Voriconazole-induced inhibition of the fungicidal activity of amphotericin B in Candida strains with reduced susceptibility to voriconazole: an effect not predicted by the MIC value alone

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Abstract

An antagonistic effect of voriconazole on the fungicidal activity of sequential doses of amphotericin B has previously been demonstrated in *Candida albicans* strains susceptible to voriconazole. Because treatment failure and the need to switch to other antifungals are expected to occur more often in infections that are caused by resistant strains, it was of interest to study whether the antagonistic effect was still seen in *Candida* strains with reduced susceptibility to voriconazole. With the hypothesis that antagonism will not occur in voriconazole resistant strains, *C. albicans* strains with characterized mechanisms of resistance against voriconazole, as well as *C. glabrata* and *C. krusei* strains with differences in degree of susceptibility to voriconazole were exposed to voriconazole and amphotericin B alone, simultaneously or sequentially in an *in vitro* kinetic model. Amphotericin B administered alone or simultaneously with voriconazole resulted in fungicidal activity. When amphotericin B was administered after voriconazole, its activity was reduced in median 61% (range 9-94%). Voriconazole-dependent inhibition of amphotericin B activity differed significantly among the strains but was not correlated with the MIC values (correlation coefficient -0.19; P=0.65). Inhibition was found in *C. albicans* strains with increases in *CDR1* and *CDR2* expression but not in the strain with an increase in *MDR1* expression. In summary, decreased susceptibility to voriconazole does not abolish voriconazole-dependent inhibition of the fungicidal activity of amphotericin B in voriconazole resistant *Candida* strains. The degree of interaction could not be predicted by the MIC value alone.
Introduction

Combination antifungal therapy may be one method to improve outcome in invasive *Candida* infections. Because animal model pharmacodynamic studies may have difficulties in simulating human pharmacokinetics, *in vitro* pharmacodynamic studies with simulated human pharmacokinetics represent an additional tool in the study of antifungal combinations in that they can yield data on drug-specific antifungal activities that will be of interest before initiating clinical trials.

Recently, an *in vitro* kinetic model for the study of combination treatment with drugs having different elimination rates has been presented and validated (12). Using this model, an antagonistic effect of voriconazole on the fungicidal activity of sequential doses of amphotericin B against *Candida albicans* has been demonstrated (12, 13). These studies investigated *C. albicans* strains fully susceptible to voriconazole. The postulated mechanism has been that inhibition of ergosterol synthesis leads to decrease of ergosterol content and thus diminishes the effect of amphotericin B which uses this sterol to mediate its inhibitory effect.

Consequently, it is reasonable to hypothesize that voriconazole-induced inhibition of the fungicidal activity of amphotericin B will not be observed in voriconazole resistant strains. If so, this would question the potential clinical relevance of this interaction because the need to switch to amphotericin B will be more likely in patients with infections caused by voriconazole resistant strains. Therefore, the aim of the present study was to test this hypothesis in an *in vitro* kinetic model in which the effect of voriconazole on fungal killing of amphotericin B was tested in *C. albicans* strains with known mechanisms of resistance against voriconazole and in *C. glabrata* and *C. krusei* strains with varying degrees of susceptibility to voriconazole.
Materials and methods

Fungal strains. Strains of *C. albicans*: C26, C40, C56 (Institute of Microbiology, University of Lausanne and University Hospital Center, Switzerland) and 20288.030 (Department of Pathology, University of Iowa, IA, USA), *C. glabrata*: B346 4018 (Department of Clinical Microbiology, Uppsala University Hospital, Sweden) and 263 SMI (Swedish Institute for Infectious Disease Control, Solna, Sweden) and *C. krusei*: B336 4026 (Department of Clinical Microbiology, Uppsala University Hospital, Sweden) and 20550.054 (Department of Pathology, University of Iowa, IA, USA) were studied.

In addition, one voriconazole susceptible wild-type strain of *C. albicans* CCUG 32723 (Culture Collection University of Göteborg, Sweden) was included as a quality control strain and for illustration of the voriconazole-dependent inhibition of amphotericin B activity as has previously been demonstrated in fully voriconazole susceptible strains (13).

