination is an invaluable attribute of *S. cerevisiae*. This trait allows for targeted gene replacement, where a DNA construct contains a gene of interest, a selectable marker and matching sequences to the upstream and downstream of the target sequence in the genome. The ends of the designed DNA fragment interact with the homologous regions in the genome, replacing the intervening chromosomal sequence (Figure 1.10; Langston and Symington, 2005). *S. cerevisiae* can be transformed with foreign DNA in the form of a plasmid or with linearised DNA to disrupt or replace defined genomic sequences.

**Figure 1.10 Schematic representation of homologous recombination.** Upstream region is denoted as US, downstream as DS and gene of interest as GOI.

1.13.2 The Pleiotropic Drug Resistance Network

In yeast, MDR is referred to as pleiotropic drug resistance (PDR). Aspects of PDR in the model yeast *S. cerevisiae* were described at the molecular level more than three decades ago. The phenomenon of PDR is thought to be ancient in origin and to have evolved long before the modern antifungal drugs were applied in the clinic (Gbelska et al., 2006). In PDR, transcriptional regulators play a crucial role by acting as sensors of chemically unrelated foreign toxins - xenobiotics (Kolaczkowska and Goffeau, 1999). Currently, about 10 transcriptional regulators are thought to be associated with the PDR network and these target about 70 different genes (Kolaczkowska and Goffeau, 1999). Pdr1p is a key transcriptional regulator involved in PDR and appears to have the most target genes (Balzi and Goffeau, 1995). Pdr1p is a promoter resident regulator, which means it is
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constitutively bound to its promoters. This allows for the basal transcription of the PDR genes as well as the induction of early drug-dependent responses (Fardeau et al., 2007).

The early drug-dependent responses are key for the development of genetic modifications, which lead to PDR. The main mechanisms by which the PDR network confers drug resistance involve the overexpression of ABC and MFS transporters. The ABC transporters in the *S. cerevisiae* PDR network include Pdr5p, Snq2p, Pdr10p, Pdr15p and Ycf1p reviewed by Prasad and Goffeau (Prasad and Goffeau, 2012) and the MFS transporters include Tpo1p and Flr1p reviewed by Dos Santos et al. (Dos Santos et al., 2014).

1.13.3 *Pdr1*-3 mutation

Certain mutations in *PDR1* confer high levels of resistance by causing constitutive overexpression of target genes. The *pdr1*-3 mutation in *PDR1* is the F815S gain-of-function mutation in Pdr1p (Carvajal et al., 1997). This mutation occurs in a negative regulatory domain of the protein and enables functional hyper-expression of efflux pumps Pdr5p, Snq2p, Yor1p, Pdr10p and Pdr15p (Carvajal et al., 1997, Decottignies et al., 1998). Pdr5p is the most prominent and extensively studied ABC transporter in *S. cerevisiae* that is regulated by Pdr1p (Balzi et al., 1994).

1.13.4 A membrane protein overexpression system

*S. cerevisiae* was used to develop a system for heterologous expression of membrane and soluble proteins. This system was initially established by Decottignies et al. (Decottignies et al., 1998) to study the yeast ABC transporters Pdr5p and Yor1p and has been further developed and utilised for characterisation of the membrane proteins involved in drug resistance (Nakamura et al., 2001, Lamping et al., 2007, Monk et al., 2014a). The *S. cerevisiae* host strain ADΔ has seven ABC transporter genes, chromosomal uracil selection marker (*URA3*) and the *PDR3* transcriptional regulator genes deleted. The *pdr1*-3 gain-of-function mutation is used to give constitutive overexpression from the *PDR5* locus. The ADΔ host strain is essentially identical to the AD1-8u strain but the *URA3* open reading frame has been completely deleted rather than containing an inactivating single nucleotide
mutation. The AD2Δ was developed later and is identical to ADΔ, but with the histidine marker (HIS1) deleted. The AD1-8u, ADΔ and AD2Δ host strains have been used to overexpress proteins of interest from the PDR5 locus. The transformation cassette contains the PDR5 promoter, the gene of interest with affinity tag such as a hexaHistidine(His)-tag, a 3-phosphoglycerate kinase (PGK) terminator and the URA3 selection marker plus PDR5 downstream sequence (Figure 1.11; Lamping et al., 2007).

**Figure 1.11 Membrane protein hyper-expression system.** A *S. cerevisiae* strain deficient in 7 ABC transporters and the PDR3 transcriptional regulator is used to overexpress ScErg11p from the PDR5 locus. The transformation cassette contains the PDR5 promoter, ScERG11 with a C-terminal His-tag, a 3-phosphoglycerate kinase (PGK) terminator, the URA3 selection marker and a PDR5 downstream sequence. Modified from (Lamping et al., 2007).

### 1.14 Aims of this project

The emergence of resistant fungal pathogens is a threat that calls for the identification of more effective drugs that can overcome resistance. Mutations in fungal Erg11p/CYP51s play an important role in the development of resistance to the triazole drugs. Understanding how these mutations affect the drug binding at the structural level is expected to give insight that will aid the design of more effective drugs.
At the commencement of this project (June 2012), the ability to overexpress, purify, crystallise and resolve the structure of *S. cerevisiae* Erg11p, as subsequently published in Monk et al., 2014 (Monk et al., 2014b), had been achieved. Important limitations of this work were that while structures with the substrate lanosterol and the long-tailed triazole inhibitor had been obtained at high resolutions (1.95-2.1 Å), the co-crystal structures obtained in complex with FLC and VCZ were at much lower resolutions (>2.6 Å). The low-resolution structures were of insufficient resolution and quality to detect important features in the active site that might influence the affinity of drug binding.

The first aim of this project was to obtain a high-resolution structure of ScErg11p in complex with FLC that would reveal features such as the presence of water-mediated hydrogen bond networks.

The first part of the research predicted how some mutations might impact antifungal resistance to short- and long-tailed triazoles.

The second aim of the project was to investigate the effect of CYP51 mutations on enzyme structure and function by using *S. cerevisiae* Erg11p as a model for pathogenic yeast. The Erg11p Y132F/H, K143R and G464S mutations of *C. albicans* and the CYP51A G54E/R/W mutations of *A. fumigatus* are the focus of this thesis (Table 1.2). In addition, the innate resistance of *A. fumigatus* CYP51A to FLC was investigated using the *S. cerevisiae* Erg11p T322I mutant.
Chapter 2

Materials and Methods
2.1 Yeast strains

The *S. cerevisiae* strain AD2Δ was used as the host to engineer strains hyper-expressing wild type and mutant ScErg11p. The strains generated and/or used in this study are listed in Appendix A.1 (Table A-1).

2.2 Chemicals

The triazole drugs FLC, ITC, VCZ, PCZ along with polyethylene glycol-400 (PEG-400), imidazole, L-histidine, luminol, p-coumaric acid and sodium dithionite were purchased from Sigma-Aldrich Ltd (St. Louis, MO, USA). Glycerol was purchased from Merck (Summit, NJ, USA). Acrylamide/bis-acrylamide (40%; 19:1) solution, ammonium persulfate (APS) and *N*,*N*,*N',*N'-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Hercules, CA, USA). Phenylmethanesulfonyl fluoride (PMSF) and Roche EDTA-free protease inhibitor pills were obtained from Roche Diagnostics (Basel, Switzerland). The detergent *n*-decyl-β-D-maltoside (DM) was purchased from Affymetrix Inc. (Santa Clara, CA, USA).

2.3 Culture media and growth conditions

The culture media used in the present study are shown in Table 2.1. Yeast strains were maintained on yeast peptone dextrose (YPD) medium solidified with 2% agar and cultured in liquid YPD medium (Table 2.1). Synthetic defined (SD) dropout medium was used for selection of transformants. SD media with complete supplement mixture was used for minimum inhibitory concentration (MIC) assays of drug efficacy with yeast strains (see below). Culture media were prepared using deionised distilled water (ddH₂O). YPD medium was autoclaved at 121 °C for 15 min and SD medium was autoclaved at 117 °C for 15 min.
## Table 2.1 Culture medium composition

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Component (wt/vol)</th>
<th>Supplier*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>YPD medium</strong></td>
<td>1% bacto-yeast extract</td>
<td>BD Difco™</td>
</tr>
<tr>
<td></td>
<td>2% bacto-peptone</td>
<td>BD Difco™</td>
</tr>
<tr>
<td></td>
<td>2% glucose</td>
<td>Merck</td>
</tr>
<tr>
<td></td>
<td>2% agar (with or without)</td>
<td>Oxoid Ltd</td>
</tr>
<tr>
<td><strong>SD uracil dropout</strong></td>
<td>0.67% yeast nitrogen base (without amino acids)</td>
<td>BD Difco™</td>
</tr>
<tr>
<td>medium</td>
<td>0.077% complete supplement mixture (uracil dropout)</td>
<td>Formedium™</td>
</tr>
<tr>
<td></td>
<td>2% agar</td>
<td>Oxoid Ltd</td>
</tr>
<tr>
<td></td>
<td>2% glucose</td>
<td>Merck</td>
</tr>
<tr>
<td><strong>SD histidine</strong></td>
<td>0.67% yeast nitrogen base (without amino acids)</td>
<td>BD Difco™</td>
</tr>
<tr>
<td>dropout medium</td>
<td>0.077% complete supplement mixture (histidine dropout)</td>
<td>Formedium™</td>
</tr>
<tr>
<td></td>
<td>2% agar</td>
<td>Oxoid Ltd</td>
</tr>
<tr>
<td></td>
<td>2% glucose</td>
<td>Merck</td>
</tr>
<tr>
<td><strong>Buffered SD</strong></td>
<td>0.67% yeast nitrogen base (without amino acids)</td>
<td>BD Difco™</td>
</tr>
<tr>
<td>medium (pH 6.8)</td>
<td>0.079% complete supplement mixture</td>
<td>Formedium™</td>
</tr>
<tr>
<td></td>
<td>2% glucose</td>
<td>Merck</td>
</tr>
<tr>
<td></td>
<td>10 mM MES and 20 mM HEPES buffered with Tris</td>
<td>Sigma-Aldrich Ltd</td>
</tr>
</tbody>
</table>

*The addresses of suppliers are as follows: BD Difco™ Laboratories Inc (Franklin Lakes, NJ, USA), Formedium™ (Norfolk, UK), Oxoid Ltd (Hampshire, UK).
2.4 Cloning of ScErg11p6×His

2.4.1 Oligonucleotides

Desalted oligonucleotides purchased from Sigma-Aldrich Ltd were prepared as 100 µM stocks in elution buffer (EB, 10 mM Tris-HCl pH 8.5) from the QIAquick PCR purification kit (Qiagen Ltd, Limburg, Netherlands). Stock solutions were diluted in molecular biology grade RNase and DNase free H₂O (5 PRIME Inc., Gaithersburg, MD, USA) to make 3.2 µM working solutions. Stock and working solutions were stored at -20 °C.

2.4.2 Agarose gel electrophoresis

DNA fragments were separated according to size, using agarose gel electrophoresis. DNA (10×) gel loading dye (Appendix A.2.2) was mixed with samples prior to loading onto the agarose gel (Appendix A.2.5). A 1 kb Plus DNA molecular ladder (Invitrogen, Carlsbad, CA, USA; Appendix A.2.4) was used to estimate the sizes of DNA fragments. The gel consisted of solidified 0.8% (wt/v) agarose (Invitrogen) containing Tris-acetate EDTA (TAE) running buffer (Appendix A.2.3). The gel was subject to electrophoresis at 100 V for 60 min. Ethidium bromide (3-5 µL of 2 mg/mL) was added to the gel and to the TAE running buffer in the tank to enable visualisation of DNA bands under UV illumination after electrophoresis.

2.4.3 Polymerase Chain Reaction

All polymerase chain reactions (PCRs), except colony PCR, were carried out using KOD Hot Start DNA polymerase (Novagen, Madison, WI, USA). Molecular biology grade RNase and DNase free H₂O (5 PRIME Inc.) was used in all molecular biology manipulations and PCR reactions. Each PCR reaction contained 5 µL of 10× reaction buffer, 5 µL of 2 mM dNTPs, 2.4 µL of 25 mM MgSO₄, 5 µL of each oligonucleotide primer (3.2 µM), 1 µL of KOD DNA polymerase (1 U/µL) and 10 – 60 ng of template DNA in a total volume of
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50 μL. The thermocycling conditions are described in Table 2.2. All PCR reactions were carried out using the DNA Engine® Thermal Cycler (Bio-Rad).

2.4.4 Recombinant PCR

Recombinant PCR was used to fuse together 2 or 3 fragments of DNA in order to prepare transformation cassettes. The reaction conditions are as described in Section 2.4.3 and the thermal cycling conditions are as indicated in Table 2.2, but with the number of cycles reduced to 20. Two-fragment recombinant PCR used equimolar amounts of template. Three-fragment recombinant PCR used equimolar amounts of the bordering fragments and a 4-fold greater amount of the central fragment.

Table 2.2 PCR Thermocycling conditions

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>KOD</th>
<th>ExTaq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Temperature</td>
<td>Duration</td>
</tr>
<tr>
<td>1</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>2</td>
<td>95 °C</td>
<td>20 sec</td>
</tr>
<tr>
<td>3</td>
<td>55 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>4</td>
<td>68 °C</td>
<td>1 min / 1 kb</td>
</tr>
<tr>
<td>Steps 2 – 4 repeated for a total of 20* or 35 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>68 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>6</td>
<td>4 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

*20 cycles was used for recombinant PCR reactions.

2.4.5 Colony PCR

Colony PCR was carried out using TaKaRa ExTaq DNA polymerase (Takara Bio Inc., Shiga, Japan). Each PCR reaction contained 2 μL of 10× buffer, 1.8 μL of 2.5 mM dNTPs, 2 μL of each oligonucleotide primer (3.2 μM), 0.15 μL of DNA polymerase (5 U/μL) and 1 – 2 μL of yeast suspension as DNA template in a total volume of 20 μL. Template DNA was obtained directly from yeast.
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colonies. A yeast suspension was prepared as follows: sufficient cells to fit on the tip of a toothpick were obtained from a yeast colony, resuspended in 10 µL of Milli-Q H₂O (Appendix A.2.1) and heat treated at 95 °C for 5 min to release the DNA. The thermocycling conditions used for colony PCR are described in Table 2.2.

2.4.6 PCR purification and DNA gel extraction

QIAquick® PCR clean up and DNA gel extraction kits (Qiagen Ltd) or the NucleoSpin® PCR and Gel clean up kit (Macherey-Nagel, Düren, Germany) were used to purify PCR products and DNA fragments. The DNA purifications were carried out according to each manufacturer’s protocol.

2.4.7 Preparation of competent yeast cells

Competent *S. cerevisiae* cells were prepared fresh for each transformation. Preparation of competent yeast cells and their transformation was carried out using an Alkali-Cation™ Yeast Transformation kit from Qbiogene (Irvine, CA, USA). A 10 mL overnight (o/n) culture was grown in YPD from a single yeast colony. A flask containing 250 mL of YPAD medium (YPD with 30 mg/L of adenine sulphate added prior to autoclaving) was inoculated with an aliquot of the overnight culture to OD₆₀₀nm = 0.1. The cells were cultured for 5 – 6 hours to OD₆₀₀nm = 0.4 – 0.6 and harvested by centrifugation at 3820 g (5000 rpm) for 5 min in a Sorvall® RC6 centrifuge using a FiberLite™ F14-6 rotor. The cell pellet was washed in 10 mL of TE buffer and pelleted again at 3820 g for 5 min. The pellet was resuspended in 5 mL of lithium/cesium acetate solution, incubated for 30 min at 30 °C with shaking at 100 rpm, centrifuged at 3820 g for 5 min and resuspended in 1 mL of TE buffer. The resultant competent cells were used the same day for transformation.

2.4.8 Yeast transformation

Competent *S. cerevisiae* cells (100 µL) were mixed with 5 µL of carrier DNA, 5 µL of histamine solution and 800 – 1000 ng of DNA transformation cassette in a total volume of 10 µL. Salmon sperm carrier DNA (10 mg/mL) was heat
denatured for 3 min at 94 °C and immediately placed on ice. The mixture was incubated at room temperature for 15 min with gentle mixing. PEG (0.8 mL) was added to 0.2 mL of TE/Cation MIXX and mixed with the cell suspension. The solution was incubated at 30 °C for 10 min, subject to heat shock at 42 °C for 10 min in a water bath and the cells pelleted at 11,000 g for 10 sec in a microcentrifuge. The supernatant was discarded, the pellet of cells resuspended in 200 μL of SOS media (sterile 1 M Sorbitol, 0.3× YPD and 10 mM CaCl₂) and spread on agar plates containing SD dropout medium (Table 2.1). The plates were incubated for 2 – 3 days at 30 °C. Colony PCR was used to identify transformants containing DNA inserts of desired size and at the correct chromosomal location. Yeast genomic DNA was isolated using Y-DER kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer’s instructions. Cassette sequencing of genomic DNA (see below) was used to confirm insertion of the open reading frame at the expected location and in the correct orientation.

2.4.9 DNA sequence analysis

The *ScERG11* DNA cassettes were amplified using primers PDR5_US and PDR5_288_DS_Rev (Appendix B.1, Table B-1). The *Hisl* disruption cassette was amplified using primers ScErg11_US_801 and ScEr11_ter_rev (Appendix B.1, Table B-2). Genetic manipulations are described in more detail in Sections 5.3.1 and 5.3.2. All DNA cassettes were verified by DNA sequence analysis carried out at the Genetic Analysis Services facility in the Department of Anatomy, University of Otago, Dunedin, New Zealand. At the facility, Sanger sequencing is carried out using BigDye® Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit. DNA sequences were analysed using FinchTV software (Geospiza) and BLAST algorithms for comparison with the expected sequence etc.
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2.5 Determination of azole susceptibility

2.5.1 MIC$_{80}$ assay

Minimum inhibitory concentrations at 80% growth inhibition (MIC$_{80}$) were measured using liquid microdilution assays in order to determine the susceptibility patterns of mutant and wild type ScErg11p overexpressing strains to triazole drugs. Triazole drugs are fungistatic rather than fungicidal and can show trailing growth at drug concentrations several-fold in excess of the MIC$_{80}$. Therefore MICs were measured at 80% growth inhibition (MIC$_{80}$) compared to no drug controls, even though trailing growth is not generally observed for the S. cerevisiae strains used in the present study when they are cultured in SD medium.

Two-fold serial dilutions of FLC, ITC and VCZ were prepared in 96-well microtiter plates using SD medium (Table 2.1; Niimi et al., 2004). Roswell Park Memorial Institute medium (RPMI) is routinely used for standardised microdilution assays with C. albicans (Clinical and Laboratory Standards Institute M27-A3 method for azole drugs). However S. cerevisiae grows poorly in this medium and SD media is used instead (Niimi et al., 2004). Each well was inoculated at a cell density of OD$_{600\text{nm}}$ = 0.005 in a total volume 200 µL. The microtiter plates were incubated in 30 °C for 48 hours with shaking at 200 rpm. A BioTek Synergy™ 2 multi-mode plate reader (BioTek Instruments, Winooski, VT, USA) was used to determine OD$_{600\text{nm}}$ as a measure of cell growth. Each MIC$_{80}$ was determined in triplicate for three clones of each strain in three separate experiments (27 separate measurements).

2.6 ScErg11p6×His purification

2.6.1 Cell culture and harvesting

ScErg11p6×His was prepared according to the method described by Monk et al. (Monk et al., 2014b). Yeast cells were grown in 1.5 L YPD liquid cultures in 5 L flasks to OD$_{600\text{nm}}$ ~10 at 30 °C with shaking at 200 rpm. The cells were
harvested by centrifugation with a Sorvall® RC6 centrifuge using a FiberLite™ F14-6 rotor at 3820 g for 5 min at 4 °C. The cells were washed by centrifugation in ice-cold ddH₂O and resuspended in cold cell breakage buffer (Appendix A.3.1). Cell pellets were stored at -80 °C.

### 2.6.2 Preparation of a crude membrane fraction from yeast

Cell pellets were thawed on ice. The pH of the ice-cold cell suspension was adjusted to 7.5 on with 1 M Tris. The 350 mL chamber of a Beadbeater (BioSpec Products Inc.) was half-filled with cold glass beads and then filled completely with the cell suspension and additional buffer. The Beadbeater, with its chamber surrounded by ice, was run for five 1 min bursts, with the cells cooled for 3 – 4 min intervals in between. Cell disruption was evaluated by light microscopy with 70 – 80% breakage usually achieved. The pH of the homogenate was readjusted on ice to 7.2 with 1 M Tris after the breakage. Nuclei and cell debris were removed by twice pelleting the suspension at 3820 g for 10 min at 4 °C. The supernatant was recovered and crude membranes were pelleted by centrifugation at 205,000 g (42,000 rpm) for 70 min at 4 °C using a Ti45 rotor in Beckman Coulter Optima™ L-100K Ultracentrifuge. The supernatant was removed by aspiration and the membrane pellets washed by centrifugation in GTED-20 buffer (Appendix A.3.2) at 205,000 g for 45 min at 4 °C. The washed pellets were resuspended in a minimal volume of GTED-20 and stored at -80 °C. The amount of protein in the membrane preparation was estimated using the Lowry method (Bio-Rad) (Lowry et al., 1951) with bovine serum albumin (Thermo Fisher) as a standard.

### 2.6.2.1 Miniaturised method for crude membrane preparations

A 5 mL overnight culture was pelleted at 3000 g for 5 min at 4 °C. Cell pellet was resuspended in 1 mL of cold ddH₂O and transferred to a 1.5 mL Eppendorf tube. The cells were pelleted by centrifugation at 3000 g for 5 min in a benchtop refrigerated centrifuge (Sigma 1-15pk) and resuspended in 250 µL of cold cell breakage buffer (Appendix A.3.1). 400 µL of glass beads were added to the cell suspension. The cells were homogenised using a shaker-vortex for 10 min at a speed of ~1000 rpm. The cell homogenate was centrifuged at 2000 g for 10 min
to remove cell debris and the remainder of glass beads. The supernatant was centrifuged at 15,000 g for 1 hour. The membrane pellet was washed with 1 mL GTED-20 buffer (Appendix A.3.2) and precipitated at 15,000 g for 1 hour. The washed pellets were resuspended in 100 µL of GTED-20 and stored at -80 °C.

### 2.6.3 Crude membrane solubilisation

Crude membranes were washed with protein solubilisation buffer (Appendix A.3.3) and pelleted by centrifugation at 105,000 g (30,000 rpm) for 70 min at 4 °C. This step removes the bulk of the EDTA that can interfere with the Ni-NTA-agarose column chromatography. The supernatant was removed by aspiration and the membrane pellets were resuspended in solubilising buffer containing 10× critical micelle concentration (CMC) of the detergent n-decyl-β-D-maltoside (DM, Appendix A.3.4). The membranes were solubilised for 1 hour on a rotating mixer in the detergent-containing solubilisation buffer (1 mL per 5 mg protein). The detergent-insoluble fraction was removed by centrifugation at 105,000 g for 70 min at 4 °C in a Ti45 rotor. The supernatant was used for Ni-NTA affinity purification.

### 2.6.4 Affinity chromatography

#### 2.6.4.1 Imidazole elution

Ni-NTA affinity chromatography was used to purify ScErg11p6×His from DM-solubilised membranes. The Ni-NTA-agarose beads (Qiagen) were washed twice with 5 mL of binding buffer (Appendix A.3.5) per 0.5 mL of packed beads to replace the bead storage solution. The solubilised membrane fraction was added to the washed Ni-NTA-agarose affinity matrix (2 mL of packed matrix per 1 g of crude membrane protein) in 50 mL falcon tubes and rotated end over end overnight at 4 °C. The resultant mixture was added to a Bio-Rad Econocolumn and the matrix allowed to settle. The column was washed with 10 mL of binding buffer per 0.5 mL of matrix to remove non-specifically bound protein. The ScErg11p6×His was eluted with affinity column elution buffer (Appendix A.3.6). If the protein was to be co-purified with a drug, it was added
2.6.4.2 Histidine elution

For characterisation by spectrophotometric analysis (cytochrome P450 type II difference spectra), ScErg11p6×His was eluted with L-histidine instead of imidazole during affinity purification. The Ni-NTA-agarose beads were pre-washed and the packed column with ScErg11p6×His bound to the beads were washed with solubilisation buffer containing 16 mM DM detergent (Appendix A.3.4) and 2 mM L-histidine. The protein was eluted with solubilisation buffer containing 16 mM DM and 50 mM L-histidine. The L-histidine was removed from the sample by washing the enzyme with solubilisation buffer containing 16 mM DM by using 50 kDa molecular-weight cut-off Amicon Ultra-4 centrifugal filters (Merck Millipore Ltd, Cork, Ireland). The removal of L-histidine was checked by taking the absolute spectra of the sample using the UltraSpec™ 6300 pro UV/Visible spectrophotometer as described in Section 2.7.2. The heme peak for wild type protein with no ligand was at ~417 nm. With L-histidine bound the peak was detected at ~420 nm. The samples recovered were refrigerated at 6 °C.

2.6.5 Size exclusion chromatography

Affinity purified fractions were further purified by size exclusion chromatography (SEC) on a Superdex 200 10/300 GL column (GE Healthcare, Buckinghamshire, UK) using the Äkta fast protein liquid chromatography (FPLC) purifier 10 (GE Healthcare). The column, which was stored in 20% ethanol, was prewashed with ddH₂O and then equilibrated with 2 column volumes (~60 mL) of SEC buffer (Appendix A.3.7) at a flow rate of 0.4 mL per min. If the enzyme was to be co-purified with a drug, the appropriate drug (10 µM for FLC, VCZ and 2 µM for PCZ and ITC) was added to the SEC buffer.
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The column was eluted at a flow rate of 0.4 mL per min and 0.5 mL fractions were collected. The red-coloured fractions containing the 62 kDa ScErg11p6×His were pooled and concentrated using 50 kDa molecular-weight cut-off Amicon Ultra-4 centrifugal filters.

2.7 Biochemical characterisation of ScErg11p6×His

2.7.1 Protein concentration estimation

For crystallisation, the concentration of the purified protein was estimated using the 280 nm protein peak in an absorbance spectrum obtained with an Ultrospec™ 6300 pro UV/Visible spectrophotometer. The absolute absorbance spectrum of the purified protein was measured between 250 nm and 500 nm. The protein concentration was calculated using the Beer-Lambert Law (Equation 2.1) where $A_{280}$ is the absorbance at 280 nm, $c$ is the protein concentration, $l$ is the cuvette path length and $\varepsilon$ is the extinction coefficient.

**Equation 2.1 Beer-Lambert Law**

$$c = \frac{A_{280}}{\varepsilon \times l}$$

The extinction coefficient ($\varepsilon$) of a protein is calculated based on the number of tryptophans, tyrosines and cysteines present in the protein (Equation 2.2) (Pace et al., 1995). The extinction coefficient of ScErg11p6×His was calculated to be 81960 M$^{-1}$ cm$^{-1}$.

**Equation 2.2 Extinction coefficient calculation**

$$\varepsilon = 5500 \times (\#\text{Trp}) + 1490 \times (\#\text{Tyr}) + 125 \times (\#\text{Cys})$$

2.7.2 Absolute absorbance spectra of ScErg11p6×His

The absolute spectrum of the purified protein was determined using Ultrospec™ 6300 pro UV/Visible spectrophotometer using the buffer that the protein is suspended in as a blank e.g. SEC buffer. The absolute absorbance spectra for SEC and affinity purified protein preparations were routinely obtained to
determine the heme to protein peak ($A_{280nm}$) ratio as well as whether the protein has bound the appropriate ligand. The heme of the ScErg11p6×His produces peaks at ~424 nm with imidazole bound, 421 nm with ITC and 417-418 nm when its ligand free.

### 2.7.3 SDS-polyacrylamide gel electrophoresis

ScErg11p6×His protein preparations were analysed by SDS-PAGE in the Protean mini gel system (Bio-Rad) using the method described by Laemmli (Laemmli, 1970). The components of the stacking and separating gels (8% acrylamide) are listed in Appendix A.4, Table A-2. The gel tank was filled with running buffer (Appendix A.4.1) and the PageRuler™ Plus prestained protein standards (5 µL, Fermentas Life Sciences, Waltham, MA, USA) were added to bordering sample wells to estimate the sizes of the proteins in samples. Prior to loading, protein samples were treated with 6× protein loading buffer (Appendix A.4.2). The samples were subject to electrophoresis for 90 min at 100 V. The gels were stained with Coomassie blue R250 (Appendix A.4.3) for at least 1 hour and destained for at least 2 hours in destaining solution (Appendix A.4.4). The destained gels were stored in 10% acetic acid. Gels were photographed using the Gel Doc imaging system (Bio-Rad).

### 2.7.4 Western blot analysis

#### 2.7.4.1 Preparation of protein samples for quantitation of expression

Cells were harvested from strains in the logarithmic growth phase ($OD_{600nm} = 6-7$). Crude membrane samples were obtained using a miniaturised method described in Section 2.6.2.1. In brief, cell cultures (5 mL) were harvested by centrifugation, broken using glass beads and a crude membrane fraction obtained by differential centrifugation. Crude membrane samples (10 µg of protein) and coloured protein markers were separated by SDS-PAGE in pairs, on 8% acrylamide gels (Section 2.7.3). One gel was immediately stained with Coomassie R250 and the other was electrotransferred to nitrocellulose.
2.7.4.2 Electrotransfer and Immunostaining

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare) using 20 mM Tris-HCl pH 8.5, 192 mM glycine and 20% methanol electrotransfer buffer. The acrylamide gel, nitrocellulose membrane, blotting paper and scrubber pads were briefly soaked in the electrotransfer medium and a sandwich comprising a scrubber pad, 3 layers of blotting paper, the gel, the nitrocellulose membrane and a further 3 layers of blotting paper and another scrubber pad was placed into a blotting cassette that was inserted into a protein transfer apparatus (Mini Trans-Blot®, Bio-Rad). The tank was filled with cold electrotransfer buffer and the proteins transferred at 100V for 1.5 hours at 4 °C.

The nitrocellulose membrane was incubated for 1 hour at room temperature in blocking solution (2.5 mg/mL skim milk, 0.02% Tween-20 in phosphate buffered saline [PBS]). The blot was then immunodecorated by incubation in blocking solution containing a 1:3000 dilution (16 mU/mL) of anti-6×His-peroxidase antibody (mouse monoclonal antibody, Roche) for 1 hour at room temperature. The membrane was then washed 3 times for 5 min in washing solution (0.1 % Tween-20 in PBS).

2.7.4.3 Detection of immunocomplexes by chemiluminescence

The nitrocellulose membrane was incubated for 2 min in freshly prepared detection solution which contained 5.5 mg of Luminol dissolved in 60 µL of DMSO, 0.28 mg of p-coumaric acid dissolved in 10 µL of DMSO and 7.7 µL of 30% hydrogen peroxide added to 25 mL of 0.1 M Tris-HCl pH 8.6 buffer. The nitrocellulose membrane covered in transparent plastic wrap was exposed in the dark to X-ray film (Amersham Hyperfilm ECL, GE Healthcare) for 30 sec or 60 sec. The X-ray film was developed, briefly washed with water and fixed according to manufacturers specification (Kodak, Rochester, NY). The amount of ScErg11p6×His protein in each lane was analysed using Image Lab 3.0 software (Bio-Rad). The amount of protein in the wild type strain (MMLY 941 or AD3Δ ScErg11) was normalised to 1 and compared to mutant enzyme overexpressing strains.
2.7.5 Mass spectrometry

The presence of wild type or mutant ScErg11p6×His was verified by mass spectrometry of tryptic fragments at the Centre for Protein Research (University of Otago, New Zealand). Purified protein preparations were separated by SDS-PAGE and the Coomassie stained protein bands of interest were excised from the gel. The protein band was digested with trypsin, which cleaves at an arginine or a lysine, unless followed by a proline. Protein digestion using a robotic workstation DigestPro MSi (Intavis AG, Cologne, Germany) was carried out according to the protocol by Shevchenko et al. (Shevchenko et al., 1996). A full mass spectrum was acquired in the mass range of 300-2000 mass to charge ratio (m/z) on an Orbitrap mass analyser with a resolution of 60,000 at m/z 400 and an automatic gain control target of 5e5. The data obtained was searched against the SWISS-PROT amino acid sequence database using the Mascot search engine. The search was set up for tryptic peptides with a maximum of 3 missed cleavage sites. Protein sequence coverage of at least 75% was obtained in all cases.

2.7.6 Determination of CYP450 concentration

Carbon monoxide (CO) binding spectra was used to calculate the concentration of functional cytochrome P450 enzyme in affinity purified protein preparations. The measurements were carried out according to the protocol described by Guengerich et al. (Guengerich et al., 2009). A 2 mL solution of enzyme diluted in solubilisation buffer with detergent DM (~1 µM; Appendix A.3.4) was split between 2 cuvettes. CO gas (BOC Gases Ltd, Dunedin, NZ) was bubbled through the enzyme solution in the sample cuvette. Sodium dithionite (~1 mg) was added to the sample cuvette after CO bubbling and to the reference cuvette. The CYP450 concentration was calculated using the resulting difference spectrum, which gave a peak at 445 nm (Equation 2.3). The extinction coefficient of 91 mM$^{-1}$ cm$^{-1}$ was used for the difference in absorbance between 450 and 490 nm (Omura and Sato, 1964a).
Chapter 2: Materials and Methods

Equation 2.3 CYP450 concentration calculation

\[(\Delta A_{450} - \Delta A_{490})/0.091 = \text{nmol of CYP450 per mL i.e } \mu M\]

The difference spectra were recorded with a Cary 1 Bio UV-visible spectrophotometer using 10 mm light path UV transparent plastic cuvettes (GE Healthcare Life Sciences).

2.7.7 CYP450 type II difference spectra

L-histidine-affinity purified ScErg11p6×His enzyme was used to measure the binding of triazole drugs. Titrations with drugs were carried out using 1 µM of ScErg11p6×His enzyme with the concentration of CYP450 determined using CO binding spectra. The triazole drugs ITC, PCZ, VCZ and FLC were dissolved in DMSO (200 mM) and added to the sample cuvette and a corresponding amount of DMSO was added to the reference cuvette. The concentration range of the triazole drugs was 0.1 – 3 µM. The total amount of DMSO added was <2% of the total volume in the cuvette. Type II difference spectra (Warrilow et al., 2010b) were recorded between 350 – 500 nm using a Cary 1 Bio UV-visible spectrophotometer. The difference in absorbance between the trough at ~410 nm and the peak at ~428 nm was plotted against drug concentration. The dissociation constants \((K_d)\) were calculated by fitting obtained data to either the Hill equation (Equation 2.4) or the rearrangement of the Morrison equation (Equation 2.5). In these calculations \(\Delta A_{max}\) is the maximum change in absorbance, \([\text{Azole}]\) is the azole concentration, \(E_t\) is the total amount of enzyme present and \(n\) is the Hill coefficient. The best fitting equation was chosen by using the Akaike information criterion (Akaike, 1974).

Equation 2.4 The Hill equation

\[\Delta A = \Delta A_{max} [\text{Azole}]^n / ([\text{Azole}]^n + K_d^n)\]

Equation 2.5 The rearrangement of the Morrison equation

\[\Delta A = \Delta A_{max} \times \{((E_t + [\text{Azole}] + K_d) - ((E_t + [\text{Azole}] + K_d)^2
- (4 \times E_t \times [\text{Azole}])^{10.5})/2 \times E_t\]

All calculations for the dissociation constant \((K_d)\) were carried out using GraphPad Prism 6 Software.
2.8 Crystallisation of ScErg11p6×His

2.8.1 Crystallisation conditions

A hanging-drop vapour-diffusion method was used to crystallise ScErg11p6×His (Monk et al., 2014b). The reservoir solution contained 45% PEG-400 in 100 mM glycine-NaOH buffer in the pH range of 9.30 - 9.55. The drop volume was 4 µL in a 1:1 ratio of reservoir solution and ~20 mg/mL of protein in SEC buffer. The drops were thoroughly mixed on a round cover slide (Hampton Research, Aliso Viejo, CA, USA) prior to sealing the slide over the appropriate well of a 24 well plate (Hampton Research). Crystals started to form after approximately one week of incubation at 18 °C. Crystals were picked using an appropriate size nylon loop (MiTeGen, Ithaca, NY, USA) and flash-cooled in liquid nitrogen.

2.8.2 Data collection

Crystals were pre-screened at the Macromolecular X-ray Crystallography Suite in the Department of Biochemistry (University of Otago, New Zealand). The copper X-ray radiation anode tube and the Rigaku R-AXIS IV++ area detector were used to screen the crystals. Crystals diffracting to a resolution of 3 Å or higher were subsequently sent to the Australian Synchrotron. Full datasets were collected at the Australian Synchrotron (Clayton, Vic, Australia). The macromolecular crystallography beamlines used for data collection were the MX1 beamline (ADSC Quantum 210r detector) and the MX2 microbeam (ADSC Quantum 315r detector). The crystals were kept frozen under an N₂ cryostream at -180 °C during data collection. The complete datasets were collected at a wavelength of 0.954 Å.

2.8.3 Data processing

Diffraction data processing was carried out using iMosflm (Batty et al., 2011) and SCALA (Evans, 2006) from the CCP4 suite (Winn et al., 2011). Phaser-MR (McCoy et al., 2007) from Phenix software suite (Adams et al., 2010) was used.
for molecular replacement using ScErg11p6×His complexed with lanosterol (PDB ID: 4LXJ) as a template (Monk et al., 2014b). Phenix.refine (Afonine et al., 2012) was used for the refinement. COOT (Emsley et al., 2010) was used to visualise the structure after each round of refinement. The triazole inhibitors were modelled into the appropriate density near the heme in the active site. Water molecules were modelled if at least one hydrogen bond was detected (2.5 – 3.3 Å). Bond length restraints were applied for Fe – nitrogen (2.15 Å) and Fe – sulfur (2.33 Å) distances during refinement (phenix.refine). The constraints were based on the average coordinate bond distance of more than 80 known Fe – N (triazole) complexes and 4 heme Fe – S complexes in the Cambridge Structural Database. The crystallographic information files (.cif) for triazole inhibitors were obtained from running the Grade Global Phasing online tool (Global Phasing Ltd, Cambridge, UK). The crystallographic information files for ITC and PCZ were altered. To obtain the correct conformation of the piperazine ring in either the chair or the twisted boat shapes the crystallographic information file had extra chiral restraints for nitrogen atoms of the ring added to it, otherwise the puckering of the ring did not assume either of those shapes (Appendix F).
Chapter 3

Structural insights into binding of the antifungal drug fluconazole to

Saccharomyces cerevisiae

lanosterol 14α-demethylase
3.1 Introduction

Until 2014, X-ray structures of eukaryotic CYP51s were obtained by deletion of the transmembrane helix to improve expression and crystallization (Strushkevich et al., 2010). These include the human CYP51 enzyme (PDB IDs: 3LD6, 3JUV, 3JUS) (Strushkevich et al., 2010) and CYP51 enzymes from the protozoa *T. cruzi* and *T. brucei* (PDB IDs: 2WX2, 2WV2, 2X2N; Chen et al., 2010). Other CYP51 structures available include *M. tuberculosis* CYP51 (e.g. PDB ID: 1EA1; Podust et al., 2001) and *L. infantum* CYP51 (PDB ID: 3L4D; Hargrove et al., 2011). The only structural information available on fungal CYP51s was from homology models based on the truncated structures of other P450s and more recently human CYP51 and *M. tuberculosis* CYP51 (Boscott and Grant, 1994, Fraczek et al., 2011, Xiao et al., 2004).

