Title: Position and numbers of FKS mutations in C. albicans selectively influence in vitro and in vivo susceptibility to echinocandin treatment

Running title: Heterozygous double mutations in C. albicans

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Abstract

Candidemia is the fourth most common microbial bloodstream infection, with *Candida albicans* being the most common causative species. The echinocandins are employed as first line treatment for invasive candidiasis until fungal speciation is confirmed by clinical diagnosis. Echinocandins block the FKS glucan synthases responsible for embedding β-(1,3) D-glucan into the cell wall. The increasing use of these drugs has led to the emergence of antifungal resistance, and elevated MICs have been associated with single residue substitutions in specific hot spot regions of *FKS1* and *FKS2*.

Here, we show for the first time, the caspofungin-mediated *in vivo* selection of a double mutation within one allele of the FKS1 hot spot 1 in a clinical isolate. We created a set of isogenic mutants and used a haematogenous murine model to evaluate *in vivo* outcome of echinocandin treatment. Heterozygous and homozygous double mutations significantly enhance the *in vivo* resistance of *C. albicans* when compared with heterozygous single mutations. The various FKS1 hot spot mutations differ in their MIC increase, substance-dependent *in vivo* response, and impact on virulence. Our results demonstrate that echinocandin EUCAST breakpoint definitions correlate with *in vivo* response at standard dosing regimen, but cannot predict *in vivo* response at dose escalation. Moreover, patients colonized by a *C. albicans* strain with multiple mutations in FKS1 have a higher risk for therapeutic failure.
Introduction

Fungal infections have emerged over the last decades as a consequence of increasing cohorts of at-risk individuals. *Candida albicans* is the most important opportunistic fungal pathogen for humans (1). Mortality rates are estimated to be as high as 45% (2), partly due to delayed or inaccurate diagnosis and inappropriate antifungal therapies.

Echinocandin drugs are recommended as the first line treatment for invasive candidemia (3, 4). Anidulafungin (ANI), caspofungin (CAS), and micafungin (MICA) are lipopeptidic antifungal agents that inhibit the synthesis of fungal wall component β-(1,3)-D-glucan by noncompetitively blocking the β-(1,3) D-glucan synthase. Drug resistance is emerging in patients with *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* infections (5-7), which complicates disease monitoring and patient management (8). Presently, echinocandin-resistance in *C. albicans* occurs with an incidence <1% (9).

Single residue substitutions located in two hot spot regions of each of the genes *FKS1* and *FKS2* (10), but not *FKS3*, have been associated with elevated echinocandin MICs (11) in *Candida* species. So far in *C. albicans*, only amino acid substitutions within *FKS1*, encoding the β-(1,3)-D-glucan synthase complex, were found in echinocandin resistant isolates (12). The most frequent amino acid changes reported were F641, L644, S645, R647, D648, P649 (10), and W1358 and R1361 (13) within host spot 1 (HS1; amino acid position 641-649; FLTLSLRDP) (12) and hot spot 2 (HS2; amino acid position 1357-1364;DWIRRYTL) (14), respectively. HS1 and HS2 alterations leading to F641, S645, and R1361 changes were associated with pronounced MIC elevations. Other HS mutations (L644, R647, D648, P649, and W1358) confer to a discrete MIC increase (14). *C. albicans* is a diploid fungus carrying a pair of each chromosome and at least two alleles of each gene locus (15). An amino acid substitution in one allele (heterozygous) affects only half of the total cellular glucan synthase pool that can be targeted by echinocandins (5), while substitutions occurring in both alleles (homozygous) affect the whole glucan synthase pool.
Here, we identify and report the first in vivo selected HS1 FKS1 double mutation (R647R/G and P649P/L) in one allele in *C. albicans* after long-term caspofungin (CAS) therapy (including detailed case report and therapeutic regimes). The treatment response of a clinical wild type (WT) strain and the corresponding sequential heterozygous derivative mutant (R647R/G and P649P/L) is compared using a well-validated murine model of disseminated candidiasis. ANI, CAS, or MICA were applied in standard (AUC100) or elevated dose (AUC500) and compared with each other and the placebo control group.

The impact of heterozygous mono- (R647R/G or P649P/L) and double mutations (R647R/G and P649P/L), and homozygous double mutations (R647G and P649L) in *FKS1* on in vitro and in vivo susceptibility, as well as their influence on virulence were tested in isogenic (identical genetic background) mutant strains.

The hypothesis addressed in this study was that heterozygous double mutation, as well as homozygous double mutations within one allele enhances the in vivo therapeutic resistance of *C. albicans* strains even at dose escalation. We were able to (1) confirm this hypothesis, (2) show significant differences in the activity of the three echinocandins, and (3) demonstrate that the reported single nucleotide polymorphisms (SNPs) have no impact on virulence.
Materials and Methods

Patient Case

A 27-year-old female patient is reported with CMC, suffering from persistent and recurrent infections of the skin, nails, and oral mucosa. In 2005, C. albicans (azole-resistant isolate) infections was successfully treated by 12 months CAS (50 mg/d) administration(16). In 2007, the patient presented with a relapse of CMC. Caspofungin (50 mg/d) was resumed in combination with terbinafine (250 mg/d) and topical Ciclopirox-crème for another 12 months, resulting in a marked improvement.

During 2007-2010, variousazole regimens consisting of VOR (2 x 200 mg/d) or POS (2 x 800 mg/d) were attempted with partial effect. In March 2010 retreatment with CAS iv (50 mg/d) for two years. Onychomycosis improved slightly (Fig.1C). In 2012, while still on CAS, fulminant infection appeared with perioral/periorbital erythematous and erosive lesions accompanied with plaques of pustules at the forehead being clinically significant (Fig.1A) and nail-destructive onychomycosis (Fig.1B). In vitro susceptibility testing according to E-test® and EUCAST classified C. albicans as CAS resistant (Table 1). Antifungal treatment was immediately changed to ANI (100 mg/d) and infections improved significantly with full healing being achieved by October 2012.