A suspension of *Candida* spp. was prepared from a 24-48 h culture from which 1-2 colonies were transferred into test tubes with 4 ml of RPMI 1640 (Invitrogen AB, Täby, Sweden), placed on a shaker and cultured at 35°C for 6 h. This process resulted in a standardized concentration of approximately $10^7$ CFU/ml determined by plating on Sabouraud dextrose agar plates and colony counting as described below.
MIC determination. MICs were determined in duplicate using CLSI (formerly NCCLS) methodology for broth dilution antifungal susceptibility testing of yeasts (18). The susceptibility endpoint for voriconazole was defined as the lowest concentration of the drug that resulted in an 80% reduction in visible growth. The MIC of amphotericin B was defined as the lowest concentration that resulted in total inhibition of visible growth.

After MIC determination, the Candida strains were re-named with a number and corresponding voriconazole MICs (Table 1). These names are used in the text below.

Characterization of mechanisms of resistance of C. albicans strains.

The C. albicans strains CA1_{MIC8}, CA2_{MIC8} and CA3_{MIC2} have previously been genetically characterized. These strains are known to exhibit overexpression of the genes encoding for drug efflux pumps (MDR1, CDR1 and CDR2) and possessing mutations in the azole target gene ERG11 (Table 1) (26, 28).

Strain CA4_{MIC256} was analyzed for the expression of genes being major mediators of azole resistance, i.e. CDR1, CDR2, MDR1 and ERG11. The expression of these genes was compared with isolate CAF2-1, which is described as an azole-susceptible strain derived from Saccharomyces cervisiae SC5314 (9). RNA was extracted and separated by electrophoresis (5) and radiolabelled probes were generated (27, 28). Signals obtained were quantified by Phospho-Imaging using a Typhon Trio (GE Healthcare). 28S and 18S RNA served as controls for identical RNA loading.

Medium. Sterile RPMI 1640 buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) was used as the growth medium.
Antifungal agents. Voriconazole (Pfizer AB, Täby, Sweden) and amphotericin B (Sigma-Aldrich AB, Stockholm, Sweden) were obtained as sterile powders, dissolved in 1 ml dimethyl sulfoxide (Sigma-Aldrich AB, Stockholm, Sweden) and diluted in RPMI 1640 to obtain the desired concentrations. The antifungal solutions were prepared immediately before each experiment.

In vitro kinetic model. A previously described one-compartment in vitro kinetic model was used allowing exposure of Candida spp. to simulated human serum pharmacokinetics for voriconazole and amphotericin B (12). The culture vessel (Bellco Glass Inc., Vineland, NJ, USA) with a total volume of 115 ml contained a magnetic stir bar for continuous mixing and two side arms, one with a silicone membrane for sampling and injection of the studied drugs and one connected by a thin plastic tubing to a vessel for waste. Fresh medium was supplied to the culture vessel with a peristaltic pump (Watson-Marlow Alitea 403U/VM4, Watson-Marlow Alitea, Stockholm, Sweden) via latex tubing connected to the culture vessel. The flow rate was adjusted to obtain the desired half-life of the antifungal agent. In regimens in which two agents with different elimination rates were used a computer-controlled dosing pump (Harvard dosing-pump 55-2222, Harvard Apparatus Inc., Holliston, MA, USA) was employed to infuse the agent with the longer half-life through a syringe connected via a plastic tubing to the culture vessel. This dosing pump also made it possible to compensate for the spontaneous degradation of the antifungals that may occur during the experimental conditions. Computer software (ARUComb 1.5, Snowfall Communications, Uppsala, Sweden) was applied for programming the pump to infuse the drug at an exponentially decreasing rate. Pharmacokinetic analysis of \( C_{\text{max}} \), \( C_{\text{min}} \) and \( t_{1/2} \) of regimens with voriconazole and amphotericin B has previously shown results close to target values (13). The in vitro model
was placed in a thermostatic room at 35°C during the experiments. Between each experiment, the equipment was sterilized by autoclaving.