The first full-length structure of a fungal CYP51 was determined by X-ray crystallography of Erg11p from the yeast *S. cerevisiae* (Monk et al., 2014b). Two structures were deposited in the PDB; one with the native substrate lanosterol (PDB ID: 4LXJ) bound in the active site in a pre-catalytic state and the other with the long-tailed triazole drug ITC (PDB ID: 4K0F) at resolutions of 2.19 Å and 1.90 Å, respectively. Structures of Erg11p complexed with FLC or VCZ could only be refined at significantly lower resolution (≥2.5 Å) and were not deposited in the PDB (Appendix G, Table G-2; Monk et al., 2014b).

Elucidation of the structure of the complete enzyme identified the likely orientation of the enzyme in relation to the lipid bilayer. The amphipathic N-terminal membrane helix 1 (MH1) and adjacent transmembrane helix (TMH1) are oriented at about 60º to each other, and contacts between TMH1 and the catalytic domain help position the catalytic domain partly in the lipid bilayer. The enzyme has a common P450 fold with α-helices (A-L) and β-sheets (1-4) constituting the catalytic domain with the heme cofactor forming the active site. The entrance to the substrate channel is in direct contact with the surface of the lipid bilayer. This ensures that the entry of the lipidic substrate is directly from the surface of the membrane. In this report, we present the high-resolution crystal structure of full-length *S. cerevisiae* Erg11p in complex with the short-tailed triazole FLC that reveals key hydrogen bond networks in the active site.
3.2 Materials and methods

The materials and methods used in this chapter are described in Chapter 2. The strains used in this chapter are listed in Appendix A.1 (Table A-1).

3.3 Results and Discussion

3.3.1 Drug susceptibility of S. cerevisiae strains expressing Erg11p

As expected, deletion of the HIS1 ORF in the ADΔ strain did not alter the azole susceptibility pattern in the derivative strain AD2Δ. Both the parent and derivative strain were equally highly sensitive to the triazoles tested (Table 3.1). Constitutive hyper-expression of ScErg11p6×His from the PDR5 locus under control of the gain-of-function pdr1-3 mutation increased resistance for the AD2Δ to FLC 6-fold, ITC 3.5-fold and VCZ 5-fold. Removal of the endogenous ScERG11 in the AD3ΔScErg11p strain did not significantly increase susceptibility to these triazoles. This showed that the azole resistance was dependent on the recombinant ScErg11p6×His and not the native ScErg11p.
Table 3.1 MIC$_{80}$ values for *S. cerevisiae* strains to FLC, ITC and VCZ

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC$_{80}$ µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
</tr>
<tr>
<td>ADA</td>
<td>0.4 (± 0.04)</td>
</tr>
<tr>
<td>AD2Δ</td>
<td>0.4 (± 0.04)</td>
</tr>
<tr>
<td>AD2Δ ScErg11p</td>
<td>2.4 (± 0.13)</td>
</tr>
<tr>
<td>AD3Δ ScErg11p</td>
<td>2.1 (± 0.02)</td>
</tr>
</tbody>
</table>

MIC$_{80}$ values were determined as described in Chapter 2. The table shows the mean for 3 separate clones of each strain using data obtained in triplicate measurements from at least 3 different experiments (a total of 9 determinations per strain). SEM indicated in brackets.

### 3.3.2 Spectral characteristics of purified ScErg11p

The absorbance spectrum for ScErg11p6×His in its low spin ferric state showed a heme Soret (γ) band at 417 nm and δ, β and α peaks at 350, 535 and 571 nm, respectively (Figure 3.1). One electron reduced spectra obtained using sodium dithionite gave a blue shift of the Soret peak from 417 nm to 414 nm. CO binding to the ferrous ScErg11p6×His produced a characteristic red shift to 445 nm. The minor shoulder at the 420 nm indicated the presence of a small amount of inactive P420 complex in the purified enzyme preparation. The absolute, reduced and CO spectra for purified ScErg11p6×His were comparable to those obtained previously for *S. cerevisiae* microsomal preparations by Yoshida and Aoyama (Yoshida and Aoyama, 1987) and indicated that the ScErg11p6×His preparation was active.
Figure 3.1 Spectral characterization of ScErg11p6×His. The absolute spectra of the ferric protein (solid trace), the ferrous protein reduced with sodium dithionite (dashed trace) and the reduced protein with bound carbon monoxide (dotted trace). Spectra were recorded using 1.5 µM of ScErg11p6×His.

3.3.3 Binding of triazole drugs to ScErg11p6×His

The binding of the FLC (Figure 3.2a) and VCZ (data not shown) to ScErg11p6×His gave type II difference spectra caused by the coordination of the nitrogen atom in the triazole ring to the heme iron, replacing the water ligand and stabilising the low-spin form (Locuson et al., 2007). The absorbance spectra of ScErg11p6×His in the presence of the triazoles caused a red shift of the heme Soret peak from 417 nm to 421 nm for both VCZ and FLC. The difference spectra obtained with 1 µM ScErg11p6×His enzyme in the presence of excess FLC and VCZ were very similar, with a peak at 428 nm and a trough at 410 nm (Table 3.2). The binding is normally characterized as tight if the $K_d$ values are lower or equal to the enzyme concentration and the Morrison equation is generally used to fit the data (Morrison, 1969). However, analysis of the change in absorbance caused by drug binding versus the concentration of triazole drug added, gave the best fit using the Hill equation and not the
rearrangement of the Morrison equation (Figure 3.2b and Table 3.2). The 
[Azo]_{0.5} values were similar for the two drugs as were the \( K_d \) values (Table 3.2).

![Figure 3.2 Binding of FLC and VCZ to ScErg11p×His.](image)

(a) Difference spectra demonstrating type II binding of FLC obtained by incremental additions of the drug to 1 \( \mu \)M of ScErg11p×His. (b) Saturation curves for FLC (filled circles) and VCZ (hollow circles) fitted to the Hill equation with \( \Delta A \) plotted against azole concentration.

Similar to previous studies with *C. albicans* CYP51, the [Azo]_{0.5} values were found to be about half the P450 concentration, indicating tight binding for VCZ and FLC (Warrilow et al., 2010a, Lamb et al., 1997). The \( K_d \) value for FLC of 74 (±15) nM is comparable to those previously obtained using the Morrison equation for CaCYP51 in two different studies i.e. 46.6 (±10.6) nM (Warrilow
et al., 2010a) and 60 (±10) nM (Park et al., 2011). The apparent Hill numbers of 3 for FLC and and 2.7 for VCZ, are unlikely to be due to positive cooperativity between ScErg11p monomers. They are more likely to result from multiple interactions between the ligand and the internal active site of an enzyme (Prinz and Schonichen, 2008) that behaves as monomer within its own detergent vesicle. Consistent with this hypothesis, size exclusion chromatography of the affinity purified enzyme gives enzyme-containing detergent vesicles that migrate with an apparent molecular size of <100 kDa, (Monk et al., 2014b).

### Table 3.2 Binding of triazole drugs to affinity purified ScErg11p6×His

<table>
<thead>
<tr>
<th>Triazole</th>
<th>ΔA max</th>
<th>λ_{trough}</th>
<th>λ_{peak}</th>
<th>K_d (µM)</th>
<th>Hill number</th>
<th>[Azole]_{0.5} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLC</td>
<td>0.048</td>
<td>410</td>
<td>428</td>
<td>0.074 (± 0.015)</td>
<td>3.0</td>
<td>0.43</td>
</tr>
<tr>
<td>VCZ</td>
<td>0.051</td>
<td>410</td>
<td>428</td>
<td>0.082 (± 0.018)</td>
<td>2.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The binding of azole drugs to 1 µM Ni-NTA affinity purified ScErg11p6×His was determined as described in Materials and methods. IC_{50} value is denoted as [Azole]_{0.5}.

#### 3.3.4 X-ray crystal structure of S. cerevisiae Erg11p in complex with FLC

Full-length S. cerevisiae lanosterol 14α-demethylase (ScErg11p6×His) co-purified with FLC during SEC was used for crystallization. Data collection parameters and refinement statistics are presented in Appendix G, Table G-1. Molecular replacement was carried out using the full length ScErg11p6×His (PDB ID: 4LXJ) and revealed a single monomer in the asymmetric unit (PDB ID: 4WMZ) comprising MH1, TM1 and the catalytic domain as seen previously (Monk et al., 2014b). The enzyme appears to be a rigid molecule, as the tertiary structure and the shape of the substrate channel is unchanged, regardless of the size of the ligand (lanosterol, ITC or FLC) bound in the active site (Monk et al., 2014b). Some differences in the ScErg11p6×His in complex with FLC, compared with the two previously deposited structures, were detected in side chains of residues of the transmembrane helix, in side chains of residues of helix-F’, helix-G and the F’/G loop that connects them, and in some parts of the
enzyme exposed to the solvent. The F/G loop is buried in the membrane and this region has the highest β-factors together with the transmembrane helix and three residues Y439, S440 and V441 in all three structures, as previously discussed (Monk et al., 2014b). The residues Y439-V441 are within the N-terminal portion of a fungus-specific loop and are located at the surface of the protein.

The proposed egress channel that bifurcates from the substrate channel shows some density in the present structure like the two previously published structures (Monk et al., 2014b). The density has been proposed to be the zymosterol rather than the immediate product 4,4-dimethylcholesta-8,14,24-trienol, based on mass spectrometry of the purified enzyme (Monk et al., 2014b). The side chains of residues around this secondary vestibule are positioned similarly amongst all three structures except H128, which has been modelled into different positions due to the limited density. This flexibility may be required to accommodate the exit of the product or interaction with subsequent enzymes in the ergosterol biosynthesis pathway e.g. Erg24 and the Erg25-Erg28 complex (Mo and Bard, 2005). The aromatic ring of the F241 is in an unfavourable position in the ScErg11p FLC structure like the lanosterol and ITC structures (PDB IDs: 4LXJ and 4K0F), possibly to accommodate the product molecule in the egress channel.

### 3.3.5 FLC binding

FLC binds within the active site of the enzyme, with clear evidence of the ligand apparent immediately following the solution from molecular replacement (Figure 3.3a). The triazole ring of FLC is coordinated to the iron within the heme cofactor (Fe---N distance 2.13 Å) corroborating the spectrophotometric data showing type II binding. The immediate environment surrounding FLC is hydrophobic as would be expected from the nature of the substrate lanosterol. Residues within 4 Å of FLC are illustrated in Figure 3.3b and include Y126, F134, I139, Y140, F236, G310, V311, G314, G315, T318, L380 and M509. The 2,4-difluorophenyl ring of FLC lies adjacent to G310 and the coordinated triazole ring abuts G314 on the consensus motif GXXXG for sterol binding on helix-I. Consistent with other eukaryotic CYP51 structures, such as the Trypanosoma CYP51s, the bend in helix-I of ScErg11p at this motif is less
acute than in the *M. tuberculosis* CYP51 structure (Podust et al., 2001). Two water molecules form hydrogen bond networks with FLC (Figure 3.3c). The first water (743) mediates hydrogen bonds between the hydroxyl groups of FLC and Y140 as well as a propionate of the heme cofactor. The second water (790) forms hydrogen bonds with the carbonyl oxygen of S382, the hydroxyl of Y126 and N4 of the second triazole ring which points away from the heme (Figure 3.3c). In the previously published structures of ScErg11p6×His complexed with lanosterol and ITC neither water 743 nor 790 was observed. Previously reported structures of other CYP51s complexed with FLC have the inhibitor bound in a similar manner. The orientation of the 2,4-difluorophenyl ring matches that seen in the *M. tuberculosis* (PDB ID: 1EA1) and *T. cruzi* (PDB ID: 2WX2) CYP51 structures (Chen et al., 2010). The 2,4-difluorophenyl ring is rotated 180° in several other structures but this would appear to be potentially unfavourable as the 2-fluoro substituent and the hydroxyl group of FLC are in close proximity (PDB ID: 2WV2, 2WUZ; Chen et al., 2010). This alternate orientation (2WUZ) is stabilized by a hydrogen bond between the fluorine substituent and the hydroxyl of Y103 (corresponding to Y126 in ScErg11p6×His; Chen et al., 2010). This residue (Y103) shows a great deal of flexibility amongst the trypanosomal structures including occupying the site for the key water 743.
Figure 3.3 FLC binding in the active site of ScErg11p. (a) OMIT map for FLC [$F_o - F_c$ map (green mesh) contoured at 3σ, $2F_o - F_c$ map (blue mesh) contoured at 1σ]. The $F_o - F_c$ map was calculated using $F_{calc}$ refined from coordinates with no ligand at the active site. The $2F_o - F_c$ map is that following final refinement. The main chain is indicated in grey and the fluconazole is indicated as sticks, with C atoms cyan, N atoms blue, O atoms red and F atoms pale blue. The heme is shown as sticks with C atoms coloured magenta. (b) Side chains of amino acid residues within 4 Å of FLC are indicated in grey. Main chain atoms are shown for G310, G314 and G315. (c) Water mediated hydrogen bonding (yellow dashed lines) between HOH743, FLC, heme and Y140, as well as between HOH790, FLC, and S382.
VCZ, another short-tailed antifungal agent, is similar to FLC and only differs in that the second non-coordinating triazole is replaced with a fluoropyrimidine group along with the addition of a methyl group. In the previous modelled structure obtained with a lower resolution data set (2.8 Å), the fluoropyrimidine ring is positioned in the same plane as the triazole with the fluoro substituent positioned in the opposite direction to the hydroxyl of the inhibitor and the methyl group (Monk et al., 2014b). The presence of the key water (743) would also provide the potential for a hydrogen bond with N3 of the fluoropyrimidine in this orientation over the flipped conformation.

3.3.6 Antifungal resistance

Mutations, which reduce the effectiveness of azole drugs, are frequently found in resistant pathogenic fungi. Of the residues within 4 Å of FLC in ScErg11p6×His that have residues equivalent to those found to be mutated in C. albicans Erg11p, only Y132F/H (Y140F/H in ScErg11p) is known to confer resistance to FLC as a single mutation (Sanglard et al., 1998, Flowers et al., 2015). The others confer resistance in combination with additional mutations or confer susceptible phenotypes alone (Morio et al., 2010). The single amino-acid substitutions Y132F, K143R, F145L, S405F, D446E, G448E, F449V, G450E, and G464S in C. albicans Erg11p have been found to confer resistance to FLC (Flowers et al., 2015). The FLC MICs for those mutants were increased ~4-fold compared to those for the susceptible strain. Our structure shows that only Y132F, K143R and G464S are located close enough to the active site and the heme cofactor to directly affect the binding of FLC. Residues D446, G448, F449, and G450 (fungus-specific loop) as well as F145 and S405 are too far away to directly affect the heme or interfere with FLC binding.

The Y132F/H mutation in C. albicans is commonly found in clinical isolates of other fungal pathogens of humans and plants that have reduced susceptibility to FLC and other azoles (Becher and Wirsel, 2012, Wheat et al., 2006a). As described above, a key water molecule (743) mediates hydrogen bonds between the hydroxyl group of the inhibitor, the hydroxyl group of Y140, and the propionate group of the heme (Figure 3.3c). Homologous mutations at Y140 to
either phenylalanine or histidine could disrupt this hydrogen-bonding network and therefore reduce susceptibility to FLC.

Mutations equivalent to G464S (\textit{S. cerevisiae} and \textit{C. albicans} numbering) have been found in both \textit{C. albicans} and \textit{A. fumigatus} (Figure 3.4). The \textit{C. albicans} G464S mutation has been found in clinical isolates and gives rise to FLC resistance (Sanglard et al., 1998, Flowers et al., 2015, Chau et al., 2004), while the equivalent G448S mutation in \textit{A. fumigatus} CYP51B is thought to confer resistance to the long-tailed drugs ITC and PCZ but not resistance to the short-tailed VCZ drug (Bueid et al., 2010). It has been suggested that this substitution would disrupt the positioning of the heme, reduce the binding of theazole, and increase resistance (Fraczek et al., 2011). However, its differential effect on short-tailed azoles compared to long-tailed azoles is not understood. G464 is located on the same face of the heme as the coordinating cysteine residue and is close to a propionate group of the cofactor that makes a hydrogen bond to a water molecule (Figure 3.4). The G464S mutation may replace this water and alter the interaction with the heme carboxylate, thus potentially tilting the heme.
Figure 3.4 Sites of mutation in C. albicans and A. fumigatus CYP51 (residues G464 and K151 in ScErg11p). The main chain is indicated as a grey ribbon and fluconazole is indicated as sticks (C atoms cyan, N atoms blue, O atoms red and F atoms pale blue). The heme cofactor is also shown as sticks (magenta C atoms). Hydrogen bonds between the water molecule at G464 and the heme propionate as well as K151 and the second heme propionate are shown (yellow dashed lines). Hydrogen bonding is also shown between HOH743, FLC, heme and Y140, as well as between HOH790, FLC, and S382.

The K143R mutation in C. albicans CYP51 (K151 S. cerevisiae numbering) confers resistance to FLC (Manavathu et al., 1999). This residue is also located on the same side of the heme cofactor as the coordinating cysteine (Figure 3.4). In the current crystal structure, the side chain of K151 forms an ionic interaction with the carboxylate group of the heme. The change from lysine to arginine, while being functionally conservative, results in the addition of a large guanidinium group in an environment optimized for lysine. The change causes local disruption, but its extent is unknown.

A. fumigatus has innate resistance to FLC, which may be a result of sequence differences between the two homologues of CYP51 present in this species. Most fungal species have a threonine at the position corresponding to T322 on helix I (S. cerevisiae numbering). A. fumigatus CYP51A has an isoleucine at this position (A. fumigatus CYP51A numbering, I301) whereas A. fumigatus
CYP51B maintains the threonine (\textit{A. fumigatus} CYP51B numbering, T315). It has been hypothesized that this difference confers resistance to FLC (Edlind et al., 2001). Alanine mutagenesis at the homologous T315 position in \textit{C. albicans} has been shown to result in increased resistance to FLC CYP51 (Lamb et al., 1997). The current structure of ScErg11p shows that T322 is located adjacent to the active site on helix-I and close to an ethylene group of the heme but about 10 Å from the iron coordinated triazole group. G310 (\textit{S. cerevisiae} numbering) is within a distance of 4 Å of the drug. Glycine or alanine is accommodated in this position in fungal CYP51s, apart from \textit{A. fumigatus} CYP51A, which has threonine (T289), and in one of two sequences available for \textit{Coccidioides immitis} RS CYP51 (T303). This residue appears to be in better position than T322 to alter the binding of FLC (Figure 3.3b).

### 3.3.7 Future prospects

The high resolution X-ray crystal structure of full-length recombinant \textit{S. cerevisiae} CYP51 (ScErg11p6×His) complexed with FLC and the resultant discovery of hydrogen bonding networks involving key waters within the active site provides the basis for detailed exploration of both the binding and resistance mechanisms of azole antifungal drugs, in particular FLC and VCZ, in fungal pathogens. Due to the strong homology of fungal CYP51s, ScErg11p can be used as a model, both structurally and experimentally, to study mutations that have been observed in resistant clinical isolates of fungal pathogens. The presence of a water-mediated hydrogen bond network between inhibitor and Y140 in the active site of lanosterol 14α-demethylase when complexed with the short chain FLC but not with the long chain ITC, as well as the interactions of the long tail of ITC in the enzyme entrance channel (Monk et al., 2014b), may explain the differential susceptibility to FLC and ITC conferred by mutations equivalent to Y140F/H. Structural and functional analysis will enable rigorous determination of the effects of these mutations on the protein and its affinity for these drugs.
Chapter 4
Resistance to triazoles
mediated by mutation of an active site tyrosine
in fungal lanosterol 14α-demethylase
4.1 Introduction

Fungal infections of plants, crops, fruit and stored produce are a major problem, especially in temperate and tropical climates. This problem is likely to be compounded by extensive monoculture, where natural barriers to fungal infection are insufficient and the widespread application of fungicides is required e.g. for control of the soybean phytopathogen *Phakopsora pakyhizi* or the wheat pathogen *Zymoseptoria tritici* (also known as *Mycosphaerella graminicola*) (Gianessi and Reigner, 2006, Fry, 2012). Several generations of triazole agrochemicals have been used since the 1970s to target phytopathogens and to counter shifts in susceptibility that often follow the introduction of new fungicides (Klittich, 2008). Similarly, multiple generations of azole drugs have been developed to circumvent non-specific interactions with liver drug metabolising enzymes and the emergence of drug resistance by fungal pathogens of humans.

Triazole drugs are becoming less effective in treating mycoses of humans and animals and in preventing the devastating effects of phytopathogens on crop production (Pfaller and Diekema, 2007, Pfaller et al., 2010, Price et al., 2015a). This has occurred because of the emergence of less susceptible fungal strains (Parker et al., 2014). Resistance to azoles used in medicine has arisen, in part, because of the use of prophylaxis with susceptible patients and prolonged treatment courses (Tashiro et al., 2012, Howard et al., 2009). A contribution to this problem by azole-based agrochemicals has been recognised recently, with these fungicides shown to induce cross-resistance to medical triazoles in *A. fumigatus* and *C. glabrata* strains (Snelders et al., 2012, Faria-Ramos et al., 2014). There is an increasingly urgent need to design antifungals and agrochemicals capable of overcoming triazole resistance. This can be best achieved by understanding the molecular basis of susceptibility and resistance to existing antifungal agents.

A common mechanism of resistance occurs due to mutations in the enzymatic target of the azole drugs, lanosterol 14α-demethylase. The *C. albicans* CYP51 (CaCYP51) Y132F/H mutations have been detected frequently in clinical isolates and lead to a 4-fold increase in resistance to FLC (determined by MIC
assays) (Sanglard et al., 1998, Flowers et al., 2015). Homologous mutations occur in the CYP51 genes of other fungal pathogens of man and plants (Figure 4.1). For example, the Y145F mutation in C. neoformans CYP51 (Sionov et al., 2012) and Y136F mutation in H. capsulatum CYP51 (Wheat et al., 2006b) both confer resistance to the short-tailed triazoles FLC and VCZ but not to the long-tailed triazoles PCZ or ITC. Similarly, the mutations Y137F in Z. tritici CYP51 (Leroux and Walker, 2011) and Y136F in Uncinula necator CYP51 (Delye et al., 1997) confer reduced susceptibility to the short-tailed azole fungicide triadimenol. The structurally homologous amino acid residue in S. cerevisiae is Y140. This tyrosine residue appears to be conserved among the CYP51 genes of humans and most fungal pathogens but in plants it is a conserved phenylalanine (Lepesheva and Waterman, 2011).

Figure 4.1 Primary sequence alignment of fungal, human and plant CYP51s in the region of ScErg11p Y140. Alignment of fungal enzymes H. capsulatum CYP51 (HcCYP51), Z. tritici CYP51 (ZtCYP51), U. necator CYP51 (UnCYP51), A. fumigatus CYP51A (AfCYP51A), C. neoformans CYP51 (CnCYP51), C. albicans CYP51 (CaCYP51), and S. cerevisiae CYP51 (ScErg11p). The human (HsCYP51) and a plant sequences Arabidopsis thaliana (AtCYP51) were compared to the fungal enzyme sequence. The frequently mutated tyrosine residue, equivalent to Y132 in CaCYP51 and Y140 in ScErg11p is highlighted in grey. The asterisk indicates conserved residues, the colon represents conservative mutations and the full stop indicates semi-conservative mutations. Alignment was carried out using ClustalW sequence alignment program.
The selection pressure exerted by individual triazole drugs can differ. For example, the *Z. tritici* CYP51 Y137F mutation arose after the introduction of triadimenol but essentially disappeared in the field following the introduction of prothioconazole (Cools and Fraaije, 2013), a pro-drug that is metabolised to the active short-tailed triazole agrochemical prothioconazole-dethio (Figure 4.2; Parker et al., 2013).

![Figure 4.2 Triazole fungicides prothioconazole-dethio, prothioconazole and triadimenol.](image)

The accumulation of multiple mutations in CYP51 can lead to significant reductions in triazole susceptibility. For example the Y131F I475T combination of mutations has been detected in CYP51 in strains of the phytopathogen *P. pachyrhizi* (Schmitz et al., 2014). The comparable double mutation Y132H I471T is found in CYP51 of the triazole resistant Darlington strain of *C. albicans* (Kakeya et al., 2000). The mutations of the Darlington strain have a
synergistic effect. The Y132H CaCYP51 expressed in a Pdr5p deficient \textit{S. cerevisiae} strain had an MIC of 16 \( \mu \text{g/mL} \) to FLC, the I471T mutant had an MIC of 6 \( \mu \text{g/mL} \) and the Y132H I471T double mutant has an MIC of \( >256 \mu \text{g/mL} \) compared to an MIC of 1.5 \( \mu \text{g/mL} \) in a control strain (Kakeya et al., 2000).

In \textit{A. fumigatus} the corresponding mutation in CYP51A is Y121F. This mutation can occur alone (Lescar et al., 2014) or together with the T289A mutation and tandem repeat 46 (TR\textsubscript{46}) in the promoter region (van der Linden et al., 2013). The Y121F mutation confers increased resistance to VCZ but not ITC or PCZ (Lescar et al., 2014) while the TR\textsubscript{46} Y121F T289A mutation is associated with failure of VCZ therapy plus a slightly reduced susceptibility to ITC and PCZ (van der Linden et al., 2013).

At the commencement of this project in 2012, a paucity of structural information on fungal lanosterol 14\( \alpha \)-demethylase had meant that the molecular mechanisms that determine the reduced susceptibility/resistance conferred by mutations in this protein were not well understood. In 2014, Monk et al. reported high-resolution structures of full-length \textit{S. cerevisiae} hexahistidine-tagged Erg11p (ScErg11p\textsubscript{6×His}) in complex with its substrate lanosterol (1.9 Å, PDB ID: 4LXJ) and the long chain azole itraconazole (ITC, 2.1Å, PDB ID: 4K0F) as well as structures with the substrate analogue estriol (2.1 Å) and the short chain triazole drugs VCZ (2.8 Å) and FLC (2.4 Å) (Appendix G, Table G-2; Monk et al., 2014b). More recently, and in Chapter 3, we published a higher-resolution (2.05 Å) structure of ScErg11p\textsubscript{6×His} in complex with FLC (PDB ID: 4WMZ; Sagatova et al., 2015). Based on this structure, the ScErg11p Y140F/H mutations were proposed to modify a water-mediated hydrogen bond network involving the hydroxyl group of FLC, thus weakening the binding of the drug. The aim of the present chapter was to test this hypothesis.

In the present study, high-resolution X-ray crystal structures of the ScErg11p\textsubscript{6×His} Y140F mutant in complex with ITC, FLC, VCZ and PCZ and the Y140H mutant in complex with FLC and ITC provide evidence that disruption of this hydrogen bond network leads to weaker drug binding for the short-tailed triazoles but not the long-tailed triazoles studied in this chapter.
4.2 Materials and Methods

The materials and methods used in this chapter are described in detail in Chapter 2. Materials and methods specific to this chapter are described in the following sections.

4.2.1 Construction of yeast strains overexpressing ScErg11p6×His

Y140F/H

ScErg11p Y140F/H constructs were made by recombinant PCR using genomic DNA from the ADΔ ScErg11p6×His overexpressing strain (MMLY 941; Appendix A.1, Table A-1) as template. Together with standard outside primers (PDR5F and PDR5DS; Appendix B.1, Table B-1) the forward primer ScErg11_Y140F_f (AAGGTGTTATTTCGATTGTCCAAATTCC) and the reverse primer ScErg11_Y140F_r (TTGGACAATCGAAATAACACCTTTACC) were used to create fragments for recombinant PCR to introduce the Y140F mutation. Similarly, the forward primer ScErg11_Y140H_f (AAGGTGTTATTCATGATTGTCCAAATTCC) with reverse primer ScErg11_Y140H_r (TTGGACAATCATGAATAACACCTTTACC) were used to introduce the Y140H mutation. The AD2Δ strain was transformed with linear ScERG11 DNA transformation cassettes that included a C-terminal hexahistidine tag, together with a transcriptional terminator and a URA3 selection marker, bordered by sequences from the PDR5 locus for integration via heterologous recombination as described by Lamping et al. (Lamping et al., 2007). Transformants expressing the URA3 marker were selected using SD-ura agar plates incubated for 48-72 hours at 30°C. Colony PCR performed on the resultant transformants was used to identify clones with the transformation cassette inserted at the correct position and in the correct orientation. The ScERG11 open reading frame and the presence of the expected mutation were confirmed by DNA sequence analysis. The resulting strains used for subsequent analysis were denoted AD2ΔScErg11_Y140F and AD2ΔScErg11_Y140H (Appendix A.1, Table A-1). The native ERG11 was deleted from mutant strains by replacement with a disruption cassette containing the His1 marker. Transformants were selected on SD-his agar plates. Colony PCR and DNA
sequence analysis were used to confirm the correct inserts. The resulting strains were designated AD3ΔScErg11_Y140F and AD3ΔScErg11_Y140H (Appendix A.1, Table A-1).

4.3 Results

4.3.1 Quantitation of ScErg11p6×His expression in crude membrane fractions

Coomassie blue R250 stained SDS-polyacrylamide gel profiles (Figure 4.3a) and western blot analysis (Figure 4.3b) of crude membrane fractions obtained from cells overexpressing ScErg11p6×His, ScErg11p6×His Y140F or ScErg11p6×His Y140H from the PDR5 locus show that the mutant enzymes were expressed in this fraction at levels near to but slightly lower than the wild type enzyme. The Coomassie blue stained gels show, as expected, that the 62 kDa native enzyme was expressed at several-fold lower levels than the recombinant hexahistidine-tagged enzyme expressed from the PDR5 locus. The Coomassie stained gels indicated the lanes containing mutant Erg11p contained a slightly reduced 62 kDa band than the control MMLY941 strain overexpressing wild type ScErg11p6×His from the PDR5 locus and native enzyme from ERG11 locus. While the comparison must be indirect, as other proteins may contribute to this band, reduction of the 62 kDa band compared to AD2Δ crude membrane preparation suggests that the mutant Y140F/H mutant enzymes were expressed at comparable levels to the wild type ScErg11p6×His expressed from the PDR5 locus. The western blots showed that the mouse anti-6×His-peroxidase antibody was specific for the hexahistidine tag found on the constructs expressed from the PDR5 locus and did not recognise the endogenous non-tagged native enzyme expressed from the ERG11 locus (Figure 4.3b). Quantitation of the digitally captured western blot by Image Lab 3.0 (Bio-Rad) software showed that ScErg11p6×His Y140F and ScErg11p6×His Y140H were expressed at 96% and 83%, respectively, of the level of the wild type ScErg11p6×His enzyme.
Figure 4.3 SDS-PAGE and western blot analysis of ScErg11p6×His wild type (WT) and Y140F/H mutant proteins. A Coomassie stained 8% acrylamide SDS-PAGE gel of samples of crude membranes (10 g) from strains overexpressing wild type and mutant ScErg11p6×His, with the AD2Δ strain used as a negative control. Ten μL of each sample at 1 μg/μL mixed with 2 μL of 6× SDS loading dye was separated on each lane. (b) Proteins electrotransferred to nitrocellulose membranes were decorated with a mouse anti-6×His-peroxidase monoclonal antibody and visualised using chemiluminescence as described in Section 2.7.4.

Analysis of the Ni-NTA and SEC purified 62 kDa band by tryptic digestion and mass spectrometry showed at least 78% coverage of the primary sequence of ScErg11p6×His and confirmed the presence of the expected mutations at Y140 (Appendix D.8, D.9).

Carbon monoxide spectra obtained with dithionite-reduced wild type and mutant enzymes gave a peak at 445 nm, which showed that the bulk of each enzyme was functional. The presence of a slightly larger shoulder at 420 nm for the mutant enzymes indicated that each mutant enzyme had more non-functional enzyme compared to the wild type (Figure 3.1). The spectral profiles for both mutant enzymes were the same and hence only a representative profile is shown in Figure 4.4.
Figure 4.4 The absolute and CO bound spectral profile of ScErg11p6×His Y140F mutant. The absolute spectrum profile is shown as a continuous line. The spectrum obtained with a dithionite-reduced and CO bound sample is presented as a dotted line.

4.3.2 Azole susceptibilities of strains overexpressing ScErg11p6×His Y140F/H

The susceptibilities of *S. cerevisiae* AD3Δ strains (native *ERG11* deleted) overexpressing Erg11p6×His Y140F/H mutants to triazole drugs were measured as MIC$_{80}$ values (Table 4.1). Overexpression of ScErg11p6×His Y140F in the AD3ΔScErg11p_Y140F strain conferred two-fold lower susceptibility to FLC than strain AD3ΔScErg11p that overexpressed wild type ScErg11p6×His i.e. MIC$_{80}$ = 4.0 µg/mL and 2.1 µg/mL, respectively. The same mutant conferred a less than 2-fold reduction in susceptibility to VCZ. The MIC$_{80}$ values were 0.42 µg/mL for the strain overexpressing ScErg11p6×His Y140F and 0.25 µg/mL for the strain overexpressing wild type ScErg11p6×His. Strain AD3ΔScErg11p_Y140H showed slightly greater susceptibilities to FLC (3.4 µg/ml) and VCZ (0.29 µg/mL) than the Y140F mutant. Although strain
AD3ΔScErg11p_Y140H showed a reduction in susceptibility to FLC (3.4 μg/mL) compared to the control strain (2.1 μg/mL) overexpressing the wild type enzyme, it gave a susceptibility to VCZ (0.29 μg/mL) that was comparable to the wild type enzyme (0.25 μg/mL).

Susceptibilities to ITC were comparable for the strains overexpressing the ScErg11p6×His Y140F, ScErg11p6×His Y140H and the wild type enzyme i.e. 0.090 μg/mL, 0.077 μg/mL and 0.105 μg/mL, respectively.

Table 4.1 MIC₈₀ values for wild type and mutant strains overexpressing ScErg11p6×His

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC₈₀ μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
</tr>
<tr>
<td>AD3ΔScErg11p</td>
<td>2.1 (±0.02)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_Y140F</td>
<td>4.0 (±0.20)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_Y140H</td>
<td>3.4 (±0.02)</td>
</tr>
</tbody>
</table>

MIC₈₀s are shown as the mean value for 3 separate clones of each strain using data obtained in triplicate measurements from at least 3 different experiments (a total of 9 determinations per strain). SEM values are indicated in brackets.

4.3.3 Spectral characterisation of triazole binding to wild type and mutant ScErg11p6×His

Ni-NTA affinity-purified mutant and wild type preparations of ScErg11p6×His (1 μM) were used to detect and quantitate type II azole binding (Warrilow et al., 2010a). The preparations used for this analysis were eluted from the affinity column using histidine and washed by centrifugal filtration to remove bound histidine. The absolute spectra showed an oxidised Soret peak at ~417 nm for wild type ScErg11p6×His and at 420 nm for the Y140F/H enzymes. Type II difference spectra were obtained for all triazoles tested. The titrations of each mutant with FLC, VCZ, ITC and PCZ are shown in Figure 4.5 and titration characteristics are listed in Table 4.2.
Chapter 4: Y140F/H mutations in ScErg11p

Table 4.2 Type II difference spectra characteristics of ScErg11p6×His wild type and Y140F/H mutants

<table>
<thead>
<tr>
<th>ScErg11p</th>
<th>Wild-type</th>
<th>Y140F</th>
<th>Y140H</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triazole</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLC</td>
<td>0.048</td>
<td>410</td>
<td>428</td>
</tr>
<tr>
<td>VCZ</td>
<td>0.051</td>
<td>410</td>
<td>428</td>
</tr>
<tr>
<td>ITC</td>
<td>0.037</td>
<td>410</td>
<td>428</td>
</tr>
<tr>
<td>PCZ</td>
<td>0.031</td>
<td>410</td>
<td>428</td>
</tr>
</tbody>
</table>

For each mutant enzyme-drug complex, the difference in absorbance between the λ_{peak} at 424 – 428 nm and the λ_{trough} at 406 – 410 nm was 2-3-fold less than that obtained for the wild type enzyme which showed a λ_{peak} at 428 nm and λ_{trough} at 410 nm. It is of note that although difference spectra obtained for the mutant enzymes were pronounced and well-defined peaks, the Y140F enzyme showed troughs that were shallower and broader (Figure 4.5). The Y140H enzyme gave a narrower trough than the Y140F preparation for the binding of FLC, PCZ and VCZ. In contrast, this mutant enzyme gave a broader trough, similar to that of the Y140F mutant, for the binding of ITC.
Figure 4.5 Type II difference spectra for FLC binding to ScErg11p6×His wild type and Y140F/H mutant enzymes. (a) Wild type ScErg11p6×His, (b) ScErg11p6×His Y140F and (c) ScErg11p6×His Y140H mutant enzyme type II difference spectra. The range of FLC concentrations used was 0.2 – 3.0 µM.

The type II binding obtained with 1 µM wild type ScErg11p6×His or Y140F/H enzymes showed sigmoidal dose-response curves (Figure 4.6). The binding curves were analysed using the Hill equation and the resultant $K_d$ values presented in Table 4.3. These values indicate tight binding of each drug i.e. the $K_d$ for each ligand was lower than the concentration of the enzyme present in
Chapter 4: Y140F/H mutations in ScErg11p

the assay. The $K_d$ values for the mutant enzymes were similar to ones obtained for the wild type enzyme i.e. within the margin of the standard error.

![Figure 4.6 Triazole binding to ScErg11p6×His and Y140F/H mutant enzymes.](image)

Figure 4.6 Triazole binding to ScErg11p6×His and Y140F/H mutant enzymes. Triazole binding for (a) fluconazole, (b) voriconazole, (c) posaconazole and (d) itraconazole to affinity purified wild type ScErg11p6×His (■), the ScErg11p6×His Y140F (●) and ScErg11p6×His Y140H (▲). All the curves are best fit by the Hill equation.
### Table 4.3 Triazole binding to affinity purified wild type and ScErg11p<sub>6</sub>×His Y140F/H

<table>
<thead>
<tr>
<th>ScErg11p</th>
<th>Triazole</th>
<th>$K_d$ (µM)</th>
<th>Hill number</th>
<th>[Azole]&lt;sub&gt;0.5&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>FLC</td>
<td>0.074 (±0.015)</td>
<td>3.0</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.082 (±0.018)</td>
<td>2.7</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.123 (±0.027)</td>
<td>1.6</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.078 (±0.023)</td>
<td>2.2</td>
<td>0.32</td>
</tr>
<tr>
<td>Y140F</td>
<td>FLC</td>
<td>0.13 (± 0.05)</td>
<td>1.86</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.03 (± 0.02)</td>
<td>2.60</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.13 (± 0.07)</td>
<td>1.83</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.15 (± 0.07)</td>
<td>1.57</td>
<td>0.29</td>
</tr>
<tr>
<td>Y140H</td>
<td>FLC</td>
<td>0.11 (± 0.06)</td>
<td>1.68</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.22 (± 0.07)</td>
<td>1.23</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.13 (± 0.06)</td>
<td>1.48</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.10 (± 0.04)</td>
<td>1.57</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Values in brackets indicate standard errors. IC<sub>50</sub> value is denoted as [Azole]<sub>0.5</sub>.

