Increase of virulence and its phenotypic traits in drug-resistant strains of *Candida albicans*

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ABSTRACT

There is concern about the rise of antifungal drug resistance but little is known about comparative biological properties and pathogenicity of drug-resistant strains. We generated fluconazole (FLC; CO23RFCLC) or micafungin (FK; CO23RFK)-resistant strains of Candida albicans by treating a FLC- and FK-susceptible strain of this fungus (CO23S) with stepwise increasing concentrations of either drug. Molecular analyses showed that CO23RFCLC had acquired markedly increased expression of the drug-resistance MDR1 efflux pump MDR1 gene, whereas CO23RFK had a homozygous mutation in the FSK1 gene. These genetic modifications did not alter to any extent the growth capacity of the drug-resistant strains, either at 28°C or at 37°C in vitro, but markedly increased their experimental pathogenicity in a systemic mouse infection model, as assessed by the overall mortality and target organ invasion. Interestingly, no apparent increase in the vaginopathic potential of the strains was observed in a oestrogen-dependent rat vaginal infection. The increased pathogenicity of drug-resistant strains for systemic infection was associated with a number of biochemical and physiological changes, inclusive of i) marked cellular alterations associated with a different expression and content of major cell wall polysaccharides; ii) more rapid and extensive hyphae formation both in liquid and solid media; iii) increased adherence to plastic and propensity for biofilm formation. Overall, our data demonstrate that experimentally-induced resistance to antifungal drugs, irrespective of drug family, can substantially divert C. albicans biology, particularly affecting biological properties of potential relevance for deep-seated candidiasis.

Key words: Candida albicans, Micafungin, Fluconazole, resistance, virulence.
INTRODUCTION

*Candida albicans*, a dimorphic opportunistic human pathogen, is the most prominent cause of oro-pharyngeal, vaginal and invasive candidiasis in man (3). In particular, oro-pharyngeal infections are very common in human immunodeficiency virus (HIV)-infected individuals and patients with AIDS, while deep-seated infections are frequent in neutropenic patients (27, 31). The incidence of candidiasis has dramatically increased in the last decades and bloodstream infections due to *Candida* spp. are becoming a prime cause of morbidity and mortality in different types of immunocompromised patients (33). Host immunosuppression and possession by *C. albicans* of defined virulence traits makes it an ideal mixture to favour the emergence of this fungus as important agent of disease in humans.

The azoles, particularly fluconazole, remain among the most common antifungal drugs but their intensive clinical use for both therapy and prophylaxis has favoured the emergence of resistant strains (42). The threat of increasing resistance to azole drugs, associated with the relative scarcity of antifungal drugs prompted the development of new drugs such as the echinocandins (e. g. micafungin). These cytocidal drugs inhibit cell wall synthesis through the inhibition of β-1,3 glucan synthase and have rapidly become an important therapeutic option in several fungal infections (25).

Most of the biological functions related to pathogenicity and virulence of *C. albicans* reside in the fungal cell wall, which, as the rigid external organelle of the fungus, is critical for fungal morphogenesis and host–fungus interplay (8,12). Among the numerous factors associated with virulence in *C. albicans*, hyphal morphogenesis is likely to be one of the most important (22). Hyphae development from yeast cells is critical for adherence, an essential first step in microbial colonization, which is in turn a key event in the initiation of the pathogenic process (5,41). Adherence may involve both glycosylated and non-glycosylated cell wall proteins acting as adhesins (21, 41). In response to attachment to a
surface, fungal cells produce biofilm, three-dimensional structures made up of cells surrounded by exopolymeric, mostly polysaccharide matrices that contribute to the infectious process and antibiotic resistance (18,23).

Overall, antifungal drug resistance (ADR) and fungal virulence are critical issues for host-parasite relationship in candidiasis. However, very little is known about any interrelation between drug resistance and virulence of *C. albicans*. Thus, we investigated whether acquisition of resistance to fluconazole or micafungin was reflected into measurable effects on pathogenicity by the fungus. To this purpose, we generated two strains resistant to fluconazole or micafungin (strains CO23RFLC and CO23RFK, respectively) from a vaginal clinical isolate of *C. albicans* (CO23s) which was fully susceptible to azoles and echinocandins (1). These three strains were compared for expression and/or search for mutations of drug resistance-related genes, and for morphological and ultrastructural characteristics inclusive of putative virulence traits in vitro and in vivo.
MATERIALS AND METHODS

Yeast strains and growth conditions.

The strain CO23S of *C. albicans* was isolated from a subject with vulvo-vaginal candidiasis, and was originally susceptible to micafungin and fluconazole. It was made resistant to FK463 or to FLC by ten growth passages in stepwise-increasing concentrations (0.01 to 8 µg/ml or 0.32 to 128 µg/ml of the respective drug) in agar solidified Yeast Nitrogen Base (YNB) medium, at 28°C. Resistance phenotype has maintained stable after multiple (fifty) passages in culture. Strains CO23S, CO23RFLC and CO23RFK had identical electrophoretic karyotypes, as determined by pulse-field gel electrophoresis, performed as reported in previous studies (1).

For the determination of growth curves, the three strains were incubated at 37°C or 28°C, for 48 hours, in 200 ml flasks containing liquid YNB medium, under slight agitation, using an initial inoculum density of 0.1 (as measured spectrophotometrically, 560 nm). Growth was assessed by harvesting 1 ml of the culture at 2, 4, 6, 8, 24 and 48 hours, and measuring its OD.