Random amplified polymorphic DNA (RAPD)-typing of clinical isolates

Clinical isolates (n = 5) were RAPD-typed using the published primer pairs M13, OPA18, OPE18, CA2, RSD10, and RSD12 together with already published PCR conditions (17-20). For DNA extractions all strains were cultured on Sabouraud 2% glucose agar for 24 h at 37 °C. DNA was extracted from pure cultures using UltraClean®Microbial DNA Isolation Kit (MO BIO Laboratories Inc.) according to manufactures instructions. The DNA concentration of DNA extract was determined using NanoVue (GE Healthcare Life Sciences) according to manufactures instructions. Genomic DNA extracts were diluted to 50 ng/μL for PCR. A volume of 18 μL of all PCR products were loaded together with 3 μL of
Gel Loading Dye, Orange (6X) (New England BioLabs® Inc.) on an 1.8 % agar (Roti®garose; Roth) gel with ethidium bromide in a concentration of 0.5 µg/mL. As reference the SimplyLoad 100 bp Extended Range DNA Ladder (Lonza Cologne GmbH) was used. Electrophoresis conditions were three hours at 80 Volt and 100 mA. Electrophoresis gels were visualised in a Gel Doc™ EZ System (BIO-RAD). Typing assay was performed twice double-blinded by two different investigators. The following C. albicans strains ATCC 90028 (= American type culture collection), ATCC 64550 and ATCC 64548 were used as population control.

In vitro susceptibility testing

Minimal inhibitory concentration (MICs) for azoles (voriconazole, isavuconazole, and fluconazole) and echinocandins (ANJ and MICA) were tested for the echinocandin-susceptible strains isolated in 2004 (S CL 1130.04, S CL 952.04, S CL 5104.04, and S CL 111.12), and the echinocandin-resistant (RR CL 110.12) carrying the mutations R647R/G and P649P/L, the parental strain SS WT SC5314 of the isogenic mutants and the mutants RR MH2 (R647R/G and P649P/L), RS MH1 (R647R/G), SR MH1 (P649P/L), and RR MH2 (R647G and P649L) according to EUCAST methodology (EDEF 7.2) (21) (see Table 1). In addition, CAS and amphotericin B were tested using CLSI and Etest® (biomerieux). As quality control strains served C. krusei ATCC 6258 or C. parapsilosis ATCC 22019 (21, 22).

Sequencing of gene regions FKS1 hot spot 1 and hot spot 2

Genomic DNA was extracted using UltraClean®Microbial DNA Isolation Kit (MO BIO Laboratories Inc.) according to manufactures instructions. Sequencing was performed using the same primers and conditions as published by Garcia-Effron (23). In short, the Candida universal FKS1 primer 1HS1F (AAT GGG CTG GTG CTC AAC AT) and 1HS1F (CCT TCA ATT TCA GAT GGA ACT TGA TG) were used for amplifying FKS1 HS1. While for FKS1 HS2 the primers 1HS2F (AAG ATT GGT GCT GGT ATG GG) and 6
1HS2R (TAA TGG TGC TTG CCA ATG AG) were used. As PCR master mix QIAGEN LongRange PCR Kit (QIAGEN) and conditions were as follows: initial denaturation 96°C for 4 min, followed by 40 cycles of 94 °C for 30 sec, 55° for 30 sec, 74° C for 90 sec, and a final elongation step of 74°C for 4 min. For cleaning PCR products ExoSAP-IT® was used. For sequencing Big Dye® Terminator v.3.1 Cycle Sequencing Kit was used in combination with Genetic Analyser 3500 (former Applied Biosystems®) according to manufactures instructions.

construction of isogeneic C. albicans FKS1-mutants

An overview of generated isogeneic mutants is given in Table S1, of used plasmids in Table S1 and of used primers in Table S2. A FKS1 deletion cassette was constructed using the SAT flipper technology (24) with primers FKS1_55 and FKS1KO_53 for the upstream homologous region and FKS1_35 and FKS1_33 for the downstream homologous region. Homologous regions were cloned into pSFS2a (24) using restriction sites ApaI/XhoI and SacII/Sacl, respectively. The resulting plasmid was linearized with PvuI and transformed into C. albicans SC5314 (25) to replace the FKS1 coding sequence and to get a heterozygous FKS1/fks1 knockout (Table S3). The SAT1 marker was recycled by overnight cultivation in maltose containing media.

For reintegration of the FKS1 gene at its endogenous locus, the FKS1 coding sequence was cloned into pSFS2a-FKS1dr (pSFS2a containing the downstream homologous region of the deletion cassette) with primers FKS1_55 and FKS1clo_53 using restriction sites Apal/Xhol. The resulting plasmid (pSFS2a-FKS1reint) was linearized with PvuI and transformed into CA-MT424 (FKS1/fks1). Correct integration was checked by colony PCR.

For mutagenesis of FKS1, PCRs were performed using pSFS2a-FKS1reint with primers FKS1_55 and the corresponding mutagenesis reverse primer as well as with the corresponding mutagenesis forward primer and FKS1_33, respectively (Table S3). The purified PCR products were mixed and the whole reintegration cassette was amplified using the primers FKS1_55_2 and FKS1_33_2. The
cassette was transformed into CA-MT424 to integrate the mutated FKS1 allele at the deleted locus.

Correct integration was checked by colony PCR and the SAT1 marker was recycled.

For the construction of homozygous mutants, PCRs were performed with primers FKS1_55 and the corresponding mutagenesis reverse primer, as well as with the corresponding mutagenesis forward primer and FKS1clo_53, respectively. The purified PCR products were mixed, the mutated FKS1 coding sequence was amplified using the primers FKS1_55 and FKS1clo_53 and cloned into pSFS2a-FKS1dr as described above for the reintegration plasmid. Prior to transformation the plasmids were linearized with PvuI. For construction of the homozygous double mutant, pSFS2a-FKS1_2mut_reint was used to replace the wild type allele in CA-MT441 (Table S2). Correct integration was checked by colony PCR and the SAT1 marker was recycled. For construction of the homozygous single mutants, the corresponding plasmids containing mutated FKS1 alleles were used for integration at the deleted alleles of strains CA-MT457 and CA-MT458, respectively. In these two strains, the wild type allele in CA-MT424 had been directly replaced by the corresponding mutated allele before. The presence of the introduced point mutations was confirmed by sequencing of the corresponding region in all mutants.