#### 4.3.4 X-ray crystal structures of mutant ScErg11p<sub>6</sub>×His in complex with triazole drugs

The data collection and refinement parameters obtained for X-ray crystal structures described in this report are presented in Appendix G, Tables G-3 and G-4. The presence of the ligands in the active site is shown by the OMIT maps in Figure 4.7.
Figure 4.7 OMIT maps for triazole binding to ScErg11p6×His Y140F/H mutants. Triazole drugs are shown as sticks, C atoms yellow, N atoms blue, O atoms red, Cl atoms green and F atoms pale blue. The heme is shown with C atoms in magenta. The OMIT maps 2Fo - Fc map (blue) are contoured at 1σ and the Fo - Fc map (green) are contoured at 3σ. Both maps were calculated using Fcalc refined from coordinates with no ligand at the active site. The electron density is shown for (a) FLC, (b) VCZ, (c) ITC and (d) PCZ binding in the active site of ScErg11p6×His Y140F mutant. The electron density is shown for (e) FLC and (f) ITC in the active site of ScErg116×His Y140H mutant.
4.3.4.1 Binding of the long-tailed drugs ITC and PCZ to ScErg11p6×His Y140F/H mutants

X-ray crystal structures were obtained for ScErg11p6×His Y140F in complex with ITC and PCZ (PDB IDs: 4ZDY and 4ZE1, respectively) in the active site at resolutions of 2.02 Å and 2.05 Å (Appendix G, Table G-3), respectively, and for ScErg11p6×His Y140H in complex with ITC at a resolution of 2.30 Å (Appendix G, Table G-4). Both the ScErg11p6×His Y140F and Y140H structures showed binding of the long-tailed triazole ITC essentially identical to that seen previously with wild type ScErg11p×His (PDB ID: 4K0F) (Figure 4.8). The most significant feature detected in the active site, apart from the mutated residues, was the presence of a water molecule (843) between a heme propionate and the aromatic side chain of the F140 (Figure 4.8a; arrow) that was not seen in the wild type structure (PDB ID: 4K0F; Monk et al., 2014b). Water 843 replaced the hydrogen bond between the hydroxyl group of Y140 and the propionate group of the heme (water 743 is in a nearby position in wild type ScErg11p6×His FLC structure PDB ID: 4WMZ) but it makes no polar contacts with the inhibitor and it is unlikely to substantially affect ITC binding. The equivalent water molecule was not detected in the structure of ScErg11p6×His Y140H in complex with ITC or in the crystal structure of ScErg11p Y140F in complex with PCZ. The absence of this water is unlikely to be due to limited resolution as the mutant ScErg11p6×His Y140F complexes with ITC and PCZ and ScErg11p6×His Y140H complex with ITC were of comparable resolution, i.e. 2.02 Å, 2.05 Å and 2.30 Å, respectively. Each crystal structure of the mutant enzyme showed the long tail of each triazole inhibitor bound within the substrate channel in an extended conformation, with the piperazine ring in the chair conformation. This conformation was stabilised via a water-mediated hydrogen bond network between the nitrogen atom of the piperazine ring (N20 in ITC and NBT in PCZ), the main chain amide of H381 and the main chain amide of S382.
4.3.4.2 Binding of the short-tailed triazoles FLC and VCZ to ScErg11p6×His Y140F/H

The structure of ScErg11p6×His Y140F in complex with FLC (PDB ID: 4ZDZ) showed the drug bound to the wild type ScErg11p6×His structure in a similar conformation (PDB ID: 4WMZ), with any differences in the interactions between the drug and the enzyme ascribed to the mutation. As described in our
previous report (Sagatova et al., 2015; Chapter 3), the structure of ScErg11p6×His in complex with FLC included a water molecule (743) that hydrogen bonds with the hydroxyl group of FLC, the hydroxyl of Y140 and one of the propionates of the heme (Figure 4.9a). In addition, the non-coordinated triazole of FLC and the main chain carbonyl of S382 formed a hydrogen bond network mediated by water 790. This water was not detected in mutant ScErg11p6×His in complex with ITC or PCZ because the tails of these ligands occupy that space. A water molecule equivalent to water 743 (numbered 843 in the mutant structures) was found in ScErg11p6×His Y140F in complex with FLC but the hydrogen bond with Y140 was abolished as a result of the Y140F mutation (Figure 4.9b). In addition, the Y140F mutation prevented formation of a hydrogen bond between the propionate group of the heme and the tyrosine hydroxyl. A new hydrogen bond between the propionate and water 843 now exists. The hydrogen bond network disrupted by the Y140F mutation results in the presence of an additional water molecule (numbered 844), which is hydrogen bonded to water 843 and the second propionate group of the heme (Figure 4.9b).
Figure 4.9 Binding of short chain azoles to ScErg11p×His wildtype and Y140F/H mutant enzymes. Water-mediated interactions are shown for (a) wildtype ScErg11p×His in complex with FLC, (b) ScErg11p×His Y140F in complex with FLC, (c) ScErg11p×His Y140F in complex with VCZ, and (d) ScErg11p×His Y140H in complex with FLC. The wild type protein is depicted in grey cartoon (PDB ID: 4WMZ), the Y140F mutant in lilac (PDB ID: 4ZDZ, 4ZE0) and the Y140H mutant in yellow (PDB ID: 4ZE3). Hydrogen bonds are shown with dashed lines and water molecules as red spheres. FLC (cyan), VCZ (green), the side chains of residue Y/F/H140, Y126, the backbone of S382 and F384 and the heme (magenta) are shown as sticks. Water 844 is indicated with an arrow. Note: the hydrogen bonds between Y126 and F384 are not shown in panels (a), (b) and (d).

The crystal structure of the Y140F mutant in complex with VCZ showed that water 843 was the only water hydrogen bonded to the ligand (Figure 4.9c). The position of the fluorine atom on the pyrimidine ring prevents the presence of the additional water (844) seen with FLC.
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FLC showed similar binding to the wild type ScErg11p6×His and ScErg11p6×His Y140H structures (Figure 4.9d). Water molecule 843 is present in the mutant structure and forms a single short hydrogen bond with the hydroxyl group of FLC. This water displays a higher B-factor of 49 compared to 30 for water 743 in the wild type structure and 37 for water 843 in the Y140F mutant structure. However, it is unlikely to form a hydrogen bond with the imidazole of H140, as the orientation of the nitrogen atom lone pair of electrons is not appropriate for this to occur. A new polar contact (bond distance 2.7 Å) observed between N2 of the imidazole of H140 and the side chain hydroxyl of T130 orients the H140 side chain closer to T130 than is seen with Y140 or F140 side chains (Figure 4.9d). For ScErg11p6×His Y140H in complex with ITC, the orientation of the imidazole ring was similar but the N2 of the H140 imidazole and the hydroxyl of T130 are separated by 3.4 Å (Figure 4.8c).

4.4 Discussion

Triazole resistant phenotypes in the yeast S. cerevisiae have been obtained by creating the single site Y140F/H mutations in lanosterol 14α-demethylase that have homologues in numerous species of pathogenic fungi (Becher and Wirsel, 2012). Whole cell assays (MIC\textsubscript{80} measurements) were used to confirm the reduced susceptibility of ScErg11p6×His Y140F/H mutants to FLC and VCZ. The <2-fold differences in MIC\textsubscript{80} values detected using overexpression of wild type and mutant ScErg11p6×His in the yeast system were not as dramatic as previously reported for the C. albicans CYP51 Y132H mutation but show a similar trend (Sanglard et al., 1998). In addition potential structural differences between CaCYP51 and ScErg11p, which have a sequence similarity of 65%, and a reduction in the amount of overexpressed ScErg11p6×His Y140H mutant by up to 17% compared with the overexpressed wild type enzyme may explain this observation for this mutant. Structural and functional analysis of these mutants demonstrated how water-mediated hydrogen bonds between a drug and the target protein within the active site, can affect the binding of the short-tailed triazole drugs FLC and VCZ but not the long long-tailed triazoles ITC and PCZ.

The affinity purified ScErg11p6×His Y140F/H enzymes differed from the wild type enzyme in both their absolute spectra and the type II difference spectra that
resulted from azole binding. The difference in the wavelength for maximum absorbance for the oxidised heme Soret peak between the Y140F/H mutant enzymes (420 nm) and the wild type enzyme (417 nm) is consistent with these mutations altering the electronic environment of the heme. Disruption of the short hydrogen bond between Y140 and the heme propionate is the common feature that may explain this effect in both the Y140F and Y140H mutants. The detection of wider troughs in the type II absorbance spectra, with the minima around 410 nm indicated that most of the iron in the Y140F/H mutant enzymes is in a low spin state prior to drug addition (Locuson et al., 2007). The different environments in the active site that result from the more polar side chain of the Y140H mutant and the aromatic group of the Y140F mutant may explain the subtle differences in triazole susceptibility and type II difference spectra of the two mutant enzymes.

Binding of the triazole drugs shifted the Soret peak of the wild type enzyme from 417 nm to 421 – 422 nm due to their replacement of heme iron bound water molecule that is thought to stabilise the low spin state (described in Section 1.9.1). The binding of the triazole drugs to ScErg11p6×His Y140F/H shifted the Soret peak from 420 to 421 nm and therefore produced a significantly smaller shift in the wavelength of the Soret peak. This mutant phenotype also gave a lower $\Delta A_{\text{max}}$ in the type II difference spectra than the wild type enzyme (Table 4.2). However, the $K_d$ values obtained in the presence of 1 µM wild type or mutant enzyme for each triazole drug indicated “tight” binding with affinities in the nanomolar range (Table 4.3). In addition, the crystal structures obtained with the mutant enzymes in complex with all four triazole drugs did not indicate limited occupancy (Figure 4.7).

A correlation between resistant phenotype and azole affinity for CYP51 enzyme was previously reported for the C. albicans CYP51 G464S (Kelly et al., 1999b), T315A (Lamb et al., 1997) and S279F (Warrilow et al., 2012) mutants but not for the I471T mutant (Warrilow et al., 2010a). Previous reports of the C. albicans CYP51 Y132H mutant have provided conflicting results. One study reported that FLC binding to Y132H CaCYP51 elicited a type I response instead of the usual type II response (Kelly et al., 1999a). Another study
reported a type II response and a $K_d$ value similar to the wild type enzyme (Park et al., 2011). Our studies show that affinities between the wild type enzyme and the Y140F/H mutants are within the error margins of the determined values for all triazoles tested. This observation may reflect the intrinsic difficulties in extracting high precision binding information needed to calculate inhibitor affinities when tight enzyme-inhibitor complexes are formed in a near stoichiometric manner. Alternatively the similarity in $K_d$s might be a coincidental outcome of compensatory changes in inhibitor on and off rates caused by the mutations. Such effects may not be observed in equilibrium binding studies or in the crystal structures prepared in the presence of excess inhibitor. Further discussion on this is covered in Chapter 6.

The crystal structures previously obtained for wild type ScErg11p6×His in complex with the substrate lanosterol (PDB ID: 4XLJ) or the long-tailed inhibitor ITC (PDB ID: 4K0F, Monk et al., 2014b) showed a hydrogen bond between one of the heme propionates and the hydroxyl group of Y140 and no water molecules in proximity (5 Å) of this substrate or inhibitor within the active site. The previously reported structure of ScErg11p6×His in complex with the short-tailed FLC (PDB ID: 4WMZ) showed that Y140, located on the B’C loop, forms a hydrogen bond network with the heme propionate group and the hydroxyl group of FLC via a water molecule (Figure 4.9a). A limitation of the present study is the lack of high-resolution structures of ScErg11p6×His and the Y140H mutant in complex with VCZ. A structure of ScErg11p6×His in complex with VCZ was previously noted by Monk et al. (Monk et al., 2014b) but the resolution of this structure (2.8 Å) was insufficient to visualise any water network. VCZ is structurally similar to FLC and contains a hydroxyl group that should interact with the Y140 residue via a comparably located water molecule. We have shown that the ScErg11p6×His Y140F mutation disrupts this hydrogen bond network between enzyme and both FLC and VCZ. These results appear, at least in part, to explain the FLC and VCZ resistance of this mutant and the retention of susceptibility to ITC and PCZ, because the key hydroxyl is replaced by a 1,3-dioxolane moiety in both long-tailed triazoles.
Calorimetric studies have shown that it is energetically favourable to include a water molecule at the protein-ligand interface, providing evidence that water-mediated interactions can stabilise protein-ligand complexes (Ladbury, 1996). Sharrow et al. demonstrated that the loss of water-mediated hydrogen bonding due to a tyrosine to phenylalanine mutation reduced favourable enthalpy and the binding affinity of a ligand to its target protein (Sharrow et al., 2005). The water-mediated polar interactions between Y140 and the hydroxyl of FLC and VCZ optimally stabilise the binding of those drugs. Fungi have evolved a mechanism of resistance, shared with plants, that disrupts this stabilising interaction.

The binding of the long-tailed triazoles ITC and PCZ to wild type ScErg11p6×His and the Y140F/H mutants pushes Y126 to a position so it interacts with the second propionate group of the heme and the main chain amide of F384 (Figure 4.8). This overall configuration is less constrained when the short-tailed triazole FLC occupied the active site providing more space (Figure 4.9). Y126 maintains its hydrogen bond with the main chain amide of F384 but is positioned further from the heme propionate. Water 790 forms hydrogen bonds with the main chain carboxyl of S382, the hydroxyl of Y126 and N3 of the non-coordinating triazole ring of FLC. How this conformation, together with the loss of the hydrogen bond between Y126 and the heme propionate, might affect the electronic configuration of the heme is not understood.

In the ScErg11p6×His Y140F structure complexed with VCZ there was only one water molecule found at the mutation site and the orientation of fluoropyrimidine ring of VCZ excluded water 790 (Figure 4.9). A hydrogen bond was maintained between Y126 and the main chain amide of F384 but a water-mediated polar interaction between the fluoropyrimidine ring and the carbonyl of S382 was not detected.

As seen in the X-ray crystal structures of ScErg11p6×His, the enzyme forms 1-1 complexes with the long-tailed triazoles PCZ and ITC in essentially identical

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1 Water 790 in PDB ID: 4WMZ, 811 in PDB ID: 4ZE3 and 839 in PDB ID: 4ZDZ
conformations that extend from the active site through the channel to the membrane-associated entry site. In the three structures of ScErg11p6×His in complex with ITC or PCZ presented here the piperazine ring is in a chair conformation. This accommodates a water molecule that forms a hydrogen bond network with the piperazine N1, the main chain amide of H381 and the main chain amide and carbonyl of S382. Structures of *T. brucei* CYP51 in complex with PCZ show the ligand in two different conformations (Chen et al., 2010). An extended conformation of the triazole tail resulted when the PCZ piperazine ring was in a chair conformation while a twisted boat shape accommodated a bent conformation. The two different conformations of PCZ may potentially be accommodated by a difference in the conformation at the opening to the channel of the N-terminal truncated *T. brucei* CYP51 (PDB ID: 2X2N; Chen et al., 2010) compared with full-length ScErg11p6×His (PDB: 4K0F). Helix F’ and the F’G loop allow for a slightly bigger opening to the substrate channel in the *T. brucei* CYP51 structure than in ScErg11p. Whether the larger opening is independent of the N-terminus truncation has yet to be determined.

Triazole drugs currently in either clinical trials or clinical use include VT-1161, ravuconazole, albaconazole and isavuconazole (ISA; Figure 1.3; Pasqualotto et al., 2010, Hoekstra et al., 2014, Warrilow et al., 2014). All four of these compounds have the hydroxyl group that could interact with Y140 (*S. cerevisiae* numbering) via a water mediated hydrogen bond network. Therefore, we postulate that these drugs may prove less effective against CYP51 mutations equivalent to Y140F/H. ISA has been approved for treatment of invasive aspergillosis in the US (McCormack, 2015). However clinical isolates of *A. fumigatus* with the TR46/Y121F/T289A CYP51 mutations are found in many parts of the world (Chowdhary et al., 2014b, Chowdhary et al., 2014a, Lavergne et al., 2015). Such mutants have the potential to be resistant to ISA due to the Y121F mutation, which confers resistance to VCZ (Lescar et al., 2014, van der Linden et al., 2013). The novel tetrazole compound VT-1161 is currently in phase II clinical trials. It was shown to be a potent inhibitor of *C. albicans* CYP51 (Warrilow et al., 2014). Thus mutations equivalent to ScErg11p Y140F/H have the potential to confer resistance to this drug. However, it is
worth noting that the longer tails of those azoles (VT-1161 and ISA) may provide extra interactions not present in FLC and VCZ binding.

The agrochemicals tebuconazole and prothioconazole-dethio both have a similarly located hydroxyl group that may be expected to generate resistance via the equivalent mutations in fungal phytopathogens e.g. Z. tritici CYP51 Y137F and P. pachyrhizi CYP51 Y131F I475T (Leroux and Walker, 2011, Schmitz et al., 2014). This characteristic is likely to be shared by the C. albicans Darlington strain, which expresses the Y132H I471T mutations in lanosterol 14α-demethylase (Kakeya et al., 2000). Finally, plant CYP51s normally have a conserved phenylalanine residue at the position equivalent to Y140 (e.g. F124 in A. thaliana; (Lepesheva and Waterman, 2011), which may contribute to their intrinsic resistance to these agrochemical fungicides.

In conclusion, ScErg11p6×His crystal structures have revealed that the resistance conferred by the Y140F/H mutations is due to disruption of a water-mediated hydrogen-bond network between the hydroxyl groups of FLC and VCZ and the enzyme plus a modification in the electronic environment of the heme. We predict that this type of resistance could be avoided by substituting the hydroxyl of group of FLC and VCZ with the 1,3-dioxolanyl group of ITC or PCZ in order to minimise those water-mediated interactions. It may also be possible to replace the hydroxyl group in antifungals like FLC, VCZ, VT-1161, prothioconazole and tebuconazole with alternative substituents that will give more favourable binding to fungal CYP51s and circumvent the impact of mutations equivalent to Y140F/H.

Note: Based on the work described in this chapter an article entitled “Triazole resistance mediated by mutations of a conserved active site tyrosine in fungal lanosterol 14alpha-demethylase” was published in Scientific Reports. This article is included in the final Appendix of this thesis under section Attached Articles.
Chapter 5

Investigation of triazole susceptibilities conferred on *Saccharomyces cerevisiae* lanosterol 14α-demethylase by homologous mutations found in fungal pathogens
5.1 Introduction

Mutations in CYP51(s) found in fungal pathogens can confer resistance to azole-based antymycotics. CYP51 mutations of the prominent fungal pathogen *C. albicans* reported in the literature have been summarised in reviews by Morio et al. and Ying et al. (Morio et al., 2010, Ying et al., 2013). These mutations are dispersed throughout the primary sequence but Marichal et al. were able to identify three hotspots; amino acids 105-165, 266-287 and 405-488 (Marichal et al., 1999). Flowers et al. have identified several single site mutations in CaCYP51 that give at least 4-fold resistance to FLC (Flowers et al., 2015). They used the full-length crystal structure of ScErg11p6×His in complex with lanosterol (PDB ID: 4LXJ; Monk et al., 2014b) to show that those resistance-conferring mutations are located in proximity to the heme, the substrate channel or in the fungus-specific external loop. Mutations close to the heme are thought to alter its tilt, hydrophobic or electronic environment and thus potentially affect the binding of the azoles in the active site. Mutations in the substrate channel may directly interfere with access to the active site or the binding of long-tailed triazoles such as ITC and PCZ e.g. the G54 mutations in *A. fumigatus* CYP51A discussed below (Section 5.1.2). How the fungus-specific loop (residues 428 – 459 in CaCYP51, 432 – 457 in ScErg11p) affects azole binding has yet to be established, despite this region bearing several single site mutations that confer resistance to FLC e.g. D446E, Y447H, G448E, F449V, G450E and V456I (*C. albicans* numbering; Flowers et al., 2015, Alvarez-Rueda et al., 2011). The CaCYP51 mutations Y447H and V456I allow the fungal strain to retain its catalytic activity in the presence of FLC (Alvarez-Rueda et al., 2011). This suggests that the mutants could have preferential binding for lanosterol over FLC or more efficient catalysis. However, since the amino acid residues of the fungus-specific loop are too far away from the active site cavity to directly affect the binding of azoles, resistance caused by mutations in this region may be due to improved catalytic efficiency resulting from enhanced interactions with other enzymes, for example NADPH-cytochrome P450 reductase. In mammalian CYP450s the reductase is known to interact with the neighbouring helix C (Bridges et al., 1998).
Chapter 5: Biochemical and structural characterisation of ScErg11p mutants

The focus of this chapter is on mutations in fungal CYP51s which, based on our previous crystal structures, appear to be close enough to the heme, the active site or the substrate entry channel to either alter the environment of the heme or directly disrupt the binding of triazole drugs. Structural and functional analysis of these mutations recreated in *S. cerevisiae* Erg11p was expected to provide insight into their effect on enzyme conformation and its affinity for the triazole antifungals.

5.1.1 Triazole resistance in *Candida albicans*

The CaCYP51 Y132F/H, K143R and G464S mutations have been discussed in Section 3.3.6. The in-depth structural and functional analysis of the Y132F/H CaCYP51 mutation recreated as ScErg11p6×His Y140F/H has been described in Chapter 4. The CaCYP51 G464S mutation has been found to confer resistance to FLC either alone or in combination with other mutations (Sanglard et al., 1998, Chau et al., 2004, Flowers et al., 2015). Spectrophotometric analysis using microsomal preparations from *S. cerevisiae* cells expressing the CaCYP51 G464S mutant found that the mutant enzyme had reduced affinity for FLC and lower catalytic activity than the wild type enzyme (Kelly et al., 1999b). It was suggested that the positioning of the heme within the enzyme could be altered due to the mutation. The CaCYP51 G464S mutation corresponds to G448S in *A. fumigatus* CYP51A. In *A. fumigatus* this single site mutation has been shown to confer resistance to the short-tailed triazole VCZ but not the long-tailed triazoles ITC or PCZ (Howard et al., 2009, Pelaez et al., 2012). Pelaez et al. (Pelaez et al., 2012) analysed the resistance of the clinical *A. fumigatus* mutant strain with microdilution assays. To my knowledge the AfCYP51 G448S mutant was not expressed and tested in *S. cerevisiae*.

The G464S mutation has been found in combination with Y132H and R467K, as well as with Y132H and H283R (Sanglard et al., 1998, Chau et al., 2004). These combinations of mutations can have synergistic effects. For example, the Y132H and G464S mutations each conferred a 4-fold increase in resistance to FLC compared to the wild type enzyme. However, when both mutations were present in CaCYP51 the resistance of the strain to FLC was increased to 32-fold (Sanglard et al., 1998). It is interesting to note that G464S and Y132H are
located on the opposite sides of the heme (Figure 5.1). As described in Chapter 3, the Y140F/H mutations of ScErg11p abolish the hydrogen bond between the heme propionate group and the hydroxyl group of the Y140. Based on the structure of ScErg11p6×His in complex with FLC (PDB ID: 4WMZ; Sagatova et al., 2015), the mutant G464S hydroxyl group was proposed to replace a water molecule that makes a hydrogen bond to the second propionate group (Figure 3.4). As well as testing this hypothesis by recreating the G464S CaCYP51 mutation in ScErg11p6×His, it was of interest to investigate whether the Y140F and G464S mutations cumulatively or synergistically affect FLC resistance and how the binding of this drug is affected on the structural level.

Figure 5.1 S. cerevisiae Erg11p with ITC bound in the active site. Residues with carbons coloured green are structurally homologous to those thought to confer reduced drug susceptibility in pathogenic fungi.

Two other mutations, which can be found in association with Y132H and G464S in CaCYP51, are R467K and H283R. CaCYP51 R467 is homologous to the ScErg11p R467 residue that is located close to G464 but does not interact with the heme. H283 in CaCYP51 is homologous to N290 in ScErg11p. It is
located in the C-terminal end of helix H in a position too distant from the heme to directly interact with it. Therefore the effect of those mutations was not pursued in this study.

5.1.2 Triazole resistance in \textit{Aspergillus fumigatus}

\textit{A. fumigatus} is intrinsically resistant to FLC and KTC but the molecular basis for the resistance has yet to be determined. This pathogen has two isoforms of the CYP51 enzyme, CYP51A and CYP51B (Mellado et al., 2001). Both isozymes catalyse sterol demethylation and neither is essential for cell growth. However, deletion of both CYP51 genes is lethal (Hu et al., 2007). Disruption of \textit{cyp51A} but not \textit{cyp51B} significantly reduced the minimal inhibitory concentration (MIC) for both KTC and FLC (Mellado et al., 2005). Therefore, it is thought that CYP51A is responsible for the intrinsic resistance to these azoles. VCZ is not susceptible to this intrinsic resistance mechanism and has been a drug of choice in the treatment of invasive aspergillosis since its introduction to the clinic in 2002 (Denning and Bromley, 2015). VCZ also crosses the blood brain barrier and can be used for treatment of cerebral aspergillosis, a previously incurable disease. Despite being more effective than AMB at treating invasive aspergillosis, the use of VCZ has significant toxicity and pharmacokinetic issues (Table 1.1).

ITC and PCZ are used as prophylactic treatments of aspergillosis. PCZ is only available in oral formulations and has limited absorption. The more recently released triazole drug ISA has better properties such as increased water solubility and a longer half life (Table 1.1) than VCZ and has been approved for the treatment of invasive aspergillosis in the USA (Section 1.7.5.1; McCormack, 2015).

One of many primary sequence differences between the CYP51A and CYP51B isoforms is I301 in CYP51A, which corresponds to T315 in CYP51B. It was proposed that I301 of CYP51A could be responsible for the reduced susceptibility of \textit{A. fumigatus} to FLC because this residue was thought to be located in the active site near the heme (Edlind et al., 2001). This idea was
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discussed in Section 3.3.6. The residue homologous with I301 in ScErg11p is T322I (Figure 5.1).

One mechanism that confers azole resistance in *A. fumigatus* with a high prevalence among clinical isolates involves point mutations in the *cyp51A* gene. Three amino residues in CYP51A frequently associated with mutations conferring azole resistance are G54, M220 and L98 (Rodriguez-Tudela et al., 2008). The L98H mutation is always accompanied by a 34 bp tandem repeat (TR34) in the promoter region of the *cyp51A*. TR34 increases the levels of *cyp51A* mRNA transcripts (Snelders et al., 2010). Several different amino acid substitutions occur at the G54 (E/K/V/R/W) and M220 (K/I/T/V) positions. The full-length structure of ScErg11p6×His enzyme (Monk et al., 2014b) shows that both sets of mutations are located near the mouth of the substrate channel.

Mutations in CYP51A at position G54 are thought to confer resistance to ITC and PCZ by disrupting the interaction of long-tailed azoles with the mouth of the substrate channel. A recent study found that long-term prophylactic treatment with ITC induces mutations at G54 in *A. fumigatus* CYP51A (Tashiro et al., 2012). The G54R/W/E mutants appear to be susceptible to the short-tailed triazole VCZ (Mann et al., 2003). In addition, the latest generation triazole, ISA, has been found to be effective against those mutants (Howard et al., 2013). This implies that these mutations do not interfere with the binding of this triazole, which has a medium length tail.

The resistance of CYP51A G54 mutants has been investigated by expressing the *A. fumigatus* CYP51A in *S. cerevisiae* (Alcazar-Fuoli et al., 2011). The *S. cerevisiae* strain used was deficient in the dominant multidrug efflux pump Pdr5p while expression of the endogenous ScERG11 was repressed by using a tetracycline-regulated promoter. This study confirmed the reduced susceptibility of these strains to ITC and PCZ and their retention of susceptibility to VCZ. Of all the mutations at this position it was reported that the G54W mutant was least susceptible to both ITC and PCZ (Alcazar-Fuoli et al., 2011). The paper of Alcazar-Fuoli et al. concluded that characteristics of specific *A. fumigatus* CYP51A alleles could be investigated in the heterologous host *S. cerevisiae*. As this laboratory has not been able to express the *A. fumigatus* CYP51A enzyme
in yeast, it was decided to use overexpressed ScErg11p as a surrogate. The structural homolog of G54 in *A. fumigatus* CYP51A is G73 in *S. cerevisiae*.

### 5.1.3 Aims of this chapter

Understanding of the mechanistic basis of resistance mutations in the CYP51s of fungal pathogens has been limited due to a paucity of structural information for this enzyme. This study investigated the molecular mechanisms of resistance at the structural and biochemical level by using *S. cerevisiae* Erg11p as a model for fungal CYP51s. The aims of this study were:

2. To determine the triazole susceptibilities of the *S. cerevisiae* strains harbouring mutations in ScErg11p.
3. To purify and biochemically characterise the mutant ScErg11p enzymes, including their stability and drug binding affinities.
4. To crystallise the mutant ScErg11ps in complex with triazole drugs.
5. To visualise how the mutations in the structure of ScErg11p affect drug binding.

### 5.2 Materials and methods

#### 5.2.1 General

The general materials and methods used in this chapter are described in Chapter 2. The strains used in this chapter are listed in Appendix A.1 (Table A-1). Materials and methods specific to this chapter are described in the following sections.

#### 5.2.2 Oligonucleotide primers

The primers used in recombinant PCR to introduce ScErg11p mutations are listed in Table 5.1. The general primers used for making the *ScERG11* mutant transformation cassettes are listed in Appendix B.1.
Table 5.1 Oligonucleotide primers used to make mutations in ScERG11

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer name*</th>
<th>Primer sequence (5’-3’)</th>
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<tr>
<td><strong>G73E</strong></td>
<td>ScErg11_G73E_f</td>
<td>GCAGTTGTTGTACGAATGAAGCCATACGAGTTTTTCG</td>
</tr>
<tr>
<td></td>
<td>ScErg11_G73E_r</td>
<td>CGTATGGCTTCATTTGTACACAAGCTGCACTACC</td>
</tr>
<tr>
<td><strong>G73R</strong></td>
<td>ScErg11_G73R_f</td>
<td>GCAGTTGTTGTACAGAATGAAGCCATACGAGTTTTTCG</td>
</tr>
<tr>
<td></td>
<td>ScErg11_G73R_r</td>
<td>GTATGGCTTCATTTGTACACAAGCTGCACTACC</td>
</tr>
<tr>
<td><strong>G73W</strong></td>
<td>ScErg11_G73W_f</td>
<td>GCAGTTGTTGTACAGGATGAAGCCATACGAGTTTTTCG</td>
</tr>
<tr>
<td></td>
<td>ScErg11_G73W_r</td>
<td>GTATGGCTTCATTTGTACACAAGCTGCACTACC</td>
</tr>
<tr>
<td><strong>K151R</strong></td>
<td>ScEgr11_K151R_f</td>
<td>GATGGAGCAAAGAAAGTTTTGTTAAGGGTGCTC</td>
</tr>
<tr>
<td></td>
<td>ScEgr11_K151R_r</td>
<td>CCTTAACAAACTTCTTTGTCCATCAATCTAGAAT</td>
</tr>
<tr>
<td><strong>T322I</strong></td>
<td>ScEgr11_T322I_f</td>
<td>CTTCTGCTGCCATTCTGCTTTGGAGTTTTTG</td>
</tr>
<tr>
<td></td>
<td>ScEgr11_T322I_r</td>
<td>CCAAGCAGAAATGGCAGCAGAAGTATG</td>
</tr>
<tr>
<td><strong>G464S</strong></td>
<td>ScEgr11_G464S_f</td>
<td>CTTACCTTTCTCTGTGTTGAGACACAGATGTATC</td>
</tr>
<tr>
<td></td>
<td>ScEgr11_G464S_r</td>
<td>GTGTCTACCACCAAGAAGGTAAAGTTATGGAGAGC</td>
</tr>
</tbody>
</table>

*f denotes a forward primer and r denotes a reverse primer. Mutated codons are highlighted in bold.

5.2.3 Streak microseeding

The ScErg11p6×His K151R enzyme did not form crystals under the conditions used for the wild type enzyme or the other mutants studied. The streak seeding method was used in an attempt to introduce nucleation sites in drops of the mutant protein and encourage crystallisation (Bergfors, 2003). Several crystals of wild type ScErg11p6×His were washed in reservoir solution to remove precipitate. Microcrystals obtained by smashing the washed crystals into small fragments with a pipette tip were briefly centrifuged in a microcentrifuge to settle any large fragments. The hanging drop vapour diffusion method was used...
for crystallisation with microseeding. The reservoir solution contained 40 or 50% PEG-400 in 100 mM glycine in the pH range 9.3 - 9.5. The drop volume was 2 µL with a 1:1 ratio of reservoir solution and purified mutant protein at 20 mg/mL. After overnight pre-equilibration at 4 °C, cover slips were opened and drops were seeded with wild type ScErg11p6×His microcrystals. A cat whisker was dipped into a seeding solution and dragged through the pre-equilibrated drop. The coverslip containing the drop was sealed onto the reservoir plate and incubated at 18 °C.

5.3 Results

5.3.1 Cloning of ScErg11p6×His mutants

Mutant ScERG11 transformation cassettes were prepared by amplifying three DNA fragments from the PDR5 locus of the genomic DNA of the ADA ScErg11p6×His overexpressing strain (MMLY 941). The fragments were fused together and amplified by using recombinant PCR with the outside primers 1 and 6 as illustrated in Figure 5.2. The G73E mutant is used as an example in this figure. The transformation cassettes for the other mutants described were made in the same way using mutation specific primers (Table 5.1). The remaining oligonucleotide primer sequences are listed in Appendix B.1, Table B-1. The DNA sequence of the wild type ScERG11 cassette is given in Appendix B.1.1.
Genomic DNA of strain MMLY 941 was used as a template for the introduction of a DNA change into a transformation cassette. The transformation cassette contains the *PDR5* upstream region, *ScERG11* with a C-terminal His-tag, the 3-phosphoglycerate kinase (PGK) terminator, the *URA3* selection marker and a *PDR5* downstream sequence. The upstream region of the *ScERG11* G73E transformation cassette was amplified using primers PDR5F (1) and ScErg11_G73E_r (2), the middle region using primers ScErg11_G73E_f (3) and ScUr3a3-rev (4) and the downstream region using primers Ura3_PDR5DS (5) and PDR5DS (6). Recombinant PCR using the outside primers 1 and 6 were used to fuse the fragments and generate the full-length cassette.

An example of the preparation of a transformation cassette containing single mutations in *ScERG11* as described in Figure 5.2 and the agarose gel electrophoresis profiles obtained for two mutations (G73E and T322I) are shown in Figure 5.3. The amplified cassettes were separated by agarose gel electrophoresis and purified using a gel extraction kit. The purified transformation cassette for each mutant, which also contained the *URA3* selection marker was used to transform the AD2Δ host strain. Solidified SD uracil dropout medium was used to select *ura*⁺ transformants.
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Figure 5.3 Construction of ScERG11 G73E and T322I transformation cassettes by recombinant PCR. Individual DNA fragments, amplified from genomic DNA of strain MMLY 941 to introduce mutations, together with a 407 bp downstream region, were fused by recombinant PCR. The DNA fragments were separated by electrophoresis on a 0.8 % agarose gel. Lane M: Molecular markers (1 kb Plus DNA molecular ladder, Invitrogen). Lane 1: the upstream region of the ScERG11 G73E transformation cassette obtained with primers PDR5F and ScErg11_G73E_r (917 bp). Lane 2: the middle region of the ScERG11 G73E transformation cassette obtained with primers ScErg11_G73E_f and ScUra3-rev (2797 bp). Lane 3: the upstream region of ScERG11 T322I transformation cassette (1660 bp) obtained with primers PDR5F and ScErg11_T322I_r. Lane 4: the middle region of ScERG11 T322I transformation cassette (2049 bp) obtained with primers ScErg11_T322I_f and ScUra3-rev. Lane 5: the ScERG11 G73E transformation cassette obtained by recombinant PCR using primers PDR5F and PDR5DS. Lane 6: the ScERG11 T322I transformation cassette obtained by recombinant PCR using primers PDR5F and PDR5DS. The size of the ScERG11 G73E and T322I transformation cassettes was expected to be 4117 bp.

The ScErg11p6×His Y140F G464S mutant was created using template genomic DNA obtained from the strain overexpressing ScErg11p6×His Y140F (AD3ΔScErg11p_Y140F; described in Chapter 4), with the G464S mutation introduced using primers specific for this mutation (Table 5.1).

Correct integration of the transformation cassettes was confirmed by colony PCR using primers PDR5_US and ScErg11_ORF_183R (Appendix B.1, Table B-1). Fragments of the expected size (1417 bp) were detected on 0.8 % agarose gels in clones that were positive for the insert (Appendix B.1, Figure B-1). Genomic DNA was extracted from colonies positive for the insert and the ScERG11 cassette was amplified using primers PDR5_US and PDR5_288_DS_Rev. The complete open reading frame of 6×His tagged
ScERG11 was sequenced and the expected mutations confirmed for each mutant. The strains prepared in this study are listed in Appendix A.1, Table A-1. The strains were named according to the mutation in ScERG11 gene and whether or not the native ERG11 was intact or removed by insertion of the ScHis1 gene disruption cassette. For example, the G73E mutant strain with native ERG11 intact was named AD2ΔScErg11p_G73E and with native ERG11 removed AD3ΔScErg11p_G73E.

5.3.2 Deletion of ERG11 native to the S. cerevisiae host

The ScHis1 disruption cassette specific for the native ERG11 open reading frame was constructed from 3 DNA fragments (Figure 5.4) obtained by PCR using the oligonucleotide primer sequences listed in Appendix B.2, Table B-2. Sequences immediately upstream and downstream of the ERG11 open reading frame and the ScHIS1 gene together with its endogenous promoter were amplified. The three DNA fragments were amplified from the genomic DNA of S. cerevisiae strain ADΔ-pABC5’ (MMLY 1760; Lamping et al., 2007).

**Figure 5.4 Construction of the ScHis1 disruption cassette.** Genomic DNA of strain MMLY 1760 was used to amplify 3 separate fragments using primers 1 – 6. The ScHis1 disruption cassette contains the ScERG11 upstream region (ERG11 US), ScHIS1 open reading frame (ScHIS1 ORF) and ScERG11 downstream sequence (ERG11 DS). The ERG11 US was amplified using primers ScErg11_US-773 (1) and ScErg11US-387R (2), the middle region comprising the ScHIS1 promoter and ORF using primers ScErg11xHisF (5) and ScErg11xHisR (6) and the ERG11 DS region using primers ScErg11DS60 (3) and ScErg11DS346R_Δ (4). Recombinant PCR outside primers 1 and 4 were used to fuse and amplify the full-length gene disruption cassette.
The *ScHis1* gene disruption cassette, designed to delete native *ERG11* in strains created using AD2Δ as host, was prepared by recombinant PCR using the external primers ScErg11_US-773 and ScErg11DS346R_Δ to fuse the fragments generated as described in Figure 5.5. Amplimers of the cassette were separated by agarose gel electrophoresis (Figure 5.5) and purified using a gel extraction kit. Strain AD2ΔScErg11p6×His was transformed with the gel purified transformation cassette and his+ transformants selected using solidified SD histidine dropout media.

![Figure 5.5 Construction of the *ScHis1* disruption cassette for native *ERG11*.](image)

The DNA fragments shown in lanes 1-3 were amplified by PCR from the genomic DNA of strain MMLY 1760. PCR fragments were separated by electrophoresis on a 0.8% agarose gel. Lane M: molecular markers (1 kb Plus DNA molecular ladder, Invitrogen). Lane 1: the upstream region of the *ScHis1* transformation cassette (387 bp). Lane 2: the middle region of the *ScHis1* transformation cassette (1753 bp). Lane 3: the downstream region of the *ScHis1* transformation cassette (287 bp). Lane 4: the *ScHis1* transformation cassette created by recombinant PCR (2379 bp).

Colony PCR using primers ScErg11_US_801 and ScHis1_ORF_493R followed by electrophoresis on 0.8% agarose gels (Appendix B.2, Figure B-2) detected the presence of a DNA fragment of the size (1604 bp) expected for insertion of the disruption cassette into the genome at the *ERG11* locus. Genomic DNA of his+ colonies indicating the presence of the cassette was extracted, the *ScHIS1* disruption cassette amplified using primers ScErg11_US_801 and
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ScErg11_ter_rev and sequenced. The oligonucleotide primer and ScHIS1 cassette sequences are listed in Appendix B.2. The ScERG11 mutant cassette at the PDR5 locus was also amplified from genomic DNA and sequenced to ensure that it had not been further modified due to deletion of the native ERG11. Deletion of native ERG11 gave viable transformants for all strains apart from the ScErg11p6×His G73W mutant. Multiple attempts to delete native ERG11 gene from this strain were unsuccessful. The strains created in this study are listed in Appendix A.1, Table A-1.

5.3.3 Azole susceptibility assays

Microdilution drug susceptibility assays (MIC_{80} determinations) were performed on wild type and mutant *S. cerevisiae* strains that expressed mutant ScERG11 from the PDR5 locus, without (AD2Δ background) and with (AD3Δ background) the native ERG11 deleted. A comparison of the susceptibilities of these strains to selected triazole drugs were used to evaluate the contribution of the native enzyme to the wild type and mutant phenotypes.