Pharmacodynamic experiments with voriconazole and amphotericin B in C. albicans, C. glabrata and C. krusei with simulated human pharmacokinetics. Different antifungal regimens were simulated in the in vitro kinetic model. These included (I) the control (no drug), (II) voriconazole at 0 h, (III) amphotericin B at 0 h, (IV) voriconazole + amphotericin B at 0 h and (V) voriconazole at 0 h and sequential doses of amphotericin B at 24, 48 and 72 h. Regimens II-V were performed with a starting concentration of voriconazole and amphotericin B of 5.0 and 2.0 mg/l, respectively.

The half-life of voriconazole was adjusted to 6 h and amphotericin B to 7 h (3, 23). Target free concentrations of 5.0 mg/l of voriconazole and 2.0 mg/l of amphotericin B were simulated. The initial voriconazole concentration represented a high drug level in the range of those observed as peak concentrations in plasma after clinically relevant doses (22), whereas the starting concentration of amphotericin B was somewhat higher to assure a maximal fungicidal activity of amphotericin B, which has been shown to be increased at higher concentrations (3, 6).

A volume of 0.25 ml of Candida suspension was added to 110 ml of RPMI 1640 through one side arm to yield a starting inoculum of approximately $10^4$ CFU/ml. To study the effect of inoculum size amphotericin B was administered alone (regimen III), also against $10^6$ CFU/ml of the resistant C. albicans strains. Volumes of 0.25-0.5 ml of voriconazole, amphotericin B, or both solutions were added through one side arm to yield the desired starting concentration. In regimens including amphotericin B the computer-controlled dosing pump was prepared with an amphotericin B suspension concentration of 200 mg/l which was infused with an exponentially decreasing flow rate. This procedure caused a negligible increase in the total
flow rate (<1%). Repeated sampling before and after each antifungal dose at 0, 1.5, 3, 6, 12 and 24 h for viable counting was performed through the silicone membrane and repeated for every 24-h period of the experiment. The samples were serially diluted 10-fold in phosphate-buffered saline (PBS). At least three samples (10 or 100 µl) from the original fungal suspension and dilutions were subsequently spread on Sabouraud dextrose agar plates, incubated at 35°C and counted after 24-48 h. To avoid antifungal carryover all samples were placed on the same spot on the Sabouraud dextrose agar plates and then allowed to diffuse into the agar for 3-5 min before spreading. If there was a clear zone where the sample had been placed after growth, the agar plate was divided into sections and the clear section was excluded from counting. To study the effect of carryover with this method undiluted and 100-fold diluted samples containing 2.5 and 5.0 mg/l of amphotericin B and voriconazole, respectively, were spread on Sabouraud dextrose agar plates. This process resulted in negligible carryover with a mean log_{10} CFU/ml that was 1.5% higher in the diluted samples for amphotericin B and 1.0% for voriconazole (13). The limit of detection of the viable counts was 1 × 10^1 CFU/ml.

All regimens performed with C. albicans strains were tested once, except for the high inoculate regimen III and regimen V, which were tested in triplicate. In the C. glabrata and C. krusei experiments all regimens, except the control, were tested in triplicates.

**Effect of pre-exposure to voriconazole on the fungicidal activity of amphotericin B in C. albicans.** To test whether the fungicidal activity of amphotericin B would be retrieved after removal of voriconazole, the effect of pre-exposure was investigated. A C. albicans suspension with a concentration of approximately 10^7 CFU/ml was diluted 1:10. 0.1 ml of the diluted fungal suspension and 0.1 ml of the voriconazole solution were subsequently added to test tubes containing 9.8 ml of RPMI 1640, resulting in a C. albicans concentration of 10^4
CFU/ml and a voriconazole concentration of 5.0 mg/l. The test tubes were placed on a shaker in a thermostatic room at 35°C for 24 h and then washed three times by centrifugation at 1400 × g for 10 min. The fungal pellet was resuspended in RPMI 1640 after each centrifugation. After wash, 9.9 ml of RPMI 1640 and 0.1 ml of amphotericin B solution were added to yield a final amphotericin B concentration of 2.0 mg/l. The test tubes were again placed on a shaker and incubated at 35°C for 24 h. Repeated sampling for viable counting was performed and samples were processed as described above. Each strain was tested in triplicate; for each strain, one control was pre-exposed to voriconazole but without the subsequent addition of amphotericin B.

