Ergosterol biosynthesis in Aspergillus fumigatus: its relevance as an antifungal target and role in antifungal drug resistance

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Antifungal Targeting Ergosterol and Sterol Biosynthesis

Sterols are neutral lipids of eukaryotic cells, among which ergosterol is the main component of fungal membranes. Ergosterol is involved in numerous biological functions such as membrane fluidity, regulation, activity and distribution of integral membrane proteins, and control of the cell cycle (Bard et al., 1993; Gooday, 1995). The essential role of sterols in maintenance of cell membranes make ergosterol and its biosynthetic pathway essential for fungal growth, and a primary target for most, currently available, antifungal drugs to treat severe human fungal infections. Three main classes of antifungal drugs, namely polyenes, allylamines, and azoles, directly target ergosterol itself, or enzymatic steps of its biosynthetic pathway. The polyene amphotericin B (AMB) has represented, for more than 30 years, the standard of antifungal therapy for invasive aspergillosis (IA). The AMB mode of action is still poorly understood. It is widely accepted that AMB kills yeast primarily via channel-mediated membrane permeabilization leading to fungal cell death (Ermischkin et al., 1976). However, recent research suggests that AMB kills yeast by simply binding ergosterol so that the channel formation represents a second complementary mechanism that further increases drug potency and the rate of cell death (Gray et al., 2012). The allylamine group of antymycotics interferes at the early-stage of ergosterol biosynthesis by inhibiting the enzyme squalene epoxidase (Egz1). Although squalene epoxidases from various origins have been investigated with respect to substrate requirements, cofactors, and inhibitors, no structural model is available, and the domains responsible for enzymatic activity and inhibitor interactions are not well understood (Ruckenstuhl et al., 2007). The cidal action of allylamines is closely associated with the development of high intracellular squalene concentrations, which are believed to interfere with fungal membrane function and cell wall synthesis (Ryder, 1992). Allylamines are not used to treat IA, however potential use in combination with azoles, polyenes, or echinocandins in the management of severe drug-resistant or refractory mycoses has been proposed (Krishnan-Natesan, 2009). Finally, the main category of antifungal agents used against Aspergillus is azole drugs, such as voriconazole (VCZ) and itraconazole, and a new generation of azoles that includes posaconazole, isavuconazole, and albaconazole. Azoles revolutionized medical mycology due to their broad spectrum and reduced toxicity compared to AMB. Azoles block the ergosterol biosynthesis pathway via inhibition of 14α-sterol demethylase (Cyp51/Erg11), a key enzyme that removes the methyl group at position C-14 of precursor sterols. Inhibition of ergosterol synthesis at this biochemical level results in toxic sterol accumulation and cell death (Ghannoum and Rice, 1999).

However, although the use of antifungal drugs has increased survival of patients with invasive fungal disease, the mortality rate associated with these infections remains extremely high. This situation becomes more complicated because emerging resistance to the existing antifungals is a current problem and secondary resistance mechanisms have been described in clinical isolates (Howard and Arendrup, 2011; Shapiro et al., 2011; van der Linden et al., 2011a). Full understanding of the ergosterol biosynthesis pathway in A. fumigatus is essential to new antifungal drug design and for improving the activity of existing ones. This review summarizes current knowledge about the enzymatic sequence and regulation of the biochemical reactions involved in ergosterol biosynthesis with a view to understanding the main mechanisms of resistance and the potential for new antifungal therapies.

Keywords: Aspergillus, ergosterol biosynthesis, antifungal drugs, resistance mechanisms, transcriptome
of antifungal resistance to drugs which target ergosterol or its biosynthesis.