5.3.3.1 Single mutations in ScErg11p expressed from the PDR5 locus

The MIC_{80} values for the strains carrying a single mutation in ScErg11p expressed from the PDR5 locus are presented as a histogram in Figure 5.6 and tabulated in Table 5.2. It was expected that each mutant strain would show comparable or reduced susceptibility relative to the wild type ScErg11p6×His overexpressing strain, to some or all the triazoles tested, regardless of whether the native enzyme was expressed. In Chapter 3, we reported that removal of the endogenous ScErg11p from the wild type overexpressing strain did not significantly alter susceptibilities to the tested triazole drugs. This finding was interpreted as showing that the reduced triazole susceptibility of the strain was due to the constitutive overexpression of hexahistidine-tagged ERG11 from the pdr1-3 regulated PDR5 locus and was essentially unaffected by the expression of the native ERG11.

Strains overexpressing ScErg11p6×His containing the T322I and G464S mutations gave susceptibilities comparable to the wild type strain for each of the triazole drugs tested (FLC, VCZ and ITC). Deletion of the endogenous 108
ScERG11 from the AD2ΔScErg11p_T322I and AD2ΔScErg11p_G464S strains did not significantly alter susceptibility to any of these triazoles. This indicates that these mutant enzymes are fully functional and do not confer significant resistance when overexpressed.

In several instances, co-expression of the wild type and mutant enzymes led to increased susceptibility to all the triazole drugs compared to co-expression of the wild type enzyme from both the native and PDR5 loci. This was observed with the G73 series of mutants and the K151R mutant in the AD2Δ background. For the co-expressed K151R mutant the MIC$_{80}$ was reduced by about 0.2-0.5-fold for all three triazoles tested. For the G73 mutants the MIC$_{80}$ was reduced approximately 2.5-fold for FLC and VCZ but not ITC. These results suggested reduced functional expression of the co-expressed mutant enzymes relative to the wild type enzyme. Alternatively, the mutant enzymes could be hypersensitive to the triazoles. For the G73 mutants, there appeared to be a significant contribution to susceptibility patterns from the native enzyme, because its deletion to give the AD3Δ background reduced the azole MIC$_{80}$ at least 2-fold. The MIC$_{80}$s of the AD3ΔScErg11p strain for FLC, VCZ and ITC were 1.9 µg/mL, 0.248 µg/mL, and 0.105 µg/mL, respectively (Table 5.2). The AD3ΔScErg11p_G73E and AD3ΔScErg11p_G73R strains were 4 times more susceptible to FLC with an MIC$_{80}$ value of 0.5 µg/mL. The MIC$_{80}$s of the AD3ΔScErg11p_G73E/R strains were ~2-fold lower for ITC (0.05 and 0.06 µg/mL) and ~5-fold lower for VCZ (0.038 and 0.047 µg/mL) than for the control AD3ΔScErg11p strain (Table 5.2). These results are consistent with the expression of less functional mutant Erg11p or the mutant enzyme could be hypersensitive to the triazole drugs. Furthermore, we were unable to obtain a viable phenotype by deletion of native ScERG11 from AD2ΔScErg11p_G73W. This result showed that the native enzyme contributed to the active enzyme detected in the AD2ΔScErg11p_G73W strain and that the G73W mutation produces an inactive enzyme.

Strains AD2ΔScErg11p_K151R and AD3ΔScErg11p_K151R, with the native ERG11 deleted, were more susceptible to FLC, VCZ and ITC than AD2ΔScErg11p or AD3ΔScErg11p. The AD2ΔScErg11p_K151R strain had
higher MIC\textsubscript{80} values for FLC and VCZ than AD3\Delta ScErg11p\_K151R, while the MIC\textsubscript{80} for ITC was 0.064 \( \mu \text{g/mL} \) for both strains. The AD3\Delta ScErg11p\_K151R strain was twice as susceptible to FLC, VCZ and ITC (MIC\textsubscript{80} = 1.2 \( \mu \text{g/mL} \), 0.117 \( \mu \text{g/mL} \), and 0.064 \( \mu \text{g/mL} \), respectively) than AD3\Delta ScErg11p (MIC\textsubscript{80} = 1.9 \( \mu \text{g/mL} \), 0.248 \( \mu \text{g/mL} \), and 0.105 \( \mu \text{g/mL} \), respectively; Table 5.2). These data indicate that the native Erg11p contributes significantly to triazole sensitivity in the AD2\Delta ScErg11p\_K151R strain and that the mutant enzyme in the AD3\Delta ScErg11p\_K151R is either less well functionally expressed or hypersensitive relative to the control overexpressed wild type enzyme.
Figure 5.6 Triazole susceptibilities conferred by expression of wild type and mutant ScErg11p6×His. MIC₈₀ s for (a) FLC, (b) ITC and (c) VCZ. Strains retaining native wild type ERG11 (2Δ) or with the native ERG11 deleted (3Δ) overexpress either wild type or mutant hexaHis-tagged ScERG11 from the PDR5 locus. The following mutations were introduced into ScErg11p6×His: G73E, G73R, G73W, T322I, K151R and G464S. The MIC₈₀ s were determined as described in Section 2.5.1 after 48 hours at 30 °C. The histogram gives the mean MIC₈₀ for each clone using data obtained in triplicate measurements from at least 3 separate experiments. Error bars represent SEM. * A strain expressing ScErg11p6×His G73W with the native ERG11 deleted could not be obtained.
### Table 5.2 Liquid MIC<sub>80</sub> values for strains overexpressing wild type ScErg11p6×His or ScErg11p6×His with single mutations

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;80&lt;/sub&gt; µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
</tr>
<tr>
<td>AD2ΔScErg11p</td>
<td>1.90 (±0.10)*</td>
</tr>
<tr>
<td>AD3ΔScErg11p</td>
<td>1.90 (±0.02)</td>
</tr>
<tr>
<td>AD2ΔScErg11p_G73E</td>
<td>0.83 (±0.02)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_G73E</td>
<td>0.50 (±0.06)</td>
</tr>
<tr>
<td>AD2ΔScErg11p_G73R</td>
<td>0.91 (±0.03)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_G73R</td>
<td>0.50 (±0.01)</td>
</tr>
<tr>
<td>AD2ΔScErg11p_G73W</td>
<td>0.95 (±0.05)</td>
</tr>
<tr>
<td>AD2ΔScErg11p_T322I</td>
<td>2.10 (±0.17)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_T322I</td>
<td>2.02 (±0.06)</td>
</tr>
<tr>
<td>AD2ΔScErg11p_K151R</td>
<td>1.67 (±0.06)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_K151R</td>
<td>1.20 (±0.07)</td>
</tr>
<tr>
<td>AD2ΔScErg11p_G464S</td>
<td>1.82 (±0.10)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_G464S</td>
<td>1.80 (±0.10)</td>
</tr>
</tbody>
</table>

*Standard error of the mean is shown in parentheses. Cells were grown in SD media. MIC<sub>80</sub> values were determined as after 48 hours at 30°C.

#### 5.3.3.2 The ScErg11p6×His Y140F G464S double mutant

The strains AD2ΔScErg11p_D/M and AD3ΔScErg11p_D/M, which overexpress ScErg11p6×His Y140F G464S, were prepared and analysed separate from the rest of the mutants described in this chapter. Therefore their drug susceptibilities are presented separately. The AD2Δ and AD3Δ strains expressing the ScErg11p6×His Y140F mutant from the PDR5 locus have been described in Chapter 4. They showed significantly greater resistance to FLC and VCZ but not ITC than control strains overexpressing wild type ScErg11p×His. For the experiment shown in Figure 5.7 and tabulated in Table 5.3, strains AD3ΔScErg11p_Y140F and AD3ΔScErg11p were used as controls. Deletion of native ERG11 from AD2ΔScErg11p_D/M gave strain AD3ΔScErg11p_D/M with similar or slightly increased susceptibility to all three triazoles (Figure 5.7). This indicates that the native Erg11p makes a minimal contribution toazole sensitivity in these strains. The MIC<sub>80</sub> of the Y140F G464S strain AD3ΔScErg11p_D/M to FLC (5.5 µg/mL) was ~4-fold higher than for the AD3ΔScErg11p6×His (1.4 µg/mL) control strain and only slightly higher than for the ScErg11p6×His Y140F (4.3 µg/mL) strain (Figure 5.7; Table 5.3), despite the G464S mutation alone having no effect on FLC resistance (Figure
Similarly the MIC$_{80}$ of AD3ΔScErg11p_D/M for VCZ was 0.185 µg/mL compared to the wild type and Y140F values of 0.08 µg/mL and 0.131 µg/mL, respectively (Table 5.3). Thus, the ScErg11p6×His Y140F G464S enzyme is functional and confers enhanced resistance to the short-tailed triazoles FLC and VCZ but does not confer resistance to the long-tailed triazole ITC.

**Table 5.3 Liquid MIC$_{80}$ values for strains overexpressing ScErg11p6×His Y140F with single and double mutations (D/M, Y140F G464S)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC$_{80}$s µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
</tr>
<tr>
<td>AD3ΔScErg11p</td>
<td>1.4 (±0.2)*</td>
</tr>
<tr>
<td>AD3ΔScErg11p_Y140F</td>
<td>4.3</td>
</tr>
<tr>
<td>AD2ΔScErg11p_D/M</td>
<td>6.1 (±0.4)</td>
</tr>
<tr>
<td>AD3ΔScErg11p_D/M</td>
<td>5.5 (±0.08)</td>
</tr>
</tbody>
</table>

*Standard error of the mean is shown in parentheses. Cells were grown in SD media. MIC$_{80}$s were determined as after 48 hours at 30°C.
Figure 5.7 Triazole susceptibility of Y140F G464S ScErg11p6×His overexpressing strains to triazole drugs. (a) FLC, (b) ITC and (c) VCZ. Strains retaining wild type ERG11 (2Δ) and with the wild type ERG11 deleted (3Δ) overexpress either wild type or mutant hexaHis-tagged ERG11 from the PDR5 locus. The ScERG11 G464S Y140F double mutation was introduced as described in Section 5.3.1. Cells were seeded at OD$_{600nm}$ = 0.005. The MIC$_{80}$ values were determined as described in Section 2.5.1 after 48 hours at 30 °C. The histogram gives the mean MIC$_{80}$ for each clone using data obtained in triplicate measurements from at least 3 separate experiments. Error bars represent SEM.
5.3.4 Purification of ScErg11p6×His from mutant strains

The strains overexpressing wild type and mutant ScErg11p6×His used for protein purification had the endogenous ScERG11 removed (AD3Δ) in all cases except the G73W mutant. The AD2ΔScErg11p_G73W mutant strain was used for protein purification as deletion of native ERG11 gave a non-viable phenotype. This approach did not affect the quality of the protein preparation as the Ni-NTA affinity purification step binds His-tagged protein only. Crude membranes were routinely prepared from 6 L cultures grown on YPD to OD$_{600\text{nm}}$ = 9 – 12 in baffled flasks as described in Section 2.6.2. The Ni-NTA affinity chromatography and SEC purification was carried out as described in Sections 2.6.4 and 2.6.5, respectively. SDS-PAGE was used to determine the relative amount of protein recovered and the quality of the preparation at each stage of purification.

All mutant enzymes were purified either without added ligand (apo-enzyme) or co-purified with a triazole drug during the SEC step. Samples obtained during affinity chromatography and SEC purification gave Coomassie stained SDS-PAGE profiles that were similar for the wild type enzyme and the G73E/R/W, K151R and T322I mutant enzymes. In contrast, both the ScErg11p6×His G464S and the Y140F G464S double mutant preparations showed signs of denaturation/degradation. In the interest of brevity, the following sections show results for the G73R mutant, representing mutants with normal purification profiles, and for the G464S mutant and the Y140F G464S double mutant. Samples obtained during purifications from ~1 g of crude membrane protein, were retained for analysis by SDS-PAGE on 8% acrylamide gels (Figure 5.8). The molecular mass of ScErg11p6×His is ~62 kDa (arrow). The Coomassie stained gel shows clear overexpression of ScErg11p6×His in the lane with the crude membranes. The solubilisation of ScErg11p6×His from crude membranes with DM was efficient as most of the enzyme represented by the ~62 kDa band was found in the supernatant and not the insoluble fraction pelleted by centrifugation. There was some loss of co-migrating protein species during affinity purification. The red coloured fraction L, eluted from the Ni-NTA
matrix with 200 mM imidazole, was pooled, concentrated to 1 mL by centrifugal filtration and further purified by SEC.

![Figure 5.8 Purification of the ScErg11p6×His G73R (a) and G464S (b) mutant proteins. A Coomassie stained 8% acrylamide SDS-PAGE gel of samples of crude membranes (CM; 40 µg), supernatant (S) and pellet (P) obtained after solubilising crude membranes and precipitating the insoluble fraction by centrifugation, flow through (FT) after the supernatant (S) was incubated with Ni-NTA agarose beads, washes of ScErg11p6×His bound to the Ni-NTA column (W1 and W2), eluted fractions that were clear (E1 and E3) and the red coloured fractions pooled for further purification by SEC (L). The ScErg11p6×His band migrating just above the 55 kDa molecular weight (MW) marker is indicated by an arrow. 10 µL of sample mixed with 2 µL of 6× SDS loading dye was loaded on each lane.](image)

The volume of samples applied to both gels in Figure 5.8 was identical but the 62 kDa protein band in the L fraction obtained with the G464S mutant stained less intensely than that of G73R mutant. SEC of ScErg11p6×His G73R purified without added ligand gave a different elution profile at $A_{280\text{nm}}$ compared to enzyme purified with ITC (Figure 5.9 a,c). Despite the protein elution profiles being otherwise essentially identical, the addition of ITC to Ni-NTA purified protein before SEC purification gave a third $A_{280\text{nm}}$ peak on the SEC chromatogram. The additional peak is probably due to DMSO used to dissolve ITC, as related ligands such as FLC that are dissolved in water do not give this peak.

SDS-PAGE of peak 1 (up to 13 mL) of the SEC elution profiles (Figure 5.9a, c), included ScErg11p6×His plus bands migrating faster and slower (Figure 5.9b, d), indicating the presence of contaminants or aggregated and/or degraded material. The fractions eluted after the peak 2 (15 mL) contained bands of smaller size than ScErg11p6×His, indicating impurities or degraded protein.
Fractions eluting between 13.5 mL and 15 mL in the SEC chromatogram were dominated by the 62 kDa band and were combined and used for crystallisation. Those fractions contained impurities but at least 90% of the protein appeared to be ScErg11p6×His.

**Figure 5.9 SEC purification of ScErg11p6×His G73R.** (a) SEC elution profile of ScErg11p6×His G73R apo-enzyme and (b) the corresponding SDS-PAGE gel showing selected fractions from the SEC elution profile. (c) SEC chromatogram of ScErg11p6×His G73R co-purified with ITC (2 μM) and (d) the corresponding SDS-PAGE gel showing selected fractions from the SEC elution profile. In both (a) and (c), the majority of the G73R mutant enzyme eluted in peak 2 (13.5 – 15 mL fraction). Peak 3 is in the 15 – 17 mL fraction (c). MW, protein molecular weight markers. L, sample of the Ni-NTA affinity purified preparation loaded onto the SEC column. 10 μL of sample mixed with 2 μL of 6× SDS loading dye was loaded in each lane. Fractions eluted in peak 2 were diluted 2-4-fold while the rest were loaded without dilution.

The SEC chromatogram for ScErg11p6×His G464S showed a lower protein yield than the G73R mutant (compare Figure 5.9 and Figure 5.10). The lower absorbance (A<sub>280nm</sub>) profile of the red, heme-containing peak 2 for
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ScErg11p6×His G464S indicated a lower concentration of intact protein in this fraction. Peak 1 indicated protein aggregation and denaturation occurred to comparable extents for both mutants. SDS-PAGE did not reveal significant degradation products throughout the entire elution profile (Figure 5.10). Collectively, these results indicate that the G464S mutation in ScErg11p6×His leads to a smaller amount of enzyme in the membrane.

Figure 5.10 SEC purification of ScErg11p6×His G464S. (a) SEC chromatogram of ScErg11p6×His G464S apo-enzyme and (b) the corresponding SDS-PAGE gel with samples of selected SEC fractions. (c) SEC chromatogram of ScErg11p6×His G464S co-purified with ITC (2 μM) and (d) the corresponding SDS-PAGE gel. In both (a) and (c), the majority of the protein was recovered in peak 2 (the 13.5 – 15 mL fraction). Peak 3 is the 15 – 17.5 mL fraction. MW, protein molecular weight markers. L, sample of the Ni-NTA affinity purified preparation loaded onto the SEC column.

The ScErg11p6×His Y140F G464S band appeared to be present in crude membranes in amounts consistent with ScErg11p6×His G464S but the double mutation gave an enzyme that appeared to be significantly degraded/denatured during preparation (Figure 5.11). On detergent extraction and elution from the Ni-NTA matrix, an additional band was detected at ~55 kDa (Figure 5.11). Finally, the affinity purified ScErg11p6×His Y140F G464S protein preparation
appeared yellow rather than the usual red colour. These results indicated that ScErg11p6×His Y140F G464S may be unstable and degrades during purification. However, the enzyme is functional within the cell since deletion of the native ERG11 gives viable mutant strains in the AD3Δ background.

**Figure 5.11 Purification of ScErg11p6×His Y140F G464S.** (a) Detergent solubilisation and Ni-NTA affinity purification, (b) SEC purified protein. Analysis on 8% acrylamide SDS-PAGE gels of samples of crude membranes (CM; 40 µg), supernatant (S) and pellet (P) obtained after solubilising crude membranes and precipitating the insoluble fraction by centrifugation, flow through (FT) after the supernatant (S) was incubated with Ni-NTA agarose beads, washes of ScErg11p6×His bound to Ni-NTA column (W1, W2 and W3), eluted fractions (E1 and E3) that were clear. The yellow fraction pooled for further purification by SEC (L). Twice affinity purified protein (SEC). The ScErg11p6×His band indicated by an arrow migrates just above the 55 kDa molecular weight marker (MW). 10 µL of sample mixed with 2 µL of 6× SDS loading dye was loaded in each lane.

The ScErg11p6×His Y140F G464S double mutant was co-purified by SEC with ITC. The SEC purification confirmed that much of the affinity-purified protein was aggregated or degraded as evidenced by the presence of more intense peaks 1 and 3 in the elution profile (Figure 5.12a). Absorption spectra of the SEC purified fractions revealed that the majority of ScErg11p6×His Y140F G464S, which retained the heme (judged by the intensity of the heme peak, data not shown) was eluted in peak 2 (Figure 5.12a, fractions 13 – 15 mL). The ratio of the heme Soret peak at ~420 nm to the protein peak at 280 nm was much lower for the purified ScErg11p6×His Y140F G464S than the wild type enzyme (Figure 5.13) or any of the other mutants studied (Appendix C, Figure C-2).
Figure 5.12 SEC of ScErg11p6×His Y140F G464S co-purified with ITC. (a) SEC elution profile of ScErg11p6×His Y140F G464S. The protein in fraction 2 (13.5 – 15 mL) was pooled and concentrated to 1 mL and separated by SEC. (b) SEC chromatogram of the second SEC purification of peak 2 fraction of (a).

A highly purified sample of ScErg11p6×His Y140F G464S was obtained by using two rounds of SEC purification in the presence of ITC (Figure 5.12b). There was significant loss of protein using this approach and the preparation had a low heme content judging by its absolute absorption spectra Figure 5.13b. Control wild type protein co-purified with ITC shows a strong type II binding heme Soret peak with maximum absorbance at ~421 nm (Figure 5.13a). The absorbance spectrum of the ScErg11p6×His Y140F G464S preparation showed a weak heme Soret peak with maximum absorbance at ~417 nm (Figure 5.13b), indicating less heme is present and that ITC is not bound. CO binding studies confirmed that most of the affinity purified ScErg11p6×His Y140F G464S was denatured (Figure 5.14b).
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Figure 5.13 Absolute absorbance spectra of SEC purified (a) ScErg11p6×His and (b) ScErg11p6×His Y140F G464S double mutant. The wild type and the double mutant protein were co-purified with ITC. The heme Soret peak maxima were at ~421 nm and ~417 nm respectively.

5.3.5 Mass spectrometry

All mutations in ScErg11p6×His were confirmed by mass spectrometry of the SDS-PAGE purified 62 kDa band which was subjected to tryptic digestion. The peptide coverage for each mutant is described in Appendix D.

5.3.6 Spectral characteristics of mutant ScErg11p6×His

The absorption maximum of the heme peak (Soret band) in wild type ScErg11p6×His without added ligand is usually seen at ~417 nm (Figure 3.1). As discussed in Chapter 4 the Y140F/H mutation changed the electronic environment of the heme such that the absorption maximum of apo-enzyme is ~420 nm. The heme Soret peak maxima obtained for apo-protein samples are shown in Table 5.4.
Table 5.4 Soret peak maximum of wild type and mutant ScErg11p6×His without added ligand

<table>
<thead>
<tr>
<th>ScErg11p6×His</th>
<th>Soret peak maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>417.3 (± 0.18)*</td>
</tr>
<tr>
<td>G73E</td>
<td>418.0 (± 0.14)</td>
</tr>
<tr>
<td>G73R</td>
<td>418.8 (± 0.24)</td>
</tr>
<tr>
<td>G73W</td>
<td>418.0 (± 0.28)</td>
</tr>
<tr>
<td>T322I</td>
<td>418.5 (± 0.42)</td>
</tr>
<tr>
<td>K151R</td>
<td>419.2 (± 0.24)</td>
</tr>
<tr>
<td>G464S</td>
<td>416.5 (± 0.18)</td>
</tr>
<tr>
<td>Y140F G464S</td>
<td>414</td>
</tr>
</tbody>
</table>

* Numbers in brackets indicate standard deviation after 6 measurements for all enzymes apart from the double mutant, which was measured once.

5.3.6.1 Determination of cytochrome P450 concentration

The concentration of functional enzyme in ScErg11p6×His preparations was determined by carbon monoxide binding studies as described in Section 2.7.6. The ScErg11p6×His G73E/R/W, K151R and T322I mutants gave carbon monoxide difference spectra profiles similar to the wild type enzyme. Figure 5.14a, c shows the difference spectra obtained for the wild type enzyme and the G73R mutant, as a representative of the aforementioned mutants. The absorbance peak for the functional CYP450 enzyme should be at about 450 nm. In the case of ScErg11p6×His the reduced carbon monoxide bound protein gave a peak at 445 nm. The wild type and the G73R mutant enzymes with carbon monoxide bound, had a small peak at 417 nm, indicating the presence of some denatured CYP450 enzyme in the protein preparation. The majority of purified Y140F G464S double mutant enzyme was non-functional as indicated by the large peak at 417 nm Figure 5.14b and the slight shoulder at 445 nm. The G464S single mutant has a slightly bigger shoulder at 445 nm than the double mutant, but most of the protein was non-functional (Figure 5.14d). This result is consistent with weak absolute spectra Soret peaks obtained for the G464S single mutant and the Y140F G464S double mutant (Table 5.4). Consequently, the preparations provided insufficient functional enzyme to obtain type II difference spectra.
Figure 5.14 Carbon monoxide difference spectra for ScErg11p6×His wild type and mutant protein. The difference spectra are shown for ScErg11p6×His (a) wild type protein and the mutants (b) Y140F G464S, (c) G73R and (d) G464S. The difference spectra were obtained using equal amounts of ScErg11p6×His protein in a control sample, which was reduced by sodium dithionite, and a test sample, which was reduced by sodium dithionite after the solution was saturated with carbon monoxide. The peak at 445 nm represents functional CYP450 and a peak at 417 nm is non-functional CYP450.

5.3.6.2 Type II binding characteristics of ScErg11p6×His

Characteristic type II difference spectra were obtained as a result of triazole binding to ScErg11p6×His. The type II difference spectra had well defined peaks at 428 nm and well defined troughs at 410 nm (Figure 3.2). The $K_d$ values for wild type enzyme were calculated using the Hill equation (Table 5.5). The type II binding experiments were repeated and showed consistent $K_d$ values for ITC, VCZ and PCZ binding, but FLC binding showed slightly greater variation due to differences in the Hill number generated from the binding curves (Table 5.5). The maximum difference between the peak and the trough ($\Delta A_{\text{max}}$) showed some variation, which did not affect the $K_d$ values (Table 5.5). The $K_d$ values...
indicate that the binding is tight for all triazoles tested but VCZ and PCZ bind
tighter to ScErg11p6×His than FLC and ITC.

### Table 5.5 Type II binding characteristics of triazoles to ScErg11p6×His

<table>
<thead>
<tr>
<th>Triazole</th>
<th>ScErg11p6×His</th>
<th>(\Delta A_{\text{max}})</th>
<th>(K_d) (µM)</th>
<th>Hill no.</th>
<th>([\text{Azole}]_{0.5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLC 1*</td>
<td></td>
<td>0.048</td>
<td>0.074 (± 0.015)</td>
<td>3</td>
<td>0.43</td>
</tr>
<tr>
<td>FLC 2</td>
<td></td>
<td>0.044</td>
<td>0.141 (± 0.028)</td>
<td>1.5</td>
<td>0.27</td>
</tr>
<tr>
<td>FLC 3</td>
<td></td>
<td>0.049</td>
<td>0.211 (± 0.031)</td>
<td>2</td>
<td>0.46</td>
</tr>
<tr>
<td>VCZ 1*</td>
<td></td>
<td>0.051</td>
<td>0.082 (± 0.018)</td>
<td>2.7</td>
<td>0.40</td>
</tr>
<tr>
<td>VCZ 2</td>
<td></td>
<td>0.036</td>
<td>0.051 (± 0.019)</td>
<td>2</td>
<td>0.23</td>
</tr>
<tr>
<td>VCZ 3</td>
<td></td>
<td>0.037</td>
<td>0.069 (± 0.018)</td>
<td>2.1</td>
<td>0.28</td>
</tr>
<tr>
<td>ITC 1*</td>
<td></td>
<td>0.037</td>
<td>0.123 (± 0.027)</td>
<td>1.6</td>
<td>0.28</td>
</tr>
<tr>
<td>ITC 2</td>
<td></td>
<td>0.047</td>
<td>0.177 (± 0.062)</td>
<td>1.7</td>
<td>0.36</td>
</tr>
<tr>
<td>PCZ 1*</td>
<td></td>
<td>0.034</td>
<td>0.078 (± 0.023)</td>
<td>2.2</td>
<td>0.32</td>
</tr>
<tr>
<td>PCZ 2</td>
<td></td>
<td>0.031</td>
<td>0.057 (± 0.013)</td>
<td>2.3</td>
<td>0.29</td>
</tr>
</tbody>
</table>

* FLC 1 and VCZ 1 values are from Chapter 3, PCZ 1 and ITC 1 values are from Chapter 4. The rest are repeats carried out for this Chapter. Values in brackets indicate standard errors. \(^{a}\) Hill coefficient. IC\(_{50}\) value is denoted as \([\text{Azole}]_{0.5}\).}

### 5.3.6.3 Type II binding characteristics of ScErg11p6×His mutants

The binding of the triazole drugs FLC, VCZ, ITC and PCZ to ScErg11p6×His G73E/R/W, K151R, T322I mutant enzymes produced type II difference spectra. The type II binding spectra were obtained as described in Section 2.7.7. Representative difference spectra for FLC binding are presented in Figure 5.15.
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Figure 5.15 Type II difference spectra of FLC binding to ScErg11p6×His (a) G73E, (b) K151R, (c) G73W and (d) T322I mutants. The difference spectra was obtained by incremental additions of FLC dissolved in DMSO to 1 µM ScErg11p6×His mutant enzyme in the sample cuvette and a corresponding amount of DMSO added to 1 µM enzyme in the reference cuvette.

The G73E/W ScErg11p6×His mutants gave ΔA_{max} values (Table 5.6) and the peak (428 nm) and the trough (410 nm) wavelengths that were comparable to the wild type ScErg11p6×His (Table 5.5). The G73R ScErg11p6×His mutant had a slightly smaller overall ΔA_{max}, with the peak and the trough shifted slightly to 413 nm and 430 nm, respectively, for FLC binding and the trough only shifted to 415 nm for VCZ binding. The K151R and T322I ScErg11p6×His mutants showed smaller ΔA_{max} values compared to the wild type enzyme (Table 5.6). In addition, the peak and the trough values were
slightly shifted for the K151R mutant to 424 nm and to 408 nm for ITC and PCZ binding and the peak only to 426 nm with VCZ binding.

The triazole drugs were titrated against 1 µM ScErg11p6×His mutant enzyme. The absorbance difference between the peak and the trough of the type II spectra was plotted against the triazole concentration to generate binding curves (Figure 5.16). The resultant $K_d$ values are presented in Table 5.6. The obtained $K_d$ values are in the nanomolar range indicating that the binding is tight.

<table>
<thead>
<tr>
<th>ScErg11p</th>
<th>Triazole</th>
<th>Δ$A_{\text{max}}$</th>
<th>Equation*</th>
<th>$K_d$ (µM)</th>
<th>Hill no.</th>
<th>[Azole]$_{0.5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>FLC</td>
<td>0.044</td>
<td>Hill</td>
<td>0.141 (± 0.028)</td>
<td>1.5</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.036</td>
<td>Hill</td>
<td>0.051 (± 0.019)</td>
<td>2</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.033</td>
<td>Hill</td>
<td>0.123 (± 0.027)</td>
<td>1.6</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.034</td>
<td>Hill</td>
<td>0.078 (± 0.023)</td>
<td>2.2</td>
<td>0.32</td>
</tr>
<tr>
<td>G73E</td>
<td>FLC</td>
<td>0.043</td>
<td>Morrison</td>
<td>0.008 (± 0.007)</td>
<td>n/a</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.043</td>
<td>Hill</td>
<td>0.08 (± 0.025)</td>
<td>2.5</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.034</td>
<td>Hill</td>
<td>0.07 (± 0.04)</td>
<td>1.9</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.042</td>
<td>Hill</td>
<td>0.10 (± 0.04)</td>
<td>2.1</td>
<td>0.36</td>
</tr>
<tr>
<td>G73R</td>
<td>FLC</td>
<td>0.030</td>
<td>Hill</td>
<td>0.08 (± 0.02)</td>
<td>2.6</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.028</td>
<td>Hill</td>
<td>0.12 (± 0.10)</td>
<td>1.7</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.030</td>
<td>Hill</td>
<td>0.08 (± 0.04)</td>
<td>2.2</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.031</td>
<td>Hill</td>
<td>0.04 (± 0.02)</td>
<td>2.3</td>
<td>0.25</td>
</tr>
<tr>
<td>G73W</td>
<td>FLC</td>
<td>0.051</td>
<td>Hill</td>
<td>0.47 (± 0.097)</td>
<td>2.3</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.053</td>
<td>Morrison</td>
<td>0.014 (± 0.007)</td>
<td>n/a</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.047</td>
<td>Morrison</td>
<td>0.023 (± 0.009)</td>
<td>n/a</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.047</td>
<td>Morrison</td>
<td>0.005 (± 0.006)</td>
<td>n/a</td>
<td>0.44</td>
</tr>
<tr>
<td>K151R</td>
<td>FLC</td>
<td>0.032</td>
<td>Morrison</td>
<td>0.038 (± 0.018)</td>
<td>n/a</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.034</td>
<td>Morrison</td>
<td>0.007 (± 0.008)</td>
<td>n/a</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.025</td>
<td>Morrison</td>
<td>0.008 (± 0.007)</td>
<td>n/a</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.039</td>
<td>Morrison</td>
<td>0.323 (± 0.168)</td>
<td>n/a</td>
<td>0.79</td>
</tr>
<tr>
<td>T322I</td>
<td>FLC</td>
<td>0.028</td>
<td>Hill</td>
<td>0.12 (± 0.03)</td>
<td>2.0</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>VCZ</td>
<td>0.028</td>
<td>Hill</td>
<td>0.10 (± 0.04)</td>
<td>2.2</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>ITC</td>
<td>0.024</td>
<td>Hill</td>
<td>0.12 (± 0.03)</td>
<td>1.9</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td>0.027</td>
<td>Hill</td>
<td>0.09 (± 0.04)</td>
<td>2.1</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* The preferred equation to fit the data was chosen using the Akaike information criterion. Values in brackets indicate standard errors. Hill coefficient. IC$_{50}$ value is denoted as [Azole]$_{0.5}$.

The majority of the data fitted the Hill equation best. The Hill coefficients were in the range between 1.5 and 2.6. The use of the Morrison equation resulted in
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*K*ₐ values that were lower than when the data was fitted to the Hill equation. The *K*ₐ values were mostly in the range of 40 – 120 nM for the G73E/R and T322I mutants, which is comparable to the wild type enzyme. A *K*ₐ of 8 nM was calculated for FLC binding by the G73E mutant. All triazoles tested showed high binding affinity with the G73W mutant (5 – 23 nM) apart from FLC, which had a *K*ₐ value of 470 nM. The *K*ₐ values estimated for the K151R mutant ranged from 7 – 38 nM for the binding of FLC, VCZ and ITC with 323 nM for PCZ. However the high standard error values indicate that the curve was poorly fitted by the equation (Figure 5.16d).

Figure 5.16 Triazole binding curves to ScErg11p6×His wild type and mutants based on type II difference spectra. The triazole drugs (a) FLC, (b) VCZ, (c) ITC and (d) PCZ were titrated against 1 µM ScErg11p6×His enzyme. The absorbance difference between the trough and the peak was plotted against the triazole concentration to generate binding curves. The wild type enzyme is represented in solid circles (●), the G73E mutant solid squares (■), G73R mutant solid triangles (▲), G73W mutant solid diamonds (♦), K151R mutant hollow squares (□) and T322I mutant hollow triangles (△).
5.3.7 X-ray crystallographic analysis of ScErg11p6×His mutants

The X-ray crystal structures obtained for the ScErg11p6×His mutants described in this chapter are listed in Table 5.7. The data collection and refinement statistics are listed in Appendix G, Tables G-5 and G-6. Structure coordinate files (.pdb and .mtz) are available in Appendix G on a CD attached to the thesis. Protein purified without ligand (apo) did not form crystals as readily as protein with ligands (FLC or ITC), presumably due to ligand stabilisation of the protein.

Crystals picked less than a month after forming gave better diffraction patterns than the crystals that were left in the drops for over a month. In addition, freshly purified protein was usually more successful in producing well diffracting crystals than protein preparations that were left refrigerated for a period of time (over one week) prior to setting up crystallisation plates. The mutant structures are isomorphous to the previously obtained ScErg11p6×His structures PDB IDs: 4LXJ and 4K0F (Monk et al., 2014b) and 4WMZ (Chapter 3; Sagatova et al., 2015).

**Table 5.7 ScErg11p6×His mutant crystal structures.**

<table>
<thead>
<tr>
<th>ScErg11p6×His mutant</th>
<th>Apo*</th>
<th>FLC</th>
<th>ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>G73E</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>G73R</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>G73W</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>G464S</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>T322I</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

*No ligand was added during purification/crystallisation.

The crystal structures of mutants that had no ligand added are referred to as apo in this study, however there was density in the active sites of the apo ScErg11p6×His G73W and T322I structures. Monk et al., (Monk et al., 2014b) previously detected some density in the active site of the wild type ScErg11p6×His ‘empty pocket’ structure. The ScErg11p6×His K151R enzyme did not produce any crystals after several attempts using the vapour diffusion method with regular (45%) and increased (up to 50%) concentration of the precipitant PEG-400, as well as use of the microseeding technique described in Section 5.2.3.
5.3.7.1 ScErg11p6×His G73E and G73W in complex with ITC

The structures of ScErg11p6×His G73E and G73W in complex with ITC were obtained to the resolution of 1.98 Å and 2.15 Å, respectively (Appendix G, Table G-5). Molecular replacement showed that the complexes were essentially identical to the wild type structure but with evidence for the presence of the mutant residues, the ligand and changes in the conformation of some residues, as discussed in the following sections.

Both mutant structures showed a conformation of ITC different to that reported for the wild type (PDB ID: 4K0F; Monk et al., 2014b) and ScErg11p6×His Y140F/H in complex with ITC (PDB ID: 4ZDY and 4ZE3) structures (Chapter 4). The piperazine ring of ITC, which has been modelled as either a chair or twisted boat conformation, accommodates this difference as a type of hinge (Figure 5.17). The chair conformation of the six membered piperazine ring allows for the extended conformation of the drug.

![ITC](image)

Figure 5.17 Three conformations of ITC molecule detected in ScErg11p. The three conformations of ITC detected in three different structures are overlaid. The extended ITC conformation (C atoms green) with the piperazine ring in a chair conformation found in the wild type ScErg11p6×His (Monk et al., 2014b). The bent ITC conformation (C atoms yellow) with the piperazine ring modelled in a twisted boat shape found in the ScErg11p6×His G73E structure. The twisted ITC conformation (C atoms cyan) with the piperazine ring in a chair shape but with the tail of the molecule twisted found in the ScErg11p6×His G73W structure.
In the structure of ScErg11p6×His G73E in complex with ITC the piperazine ring adopts the twisted boat shape, which facilitates the bent shape of ITC. The tail of the ITC molecule was bent away from the E73 residue. There are possible \(\pi\)-anion interactions between the carboxylate group of the E73 residue and the triazole-5-one group of the ITC molecule (Mascal et al., 2002, Schwans et al., 2013). Chen et al. observed the same conformation for PCZ in complex with \(T. brucei\) CYP51 (resolution 2.6 Å and 2.7 Å; Chen et al., 2010). Due to the scattered \(Fo-Fc\) electron density maps obtained with two of their structures, they suggested that PCZ has two conformers in a dynamic equilibrium.

In the ScErg11p6×His G73E structure in complex with ITC no density was detected initially following phase solution for the \(\beta\) or \(\gamma\) carbons for the E73 but there was some density for the carboxyl group (Figure 5.18). After refinement, the \(2Fo-Fc\) density at the mutation site showed all the atoms of the glutamic acid residue.
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Figure 5.18 OMIT maps for ITC in complex with ScErg11p6×His (a) G73E and (b) G73W mutants. Electron density is shown for ITC and the site of the mutations G73E and G73W immediately following phasing and prior to modelling of the inhibitor. The final modelled ITC and the mutated residues are shown as sticks with C atoms represented in yellow. For all structures represented as sticks the N atoms are in blue, O atoms in red, Cl atoms in green and F atoms are in light blue. The heme is shown with C atoms in magenta in all subsequent figures. Unless otherwise stated the electron density map $2F_o - F_c$ (blue) is contoured at 1σ and the $F_o - F_c$ map is contoured at 3σ (green) or -3σ (red). Maps are CCP4 maps generated by Phenix for visualisation in PyMOL. Both maps were calculated using $F_{calc}$ refined from coordinates with no ligand or mutant residue present at the active site. All OMIT maps show the overlay of the final structure with the maps after molecular replacement (phaser). Arrows indicate the piperazine ring.

ITC in complex with ScErg11p6×His G73W adopted a different conformation to that seen with ScErg11p6×His G73E (Figure 5.17). The piperazine ring was modelled as the chair conformation but the tail of the ligand was slightly twisted in order to accommodate the W73 residue. There are π-stacking interactions between the W73 and the triazol-5-one group of ITC.

Structures of ScErg11p6×His G73W and G73E in complex with ITC showed no electron density in the egress channel, but some residues around the egress channel had different conformations compared to the wild type structure complexed with ITC (PDB ID: 4K0F). These residues had different rotamers and positions (Figure 5.19), particularly the side chains of the residues F241,
L129, F384, H128 and R98. In the G73E/W ITC structures those residues point into the egress channel and the F241 side chain occupies space within the “egress channel”. The same residues in the structure of the wild type enzyme in complex with ITC point away from the channel to accommodate the ligand in it.