Electron Microscopy (EM)

For scanning EM, *C. albicans* cells were grown in glucose supplemented YNB medium (see above) at 28°C for 24 hours. After washing twice in calcium and magnesium-free phosphate-buffered saline (PBS), the cellular pellets resulting from centrifugation were fixed for 20 min at room temperature with 2.5% (v/v) glutaraldehyde in 0.01 M cacodylate buffer (pH 7.4) containing 2% (w/v) sucrose. After 3 washes in the same buffer, the cells were post-fixed with 1% (w/v) OsO4 for 1 hour, dehydrated on an ethanol gradient, critical point dried in CO2 and gold coated by sputtering. The samples were examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments,
For a quantitative analysis of cell volume distribution on SEM images, both the major (a) and the minor (b=c) axes of randomly chosen 100 yeast cells of each of the three strains, were measured. Cell volume was calculated by assuming that the yeast cells have an ellipsoid shape, using the formula $\frac{4}{3}\pi(a \cdot b \cdot c)$. The values shown on the histograms are means ± SD.

For transmission EM, cells were prefixed with glutaraldehyde, as above, and then post-fixed with the OsO$_4$ solution overnight, at 4°C. The cells were then dehydrated in acetone gradient and embedded in epoxy resin (Agar 100 resin, Agar Scientific Ltd, Stansted, UK) as per routine procedures. Ultrathin sections, obtained with a LKB Ultrotome Nova, were stained with uranyl acetate and lead citrate, and examined with Philips 208 transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

**Determination of dry weight and polysaccharides content**

For dry weight determination, cells grown for 24 h at 30°C with shaking. Each culture (50 ml) was filtered through 0.45 µm membrane filters (Millipore). Filters were washed with 50 ml of distilled water, dried at 80°C for 12 h and the dry weight was calculated for each strain. Each assay was performed in triplicate. For determination of alkali-acid soluble and insoluble cell wall components, the strains CO23s, CO23RFK and CO23RFLC were grown in YPD at 28°C for 24h. The cells were hydrolysed as described by Flett and Manners (18,19). An aliquot of the cells were treated with 0.5 M acetic acid at 60°C for 3h, centrifuged and the resultant pellet treated with 0.5 M NaOH, at 90°C for 6h. The insoluble residue was treated with 20 Units/ml of a purified β 1-3 glucanase (Zymoliase 100T) overnight at 37°C. Carbohydrates were assayed by the method of Dubois (16).
Monoclonal antibodies (mAbs)

mAb AF1 was produced in mice immunized with a crude mannoprotein preparation (GMP) from yeast cells of *C. albicans* (6). The mAb was purified from mouse ascitic fluids by affinity chromatography on Affiprep-proteinA column equilibrated with MAPS II buffer (Bio-Rad, Richmond, CA/USA). Titer of the purified mAb preparation was assessed by indirect enzyme-linked immunosorbent assays (ELISA), employing GMP as the solid-phase antigens.

Immunoelectron microscopy

For AF1 epitope localization in the post-embedding procedure, thin section, obtained as described above and collected on gold grids were treated for 3 min with 0.5 mg of sodium borohydride per ml of ice-cold distilled water. After washing in ice-cold distilled water (3 times, 5 min) and in phosphate-buffered saline (PBS) containing 0.5 % (wt/vol) bovine serum albumin (BSA), 0.05 % Tween 20, and 5% foetal serum (3 times, 5 min), the sections were incubated with mAb AF1 (diluted 1:10) overnight at 4°C. After washing for 2h at room temperature, by floating the grids on PBS drops, samples were labelled with rabbit anti-mouse IgM gold conjugate (1:10 diluted; Sigma) and then washed in PBS buffer for 3h at room temperature. For negative control, the sections were incubated with the irrelevant IgG2a monoclonal antibody or with a goat anti mouse IgG-gold alone.

Quantitative real-time reverse transcription (RT)-PCR

Quantitative expression of the *CDR1* (31), *CDR2* (37), *MDR1* (31), *ERG11* (31) and *FLU1* (4) genes was performed by real-time RT-PCR with an i-Cycler iQ™ system (Bio-Rad Laboratories, Hercules, CA), using total RNAs extracted from the exponential-phase cultured *C. albicans* strains as described previously (39). Primer pairs and Taqman probes
for the target and reference *TEF3* genes (37), were designed using Beacon Designer 3 v. 3.00 software (Premier Biosoft International, Palo Alto, CA) and synthesized by MWG Biotech (Florence, Italy) (Table 2). RT-PCRs were carried out using reagents and conditions as those reported elsewhere (39). Each reaction was run in quadruplicate. In each sample, relative mRNA expression levels of the target genes were normalized for input RNA against the level of *TEF3* gene transcripts and calculated using the comparative cycle time (*Ct*) method (29).

**PCR amplification and DNA sequencing**

Purified genomic DNA was obtained from each *C. albicans* strain using the EZ1 DNA Tissue kit (Qiagen, Milan, Italy) and the BioRobot EZ1 workstation (Qiagen) in accordance with the manufacturer’s instructions. The entire *FSK1* and *ERG11* open reading frames were PCR amplified using primers and reaction conditions described previously (2, 31). PCR products were purified with the Minielute PCR purification kit (Qiagen) and sequenced on both strands with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using primers and cycling conditions already reported (2, 31).

