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Stepwise emergence of azole, echinocandin and amphotericin B multidrug resistance *in vivo* in *Candida albicans* orchestrated by multiple genetic alterations

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Objectives: The objective of this study was to characterize the underlying molecular mechanisms in consecutive clinical *Candida albicans* isolates from a single patient displaying stepwise-acquired multidrug resistance.

Methods: Nine clinical isolates (P-1 to P-9) were susceptibility tested by EUCAST EDef 7.2 and Etest. P-4, P-5, P-7, P-8 and P-9 were available for further studies. Relatedness was evaluated by MLST. Additional genes were analysed by sequencing (including *FKS1*, *ERG11*, *ERG2* and *TAC1*) and gene expression by quantitative PCR (*CDR1*, *CDR2* and *ERG11*). UV-spectrophotometry and GC-MS were used for sterol analyses. *In vivo* virulence was determined in the insect model *Galleria mellonella* and evaluated by log-rank Mantel–Cox tests.

Results: P-1+P-2 were susceptible, P-3+P-4 fluconazole resistant, P-5 pan-azole resistant, P-6+P-7 pan-azole and echinocandin resistant and P-8+P-9 MDR. MLST supported genetic relatedness among clinical isolates. P-4 harboured four changes in Erg11 (E266D, G307S, G450E and V488I), increased expression of *ERG11* and *CDR2* and a change in Tac1 (R688Q). P-5, P-7, P-8 and P-9 had an additional change in Erg11 (A61E), increased expression of *CDR1*, *CDR2* and *ERG11* (except for P-7) and a different amino acid change in Tac1 (R673L). Echinocandin-resistant isolates harboured the Fks1 S645P alteration. Polyene-resistant P-8+P-9 lacked ergosterol and harboured a frameshift mutation in *ERG2* (F105SfsX23). Virulence was attenuated (but equivalent) in the clinical isolates, but higher than in the azole- and echinocandin-resistant unrelated control strain.

Conclusions: *C. albicans* demonstrates a diverse capacity to adapt to antifungal exposure. Potentially novel resistance-inducing mutations in *TAC1*, *ERG11* and *ERG2* require independent validation.

Keywords: mycology, molecular typing, antifungal resistance, resistance mechanisms

Introduction

Candida albicans is inherently susceptible to all antifungal drugs. Monoresistance to azoles or echinocandins and a few cases of combined azole and amphotericin B resistance have been reported, but multidrug resistance covering all three drug classes is a rare and, to our knowledge, previously unreported phenomenon in *C. albicans*.[1]

Azole resistance in *C. albicans* is often an interplay of: (i) structural changes of Erg11 (14α-methyl sterol demethylase), the target of azoles; (ii) overexpression of *ERG11*; and (iii) increased cellular export of azoles by up-regulated drug efflux transporters.[2] The genetic regulation of azole resistance involves *ERG11* up-regulation linked to specific gain-of-function (GOF) mutations in zinc cluster transcription factor *UPC2*[3] as well as increases in copy number due to isochromosome formation or duplication of chromosome 5.[4] Likewise, GOF mutations in transcription factors *TAC1* and *MRR1* lead to up-regulation of drug efflux pumps *CDR1*/*CDR2* and *MDR1*, respectively.[5] Acquired echinocandin resistance in *C. albicans* has been linked to structural alterations of the target enzyme Fks1 (1,3-β-D-glucan synthase), which is essential for cell wall synthesis.[6] Resistance to polyenes in *C. albicans* is rare, but has been linked to inactivation of essential proteins in ergosterol biosynthesis leading to ergosterol depletion (the target of amphotericin B) and the formation of other sterols.[2]

Here, we present a detailed molecular assessment of the underlying genetic mechanisms contributing to the sequential development of unique MDR strains by evaluation of consecutive *C. albicans* isolates from a single patient.