Figure 5.19 Conformation of amino acid residues surrounding the egress channel. The structure of ScErg11p6×His G73E in complex with ITC was aligned with wild type ScErg11p6×His in complex with ITC (PDB ID: 4K0F). ITC and the residues around the egress channel are shown as sticks. C atoms are represented in yellow for the G73E structure with C atoms in grey for ITC molecule. C atoms are cyan for the wild type egress channel residues and the ITC molecule. ITC is in the bent conformation (piperazine ring as a twisted boat) in the G73E structure and in an extended conformation (piperazine ring as a chair) in the wild type structure.

The bend in the ITC molecule is more acute in the G73E structure than the G73W structure. The bend in ITC in the mutant structures shifts the tail of the ITC molecule closer to the egress channel, pushing out any potential product and allowing the side chain of F241 to occupy that region.
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There are three water molecules in the substrate channel close to the piperazine ring of ITC in the structure of ScErg11p6×His Y140F in complex with ITC (PDB ID: 4ZDY). Those waters form a hydrogen-bonding network with each other and the surrounding amino acid residues, which form a hydrophilic pocket. Residues S382, H381, S508 and F506 all interact with those water molecules via hydrogen bonds. One of the water molecules is hydrogen bonded to the piperazine ring of ITC. The piperazine ring acts as a hinge in order for the two more rigid components to fit into the substrate channel. In the presence of mutation at G73, this hinge adopts more unusual conformations in order to fit the channel when a larger residue compared to the wild type glycine occupies it. This prevents the piperazine from forming a hydrogen bond to the water molecule in both mutant structures.

5.3.7.2 ScErg11p6×His G73E and G73R mutant structures in complex with FLC

Structures of ScErg11p6×His G73E and G73R in complex with FLC were obtained at resolutions of 2.25 Å and 2.20 Å, respectively (Appendix G, Table G-5). The OMIT maps show good density for the ligand after molecular replacement in both structures (Figure 5.20). The side chains E73 and R73 in both FLC structures showed limited density after refinement. The E73 had no density immediately after molecular replacement and the 2Fo-Fc density only covered the β-carbon of the residue after refinement. The R73 residue lacks density for the β and γ carbons (Figure 5.20c). As described in Section 3.3.5 the binding of the short-tailed ligand FLC to wild type ScErg11p6×His (PDB ID: 4WMZ; Sagatova et al., 2015) involves a network of water-mediated hydrogen bonds. Both waters found in the wild type structure are present in these mutant structures in the same positions i.e. water molecules 743 and 790 are hydrogen bonded to FLC in both mutant structures.
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Figure 5.20 OMIT maps for FLC binding to ScErg11p6×His G73E and G73R. Electron density for (a) FLC in the ScErg11p6×His G73E structure, (b) FLC in the ScErg11p6×His G73R structure and (c) R73 in the G73R structure. The density E73 was not detected after molecular replacement. FLC and the R73 are shown as sticks with C atoms depicted in yellow.

There is density present in the egress channel in both the ScErg11p6×His G73E and G73R mutant-FLC structures but it is smaller than that previously reported in the wild type lanosterol structure (PDB ID: 4LXJ). The residues around the egress channel are in similar conformations to those in the wild type protein in complex with FLC. There are unmodelled densities in the substrate channels of both mutants, which are also present in the wild type ScErg11p6×His FLC structure (Figure 5.21). In the wild type structure the density is modelled as a water molecule, however it could possibly be a bigger molecule with a polar group (Figure 5.21a) as it is close enough to the peptide chain to make hydrogen bonds with the carbonyl group of residue P379 and amide group of H381. The structure of ScErg11p6×His G73R in complex with FLC has several smaller unmodelled blobs in the same region (Figure 5.21b) that are similar to those detected in the structure of G73E ScErg11p6×His in complex with FLC.
Figure 5.21 Unmodelled blobs of electron density in the substrate channels of (a) ScErg11p6×His and (b) ScErg11p6×His G73R in complex with FLC. C atoms of FLC (sticks) and amino acid side chains (lines) are presented in yellow and the secondary structure is presented as grey cartoon. The electron density maps after final refinement are shown in this figure.

Several amino acid residues in the fungus-specific loop (amino acids 432 - 457) have limited density (438 – 441) in all of the structures along with high B-factors. The structure of ScErg11p6×His G73E in complex with FLC had no density for those residues (Figure 5.22a) so they were not included in the model. In contrast, the ScErg11p6×His G73R structure in complex with FLC had reasonable density in that region (Figure 5.22b). In the rest of the mutant structure this region has been modelled in similar to that presented in Figure 5.22b.
Figure 5.22 Electron density maps for residues of the fungus-specific loop in ScErg11p6×His G73E (a) and G73R (b) mutants. Electron density is shown for residues S437, S438, Y439, S440, V441 and G442. Residues are depicted as sticks with C atoms in yellow. The density maps after final refinement are shown in this figure.

5.3.7.3 ScErg11p6×His T322I and G73W mutant apo structures

The ScErg11p6×His G73W and T322I apo structures have been determined to the resolutions of 2.10 Å and 2.35 Å, respectively (Appendix G, Tables G-5 and G-6). There was clear crystallographic evidence for the presence of the mutations (Figure 5.23), which was confirmed by the data obtained using mass spectrometry (Section 5.3.5).

Figure 5.23 OMIT maps of mutation sites in ScErg11p6×His (a) T322I and (b) G73W apo structures. Residues are depicted as sticks with C atoms in yellow.
Both apo structures showed some density in the active site (Figure 5.24). There is difficulty in determining what this density represents. However, the density at the heme iron could represent molecular oxygen (Figure 5.24), which was modelled into the ScErg11p6×His structure in complex with lanosterol (PDB ID: 4LXJ; Monk et al., 2014b).

Figure 5.24 Substrate channel density of (a) G73W and (b) T322I ScErg11p6×His apo structures. The density maps after final refinement are shown in this figure. Arrows point to the density beside the heme iron.

The density in the substrate channel of both apo structures did not accommodate water molecules. Following refinement of the structures with waters modelled into the substrate channel, there was considerable $Fo-Fc$ density around the waters (Figure 5.25).
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Figure 5.25 The (a) T322I and (b) G73W ScErg11p6×His apo structures with water molecules modelled into the substrate channel density. Density maps obtained after refinement with water molecules in the substrate channel are shown.

The carbonyl group of residue G314 in the ScErg11p6×His T322I and G73W apo structures occupies a similar position in the ScErg11p6×His lanosterol structure (PDB ID: 4LXJ). An overlay of the mutant apo structures with ScErg11p6×His in complex with FLC (PDB ID: 4WMZ) showed that the G314 carbonyl group is closer (2.8 Å) to the hydroxyl group of T318 and the occupant of the density at the heme iron in the apo structures compared its position in the triazole bound structure (Figure 5.26). The coordination of the triazole ring to the heme iron pushes G314 away from the heme and the T318 hydroxyl group (3.1 Å). The G314 carbonyl in the apo structure is shifted by 1.7 Å upon binding of the triazole drug. The same overlay also reveals that the distance between the carbonyl of G314 in the apo structure and the closest carbon atom of FLC in the ScErg11p6×His FLC structure is 1.9 Å. In the ScErg11p6×His FLC structure this distance is 3.4 Å.
Figure 5.26 Positions of residues G314 and T318 in ScErg11p6×His in complex with FLC and the ScErg11p6×His G73W apo structure. (a) Front view, (b) side view (rotated approximately 90°). Helix I residues are presented in cyan for the ScErg11p6×His in complex with FLC (PDB ID: 4WMZ) and the hydrogen bond distance between the carbonyl of G314 and the hydroxyl of T318 is 3.1 Å. Helix I residues are presented in yellow for the ScErg11p6×His G73W apo structure, and the hydrogen bond distance between the carbonyl of G314 and the hydroxyl of T318 is 2.8 Å. Hydrogen bonds are represented by yellow dashed lines.

The residue M509 located in the substrate channel is in a different conformation in the ScErg11p6×His G73W apo structure compared to the wild type structure (Figure 5.27) and the rest of the mutants, including the ScErg11p6×His T322I apo structure. This indicates that there is flexibility of this and surrounding residues in the substrate channel. In the ScErg11p6×His G73W structure M509 occupies space in the substrate channel, pointing towards the egress channel (Figure 5.27, M509 in cyan), whereas in the structure of wild type ScErg11p6×His in complex with FLC this residue is tucked away from occupying space in the channel (Figure 5.27, M509 in yellow). There is also a small overlap between the blobs of density in the substrate channel of wild type ScErg11p6×His in complex with FLC (Figure 5.21) and the positioning of the M509 residue in the G73W apo structure. However, that density is much larger than the side chain of M509. This may either indicate this residue exists in multiple conformations or that there is an additional ligand present at this residue.
Figure 5.27 Conformation of the M509 residue in the ScErg11p6×His G73W apo structure compared to the ScErg11p6×His apo structure. The ScErg11p6×His G73W apo structure was aligned to the structure of wild type ScErg11p6×His in complex with FLC structure. The M509 and S508 residues are shown as sticks with C atoms coloured cyan in the G73W apo substrate channel and in yellow in the wild type FLC structure.

The amino acid residues around the egress channel in both apo structures were found to be in similar conformations to those found with wild type ScErg11p6×His in complex with FLC.

5.3.7.4 ScErg11p6×His T322I mutant structures

The structures of ScErg11p6×His T322I mutant enzyme in complex with ITC and FLC were determined at resolutions of 2.35 Å and 2.00 Å, respectively (Appendix G, Table G-6). There is clear evidence for the presence of the mutation and the ligand in both structures (Figure 5.28). The binding of the ligands ITC and FLC was found to be similar to that seen in the wild type ScErg11p6×His structures (PDB ID: 4WMZ and 4K0F).
Figure 5.28 OMIT maps for the mutation site T322I and the ligands (a) FLC and (b) ITC in structures of ScErg11p6×His T322I. C atoms are shown in yellow for I322, ITC and FLC. Helix I is presented as yellow cartoon.

The I322 residue is located on helix I. In both structures the distance between the closest atoms of I322 (Cγ1) and heme (CBB carbon) was found to be ~4 Å and the δ carbon of I322 is pointing away from the heme (Figure 5.28). In the wild type ScErg11p6×His FLC structure (PDB ID: 4WMZ) the side chain hydroxyl of T322 makes a hydrogen bond to the carbonyl group of T318 and a water molecule (Figure 5.29b). These interactions were disrupted due to the mutation to a non-polar residue (Figure 5.29a). The hydrogen bond between the amide of residue 322 and carbonyl of T318 was the same in both structures, maintaining the integrity of the helix.
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5.3.7.5 ScErg11p6×His G464S mutant structures

The ScErg11p6×His G464S mutant structures in complex with ITC and FLC have been obtained at the resolution of 2.24 Å and 2.15 Å, respectively (Appendix G, Table G-6). There was clear evidence for the presence of the mutation and the ligand in both structures (Figure 5.30). The binding of ITC and FLC was found to be similar to the binding of these ligands in the structures of wild type ScErg11p6×His (PDB ID: 4WMZ and 4K0F).

Residue S464 is located on the K’L loop several residues away from the conserved cysteine 470. The S464 is hydrogen bonded to the propionate group of the heme, replacing the water molecule, which is present in the structure of wild type ScErg11p6×His (PDB ID: 4WMZ; Figure 3.4). The hydrogen bond of S464 to the heme propionate did not noticeably alter the tilt of the heme. A

Figure 5.29 Residue 322 interactions in the (a) T322I and (b) wild type ScErg11p6×His structures in complex with FLC. C atoms are shown in yellow for I322, T322 and T318 and in cyan for FLC. Helix I is presented as yellow cartoon. Hydrogen bonds are presented as yellow dashed lines.
Ramachandran plot indicates that the conformation of this residue is an outlier in both structures. The G464 residue in the wild type structures is in the allowed region of the Ramachanran plot.

Figure 5.30 OMIT maps for mutation site G464S and ligands (a) FLC and (b) ITC in ScErg11p6×His G464S structures. C atoms are shown in yellow for S464, ITC and FLC. Helix L and the K’L loop are presented in yellow cartoon. The yellow dashed line represents a hydrogen bond.
5.4 Discussion

The aim of this study was to reproduce and characterise resistance-conferring CYP51 mutations from clinical isolates of fungal pathogens using *S. cerevisiae* Erg11p as a surrogate. All chosen mutations were successfully introduced into the *S. cerevisiae* overexpression system. All strains with mutant enzymes, apart from the ScErg11p6×His G73W, were viable when the native ERG11 was deleted. Contrary to expectation, the introduction of single mutations into ScErg11p6×His did not produce strains with triazole resistant phenotypes. However, biochemical and crystallographic investigation of the mutant enzymes showed that each mutation modified the enzyme when compared to wild type ScErg11p6×His.

As noted in the Section 5.3.7.2, residues 438 – 441 in the fungus-specific loop (amino acids 432 - 457) have limited density and have high B-factors in all of the mutant structures. This indicates that the fungus-specific loop is a flexible entity. The flexibility of the loop may be involved with interactions with its cognate CYP450 reductase. In the ScErg11p6×His structures there are two charged residues R469 (K’L loop) and E473 (helix L), which interact directly and indirectly, respectively with the conserved cysteine (470), as well as two residues, S437 and S438, from the fungus-specific loop (Figure 5.31). The two serine residues of the fungus-specific loop may interact with the charged residues and move away upon the binding of the reductase. Hargrove et al. also see a flexible fungus-specific loop in the structures of the *A. fumigatus CYP51B* (PDB IDs: 4UYM, 4UYL; Hargrove et al., 2015). They propose that the fungus-specific loop is involved in the regulation of the electron transfer from the CYP450 cognate reductase. Their findings are consistent with what is seen in our structures.
Figure 5.31 Conformation of amino acid residues potentially involved in electron transfer. The ScErg11p6×His in complex with FLC structure (PDB ID: 4WMZ) is shown as a representative in this image. Fungus-specific loop residues (FSL) S437 and S437, helix L residue E473, loop K'L residues R469 and C470 and the heme are shown in sticks. C atoms of the main chain are grey. Electrostatic interaction and hydrogen bonds are shown as yellow dashed lines.

5.4.1 Technical considerations

We initially used Soret peak shift experiments to estimate the dissociation constants ($K_d$) for triazole drug binding to ScErg11p6×His wild type and mutant enzymes (Appendix E). The enzyme concentration for those experiments was determined using the $A_{280}$ peak of an SEC purified preparation. This approach provided an estimate of the total amount of protein rather than the amount of functional CYP450 protein in the preparation, unlike the carbon monoxide binding spectral assay (Section 5.3.6.1). The Hill equation (Equation 2.4), which does not take into account the enzyme concentration along with the
rearrangement of the Morrison equation (Equation 2.5), which does, were both used to estimate the $K_d$ values. Since the enzyme concentration was inaccurate this may have resulted in an inappropriate equation being used to fit the data. Hence, the results for the Soret peak shift experiments are presented in the Appendix E.

Given these problems, it was decided to use the carbon monoxide spectral assays to more accurately estimate the concentration of functional CYP450 enzyme present in protein preparations (Omura and Sato, 1964a). Still, Soret peak shift experiments can give reliable $K_d$ values if the protein concentration of functional CYP51 is estimated using the carbon monoxide method. Locuson et al. (Locuson et al., 2007) compared the accuracy of the type II difference spectra to the Soret peak shift. They have concluded that although both methods can give similar $K_d$ values, the type II difference spectra are more informative than the heme Soret peak shift, especially in the case of human drug metabolising CYP450s. The type II difference spectra can reveal if there is more high spin or low spin CYP450 present prior to addition of the drug based on the trough. A trough at 410 nm indicates that the CYP450 iron is mostly in low spin form while a trough at 390 nm represents high spin. All of the type II binding studies in this thesis revealed a trough around 410 nm, consistent with a low spin state.

The majority of studies on CYP51 enzymes use type II binding studies to characterise azole binding (Park et al., 2010, Warrilow et al., 2010a, Warrilow et al., 2010b, Zelenko et al., 2014, Podust et al., 2007). In the present study type II difference spectra were used to estimate the $K_{d8}$ for triazoles. A recent study by Warrilow et al. shows that there are significant problems associated with estimation of $K_d$ values using purified enzyme (Warrilow et al., 2015). This is further discussed in Chapter 6.

**5.4.2 Apo enzyme mutant structures**

Structures were obtained for the ScErg11p6×His G73W and the T322I without added ligands. They reveal that there is some movement of helix I residue G314 (Figure 5.26). The position of the carbonyl group of residue G314 found in
those structures is similar to the wild type lanosterol structure (PDB ID: 4LXJ) (Monk et al., 2014b). Residue G314 is part of the sequence G310VLMG314, which is a consensus motif (GXXXG) involved in binding lanosterol (Monk et al., 2014b). G314, together with T318, is involved in creating the characteristic kink of helix I. The hydrogen bond between the side chain of T318 and carbonyl of G314 disrupts the regular hydrogen-bonding pattern of an $\alpha$-helix ($i, i+4 \text{ NH} \cdots \text{O=C}$). In cytochrome P450$_{\text{cam}}$ of $P. \text{putida}$ helix I residue T252, which corresponds to T318 of ScErg11p, has been implicated in protonation of molecular oxygen. T252 is not the direct proton donor, but it is postulated to stabilise the Fe-OOH complex by acting as a H-bond acceptor (Poulos, 2007). The apo structures discussed here reveal that the movement of the carbonyl group of G314 places it closer to the heme iron than the side chain of residue T318. In addition, a rotation of approximately 180° of the side chain T318 would be required in order to interact with any oxygen species. This indicates that G314 in ScErg11p could be involved in catalysis by stabilising the Fe-OOH complex.

Both apo structures are not completely without ligand. Weak electron density in the active site indicates presence of a ligand (Figure 5.24). The ligand seems to have low occupancy, presenting a challenge in its identification. The ligand could be imidazole as it is used in affinity purification. However, prior to setting crystals the protein preparation is checked spectrophotometrically. The presence of significant iron coordinated imidazole would be obvious as this should move the heme Soret peak from ~417 nm to ~425 nm. Also, as indicated by Figure 5.25, water molecules do not occupy the density. Other possibilities include spectrophotometrically undetectable levels of triazole contamination from the SEC column or the presence of traces of lanosterol, the natural ligand of ScErg11p that might have remained in the active site of a portion of the enzyme during purification.

5.4.3 G73E/R/W ScErg11p6×His mutants

Collectively the G73E/R/W ScErg11p6×His mutant-overexpressing strains conferred increased susceptibility to FLC, VCZ and ITC. These susceptibility patterns do not appear consistent with previous literature for strains of $A$. 
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*fumigatus* CYP51A G54 mutants (Escribano et al., 2012, Tashiro et al., 2012, Warrilow et al., 2015, Mann et al., 2003). The G54 mutants of *A. fumigatus* have also been reported to confer resistance to ITC and PCZ when expressed in *S. cerevisiae* from a plasmid, with levels of the native enzyme regulated by a TET-promoter system (Alcazar-Fuoli et al., 2011). It would be interesting to partially replicate this approach by overexpressing AfCYP51A in our yeast expression system and abolishing expression of the native enzyme by deleting *ScERG11*. Unfortunately, AfCYP51A has yet to be successfully expressed in our expression system.

Deletion of the native *ERG11* ORF from AD2ΔScErg11p_G73W resulted in a non-viable strain. This suggests that the presence of the bulky tryptophan residue at this position in ScErg11p6×His disrupts the catalytic activity of the enzyme. The possibility of G73W mutant enzyme being misfolded or not expressed in sufficient amounts is excluded as the enzyme was purified from crude membranes of AD2ΔScErg11p_G73W strain and characterised by X-ray crystallography and with the carbon monoxide and type II spectral studies. Reduction in the catalytic activity of ScErg11p6×His by the mutations at G73 may explain the susceptible phenotypes of those mutants. It may also explain why the native *ERG11* contributes more significantly to resistance levels in the G73E/R mutant strains than in other mutants (Figure 5.6).

The MIC80 values obtained for the G73E/R/W mutant strains show they are more susceptible to FLC and VCZ than to ITC compared to the wild type enzyme overexpressing strain. However, Soret heme peak shift and type II binding studies did not reveal significant differences between the binding affinities of short-tailed and long-tailed triazoles to the G73E/R/W mutant enzymes. Our crystallographic data indicates that there are extra interactions between the site of the G73E/W mutations and the triazole-5-one ring in the tail of the long-tailed triazole ITC (Figure 5.18). The extra interactions of the G73E/W enzymes with long-tailed triazoles may enable the drug to dissociate less quickly after it binds. However, as previously stated, there are no obvious differences in the *Kd* values between short and long triazole drug binding for these mutants. Compared to the wild type enzyme the G73E/R/W mutants bind
triazoles with similar affinity or tighter, apart from G73W mutant FLC affinity (470 nM). It is noted that all enzyme preparations showed tight binding. This may have masked mutation-specific differences in affinities in assays that required 1 μM enzyme. The development of an assay of fungal CYP51 activity that used concentrations of enzyme well below $K_d$ values would be of considerable value in resolving this problem. It is therefore of considerable interest that Riley et al. (Riley et al., 2015) have recently developed a fluorescence-based assay for T. cruzi CYP51 that uses benzyloxymethyloxycyanocoumarin (Vivid® BOMCC) as a substrate that yields the fluorescent product 3-cyano-7-hydroxycoumarin.

Egress channel residues have been found to be in a different conformation in two mutant structures (ScErg11p6×His G73E/W) in complex with ITC compared to the wild type structure. The flexibility of these residues around the egress channel suggests that they have to move in order to accommodate the demethylated product.

*A. fumigatus* has two CYP51 homologues and, as far as we are aware, the G54 mutation has been found only in CYP51A (Mann et al., 2003). One homology modelling study of AfCYP51A, based on the human CYP51 structure (PDB ID: 3JUS; Strushkevich et al., 2010), was used to suggest that G54 mutations have the potential to block the channel entry or directly interact with the long-tailed triazoles (Fraczek et al., 2011).

Our findings indicate that the mutation in the mouth of the substrate channel of ScErg11p×His may reduce the catalytic activity of the enzyme and provide extra interactions for the binding of the long-tailed triazoles. As the writing of this thesis was in the final stages Warrilow et al. (Warrilow et al., 2015) published a study on the AfCYP51A G54W mutant. The assay of AfCYP51A G54W mutant reconstituted with the cognate reductase has demonstrated that the mutation reduces the catalytic activity of AfCYP51A 3-fold compared to the wild type enzyme. In addition this mutation was shown to confer 11- and 34-fold higher IC$_{50}$ values to ITC and PCZ, respectively, compared to a moderate 2-fold increase to VCZ.
One way the differential effect of mutations in the mouth of the substrate channel on ScErg11p and AfCYP51 could be explained is if the AfCYP51A substrate channel opening is narrower than it is in ScErg11p. The presence of a bulky residue at the G54 mutation site of AfCYP51A could provide insufficient space to accommodate the tail of a long-tailed triazole. The result would be reduced binding of long-tailed triazoles and without affecting the binding of short-tailed triazoles. However, homology models of AfCYP51A based on the ScErg11p provide no convincing evidence for this hypothesis.

5.4.4 T322I and K151R ScErg11p6×His mutants

The triazole susceptibilities of the mutant strains overexpressing ScErg11p6×His T322I was comparable to the strains overexpressing wild type ScErg11p6×His. The type II binding studies also revealed that the affinity of the triazole drugs to the T322I mutant enzyme are comparable to those of the wild type enzyme. The $K_d$ values obtained for this mutant from the heme Soret peak shift experiments show too much variation to be reliable, for reasons discussed above. However, the heme Soret peak shift was smaller at the binding of FLC, ITC and PCZ compared to the wild type enzyme. In addition the $\Delta A_{\text{max}}$ from the type II binding spectra was smaller for all triazoles tested with this mutant enzyme. This may indicate that the heme environment is somehow altered, even though the T322 residue does not interact directly with the heme. The change from a hydrophilic to a hydrophobic residue may have had an influence on the heme as it is a conjugated molecule. Crystallographic data also shows that the mutation alters some interactions of the helix I. The abolition of polar interactions between T322 and T318 seen in the wild type structure (PDB ID: 4WMZ; Figure 5.29) may have also had an effect on the heme.

Perhaps I301 of AfCYP51A (T322I in ScErg11p6×His) is involved in the intrinsic FLC resistance, but in combination with other factors not present in the ScErg11p6×His. This may explain why the ScErg11p6×His T322I mutant showed an unchanged pattern of triazole susceptibility.

The K151R mutant strain had slightly increased susceptibility to all triazoles tested, specifically after removal of the native ERG11. This mutant failed to
produce crystals even though the K151R mutation is adjacent to the active site (K151 forms a salt bridge with a heme propionate), which should have allowed for all the contacts involved in the formation of the crystal lattice to remain as they are in the wild type enzyme. The mutation may have had an effect on the overall fold of the protein despite the purification profile and the carbon monoxide binding studies showing that the mutant protein retained functional heme after purification. In the wild type ScErg11p6×His enzyme K151 is adjacent to the propionate group of the heme and forms hydrogen bonds with the carbonyl groups of R469 and R467, residues that are located on the same loop as the conserved C470 (Figure 5.32). Replacement of K151 with a bulkier residue might result in the complete loss of these interactions if the arginine adopts a different conformation to the lysine. Alternatively, the arginine could retain a similar conformation to the lysine and push residues R469, R467 and the heme more towards the cavity of the substrate channel. The change of interactions with the heme may result in an altered heme environment producing the changes of the heme Soret peak shift and the $\Delta A_{\text{max}}$ observed in the type II binding studies.
5.4.5 The ScErg11p6×His G464S and Y140F G464S mutants

The strain overexpressing ScErg11p6×His G464S did not show any change in the triazole susceptibility compared with the strain overexpressing ScErg11p6×His. However introduction of the second mutation Y140F into ScErg11p6×His reduced the susceptibility of the strain to short-tailed triazoles, FLC and VCZ more than the single Y140F mutation (Table 5.3). The single G464S mutation deleteriously affected function of the purified enzyme preparation and the introduction of the second mutation appeared to make the protein even less stable, with the heme poorly retained within the protein. Carbon monoxide binding studies showed that ScErg11p6×His G464S and G464S Y140F enzymes were both recovered predominantly in non-functional and probably denatured states. The absolute spectra of the double mutant

Figure 5.32 The interactions of residue K151 in ScErg11p6×His FLC structure. C atoms are shown in yellow. Helix C is presented as a yellow cartoon. Hydrogen bonds and a salt bridge are presented as yellow dashed lines.
confirmed that the enzyme had lost the heme i.e. the intensity of the heme Soret peak was very low compared to the protein peak in enzyme preparations co-purified with ITC. The heme Soret peak of the ScErg11p6×His G464S without added ligand (apo) was detected at 416.5 nm (Table 5.4). This was lower than for the wild type enzyme (417 nm) and the rest of the mutants considered, however still within the margin of error of the spectrophotometer. The intensity of the heme Soret peak of the G464S single mutant was comparable to the wild type enzyme preparation and like the heme Soret peak shift detected was most likely due to the residual functional protein in a mixed preparation of detergent solubilised affinity-purified enzyme. The carbon monoxide binding studies confirmed the non-functional state of the majority of the ScErg11p6×His G464S mutant. The heme Soret peak of the double mutant Y140F G464S enzyme was detected at an even lower wavelength (414 nm). It is possible that microsomal preparations will give more stable mutant enzyme samples for both the single and double mutant that could then be used for Soret peak shift or type II difference spectral experiments to better indicate the affinity of triazole drugs to this mutant. This approach was used previously for the C. albicans CYP51 G464S mutant (Kelly et al., 1999b).

The G464S mutation creates a hydrogen bond to one heme propionate group (Figure 5.30) and the Y140F mutation abolishes a hydrogen bond to the other. As well as making the protein unstable this change could potentially tilt the heme producing a resistant phenotype, implying that the orientation of the heme is important in resistance and protein stability. In contrast, tilting of the heme was not observed in any of the single mutant structures in complex with triazole drugs.

5.4.6 Summary

The G464S mutation, which excluded a water molecule and led to a new hydrogen bond with a heme propionate, synergised with the triazole resistance phenotype of ScErg11p6×His Y140F. The double mutation resulted in the enzyme becoming unstable for purification. In contrast, all single residue mutants tested in this chapter gave enzymes stable to purification and that could be crystallized, apart from K151R. However, in whole cells the overexpressed
mutant ScErg11ps were either more susceptible to triazole drugs or showed resistance levels similar to the strain overexpressing the wild type enzyme. This finding, despite the relatively high sequence similarity between the fungal CYP51s, indicates ScErg11p to be a model that may not always adequately reflect the phenotypes of the wild type and mutant CYP51s of pathogenic fungi. Several mutations in proximity of the heme, notably G464S, K151R and the double mutation Y140F G464S, alter hydrogen bonding and electrostatic interactions to a heme propionate, thus changing the environment of the heme and affecting enzyme stability. The T322I mutation of helix I alters the environment of the heme through indirect interactions, probably via T318 residue in ScErg11p. The G73E/R/W mutations in the substrate channel appear to reduce the catalytic activity of the ScErg11p6×His enzyme but have no effect on the heme environment. The E/W73 residues interact with the tail of the long-tailed triazole ITC but how this affects enzyme affinity for this drug requires further experimentation.
Chapter 6

Conclusions and future work
The endoplasmic reticulum-associated enzyme lanosterol 14α-demethylase (Erg11p or CYP51) catalyses a key step in fungal ergosterol biosynthesis and is a major drug target used in the treatment of fungal disease. Mutations in CYP51 that reduce the efficacy of triazole drugs are one of several mechanisms that fungal species have evolved to overcome the mechanism of action of these antifungals. The research described in this thesis set out to investigate the molecular basis of fluconazole binding to its target and how single site mutations in CYP51 that have been reported to confer resistance to triazole antifungals in clinical isolates of fungal pathogens modify the affinity of the enzyme for these drugs. The S. cerevisiae enzyme ScErg11p has been used as a surrogate to reproduce such mutations, principally because it was the only fungal CYP51 to have its structure determined at high resolution by X-ray crystallography (Monk et al., 2014b). Previous studies that sought to interpret the molecular basis of triazole binding and resistance relied instead on the structural homology models that used, as templates, crystal structures of CYP51 enzymes from unrelated organisms such as M. tuberculosis (26-28% sequence identity with fungal CYP51s; Xiao et al., 2004, Sheng et al., 2004, Sheng et al., 2009). The sequence similarity of CYP51s in fungi varies considerably (Lepesheva and Waterman, 2011), for example the sequence identity between ScErg11p and CaCYP51 is 65% and ScErg11p and AfCYP51A is only 51%. While the overall sequence similarity among CYP51s of fungal pathogens is not high, the substantial conservation of amino acid residues within the active site and entry channel that are likely to affect interactions with triazole drugs led us to believe that the yeast enzyme could be used as a relevant and informative experimental model. Since resistant phenotypes found in fungal pathogens could be reproduced in the ScErg11p Y140F/H and Y140F G464S mutants, albeit not as strongly, it is likely that the mechanism of action of these mutations is conserved across a variety of fungal species. On the other hand, the rest of the CYP51 resistance mutations reproduced in this study either had no effect on drug susceptibility or conferred increased susceptibility in our yeast expression system. Possible reasons for these apparent discrepancies are discussed in the next section.
All ScErg11p6×His mutant enzymes were successfully expressed in a S. cerevisiae overexpression system. The mutant strains were characterised by determining triazole susceptibilities and the purified mutant enzymes were investigated using biochemical and crystallographic techniques. High-resolution ScErg11p6×His mutant structures have been obtained in the “apo” state by not adding ligands or in complex with added triazole drugs. The “apo” state was not entirely ligand free as discussed in Section 5.4.2.

The effects of specific mutations on protein structure and function have been discussed in previous chapters. The structural impact of each mutation analysed appeared to be localised, with all mutant enzyme structures obtained being isomorphous to the wild type enzyme (Monk et al., 2014b, Sagatova et al., 2015). The wild type and the mutant enzymes were all crystallised in a monoclinic space group P2₁, all enzymes adopted the same fold and only in a few instances were residues at distances greater than 5 Å from the site of the mutation significantly altered in conformation compared to the wild type enzyme.

6.1 Limitations of this study

The S. cerevisiae system applied in this study used the pdr1-3 gain of function mutation in the Pdr1p transcriptional regulator to constitutively overexpress open reading frames of interest from the PDR5 genomic locus (Lamping et al., 2007, Monk et al., 2014a). This overexpression system allowed full-length, properly folded ScErg11p6×His to be hyper-expressed in a host that is hypersensitive to triazole drugs due to the deletion of 7 ABC transporters, including those primarily responsible for the efflux of triazole drugs in this organism. This system had the following advantages:

1. Ease of genetic construction via insertion of a gene for overexpression into the PDR5 locus via homologous recombination.
3. The recombinant protein was expressed at levels that gave a high signal-to-noise ratio versus the native enzyme.
4. Deletion of the ABC transporters allowed the accumulation of triazole drugs in the cell without the complication of drug efflux, giving unambiguous growth inhibition curves with the triazole drugs and allowing MIC changes to be attributable to the overexpressed ScErg11p.

The disadvantages of this system included:

1. A major portion of the overexpressed enzyme in ScErg11p-eGFP constructs (Monk et al., 2014b) appeared to be clumped near the nuclear membrane (the rest localises in the endoplasmic reticulum), possibly meaning that a significant fraction of the enzyme may be isolated from the ergosterol biosynthetic pathway or not physiologically active.

2. The overexpression of ScErg11p resulted in only a 3-4-fold increase in triazole resistance (whole cell assays), which was at a level significantly lower than might be expected for the observed increase in expression of the recombinant protein compared with the native protein.

CYP450 proteins require reduction by their cognate NADPH-cytochrome P450 reductase. If the endogenous reductase becomes rate limiting it is possible that only a fraction of the overexpressed enzyme is physiologically active and able to contribute to ergosterol metabolism. An expression system that simultaneously overexpresses ScErg11p with its cognate NADPH-P450 reductase may help overcome this problem. An alternative is to use reconstitution experiments with recombinant enzymes and/or membrane fractions as described below (Section 6.3). In the present study, it is also possible that enzyme overexpression might have led to inhibitor sequestration by molecules of Erg11p enzyme outside the multienzyme “ergosome” complex in the ergosterol biosynthesis pathway described by Mo and Bard (Mo and Bard, 2005). The ergosome complex includes Erg11p and Erg24p together with Erg25p-Erg26p mounted of the scaffold protein Erg28p. Inhibitor sequestration could lower the concentration of the drug available to inhibit the physiologically active enzyme, but the affinity of the sequestering enzyme for the drug may not be the same as that of the functional wild type or mutant enzyme.
In this thesis comparisons were always made between strains with identical genetic backgrounds i.e. the wild type ScErg11p overexpressing strain was compared to a mutant ScErg11p overexpressing strain either with the native ERG11 intact or deleted. Perhaps a more physiologically relevant way to compare phenotypes of strains bearing mutant and wild type enzyme would be to express the mutant enzymes from the native ERG11 locus. The recombinant enzyme would then be expected to distribute normally in the endoplasmic reticulum and behave in a more physiologically relevant manner. On the other hand, overexpression of CYP51 is an important mechanism of resistance in itself. For example, in *A. fumigatus* resistant clinical isolates, tandem repeats in the CYP51A promoter cause increased expression in addition to the effects of single or multiple mutations in the CYP51A ORF (van der Linden et al., 2013, Snelders et al., 2008).

In order to evaluate the physiological impact of resistance mutations, the triazole susceptibilities of strains overexpressing mutant ScErg11p6×His were compared to those of strains overexpressing the wild type enzyme. In some cases the mutant enzyme may not have been overexpressed at the same level as the wild type enzyme, may have been intrinsically unstable or turned over more rapidly at different stages of the growth cycle. This may have led to differences in the triazole susceptibilities observed between *S. cerevisiae* and those that occur in fungal pathogens. For example, if a mutant enzyme is overexpressed at a lower level than the wild type enzyme and confers reduced susceptibility to a drug, the contribution to resistance (per molecule of Erg11p) caused by a mutation may actually be higher than that inferred from MIC determinations. It is suggested that future studies require quantitation of functional enzyme in membrane preparations rather than the crude, activity-independent measures used in aspects of the present study that were based on the detection of the Coomassie stained or His-tagged protein. This could be achieved by measuring carbon monoxide difference spectra of the reduced enzyme in such preparations, provided the mutation does not affect the extinction coefficient of the heme.
Chapter 6: Conclusions and future work

There are difficulties intrinsic to determining precise inhibitor affinities when tight enzyme-inhibitor complexes are formed in a near stoichiometric manner. As a result, this thesis has used a modified approach to the estimation of $K_d$ values for type II binding of triazole drugs compared to previous studies with fungal CYP51s. In the studies by Warrilow and colleagues, a rearrangement of the Morrison equation was used to estimate the $K_d$ values if tight binding was observed i.e. enzyme saturation occurred at a concentration of the drug equal to or less than the concentration of the enzyme (Warrilow et al., 2010b, Warrilow et al., 2010a). In the present study some datasets consistent with tight binding did not fit the rearrangement of the Morrison equation. In such instances the Hill equation gave a better fit and was used instead. In addition, solubilisation of the enzyme from the membranes can also change the ligand-binding properties of CYP51s as demonstrated by Warrilow et al. (Warrilow et al., 2015). Assays using *A. fumigatus* NADPH cytochrome P450 reductase reconstituted with membrane preparations of *A. fumigatus* CYP51A or CYP51B enzymes heterologously expressed in *E. coli* (discussed below, Section 6.3) gave IC$_{50}$ values 34-fold higher for FLC binding to AfCYP51A (17 µM) compared to AfCYP51B (0.5 µM). However, a comparison of FLC binding by these two CYP51 enzymes estimated using type II binding with the Ni-NTA affinity purified enzymes gave only a 3-fold difference in $K_d$ values i.e. 12 µM for AfCYP51A and 4 µM for AfCYP51B. These differences, presumably caused by the solubilisation of the enzyme with detergents, highlight the importance that the lipid bilayer plays in positioning the enzyme, determining its conformation and the access of triazole inhibitors to the active site. Reconstitution of the affinity purified enzyme in lipid vesicles may provide a way forward towards obtaining more reliable and physiologically relevant estimates of the binding affinities of CYP51 inhibitors. We conclude that the type II binding studies presented in this thesis using affinity purified enzyme preparations may only have value in discriminating between drugs that bind tightly and weakly to the active site.

During crystallographic refinement of structural models specific constraints were applied. The distance between the heme iron and the nitrogen atom of the
triazole ring was set at 2.15 Å and the distance between the sulphur atom of C470 and the heme iron set at 2.33 Å, as described in Section 2.8.3. As expected, examination of electron density maps obtained at resolutions of around 2 Å was unable to detect significant changes in heme-ligand distances. A resolution of 1.5 Å is required to establish the position of individual atoms. Disruption in the position of the heme has been proposed to be one of the ways resistance mutations could affect the structure and function of CYP51 enzymes (Fraczek et al., 2011). Mutations Y140F/H, K151R and T322I in ScErg11p6×His were found to induce changes on the electronic environment of the heme as judged by the longer wavelength of the Soret peak in mutant “apo enzyme” preparations or the smaller ΔA_max of mutants that were detected in type II binding studies with triazole drugs. An altered tilt of the heme was not observed in any of the mutant structures and, given the structural resolutions (<2 Å) achieved with the structures, it was not possible to determine whether the mutations induced changes that altered the distance between the heme iron and the triazole drug. If the distance was altered in ScErg11p6×His mutants, the change is likely to be a fraction of an angstrom. Molecular dynamics could be used to investigate whether there are differences in the distances between the heme iron and the coordinated nitrogen atom of the triazole drug in mutant enzymes compared to the wild type. Changes in the nature of enzyme-heme and enzyme-drug interactions in the case of the ScErg11p6×His Y140F/H mutants might be enough to alter this distance.