Statistics and calculations. Log_{10} CFU counts that were due to fungal growth or killing approximated to normal distribution. Fungicidal effect was defined as a reduction in CFU/ml from the starting inoculum of ≥99.9% (21). Fungal killing was defined as the difference in log_{10} CFU/ml immediately before administration of amphotericin B and 6 h thereafter and expressed as the mean ± standard error (SE). A repeated measures ANOVA (analysis of variance) was carried out to compare the fungal killing between the antifungal regimens and Candida strains over the 6 h period. Spearman rank correlation was used to correlate the reduction of fungal killing of amphotericin B after voriconazole exposure (compared with amphotericin B alone) with the MIC. The voriconazole susceptible quality control strain CA5 MIC 0.004 was not included in the correlation analysis. In the statistical analysis colony count values below the detection limit were set to 1.0 log_{10} CFU/ml. Statistica 8.0 (StatSoft, Inc, Tulsa, OK, USA) was used for all analyses.
Results

MIC determination. The MICs of voriconazole and amphotericin B for *C. albicans*, *C. glabrata* and *C. krusei* strains are shown in Table 1.

Characterization of mechanisms of resistance in strain CA4<sub>MIC256</sub>

*CDR1* and *ERG11* were slightly more upregulated in CA4<sub>MIC256</sub> than the azole-susceptible isolate CAF2-1 (Figure 1). However, no *CDR2* and *MDR1* expression could be detected. This feature is typical for most azole-susceptible isolates and suggests an absence of mutations in the regulators *TAC1* and *MRR1* (4, 7), which otherwise contribute to high expression levels of efflux pump genes. Even if no matched susceptible isolate exists for CA4<sub>MIC256</sub>, the slight upregulation of *CDR1* and *ERG11* may partially contribute to the phenotype of CA4<sub>MIC256</sub>. However, it cannot be excluded that other hitherto unknown mechanisms of resistance in addition may play a role in this isolate.

Pharmacodynamic experiments with voriconazole and amphotericin B in *C. albicans*, *C. glabrata* and *C. krusei* with simulated human pharmacokinetics. Time-kill curves (0-48 h) for regimens in which *C. albicans*, *C. glabrata* and *C. krusei* were exposed to voriconazole and amphotericin B with starting concentrations of 5.0 and 2.0 mg/l, respectively, or combinations thereof, are depicted in Figure 2.

For the unexposed controls, there was an approximately 3-log<sub>10</sub> increase in CFU/ml. In *C. albicans* exposure to voriconazole resulted in 1.2 and 1.4 log<sub>10</sub> CFU/ml reduction compared with the controls at 24 h against strains CA2<sub>MIC8</sub> and CA3<sub>MIC2</sub>, respectively, whereas only a minimal effect was observed in strains CA1<sub>MIC8</sub> and CA4<sub>MIC256</sub> (0.4 and 0.1 log<sub>10</sub> CFU/ml reduction at 24 h, respectively). The regimens with amphotericin B and
simultaneous administration of amphotericin B and voriconazole against *C. albicans* resulted in a fungicidal activity within 1.5 h. No difference was noted when the starting inoculum was increased to 6 log_{10} CFU/ml (data not shown). When *C. albicans* was exposed to voriconazole for 24 h before administration of amphotericin B, fungal killing of amphotericin B was significantly reduced compared with that of amphotericin B alone, not only in the fully susceptible strain CA5_{MIC0.004}, but also in the CA1_{MIC8}, CA3_{MIC2} and CA4_{MIC256} strains (P<0.01). However, fungal killing of amphotericin B was not reduced by voriconazole in the CA2_{MIC8} strain (P=0.22). The reduction of the fungal killing of amphotericin B after voriconazole exposure is summarized in Table 2. Except for the comparison between strains CA1_{MIC8} and CA3_{MIC2}, the resistant *C. albicans* strains differed significantly in the voriconazole-induced inhibition of the fungicidal killing of amphotericin B at 24 h when compared pairwise (P<0.05). There was also a significant difference between the strains CA1_{MIC8}, CA2_{MIC8} and CA3_{MIC2} when compared pairwise with the susceptible isolate CA5_{MIC0.004} (P<0.05). However, no difference was detected between the strain with the highest MIC value (CA4_{MIC256}) and the voriconazole susceptible strain (P=0.12). Inhibition of amphotericin B by voriconazole was detected in the *C. albicans* strains with increases in *CDR1* and *CDR2* expression but not in the strain with increased *MDR1* expression.