**Experimental Infections**

1. **Mouse systemic model.** *C. albicans* strains were grown to stationary phase in YNB medium at 28°C. Cells were harvested by centrifugation, washed twice in calcium and magnesium-free phosphate-buffered saline (PBS), and suspended to a density of 5x10^7 cells/ml enumerated by haemocytometer counts. Subsequently, group of ten male Balb/C mice (18 to 20 g each; Charles River Laboratories, Calco, Italy) were injected intravenously with 0.2 ml (1x10^7 cells) of a suspension of each strain. Mice were observed
daily for signs of morbidity and mortality for a period of 30 days. All dead animals were
autopsied and internal organ invasion by the fungus was assessed. In a separate experiment,
the fungus burden in the left kidney, taken as representative target organ, was also assessed.
Differences in median survival time (MST, in days) and in the ratio between dead over
total challenged animals (D/T) were statistically assessed by non-parametric Mann-
Whitney test (for MST) and Fisher’s exact test (for D/T). Differences in survival curves
were assessed by the Log-rank test.

2. Rat vaginal model. A rat vaginal model was used for the experimental vaginal infection,
as previously described (14). Two independent experiments with each fungal strain were
conducted and in each experiment groups of five rats were used. Oophorectomized female
Wistar rats (80–100 g; Charles River Calco, Italy) were injected subcutaneously with
estradiol benzoate 0.5 mg ( Estradiolo, Amsa Farmaceutici srl, Rome, Italy). Six days after
the first estradiol dose, all animals were inoculated intravaginally with $10^7$ yeast cells of
each *C. albicans* strain tested in 0.1 mL of saline. The inoculum of each strain used for
challenge was dispensed into the vaginal cavity through a syringe equipped with a
multipurpose calibrated tip (Combitip; PBI, Milan, Italy). The strains had been grown
previously in YPD broth (yeast extract 1%, peptone 2%, dextrose 2%) at 28°C on a gyrator
shaker (200 rpm), harvested by centrifugation (1500 g), washed, counted in a
haemocytometer, and suspended to the required number in saline solution. The number of
cells in the vaginal fluid was counted by culturing 1 µL samples (using a calibrated plastic
loop, Disponoic; PBI) taken from each animal, on SDA containing chloramphenicol (50
mg/L) as previously described. The kinetics of *Candida* vaginal infection were monitored
by the number of cfu/mL of vaginal lavage fluid. The infection was monitored for at least
21 days after the challenge, with vaginal fluid sampling usually being made at 1, 24 and 48
hr, then on days 5, 7, 14 and 21. The animal experimentation referred to in this paper was approved by the ad hoc committee of the Istituto Superiore di Sanità, Rome, Italy.

**Mycelial development and agar-invasive hyphal growth**

The cells were grown in YNB supplemented with 0.1% (w/v) N-acetyl-D-glucosamine (GlcNac; Sigma), or in RPMI 1640 medium (Sigma Chem.Co, Detroit,MI) added with 10% foetal calf serum), and incubated for 24 hours at 37°C.

Hyphal growth was induced on 4% bovine calf serum containing various concentrations (0.5 to 3%) of bacteriological agar as indicated. Cells grown overnight at 28°C were diluted to 5x10^6 in YPD, and 2 µl (10^4 cells) were spotted on the surface of the serum agar and incubated at 37°C for 7 days. Plates were monitored daily for invasive growth.

**Adherence assay and biofilm formation**

Cells were grown for 24 hrs at 28°C in YPD broth, washed twice with sterile phosphate buffered saline (PBS; 10 mM phosphate buffer, 2,7 mM potassium chloride, 137 mM sodium chloride pH 7,4, and resuspended in RPMI1640 supplemented with morpholineproanesulfonic acid (MOPS) at 1.5x10^3 cells/ml. After incubation for 3 hrs at 37°C in 6-wells polystyrene plates (Corning Incorporated, Corning, NY) followed by extensive washing, 1 ml of Sabouraud dextrose agar was poured in each well and allowed to solidify. After incubation at 37°C for 24 hrs, colonies were counted and the results expressed as percentage of the inoculum. The inoculum size of each cell suspension was confirmed by plating aliquots of the culture directly in Sabouraud dextrose agar plates.

For biofilm formation, cells grown as above were seeded at a density of 1x10^6 cells/ml in pre-sterilized, polystyrene, flat bottom 6-well microtiter plates (Corning, N.Y) and incubated for 48h at 37°C (33). After biofilm formation, the medium was aspirated, and
non-adherent cells were removed by thoroughly washing the biofilms three times with sterile PBS.

A semiquantitative measurement of biofilm formation was made by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetra-zolium-5-carboxanilide]-reduction assay. XTT (Sigma) was dissolved in PBS at 0.5 g/liter. The solution was sterilized filter through a 0.22 µm pore size filter. Prior to each assay, the XTT solution was thawed and supplemented with menadione (10 mM stock dissolved in acetone; final concentration 1 µM; Sigma), an aliquot of 1 ml of the XTT-menadione solution was added per well, and the plates were incubated in the at 37°C for 2 h. A sample (500 µl) was then transferred from each well into a fresh 12-well plate (to eliminate interference of cells with colorimetric readings) and the colorimetric change resulting from XTT reduction was measured at 490 nm (34). Biofilm cultures were grown in triplicate, and each assay performed six times. For photographs, the biofilms were stained with crystal violet (35).
RESULTS

Growth, ultrastructure and biochemical characteristics of CO23_{RFLC} and CO23_{RFK} strains.