**Aspergillus fumigatus ERGOSTEROL BIOSYNTHESIS PATHWAY**

The biosynthesis of ergosterol involves about 20 enzymes and includes the synthesis of squalene from mevalonate (Ferreira et al., 2005; Alcazar-Fuoli et al., 2008). This route is well known in Saccharomyces cerevisiae which provides a model for studying this pathway in other eukaryotes (Fryberg et al., 1973). Depletion of ergosterol biosynthetic gene functions is lethal in S. cerevisiae, due to complete prevention of ergosterol production. Deviating from the prototypical pathway described in S. cerevisiae, other more complex biosynthetic pathways have been proposed in some fungi and also in plants, suggesting that the enzymatic pathways of ergosterol biosynthesis are specific to fungal taxa (Fryberg et al., 1973; Nes et al., 1989). In A. fumigatus, the study of this pathway at a genomic level, demonstrated the existence of multiple genes encoding key enzymes such as two distinct 14-α sterol demethylases (Cyp51A and Cyp51B; Mellado et al., 2001) and three C-5 sterol desaturases (Erg3; Alcazar-Fuoli et al., 2006). The classical ergosterol biosynthesis route was reconsidered in A. fumigatus based on the existence of multiple genes which putatively encode enzymes with the same or duplicated function in the pathway (Figure 1). Genetic and biochemical analysis of azole susceptible wild-type strains and a panel of clinical azole resistant strains identified a total of 14 sterols showing a similar sterol pattern for all of them (Table 1). Each of theazole resistant strains had a different antifungal susceptibility pattern that correlated with different mutations in the cyp51A gene. The lack of differences between sterols highlights the fact that the resistance mechanism in those strains was target-dependent and not sterol-dependent, as will be discussed later. Instead, the sterol composition of single enzyme defective A. fumigatus mutants was rather different depending on the lacking enzyme (Alcazar-Fuoli et al., 2008). Mutants deleted in 14-α sterol demethylases (cyp51A and cyp51B) had less ergosterol than the parental strain and accumulated C-4, and C-14 methyl sterols, mainly eburicol (Figure 1). Accumulation of eburicol, together with a decrease in ergosterol, was much more prominent when the cyp51B gene was deleted compared to Δcyp51A strain. Downstream in the pathway, the synthesis of ergosterol involves the transformation of fecosterol, consisting of double-bond rearrangements in the steroid nucleus and in the side chain, isomerization of the double connection in the C-8 to the C-7 followed by the desaturation at C-5 and C-22, and the reduction of the C-24. The enzymatic sequence of these three last steps could differ between fungal taxa and growth conditions (Nes et al., 1989). Single gene deletions of each of the C-5 sterol desaturases (Erg3A, Erg3B, and Erg3C) revealed a different sterol profile in terms of total amount of ergosterol and sterol composition. Again this phenotype was different depending on the deleted enzyme. While the absence of the genes erg3A and erg3C did not significantly affect total ergosterol biosynthesis, the deletion of erg3B caused a dramatic ergosterol decrease (70%) and a vast accumulation of the A7 sterols (Figure 1). Although the sterol composition revealed differences according to the enzyme that was deleted, none of the single gene deletions, at 14-α sterol demethylase or C-5 sterol desaturase level, was lethal for A. fumigatus or influenced its virulence (Mellado et al., 2005; Alcazar-Fuoli et al., 2006). This suggests that although A. fumigatus might use one of the enzymes Cyp51 or Erg3 for normal growth, this fungal pathogen is able, including during infection, to adapt and compensate that lack of an enzyme function.

**ANTIFUNGAL RESISTANCE MECHANISMS RELATED TO ERGOSTEROL OR ITS BIOSYNTHETIC PATHWAY**

Three of the most widely used antifungal drugs, triazoles, polyenes, and allylamines, are aimed at ergosterol, and they are either fungicidal but toxic to the host (polyenes) or fungi static and more vulnerable to resistance (triazoles). Triazoles are inhibitors that target the ergosterol biosynthetic pathway by binding to the Cyp51 family of cytochrome P450s (14-α sterol demethylases) causing the depletion of ergosterol biosynthesis and the accumulation of lanosterol or eburicol (Kelly et al., 1993). A. fumigatus contains two different, but related Cyp51 proteins, encoded by cyp51A and cyp51B (Mellado et al., 2001) that are functional 14-α sterol demethylases (Martel et al., 2010; Warrilow et al., 2010). The lethality caused by double gene deletion has been shown, although neither gene is itself essential (Mellado et al., 2005; Hu...
Table 1 | Relative amount of sterols (% of total sterols) in different A. fumigatus wild-type (CM-237), cyp51Δ deleted strains (A. cyp51ΔF), and azole resistance mutants strains with different cyp51A point mutations (CM-796; G54V; CM-3269: ITC185H; CM-2159: M220K).