In the *C. glabrata* and *C. krusei* strains amphotericin B alone resulted in fungicidal activity within 1.5-6 h. Simultaneous administration of amphotericin B and voriconazole significantly delayed the fungicidal killing compared with amphotericin B alone as reflected by a median 4.5 h delay of the fungicidal effect (P<0.01) (Figure 2f-i). When *C. glabrata* and *C. krusei* were exposed to voriconazole for 24 h before amphotericin B, fungal killing of amphotericin B was significantly reduced in all strains (P<0.01 for each of the strains) which is summarized in Table 2.
The reduction of the activity of amphotericin B after voriconazole exposure compared with amphotericin B alone did not correlate with the MIC value (\( r=-0.19, P=0.65 \) for all strains tested and \( r=0.32, P=0.68 \) for the \( C. albicans \) strains).

The \( Candida \) strains in regimen V were exposed to voriconazole at 0 h and thereafter exposed to sequential doses of amphotericin B at 24, 48 and 72 h. Despite a gradually decreasing concentration of voriconazole over time in the culture vessel, the fungicidal activity of amphotericin B did not recover in any strain. Data on the fungal killing at different time points are summarized in Table 2.

**Effect of pre-exposure to voriconazole on the fungicidal activity of amphotericin B in \( C. albicans \).** In Figure 3 time-kill curves are given for \( C. albicans \) strains exposed to 2.0 mg/l of amphotericin B after 24 h pre-exposure to 5.0 mg/l of voriconazole. After elimination of voriconazole, an early fungicidal activity of amphotericin B was detected against four of the five \( C. albicans \) strains. However, even after three times washing, the fungicidal activity of amphotericin B was still abolished in the strain \( CA4_{MIC 256} \) after 24 h of voriconazole pre-exposure.
Discussion

We have previously shown that voriconazole reduced the amphotericin B activity in *C. albicans* strains fully susceptible to voriconazole (12, 13). Moreover, it was shown that when the concentration of voriconazole had fallen below the MIC or when voriconazole was washed away, amphotericin B regained its fungicidal effect (13). In the strains of *C. albicans* resistant to voriconazole and those of *C. glabrata* and *C. krusei* with varying susceptibility to voriconazole investigated in the present study, exposure to voriconazole followed by amphotericin B resulted in reduced activity of amphotericin B in all but one strain. Our hypothesis was that the voriconazole-induced inhibition of the fungicidal activity of amphotericin B would not be observed in voriconazole resistant strains but, in contrast, this negative interaction occurred despite reduced voriconazole susceptibility. However, in comparison with the susceptible isolates studied previously, the inhibition was less pronounced. At 24 h when amphotericin B was administered, the level of voriconazole had reached values far below the MIC in the majority of strains, suggesting that also sub-MIC levels of voriconazole negatively influence the activity of subsequent doses of amphotericin B. Furthermore, in contrast to other *C. albicans* strains tested here and in our previous study, this negative interaction was prolonged in the CA4\text{MIC256} strain despite three washes which should have removed all voriconazole. This finding suggests that the voriconazole inhibitory effect might be sustained in some isolates.