Brief case report

A man in his early sixties with angioimmunoblastic T cell lymphoma underwent stem cell transplantation in 2006 followed by long-term pancytopenia and recurrent infections including numerous episodes of oropharyngeal and oesophageal candidiasis despite various courses of antifungal treatment (Figure S1, available as Supplementary data at *JAC* Online). Increasingly resistant *C. albicans* isolates were found in oesophageal and colon biopsies and faeces before the patient died in 2011.

Materials and methods

Strains and susceptibility testing

Nine clinical isolates obtained in 2006–11 underwent susceptibility testing at the Statens Serum Institut according to EUCAST EDef 7.2 (azoles and anidulafungin) and by Etest (amphotericin B and caspofungin).[7] Susceptibility was interpreted by using the established EUCAST breakpoints (http://www.eucast.org/clinical_breakpoints/) and CLSI breakpoints for Etests.[8–11]

Sequencing and gene expression analysis

Five isolates (P-4, P-5, P-7, P-8 and P-9) were available for molecular analyses (Table 1). MLST was performed as described previously[12] and additional genes were sequenced (notably *FKS1*, *ERG11*, *ERG2*, *UPC2* and *TAC1*).[13–15] Gene expression analysis was performed for *ERG11* and drug efflux pumps *CDR1* and *CDR2* by RNA quantification using quantitative PCR (qPCR) as described previously.[15] All primers used in this study are provided in Table S1.

Sterol composition

Sterol analysis was performed by: (i) spectrophotometric UV absorption profiles on non-saponifiable fractions of lipids extracted with *n*-heptane through vigorous vortex agitation by a simplified protocol from a previous study and measured between 240 and 320 nm;[16] and (ii) GC-MS. Sterols were extracted with NaOH and methanol at 90°C and further by pentane phase separation and dissolved in 2-propanol. Next, 50 μL of the extract was further evaporated to dryness, derivatized to trimethylsilyl ethers and analysed as described previously.[17]

Virulence determination in the Galleria mellonella larvae model

Virulence was evaluated in the insect model *G. mellonella* caterpillars as described previously.[18] Caterpillars (250–325 mg, HPReptiles, Copenhagen, Denmark) were inoculated in groups of 20 or 25 and the inocula were standardized to achieve an 80% mortality of WT isolates within 5 days ($\sim 5 \times 10^5$ cells/larvae). Caterpillars were incubated at 37°C for up to 5 days after inoculation. Groups were compared using the log-rank Mantel–Cox test (Prism 6.05, GraphPad). *P* values of <0.05 were considered significant.

Results and discussion

Antifungal treatment and susceptibility profiles

During long-term antifungal treatment (Figure S1), antifungal resistance emerged in a stepwise manner (Table 1). Fluconazole resistance was found in P-3 (after a total of 34 weeks of fluconazole therapy), pan-azole resistance in P-5 (after 8 weeks of voriconazole and 96 weeks of posaconazole exposure), echinocandin resistance in P-6 (after ∼6 weeks of caspofungin and anidulafungin exposure) and, finally, multidrug resistance emerged in P-8 (after 9 weeks of either nystatin or amphotericin B treatment).

MLST analysis

Identical diploid STs were found for isolates P-7, P-8 and P-9 (21-26-14-19-72-102-84), suggesting that they were isogenic. P-4 (21-26-14-18-76-102-84) and P-5 (21-26-14-18-72-102-84) deviated in the *MP1b* and *SYA1* alleles. When including additional sequenced genes such as *FKS1*, *ERG11* and *ERG2* in an expanded MLST analysis, there was an even stronger hereditary link suggesting a common progenitor and the stepwise selection of isogenic MDR offspring.

Fluconazole resistance

Among four amino acid changes identified in Erg11 of the fluconazole-resistant isolate P-4 (Table 1), G307S and G450E have previously been associated with fluconazole resistance and confirmed in genetically engineered *C. albicans*.[19] Hence, these mutations may have been significant drivers of fluconazole resistance in P-4 and probably further potentiated by elevated expression levels of *ERG11* and particularly *CDR2* (Table 1).