Haematogenous mouse model

Pilot study for inoculum determination. To evaluate the in vivo virulence of isogeneic mutant strains, a total of 24 NRMI mice were challenged in groups of three with four isogeneic mutant strains (SSMH2, RSMH1, SRMH1, and SSMHO2; detailed information Table 1 and Table S1) and two inoculum sizes (5 x 10⁵ CFU/murine and 5 x 10⁶/murine). Aim was to get sufficient infection without mortality. Using a C. albicans cell concentration for infection of 5 x 10⁵/murine, nine out of twelve mice died. Challenging the mice with a dose of 5 x 10⁵ CFU/murine one out of three mice per group died and kidney burden in surviving mice was found to be between 10⁴ to 10⁵ CFU/mL kidney homogenate. Hence, an inoculum of 1*10⁵/murine was used for the treatment studies.
In vivo treatment studies. Evaluation of in vivo resistance of *C. albicans* clinical isolates (SCL and RRCL; Table 1 and Table 2) against ANI, CAS, MICA, and placebo (0.09 % NaCl) was performed in a haematogenous mouse model. 84 NMRI mice (weight 26.0 g – 30.0 g, Harlan Scandinavia, Allerød, Denmark) were kept with free access to food and water. On day 0 mice were challenged by intravenous injection (200 µl administered with a 25-gauge syringe) of *C. albicans* (5 x 10^5 CFU/mL; final concentration per murine 1 x 10^5) suspension. Mice were challenged with either of the two strains SCL (n = 42 mice) or RRCL (n = 42 mice) and treated by the intraperitoneal route (i.p.) on days 1-3 with 0.5 mL of either ANI, CAS or MICA in standard AUC_100 and high doses AUC_500 or with placebo (see below).

Treatment studies of isogeneic mutant strains and their parental strain were divided into three animal experiments (per antifungal compound tested), as a total of 312 NMRI mice (weight between 26.0 g and 30.0 g) were used. Inoculation of mice and treatment scheme as outlined above and dosing as described below. Treatment groups consisted of six mice and control groups of eight mice. As backup, four additional mice were available to replace mice which were inoculated outside the tail vein and therefore excluded; if the backup mice were not needed they were allocated to the control groups (hence some control groups include nine mice).

Echinocandin treatment doses used in murine model: a) the standard dose (x_100) was the dose that resulted in human equivalent exposure (AUC of a 100) and calculated using our echinocandin pharmacokinetic/pharmacodynamic studies in this exact mouse model previously reported (26). The doses were as follow: ANI_100 (2.16 mg/kg), CAS_100 (0.76 mg/kg), and MICA_100 (2.71 mg/kg). The high dose (x_500) was five-time the human equivalent exposure dose and was included in order to examine if a dose escalation would be useful to overcome resistance of *C. albicans*.

For the determination of the fungal burden (CFU/mL tissue homogenate), kidneys were aseptically removed at day 4. Organ weight was determined and kidneys were placed in sterile physiological
saline (750 µl per pair of kidneys) and organs were stored at -80°C prior to CFU count. Fungal burden was determined using the spot technique: plating two 20 µL spots of 10-fold dilutions of tissue homogenate (homogenization performed with a homogenizer (RW 16 Basic, IKA Labortechnik, Bie & Berntsen, Denmark). Colony forming unit count was expressed as \( \log_{10} \) of CFU/mL kidney homogenate. The lower limit of detectable CFU was 25 CFU/mL tissue homogenate. All murine experiments reported in this study were approved by the Danish Animal Experimentation Committee under the Ministry of Justice (number 2009/561-1637).

Statistical analyses

To check for normal distribution of fungal burden data, a normality test was performed using Shapiro-Wilk Normality test. Kruskal-Wallis test was applied, as data were found to be not normally distributed. Outliers (maximum one per control group with a < 100 CFU/mL kidney homogenate) in the control groups (each eight placebo-treated mice) were removed for statistical significance calculation, as these mice did not successfully establish a manifest \( C. \text{ albicans} \) infection or cleared the infection. Outliers were identified by Prism Graph Pad version 5. To guarantee transparency of all data and to provide the readers a complete image, outliers are included in all tables and figures. \( P \) values \( \leq 0.05 \) were regarded as statistically significant in all analyses.

Filamentation ability tests

All studied \( C. \text{ albicans} \) strains were overnight grown on Sabouraud 2% Glucose Agar (Carl-Roth®) at 37°C. Cells were harvested in 0.9 % NaCl. Cell number was adjusted using the haemocytometer Neubauer CE chamber to \( 5 \times 10^5 \) cells/mL. A volume of 500 µL were centrifuged at 13,000 rpm for 5 min in a micro 20 (Hettrich) centrifuge, supernatant was discarded and cell pellet was suspended in 500 µL FBS (GIBCO®, Invitrogen). FBS with cells were transferred into Nikon BioStation IM (Nikon)
cell incubator and monitoring system. Cells were evaluated by microscopy after 0.5 h, 1 h, 2 h, and 3 hours incubation at 37°C in Nikon BioStation IM using Bio Station software Ver. 2.1. In addition, for long time comparison (4 h) of the germ tube formation experiment, movies were captured for SC5314, RRMM2, SCL, and RRCL in four individual experiments per strain. Images were captured all 10 min at 200 times and 400 times magnification.