The reason for not recovering the fungicidal activity of amphotericin B after voriconazole exposure despite repeated doses of amphotericin B at 48 h and 72 h in any of the strains is difficult to explain but has also been seen in a previous study (13). It seems unlikely that this phenomenon is caused by voriconazole because of the gradually decreasing voriconazole concentration that at the time of these amphotericin B exposures should be extremely low. As
discussed previously, a more likely mechanism could involve an adaptive resistance against
amphotericin B because of repeated amphotericin B administration (34). However, this
hypothesis needs to be tested by further experiments.
Simultaneous exposure of amphotericin B and voriconazole delayed the fungicidal killing for
the *C. glabrata* and *C. krusei* strains compared with that of amphotericin B alone, which was
not observed in *C. albicans*. Whether this result is a consequence of species-specific features
or that the assay was not sensitive enough to discover a difference in fungal counts between
the regimens in *C. albicans* is not known.
Our results are in part consistent with those of Louie *et al.*, who evaluated the interaction
between fluconazole and amphotericin B in a murine model of candidiasis (14). For
fluconazole-susceptible and mid-resistant strains, the combination of fluconazole and
amphotericin B was antagonistic, as shown by both quantitative culture results and survival
experiments. However, antagonism was not observed in the highly fluconazole-resistant strain
(14). In contrast, no correlation was found in our study between the degree of interaction and
the MIC value of the *C. albicans* strains. The mechanism of resistance may be of importance
because inhibition of amphotericin B was observed in strains with increases in *CDR1* and
*CDR2* expression (CA1\textsubscript{MIC8} and CA3\textsubscript{MIC2}) and *CDR1* expression (CA4\textsubscript{MIC256}), but not in the
strain with an increase in *MDR1* expression (CA2\textsubscript{MIC8}). Still, it is difficult to postulate a
mechanism of how differences in efflux pump systems would cause dissociation between the
degree of voriconazole-amphotericin B interaction and the MIC value. The antifungal effects
mediated by voriconazole and other azoles consist of inhibition of ergosterol synthesis leading
to depletion of ergosterol in the cell membrane and accumulation of toxic sterols (1). Both
mechanisms affect theazole MIC, whereas only ergosterol depletion might have the ability to
modify the interaction with amphotericin B. It can be speculated that the various mutations in
*ERG11* (or other unknown differences) in the strains tested may affect these two mechanisms
differently and thus contribute to the dissociation between the MIC and the voriconazole-amphotericin B interaction. Furthermore, voriconazole-induced accumulation of non-ergosterol sterols may also affect amphotericin B action since amphotericin B has different affinity for various sterols (1). Similarly to the *C. albicans* strains, inhibition was more pronounced in the *C. glabrata* strain with the higher MIC and a 24-h growth comparable to the control than in the strain with the lower MIC, suggesting that other mechanisms than those reflected merely by the MIC value are of importance for the magnitude of the interaction also in non-*albicans* strains.

During the past decades, the potential antagonism between azoles and amphotericin B has been intensively discussed. Several *in vitro* studies have shown decreased activity of amphotericin B in combination with an azole or if azoles and amphotericin B are used sequentially (8, 11-13, 15, 29, 33). In some of the studies that failed to identify a negative interaction between azoles and amphotericin B the drugs were administered simultaneously (2, 10, 19). The mechanism for this might be that there is not enough time for the azole to affect its target before the fungal cell is killed by the action of amphotericin B. Animal models have revealed either no interaction or antagonism (15, 16, 25, 30, 31). To our knowledge, the effect of the combination of azoles and amphotericin B has never been shown to be more effective than amphotericin B alone provided that fungicidal doses of amphotericin B were used. In the only clinical trial addressing the combination of amphotericin B and fluconazole, Rex et al. found a trend toward a better outcome as compared with fluconazole alone (24).

However, the lack of a comparison arm with amphotericin B alone, which in some of the experimental models has been the most active regimen, implies that this clinical trial does not exclude that azoles can negatively interact with amphotericin B (24).
Voriconazole is considered one of the treatment options for non-neutropenic and neutropenic patients suffering from invasive *Candida* infections (20, 32). Furthermore, voriconazole has recently been recommended for prophylactic use against invasive fungal infections (17). Voriconazole resistance may be one reason for treatment failure or breakthrough fungal infections. In such situations amphotericin B would be one of the therapeutic alternatives. Until now, an argument has been that the azole-polyene interaction is of limited importance because the voriconazole-induced antagonistic effect would only be found in susceptible strains that should be eliminated by the azoles. However, our data clearly demonstrate that this effect is observed also in isolates that are more prone to cause treatment failure because of their reduced susceptibility.