Pan-azole resistance

Subsequent pan-azole-resistant clinical isolates possessed an additional alanine to glutamic acid change (A61E) in Erg11 (Table 1). A61E is novel, but involves a codon (A61V) previously indicated to slightly impact binding of and susceptibility to itraconazole and posaconazole.[20] Compared with the A61V alteration, a change from the hydrophobic alanine to the acidic and much larger glutamic acid found here would be expected to have a stronger effect on interference with long-tailed azoles. Protein modelling (Figure S2) illustrates steric interference of A61E with the tail of itraconazole and thus is consistent with this hypothesis, although independent validation remains necessary. Overexpression of *ERG11* (except for P-7), *CDR1* and *CDR2* may have contributed to azole resistance, especially to voriconazole in P-5 through P-9.

Elevated gene expression and genetic precursors

ERG11 up-regulation may have been a compensatory mechanism (independent of *UPC2* mutations) to avoid ergosterol depletion associated with the potentially reduced catalytic activity of the Erg11 variants.[21,22]

CDR1 and especially *CDR2* up-regulation was detected in high relative levels in all azole-resistant clinical isolates (Table 1). *TAC1* sequencing revealed a novel amino acid change (R688Q) in P-4. Whether this alteration mediated the up-regulation particularly of *CDR2* in P-4 deserves further investigation.[23] The allelic state of *TAC1* was heterozygous in P-4, but homozygous in P-5 through P-9; thus, it is likely that the *TAC1* locus underwent a 'loss of heterozygosity' from P-4 to P-5, which is a phenomenon frequently associated with azole resistance.[4] Additionally, another novel *TAC1* change (R673L) was found in P-5 through P-9 (Table 1),

Table 1. Characteristics of nine clinical isolates: site and date obtained, susceptibility, gene products and relative gene expression levels

	P-1 (WT)	P-2 (WT)	P-3 (F)	P-4 (F)	P-5 (A)	P-6 (A+E)	P-7 (A+E)	P-8 (MDR)	P-9 (MDR)
Site	oesophagus[a]	oesophagus[a]	oropharynx[b]	oropharynx[b]	oesophagus[a]	oesophagus[a]	faeces[b]	faeces[b]	colon biopsy[a]
Date	25.04.06	11.07.06	28.01.08	01.04.08	21.04.10	17.08.10	10.04.11	10.04.11	06.05.11
FLC[c]	0.125	0.25	16	8	>16	>16	>16	>16	16
ITC[c]	≤0.03	≤0.03	≤0.03/4[d]	≤0.03	16	>4	16	16	>16
VRC[c]	≤0.03	≤0.03	≤0.03/4[d]	≤0.03	1	0.5	0.25	0.125	0.125
POS[c]	NA	NA	≤0.03/4[d]	≤0.03	>4	>4	4	4	0.5/4[d]
ANI[c]	NA	NA	NA	0.015	0.015	0.25	1	1	0.5
CAS[e]	0.06	0.25	0.25	0.25	0.50	>32	>32	>32	>32
AMB[e]	0.25	0.5	0.38	0.5	0.5	0.5	0.5	>32	>32
Erg11[f]	NA	NA	NA	E266D G307S G450E V488I	A61E E266D G307S G450E V488I	NA	A61E E266D G307S G450E V488I	A61E E266D G307S G450E V488I	A61E E266D G307S G450E V488I
ERG11(expr.)[g]	NA	NA	NA	4.85	12.3	NA	0.43	5.70	3.44
CDR1(expr.)[g]	NA	NA	NA	1.69	7.40	NA	2.95	4.73	1.45
CDR2(expr.)[g]	NA	NA	NA	69.2	868.1	NA	194.8	132.5	14.5
Tac1[f,h]	NA	NA	NA	R688Q[i]	R673L	NA	R673L	R673L	R673L
Fks1[f]	NA	NA	NA	V661F[i]	V661F[i]	NA	S645P[i] V661F[i]	S645P[i] V661F[i]	S645P[i] V661F[i]
Erg2[f]	NA	NA	NA	WT	WT	NA	F105fs[i,j]	F105fs[j]	F105fs[j]