Chitin content measurement by FACS analyses

All studied C. albicans strains were overnight grown on Sabouraud 2% Glucose at 37°C. Cells were harvested in 0.9 % NaCl. Cell number was adjusted using the haemocytometer Neubauer CE chamber to 5 x 10^6 cells/mL. Cells were inoculated into fresh Sabouraud 2 %. Cells were harvested after 4 h incubation at 37°C shaking at 200 rpm. Samples were fixed in 10% (v/v) neutral buffered formalin for 15 min. Cells were washed with sterile 0.9 % NaCl and stained with 25 µg/mL calcofluor white (Sigma-Aldrich) for 15 min at room temperature. Cells were washed twice with 0.9 % NaCl to remove excessive stain. Cells were filtered using CellTrics filter ® (5 µm pore size, PARTEC) to remove cell clumps. Cells were prepared in standard procedure for FACS analysis. Measurement of 10,000 events was carried out by flow cytometer FACSVerse (BD Biosciences). Results were given as fluorescence intensity (Calcofluor-A). SSWT (SC5314) unstained was used as reference strain for gating cell population. For statistical analyses minimum, median and maximal fluorescence signal was determined. SSWT stained with calcofluor white served as wild type reference. Fluorescence peaks of clinical isolates (SCL and RRCL) were compared to the fluorescence peak of the SSWT population. All FACS experiments were performed in independent triplicates.

Results

In vivo emergence of heterozygous double mutants of C. albicans and CAS medication
Five clinical isolates obtained between 2004 and 2012 from a female patient suffering from chronic mucocutaneous candidiasis (CMC) (case report available in the material and methods section) were tested for their in vitro susceptibility against ANI, CAS, MICA, fluconazole (FLU), isavuconazole, voriconazole (VOR), and amphotericin B. Isolates from 2004 were found FLU- and VOR-resistant according to EUCAST breakpoints, but CAS-susceptible according to CLSI breakpoints, and echinocandin susceptible according to the EUCAST breakpoints (using ANI as a marker for ANI and CAS susceptibility as currently recommended) (22, 27). By contrast, isolates gained in 2012 were micafungin resistant and showed an elevated ANI minimal inhibitory concentration (MIC) when compared to the initial isolate, but not exceeding the EUCAST breakpoint (MIC 0.03 mg/L and ANI breakpoint: S <0.03 mg/L) and were therefore classified as anidulafungin and caspofungin susceptible using the EUCAST breakpoint and anidulafungin as a marker for caspofungin. In contrast, these isolates were classified as caspofungin resistant using Etest and CLSI breakpoints. Detailed data on MICs are given in Table 1.

To investigate the underlying molecular resistance-mechanism, FKS1 HS1 and HS2 were sequenced. Notably, strains isolated in 2004 carried four silent mutations (no AA change, also called synonymous substitution), one within the FKS1 HS1 A1929T (T643) and five outside the FKS1 HS regions located at nucleic acid positions A1641A/T (P547), A1653A/G (T551), T1662T/C (I554), C4125C/T (I1375), and T4230T/C (A1410). In addition, two missense (AA change, also called nonsynonymous mutation) mutations in the same allele (heterozygous) were found at nucleic acid position A1939A/G and C1946C/T leading to residue changes R647R/G and P649P/L, respectively (Table 2). Identical silent mutations found in FKS1 HS1 and HS2 were shared by all isolates, strongly suggesting their clonal origin and in vivo development of resistance. To verify this hypothesis, randomly amplified polymorphic DNA typing (RAPD-typing) was performed with five markers (M13, CA2, OPA18, OPE18, and RSD10). As displayed in Fig. 2, the RAPD genotype was identical for the clinical isolate. Data herein support the development of acquired CAS-resistance in C. albicans during a 6-year treatment period with CAS.
To evaluate the impact of the double mutation on the therapeutic response in vivo, the in vitro echinocandin-resistant clinical isolate carrying the R647R/G and P649P/L mutations (RRCL) and the susceptible wild type clinical isolate (SCL) were compared in a murine haematogenous candidiasis model. ANI, CAS, and MICA were applied at two doses (AUC100 and AUC500). The fungal burden in kidney homogenates of animals infected with the susceptible wild type strain were significantly reduced (Fig. 3). Comparing the control mouse group with each of the treatment groups, the reduction in kidney burden were found to be highly significant in all treated groups (P < 0.01), with the exception of MICA100 for which the reduction was less pronounced, albeit still statistically significant (P< 0.05) (Table S4).

In contrast, the reduction of kidney burdens failed to reach statistical significance (between the different treatment groups and between the placebo with the treatment groups) in mice infected with RRCL (MICs of ANI < 0.03 µg/mL, MICA > 1 µg/mL, CAS > 2 µg/mL). By comparing the median of different treatments with placebo groups, it is obvious that the fungal burden is lowest in ANI500, CAS500, respectively followed by ANI100, CAS100, respectively and placebo control (Table S5). Significant differences of CFUs/g kidney within the control groups of mice infected with either SCL or RRCL were observed, as well as differences between standard and high medications (Table S4). By comparing the median values of SCL (MICs of ANI and MICA ≤ 0.008 µg/mL, CAS of 0.125 µg/mL) and RRCL, a reduction of the latter is obvious even if not statistically significant (Table S5 and Fig. 3). These in vivo data fully support and correlate with in vitro data, as ANI was found to be the most active echinocandin against RRCL in vivo.