A lack of a fungicidal activity of amphotericin B may be of concern in various clinical situations. In these situations a switch from azoles to amphotericin B may, at least initially, result in a reduced antifungal effect. Therefore, until more data are available, a reasonable recommendation would be to use the combination of azoles and amphotericin B for the treatment of *Candida* infection with caution and possibly consider another drug than amphotericin B as the preferred fungicidal agent when switching from an azole agent.

In conclusion, a reduction of the fungicidal activity of amphotericin B against *C. albicans* strains previously exposed to voriconazole was demonstrated despite reduced voriconazole susceptibility. The degree of interaction was not predicted by the MIC value alone and mechanisms of resistance may be of importance. Furthermore, a reduction of the activity of amphotericin B by voriconazole was also demonstrated in strains of *C. glabrata* and *C. krusei*, regardless of whether exposed sequentially or simultaneously.
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multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the
main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains.


Table 1. Summary of MIC values and resistance mechanisms against voriconazole for the Candida strains

<table>
<thead>
<tr>
<th>Candida strain</th>
<th>Name in current work</th>
<th>Voriconazole MIC (mg/l)</th>
<th>Amphotericin B MIC (mg/l)</th>
<th>Voriconazole resistance mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans C26</td>
<td>CA1&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>8</td>
<td>0.5</td>
<td>Increase in CDR1 and CDR2 mRNAs; mutations of ERG11: S405F, Y132H (26)</td>
</tr>
<tr>
<td>C. albicans C40</td>
<td>CA2&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>8</td>
<td>0.5</td>
<td>Increase in MDR1 mRNA; mutations of ERG11: G464S, R467K, Y132H (26)</td>
</tr>
<tr>
<td>C. albicans C56</td>
<td>CA3&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>2</td>
<td>0.5</td>
<td>Increase in CDR1 and CDR2 mRNAs; mutations of ERG11: G129A, G464S (26)</td>
</tr>
<tr>
<td>C. albicans 20288.030</td>
<td>CA4&lt;sub&gt;MIC256&lt;/sub&gt;</td>
<td>256</td>
<td>0.5</td>
<td>Increase in CDR1 mRNA; upregulation of ERG11</td>
</tr>
<tr>
<td>C. glabrata B346 4018</td>
<td>CG1&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>0.5</td>
<td>0.5</td>
<td>NA</td>
</tr>
<tr>
<td>C. glabrata 263 SMI</td>
<td>CG2&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>4</td>
<td>0.5</td>
<td>NA</td>
</tr>
<tr>
<td>C. krusei B336 4026</td>
<td>CK1&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>0.5</td>
<td>0.5-1</td>
<td>NA</td>
</tr>
<tr>
<td>C. krusei 20550.054</td>
<td>CK2&lt;sub&gt;MIC&lt;/sub&gt;</td>
<td>4</td>
<td>0.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

C. albicans CCUG 32723: Voriconazole susceptible strain included as a quality control

NA, not analyzed
Table 2. Fungal killing of sequential doses of amphotericin B after voriconazole exposure and reduction of fungal killing in percent compared with amphotericin B alone

| Time point for the amphotericin B administration after the initial voriconazole dose |
|----------------------------------|----------------------------------|----------------------------------|
|                                  | 24 h                             | 48 h                             | 72 h                             |
| Fungal killing (log<sub>10</sub> CFU/ml) | Reduction of fungal killing (%) | Fungal killing (log<sub>10</sub> CFU/ml) | Reduction of fungal killing (%) | Fungal killing (log<sub>10</sub> CFU/ml) | Reduction of fungal killing (%) |
| CA1<sub>MIC8</sub>               | 2.6±0.4                          | 44                               | 0.8±0.2                          | 83                               | 1.1±0.2                          | 76                               |
| CA2<sub>MIC8</sub>               | 4.4±0.8                          | 9                                | 0.4±0.2                          | 92                               | 1.1±0.6                          | 78                               |
| CA3<sub>MIC2</sub>               | 2.0±0.3                          | 59                               | 0.8±0.6                          | 84                               | 1.1±0.2                          | 78                               |
| CA4<sub>MIC256</sub>             | 0.3±0.1                          | 94                               | 0.2±0.0                          | 96                               | 0.4±0.4                          | 92                               |
| CG1<sub>MIC0.5</sub>             | 1.3±0.1                          | 63                               | 1.4±1.2                          | 60                               | 0.0±0.9                          | 100                              |
| CG2<sub>MIC4</sub>               | 0.7±0.3                          | 83                               | 1.9±0.3                          | 49                               | 0.8±0.2                          | 78                               |
| CK1<sub>MIC0.5</sub>             | 0.4±0.2                          | 86                               | 1.0±0.2                          | 69                               | 1.1±0.2                          | 66                               |
| CK2<sub>MIC4</sub>               | 1.7±0.4                          | 47                               | 1.0±0.7                          | 69                               | 1.5±0.5                          | 53                               |
| CA5<sub>MIC0.004</sub>           | 0.0±0.1                          | 100                              | 0.0±0.1                          | 100                              | 1.1±0.7                          | 59                               |