| Sterol names | Strains |
|--------------|---------|
|              | CM-237* | A cyp51Δ | A cyp51ΔF | CM-796* | CM-3269* | CM-2159* |
| 1) 24-methylcholesta-5,22(19)-en-3β-ol | 1.09 | 3.89 | 2.74 | 0.85 | 1.18 | 0.92 |
| 2) 24-methylcholesta-5,22-trien-3β-ol | 0.91 | 0.87 | 0.77 | 0.90 | 0.80 | 0.85 |
| 3) 24-methylcholesta-5,23,22-tetraen-3β-ol | 0.71 | 5.69 | 4.57 | 0.00 | 0.00 | 0.00 |
| 4) 24-methylcholesta-5,22-tetraen-3β-ol (Ergosterol) | 76.12 | 59.48 | 48.82 | 64.08 | 63.70 | 86.44 |
| 5) 24-methylcholesta-22(28)-di-en-3β-ol | 2.62 | 1.84 | 1.18 | 1.51 | 1.67 | 1.09 |
| 6) 24-methylcholesta-5,22,24(28)-tri-en-3β-ol | 0.48 | 0.41 | 0.25 | 0.32 | 0.21 | 0.26 |
| 7) 24-methylcholesta-22,24(28)-tri-en-3β-ol | 0.66 | 1.44 | 0.71 | 0.82 | 0.51 | 0.20 |
| 8) 24-methylcholesta-5,22,24(28)-tri-en-3β-ol | 0.55 | 0.14 | 0.34 | 0.06 | 0.19 | 0.16 |
| 9) 24-methylcholesta-24,28-di-en-3β-ol (Episterol) | 1.51 | 2.55 | 2.01 | 3.59 | 1.84 | 1.08 |
| 10) 24-Ethylcholesta-5,22-trien-3β-ol | 74.0 | 5.48 | 9.26 | 2.43 | 6.46 | 5.10 |
| 11) 4,4,14-trimethylcholesta-8,24-dien-3β-ol | 1.06 | 1.39 | 2.87 | 3.63 | 0.29 | 0.18 |
| 12) 4,4,23,24-dimethylcholesta-8,24-dien-3β-ol | 0.50 | 2.03 | 0.98 | 0.73 | 0.00 | 0.82 |
| 13) 4,4,14-trimethylcholesta-8,24-dien-3β-ol (Eburicol) | 1.14 | 9.54 | 3.65 | 4.01 | 1.30 | 0.23 |
| 14) 4,4,23,24-dimethylcholesta-8,24-dien-3β-ol | 2.04 | 5.44 | 3.36 | 0.66 | 0.42 | 0.65 |

*Geometric mean of MICs to antifungal agents of A. fumigatus strains: CM-237: ITC (0.25 μg/ml), VRC (0.48 μg/ml), POS (0.08 μg/ml), FLC (1094.55 μg/ml), AMB (0.26 μg/ml), TRB (0.4 μg/ml), CM-796: ITC (1.99 μg/ml), VRC (1.79 μg/ml), POS (1.48 μg/ml), FLC (1094.55 μg/ml), AMB (0.15 μg/ml), TRB (2.38 μg/ml), CM-3269: ITC (18.06 μg/ml), VRC (1.19 μg/ml), POS (2.0 μg/ml), FLC (1094.55 μg/ml), AMB (2.3 μg/ml), POS (0.15 μg/ml), FLC (1094.55 μg/ml), AMB (0.2 μg/ml), TRB (2.9 μg/ml).

et al., 2007). Moreover, the importance of A. fumigatus Cyp51 proteins in sterol biosynthesis and their significance in azole susceptibility have been extensively studied (Alcazar-Fuoli et al., 2008, 2011; Martel et al., 2010). Also, the in vitro and in vivo correlation of azole resistance has been widely documented; with a clear association of resistant A. fumigatus strain isolation and high level of patient response to therapy (van der Linden et al., 2011a). A. fumigatus azole resistance can arise either from modification of the Cyp51A target site, or by its overexpression. Resistance acquired through exposure to azoles in the patient is correlated with heightened levels of cell wall lipids and an absence of ergosterol (Kelly et al., 1997; Morio et al., 2012). The deletion of three independent cyp51Δ strains bearing different Cyp51A point mutations suggests that their azole resistant phenotypes are not the result of perturbations of the ergosterol biosynthetic pathway (Table 1). It is more likely that azole resistance in these variants results from a reduced affinity of drug binding to the Cyp51A enzyme. In this sense, 3D protein models of Cyp51A in combination with azoles have provided the basis to address how point mutations can affect azole drug resistance (Alcazar-Fuoli et al., 2011; Fraczek et al., 2011).