Influence of heterozygous single and double, as well as homozygous double mutations on virulence and in vivo treatment response

To evaluate the influence of both mutations individually and in combination, in vitro and in vivo, isogenic mutants (identical genetic background) were generated (Table S1). An overview of the in
vitrō susceptibility results of parental strain and isogenic mutants are given in Table 1. Parental
strain, as well as SRMH1 (heterozygous P649P/L) were classified as in vitrō susceptible against all
echinocandins. RS MH1 (heterozygous R647R/G) was classified as resistant to MICA only, since it was
only one dilution step above the breakpoint. Strain RR MH1 (heterozygous double mutation; P649P/L
and R647R/G) and RR MH2 (homozygous double mutation; P649L and R647G) were classified as in
vitrō pan-echinocandin resistant. Notably, ANI, as well as MICA MICs were only one dilution above
the respective breakpoints for the heterozygous double mutant RR MH2. But the homozygous double
mutant RR MH2 had a MICA MIC six dilutions above the breakpoint. Thus, a stepwise increase in MICs
and development from single echinocandin to multiple echinocandin resistance was observed
comparing single to double codon alterations and hetero- to homozygous mutations, respectively.
Comparing the clinical heterozygous double mutant RR CL with the heterozygous isogenetic mutant
RR MH2, it is obvious that these two mutations apparently affect ANI susceptibility to a much lesser
extent than they affect MICA and CAS. MICs for MICA and CAS appeared several times higher.
However, the caspofungin testing was performed using the commercial Etest and therefore may not
be directly comparable (28) and interpretation of MICA susceptibility data should be done with
cautions, as both isolates showed a trailing phenotype, the implication of which is not elucidated for
the echinocandins (Table 1).

To evaluate the differences in in vitrō response towards the different echinocandins in vivō, a murine
model was again used (Fig.4). A statistical significant reduction of fungal burdens in kidneys was
observed for ANI, CAS, and MICA (AUC100 and AUC500) and mutant strains when compared to placebo-
treated controls. The median of fungal burdens per treatment arm and placebo group displays the
parental (SS WT ) and the two strains carrying a heterozygous single mutation to best respond in vivō
(Table S6). This is in agreement with in vitrō data, as all strains were categorized as ANI-susceptible
(Table 1). Dose escalation markedly reduced the fungal burden of the heterozygous double mutant
RR MH2, whereas the homozygous double mutant RR MH2 reduction was less prominent (Table S6). All
CAS-treated groups showed statistically significant lower fungal burdens than mice in the placebo
control groups. By comparing the median of fungal burden, the treatment efficacy against the heterozygous double mutant RR_{MH2} was limited at the standard dose, but improved by dose escalation (Table S6). Whilst *in vitro* resistance to CAS was confirmed *in vivo* for the RR_{MH2}, resistance of RR_{MHO2} was not observed *in vivo*. Based on their fungal burdens, SS_{WT}, RS_{MH1}, and SR_{MH1} were found CAS-susceptible both *in vivo* and *in vitro* (Table 1 and Fig.4).

Micafungin-treated groups displayed statistically significant lower fungal burdens when compared to placebo-treated mice (P < 0.05; see Fig.4). The two double mutants RR_{MH2} and RR_{MHO2} showed only limited responses against MICA_{ldo} treatment. In contrast to RR_{MHO2}, RR_{MH2} responded to dose escalation. Both strains were resistant towards MICA *in vitro*. For all other strains, standard dosing was sufficient to clear infections (Fig. 4), even though RS_{MH1} was found MICA-resistant according to EUCAST breakpoints (Table 1). However, we would like to mention that the clinical isolates and the mutants constructed in the laboratory represent different background strains and therefore comparisons have to be made with caution.

Clinical isolates (S_{CL} and RR_{CL}) were found to differ in virulence in terms of kidney CFU count and treatment responses (Fig.3). On the contrary, isogenic mutants that carry exclusively FKS1 HS1 mutations did not vary in their virulence (Fig.4). The major difference between RR_{MH2} and RR_{CL} was that RR_{MH2} responded to doses escalation of ANI, CAS, MICA, whereas RR_{CL} did not. Thus, a clear trend between standard and escalated dosing was found for RR_{MH2} and RR_{MHO2} and ANI and MICA (Table S5 and Fig.4). In contrast to the clinical isolates, no virulence differences were observed comparing the parental strain and the isogenic mutants (Table S6, Fig.4).

Fluorescence-activated cell sorting (FACS) analyses on the chitin content of clinical isolates (S_{CL} and RR_{CL}), isogenic mutants (SR_{MH2}, RS_{MHO2}, RR_{MHO2}, and RR_{MH2}) and SS_{WT} (used as reference) resulted in highly similar fluorescence signals for all cell populations (Fig.S1 and Fig.S2). *In vitro* germination tests in fetal bovine serum showed that both clinical isolates S_{CL} and RR_{CL} showed decreased filamentation efficacy. After 1h at 37°C, about 60% of all cells formed germ tubes, while all isogenic mutants (RS_{MH1},...
SRMH1, RRMH2, RRMHO2) behaved like the parental wild type strain SC5314 (after 1h at 37°C 100% of all cells formed germ tubes) (Fig. 5). Decreased filamentation ability was most pronounced in RRCL.

For the first time it was demonstrated that these SNPs in FKS1 HS1, do not necessarily have an impact on (a) the chitin content, (b) the filamentation ability, and (c) the virulence of C. albicans strains, when only these SNPs are integrated into a wild type strain.
Discussion

In the current study, a female patient suffering from CMC due to MICA- and CAS-resistant *C. albicans* (Table 1) failed to be cured by long-term CAS medication, but was cured by ANI. To determine whether strain RR_{CL} evolved from S_{CL} or replaced S_{CL} under selection pressure of CAS, RAPD genotyping of the clinical isolates was performed. The efficacy of RAPD as typing method for clinical *C. albicans* isolates was previously demonstrated (17, 18). RAPD revealed a clonal relationship between the isolates (Fig.2). Clonal relationship was additionally supported by the presence of six identical synonymous substitutions, one of those located in the FKS1 HS1 (Table 2). Based on this data, we conclude that echinocandin resistance of *C. albicans* emerged in the patient.

Molecular analyses demonstrated that RR_{CL} carried amino acid substitutions R647R/G and P649P/L in FKS1 HS1 in the same allele (Table 2), the occurrence of such a double amino acid substitution in FKS1 HS1 is new and their impact on *in vitro* and *in vivo* echinocandin susceptibility is demonstrated for the first time.