WT, WT susceptibility; F, fluconazole resistant; A, azole resistant; E, echinocandin resistant; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ANI, anidulafungin; CAS, caspofungin; AMB, amphotericin B; NA, not available.
MIC values above clinical breakpoints and regarded as resistant are highlighted grey.
Underlined amino acid changes are considered to be associated with resistance.
[a]Primary specimen.
[b]Culture.
[c]EUCAST (EDef 7.2) MIC (mg/L).
[d]Trailing phenotype with ∼50% growth inhibition in the concentration range 0.5–4 mg/L.
[e]Etest MIC (mg/L).
[f]Amino acid change.
[g]Changes in gene expression (fold) normalized to *ACT1* expression and relative to that of control wild-type strain SC5314.
[h]The *TAC1* gene sequence harboured multiple non-synonymous mutations, but only potential GOF mutations are shown.
[i]Heterozygous.
[j]Frameshift mutation F105SfsX23 due to a 1 bp deletion (314delT).

which displayed even higher expression levels of *CDR1* and *CDR2*, indicating the potential of another novel GOF variant and should be independently validated in future studies.[24]

Echinocandin resistance and FKS1 mutations

Echinocandin-resistant clinical isolates harboured a (heterozygous) mutation in *FKS1* leading to the amino acid change S645P (Table 1), which is well known to confer high-level echinocandin resistance.[25]

Amphotericin B resistance

Sequencing of essential genes in the ergosterol biosynthetic pathway revealed a heterozygous frameshift mutation in *ERG2* (encoding $\Delta^{8\rightarrow7}$ isomerase) at amino acid position Phe-105 (F105SfsX23) in P-7, but homozygous in the amphotericin B-resistant isolates P-8 and P-9. This caused a truncated protein sequence reduced from 217 to 126 amino acids (Table 1), which may presumably be associated with protein function inactivation. In support, an amino acid disruption in Erg2 corresponding to Thr-115 in *C. albicans* was critical for sterol $\Delta^{8\rightarrow7}$ isomerization in other *Candida* species.[26,27] GC-MS analysis showed that P-8 and P-9 were depleted of ergosterol and instead displayed an accumulation of ergosta-8-enol, ergosta-8,22-dienol, ergosta-5,8,22-trienol and fecosterol as well as a few other sterols (Figure 1). This sterol profile matches previous findings involving *ERG2* deletion mutants.[26,28] Thus, although supported by the literature, an independent validation is necessary to address whether the *ERG2* mutation alone is able to confer polyene resistance.

Virulence of the resistant isolates

Acquired resistance in *C. albicans* may come at a fitness and virulence cost.[15,29,30] The two susceptible control strains C-1 and C-2 were equally virulent ($P=0.2$) and resulted in day 5 mortality of 88% and 98%, respectively (Figure 1). P-4 was statistically less