As first step in this investigation, the two drug-resistant strains CO23_{RFLC} and CO23_{RFK} (CO23_{RFLC}; MIC > 64 µg/ml; CO23_{RFK} MIC > 4 µg/ml) were compared to their parental strain CO23_{S} (FLC MIC 0.25 µg/ml; FK463, MIC 0.025 µg/ml) for a number of basic biochemical and physiological properties. Concerning their growth capacity at 37°C, the fluconazole-resistant strain had a prolonged lag phase, as compared to both the parental and the micafungin-resistant strain, nonetheless, the total growth yield after 24-48hrs of growth was substantially the same as that of the other two strains (1A). At 28°C, the three strains had almost overlapping growth curves in YNB medium (data not shown). At variance with growth, the three strains were quite dissimilar in growth morphology (1B), and, mostly, cell volume (1C). Particularly, scanning electron microscopy (SEM) showed that sensitive *C. albicans* cells had their typical shape, dimension and surface morphology, while CO23_{RFK} and CO23_{RFLC} cells demonstrated overall increased cell dimensions and increased propensity to clustering, suggesting an altered budding mechanism. Quantitative analysis of cell dimension (Fig.1C) revealed that the mean volume of drug-resistant cells was roughly twice that of the parental strain (9.3±3.0, 17.0±5.9 and 18.6±5.6 µm³ for CO23_{S}, CO23_{RFK} and CO23_{RFLU} strains, respectively).

The fungal cell wall is a plastic and dynamic structure, that constantly changes in response to environmental signals, with a number of highly efficient, compensatory mechanisms (28). Since this organelle could be the target of the above changes, we examined the cells of the three strains for any variation in cell extracts representative of major cell wall components. As shown in Table 1, all strains had roughly similar total hexose amount per cell, but the two drug-resistant ones had a much higher percentage of cell wall material per
cell as compared to the parent strain, indicating a marked enrichment in the carbohydrate-protein complexes (mostly mannan, β-glucan and their complexes with proteins), which was also in line with apparent abnormal cell wall deposition seen in the se strains by SEM (Figure 1 and other data not shown). Although no specific measurements of these components was attempted, the isolated, purified cell wall of each strain was grossly separated into its alkali-soluble and alkali-acid insoluble fractions which are known to contain essentially more plastic, mannoproteins and soluble β-glucan constituent in the former fraction, and more insoluble, rigid β-glucan complexes with proteins and chitin in the latter fraction, respectively (18,19). By this analysis, a marked relative decrease of the cell wall alkali-acid insoluble constituent was detected in both the drug-resistant strains as compared to the parental strain. Particularly, the content of the alkali-acid insoluble material per equal cell wall amount of the CO23RFK strain was less than half that of the parental, drug-susceptible strain (Table 1). Overall, this analysis suggested an unbalance of the respective proportions of mannoproteins, β-glucan and other constituents in the drug-resistant strains, which appear to be relatively enriched in soluble, plastic and impoverished in insoluble, rigid cell wall materials.

We also analyzed the presence and distribution of a mannoside epitope common to major mannoprotein component (MP), as a potential indicator of cell wall changes, by means of immunogold labelling with the specific monoclonal antibody AF1 (6). As shown in Figure 2 the cells of the parent strain revealed the expected intense and uniform labelling of the entire cell wall profile, with numerous gold particles randomly spanning cell wall layers. In contrast, the gold particles were less numerous and irregularly distributed throughout the cell wall of both resistant strains, particularly in the micafungin-resistant one. All this suggests that the deposition of the MP component and its organization within wall layers were changed in the drug-resistant strains. Figure 2 also shows reduced thickness of the cell wall of drug-resistant strains compared to the cell wall of the drug-susceptible strain, in line
with the reduced proportion of the alkali-acid insoluble material but in an apparent contrast with the data of total cell wall amount of these strains.

Molecular analyses of drug resistance-related genes in CO23_{RFLC} and CO23_{RFK} strains.

To investigate the molecular mechanisms of the acquisition of resistance to fluconazole and micafungin by the *C. albicans* strains CO23_{RFLC} and CO23_{RFK}, the expression of the *CDR1*, *CDR2*, *MDR1*, and *FLU1* genes, encoding efflux pumps known to be associated to azole resistance (4,31, 37), and of the *ERG11* (31) (Table 2), and mutations in the *FSK1* (2) genes was analyzed. Table 3 shows the mRNA expression levels of the target genes normalized against the *TEF3* gene as in-house transcript. As expected, expression of efflux pump genes was affected only in the fluconazole-resistant CO23_{RFLC}, compared to the wild-type susceptible strain CO23_{S}. In particular, CO23_{RFLC} exhibited strongly increased expression levels only of *MDR1* (33-fold), whereas slight increases were observed for *CDR1*, *CDR2*, and *FLU1* (3.7-, 3.0-, and 3.9-fold, respectively). Notably, no variation that led to an amino acid substitution was found in CO23_{RFLC} and CO23_{RFK} *ERG11* genes, which were expressed at levels similar to those of CO23_{S} (Table 3). In contrast, the micafungin-resistant strain CO23_{RFK} displayed a S645Y substitution which was expressed as a homozygous mutation in the *FKS1* gene.

Virulence of *C. albicans* as related to susceptible/resistant phenotype

To study whether acquisition of micafungin and fluconazole-resistance of *C. albicans* had any consequence on pathogenicity of this fungus, we used a murine systemic infection model having as endpoints mouse mortality and organ invasion following intravenous infection, as well as an estrogen-dependent rat vaginal infection to assess mucosal virulence. In the systemic infection model, both CO23_{RFK} and CO23_{RFLC} were compared to
the drug-susceptible parental strain CO23S in three repeated, independent experiments. Because of the absolute comparability of mortality data from the three independent experiments, with no statistically significant inter-experiment variability, the data were cumulated for a Kaplan-Meyer survival curve (Figure 3A).