Recently, it was shown that isolated heterozygous (29) and homozygous (23) point mutations at the amino acid position P649 refer to echinocandin resistance. In contrast, to the isolates studied by Desnos-Olivier (29) and Garcia-Effron (23), our clinical isolate carries a heterozygous P649P/L substitution which is accompanied by a second amino acid substitution (R647R/G). Also, the isolated homozygous amino acid substitution R647G was published previously by Dannaoui et al. (30). This mutation was found to only influence MICs of CAS and MICA, but not ANI. In general, mutations at position P649 and P647 in *C. albicans* were associated with discrete elevations of echinocandin MICs when compared with FKS1 HS1 alterations involving codons S645 and F641 (22). In our study, only the simultaneous presence of both mutations leads to high level MICs for CAS and MICA (Table 1). Infections caused by isolates carrying either an FKS1 mutation at position S645 or F641 could not be cleared by standard doses (10 mg/kg) of MICA and CAS (31, 32). This is similar to our data, as strain RR_{CL} was demonstrated to be *in vivo* resistant in a haematogenous murine model against all
echinocandins at standard dose (Fig.3). But, CMC was cured successfully with ANI in our case study. This discrepancy may be explained by various factors. First, the haematogenous infection in the animal model may be more difficult to cure than the less severe superficial infection in this patient. Second, the lower virulence and growth rate of the clinical isolate may lead to a slower response to treatment as a certain number of multiplication rounds are needed for activity. Thus, efficacy could possibly have been obtained, if the treatment duration had been extended beyond the standard three days in the mouse model. Moreover, when comparing the *in vitro* susceptibility of RRCL with the corresponding isogenic mutant strain RR_MH2 it is obvious, that ANI susceptibility is less influenced than that for CAS and MICA. MICs of the clinical isolate are remarkably higher for CAS and MICA. This finding correlated with the clinical response as the patient failed caspofungin, but not anidulafungin treatment. Since strain RR_MH2 displays pronounced trailing, when tested according to EUCAST, micafungin MIC data interpretation may be affected however.

A fundamental finding was that the clinical isolate RRCL and the corresponding RR_MH2 differ in their *in vivo* response, in particular at dose escalation (AUC500) (Fig.3 and Fig.4). The efficacy in mice challenged with RRCL was not improved with high doses of ANI, MICA and CAS, in contrast to what was observed for mice challenged with the isogenic mutant RR_MH2. Such differences may be explained by the different genetic background of the clinical and the laboratory derived mutant strains or by additional molecular mechanisms that were acquired by RRCL during long-term CAS exposure. Such additional adaptations associated with echinocandin resistance in *C. albicans* have been published previously; among them are: the overexpression of HSP90 (33), the overexpression of Cdr2p ATP-binding cassette (ABC) transporter (34), and elevated chitin levels in fungal cell wall (35). Beside the FKS1 HS1 mutations, additional simultaneously operating mechanisms might contribute to the altered echinocandin resistance of the clinical *C. albicans* strain RRCL. To fully address the question of alternative mechanisms that enhance echinocandin resistance a whole genome comparison and RNA sequencing will be performed on the clinical isolates SCL and RRCL.
Interestingly, others have reported that some FKS1 mutations are associated with a reduced virulence and fitness in clinical and laboratory-derived mutant strains (35-37). In the current study, fungal loads of tissue homogenates of mice infected with either the parental strain SC5314 or isogenic mutant strains were identical, suggesting that mutations do not affect virulence or fitness in vivo. Notably, the clinical isolates SC5 and RRSC show a reduced filamentation in vitro, in contrast to the parental strain SC5314 and the derived isogenic mutants (Fig.5 and Fig.1). Ben-Ami et al. (36) explained loss of fitness in homozygous FKS1 HS mutants (F641S, S645F, and S645P) with a reduced maximum catalytic capacity of glucan synthase complex and increased cell wall chitin that comes along with thickened cell walls hampered by filamentation capacities (37). Isogenic mutants investigated herein (carrying either R647R/G and/or P649P/L or R649G and P649L in FKS1 HS1) lacked any change of cell wall chitin (Fig.S1). Therefore, mutations at positions R647R/G and/or P649P/L seem not to influence filamentation of C. albicans adding to the previous observations that various resistance mutations may or may not influence virulence and fitness (38). The impact of the position of the amino acid substitution in the FKS1 gene on the fitness of C. albicans needs to be further investigated using competition experiments with bar-coded strains. Moreover, increased or decreased fitness of organisms may depend on the hosts’ immune status, as significant differences between immunocompetent and neutropenic mice were found (37).

Slater et al. (31) reported that infections due to homozygous FKS S645 mutants were not cleared with MICA at neither standard nor elevated doses (AUC200). The finding of Slater et al. might be explained by the fact that S645 is the most dominant resistance mutation described yet and in comparison to our study dose was escalated only four times. In our study, infections with RRMMO2 failed treatment with MICA100, ANI100, CAS100, MICA500 and ANI500 (Fig.4), even though a doses response was observed as CFU was decrease for escalated doses (AUC500). Compared with the study of Slater et al. our strains carry weaker mutations and therefore show partial dose-depended response. But, only CAS500 was able to clear the infection with RRMMO2 below the detection level. Hence, dose escalation may not be sufficient to fully overcome infections with double mutant strains.
Translated into clinical practice, our data suggest that the EUCAST MICs of echinocandins interpreted by the associated EUCAST breakpoints correlate with in vivo response at standard dosing regimen, but cannot predict in vivo response to dose escalation according to the murine model applied. Of note is that EUCAST and CLSI have abstained from setting caspofungin epidemiological cut-off values for Candida species, because of unacceptable high variation in MIC ranges obtained over time and between centers (27). Also commercial systems such as Etest do not overcome these variation problems (28). Therefore it is currently not recommended using caspofungin susceptibility testing in routine laboratories; instead anidulafungin and micafungin should be tested and reported as echinocandin markers (27). Nevertheless, caspofungin susceptibility testing performed for research purposes to evaluate the degree of MIC raise. The presence of simultaneous mutation within the FKS1, as well as history of long-term echinocandin therapy and the occurrence of homozygous FKS1 mutations should be treated with caution in terms of an ongoing treatment with ANI, CAS or MICA. The presence of FKS1 HS mutation in a clinical isolate does not necessarily imply that the isolate is less virulent than a wild type C. albicans strain. C. albicans strains carrying either a heterozygous/homozygous double mutation in one allele or homozygous single mutations are more likely to be therapy refractory in vivo not only at standard doses, but also at dose escalation.