### 6.2 Implications for CYP51 inhibitor design

The present study has identified several features that should be considered in the design of CYP51 inhibitors. These antifungal drugs should have a strong preferential affinity for a range of fungal CYP51s and not the human CYP51 enzyme or the human CYP450 drug metabolising enzymes such as CYP3A4, CYP2C19, CYP2C9 (Ekroos and Sjogren, 2006, Hyland et al., 2003). It is known that as well as binding to and inhibiting the drug metabolising CYP450s, some azoles can also induce their expression (Korashy et al., 2007, Sun et al., 2006). Thus treatment of fungal disease in patients with underlying conditions
can become more problematic due to drug interactions caused by polypharmacy. For example, the imidazoles clotrimazole and ketoconazole both have the disadvantage of showing tight binding to human CYP51 (Warrilow et al., 2013). Voriconazole is metabolized by both CYP2C19 and CYP3A4 and its rates of metabolism and impact on the metabolism of other drugs can vary significantly between individuals (Hyland et al., 2003). As a result, serum levels of voriconazole need to be carefully monitored during prophylaxis and the treatment of invasive aspergillosis (Ashbee et al., 2014). The tetrazole VT-1161 has the advantage of showing tight type II binding with the fungal CaCYP51 enzyme (Kd = 39 nM) but not human CYP51 i.e. no binding was obtained with human CYP51 at a VT-1161 concentration of 86 µM (Warrilow et al., 2014). A crystal structure of human CYP51 in complex with this ligand is needed to determine whether VT-1161 binds to human CYP51 without perturbing the heme, or whether it binds at all. Since the tetrazole group shows ~2,000-fold greater selectivity for fungal CYP51 than human CYP51 (Warrilow et al., 2014), it appears to be advantageous to use this substituent instead of the triazole group in future drug design. However, synthetic chemistry using tetrazole groups is considered to be both more expensive and dangerous as compounds containing such groups can be explosive.

In order to accommodate passage of its substrate lanosterol, the entry channel of ScErg11p is made up of mainly hydrophobic residues and it is isolated from solvent exposure by the lipid bilayer. The long-tailed triazoles ITC and PCZ have three aromatic rings including the halogenated aromatic ring adjacent to the heme coordinating triazole ring, making both compounds lipophilic and relatively insoluble in water. There is a hydrophilic pocket in the substrate channel of ScErg11p, which contains the three water molecules when ITC is bound, including one, which forms a hydrogen bond network with the main chain components of H381 and S382. Apart from being generally hydrophobic, the long tails of novel antifungals could be designed to target this water-containing pocket.
Chapter 6: Conclusions and future work

There are 36 residues conserved in eukaryotic CYP51s (Lepesheva and Waterman, 2011). Conserved residue Y126 (S. cerevisiae numbering) is involved in supporting the heme via a hydrogen bond to one of the heme the propionate group. Our structures show movement of this residue depending on what ligand is bound. Binding of lanosterol, ITC or PCZ maintains the hydrogen bond between the Y126 and heme propionate but the binding of VCZ or FLC abolishes that hydrogen bond, as shown in Figure 4.8. The number of fungus-specific, conserved residues are limited and several of those have similar properties to the ones conserved in animals (Lepesheva and Waterman, 2011). For example, residue F241 (S. cerevisiae numbering; located at the egress channel) is conserved in fungi, however in animals the residue in the same position is a conserved tryptophan.

New azole drugs should also be effective against azole resistant CYP51 mutants. As a result of this study, the design of the new azole drugs can now begin to consider the impact of resistance-conferring mutations in CYP51. The findings of this thesis suggest the following considerations when designing novel azole drugs targeting fungal CYP51s:

1. The hydroxyl group present in FLC and VCZ could be replaced with alternative substituents that circumvent the consequences of tyrosine to phenylalanine or histidine mutations on the B’C loop. The 1,3-dioxolane moiety present in ITC and PCZ does not interact via a water-mediated hydrogen bond network with the tyrosine and therefore mutation to phenylalanine or histidine will have no effect on binding.

2. The hydrophilic pocket in the substrate channel that accommodates three water molecules and interacts with a nitrogen atom of the piperazine ring of ITC (N20) or PCZ (NBT) could be used to provide extra interactions for a medium length azole drug. This region at the nexus of the active site, entry and exit channel of ScErg11p6×His has the advantage that it contains Y72, F241, H381 and S382. These amino acid residues are conserved among major fungal pathogens, are not found in human CYP51 and have yet to be shown to contribute to azole resistance.
3. The resistance to long-tailed triazoles PCZ and ITC appears to be due to mutations that have been mapped to the substrate channel of *A. fumigatus*. An azole drug with a tail of medium length may avoid this problem. For example, long tailed triazoles seem to be relatively flexible in their binding to CYP51s. Three distinct configurations are observed in the binding of ITC to ScErg11p6×His wild type and the G73E/W mutants. Two of those configurations have been previously observed with *T. brucei* CYP51 in complex with PCZ, which is structurally very similar to ITC (Chen et al., 2010). However, the conformation in ScErg11p6×His G73W in complex with ITC has not been observed previously.

### 6.3 Future work

Type I difference spectra, with a peak at 380 – 390 nm and a trough 415 – 420 nm, are detected when a CYP51 substrate displaces the water molecule at the heme iron, causing the iron to adopt a high spin state (Jefcoate, 1978). Such studies are used to estimate the binding affinity of the substrate to the enzyme and can give insight into the catalytic efficiency of wild type and mutant enzyme preparations. Deletion of the native *ERG11* from a strain designed to constitutively overexpress ScErg11p6×His G73W from the *PDR5* locus was unable to give a viable phenotype, indicating that the mutant enzyme is catalytically inactive. The catalytic activity of this mutant could be disrupted due to the blockage of the entry channel by this bulky residue. Another possibility could be that the substrate lanosterol was prevented from entering the substrate channel due to potential π-stacking interactions between the tryptophan and lanosterol (similar to ones observed between W73 and the tail of ITC). It is also possible that strains overexpressing ScErg11p6×His G73E/R may have reduced catalytic activity. This may have contributed to the greater susceptibility of those strains to all triazoles tested compared to strains overexpressing the wild type enzyme. Partial blockage of the substrate channel and the presence of a charged residue at the mouth of the channel may have impeded the hydrophobic lanosterol molecule from readily entering the entry
channel and reaching the active site. The use of type I binding to calculate the accessiblility and binding affinity of the substrate lanosterol to ScErg11p6×His wild type and mutant enzymes could provide insight into how the G73 series of mutations affect catalytic activity. The opposite is true for the CaCYP51 I471T and S279F mutations, which have been tested using the type I binding studies. Those mutations were found to increase product affinity, suggesting increased catalytic turn over (Warrilow et al., 2010a, Warrilow et al., 2012). This increase in product affinity and thus increase in catalytic activity was suggested to explain the reduced susceptibility to the azoles by those mutants.

There is a more direct way to determine the catalytic activity of a CYP51 enzyme. This involves the incubation of lanosterol and NADPH with CYP51 and its cognate P450 reductase reconstituted in an artificial lipid bilayer or with an E. coli membrane fraction. Estimation via gas chromatography-mass spectrometry of the amount of CYP51-related metabolites present can be used to determine kinetic parameters and incubation with a triazole drug enables estimation of $IC_{50}$ values for the inhibitor (Warrilow et al., 2010a, Parker et al., 2013, Park et al., 2011). A recent study by Warrilow et al. (Warrilow et al., 2015) has utilised this assay to demonstrate that A. fumigatus CYP51A G54W mutant confers 11- and 34-fold increases in $IC_{50}$ values to ITC and PCZ, respectively, compared to wild type preparations. This in vitro study affirmed the resistance phenotype of these mutants to long-tailed triazoles that were detected in clinical isolates as MIC values. Assays of ScErg11p mutants using this approach will enable comparative testing of the wild type and mutant enzyme function and their response to triazole drugs. However, these assays are both technically demanding and expensive. A major step forward would be the development of surrogate substrates that give more readily detectable products.

There is an extensive repertoire of convenient substrates used with cytochrome P450 enzymes (PROMEGA luminescent substrates and coumarins; Cali et al., 2008, Bell et al., 2008). In the case of CYP51 enzymes there is only one example of such an assay. A study by Riley et al. (Riley et al., 2015) has developed a coumarin-based high-throughput fluorescence assay to measure the
Chapter 6: Conclusions and future work

binding of potential drugs to *T. cruzi* CYP51. Whether the same assay works for fungal CYP51s remains to be determined.

Several azole drugs in clinical trials, such as VT-1161 and Isavuconazole have a hydroxyl group equivalent to that found in FLC and VCZ. This hydroxyl group is expected to interact with the Y140 of ScErg11p6×His via a water-mediated hydrogen bond network. This suggests that susceptibilities to VT-1161 and ISA may be reduced by mutations equivalent to ScErg11p6×His Y140F/H. It will be interesting to see if the medium length tails found in these drugs confer an advantage over the short-tailed drugs FLC and VCZ in overcoming these resistance mutations. Tests of susceptibility on strains overexpressing ScErg11p6×His wild type and Y140F/H mutants to these drugs along with X-ray crystallographic analysis are expected to provide insight into how the tails of those drugs influence susceptibility patterns and drug binding. The strain overexpressing ScErg11p6×His and the isolated ScErg11p6×His enzyme provide valuable tools to investigate the cellular activity of novel azole antimycotics and their effects at the biochemical and structural levels.

Overexpressed ScErg11p6×His may also be useful in investigating the role of the fungus-specific loop. Several mutations (D446E, Y447H, G448E, F449V, G450E and V456I; *C. albicans* numbering) found in this region of the enzyme surface confer resistance to triazole drugs thus indicating its importance in enzyme activity (Flowers et al., 2015, Alvarez-Rueda et al., 2011). Structural information is incomplete for some parts of the ScErg11p6×His fungus-specific loop in several of the crystal structures I have obtained, indicating that the loop is a flexible moiety. Mutating parts of the loop may give insight into its function. An assay using reconstituted CYP51 enzyme (Lepesheva et al., 2007) could be used to reveal if the mutations in the fungus-specific loop alter the catalytic activity of the enzyme.

6.4 Summary

The research carried out in this thesis investigated the applicability of ScErg11p as a suitable model to represent resistance mutations in CYP51 of pathogenic...
fungi. In the case of Y140F/H and Y140F G464S mutations ScErg11p was a suitable model and resistance phenotypes were reproduced, however the rest of the mutations made no difference or conferred susceptibility to triazole drugs. Thus the relevance of our model is site-specific. The high-resolution structure of wild type enzyme with FLC reveals details of how this widely used triazole drug interacts with its molecular target. Based on those findings we established a mechanism of resistance to FLC for the tyrosine to a phenylalanine or a histidine mutation at position 140 (S. cerevisiae numbering). These findings will contribute to the structure-directed design of novel azole based antimycotic drugs that aims to overcome the action of certain resistance mutations.
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References


References
References


References


Appendix A

Strains and Buffers
## A.1 S. cerevisiae strains used and prepared during this study.

Table A-1 Strains used in this study

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Appendices

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*MMLY- Molecular microbiology laboratory number.

aThose strains were not filed into the yeast strain collection.

A.2 Solutions used for DNA manipulations

A.2.1 Water purification

All solutions were prepared in either deionised distilled water ddH$_2$O or ultrapure water (Milli-Q H$_2$O). Milli-Q water was obtained by purifying the ddH$_2$O using the Simplicity Water Purification System (Merck Millipore, Billerica, MA, USA).

A.2.2 DNA loading dye

DNA loading dye (10×) contained 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF and 50% v/v glycerol in ddH$_2$O.

A.2.3 TAE running buffer

Tris-acetate EDTA (TAE) buffer (50×) contained 242 g Tris base (Invitrogen), 37.2 g Na$_2$EDTA (Merck), 114.2 mL glacial acetic acid (Merck) in 1 L of ddH$_2$O and was stored at 4 °C.

A.2.4 1 Kb plus ladder

The DNA ladder contained 50 μL of 1Kb plus DNA ladder (Invitrogen), 200 μL 50 mM NaCl and 250 μL 10× DNA loading dye.
A.2.5 Agarose gels

Agarose gels (0.8%) were prepared by adding 0.8 g of UltraPure™ agarose (Invitrogen) to 100 mL of TAE buffer. The agarose containing mix was melted using a microwave, cooled to about 45°C, and 5 µL of ethidium bromide (50 ng/mL) added. The solution was poured into a gel cast, a suitable comb was inserted and the gel was allowed to solidify at room temperature.

A.3 Protein purification buffers

A.3.1 Cell breakage medium

Cell breakage buffer consisted of 20 % glycerol (w/v), 50 mM Tris, 0.5 mM EDTA and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)* in Milli-Q H₂O. The pH was adjusted to 7.5 at room temperature with 5 M HCl. The medium was stored at 4 °C prior to the addition of PMSF (Roche).

*PMSF is unstable in aqueous solution and therefore was added to all buffers immediately prior to use. Stock PMSF (100 µM) is dissolved in isopropanol.

A.3.2 GTED-20

GTED-20 consisted of 20 % glycerol (w/v), 10 mM Tris, 0.5 mM EDTA and 0.5 mM PMSF in Milli-Q H₂O. The pH was adjusted to 7.5 at room temperature with 1 M HCl. The buffer was stored at 4 °C.

A.3.3 Protein solubilisation solution

Protein solubilisation buffer contained 10% (wt/vol) glycerol, 250 mM NaCl, 20 mM Tris, 0.5 mM PMSF and 1 Roche EDTA-free protease inhibitor pill per 200 mL in Milli-Q H₂O. The pH was adjusted to 7.5 at room temperature with 1 M HCl.

A.3.4 Protein solubilisation solution with detergent

Protein solubilisation solution containing 16 mM n-decyl-β-D-maltoside (DM, 10×CMC).
A.3.5 Ni-NTA affinity matrix binding solution

Ni-NTA affinity matrix binding solution contained 10% (wt/vol) glycerol, 250 mM NaCl, 20 mM Tris, 0.5 mM PMSF, 16 mM DM (10×CMC), 20 mM imidazole and 1 Roche EDTA-free protease inhibitor pill per 200 mL of the solution in Milli-Q H₂O. The pH was adjusted to 7.5 at room temperature with 0.1 M HCl.

A.3.6 Ni-NTA affinity column elution buffer

Ni-NTA affinity matrix binding solution containing 200 mM imidazole. The pH was adjusted to 7.5 at room temperature using 1 M HCl.

A.3.7 Size exclusion chromatography buffer

Size exclusion chromatography buffer contained 10% (wt/vol) glycerol, 150 mM NaCl, 20 mM HEPES, 0.5 mM PMSF, 6.4 mM DM (4×CMC) and 1 Roche EDTA-free protease inhibitor pills per 200 mL. The pH of the solution was adjusted to 7.5 at room temperature with 1 M NaOH. The buffer was filtered with a 0.22 µm nitrocellulose filter (Merck Millipore) and degassed prior to use in FPLC.

A.4 SDS polyacrylamide gel electrophoresis

A.4.1 SDS PAGE running buffer

SDS PAGE running buffer consisted of 12.6 g glycine (Merck), 2.7 g Tris and 0.9 g sodium dodecyl sulphate (Invitrogen) dissolved in 900 mL of ddH₂O.

A.4.2 Protein loading buffer

Protein loading buffer (6×) consisted of 7 mL of 4× upper stacking buffer, 3 mL glycerol, 1 g SDS, 0.2 g dithiothreitol (Roche Diagnostics) and 2 mg bromophenol blue. The solution was stored frozen at – 20 °C.
Table A-2 SDS PAGE components

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating gel</th>
<th>Stacking gel</th>
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<tbody>
<tr>
<td>40% acrylamide</td>
<td>2.4 mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>4× separating buffer</td>
<td>3.0 mL</td>
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</tr>
<tr>
<td>(1.5 M Tris.HCl, pH 8.8; 0.4 % SDS)</td>
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<td></td>
</tr>
<tr>
<td>4× upper stacking buffer</td>
<td>1 mL</td>
<td></td>
</tr>
<tr>
<td>(0.5 M Tris.HCl, pH 6.8; 0.4 % SDS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milli-Q H₂O</td>
<td>6.6 mL</td>
<td>2.5 mL</td>
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<tr>
<td>TEMED</td>
<td>8 µL</td>
<td>2 µL</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>60 µL</td>
<td>30 µL</td>
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</table>

A.4.3 Coomassie brilliant blue stain

Coomassie brilliant blue stain contained 0.1% Coomassie Brilliant Blue R250 (Sigma-Aldrich Ltd), 10% glacial acetic acid and 50% methanol made up in ddH₂O and filtered using Whatman No 1 (Thermo Fisher) filter paper.

A.4.4 Acrylamide gel destaining solution

The destaining solution contained 10% glacial acetic acid, 20% methanol made up in ddH₂O. Destained gels were stored in 1% acetic acid.
Appendix B

Oligonucleotide primer and transformation cassette sequences
### B.1 *ScERG11* cassette manipulations

**Table B-1 Generic oligonucleotide primers used for *ScERG11* cassette manipulations**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
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<tr>
<td>PDR5F</td>
<td>GAACATGAACGTTCCTCAAGCGCG</td>
</tr>
<tr>
<td>ScUra3-rev</td>
<td>CTGGCCGCAATCTTCTCACAATATGC</td>
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<tr>
<td>Ura3-PDR5DS</td>
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<td>PDR5DS_R</td>
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<tr>
<td>PDR5_US</td>
<td>GAGCATAAAACAGAGAGCGACATATAGG</td>
</tr>
<tr>
<td>ScErg11_ORF_183R</td>
<td>GAAAACACTAGAGGTCGGACCTCC</td>
</tr>
<tr>
<td>PDR5_288_DS_Rev</td>
<td>CCCTAGCTACATAGAGGACGGTCCTCC</td>
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**B.1.1 Sequence of the *ScERG11* transformation cassette.**

The PDR5F priming site at the 5’ end of the DNA sequence is highlighted in blue while the PDR5DS_R priming site at the 3’ end of the DNA sequence is highlighted in red. The *ScERG11* open reading frame is in bold, the PGK terminator in italics and the *URA3* selection marker highlighted in grey. The sequence upstream of *ERG11* and the sequence downstream of URA3 are *PDR5* upstream and downstream regions.

```
GAACATGAACGTTCCTCAGCGCGAACGTTCGCTTCTGGCCGAGCACAGGATAGTGGCA
GAAGCACCATCAGACATCTCTGAGATACGACAGACACACTCTGGCCGCCAGAAACGTCGCTG
GAAGACCATTGGTCAGTGTGCTCAGCTACAGTTACCGCAATCACTACAGATCAGATCAGATCAG
TTCTCGAGAGATCAGTGTGCTCAGCTACAGTTACCGCAATCACTACAGATCAGATCAGATCAG
TTCTCGAGAGATCAGTGTGCTCAGCTACAGTTACCGCAATCACTACAGATCAGATCAGATCAG
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TTCTCGAGAGATCAGTGTGCTCAGCTACAGTTACCGCAATCACTACAGATCAGATCAGATCAG
TTCTCGAGAGATCAGTGTGCTCAGCTACAGTTACCGCAATCACTACAGATCAGATCAGATCAG
TTCTCGAGAGATCAGTGTGCTCAGCTACAGTTACCGCAATCACTACAGATCAGATCAGATCAG
```

198
Figure B-1 Colony PCR analysis used to verify the presence and orientation of the *S. cerevisiae ERG11* cassette. Primers PDR5_US and ScErg11_ORF_183R were used to amplify *ERG11* from individual yeast colonies. The expected size of the amplimer was 1417 bp. The PCR fragments were separated by electrophoresis using a 0.8% agarose gel. Lane M: molecular markers (1 kb Plus DNA molecular ladder). Lanes 1–6 are clones positive for the insert. Lane 7: positive control (ADΔ ScErg11p strain). Lane 8: negative control (AD2Δ strain).
Appendices

B.2 ScHis1 disruption cassette manipulations

Table B-2 Primers used to prepare ScHis1 cassette for disruption of native ERG11

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’).</th>
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<td>ScErg11_US-759</td>
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<tr>
<td>ScErg11US-387R</td>
<td>TTCAGGCATAAAAATCGATGTCTCC</td>
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<tr>
<td>ScErg11xHisF</td>
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<td>ScErg11xHisR</td>
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<td>ScErg11DS60</td>
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<td>ScErg11DS346R_Δ</td>
<td>GACTGCTTTATTTTCTGCTGCGCTG</td>
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<tr>
<td>ScErg11_US_801</td>
<td>GCCGCTGTCCCCGTACAGACGAAC</td>
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<tr>
<td>ScHis1_ORF_493R</td>
<td>CAATTCCCGAGCAGACATGATGC</td>
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<tr>
<td>ScERG11-ter-rev</td>
<td>CACTTAAAAAAGCAGTAATGATC</td>
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B.2.1 Sequence of the ScHis1 cassette for disruption of native ERG11

The *S. cerevisiae* ERG11 upstream and downstream sequences flank ScHIS1 which is shown in bold. The ScErg11_US-759 priming site is highlighted in green, the ScErg11xHisF priming site in yellow, ScErg11xHisR priming site in blue and ScErg11DS346R_Δ priming site in grey.
Figure B-2 Colony PCR analysis used to verify the presence of ScHIS1 cassette. Primers ScErg11_US_801 and ScHis1_ORF_493R were used to amplify the disruption cassette from individual yeast colonies. The expected size of the amplimer was 1604 bp. The PCR fragments were separated by electrophoresis using a 0.8% agarose gel. Lane M: molecular markers (1 kb Plus DNA molecular ladder). Lanes 1 – 9 are clones positive for the insert. Lane 10: clone negative for the insert. Lane 11: positive control (AD3ΔScErg11p_Y140F strain). Lane 12: negative control (AD2Δ strain).
Appendix C

Protein purification figures
Figure C-1 SEC chromatograms (Superdex 200 10/300 column eluted with size exclusion chromatography buffer, Appendix A.3.7) of ScErg11p6×His mutants purified without added ligand. The SEC fractions for the ScErg11p6×His G73W (a), G73E (b), K151R (c) and T322I (d) enzyme that eluted within 11 – 15 mL were pooled, concentrated by centrifugal filtration and used for crystallisation.
Figure C-2 Absolute absorbance spectra of ScErg11p6×His mutants co-purified with ITC during SEC. The absorbance spectra of ScErg11p6×His G73R (a), the T322I (b), the G464S (c) and K151R (d) are shown.
Appendix D

Mass spectrometry results
Mass spectrometry determination of the tryptic peptide coverage and detection of the mutated residue for ScErg11p6×His mutants

Sequences identified by mass spectrometry from tryptic fragments of the 62 kDa band ScErg11p6×His recovered by SDS-PAGE are bold and highlighted in grey. The site of the mutation in each primary sequence (single letter amino acid code) is highlighted in red.

### D.1 ScErg11p6×His G73E mutation

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<tr>
<td><strong>EECQKYGDI FSFVLGRVM TVYLGPKGHE FVFNAKLADV</strong></td>
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<tr>
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</tr>
<tr>
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<tr>
<td><strong>PENPTPSAR SLLGKEMRAK LTDTFAYLYS DLDKGFPTIP</strong></td>
<td></td>
</tr>
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Protein sequence coverage is 79.2%.

### D.2 ScErg11p6×His G73R mutation

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Protein sequence coverage is 84.7%.
D.3 ScErg11p×His G73W mutation

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| 41 | YNIVWQLLIS  | LRKDRPPLVF  | YWIPWGVSAV  | VIHMKPYEFF |
| 81 | EECQKXYGDI  | FSFVLLGRNV  | TVYLGPGKGE  | FVFKAKLADV |
| 121| SAEAYAHLT   | TPVPFGKGYI  | DCPNSRLMEOQ | KFPVKGALTK |
| 161| EAFKSYVPLI  | AEEVYKYFRD  | SKNPRLNERT  | QTIDVWVQ |
| 201| PERTFTASAR  | SSLGKEMRAK  | LDGFAYLYS  | DLDGFTPIN |
| 241| FVFPNLPLEH  | YRRDHAQKA   | ISGTYMSLARK | ERRKNNQIQD |
| 281| RDLIDSLMKN  | STYKDGVKMT  | DQETIANLLIG | VLMGGQHTSA |
| 321| ATSAWILLHL  | AERPDVQOEL  | YEEQMRVLGD  | GKKELTDK |
| 361| QEMPPLNQTI  | KELRMHHPL  | HSLFRKVMKD  | MHVPNTSYVI |
| 401| PAGYHLVSP   | GYTHLRDEYF  | PNABQFNHR  | WNNDSASSYS |
| 441| VGEEDVGFPG  | AISKGVSSPY  | LPFQGGRHRC | IGEHFAYQOL |
| 481| GVLMSIFIRT  | LKWHYPEGKT  | VPPPDFTSMV  | TLPTGPAKII |
| 521| WEKRNPQKXI  | GGRHHHHHH |

Protein sequence coverage is 84.7%

D.4 ScErg11p×His K151R mutation

| 1  | MSATKSIVGE  | ALEYVNIGLS  | HFLALPLAQR  | ISLIIIIPFI |
| 41 | YNIVWQLLIS  | LRKDRPPLVF  | YWIPWGVSAV  | VIHMKPYEFF |
| 81 | EECQKXYGDI  | FSFVLLGRNV  | TVYLGPGKGE  | FVFKAKLADV |
| 121| SAEAYAHLT   | TPVPFGKGYI  | DCPNSRLMEOQ | KFPVKGALTK |
| 161| EAFKSYVPLI  | AEEVYKYFRD  | SKNPRLNERT  | QTIDVWVQ |
| 201| PERTFTASAR  | SSLGKEMRAK  | LDGFAYLYS  | DLDGFTPIN |
| 241| FVFPNLPLEH  | YRRDHAQKA   | ISGTYMSLARK | ERRKNNQIQD |
| 281| RDLIDSLMKN  | STYKDGVKMT  | DQETIANLLIG | VLMGGQHTSA |
| 321| ATSAWILLHL  | AERPDVQOEL  | YEEQMRVLGD  | GKKELTDK |
| 361| QEMPPLNQTI  | KELRMHHPL  | HSLFRKVMKD  | MHVPNTSYVI |
| 401| PAGYHLVSP   | GYTHLRDEYF  | PNABQFNHR  | WNNDSASSYS |
| 441| VGEEDVGFPG  | AISKGVSSPY  | LPFQGGRHRC | IGEHFAYQOL |
| 481| GVLMSIFIRT  | LKWHYPEGKT  | VPPPDFTSMV  | TLPTGPAKII |
| 521| WEKRNPQKXI  | GGRHHHHHH |

Protein sequence coverage is 85%.
**Appendices**

### D.5 ScErg11p6×His T322I mutation

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Protein sequence coverage is 91.4%

### D.6 ScErg11p6×His G464S mutation

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Protein sequence coverage is 82.8%.

210
### D.7 ScErg11p6×His G464S Y140F double mutation

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### D.8 ScErg11p6×His Y140F mutation

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Protein sequence coverage is 78%.
### D.9 ScErg11p×His Y140H mutation

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Protein coverage is 93%.
Appendix E

Soret peak shift measurement of type II binding affinity
E.1 Method

The SEC purified protein concentration used in this study was estimated, as described in Section 2.7.1, using the $A_{280\text{nm}}$ peak in the absorbance spectra (extinction coefficient $= 1.345 \text{ mg}^{-1}\cdot\text{mL}^{-1}\cdot\text{cm}^{-1}$). Wild type or mutant ScErg11p6×His (1 µM) was titrated using the triazole drugs FLC, VCZ, ITC and PCZ. The range of drug concentration used was $0.05 – 15$ µM. The heme Soret peak maximum was determined by scanning from 390 to 450 nm against a reagent blank using an Ultrospec™ 6300 pro UV/Vis spectrophotometer. The apparent $K_d$ values for the enzyme-drug interaction were calculated by fitting data to the Hill equation (Equation 2.4), a rearrangement of the Morrison equation (Equation 2.5) or the Michaelis-Menten equation and choosing the best fit obtained using GraphPad Prism 6 software. The Michaelis-Menten equation ($\Delta A = \Delta A_{\text{max}}[\text{Azole}]/([\text{Azole}] + K_d)$) was used when the Hill coefficient in the Hill equation was equal to 1. $\Delta A_{\text{max}}$ is the maximum change in absorbance and $[\text{Azole}]$ is the azole concentration.

E.2 Results

Heme Soret peak shift measurements were carried out for FLC, VCZ, ITC and PCZ binding to the wild type and all mutant ScErg11p6×His proteins, except for the Y140F G464S double mutant. The binding curves, fitted to the Hill equation, the rearrangement of the Morrison equation or the Michaelis-Menten, are shown in Figure E-1. The resulting $K_d$ values are presented in Table E-1.
Figure E-1 The binding of triazole drugs to ScErg11p6×His wild type and mutants based on the Soret heme peak shift. ScErg11p6×His (1 µM) was titrated with triazole drugs (a, b) FLC, (c, d) VCZ, (e, f) ITC and (g, h) PCZ. The wild type enzyme is represented in solid circles, the G73E mutant solid squares, G73R mutant solid triangles, G73W mutant solid diamonds, K151R mutant hollow squares, T322I mutant hollow triangles and G464S mutant in hollow diamonds.
The maximal red shift of the heme Soret peak was ~4 nm for the binding of triazoles FLC, VCZ, ITC and PCZ to wild type ScErg11p6×His. The $K_d$ values for FLC, ITC and PCZ binding were determined to be in the nM range for the wild type enzyme (Table E-1). The $K_d$ values in the nM range for FLC, ITC and PCZ for the wild type and mutant ScErg11p6×His indicate that the binding of these drugs is tight. Surprisingly, the binding of VCZ to wild type and mutant enzymes gives $K_d$ values that appeared to be much higher than for the rest of the triazoles tested (Table E-1).

ScErg11p6×His G73E/W produced a heme Soret peak shift of <3 – 4 nm on triazole binding (Figure E-1 a, c, e, g). However, ScErg11p6×His G73R gave a smaller shift of ~2.5 nm for binding of FLC and ITC (Figure E-1 a, e). The smaller Soret peak shift still indicated very tight binding, with $K_d$ values of 15 nM and 35 nM for FLC and ITC binding, respectively.

ScErg11p6×His K151R enzyme gave a maximum Soret peak shift of ~1.5 nm on triazole binding (Figure E-1 b, d, f, h). The ScErg11p6×His K151R apo-protein had a heme Soret peak of 419.2 nm, which is higher compared to the rest of the mutants (Table 5.2), apart from Y140F/H mutants (420 nm). The binding of FLC to K151R mutant enzyme appeared to be strongest with a $K_d$ of 13 nM determined. The ScErg11p6×His G464S mutant behaved similarly to the wild type enzyme (Figure E-1 b, d, f, h). While the VCZ binding curve for the wild type enzyme fitted the Hill equation, the binding curve for the mutant enzyme best fitted the rearrangement of the Morrison equation. The $K_d$ value for VCZ binding for the mutant enzyme (0.31 µM) was indicative of tight binding, with much weaker binding obtained with the wild type enzyme (1.37 µM).

The ScErg11p6×His T322I mutant had a maximum Soret peak shift of ~1.6 nm for the binding of FLC and ITC (Figure E-1 b, f) and 3 – 4 nm for binding of VCZ and PCZ (Figure E-1 d, h). The highest affinity binding for this mutant enzyme was shown by FLC and PCZ with $K_d$ values of 3.5 nM and 0.02 nM, respectively. The Hill coefficient values of 3.1 for FLC and 4.9 for PCZ binding to this mutant appeared unusually high.
Table E-1 Triazole binding characteristics of ScErg11p6×His wild type and mutants based on the Soret heme peak shift

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*The preferred equation to fit the data was chosen using the Akaike information criterion. M-M stands for Michaelis-Menten.

E.3 Discussion

There are inaccuracies in determining the migration of the heme Soret peak at drug addition. The differences recorded by the spectrophotometer are sometimes less than 1 nm, which is within the range of machine error. Inaccuracies in detecting small changes in wavelength of the heme Soret peak could have major effects on the shape of the binding curve and hence the Hill
Appendices

coefficient – which would also dramatically affect calculation of the apparent $K_d$.

The $K_d$ values obtained from Soret peak shift experiments for VCZ with the wild type enzyme were much higher compared to the values obtained for ITC, FLC and PCZ. The high $K_d$ values for VCZ binding from Soret peak shift experiments are most likely to result from experimental error such to the addition of an improper amount VCZ. The experiment has not been replicated. Repeating these studies with freshly made stocks may give more reliable $K_d$ values for VCZ. Otherwise the triazole binding constants indicate that the binding is tight. Other issues, which arise from using this assay, are discussed in Section 5.4.1.
Appendix F

Crystallographic information file for itraconazole (1YN)
Appendices

Modifications are highlighted in grey.

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# -------- service provided by Global Phasing Ltd. --------
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# GEN: using MOGUL 1.6(RC5), CSD as535be, with quantum mechanics RM1
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# bonds lengths set by partial QM             5
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# non-H angles set by partial QM             21
# H bond angles set by partial QM            86
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# atoms NOT trigonal from 3 mogul angles      2
# trigonal atoms from partial QM             18
# atoms not trigonal from partial QM          0
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# bonds mogul indicates 3-fold staggered      2
# bonds mogul indicates not flat               0
# in flat 5/6 membered rings after PQM        28
# other flat bonds after partial QM           1
# sp3-sp3 set 3-fold staggered by QM         6
# All other torsions (unrestrained)           14
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# Angles defined by QM have a sigma set to 3.0 degrees
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# BUSTER-KEYWORD TRUSTTORS

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Appendix G

Dataset statistics of X-ray crystal structures
Table G-1 Data collection and refinement statistics for ScErg11p6xHis in complex with FLC (Sagatova et al., 2015)

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</tr>
<tr>
<td>Unique reflections</td>
<td>47935</td>
</tr>
<tr>
<td>Average redundancy</td>
<td>6.1 (5.3)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>95.6 (84.6)</td>
</tr>
<tr>
<td>&lt;I/σ(I)&gt;</td>
<td>12.4 (1.4)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>33.4</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt; †</td>
<td>0.083 (0.953)</td>
</tr>
<tr>
<td>CC(1/2)</td>
<td>0.998 (0.634)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;cryst&lt;/sub&gt; ‡</td>
<td>0.20 (0.30)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; §</td>
<td>0.23 (0.32)</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>47899 (2351)</td>
</tr>
<tr>
<td>Number of atoms in model</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>4304</td>
</tr>
<tr>
<td>Ligand</td>
<td>22</td>
</tr>
<tr>
<td>Water molecules</td>
<td>155</td>
</tr>
<tr>
<td>Deviation from ideal bond lengths* (Å)</td>
<td>0.008</td>
</tr>
<tr>
<td>Deviation from ideal bond angles* (°)</td>
<td>1.1</td>
</tr>
<tr>
<td>Ramachandran analysis (%)</td>
<td></td>
</tr>
<tr>
<td>Preferred</td>
<td>96.5</td>
</tr>
<tr>
<td>Allowed</td>
<td>3.2</td>
</tr>
<tr>
<td>Residues in disallowed regions</td>
<td>0.2</td>
</tr>
</tbody>
</table>

† R<sub>merge</sub> = Σ<sub>hkl</sub> Σ<sub>i</sub> |I<sub>i(hkl)</sub>| - [I<sub>(hkl)</sub>]) / Σ<sub>hkl</sub> Σ<sub>i</sub> I<sub>(hkl)</sub>. ‡ R<sub>cryst</sub> = Σ<sub>hkl</sub> |F<sub>obs</sub> - F<sub>calc</sub>| / Σ<sub>hkl</sub> |F<sub>obs</sub>| computed over a working set composed of 95% of data. § R<sub>free</sub> = Σ<sub>hkl</sub> |F<sub>obs</sub> - F<sub>calc</sub>| / Σ<sub>hkl</sub> |F<sub>obs</sub>| computed over a test set composed of 5% of data.

*The parameters for ideal values for bond lengths and bond angles used were defined by Engh and Huber (Engh and Huber, 1991). The validation statistics were obtained from Aimless (Evans, 2006) and Phenix (Chen et al., 2010).