The results consistently demonstrated that CO23S caused little overall mortality in the three cumulated experiments. In contrast, 57 and 70% lethality was measured on systemic infection by mice injected with CO23RFK and CO23RFK, respectively, with a median survival time of less than 1 week, as compared with only 10% lethality on infection with the parental, drug-susceptible strain (P<0.01, Fisher exact test). In all three experiments, the autopsy of all dead mice demonstrated deep organ invasion by the fungus, with extensive mycelial growth in target organs (kidney and heart; data not shown). In another independent experiment, where a sub-lethal inoculum size (10^5 cells) was used and mice were all sacrificed on day 2 post-challenge, all mice (three per group) infected by the two drug-resistant strains had >10^4 fungal cells/kidney whereas <10^2 cells were recovered from the kidney of the animals administered with the parental strain.

The virulence of the three strains in a mucosal model of candidiasis (rat vaginal model) was also assessed. In two independent experiments, the three strains showed almost overlapping kinetics of vaginal infection, with comparable times of spontaneous healing of the infection. Thus no virulence increase was shown by the two drug-resistant strains in the above model (Fig. 3B).

In vitro mycelial morphology

The results above demonstrated that acquisition of resistance to both FK463 and FLC was associated with a selective increase in the experimental pathogenicity for systemic infection of an originally poorly virulent strain of C. albicans in the above setting.
Since: i) the “in vivo” data showed massive hyphae formation in the organs (kidney and heart) of mice injected with the drug-resistant strains; ii) hyphae formation is a major virulence trait of the fungus, additional experiments were carried out to assess the capacity of each strain to produce hyphae in vitro. As shown in Figure 4 (A), each strain was capable of producing hyphal filaments when incubated in liquid, synthetic (N-acetyl-glucosamine-based) or in rich, serum-based medium. However, the hyphae produced in both media by the drug-resistant cells after 24 h incubation were clearly more numerous and longer than those generated by the drug-susceptible parent strain. The three strains were also tested for agar penetration capacity, a property related to hyphae formation, by spotting onto the surface of plates containing agar concentrations ranging from 0.8 to 3% to provide various levels of resistance to fungal penetration. Upon observation at day 7th of culture, agar invasion by the drug-susceptible strain was completely absent beyond a slight border of the original colony, while the drug-resistant strains showed long branching filaments radiating into the agar from the cells on the surface. These resistant strains displayed significantly longer and more tangled hyphal growth on the colony periphery (Figure 4, B).

Adherence and biofilm formation

It is well known that hyphae of C. albicans are more adhesive and tissue-invasive than their yeast cell counterpart (41). Thus, from the data above, variations in adherence by the drug-resistant strains could be anticipated. We previously examined the adherence properties of the three strains to plastic surfaces, as this is an important step in the development of biofilms and may contribute to the virulence properties of the organism. As shown in Figure 5A, there was a significant difference in the adherence to plastic among the strains, with both drug-resistant strains showing almost twice number of adherent cells as compared to the control. Then, we examined biofilm formation by the adherent cells
following incubation in fresh medium for up to 24 h. Biofilms were formed by both mutant
and wild type, but the biofilm produced by the drug-resistant cells was more abundant than
that produced by the susceptible strain as consistently shown by microscopic observations
of growth in agar plates (Fig.5B) and capacity to reduce tetrazolium salt (Fig.5C).
DISCUSSION

In this study, we addressed the poorly investigated relationship between resistance to antifungal drugs (ADR) and virulence of the human opportunistic pathogen *C. albicans*. ADR, in particular azole-resistance, of this organism has become a clinically relevant issue (38) and most studies have been devoted to investigate its mechanisms (32). Wealth of studies have also addressed virulence trait expression in this fungus. There is substantial agreement that adherence properties and capacity of switching the morphology from yeast to mycelial habit of growth, possibly interrelated each other, play a substantial role for host tissue invasion (12). Moreover, the propensity for biofilm formation has recently been shown to impact both on pathogenicity and drug resistance (30). While numerous reports have elucidated mechanisms of drug resistance and its evolution in Candida, and, particularly, its relevance for fungus fitness in vitro (9,11), very little is known on the potential consequences of ADR acquisition on virulence expression in vitro and in vivo.

In the absence of consistent reports, an implicit assumption circulates that, in analogy to most cases of antibiotic resistance in bacteria, ADR would not influence, or would rather decrease, the fungus virulence, possibly associated with changes in fitness cost of resistance. Our expectation was similar, since pathogens showing a higher MIC of a drug are assumed to be less fit in vivo in the absence of the drug, as under the conditions of our experiments (10). In this line, Kurtz et al (24) reported that the virulence of spontaneous or induced echinocandin-resistant mutants of *C. albicans* was either unimpaired or greatly reduced as compared to the parental strain. In addition, the echinocandin-resistant mutant of *C. albicans* (CA2) has a very low pathogenicity in a systemic infection (13). Interestingly, the spontaneous resistant mutant with unimpaired virulence retained full ability to make hyphae in vitro while the CA2 mutant was agerminative (13,24). In this case, the mutants with reduced virulence were derived by random chemical mutagenesis, and not, as in the present investigation, by direct exposure to the antimycotics, the latter
situation more closely mimicking the in vivo one. Chemical mutagenesis is a procedure that may greatly alter many gene functions and is very unlikely to occur in vivo, during antimycotic therapy.