In conclusion, adoption of EUCAST breakpoints for the clinical strains resulted in a classification that correlated with the clinical response in the patient. In the animal model, the clinical strains with heterozygous double mutations within the FKS1 and laboratory generated strain with homozygous double mutation failed to respond to dose escalation. C. albicans carrying heterozygous double mutations have higher in vitro MIC for echinocandins than parental and heterozygous single mutation strains. Neither of heterozygous single mutations (R647R/G or P649P/L) nor the heterozygous double mutation (R647R/G and P649P/L) nor the homozygous double mutation (R647G and P649L) in the FKS1 HS1 had an impact (1) on the virulence of isogenic C. albicans in respect to CFU/mL kidney homogenates, (2) on the filamentation capacity, and (3) on chitin content. Therefore, any loss of fitness and/or virulence cannot be provided based on the presence of FKS1 HS mutations.
but probably depends on the nature and position of the specific alteration. The various FKS1 HS mutations differ in MIC elevations, in their response to ANI, CAS, and MICA, and in their impact on virulence.
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23


FIG 1 Presentation of the clinical features of the female 27 year-old patient.

A At the right forehead and the lips erythematous plaques topped with crusts and pustules are visible. B Paronychion and destruction of the nail by C. albicans. C Periodic acid-Schiff staining showing parakeratosis with multiple hyphae (arrow) present in the cornified layers.

FIG 2 Genotyping of clinical isolates of Candida albicans isolated between 2004 and 2012 using RAPD.

PCR products for the five markers M13 (A), CA2 (B), OPA18 (C), OPE18 (D), and RSD 10 (E) (17-20) were separated on 1.8 % agar-gel for a run time of 3 h at 80 V and 100 mA. Electrophoresis gels were stained with ethidium bromide. In columns: marker (0), isolate 952/04 (1), isolate 1130/04 (2), isolate 5104/04 (3), isolate 110/12 (4), and isolate 111/12 (5).

FIG 3 Evaluation of in vivo susceptibility of the two clinical strains 1130.04 (Scl; no mutation in FKS1) and 110.12 (RRcl; heterozygous double mutation in FKS1 at position R647R/G and P649P/L) and high (x₅₀) or standard doses (x₁₀₀) of anidulafungin (ANI), caspofungin (CAS), or micafungin (MICA), respectively. Treatment groups consisted of six mice and control groups of eight mice.

Infected murine treated with placebo control (C) served as control. Colony forming units (CFU) per mL kidney homogenate is depicted after the end of treatment. Limit of detection (LoD) and median with interquartile range is indicated. Statistical significance was calculated using Kruskal-Wallis test and Dunnett’s Multiple Comparison test columns marked with * P < 0.5, ** P < 0.01, and *** P < 0.001; outliers (maximum one outlier per control group) were removed for calculation only (CFU ≤ 100 in the placebo-treated groups).

FIG 4
In vivo susceptibility of isogeneic mutants derived from parental strain (SSCL).

Parental strain and the four isogeneic mutant strains (RR MH2, RS MH1, SR MH1, and RR MHO2) were evaluated for high (x_{50}) and standard (x_{100}) doses of A anidulafungin (ANI), B caspofungin (CAS), and C micafungin (MICA) and compared to placebo control (C). Treatment groups consisted of six mice and control groups of eight mice.

CFU counts per mL kidney homogenate is depicted after the end of treatment. Limit of detection (LoD) and median with interquartile range are indicated in each graph. Statistic is based on Kruskal-Wallis test and Dunnett’s Multiple Comparison tests, statistical significances are given as * is P < 0.5, ** is P < 0.01, and *** P < 0.001; outliers (maximum one per control group) were removed for calculation only (CFU ≤ 100 in the placebo-treated groups).

Strains carry mutations in their FKS gene as follows: RR MH2 heterozygous double mutation (R647R/G and P649P/L), RS MH1 heterozygous single mutation (R647R/G), SR MH1 (P649P/L), and RR MHO2 homozygous double mutation (R647G and P649L).

FIG 5 Filamentation ability of the clinical strains A of SCL and B RR CL and the corresponding laboratory strains C SS WT and D RR MH2 in fetal bovine serum after 60 min at 37°C. Delayed germination of approximately 40% of all Candida albicans cells is observed in A and B, but 100% of all cells are germinated in C and D.
FIG 1 Presentation of the clinical features of the female 27 year-old patient. 
A At the right forehead and the lips erythematous plaques topped with crusts and pustules are visible. B Paronychion and destruction of the nail by *C. albicans*. C Periodic acid-Schiff staining showing parakeratosis with multiple hyphae (arrow) present in the cornified layers.
FIG 2 Genotyping of clinical isolates of *Candida albicans* isolated between 2004 and 2012 using RAPD. PCR products for the five markers M13 (A), CA2 (B), OPA18 (C), OPE18 (D), and RSD 10 (E) (17-20) were separated on 1.8 % agar-gel for a run time of 3 h at 80 V and 100 mA. Electrophoresis gels were stained with ethidium bromide. In columns: marker (0), isolate 952/04 (1), isolate 1130/04 (2), isolate 5104/04 (3), isolate 110/12 (4), and isolate 111/12 (5).
FIG 3 Evaluation of in vivo susceptibility of the two clinical strains 1130.04 (SΔ; no mutation in FKS1) and 110.12 (RRΔ; heterozygous double mutation in FKS1 at position R647R/G and P649P/L) and high (x500) or standard doses (x100) of anidulafungin (ANI), caspofungin (CAS), or micafungin (MICA), respectively. Treatment groups consisted of six mice and control groups of eight mice. Infected murine treated with placebo control (C) served as control. Colony forming units (CFU) per mL kidney homogenate is depicted after the end of treatment. Limit of detection (LoD) and median with interquartile range is indicated. Statistical significance was calculated using Kruskal-Wallis test and Dunnett’s Multiple Comparison test columns marked with * P < 0.5, ** P < 0.01, and *** P < 0.001; outliers (maximum one outlier per control group) were removed for calculation only (CFU ≤ 100 in the placebo-treated groups).
Parental strain and the four isogenetic mutant strains (RRMHO2, RSMH1, SRMH1, and RRMHO2) were evaluated for high (x500) and standard (x100) doses of A anidulafungin (ANI), B caspofungin (CAS), and C micafungin (MICA) and compared to placebo control (C). Treatment groups consisted of six mice and control groups of eight mice.