Fungal killing was defined as the mean difference in log<sub>10</sub> CFU/ml ± SE immediately before administration of amphotericin B and 6 h thereafter.
Figure legends

Figure 1. Expression levels of drug resistance genes in *C. albicans* strain CA4\textsubscript{MIC256} compared with the azole susceptible strain CAF2-1. 28S and 18S RNA are shown as control for identical RNA loading.

Figure 2. Time-kill plots of voriconazole (VRC) and amphotericin B (AMB) regimens against *C. albicans* strains CA\textsubscript{MIC8} \textsuperscript{a}, CA\textsubscript{MIC8} \textsuperscript{b}, CA\textsubscript{MIC2} \textsuperscript{c}, CA\textsubscript{MIC256} \textsuperscript{d} and CA\textsubscript{MIC0.004} \textsuperscript{e}; *C. glabrata* strains CG\textsubscript{MIC0.5} \textsuperscript{f} and CG\textsubscript{MIC4} \textsuperscript{g}; and *C. krusei* strains CK\textsubscript{MIC0.5} \textsuperscript{h} and CK\textsubscript{MIC4} \textsuperscript{i} in the *in vitro* kinetic model. Colony count values below the limit of detection were set to 0.5 log\textsubscript{10} CFU/ml.

Figure 3. Time-kill plots of *C. albicans* CA\textsubscript{MIC8} \textsuperscript{a}, CA\textsubscript{MIC8} \textsuperscript{b}, CA\textsubscript{MIC2} \textsuperscript{c}, CA\textsubscript{MIC256} \textsuperscript{d} and CA\textsubscript{MIC0.004} \textsuperscript{e} exposed to 2.0 mg/L of amphotericin B (AMB) after pre-exposure to 5.0 mg/l voriconazole (VRC) for 24 h. Time 0 h is the time point for AMB exposure. Colony count values below the limit of detection were set to 0.5 log\textsubscript{10} CFU/ml.
Figure 1

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>ERG11</td>
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<tr>
<td>CDR2</td>
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<td>18s</td>
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</table>
Fig. 2a (CA1MIC8)

- Control
- VRC 0h
- VRC 0h, AMB 24h
- AMB 0h
- VRC+AMB 0h
- Limit of detection

Time (h)
Fungal count (log_{10} CFU/ml)

Fig. 2b (CA2MIC8)

- Control
- VRC 0h
- VRC 0h, AMB 24h
- AMB 0h
- VRC+AMB 0h
- Limit of detection

Time (h)
Fungal count (log_{10} CFU/ml)

Fig. 2c (CA3MIC2)

- Control
- VRC 0h
- VRC 0h, AMB 24h
- AMB 0h
- VRC+AMB 0h
- Limit of detection

Time (h)
Fungal count (log_{10} CFU/ml)
Fig. 3a (CA1$_{MIC8}$)

Fig. 3b (CA2$_{MIC8}$)

Fig. 3c (CA3$_{MIC8}$)

- Control
- AMB
- Limit of detection

Fungal count (log$_{10}$ CFU/ml) vs. Time (h)
Fig. 3d (CA4\textsubscript{MIC\textsubscript{256}})

- Control
- AMB
- Limit of detection

Fig. 3e (CA5\textsubscript{MIC\textsubscript{0.004}})

- Control
- AMB
- Limit of detection