Contrary to the above expectations, we have observed here a marked increase in the virulence of *C. albicans* for systemic infection when the fungus acquired ADR by exposure to the drug in vitro. More puzzling, the virulence increased to a rather equal extent when the originally susceptible strain was made resistant to either fluconazole or micafungin, two drugs belonging to distinct families, with a completely different biochemical mechanism of action and, as also shown here, different mechanism of resistance. Finally, the increase in virulence for deep-seated infection in the mouse was not paralleled by difference in the virulence in the vaginal infection in rats, a self-healing superficial infection. The data suggest that: *i)* virulence acquisition has nothing to do with any specific drug action or resistance mechanisms (see also below); *ii)* only the factors associated with the fungal capacity to invade host tissues have been altered in the drug-resistant strains.

We have not investigated all possible mechanisms reported in the literature to be relevant for deep-seated infection by *C. albicans* in normal mouse, thus a detailed picture of the virulence increase described here must await additional investigations. However, we have shown here that, among the many changes in the biological properties shown by the drug-resistant strains, there are some which are commonly considered to be virulence traits of *C. albicans*. They include, and are possibly interrelated each other, the capacity to generate long hyphal threads, a pronounced adherence to plastic and ability to invade agar medium, and greater propensity to make a biofilm.

The ability to switch from yeast to hyphal growth is likely central to all the observed phenomena and probably explains them. It is a formally-established virulence trait of the *C. albicans*, since all mutants unable to form hyphae are non-pathogenic in systemic mouse models of infection (26). Hyphae are much more adhesive and much less phagocytetable
than yeast cells. In addition, they have evolved a number of mechanisms to evade host innate and adaptive immune responses, including the demonstrated capacity to generate non-protective cytokine patterns (36).

While the above properties are relevant for virulence of C. albicans, it is quite clear from our observations that the drug-resistant strains have undergone multiple changes in the cell wall, some of which might more generally affect fungal fitness in vivo (9-11). Concerning fitness in vitro, it is of interest that the growth curves of the three strains in simple, YNB-based medium, at 37 or 28 °C, do not support any marked modification of fitness by the drug-resistant strains, nonetheless, the increased filamentation in vitro would suggest that both drug-resistant strains had increased their fitness in vitro, for a feature that, as discussed above, is related to a more aggressive behaviour in vivo. The relationship between fitness and virulence attributes clearly warrants future investigations. Cell wall changes include: 1) a marked alteration in the distribution of a major cell wall component, i.e. the mannoprotein (MP), which contains critical adhesins (37), particularly in the micafungin-resistant strain; 2) decreased amount of alkali-acid-insoluble material, to which MP is closely linked. This material substantially corresponds to the β-glucan interwoven fibrillar constituent of the cell wall. It is bound to chitin, and confers rigidity and resistance to the cell wall itself (20). Since the total cellular content of hexose (essentially, glucose plus mannose) is roughly similar in all strains, this suggests that some form of compensation might have occurred in the cell wall structure, to account for efficient growth on simple media and aggressive behaviour in vivo by the drug-resistant strains. The specific decrease of this material relative to the other cell wall constituents might account for the marked increase in the cell volume and deformation observed in the resistant strains, as if the cell had become more extensible. Interestingly, defective cell wall β-glucan incorporation is expected, and has indeed been reported in echinocandin drug-resistant mutants (15), whereas it is not an obvious expectation of fluconazole-resistant
mutants, again suggesting that the changes observed in the drug-resistant strains have nothing to do with specific antimycotic action and target.

On the other hand, increased secretion of β-glucan and other cell wall, plastic constituents, probably represents a counterpart to the lower incorporation of this critical polysaccharide into the cell wall and this could contribute to explain increased biofilm formation. C. albicans biofilm has been recently shown to consist mostly of β-glucan, which reduced the susceptibility of C. albicans cells to fluconazole by binding this drug extracellularly (30). It is of some speculative interest that drug resistance confers to the fungi increased propensity to biofilm formation, as shown here, clearly suggesting that biofilm formation may also be induced in vivo by drug treatment.

How general can be the phenomenon described here, i.e. increased virulence of ADR strains? In this context, three facts warrant careful consideration: i) virulence increase was detected only in the systemic infection model, not in a mucosal one; ii) the susceptible strain was initially isolated from a HIV-positive subject with recurrent vaginitis. In a comparison with the usual virulence of C. albicans on intravenous infection of mice (LD$_{50}$ around $10^6$ cells), it is clear that this vaginophatic strain is of relatively low virulence for systemic infection (LD$_{50}$ around $10^7$ cells). The virulence gained by the resistant strains makes it roughly equivalent to the “standard” level of virulence possessed by other strains of the fungus in systemic infection. This may also hold true for the increased expression of many virulence traits by a FLC-resistant strain of C. albicans and Cryptococcus neoformans, confirming what has recently been described (17, 40); iii) the drug resistance has been acquired by in vitro treatments, consisting in repeated passages in media with increasing drug concentrations. It is not clear to what extent this may be of significance in clinical situation. Refractory, chronic mucosal infections by C. albicans in HIV-positive subjects have originated azole-resistant strains (32) and it is possible that the mechanism of
this ADR is not very dissimilar from the one described here as these subjects are usually treated with repeated cycles of antifungals, often with increased concentrations.

Despite the above caveats, it remains of concern that intrinsically low virulence strains of *C. albicans* may become virulent as a consequence of ADR acquisition.