CFU counts per mL kidney homogenate is depicted after the end of treatment. Limit of detection (LoD) and median with interquartile range are indicated in each graph. Statistic is based on Kruskal-Wallis test and Dunnett’s Multiple Comparison tests, statistical significances are given as * is P < 0.5, ** is P < 0.01, and *** P < 0.001; outliers (maximum one per control group) were removed for calculation only (CFU ≤ 100 in the placebo-treated groups).

Strains carry mutations in their FKS gene as follows: RRMHO2 heterozygous double mutation (R647R/G and P649P/L), RSMH1 heterozygous single mutation (R647R/G), SRMH1 (P649P/L), and RRMHO2 homozygous double mutation (R647G and P649L).
FIG 5 Filamentation ability of the clinical strains A of $S_{CL}$ and B $R_{CL}$ and the corresponding laboratory strains C $S_{WT}$ and D $R_{RH}$ in fetal bovine serum after 60 min at 37°C. Delayed germination of approximately 40 % of all Candida albicans cells is observed in A and B, but 100 % of all cells are germinated in C and D.
TABLE 1

*In vitro* susceptibility (MIC, mg/L) for the clinical and mutant isolates included in the study and of quality control strains (grey) determined by EUCAST or Etest®, respectively.

Susceptibility classification (S, I, R) was performed according to the EUCAST break points (BP) except for CAS and Etest® where CLSI BP were adopted as recommended by the manufacturer.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>EUCAST MIC</th>
<th>Etest® MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANI</td>
<td>MICA</td>
</tr>
<tr>
<td>Clinical isolates and QC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scl 1130.04</td>
<td>&lt;0.008 (S)</td>
<td>&lt;0.008 (S)</td>
</tr>
<tr>
<td>RRsc 110.1</td>
<td>0.03 (S)</td>
<td>&gt;1 (R)</td>
</tr>
<tr>
<td>ATCC 22019</td>
<td>0.125 (I)</td>
<td>&gt;1 (I)</td>
</tr>
<tr>
<td>Mutant isolates and QC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSsc SC5314</td>
<td>0.015 (S)</td>
<td>&lt;0.008 (S)</td>
</tr>
<tr>
<td>RRmsc2</td>
<td>0.06 (R)</td>
<td>0.03 (R)</td>
</tr>
<tr>
<td>RSmsc2</td>
<td>0.015 (S)</td>
<td>0.03 (R)</td>
</tr>
<tr>
<td>SRmsc2</td>
<td>0.015 (S)</td>
<td>0.008 (S)</td>
</tr>
<tr>
<td>RRmsc2</td>
<td>0.06 (R)</td>
<td>1 (R)</td>
</tr>
<tr>
<td>ATCC 6258</td>
<td>0.06 (S)</td>
<td>0.125</td>
</tr>
</tbody>
</table>

ANI anidulafungin, MICA micafungin, VOR voriconazole, ISA isavuconazole, FLU fluconazole, CAS caspofungin, AMB amphotericin B. (S) susceptible, (I) intermediate, (R) resistant

a the clinical isolates 952.04 and 5104.04 revealed the same MIC values within ± one dilution step difference, these strain do not carry a coding FKS1 HS1 and HS2 mutation

b the clinical isolate 111.12 revealed the same MIC value within ± one dilution step difference, these strains carry the mutations R647R/G and P649P/L

c SC5314 is the wild type parental strain, mutations carried by the strains were as followed RRmsc2 (R647R/G and P649P/L), RSmsc2 (R647R/G), SRmsc2 (P649P/L), and RRmsc2 (R647G and P649L)
d Micafungin trailing 0.03-1 but stays below the 50% endpoint in this range

e Micafungin creeping increase 0.03-0.25

f Micafungin trailing 0.06-0.25 and just above the 50% endpoint in this range
TABLE 2

Overview on FKS mutation found in clinical isolates gained between 2004 (x.04) and 2012 (x.012) from the index/case patient.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>FKS1 hot spot 1 mutation*</th>
<th>FKS1 hot spot 2 mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1641 (A) 1653 (A) 1662 (T) 1929 (A) 1939 (A) 1946 (C)</td>
<td>4215 (C ) 4230 (T)</td>
</tr>
<tr>
<td>S. 1130.04</td>
<td>A/T A/G T/C T A C</td>
<td>C/T T/C</td>
</tr>
<tr>
<td>952.04</td>
<td>A/T A/G T/C T A C</td>
<td>C/T T/C</td>
</tr>
<tr>
<td>5104.04</td>
<td>A/T A/G T/C T A C</td>
<td>C/T T/C</td>
</tr>
<tr>
<td>RR 110.12</td>
<td>A/T A/G T/C T A/G C/T C/T T/C</td>
<td></td>
</tr>
<tr>
<td>111.12</td>
<td>A/T A/G T/C T A/G C/T C/T T/C</td>
<td></td>
</tr>
</tbody>
</table>

nucleotide acid mutation: header indicates position (wild type nucleic acid)

a amino acid change: nucleic acid change does not result in a change of amino acid (not in bold); wild type amino acid/position/heterozygous mutation (in bold)

b amino acid change arginine (R) to glycine (G); codon change AGA to GGA

c amino acid change proline (P) to leucine (L); codon change CCU to CUU