Note: The R values are calculations are the same in all tables of this Appendix.
Appendices

Table G-2 X-ray structures of *Saccharomyces cerevisiae* Erg11p dataset statistics adapted from Monk et al. (Monk et al., 2014)

<table>
<thead>
<tr>
<th>Erg11 datasets</th>
<th>Empty pocket</th>
<th>+ ITC PDB ID 4K0F</th>
<th>+ FLC</th>
<th>+ VCZ</th>
<th>+ Estriol</th>
<th>+ Lanosterol PBD ID 4IXJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>dmin</td>
<td>2.4</td>
<td>2.2</td>
<td>2.5</td>
<td>2.8</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>a</td>
<td>80.1</td>
<td>79.6</td>
<td>80.31</td>
<td>80.1</td>
<td>79.4</td>
<td>78.2</td>
</tr>
<tr>
<td>b</td>
<td>67.7</td>
<td>67.6</td>
<td>67.3</td>
<td>67.7</td>
<td>67.5</td>
<td>67</td>
</tr>
<tr>
<td>c</td>
<td>81</td>
<td>80.7</td>
<td>81.3</td>
<td>81.2</td>
<td>80.8</td>
<td>80.4</td>
</tr>
<tr>
<td>α</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>β</td>
<td>100.14</td>
<td>99.75</td>
<td>100.34</td>
<td>100.14</td>
<td>99.58</td>
<td>99.54</td>
</tr>
<tr>
<td>γ</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Completeness</td>
<td>95.8%</td>
<td>97%</td>
<td>91.7%</td>
<td>98.9%</td>
<td>97%</td>
<td>96.5%</td>
</tr>
<tr>
<td>(96.7%)</td>
<td>(99%)</td>
<td>(86.7%)</td>
<td>(86.7%)</td>
<td>(94.7%)</td>
<td>(72.8%)</td>
<td></td>
</tr>
<tr>
<td>I/σI</td>
<td>31.3 (3)</td>
<td>25 (2.6)</td>
<td>22.7 (1.9)</td>
<td>31.3</td>
<td>32.2 (2.9)</td>
<td>26.6 (1.58)</td>
</tr>
<tr>
<td>Rsym</td>
<td>0.058</td>
<td>0.052</td>
<td>0.049</td>
<td>0.069</td>
<td>0.054</td>
<td>0.056 (0.577)</td>
</tr>
<tr>
<td>(0.486)</td>
<td>(0.41)</td>
<td>(0.472)</td>
<td>(0.577)</td>
<td>(0.541)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_free ‡</td>
<td>0.237</td>
<td>0.234</td>
<td>0.294</td>
<td>0.253</td>
<td>0.237</td>
<td>0.227</td>
</tr>
<tr>
<td>R_cryst ‡</td>
<td>0.196</td>
<td>0.201</td>
<td>0.227</td>
<td>0.191</td>
<td>0.198</td>
<td>0.195</td>
</tr>
</tbody>
</table>
### Table G-3 Data collection and refinement statistics for the ScErg11p6×His Y140F mutant structures

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ITC</th>
<th>PCZ</th>
<th>FLC</th>
<th>VCZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB code</td>
<td>4ZDY</td>
<td>4ZDZ</td>
<td>4ZE1</td>
<td>4ZE0</td>
</tr>
<tr>
<td>Space group</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>34.27 – 2.02 (2.13 – 2.02)</td>
<td>37.45 – 2.05 (2.11 – 2.05)</td>
<td>51.75 – 2.30 (2.38 – 2.30)</td>
<td>51.48 – 2.20 (2.27 – 2.20)</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>a = 78.18 b = 67.07 c = 80.70</td>
<td>a = 78.79 b = 67.75 c = 81.06</td>
<td>a = 78.91 b = 67.15 c = 81.14</td>
<td>a = 77.22 b = 66.55 c = 80.90</td>
</tr>
<tr>
<td>Diffraction source</td>
<td>MX1</td>
<td>MX2</td>
<td>MX2</td>
<td>MX1</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.954</td>
<td>0.954</td>
<td>0.954</td>
<td>0.954</td>
</tr>
<tr>
<td>Total reflections</td>
<td>384013</td>
<td>285878</td>
<td>125003</td>
<td>251724</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>54071</td>
<td>52170</td>
<td>37123</td>
<td>40318</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>7.1 (6.8)</td>
<td>5.5 (5.5)</td>
<td>3.4 (3.3)</td>
<td>6.2 (5.5)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.6 (98.2)</td>
<td>98.3 (98.0)</td>
<td>99.1 (99.5)</td>
<td>97.4 (92.7)</td>
</tr>
<tr>
<td>Mean (I/σ(I))</td>
<td>15.9 (3.3)</td>
<td>12.9 (1.8)</td>
<td>3.9 (1.5)</td>
<td>10.8 (2.0)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>26.88</td>
<td>34.16</td>
<td>29.89</td>
<td>30.50</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;†</td>
<td>0.087 (0.648)</td>
<td>0.082 (0.979)</td>
<td>0.151 (0.456)</td>
<td>0.113 (0.853)</td>
</tr>
<tr>
<td>CC(1/2)</td>
<td>0.999 (0.858)</td>
<td>0.997 (0.538)</td>
<td>0.970 (0.860)</td>
<td>0.995 (0.907)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;cryst&lt;/sub&gt;‡</td>
<td>0.1906 (0.2846)</td>
<td>0.2026 (0.3739)</td>
<td>0.1966 (0.2627)</td>
<td>0.1919 (0.2997)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;§</td>
<td>0.2294 (0.3631)</td>
<td>0.2427 (0.4220)</td>
<td>0.2372 (0.3228)</td>
<td>0.2390 (0.3145)</td>
</tr>
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<td>Number of reflections</td>
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<td>40220</td>
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<td>Number of atoms in model</td>
<td>4548</td>
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<td>4532</td>
<td>4534</td>
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<tr>
<td>Protein</td>
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<td>4323</td>
<td>4319</td>
<td>4337</td>
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<tr>
<td>Ligand</td>
<td>49</td>
<td>51</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>Water molecules</td>
<td>187</td>
<td>139</td>
<td>191</td>
<td>172</td>
</tr>
<tr>
<td>Deviation from ideal bond lengths (Å)</td>
<td>0.008</td>
<td>0.008</td>
<td>0.005</td>
<td>0.008</td>
</tr>
<tr>
<td>Deviation from ideal bond angles (°)</td>
<td>1.04</td>
<td>1.079</td>
<td>0.874</td>
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<tr>
<td>Ramachandran analysis (%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred</td>
<td>97.15</td>
<td>95.45</td>
<td>96.20</td>
<td>96.22</td>
</tr>
<tr>
<td>Allowed</td>
<td>2.66</td>
<td>3.98</td>
<td>3.42</td>
<td>3.59</td>
</tr>
<tr>
<td>Residues in disallowed regions</td>
<td>0.65</td>
<td>1.08</td>
<td>1.94</td>
<td>2.15</td>
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</tbody>
</table>
### Table G-4 Data collection and refinement statistics for the ScErg11p6×His Y140H mutant structures

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ITC</th>
<th>FLC</th>
</tr>
</thead>
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<tr>
<td>PDB code</td>
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<td>4ZE3</td>
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<tr>
<td>Space group</td>
<td>P 1 2 1 1</td>
<td>P 1 2 1 1</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>80.04 – 2.30 (2.38 – 2.30)</td>
<td>80.21 – 2.20 (2.27 – 2.20)</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>a = 77.83 b = 66.67 c = 81.01</td>
<td>a = 76.49 b = 64.84 c = 81.03</td>
</tr>
<tr>
<td>Diffraction source</td>
<td>MX2</td>
<td>MX2</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.954</td>
<td>0.954</td>
</tr>
<tr>
<td>Total reflections</td>
<td>144675</td>
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</tr>
<tr>
<td>Unique reflections</td>
<td>36597</td>
<td>39850</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.0 (4.0)</td>
<td>3.8 (3.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8 (100.0)</td>
<td>99.3 (98.3)</td>
</tr>
<tr>
<td>Mean (I/σ(I))</td>
<td>8.7 (1.9)</td>
<td>11.4 (2.2)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>39.16</td>
<td>35.53</td>
</tr>
<tr>
<td>R\textsubscript{merge}\textsuperscript{†}</td>
<td>0.087 (0.643)</td>
<td>0.064 (0.628)</td>
</tr>
<tr>
<td>CC(1/2)</td>
<td>0.994 (0.752)</td>
<td>0.997 (0.718)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R\textsubscript{cryst}\textsuperscript{‡}</td>
<td>0.1881 (0.2971)</td>
<td>0.1916 (0.2952)</td>
</tr>
<tr>
<td>R\textsubscript{free}\textsuperscript{§}</td>
<td>0.2316 (0.3622)</td>
<td>0.2274 (0.3288)</td>
</tr>
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<td>Number of reflections</td>
<td>36484</td>
<td>39809</td>
</tr>
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<td>Number of atoms in model</td>
<td>4484</td>
<td>4436</td>
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<tr>
<td>Protein</td>
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<td>4294</td>
</tr>
<tr>
<td>Ligand</td>
<td>49</td>
<td>22</td>
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<tr>
<td>Water molecules</td>
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<td>120</td>
</tr>
<tr>
<td>Deviation from ideal bond lengths (Å)</td>
<td>0.009</td>
<td>0.004</td>
</tr>
<tr>
<td>Deviation from ideal bond angles (°)</td>
<td>1.182</td>
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</tr>
<tr>
<td>Ramachandran analysis (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred</td>
<td>96.40</td>
<td>96.56</td>
</tr>
<tr>
<td>Allowed</td>
<td>3.41</td>
<td>3.05</td>
</tr>
<tr>
<td>Residues in disallowed regions</td>
<td>1.29</td>
<td>1.08</td>
</tr>
</tbody>
</table>
## Table G-5 Data collection and refinement statistics for the ScErg11p6×His G73E/W/R mutant structures

<table>
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<tr>
<th>Mutation</th>
<th>G73E</th>
<th>G73W</th>
<th>G73R</th>
<th>G73W</th>
<th>G73E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand</td>
<td>ITC</td>
<td>Apo</td>
<td>FLCL</td>
<td>ITC</td>
<td>FLCL</td>
</tr>
<tr>
<td>PDB code</td>
<td>5ESG</td>
<td>5ESI</td>
<td>5ESE</td>
<td>5ESH</td>
<td>5ESF</td>
</tr>
<tr>
<td>Space group</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
<td>P 1 2 1</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>(2.09 – 1.98)</td>
<td>(2.16 – 2.10)</td>
<td>(2.27 – 2.20)</td>
<td>(2.2 – 2.15)</td>
<td>(2.32 – 2.25)</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>a = 78.24 b = 67.31 c = 80.83</td>
<td>a = 78.90 b = 67.05 c = 80.98</td>
<td>a = 78.35 b = 67.28 c = 81.02</td>
<td>a = 77.56 b = 66.85 c = 80.88</td>
<td>a = 78.49 b = 66.93 c = 81.06</td>
</tr>
<tr>
<td>Diffraction source</td>
<td>MX1</td>
<td>MX1</td>
<td>MX2</td>
<td>MX2</td>
<td>MX2</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.954</td>
<td>0.954</td>
<td>0.954</td>
<td>0.954</td>
<td>0.954</td>
</tr>
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<td>Total reflections</td>
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<td>324866</td>
<td>151591</td>
<td>313763</td>
<td>275487</td>
</tr>
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<td>Unique reflections</td>
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<td>47947</td>
<td>41538</td>
<td>44569</td>
<td>39044</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>7.3 (7.2)</td>
<td>6.8 (6.5)</td>
<td>3.6 (3.7)</td>
<td>7.0 (6.9)</td>
<td>7.0 (6.7)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.6 (95.3)</td>
<td>98.0 (92.2)</td>
<td>98.1 (97.7)</td>
<td>99.6 (99.2)</td>
<td>99.7 (99.3)</td>
</tr>
<tr>
<td>Mean (I/σ(I))</td>
<td>14.3 (2.0)</td>
<td>11.3 (2.2)</td>
<td>9.2 (1.4)</td>
<td>12.3 (2.0)</td>
<td>9.5 (1.5)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>31.40</td>
<td>31.63</td>
<td>42.3</td>
<td>36.09</td>
<td>38.65</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;†</td>
<td>0.093 (0.975)</td>
<td>0.092 (0.670)</td>
<td>0.071 (0.886)</td>
<td>0.093 (0.947)</td>
<td>0.13 (1.62)</td>
</tr>
<tr>
<td>CC(1/2)</td>
<td>0.999 (0.739)</td>
<td>0.997 (0.832)</td>
<td>0.995 (0.362)</td>
<td>0.998 (0.760)</td>
<td>0.996 (0.683)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;cryst&lt;/sub&gt;‡</td>
<td>0.1989 (0.3340)</td>
<td>0.1895 (0.2674)</td>
<td>0.2054 (0.3747)</td>
<td>0.1827 (0.2496)</td>
<td>0.2015 (0.4309)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;§</td>
<td>0.2345 (0.3727)</td>
<td>0.2261 (0.2927)</td>
<td>0.2364 (0.3943)</td>
<td>0.2221 (0.2787)</td>
<td>0.2529 (0.4569)</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>57667</td>
<td>47908</td>
<td>41478</td>
<td>44525</td>
<td>39045</td>
</tr>
<tr>
<td>Number of atoms in model</td>
<td>4507</td>
<td>4525</td>
<td>4443</td>
<td>4522</td>
<td>4417</td>
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<tr>
<td>Protein</td>
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<td>4340</td>
<td>4321</td>
<td>4324</td>
<td>4288</td>
</tr>
<tr>
<td>Ligand</td>
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<td>0</td>
<td>22</td>
<td>49</td>
<td>22</td>
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### Table G-6 Data collection and refinement statistics for the ScErg11p6×His G464S and T322I mutant structures

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<td>Preferred</td>
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References:


Attached Articles:

Structural Insights into Binding of the Antifungal Drug Fluconazole to Saccharomyces cerevisiae Lanosterol 14-Demethylase

and

Triazole resistance mediated by mutations of a conserved active site tyrosine in fungal lanosterol 14alpha-demethylase
Fungal pathogens are estimated to be responsible for ~1.5 million deaths per year worldwide, with members of the genera *Candida*, *Cryptococcus*, and *Aspergillus* being those most often associated with life-threatening disseminated disease (1). Comorbidity with infectious diseases such as malaria, tuberculosis, and AIDS, the use of therapies that leave individuals immunocompromised, and the emergence of resistant clinical isolates due to antifungal prophylaxis and antifungal agrochemicals all contribute to this problem (2). In sub-Saharan Africa, *Cryptococcus* species are most often associated with comorbidity and cause about 400,000 deaths per annum. In the United States, *Candida* species are the fourth-most-common cause of nosocomial bloodstream infections, accounting for 8% to 10% of all hospital-acquired infections and resulting in an attributed mortality rate of 40% (3). Fluconazole (FLC)-resistant *Candida* species account for 7% of bloodstream isolates of this organism. This results in significant additional hospitalization costs and about 220 deaths per annum in the United States (4). Chemotherapy and radiotherapy patients, as well as those undergoing immunosuppressive therapies associated with organ transplants, are particularly susceptible to life-threatening invasive mycoses, mainly those caused by *Candida* and *Aspergillus* species (5).

Despite the introduction and use of the echinocandin antifungals since 2001, azole drugs such as FLC remain the most widely used class of antifungals due to their efficacy and low cost. The azole drugs used in the clinic are categorized into imidazoles and triazoles, depending on the nature of the iron-coordinating heterocycle in the drug. FLC is a first-generation triazole but has a half-life longer than and bioavailability superior to those of the second- and third-generation triazoles such as itraconazole (ITC), voriconazole (VCZ; Fig. 1), and posaconazole (PCZ) (6). Despite favorable pharmacokinetic properties and limited drug interactions, FLC is less potent than other triazoles and exhibits no activity against invasive molds (7).VCZ is structurally related to FLC but has the disadvantage of showing significant toxicity and many major drug interactions and requires therapeutic drug monitoring (8). Even so, VCZ remains the drug of choice for treatment of disseminated aspergillosis (2).

The antifungal activity of the azoles depends on their ability to inhibit the activity of the enzyme lanosterol 14α-demethylase (CYP51 [or Erg11p]), a member of the CYP51 class of cytochrome P450 enzymes (9). The active site of lanosterol 14α-demethylase contains a heme cofactor where the sixth position of the octahedral coordinate geometry of the iron can be occupied by a nitrogen in either the imidazole ring or the triazole ring of theazole drugs. The 14α-demethylation of lanosterol (Fig. 1) is the rate-limiting step in ergosterol biosynthesis in fungi (10). Inhibition of Erg11p leads to a depletion of ergosterol from cell membranes. This affects the fluidity of the lipid bilayer and slows fungal growth. Inhibition of Erg11p also results in the accumulation of toxic metabolites such as the 14α-methyl-3,6-diol formed from 14-methyl-fecosterol (11). The combined effects of ergosterol depletion and toxic metabolite production are fungistatic for many pathogenic fungi, including *Candida albicans*, *Candida glabrata*, and *Aspergillus* species.

Fungi have evolved several mechanisms to circumvent the action of azoles, including mutations in the drug-binding site of Erg1p that reduce the binding affinity of triazoles and thus confer resistance (12–14). The innate resistance of *Aspergillus fumigatus* to FLC is thought to be due to the presence of two homologues of the drug target, with CYP51B appearing sensitive to fluconazole whereas CYP51A confers significantly reduced susceptibility (15).
Prokaryotic P450s are soluble cytoplasmic proteins, whereas the eukaryotic enzymes, excluding mitochondrial P450s, possess a single transmembrane helix that tethers the enzyme to the lipid bilayer. Until 2014, X-ray structures of eukaryotic CYP51s were obtained by deletion of the transmembrane helix to improve expression and crystallization (16). These CYP51s included the human CYP51 enzyme (PDB identifiers 3LD6, 3JUV, and 3JUS) (16) and CYP51 enzymes from the protozoa Trypanosoma cruzi and Trypanosoma brucei (PDB IDs 2WX2, 2WV2, and 2X2N) (17). Other available CYP51 structures include those of Mycobacterium tuberculosis CYP51 (e.g., PDB ID 1EA1) (19) and Leishmania infantum CYP51 (PDB ID 3L4D) (20). The only structural information available on fungal CYP51s was from homology models based on the truncated structures of other P450s and, more recently, on human CYP51 and Mycobacterium tuberculosis CYP51 (21–23).

The first full-length structure of a fungal CYP51 was determined by X-ray crystallography of Erg11p from the yeast Saccharomyces cerevisiae (24). Two structures were deposited in the PDB, one with the native substrate lanosterol (PDB ID 4LXJ) bound in the active site in a precatalytic state and the other with the long-tailed triazole drug ITC (PDB ID 4K0F), at resolutions of 2.19 Å and 1.90 Å, respectively. Structures of Erg11p complexed with FLC or VCZ could be refined only at a significantly lower resolution (≥2.5 Å) and were not deposited in the PDB (24). Elucidation of the structure of the complete enzyme identified the likely orientation of the enzyme in relation to the lipid bilayer. Amphipathic N-terminal membrane helix 1 (MH1) and the adjacent transmembrane helix (TMH1) are oriented at an angle of about 60° to each other, and contacts between TMH1 and the catalytic domain help position the catalytic domain such that it is partly in the lipid bilayer (Fig. 2). The enzyme has a common P450 fold with α-helices (A to L) and β-sheets (1 to 4) constituting the catalytic domain, with the heme cofactor forming the active site. The entrance to the substrate channel is in direct contact with the surface of the lipid bilayer. This ensures that the entry of the lipidic substrate is performed directly from the surface of the membrane. In this report, we present the high-resolution crystal structure of full-length S. cerevisiae Erg11p in complex with the short-tailed triazole FLC that reveals key hydrogen bond networks in the active site.

**MATERIALS AND METHODS**

**Yeast strains.** The yeast strains used in this study are described in Table 1. The ADΔ strain was used for heterologous overexpression of the full-length S. cerevisiae ERG11 gene containing a sequence encoding a C-terminal hexahistidine tag (S. cerevisiae Erg11p6×His [ScErg11p6×His]). This strain is a derivative of the ADΔ strain previously described by Lamping et al. (25, 52) but with the HIS1 gene deleted. Both strains have the

<table>
<thead>
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<th>Table 1 Yeast strains used in this study</th>
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<tr>
<td>Strain</td>
</tr>
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<tr>
<td>ADΔΔ</td>
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<tr>
<td>ADΔΔScErg11p</td>
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<td>ADΔΔScErg11p</td>
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**FIG 1** The chemical structures of the substrate lanosterol, the short-tailed triazole antifungal agents FLC and VCZ, and the long-tailed azole antifungal ITC. An asterisk (*) indicates the 14α-methyl of the lanosterol.

**FIG 2** The structure of ScErg11p6×His. The protein is colored from the N terminus to the C terminus with a gradient from blue to red. The heme (purple carbons) and FLC (green carbons) are shown in sticks. For clarity, only some of the α-helices and β-sheets are labeled. FSL, fungus-specific loop.
major ATP-binding cassette transporter pumps deleted and are therefore hypersensitive toazole drugs. The ScErg11p6×His strain was constitutively overexpressed from the PDR5 locus due to a gain-of-function pdr1-3 mutation in the Pdr1p transcriptional regulator (26). The endogenous ScERG11 gene was then replaced via homologous recombination using a histidine (His1) marker to create strain AD3ΔScErg11p (Table 1) using a previously described method (25).

MIC_{50} determinations. Triazole drugs are fungistatic and often give trailing growth even at high drug concentrations. Therefore, MICs were determined at 80% growth inhibition compared to the level seen with the nondonor controls. In addition, the RPMI medium used for standard MIC determinations with Candida albicans supports poor growth in S. cerevisiae (27) and so was not used. Instead, the MIC_{50}s of FLC, ITC, and VCZ for yeast cells were determined using 0.079% complete synthetic media (Formedium, Norfolk, United Kingdom) containing 0.67% (wt/vol) yeast nitrogen base without amino acids (BD Difco Laboratories Inc., Franklin Lakes, NJ), 2% (wt/vol) glucose, 10 mM MES (4-morpholineethanesulfonic acid), and 20 mM HEPES (buffered with Tris to pH 6.8). The 96-well microtiter plates were seeded with cells to an optical density at 600 nm (OD_{600}) of 0.005. The microtiter plates were incubated at 30°C with shaking at 200 rpm for 48 h, and OD_{600} measurements were made using a BioTek Synergy 2 multimode plate reader (BioTek Instruments, VT, USA). MIC_{50} determinations were done in triplicate for each strain and in 3 separate experiments.

Protein purification. The purification of ScErg11p6×His was carried out according to methods described previously (24). In brief, crude membrane preparations were from liquid cultures grown overnight in YPD medium (1% [wt/vol] yeast extract [BD Difco], 2% [wt/vol] peptone [BD Difco], 2% [wt/vol] dextrose) at 30°C with shaking at 200 rpm. Cells were broken by bead beating, and crude membranes were obtained by differential centrifugation. Crude membranes were solubilized with 10% critical micelle concentration (CMC) n-decyl-β-D-maltoside (DM) (Affymetrix Inc., Santa Clara, US). ScErg11p6×His was purified from the solubilized crude membrane fraction by affinity chromatography using 2 ml of packed nickel-nitrilotriacetic acid (Ni-NTA)-agarose matrix (Qiagen) per gram of protein. FLC was added at a final concentration of 40 μM to 5 ml of pooled eluate obtained with 200 mM imidazole. The affinity-purified ScErg11p6×His was concentrated by centrifugal filtration using a 50-kDa-molecular-mass-cutoff Amicon Ultra-4 centrifugal filter (Millipore). The sample was further purified by size exclusion chromatography (SEC) using a Superdex 200 10/300 GL column (GE Healthcare Life Sciences, Uppsala, Sweden) and further purified by hydroxyapatite chromatography medium (10% [wt/vol] glycerol, 150 mM NaCl, 20 mM HEPES, 0.5 mM phenylmethanesulfonyl fluoride, 16 mM DM [4× CMC], 10 μM FLC, and 1 Roche EDTA-free protease inhibitor pill per 400 ml). The pooled, colored fractions containing the 62-kDa ScErg11p6×His were concentrated using a 50-kDa-molecular-mass-cutoff Amicon Ultra-4 centrifugal filter.

Cytochrome P450 concentration. Carbon monoxide-binding spectra were used to determine the concentrations of functional and nonfunctional cytochrome P450 according to the protocol described by Guengerich et al. (28). The cytochrome P450 concentration was determined using 1.5 μM ScErg11p6×His in affinity chromatography medium (10% [wt/vol] glycerol, 250 mM NaCl, 20 mM Tris, 0.5 mM phenylmethanesulfonyl fluoride, 16 mM DM [10× CMC], and 1 Roche EDTA-free protease inhibitor pill per 400 ml). The pooled, colored fractions containing the 62-kDa ScErg11p6×His were concentrated using a 50-kDa-molecular-mass-cutoff Amicon Ultra-4 centrifugal filter.

Carbon monoxide-binding spectra were recorded using a Cary 1 Bio UV-visible spectrophotometer. The sample was equilibrated with 100% CO for 10 min before data collection. The difference absorbance spectrum was calculated according to the protocol described by Guengerich et al. (28). The carbon monoxide-binding spectra were recorded using a Cary 1 Bio UV-visible spectrophotometer. The CO-P450 complex was obtained by bubbling CO through the sample in 10-mm-path length cuvette at a pH range of 9.3 to 9.5. The drops were 4 μl in a 1:1 ratio of reservoir solution and 20 μg/ml of protein in SEC buffer. The spectra were flash-cooled in liquid nitrogen prior to data collection. A complete data set was collected on the MXI1 beamline at the Australian Synchrotron using an ADSC Quantum 210r detector. The data were indexed and integrated using Mosflm (32) and scaled with SCALA (33). Molecular replacement was carried out using Phaser-MR (34) from Phenix (35) and ScErg11p6×His cocrystallized with lanosol (PDB ID 4LXJ) as the template (24). Refinement and modeling were performed using phenix.refine (35) and Coot (36), respectively. The inhibitor was modeled into the appropriate density in the active site, and quantities of water were added if at least one hydrogen bond was detected (2.5 to 3.3 Å).

Iron-to-nitrogen and iron-to-sulfur distances were constrained during refinement to 2.15 Å and 2.33 Å based on the average coordinate bond distance for the difference in absorbance between 445 and 490 nm (29). The cytochrome P450 concentration was determined using 1.5 μM ScErg11p6×His in affinity chromatography medium (10% [wt/vol] glycerol, 250 mM NaCl, 20 mM Tris, 0.5 mM phenylmethanesulfonyl fluoride, 16 mM DM [4× CMC], 10 μM FLC, and 1 Roche EDTA-free protease inhibitor pill per 400 ml). The pooled, colored fractions containing the 62-kDa ScErg11p6×His were concentrated using a 50-kDa-molecular-mass-cutoff Amicon Ultra-4 centrifugal filter.

TABLE 2 FLC, ITC, and VCZ MIC_{50} values for S. cerevisiae strains

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<td>0.05 (± 0.001)</td>
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<tr>
<td>ADΔΔ</td>
<td>0.4 (± 0.04)</td>
<td>0.03</td>
<td>0.05 (± 0.001)</td>
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<td>0.105 (± 0.004)</td>
<td>0.25 (± 0.05)</td>
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</table>

* MIC_{50} values were determined as described in Materials and Methods. The table shows the means for 3 separate clones of each strain calculated using data obtained in triplicate measurements from at least 3 different experiments (a total of 9 determinations per strain). Values for standard errors of the means (SEM) are indicated in brackets.

RESULTS AND DISCUSSION

Drug susceptibility of S. cerevisiae strains expressing Erg1p. As expected, deletion of the HIS1 ORF in the ADΔ strain did not alter the azole susceptibility pattern in the AD2Δ derivative strain. The parent and derivative strains were equally highly sensitive to the triazole drugs (Table 2). Constitutive hyperexpression of

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ScErg1p6×His from the PDR5 locus under the control of the gain-of-function pdr1−3 mutation increased the resistance of the AD2Δ strain to FLC 6-fold, to ITc 3.5-fold, and to VCZ 5-fold. Removal of the endogenous ScERG11 in the AD3ΔScErg11p strain did not significantly increase susceptibility to these triazoles. This showed that the azole resistance was dependent on the strain to FLC 6-fold, to ITc 3.5-fold, and to VCZ 5-fold. SS. cerevisiae Erg11p in complex with FLC. Full-length S. cerevisiae lanosterol 14α-demethylase (ScErg1p6×His) copurified with FLC during SEC was used for crystallization. Data collection parameters and refinement statistics are presented in Table S1 in the supplemental material. Molecular replacement was carried out using full-length crystal structure of Cyp51A in complex with fluconazole (42). The apparent Hill number of 3 for FLC and 2.7 for VCZ are unlikely to be due to positive cooperativity between ScErg11p monomers. They are more likely to result from multiple interactions between the ligand and the internal active site of an enzyme (43) that behaves as a monomer within its own detergent vesicle. Consistent with this hypothesis, size exclusion chromatography analysis of the affinity-purified enzyme gives enzyme-containing detergent vesicles that migrate with an apparent molecular mass of 100 kDa (24).

Binding of triazole drugs to ScErg11p6×His. The binding of FLC (Fig. 4a) and VCZ (data not shown) to ScErg11p6×His gave type II difference spectra caused by the coordination of the nitrogen atom in the triazole ring with the heme iron, replacing the water ligand and stabilizing the low-spin form (39). The absorbance spectra of ScErg1p6×His in the presence of the triazoles caused a red shift of the heme Soret peak from 417 nm to 421 nm for both VCZ and FLC. The difference spectra obtained with 1 μM ScErg1p6×His enzyme in the presence of excess FLC and VCZ were very similar, with a peak at 428 nm and a trough at 410 nm (Table 3). The binding is normally characterized as tight if the $K_d$ values are lower than or equal to those of the enzyme concentration, and the Morrison equation is generally used to fit the data (40). However, using the Hill equation for analysis of the change in absorbance caused by drug binding versus the change in the concentration of triazole drug added, and not the rearrangement of the Morrison equation, gave the best fit (Fig. 4b and Table 3). The $[\text{azole}]_{0.5}$ values were similar for the two drugs, as were the $K_d$ values (Table 3). Similarly to previous studies performed with C. albicans CYP51, the $[\text{azole}]_{0.5}$ values were found to be about half the $K_d$ concentration values, indicating tight binding for VCZ and FLC (31, 41). The $K_d$ value for FLC of 74 ± 15 [standard error] nM is comparable to those previously obtained using the Morrison equation for CaCYP51 in two different studies, i.e., 46.6 ± 10.6 nM (31) and 60 ± 10 nM (42). The apparent Hill numbers of 3 for FLC and 2.7 for VCZ are unlikely to be due to positive cooperativity between ScErg11p monomers. They are more likely to result from multiple interactions between the ligand and the internal active site of an enzyme (43) that behaves as a monomer within its own detergent vesicle. Consistent with this hypothesis, size exclusion chromatography analysis of the affinity-purified enzyme gives enzyme-containing detergent vesicles that migrate with an apparent molecular mass of <100 kDa (24).

Spectral characterization of purified ScErg11p. The absorbance spectrum for ScErg1p6×His in its low-spin ferric state showed a heme Soret (γ) band at 417 nm and δ, β, and α peaks at 350, 535, and 571 nm, respectively (Fig. 3). One-electron-reduced spectra obtained using sodium dithionite gave a blue shift of the Soret peak from 417 nm to 414 nm. CO binding to the ferrous ScErg1p6×His produced a characteristic red shift to 445 nm. The minor shoulder at 420 nm indicated the presence of a small amount of inactive P420 complex in the purified enzyme preparation. The absolute, reduced, and CO spectra for purified ScErg1p6×His were comparable to those obtained previously for S. cerevisiae microsomal preparations by Yoshida and Aoyama (38) and indicated that the ScErg1p6×His preparation was almost completely active.

ScErg1p6×His from the PDR5 locus under the control of the gain-of-function pdr1−3 mutation increased the resistance of the AD2Δ strain to FLC 6-fold, to ITc 3.5-fold, and to VCZ 5-fold. Removal of the endogenous ScERG11 in the AD3ΔScErg11p strain did not significantly increase susceptibility to these triazoles. This showed that the azole resistance was dependent on the presence of recombinant ScErg1p6×His and not that of the native ScErg1p.

Figure 3: Spectral characterization of ScErg1p6×His. Data represent the absolute spectra of the ferric protein (solid trace), the ferrous protein reduced with sodium dithionite (dashed trace), and the reduced protein with bound carbon monoxide (dotted trace). Spectra were recorded using 1.5 μM ScErg1p as described in Materials and Methods.
TABLE 3 Binding of triazole drugs to affinity-purified ScErg1p6×His<sup>a</sup>

<table>
<thead>
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<th>Triazole</th>
<th>ΔA&lt;sub&gt;max&lt;/sub&gt;</th>
<th>λ&lt;sub&gt;rough&lt;/sub&gt;</th>
<th>λ&lt;sub&gt;peak&lt;/sub&gt;</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
<th>Hill no.</th>
<th>[Azole]&lt;sub&gt;IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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<td>FLC</td>
<td>0.048</td>
<td>410</td>
<td>428</td>
<td>0.074 (± 0.015)</td>
<td>3.0</td>
<td>0.43</td>
</tr>
<tr>
<td>VCZ</td>
<td>0.051</td>
<td>410</td>
<td>428</td>
<td>0.082 (± 0.018)</td>
<td>2.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> The binding ofazole drugs to 1 μM Ni-NTA affinity-purified ScErg1p6×His was determined as described in Materials and Methods. max, maximum. Standard errors are shown in parentheses.

ScErg1p6×His (PDB ID 4LXJ) and revealed a single monomer in the asymmetric unit (PDB ID 4WMZ) comprising MH1, TM1, and the catalytic domain as seen previously (24) (Fig. 2). The enzyme appears to be a rigid molecule, as the tertiary structure and the shape of the substrate channel are unchanged, regardless of the size of the ligand (lanosterol, ITC, or FLC) bound in the active site (24). Some differences in ScErg1p6×His in complex with FLC, compared with the two previously deposited structures, were detected in side chains of residues of the transmembrane helix, in side chains of residues of helix-F and helix-G and the F′/G loop that connects them, and in some parts of the enzyme exposed to the solvent. The F/G loop is buried in the membrane, and this region has the highest B-factor values together with the transmembrane helix and the three residues Y439, S440, and V441 in all three structures, as previously discussed (24). Residues Y439 and V441 are within the N-terminal portion of a fungus-specific loop and are located at the surface of the protein.

The proposed egress channel that bifurcates from the substrate channel shows some density in the present structure comparable to that seen in the two previously published structures (24). The density has been proposed to representzymosterol rather than the immediate product 4,4-dimethylcholesta-8,14,24-trienol, based on mass spectrometry analysis of the purified enzyme (24). The side chains of residues around this secondary vestibule are positioned similarly among all three structures except H128, which has been modeled into different positions due to the limited density. This flexibility may be required to accommodate the exit of the product or interaction with subsequent enzymes in the ergosterol biosynthesis pathway, e.g., Erg24 and the Erg25-Erg28 complex (44).

**FLC binding.** FLC binds within the active site of the enzyme, with clear evidence of the ligand apparent from the molecular replacement sequence immediately following the solution step of the experiment (Fig. 5a). The triazole ring of FLC is coordinated to the iron within the heme cofactor (Fe-N distance, 2.13 Å), corroborating the spectrophotometric data showing type II binding. The immediate environment surrounding FLC is hydrophobic, as would be expected from the nature of the lanosterol substrate. Residues within 4 Å of FLC are illustrated in Fig. 5b and include Y126, F134, I139, Y140, F236, G310, V311, G314, G315, T318, L380, and M509. The 2,4-difluorophenyl ring of FLC lies in a position adjacent to G310, and the coordinated triazole ring abuts G314 on the GXXXG consensus motif for sterol binding on helix-I. Consistent with other eukaryotic CYP51 structures, such as the *Trypanosoma* CYP51 structure, the bend in helix-1 of ScErg1p1 at this motif is less acute than in the *M. tuberculosis* CYP51 structure (19). Two water molecules form hydrogen bond networks with FLC (Fig. 5c). The first water (molecule 743) mediates hydrogen bonds between the hydroxyl groups of FLC and Y140 as well as a propionate of the heme cofactor. The second water (molecule 790) forms hydrogen bonds with the carbonyl oxygen of S382, the hydroxyl of Y126, and N4 of the second triazole ring which points away from the heme (Fig. 5c). In the previously published structures of ScErg1p6×His complexed with lanosterol and ITC, neither water 743 nor water 790 was observed. Previously reported structures of other CYP51s complexed with FLC have the inhibitor bound in a similar manner. The orientation of the 2,4-difluorophenyl ring matches that seen in the *M. tuberculosis* (PDB ID 1EA1) and *T. cruzi* (PDB ID 2WX2) CYP51 structures (17). The 2,4-difluorophenyl ring is rotated 180° in several other structures, but this orientation would appear to be potentially unfavorable, as the 2-fluoro substituent and the hydroxyl group of FLC are in close proximity (PDB ID 2WV2 and 2WUZ) (17). The latter alternate orientation (2WUZ) is stabilized by a hydrogen bond between the fluorine substituent and the hydroxyl of Y103 (corresponding to Y126 in ScErg1p6×His) (17). This residue (Y103) shows a great deal of flexibility among the trypanosomal structures, including occupying the site for key water molecule 743.

**VCZ,** another short-tailed antifungal agent, is similar to FLC and differs only in that the second noncoordinating triazole is replaced with a fluoropyrimidine group along with the addition of a methyl group. In the previous modeled structure obtained with a lower-resolution data set (2.8 Å), the fluoropyrimidine ring is positioned in the same plane as the triazole ring, with the fluoro substituent positioned in the direction opposite to that seen with the hydroxyl of the inhibitor and the methyl group (24). The presence of the key water (743) would also provide the potential for a hydrogen bond with N3 of the fluoropyrimidine in this orientation in preference to the flipped conformation.

**Antifungal resistance.** Mutations which reduce the effectiveness ofazole drugs are frequently found in resistant pathogenic fungi. Of the residues within 4 Å of FLC in ScErg1p6×His that have residues equivalent to those found to be mutated in *C. albicans* Erg1p1, only Y132F/H (Y140F/H in ScErg1p1) is known to confer resistance to FLC as a single mutation (13, 45). The others confer resistance in combination with additional mutations or confer susceptible phenotypes alone (46). The single-amino-acid substitutions Y132F, K143R, F145L, S405F, D446E, G448E, F449V, G450E, and G464S in *C. albicans* Erg1p1 have been found to confer resistance to FLC (45). The FLC MICs for those mutants were increased ~4-fold compared to those for the susceptible strain. Our structure shows that only Y132F, K143R, and G464S are located close enough to the active site and the heme cofactor to directly affect the binding of FLC. Residues D446, G448, F449, and G450 (fungus-specific loop) as well as F145 and S405 are too far away to directly affect the heme or interfere with FLC binding.

The Y132F/H mutation in *C. albicans* is commonly found in clinical isolates of other fungal pathogens of humans and plants that have reduced susceptibility to FLC and other azoles (12, 47). As described above, a key water molecule (743) mediates hydrogen bonds between the hydroxyl group of the inhibitor, the hydroxyl group of Y140, and the propionate group of the heme.
Homologous mutations at Y140 to either phenylalanine or histidine could disrupt this hydrogen bonding network and therefore reduce susceptibility to FLC.

Mutations equivalent to G464S (S. cerevisiae and C. albicans numbering) have been found in both C. albicans and A. fumigatus (see Fig. S2 in the supplemental material). The C. albicans G464S mutation has been found in clinical isolates and gives rise to FLC resistance (13, 45, 48), while the equivalent G448S mutation in A. fumigatus CYP51B is thought to confer resistance to the long-tailed drugs ITC and PCZ but not resistance to the short-tailed VCZ drug (49). It has been suggested that this substitution would disrupt the positioning of the heme, reduce the binding of theazole, and increase resistance (22). However, its differential effect on short-tailed azoles compared to long-tailed azoles is not understood. G464 is located on the same face of the heme as the coordinating cysteine residue and is close to a propionate group of the cofactor that makes a hydrogen bond to a water molecule (see Fig. S2). The G464S mutation may replace this water and alter the interaction with the heme carboxylate, thus potentially tilting the heme.

The K143R mutation in C. albicans CYP51 (K151 S. cerevisiae numbering) confers resistance to FLC (50). This residue is also located on the same side of the heme cofactor as the coordinating cysteine (see Fig. S2 in the supplemental material). In the current crystal structure, the side chain of K151 forms an ionic interaction with the carboxylate group of the heme. The change from lysine to arginine, while being functionally conservative, results in the addition of a large guanidinium group in an environment optimized for lysine. The change causes local disruption, but its extent is unknown. A. fumigatus has innate resistance to FLC, which may be a result of sequence differences between the two homologues of CYP51 present in this species (see Fig. S1 in the supplemental material). Most fungal species have a threonine at the position corresponding to T322 on helix I (S. cerevisiae numbering). A. fumigatus CYP51A has an isoleucine at this position (A. fumigatus CYP51A numbering, I301) whereas A. fumigatus CYP51B maintains the threonine (A. fumigatus CYP51B numbering, T315). It has been hypothesized that this difference confers resistance to FLC (51). Alanine mutagenesis at the homologous T315 position in C. albicans has been shown to result in increased resistance to FLC CYP51 (41). The current structure of ScErg11p shows that T322 is located adjacent to the active site on helix-I and close to an ethylene group of the heme but about 10 Å from the iron-coordinated triazole group. G310 (S. cerevisiae numbering) is within a distance of 4 Å of the drug. Glycine or alanine is accommodated in this position in fungal CYP51s, apart from A. fumigatus CYP51A, which has threonine (T289), and in one of two se-
quences available for *Coccidioides immitis* RS CYP51 (T303). This residue appears to be in better position than T322 to alter the binding of FLC (Fig. 5c).

**Future prospects.** The high-resolution X-ray crystal structure of full-length recombinant *S. cerevisiae* CYP51 (ScErg11p×His) complexed with FLC and the resultant discovery of hydrogen bonding networks involving key water molecules within the active site provide the basis for detailed exploration of both the binding and resistance mechanisms ofazole antifungal drugs, in particular, FLC and VCZ, in fungal pathogens. Due to the strong homology of fungal CYP51s, ScErg11p can be used, both structurally and experimentally, as a model to study the mutations that have been observed in resistant clinical isolates of fungal pathogens. The presence of a water-mediated hydrogen bond network between an inhibitor and Y140 in the active site of lanosterol 14α-demethylase when complexed with the short-chain FLC but not with the long-chain ITIC, as well as interactions of the long tail of ITIC in the enzyme entrance channel (24), may explain the differential levels of susceptibility to FLC and ITIC conferred by mutations equivalent to Y140F/H. Structural and functional analysis will enable rigorous determination of the effects of these mutations on the protein and its affinity for these drugs.

**ACKNOWLEDGMENTS**

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Triazole resistance mediated by mutations of a conserved active site tyrosine in fungal lanosterol 14α-demethylase

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Emergence of fungal strains showing resistance to triazole drugs can make treatment of fungal disease problematic. Triazole resistance can arise due to single mutations in the drug target lanosterol 14α-demethylase (Erg11p/CYP51). We have determined how commonly occurring single site mutations in pathogenic fungi affect triazole binding using Saccharomyces cerevisiae Erg11p (ScErg11p) as a target surrogate. The mutations Y140F/H were introduced into full-length hexahistidine-tagged ScErg11p. Phenotypes and high-resolution X-ray crystal structures were determined for the mutant enzymes complexed with short-tailed (fluconazole and voriconazole) or long-tailed (itraconazole and posaconazole) triazoles and wild type enzyme complexed with voriconazole. The mutations disrupted a water-mediated hydrogen bond network involved in binding of short-tailed triazoles, which contain a tertiary hydroxyl not present in long-tailed triazoles. This appears to be the mechanism by which resistance to these short chain azoles occurs. Understanding how these mutations affect drug affinity will aid the design of azoles that overcome resistance.