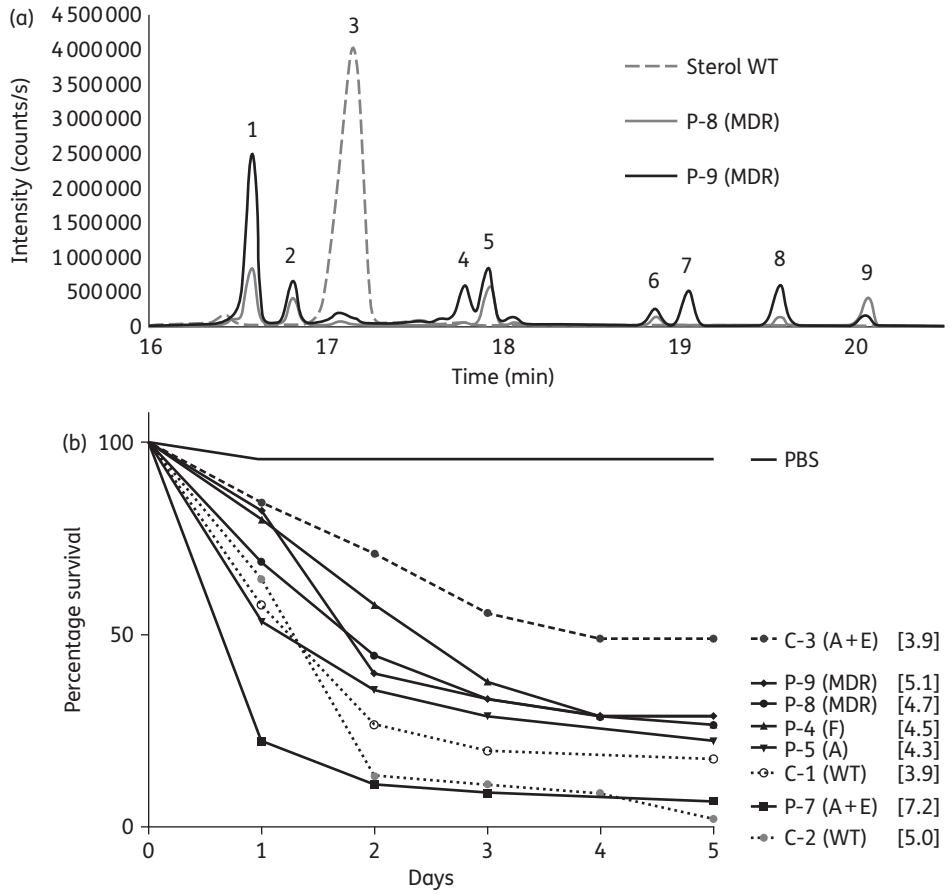

Figure 1. Strain characteristics. (a) GC-MS chromatograms for sterol in WT, P–8 and P–9 isolates following growth in yeast extract peptone dextrose (YEPD) medium. Sterol intermediates are as follows: 1, ergosta-5,8,22-trienol; 2, ergosta-8,22-dienol; 3, ergosterol; 4, fecosterol; 5, ergosta-8-enol; 6, 14α methyl ergosta 8,24(28)-dien-3β,6α-diol; 7, lanosterol; 8, ergosta-8-en-diol; and 9, unknown sterol. (b) Virulence in the *G. mellonella* larvae model. Letters in round brackets denote susceptibility profiles: WT, WT susceptibility; F, fluconazole resistant; A, azole resistant; and E, echinocandin resistant. Mean cells/larva injected ($\times 10^5$) are indicated in square brackets. Broken lines indicate reference strains and continuous lines indicate clinical isolates.

virulent than both WT control isolates, but all clinical isolates were significantly more virulent than an unrelated azole- and echinocandin-resistant control strain C-3 (displaying 51% overall-mortality). The virulence of the clinical isogenic isolates was only slightly reduced compared with that of the susceptible control strains, indicating that compensatory mechanisms may have abrogated virulence cost in these MDR isolates.

Conclusions

Here, we presented a suite of well-known and novel genetic mechanisms contributing to the sequential development of resistance to all three antifungal drug classes, which to the best of our knowledge is the first example of multidrug resistance emerging *in vivo* in *C. albicans*. Interestingly, the observed resistance came without significant virulence cost. The superficial nature of the infection, where subtherapeutic concentrations are more common, may have facilitated the emergence of resistance. Our study is associated with several limitations. Most importantly, we did not have the initial susceptible clinical isolates as such superficial WT strains are not routinely stored. Secondly, we have addressed and argued for several potential resistance mechanisms and hypothesized novel findings supported by existing knowledge. However, the significance of these findings requires independent validation.

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Supplementary data

Figure S1, Table S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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