Regarding the polyene antifungals, the association between A. fumigatus susceptibility to AMB in vitro and clinical outcome is unclear. Secondary resistance to AMB is generally not observed, even in patients whose therapy has failed (Moosa et al., 2002), yet resistant mutants have been spontaneously induced in the laboratory (Marasović et al., 1998) and resistance to AMB is well documented for many other Aspergillus species (Blum et al., 2008; van der Linden et al., 2011b; Hadrich et al., 2012). Acquired resistance to AMB has been most extensively evaluated in yeasts and is associated with mutations in the ERG3 gene, which is linked to qualitative and quantitative alterations of membrane lipids and an absence of ergosterol (Kelly et al., 1997; Morio et al., 2012). However, the deletion of three independent erg3Δ-like genes in A. fumigatus did not affect susceptibility to AMB, despite marked alterations of sterol composition and a decrease in total ergosterol (Alcazar-Fuoli et al., 2006). Other studies have determined that neither ergosterol content, cell wall composition, or lipid peroxidation levels correlate with heightened A. terreus AMB resistance, only that a higher level of catalase production in
A. terreus might contribute to AMB resistance since it promotes oxidative damage of fungal cell membranes through generation of reactive oxygen species (Blum et al., 2008).

Terbinafen belongs to the allylamine class of antifungals that inhibit squalene epoxidase (Erg1). Erg1 catalyzes the first oxygenation step in sterol biosynthesis and is suggested to be one of the rate-limiting enzymes in this pathway. Alterations in A. fumigatus erg1 gene dosage can promote terbinafine resistance (Liu et al., 2004), however, the first terbinafine resistance mechanism was described in A. nidulans and associated with the activity of salicylate 1-monooxygenase (salA), a well-characterized naphthalene-degrading enzyme, suggesting that resistance could follow degradation of the naphthalene ring contained in terbinafine (Grauinho et al., 2004). Also, a point mutation, F991L, in the squalene epoxidase enzyme was found to confer a strong terbinafine resistance phenotype. The equivalent mutation was introduced into the homologous gene of A. fumigatus resulting in terbinafine resistance (Rocha et al., 2006). Since terbinafine is not currently used in the management of IA, the appearance of terbinafine-resistant strains in the clinical setting is unlikely, however, its potential use in combination with other antifungal drugs should be considered.

**ALTERNATIVES TO IMPROVE ANTIFUNGAL EFFICACY AND TO MINIMIZE DRUG RESISTANCE**

Global gene expression studies identified that ergosterol biosynthesis is likely to be highly sensitive to environmental perturbations. Among them, adaptation to the host environment when A. fumigatus initiates disease is particularly interesting. The transcription of A. fumigatus during early stages of infection in the neutrophilic murine lung identified reduced expression of genes encoding ergosterol pathway functions (Erg11, Erg24, and Erg3) relative to laboratory cultures (McDonagh et al., 2008).

Although the target site of azole activity is well studied, the role of other proteins in the mode of action of these drugs in fungi is poorly understood. Recently, a critical role for SrbA-mediated regulation of ergosterol biosynthesis and triazole drug interactions in A. fumigatus has been reported (Blisser and Cramer, 2012). SrbA was identified by transcriptional profiling under hypoxia conditions as a regulator of ergosterol biosynthetic genes in response to low oxygen levels. The ΔsrbA mutant strain was attenuated in virulence and it was hypersusceptible to fluconazole and VCZ (Willger et al., 2008). Also, several genes encoding enzymes that require high levels of oxygen were found to be transcriptionally repressed in the absence of SrbA, including the enzymes Erg6, Erg11, Erg24, Erg25, and Erg3. The relationship between ergosterol regulation and hypoxia was also demonstrated by the analysis of the sterol profile of the ΔsrbA which showed a decrease in ergosterol levels and accumulation of C-4 methyl sterols, suggesting a blockage of C-4 demethylation. In addition, the apparent control of cyp51 transcript levels by SrbA suggests an additional target for drug development. A role for SrbA in the development of triazole resistance has also been suggested, since alterations in this transcription factor or its DNA-binding affinity could transcriptionally change interactions that could alter target abundance and would lead to triazole resistance (Blisser and Cramer, 2012).