Conversely, it could be speculated that the original isolate had lost in vivo, by some unknown mechanisms, the typical virulence asset of *C. albicans* strains, and that the in vitro passages in the presence of antimycotics brought about a recovery of the lost virulence traits. This is a distinct possibility against which, however, three observations seem to argue. First, the original isolate was of low systemic pathogenicity since its initial isolation, ruling out that long or unusual maintenance conditions in vitro before initiation of this investigation caused loss of virulence. Second, resistant strains were as vaginopathic in the rat model infection as the original susceptible isolate, demonstrating that the strain had retained the specific virulence traits for mucosal infection; third, the CO23 strain was isolated from a subject with recurrent candidal vaginitis. Although no detailed clinical history of the patient is available, it is rather common in these subjects the continuous use of vaginal medications with cycles of antimycotics. The theory of “restoration” would assume that this strain with a “normal” pathogenicity potential, suffered a reduction of this potential during in vivo therapy so as to become of little pathogenicity on the isolation. All this would contrast what we describe here, i.e. gain of systemic virulence on drug treatment.

While a selective loss of virulence in vivo cannot be ruled out, other adaptive and/or fitness factors related to the isolation source and the different host niche are to be considered. In this context, noteworthy is the remarkable difference detected between strains of *C. parapsilosis* isolated from vaginitis patients and the isolates of the same fungus from patients with candidemia: the former were more pathogenic in the rat vaginal
infection model while the latter were more pathogenic in the systemic mouse infection model (7).

Finally, noteworthy is also the observation that both FK463 and FLC-resistant strains gained equal virulence upon resistance acquisition, demonstrating that the virulence gain is independent on mechanism of drug action and resistance itself. This is well in line with the changes in glucan-associated proteins of the cell wall which, as shown elsewhere (1), are also independent on the specific drug action. Overall, it appears that the stress of the resistance acquisition upon prolonged, dosage-increasing treatments with antifungals, may induce biological compensatory changes which, by unknown mechanisms, could select for acquisition by *C. albicans* of some advantages in terms of fitness and capacity of attacking the host systemically. Whatever the explanation, our data invite to further studies in other well characterised resistant strains of *C. albicans* for a conclusion on this important clinical issue.
REFERENCES


Figure Legends

Figure 1

Growth curves (A) scanning electron micrographs (EM; B) and cell volume (C) of CO23s (wild type), CO23RFK and CO23RFLC (micafungin and fluconazole-resistant strains, respectively) of C. albicans. The growth curve were obtained growing the strains at 37°C in liquid YNB medium. The cell volumes were calculated from SEM observations, as described in Materials and Methods. In B, A,B and C indicate the strains CO23s, CO23RFK and CO23RFLC respectively with corresponding cell volume.

Figure 2

EM localization of mannoproteins constituents in freeze-substituted yeast cells of C. albicans strains CO23s (A), CO23RFK (B) and CO23RFLC (C) following post-embedding labelling with the mAb AF1 followed by gold-labelled, secondary antibody. For technical details, see text.

Figure 3

Experimental pathogenicity of C.albicans strains in a systemic (A) and mucosal (B) infection models.

A. Male Balb/C mice were infected intravenously with the indicated C.albicans strains (inoculum size: 10^7 cells). Survival was determined over the time indicated. There was a statistically significant difference (P<0.01) between the parental and each of the two drug-resistant strains. For further details, see Materials and Methods.

B. Oophorectomized, oestrogen-treated rats were infected intravaginally with the indicated C.albicans strains (inoculum size: 10^7 cells). At the indicated time intervals, the
intravaginal burden of fungal cells was measured as described in Materials and Methods. No statistically significant difference was found among the strains.

**Figure 4**

Hyphae and invasive hyphal growth of *C. albicans* strains CO23<sub>S</sub>, CO23<sub>RFK</sub> and CO23<sub>RFLC</sub>.  

**A.** Cells were induced to hyphal formation by 24 h incubation at 37 °C in YNB medium containing 0.1% (w/v) GlcNac (line 1) or RPMI1640 medium containing 10 % fetal calf medium (line 2). Photomicrographs were taken at a phase contrast microscope using 40x objective and are representative of 50% random fields observed.  

**B.** 10<sup>4</sup> cells of CO23<sub>S</sub>, CO23<sub>RFK</sub>, CO23<sub>RFLC</sub> strains were suspended in 1 µl of YPD and spotted onto the surface of 4% serum plates containing 0.8 to 3 % agar, and the plates were incubated at 37°C. (line1) Top view of spot colonies at 7 days of incubation on 0.8% agar using a 10x objective. (line 2) Images of spot colony edges at 7 days of incubation on 3% agar using a 40x objective. The photographs are representative of the whole mounted microscopic plates.

**Figure 5**

Adherence, biofilm and XTT assay of *C.albicans* strains CO23<sub>S</sub>, CO23<sub>RFK</sub> and CO23<sub>RFLC</sub>.  