Fungal infections are estimated to directly affect approximately one billion people globally and also have the potential to affect food security, especially in temperate and tropical climates. The increased incidence of potentially lethal invasive fungal infections (IFIs) and the emergence of resistant fungal pathogens are growing concerns1,2.

Fungal infections of humans that require medical intervention affect the immune-deficient, such as the very young (premature infants), females of reproductive age, the elderly and the debilitated. The most susceptible individuals have co-morbidities such as AIDS and cancer, or are patients who are medically immune suppressed. Members of the fungal species Candida, Aspergillus and Cryptococcus are the most prominent causes of IFIs in humans3,4. For example, in Sub-Saharan Africa where AIDS is endemic, infection by Cryptococcus neoformans is estimated to cause over 500,000 deaths each year5. Candida albicans is the 4th leading cause of blood stream infections detected in the United States6 while Aspergillus fumigatus infections have a high mortality rate among transplant patients7.

Fungal infection of plants, crops, fruit and stored produce is a major problem, especially in temperate and tropical climates. This problem is likely compounded by extensive monoculture, where natural barriers to fungal infection are insufficient and the widespread application of fungicides is required8 e.g. for control of the soybean phytopathogen Phakopsora pachyrhizi or the wheat pathogen Zymoseptoria tritici (previously known as Mycosphaerella graminicola). Multiple generations of phytopathogen-targeting triazole agrochemicals have been used since the 1970s to counter shifts in antifungal susceptibility elicited by exposure to earlier azoles.

The triazole drugs, the largest class of antifungals used in the clinic or as agricultural fungicides, are becoming less effective in treating mycoses of humans and animals and in preventing the devastating effects of phytopathogens on crop production. This has occurred because of the emergence of less susceptible fungal strains9. Resistance to azoles used in medicine has arisen, in part, because of the use of prophylaxis with susceptible

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patients and prolonged treatment courses\textsuperscript{10,11}. A contribution to this problem by azole-based agrochemicals was recognised recently, with these fungicides shown to induce cross-resistance to medical triazoles in \textit{A. fumigatus} and \textit{Candida glabrata} \textsuperscript{12,13}. There is an increasingly urgent need to develop antifungals and agrochemicals capable of overcoming triazole resistance.

A common mechanism of resistance among fungal pathogens occurs due to mutations in the enzymatic target of azole drugs, the cytochrome P450 lanosterol 14α-demethylase (denoted as Erg11p or CYP51). Azoles inhibit the demethylase by coordinating to the heme iron in the active site via a heterocyclic nitrogen atom of an imidazole, triazole or tetrazole ring\textsuperscript{14–16}. In \textit{C. albicans} Erg11p (CaErg11p) the Y132F/H mutations have frequently been detected in clinical isolates and lead to a 4-fold increase in resistance to fluconazole (FLC)\textsuperscript{17,18}. Homologous mutations occur in the CYP51 genes of other fungal pathogens of man and plants (Fig. 1a)\textsuperscript{8,19,20}. For example, the Y145F mutation in \textit{C. neoformans} CYP1\textsuperscript{21} and Y136F mutation in \textit{Histoplasma capsulatum} CYP1\textsuperscript{22} both cause resistance to the short-tailed triazoles FLC and voriconazole (VCZ) but not to the long-tailed triazoles posaconazole (PCZ) or itraconazole (ITC, Fig. 1b). Similarly, the mutations Y137F in \textit{Z. tritici} CYP1\textsuperscript{23} and Y136F in \textit{Uncinula necator} CYP1\textsuperscript{24} confer reduced susceptibility to the short-tailed azole fungicide triadimenol. In \textit{A. fumigatus} the homologous CYP51 Y121F mutation can occur alone\textsuperscript{25} or together with the T289A mutation and tandem repeat 46 (TR46) in the promoter region\textsuperscript{26}. The Y121F mutation confers resistance to VCZ but not ITC or PCZ\textsuperscript{25} while the TR46 Y121F T289A mutation is associated with failure of voriconazole therapy and a slightly reduced susceptibility to ITC and PCZ\textsuperscript{26}.

The selection pressure exerted by individual triazole drugs can differ. For example, the \textit{Z. tritici} CYP51 Y137F mutation arose after the introduction of triadimenol but essentially disappeared in the field following the introduction of prothioconazole\textsuperscript{27}, a pro-drug that is metabolised to the active short-tailed triazole agrochemical prothioconazole-destroil\textsuperscript{28}. The accumulation of multiple mutations in CYP51 can lead to significant reductions in triazole susceptibility. For example the Y131F I475T combination of mutations has been detected in CYP51 of the strains of the phytopathogen \textit{P. pachyrhizzi}\textsuperscript{29}. The comparable double mutation Y132H I471T is found in Erg11p of the triazole resistant Darlington strain of \textit{C. albicans}\textsuperscript{30}.

Figure 1. (a) Sequence alignment of fungal CYP51s. Alignment of \textit{Histoplasma capsulatum} CYP51 (HcCYP51), \textit{Zymoseptoria tritici} CYP51 (ZtCYP51), \textit{Uncinula necator} CYP51 (UnCYP51), \textit{Aspergillus fumigatus} CYP51A (AfCYP51A), \textit{Cryptococcus neoformans} CYP51 (CnCYP51), \textit{Candida albicans} CYP51 (CaErg11p), and \textit{Saccharomyces cerevisiae} CYP51 (ScCYP51). The frequently mutated tyrosine residue homologous to ScErg11p Y140 is highlighted in grey. (b) The chemical structures of triazole antifungals used in this study: fluconazole, voriconazole, itraconazole and posaconazole.
Until recently, a paucity of structural information on fungal lanosterol 14α-demethylase has meant that the molecular mechanisms determining the reduced susceptibility conferred by mutations in fungal lanosterol 14α-demethylyase were not well understood. We have reported high-resolution structures of full-length Saccharomyces cerevisiae hexahistidine-tagged Erg11p (ScErg11p6 × His) in complex with its substrate lanosterol (1.9 Å, PDB ID: 4XLJ) and the long chainazole itraconazole (2.1 Å, PDB ID: 5EQB)31. We have also described a high-resolution (2.05 Å) structure of ScErg11p6 × His in complex with FLC (PDB ID: 4WMZ)32. Based on the latter structure, the ScErg11p Y140F/H mutations were proposed to potentially modify a water-mediated hydrogen bond network involving the tertiary hydroxyl group of FLC, thus weakening the binding of the drug. In the present study, new high-resolution X-ray crystal structures of the ScErg11p6 × His Y140F mutant in complex with ITC, FLC, VZC and PCZ and the Y140H mutant in complex with FLC and ITC, along with the wild type ScErg11p6 × His in complex with VZC, provide evidence that disruption of this hydrogen bond network leads to weaker drug binding.

Results
Quantitation of the expression of ScErg11p6 × His in crude membrane fractions. The strains used in the present study are shown in Supplementary Information, Supplementary Table S1.

Coomassie blue R250 stained SDS-polyacrylamide gel profiles (Supplementary Fig. S1a) and western blot analysis (Supplementary Fig. S1b) of crude membrane fractions obtained from yeast overexpressing ScErg11p6 × His, ScErg11p6 × His Y140F or ScErg11p6 × His Y140H show that the mutant enzymes were expressed in this fraction at levels at or near wild type enzyme. Analysis of the Ni-NTA and affinity-purified 61 kDa band by tryptic digestion and mass spectrometry showed at least 78% coverage of the primary sequence of ScErg11p6 × His. The type II binding curves obtained using the wild type ScErg11p6 × His Y140F mutant showed a sigmoidal dose-response (Fig. 2c) that gave best fit using the Hill equation. The resultant [Azole]0.5 and Kd values are listed in Table 1. These values indicate tight binding for each drug tested. The ΔY140F in the AD3 mutants in complex with ITC and PCZ.

Azole susceptibilities of strains overexpressing ScErg11p6 × His Y140F/H. Susceptibilities to triazole drugs of S. cerevisiae AD3Δ strains overexpressing Erg11p6 × His Y140F/H, but with the native ERG11 deleted, were measured as liquid MIC values (Supplementary Table S2). Overexpression of ScErg11p6 × His Y140F in the AD3ΔScErg11p6 × Y140F strain conferred 2-fold lower susceptibility to FLC than the strain overexpressing wild type ScErg11p6 × His i.e. MIC80 values of 4.0 μg/ml and 2.1 μg/ml, respectively. The same mutant conferred a 1.7-fold reduction in susceptibility to VZC. The MIC80 values were 0.42 μg/ml for the strain overexpressing ScErg11p6 × His Y140F and 0.25 μg/ml for the strain overexpressing wild type ScErg11p6 × His. The susceptibilities to ITC were comparable for the strains overexpressing the ScErg11p6 × His Y140F, ScErg11p6 × His Y140H or wild type enzyme i.e. 0.09 μg/ml, 0.77 μg/ml and 0.105 μg/ml, respectively. Strain AD3ΔScErg11p6 × Y140H showed slightly greater susceptibilities to FLC (3.4 μg/ml) and VZC (0.29 μg/ml) than the Y140F mutant. While strain AD3ΔScErg11p_Y140H showed a reduced susceptibility to FLC, its susceptibility to VZC was comparable to the control strain overexpressing the wild type enzyme.

Spectral characterisation of triazole binding to wild type and mutant ScErg11p6 × His. The absolute absorbance spectra for wild type ScErg11p6 × His showed an oxidised Soret peak at ~417 nm and for the Y140F/H enzymes the peak is seen at 420 nm (Fig. 2a). Carbon monoxide difference spectra obtained with dithionite-reduced wild type and mutant enzymes gave a peak at 445 nm which showed that the bulk of each affinity-purified enzyme was functional (Fig. 2a). The presence of a slightly larger shoulder at 420 nm for the mutant enzymes compared to the wild type enzyme32 indicated that the affinity-purified mutant enzyme may be less stable than the wild type enzyme.

Ni-NTA affinity purified mutant and wild type ScErg11p6 × His preparations (1 μM) were used to detect and quantify type II azole binding. The spectral shift of the Soret peak from 417 nm to 421–424 nm in cytochrome P450s is also detected by the type II difference spectra. This change is associated with the replacement of a water molecule coordinated to the heme iron with theazole heterocycle. Type II difference spectra were obtained for FLC, VZC, ITC and PCZ binding to both mutant enzymes. The binding of FLC is shown as an example (Fig. 2b). The type II difference spectra for the mutant enzymes were less intense than for the wild type enzyme. They gave 2–3-fold smaller differences in absorbance (ΔAmax), with peaks shifted from 428 nm for wild type enzyme to 424–425 nm for the mutant enzymes plus less well-defined troughs between 406–410 nm (Supplementary Table S3). The type II binding curves obtained using the wild type ScErg11p6 × His and the Y140F/H mutants (1 μM) showed a sigmoidal dose-response (Fig. 2c) that gave best fit using the Hill equation. The resultant [Azole]0.5 and Kd values are listed in Table 1. These values indicate tight binding for each drug tested. The Kd values for the wild type and mutant enzymes were comparable i.e. within the margin of the standard error.

X-ray crystal structures of ScErg11p6 × His Y140F/H mutants in complex with ITC and PCZ. X-ray crystal structures were obtained for ScErg11p6 × His Y140F in complex with ITC and PCZ (PDB IDs: 4ZDY and 4ZEL) at resolutions of 2.02 Å and 2.05 Å (Supplementary Table S4), respectively, and for ScErg11p6 × His Y140H in complex with ITC (PDB ID: 4ZE2) at a resolution of 2.30 Å (Supplementary Table S5). Both the ScErg11p6 × His Y140F and Y140H structures showed the binding of ITC was essentially identical to that seen previously with wild type ScErg11p6 × His (PDB ID: 5EQB, Fig. 3)31. In addition to detecting the mutated residue, a water molecule (843) not present in the wild type structure was found between an oxygen of the ITC 1,3-dioxolane group, the propionates of heme rings A and D and the aromatic side chain of the F140 (Fig. 3a) at distances of 4.0, 3.1, 2.5 and 3.3 Å, respectively. Water 843 replaced the phenolic hydroxyl group of Y140 but, because it makes no polar contacts with the inhibitor, it is unlikely to have any effect on ITC binding. The equivalent water molecule was not detected in the structure of ScErg11p6 × His Y140F in complex with PCZ (Fig. 3b) or in ScErg11p6 × His Y140H in complex with ITC (Fig. 3c). Each mutant crystal structure showed the long tail of the triazole ligand bound within the substrate entry channel in an extended conformation, with the piperazine

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ring in the chair conformation. This conformation was stabilized via a water-mediated hydrogen bond network between the tetrahedral nitrogen atom N1 of the piperazine ring and the main chain amide nitrogens of H381 and S382 (Fig. 3d). The water mediating this hydrogen bond network resides in a hydrophilic pocket that contains

Figure 2. Spectral characterisation of ScErg11p6 × His Y140F/H mutants. (a) The absolute (continuous line) and CO bound (dotted line) spectra of ScErg11p6 × His Y140F mutant. (b) Type II difference spectra for FLC binding to ScErg11p6 × His wild type and mutant enzymes. (c) Fluconazole binding to wild type ScErg11p6 × His (●) ScErg11p6 × His Y140F (■) and ScErg11p6 × His Y140H (▲). All curves best fit the Hill equation.

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<td>0.123 (±0.027)</td>
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<td>PCZ</td>
<td>0.32</td>
<td>0.078 (±0.023)</td>
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<td>FLC</td>
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<td>0.13 (±0.05)</td>
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<td>0.10 (±0.04)</td>
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Table 1. Triazole binding to affinity purified wild type and ScErg11p6 × His Y140F/H. Values in brackets indicate standard errors. IC50 value is denoted as [Azole]0.5.
two other waters when ITC or PCZ is bound. The binding of the long-tailed triazoles ITC and PCZ to wild type ScErg11p6×His and the Y140F/H mutants pushes Y126 to a position so it forms hydrogen bonds with the heme ring D propionate group and the amide nitrogen of F384 (Fig. 3). The overall configuration of this residue was found to be less constrained in the presence of FLC, which occupies less space between the two groups (Fig. 4a).

**Figure 3. Binding of long-tailed azoles ITC and PCZ to ScErg11p6×His Y140F/H.** (a) ScErg11p6×His Y140F in complex with ITC (PDB ID: 4ZDY) and (b) PCZ (PDB ID: 4ZE1) and (c) ScErg11p6×His Y140H in complex with ITC (PDB ID: 4ZE2). Panel (d) depicts the hydrophilic pocket (surface representation) in the substrate channel of ScErg11p6×His Y140F. The hydrogen bonding interactions are shown between the water molecule (930), the N1 of the piperazine ring and the main chain amide groups of H381 and S382. The ScErg11p6×His Y140F is shown in lilac cartoon and ScErg11p6×His Y140H is depicted in yellow cartoon. Hydrogen bonds are shown as yellow dashed lines and water molecules as red spheres. ITC (green carbons), PCZ (yellow carbons), the side chains of residues 140 and 126, the backbone of F384, residues H381 and S382 and the heme (magenta) are shown as sticks.

**X-ray crystal structures of ScErg11p6 × His wild type and Y140F/H mutants in complex with FLC and VCZ.** The structures of wild type ScErg11p6×His and the Y140F/H mutant enzyme in complex with FLC (PDB IDs: 4WMZ and 4ZDZ, respectively) showed the drug to be bound in a similar conformation, with all differences in the interactions between the drug and the enzyme ascribed to the mutation. As described in our previous report8, the structure of wild type ScErg11p6×His in complex with FLC included a water-mediated (743) hydrogen bond network that involves the hydroxyl group of FLC, the hydroxyl of Y140 and the heme ring D propionate (Fig. 4a). In addition, the non-coordinated triazole of FLC and the main chain carbonyl of S382 formed a hydrogen bond network mediated by water 790. This water was not detected in mutant ScErg11p6×His in complex with ITC or PCZ because the tails of these ligands occupy that space. A water molecule equivalent to water 743 (numbered 843 in the mutant structures) was found in ScErg11p6×His Y140F in complex with FLC but the hydrogen bond with Y140 was abolished as a result of the Y140F mutation (Fig. 4b, PDB: 4ZDZ). In...
addition, the Y140F mutation prevented formation of a hydrogen bond with the ring D propionate group of the heme. A new hydrogen bond between the propionate and water now exists. Disruption of the hydrogen bond network by the Y140F mutation results in an additional water molecule (numbered 844), hydrogen bonded to water 843 and heme ring D propionate (Fig. 4b).

Wild type ScErg1p6 × His and ScErg1p6 × His Y140H complexes with FLC show the azole in similar conformations (Fig. 4a,c, PDB IDs: 4WMZ and 4ZE3). Water molecule 843 is present and forms a single short hydrogen bond between the imidazole ring of H140 and the hydroxyl of T130 orients the H140 side chain closer to T130 than with Y140 or F140 side chains (Fig. 4c). For ScErg1p6 × His Y140H in complex with ITC, the orientation of the imidazole ring was similar but the N2 of the H140imidazole and the hydroxyl of T130 are separated by 3.4 Å.

Figure 4. Binding of short-tailed azoles FLC and VCZ to ScErg11p6 × His. Water-mediated interactions are shown for binding of (a) FLC and (d) VCZ to wild type ScErg11p6 × His (PDB IDs: 4WMZ and 5HS1, respectively). The modified interactions due to the Y140F/H mutations are depicted in panels (b) and (c) for FLC binding (PDB IDs: 4ZDZ and 4ZE3) and (e) for VCZ binding (PDB ID: 4ZE0). The wild type protein is depicted in grey cartoon (PDB IDs: 4WMZ and 5HS1), the Y140F mutant in lilac and the Y140H in yellow. The hydrogen bonds are shown with dashed lines and water molecules as red spheres. FLC (cyan carbons), VCZ (green carbons), the side chains of residue 140, 126, the backbone of S382 and F384 and the heme (magenta) are shown as sticks.
The crystal structure of wild type ScErg11p6 × His in complex with VCZ (PDB: 5HS1) reveals the azole drug binding in a similar fashion to FLC but with distinct differences due to its structure (Fig. 4d). The pyrimidine ring of VCZ occupies a similar position to the non-coordinating triazole ring of FLC but projects further into the substrate channel. The 5-fluoro substituent of the pyrimidine ring projects towards Y126 forcing this residue close enough to interact with the heme ring A propionate (2.8 Å, as seen in complexes with the long-tailed azoles PCZ and ITC). In the FLC structure (PDB ID: 4WMZ) those two groups are 4.3 Å apart. The key water molecule (743) is present and forms a similar hydrogen-bonding network with the phenolic hydroxyl of Y140, the heme ring D propionate and the tertiary hydroxyl group of VCZ (Fig. 4d). An additional hydrogen bond is present between the 5-fluoro substituent and the key water 743 (3.0 Å), which is shifted slightly towards Y140. The water molecule corresponding to water 790 in the FLC structure is not present at this position due to the projection of the pyrimidine ring. However, a water molecule (750, Fig. 4d) seen in complexes with the long-tailed azoles is also seen here. It forms hydrogen bonds between the pyrimidine nitrogen (N1), the main chain nitrogens of H381 and S382 plus the carbonyl of residue M509 which projects into the binding site cavity (Supplementary Fig. S5). Such flexibility has not been observed in this region previously. In the structure of ScErg11p6 × His in complex with lanosterol the carbonyl of M509 is within 2.9 Å of the tail of the substrate (PDB ID: 4XLI) while the equivalent residue (M487) in human CYP51 has been implicated in substrate recognition due to its close proximity to the ligand ketoconazole (PDB ID: 3LD6)34.

The crystal structure of the ScErg11p6 × His Y140F mutant in complex with VCZ (Fig. 4e, PDB ID: 4ZE0) shows the ligand binds in a similar orientation but slightly deeper into the active site than in the wild type enzyme, with the quaternary carbon shifted ~0.5 Å. The mutation to phenylalanine with the corresponding removal of the hydroxyl group lets water 843 occupy the vacated space and thus allow the ligand to bind deeper. In addition, the plane of the phenyl ring of F140 is slightly shifted away from the wild type complex with VCZ. Y126 is also in a similar position to that seen in the wild type VCZ complex. The distance between the heme ring A propionate and Y126 is 4.4 Å and the additional water (750) is not seen in the mutant complex. M509 no longer projects into the active site but is adjacent to F236. In comparison to the recent structure of A. fumigatus CYP51B in complex with VCZ35, the pyrimidine ring is rotated 180°, allowing the ring to approach Y122 (A. fumigatus numbering; Y126 in ScErg11p6 × His), while the 5-fluoro substituent is oriented towards F504 in a space in the ScErg11p6 × His structure adjacent to V510.

**Discussion**

We have created and analysed the structure and function of surrogate triazole resistance phenotypes in the yeast *S. cerevisiae* by creating Y140F/H single site mutations in lanosterol 14α-demethylase that have homologues in numerous species of pathogenic fungi36–39. This study shows how alterations to a water-mediated hydrogen-bonding network, between the drug and target protein, can affect the affinity of the short-tailed triazole drugs FLC and VCZ but not the long-tailed triazoles ITC and PCZ. Whole cell assays (MIC50 measurements) were used to confirm the reduced susceptibility of ScErg11p6 × His Y140F/H mutants to FLC and VCZ. The 2-fold differences in MIC50 values detected using overexpression of wild type and mutant ScErg11p6 × His in the yeast system were not as dramatic as previously reported for the *C. albicans* Erg11p1 Y132H mutation37 but show a similar trend.

Triazole binding studies using affinity-purified wild type and ScErg11p6 × His Y140F/H enzymes gave differences in both absolute absorption spectra prior to the addition of ligand and the magnitude of the change obtained for type II difference spectra after ligand addition. The differences in the oxidised heme Soret peak between the Y140F/H mutant enzymes (420 nm) and the wild type enzyme (417 nm) indicate that the mutations alter the electronic environment of the heme. Disruption of the short hydrogen bond between Y140 and the heme ring D propionate is the common feature that may explain this effect in both the Y140F and Y140H mutants. The [Azole]0.5 and Kd values obtained from plotting the change in absorbance against theazole concentration with the wild type and mutant enzymes for each triazole drug indicated comparable high affinity binding (Table 1). The apparent lack of correlation between resistant phenotypes andazole affinity for free CYP51 enzyme was previously reported for the *C. albicans* CYP51 Y132H mutant38 and the I471T mutation37. This may reflect difficulty in extracting the information needed to calculate inhibitor affinities due to the formation of near stoichiometric enzyme-inhibitor complexes. In addition, Warrillow et al. have shown that solubilisation of the enzyme from the membranes can also change the ligand-binding properties of CYP51s38. Assays using *A. fumigatus* NADPH cytochrome P450 reductase reconstituted with membrane preparations of *A. fumigatus* CYP51A or CYP51B enzymes heterologously expressed in *E. coli* gave IC50 values 34-fold higher for FLC binding to AICYP51A (17 μM) compared to AICYP51B (0.5 μM). In contrast, FLC binding by these two CYP51 enzymes estimated using type II binding with the Ni-NTA affinity purified enzymes gave only a 3-fold difference in IC50 values i.e. 12 μM for AICYP51A and 4 μM for AICYP51B. These discrepancies, presumably caused by the solubilisation of the enzyme with detergents, highlight the importance of the lipid bilayer in positioning the enzyme, determining its conformation and the access of triazole inhibitors to the active site. Reconstitution of the affinity-purified enzyme in lipid vesicles or nanodisks38 may provide methods to obtain more reliable and physiologically relevant estimates of the binding affinities of CYP51 inhibitors.

The ScErg11p6 × His Y140F/H mutations disrupt the water-mediated hydrogen bond network between enzyme and both FLC and VCZ. These findings help explain the FLC and VCZ resistance but retention of ITC and PCZ susceptibility in Y140F/H mutants. The key tertiary hydroxyl group in FLC and VCZ is replaced by a 1,3-dioxolane moiety in both long-tailed triazoles with additional hydrophobic interactions with the entry channel exclusive to these inhibitors. Calorimetric studies have shown that it is energetically favourable to include a water molecule at the protein-ligand interface, providing evidence that water-mediated interactions can stabilise protein-ligand complexes38. Sharrow et al. demonstrated that the loss of water-mediated hydrogen bonding due to a tyrosine to phenylalanine mutation reduced favourable enthalpy and the binding affinity of a ligand to its
target protein. The water-mediated polar interactions between Y140 and the tertiary hydroxyl of FLC and VCZ optimally stabilise the binding of these drugs.

Triazole drugs currently in clinical trials include albaconazole, ravuconazole, isavuconazole and VT-1161 (Supplementary Fig. S6). All four of these compounds have the tertiary hydroxyl group that would interact with the Y140 residue. We postulate that these drugs may become less effective inhibitors of CYP51 due to mutations equivalent to Y140F/H (S. cerevisiae numbering). Isavuconazole has been approved for treatment of invasive aspergillosis in the USA. However clinical isolates of A. fumigatus with the Trp/Cys121/Thr289/S286/CYP51 mutations are found in many parts of the world. This mutant has the potential to be resistant to isavuconazole due to the Y121F mutation, which confers resistance to VCZ. The novel tetrazole compound VT-1161 is currently in phase II clinical trials and has been shown to be a potent inhibitor of C. albicans CYP51. A recent structure of the Trypanosoma cruzi CYP51 in complex with VT-1161 (PDB ID: 5AJR) places the hydroxyl group of the drug into the same position as the hydroxyl group of FLC in the ScErg11p structure (PDB ID: 4WMZ). Thus the tertiary hydroxyl of VT-1161 could interact with Y140 (S. cerevisiae numbering) via a water-mediated hydrogen bond network. Therefore, mutations equivalent to ScErg11p Y140F/H have the potential to confer resistance to albaconazole, ravuconazole, isavuconazole or VT-1161 unless their medium-length tails in the entry channel confer sufficient affinity to counteract this problem. The short-tailed agrochemicals tebuconazole and prothioconazole have a similarly located hydroxyl group and resistance can be expected to be conferred via the equivalent mutations in fungal phytopathogens e.g. Z. tritici YCP51 Y137F and P. pachyrhizi YCP51 Y131F I475T.

In conclusion, ScErg11p × His crystal structures have revealed that the resistance conferred by the Y140F/H mutations via the loss of hydroxyl group results in the disruption of a water-mediated hydrogen bond network between the tertiary hydroxyl groups of FLC and VCZ and the enzyme. This leads directly to a decrease in binding affinity. We predict that this type of resistance could be avoided by designing the drug into the same position as the hydroxyl group of FLC in the ScErg11p structure (PDB ID: 4WMZ). Thus the tertiary hydroxyl of VT-1161 could interact with Y140 (S. cerevisiae numbering) via a water-mediated hydrogen bond network. Therefore, mutations equivalent to ScErg11p Y140F/H have the potential to confer resistance to albaconazole, ravuconazole, isavuconazole or VT-1161 unless their medium-length tails in the entry channel confer sufficient affinity to counteract this problem. The short-tailed agrochemicals tebuconazole and prothioconazole have a similarly located hydroxyl group and resistance can be expected to be conferred via the equivalent mutations in fungal phytopathogens e.g. Z. tritici YCP51 Y137F and P. pachyrhizi YCP51 Y131F I475T.

Materials. Desalted oligonucleotides, FLC, ITC, VCZ and PCZ were purchased from Sigma-Aldrich Ltd (St. Louis, MO). Colony polymerase chain reactions (PCR) were carried out using TaKaRa DNA polymerase (Takara Bio Inc, Shiga, Japan). All other PCR reactions were performed using KOD Hot Start DNA polymerase (Novagen, Madison, WI). PCR clean up and DNA gel extraction was carried out using kits from Qiagen Pty Ltd (Limburg, Netherlands). Genomic DNA from yeast was isolated using the Y-DER kit from Thermo Fisher (Waltham, MA). Yeast DNA transformation was carried out using an Alkali Cation Yeast Transformation kit from Qbiogene (Irvine, CA). DNA transformation cassettes and genes inserted at the PDR1 locus for resistance to 7 major ATP-binding cassette transporters and the PDR3 gene. Both strains have the gain-of function pdr1–3 mutation in the PDR1 transcriptional regulator gene to provide constitutive overexpression of ScErg11p × His from the PDR5 locus. Yeast strains were grown on YPD medium: 1% (wt/vol) Bacto-yeast extract (BD Difco™), 2% (wt/vol) Bacto-peptone (BD Difco™) and 2% (wt/vol) glucose. Synthetic defined (SD) medium was used for selection of transformants. It contained 2% (wt/vol) glucose, 0.67% (wt/vol) yeast nitrogen base without amino acids (BD Difco™), 2% (wt/vol) agar (Oxoid Ltd., Hampshire, UK) and either uracil drop-out (Qiogene, Irvine, CA) or histidine drop-out (Formedium™, Norfolk, UK) complete supplement mixture. Liquid SD media with complete supplement mixture (Formedium™) containing 10 mM MES and 20 mM HEPES buffered with TRIS to pH 6.8 was used for MIG40 determinations.

Construction of yeast strains overexpressing ScErg11p × His Y140F/H. ScErg11p Y140F/H constructs were made by recombinant PCR using genomic DNA from the ADΔ ScErg11p × His overexpressing strain (Supplementary Table S1) as template. Together with standard flanking primers (PDR5F GAACATGAACCTTCCAGGCCG and PDR5DS TATGAGAAGACGGTTCGCCATTCGGACAG) the forward primer ScErg11_Y140F_f (AAGGTGTTATTTCGATGTCGACACG) and the reverse primer ScErg11_Y140F_r (TTGGACAATCGAAATAACACCTTTACC) were used to create fragments for recombinant PCR to introduce the Y140F mutation. Forward primer ScErg11_Y140F_f (AAGGTGTTATTTCGATGTCGACACG) and reverse primer ScErg11_Y140H_r (TTGGACAATCGAAATAACACCTTTACC) were used to introduce the Y140H mutation. The ADΔ strain was transformed with linear mutant ScERG11 DNA constructs that included a C-terminal hexahistidine tag, the PGK transcription terminator, a URA3 selection marker, bordered by sequences from the PDR5 locus for integration into the genome via homologous recombination as described by Lamping et al. Transformants were selected using SD-Ura agar plates incubated for 48–72 hrs at 30°C. Colony PCR was performed on the resultant transformants to identify clones with the insert in the correct position. The ScERG11 open reading frame and the expected presence of the expected mutation were confirmed by DNA sequencing analysis. The resulting strains were denoted ADΔScErg11_Y140F and ADΔScErg11_Y140H (Supplementary Table S1). The endogenous ScERG11 was deleted from mutant strains by replacement with a disruption cassette containing the His3 marker. Transformants were selected on SD-His agar plates. Colony PCR and DNA sequence analysis were performed at Genetic Analysis Services facility (University of Otago, Dunedin, New Zealand). The presence of the mutations Y140F/H in ScErg11p × His was verified by mass spectrometry at the Centre for Protein Research (University of Otago, New Zealand). An LTQ-Orbitrap hybrid mass spectrometer was used to obtain protein sequence coverage of at least 78% for each construct (Supplementary Figs S1 and S2).
Azole susceptibility of strains overexpressing ScErg11p. The susceptibilities of strains overexpressing wild type ScErg11p6 × His and ScErg11p6 × His Y140F/H to triazole drugs were measured as MIC₈₀ values using broth microdilution assays. The MIC₈₀s were defined as 80% growth inhibition compared to no drug controls because triazole drugs are fungistatic rather than fungicidal and can give trailing growth. MIC₈₀s to FLC, ITC and VCZ were determined in 96-well microtiter plates using SD buffered to pH 6.8 instead of RPMI³⁶. Cells were seeded at OD₆₀₀₀₉₅ = 0.005 (1.5 × 10⁴ CFU) and the plates were incubated in 30 °C with shaking at 200 rpm for 48 hrs. Cell growth was assessed by measuring the OD₆₀₀ₐₜ of wells using a BioTek Synergy² multi-mode plate reader (BioTek Instruments, Vermont, USA). Each MIC₈₀ was determined using triplicate measurements for three clones of each strain in three separate experiments.

Purification of ScErg11p. The purification of Y140F/H mutant ScErg11p6 × His was carried out according to the methods described previously by Monk et al.⁵³ using strains deleted of native ERL1 i.e. AD3ΔScErg11_ Y140F and AD3ΔScErg11_Y140H. In brief, yeast cells were grown in 1.5 L liquid cultures in YPD medium to OD₆₀₀₀₉₅ ~ 10 at 30 °C with shaking at 200 rpm. Harvested yeast cells were broken using a bead beating protocol and crude membranes were prepared by differential centrifugation. The protein concentration of crude membranes was estimated using the Lowry method⁵¹ with bovine serum albumin (Thermo Fisher) as standard. Crude membranes were solubilised with 10× critical micelle concentration (CMC) n-decyl-β-D-maltoside (DM) in a medium containing 10% (wt/vol) glycerol, 250 mM NaCl, 20 mM Tris pH 7.5, 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and 1 EDTA-free protease inhibitor pill (Roche) per 200 mL. The solubilised ScErg11p6 × His was affinity-purified using 2 mL of packed Ni-NTA-agarose matrix (Qiagen) per 1 gml of crude membrane protein. Affinity purification buffer containing 10% (wt/vol) glycerol, 250 mM NaCl, 20 mM Tris pH 7.5, 0.5 mM PMSF, 16 mM (10 × CMC) DM, 20 mM imidazole and 1 EDTA-free protease inhibitor pill per 200 mL was used to wash non-specifically bound proteins from the column. The ScErg11p6 × His enzyme was eluted from the column by using 200 mM imidazole in the affinity purification buffer. The triazole drugs ITC, FLC, VCZ or PCZ dissolved in dimethyl sulfoxide (DMSO) were added to the pooled fractions with final concentrations of 40 μM for FLC and VCZ and 20μM for PCZ and ITC.

Affinity-purified fractions were further purified by size exclusion chromatography (SEC) using a Superdex 200 10/300 GL column (GE Healthcare Life Sciences, UK). The column was equilibrated with degassed and filtered SEC buffer which contained 10% (wt/vol) glycerol, 150 mM NaCl, 20 mM HEPES, 0.5 mM PMSF, 6.4 mM DM (4 × CMC) and 2 EDTA-free protease inhibitor pills per 400 mL pH adjusted with NaOH to 7.5 at room temperature. The appropriate drug was added to the SEC buffer for co-purification; 10 μM FLC, 10 μM VCZ, 2 μM ITC or 2 μM PCZ. The coloured fractions containing the 62 kDa ScErg11p6 × His were pooled and concentrated using 50 kDa molecular-weight cut-off Amicon Ultra-4 centrifugal filters (Merck Millipore Ltd, Cork, Ireland).

Crystallisation and data collection. A hanging-drop vapour-diffusion method was used to crystallise the ScErg11p6 × His Y140F/H co-purified with a triazole drug. The reservoir solution contained 45% polyethylene glycol-400 in 100 mM glycate at a pH range of 9.3–9.55. Drops (total volume 4 μl) were in a 1:1 ratio of reservoir solution and ~20 mg/ml of the protein in SEC buffer. Red boat-shaped crystals formed after about one week of incubation at 18 °C and were collected for X-ray studies. The crystals were flash-cooled in liquid nitrogen prior to data collection. Datasets were collected on the MX1 beamline (ADSC Quantum 210r detector) or MX2 microbeam (ADSC Quantum 315r detector) at the Australian Synchrotron (Melbourne, Australia). During data collection the crystals were kept frozen under a cryostream at −180 °C. Indexing and integration of data was done using iMosflm⁵² and scaling with SCALA⁵³. Phaser-MR⁵⁴ from Phenix was used to carry out molecular replacement using ScErg11p6 × His complexed with lanosterol (PDB ID: 4LXJ) as a template. Refinement and modelling were performed using phenix.refine⁵⁵ and Coot⁵⁶. Waters were added if at least one hydrogen bond was detected (2.5–3.3 Å) and the inhibitors were modelled into the appropriate density in the active site and substrate channel. The crystallographic information files (. cif) for triazole inhibitors were obtained from the Grade website. The crystallographic information files were imported into the Global Phasing online tool (Global Phasing Ltd.). During refinement Fe – nitrogen and Fe – sulfur distances were constrained to 2.15 Å and 2.33 Å based on the average coordinate bond distance of more than 80 known Fe-N and Fe-S triazole complexes and 4 heme Fe-S in the Cambridge Structural Database as previously described. The Ramachandran statistics for residues range from 95.45–97.15% for residues in preferred regions, 2.66–3.98% for residues in allowed regions and 0.38–2.15% for residues in disallowed regions for all datasets.

Spectral binding characteristics of Y140F/H mutants. The concentration of functional cytochrome P450 for drug binding studies was determined using the carbon monoxide binding spectra according to the protocol described by Guengerich et al.⁵³. Affinity purified ScErg11p6 × His used for spectroscopic assays was eluted using affinity purification buffer containing 50 mM L-histidine instead of imidazole. L-histidine was removed from the sample by washing the enzyme with solubilisation buffer containing 16 mM DM using 50 kDa molecular-weight cut-off Amicon Ultra-4 centrifugal filters (Merck Millipore Ltd, Cork, Ireland). The removal of L-histidine was checked by taking the absolute spectra of the sample using the Ultrospec™ 6300 pro UV/Visible spectrophotometer. The heme peak for wild type protein with no ligand was at ~417 nm. With L-histidine bound the peak was detected at ~420 nm. Enzyme concentration was determined by saturating the sample cuvette with CO gas prior to the addition of sodium dithionite. The reference cuvette containing the same amount of enzyme was treated with sodium dithionite only. The P450 concentration was determined by measuring the difference in absorbance between 446 and 490 nm and using the extinction coefficient of 91 μM⁻¹ cm⁻¹. Absorption spectra were recorded with a Cary 1 Bio UV-visible spectrophotometer using 10 mm path UV transparent plastic
cuvettes (GE Healthcare Life Sciences, UK). Difference spectra were measured using 1 μM ScErg11p6 × His wild type or Y140F/H enzyme titrated with the triazole drugs ITC, PCZ, VCZ and FLC. Triazole drugs dissolved in DMSO were added to the sample cuvette, with the same amount of DMSO added to the reference cuvette. The total amount of DMSO was <2% of the total volume in the cuvettes. Difference spectra between 350–500 nm were recorded and the trough-peak absorbance changes were used to plot binding curves. The dissociation constant $K_d$ for type II binding of triazole drugs was calculated using GraphPad Prism 6 Software (GraphPad Prism, San Diego, CA) by applying the Hill equation using the formula $ΔA = ΔA_{max} [\text{Azole}]^{n} / ([\text{Azole}]^{n} + K_d^n)$, with $ΔA_{max}$ being the maximum change in absorbance and $[\text{Azole}]$ the azole concentration.

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Author Contributions
A.A.S. generated the Y140F ScErg11p mutant yeast strain, carried out the MIC<sub>90</sub> studies for this mutant strain, purified the mutant enzyme, determined the structures of six of seven ligand protein complexes for this mutant and wrote the manuscript. M.V.K. designed mutant strains, oversaw execution of some experiments and edited the manuscript. R.K.W. generated the Y140H ScErg11p mutant yeast strain, carried out the MIC<sub>90</sub> determinations, protein purification, drug binding and crystallisation studies. M.S. carried out drug binding studies, screened protein crystals and collected data for ScErg11p<sub>6</sub> × His Y140H mutant and determined the structure of ScErg11p<sub>6</sub> × His VCZ. J.D.A.T. oversaw data collection, processing and refinement and analysis of crystallographic data and edited the manuscript. B.C.M. has conceived the study, oversaw the design of the experiments and edited the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

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