In a similar manner, analysis of sterol composition in iron-depleted and replete cultures revealed decreased ergosterol biosynthesis during iron starvation and the accumulation of two types of sterol intermediates C-4 and Δ7. The genome-wide expression response of A. fumigatus in a shift from iron-depleted to iron-replete conditions (10, 30, 60, 120, and 240 min compared with that from 0 min) showed that Erg24 and Erg25 functions were down-regulated for all of the time points, and in contrast Erg11 was up-regulated at 30 and 60 min of iron exposure (Schettel et al., 2008). In conditions of iron limitation, A. fumigatus uses siderophore-assisted iron uptake, which in turn uses mevalonate as a precursor. The results described above, together with the characterization of functional siderophore deleted mutants, proved the link between fungal ergosterol and siderophore biosynthesis in A. fumigatus via the compound mevalonate. The latter would appear to feed both biochemical pathways (Yasmin et al., 2012). Also, in hypoxic conditions SrbA was found to activate siderophore-mediated iron uptake in response to hypoxia and iron starvation in part by transcriptional activation of another transcription factor, HapX (Blatter et al., 2011).

Transcriptional regulation of ergosterol biosynthesis in A. fumigatus has also been studied under antifungal exposure in laboratory cultures. Microarray analysis indicated down-regulation of the erg6 gene under both AMB and VCZ exposure (da Silva Ferreira et al., 2006; Gustam et al., 2008). Erg6 has a role in the synthesis of secondary sterols (ethyl sterols), and its down-regulation suggests that A. fumigatus alters the synthesis of secondary sterols in response to AMB or VCZ. However, different results were found for the A. fumigatus Cyp51-encoding genes, which were both down-regulated under VCZ treatment and up-regulated with AMB. These results may indicate the existence of differential responses for overcoming the distinct stresses imposed by these drugs. In addition, genes encoding for Erg24, Erg25, and Erg3 were differentially expressed when A. fumigatus cells were cultured in the presence of VCZ (da Silva Ferreira et al., 2006).

Collectively, the above observations suggest that ergosterol biosynthesis is prone to perturbation by environmental conditions, and in a manner dependent upon Erg6, Erg11, Erg24, Erg25, and Erg3 functions, thereby highlighting these gene functions as alternative or synergic inhibitors of the pathway. An important finding in A. fumigatus was the identification of C-24 ethyl steroids. These steroids can also be final metabolites of the sterol pathway and they are predominantly found in higher plants, though absent in mammalian cells, which cannot alkylate the C-24 of sterols. In plants C-24 ethyl steroids have multiple roles to play in growth and development, however, few reports exist on the detection of C-24 ethyl steroids in fungi, and their role in A. fumigatus remains unknown. C-24 alkylated is catalyzed by S-adenosyl-methionine-sterol-C-methyltransferases (SAMs). In S. cerevisiae the methylation is catalyzed by erg6 which converts zymosterol into fecosterol, and at least two methyltransferases are involved in consecutive methylation reactions leading to 24-ethyl sterols in higher plants (Bouvier-Nave et al., 1998). In A. fumigatus, only one Erg6 has been identified that could transform lanosterol to eburicol and may also be involved in the production of C-24 ethyl steroids. The sterol profile analysis of A. fumigatus strains showed that the synthesis of 24-Ethylcholesta-5,7,22-trien-3β-ol is sensitive to...
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pathway perturbations. In a Δcyp51B mutant a lack of ergosterol is compensated by an increase of 24-Epicholesterol-5,7,22-trien-3β-ol, probably because the activity of this pathway becomes diverted toward the synthesis of alkyl sterols. However, when erg3B is suppressed, ergosterol levels are decreased together with 24-ethylcholesterol-5,7,22-trien-3β-ol, confirming that this enzyme would also be involved in the deactivation of C-5,7 alkyl sterols.

Genome-wide studies are providing us with a very useful platform from which to dissect the linkages between cellular sterol biosynthesis and other cell functions or metabolic pathways. Such new findings might be further explored for novel drug development or for possible combinatorial therapeutic strategies to fight invasive fungal diseases. Among them, genes encoding enzymes involved in intracellular stress, cell wall synthesis, and transport have been found to be differentially expressed under antifungal exposure or underhost imposed stresses. To conclude, A. fumigatus has been found to be differentially expressed under antifungal exposure or underhost imposed stresses. Therefore, the precise knowledge of this complex biosynthetic pathway could facilitate the future development of novel and more selective antifungal drugs in order to improve efficacy and to minimize drug resistance.

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