**A.** Percentage of plastic adherent cells of the strains CO23<sub>S</sub>, CO23<sub>RFK</sub> and CO23<sub>RFLC</sub>. The cell were allowed to adhere to polystyrene surface , then poured with 1 ml of Sabouraud dextrose agar which was allowed to solidify, incubated at 37°C for 24 hrs. **B.** Production of biofilm onto polystyrene surfaces. Biofilms were stained with crystal violet and photographed at 10x and 40x magnification using an inverted microscope. **C.** Equal numbers of cells from CO23<sub>S</sub>, CO23<sub>RFK</sub> and CO23<sub>RFLC</sub> were suspended in 1 ml of RPMI and incubated in 12 well plates for the times indicated. Non adherent cells were then
removed by washing, and adherent cell were XTT assayed. Experiments were repeated
three times with similar results.
Figure 1
Cell wall composition of drug-susceptible and resistant *C. albicans* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Glucose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Cell wall&lt;sup&gt;b&lt;/sup&gt;</th>
<th>%AA-insoluble&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO23&lt;sub&gt;S&lt;/sub&gt;</td>
<td>58.16</td>
<td>50.0</td>
<td>27.77</td>
</tr>
<tr>
<td>CO23&lt;sub&gt;RFK&lt;/sub&gt;</td>
<td>52.51</td>
<td>86.4</td>
<td>12.97</td>
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<tr>
<td>CO23&lt;sub&gt;RFLC&lt;/sub&gt;</td>
<td>49.53</td>
<td>74.8</td>
<td>18.40</td>
</tr>
</tbody>
</table>

<sup>a</sup>: expressed as % of cell dry weight and measured by method of Dubois et al.

<sup>b</sup>: expressed as % of cell dry weight

<sup>c</sup>: expressed as % alkali-acid insoluble cell wall material/mg of cell wall dry weight
Figure 2
<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Primer or probe</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene location (5'-3')</th>
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</thead>
<tbody>
<tr>
<td><strong>CDR1</strong> (X77589)</td>
<td>CDR1a</td>
<td>AACCGTTTACGTTGAACACGATAT</td>
<td>2504-2527</td>
</tr>
<tr>
<td></td>
<td>CDR1b</td>
<td>ACCAACTTCACCATCTTCATGAC</td>
<td>2565-2588</td>
</tr>
<tr>
<td></td>
<td>CDR1pr</td>
<td>6FAM- ACTCACGCCGACACCACCGTTGTT- TAMRA</td>
<td>2535-2558</td>
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<tr>
<td><strong>CDR2</strong> (U63812)</td>
<td>CDR2a</td>
<td>TGGCTAGTGGTTATATGGGATCT</td>
<td>1725-1748</td>
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<tr>
<td></td>
<td>CDR2b</td>
<td>AAGCTTCAGCAATGGGACACTCTTT</td>
<td>1821-1844</td>
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<tr>
<td></td>
<td>CDR2pr</td>
<td>6FAM- TCACCACCGGAAACACCACGCAC- TAMRA</td>
<td>1792-1815</td>
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<tr>
<td><strong>MDR1</strong> (Y14703)</td>
<td>MDR1a</td>
<td>TCTCGGTTGGATTCGCTTAAT</td>
<td>2659-2681</td>
</tr>
<tr>
<td></td>
<td>MDR1b</td>
<td>AATGGACCAAAAATGGGACCACA</td>
<td>2775-2797</td>
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<tr>
<td></td>
<td>MDR1pr</td>
<td>6FAM- ACGGCCAACTCAAAGGACGACAC- TAMRA</td>
<td>2751-2773</td>
</tr>
<tr>
<td><strong>FLU1</strong> (AF188621)</td>
<td>FLU1a</td>
<td>TTTGTCGTTTTCTTGCTGTTTT</td>
<td>1153-1175</td>
</tr>
<tr>
<td></td>
<td>FLU1b</td>
<td>ATTAACATATCGGCCATAACCGC</td>
<td>1206-1229</td>
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<tr>
<td></td>
<td>FLU1pr</td>
<td>6FAM- AGGCCCAACACTCAGCAGCACC- TAMRA</td>
<td>1179-1202</td>
</tr>
<tr>
<td><strong>ERG11</strong> (AY856351)</td>
<td>ERG11a</td>
<td>TTATTAGGGTTCCATTCTTGTATTA</td>
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<tr>
<td></td>
<td>ERG11b</td>
<td>AAATTCATAAAGGTTTGTGCCATATG</td>
<td>188-213</td>
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<td>ERG11pr</td>
<td>6FAM- TGCAGGAACAAACCCAGGAATCCA- TAMRA</td>
<td>160-183</td>
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<tr>
<td><strong>TEF3</strong> (Z12822)</td>
<td>TEF3a</td>
<td>AACCGTTTACGTTGAACACGATAT</td>
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<tr>
<td></td>
<td>TEF3b</td>
<td>ACCAACTTCACCATCTTCATGAC</td>
<td>2565-2588</td>
</tr>
<tr>
<td></td>
<td>TEF3pr</td>
<td>Texas Red- ACTCACGCCGACACCACCGTTGTT- BHQ2</td>
<td>2535-2558</td>
</tr>
</tbody>
</table>
Abbreviations: 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-\(N,N,N',N'\)-tetramethylrhodamine; Texas Red, trademark product from Molecular Probes; BHQ2, Black Hole Quencer 2.
TABLE 3. Expression levels of the *CDR1*, *CDR2*, *MDR1*, *FLU1*, and *ERG11* genes for the *C. albicans* strains studied

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gene expressiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>CDR1</em></td>
</tr>
<tr>
<td>CO23S</td>
<td>9.0± 0.3</td>
</tr>
<tr>
<td>CO23RFLC</td>
<td>34.0± 4.0</td>
</tr>
<tr>
<td>CO23RFK</td>
<td>11.0± 4.0</td>
</tr>
</tbody>
</table>

aQuantification was performed by real-time RT-PCR. Values are averages of four independent experiments and represent *TEFx1000* -normalized levels of expression of the target genes (see Materials and Methods).
Figure 3

A

B

Days post challenge

Days post infection

% survivors

C.F.U. xml (x 10^{-3})

CO23 S

CO23Rflc

CO23Rfk
Figure 4
Figure 5