In this study, the susceptibility of three clinical isolates and one reference strain of *C. dubliniensis* (Table 1) to FLU was tested in the presence of DOX in a chequerboard experiment. In addition, gene expression analyses of those encoding for efflux pumps (*CdCDR1*, *CdCDR2*, *CdMDR1*) and enzymes involved in the ergosterol biosynthesis, i.e., C5,6-desaturase enzyme (*CdERG3*) and lanosterol demethylase (*CdERG11*), were conducted after short- and long-term DOX exposure.

**Material and methods**

For the chequerboard experiment, isolates were incubated with FLU and DOX in different concentration combinations in a 96-well microtiter plate assay. The isolates were initially inoculated onto solid agar plates and incubated overnight at 35 °C. One colony of each isolate was then transferred to 0.9% sodium chloride (1/1000 – 5/1000 cells/ml), the suspension diluted 1:100 with synthetic RPMI medium (Sigma, Steinheim, Germany) and added to the microtiter plate. The plate layout used was published previously [12]. FLU was added and sequentially diluted from 16 – 0.031 μg/ml, whereas the addition of DOX was consecutively diluted from 80 – 2.5 μg/ml. Plates were incubated at 35 °C for 48 h, after which, the presence of growth in wells above the minimal inhibition concentration (MIC) level of FLU, was considered to be an indicator of an interactive effect of DOX on FLU affecting the isolates.

**Keywords** *Candida dubliniensis*, doxorubicin, resistance, drug efflux pumps, fluconazole susceptibility

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**Introduction**

A higher mortality rate (approximately 40%) among cancer patients has been noted to be associated with *Candida* infections (overall 30-day mortality) [1]. Resistance to antifungals increases complications and treatment failures during therapy. Previously published studies have shown that resistance in yeasts may not only be due to antifungal drugs but also to antibiotics and cytostatic agents [2–5]. Our group recently demonstrated that the cytostatic agent doxorubicin (DOX) increased the gene and protein expression of efflux pumps in clinical *C. albicans* isolates and decreased their susceptibility to fluconazole (FLU) [3].

The opportunistic yeast *C. dubliniensis* was first described in 1995 and is very closely related to *C. albicans* [6]. Despite genetic and morphologic similarities, the two species differ in their prevalence and pathogenicity. In contrast to *C. albicans*, *C. dubliniensis* is responsible for approximately 2% of cases of *Candida* infections [7]. However, its prevalence increases in HIV-infected patients and those with diabetes, cystic fibrosis and cancer [6,8–11].

In this study, the susceptibility of three clinical isolates and one reference strain of *C. dubliniensis* (Table 1) to FLU was tested in the presence of DOX in a chequerboard experiment. In addition, gene expression analyses of those encoding for efflux pumps (*CdCDR1*, *CdCDR2*, *CdMDR1*) and enzymes involved in the ergosterol biosynthesis, i.e., C5,6-desaturase enzyme (*CdERG3*) and lanosterol demethylase (*CdERG11*), were conducted after short- and long-term DOX exposure.

**Material and methods**

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susceptibility to the antifungal. The fractional inhibitory concentration (FIC) index was determined as described elsewhere [12]. DOX antagonism relative to the isolates susceptibilities to FLU was defined by a FIC index of > 4, while a FIC index result of ≤ 4 was considered to be indicative of indifferent effect [13]. The checkerboard experiment was performed three times.

For gene expression analyses, *C. dubliniensis* isolates were cultured overnight in yeast-peptone-dextrose (YPD) broth (Qbiogene, Heidelberg, Germany) at 35°C. Portions of the overnight cultures were used to inoculate fresh YPD broth and cells were allowed to grow until mid-log phase. DOX was added to samples designated for treatment (20 μg/ml), incubated for 90 min and cells harvested for RNA isolation. In a second experiment, the isolates were incubated with 20 μg/ml DOX for 10 days starting on day 0, with the medium supplemented with fresh DOX on days 1, 2, 3, 6 and 8. In both types of experiments, each isolate was concomitantly cultured without the drug as a control and FLU was not added to any of the cultures. Samples were taken on the days indicated above followed by subsequent RNA isolation. RNA isolation and cDNA synthesis were performed as described previously [3] and both types of experiments were conducted on two different occasions.

**Table 1** Overview of *Candida dubliniensis* isolates used for this study.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Abbreviation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM 13268</td>
<td>–</td>
<td>DSMZ Braunschweig</td>
</tr>
<tr>
<td>RKI 01-0170-01</td>
<td>Cd1</td>
<td>Robert-Koch-Institut, Berlin</td>
</tr>
<tr>
<td>RKI 01-0265</td>
<td>Cd2</td>
<td>Robert-Koch-Institut, Berlin</td>
</tr>
<tr>
<td>RKI 01-0268-02</td>
<td>Cd3</td>
<td>Robert-Koch-Institut, Berlin</td>
</tr>
</tbody>
</table>

Gene expression analyses were carried out using the LightCycler® 480 system (Roche, Mannheim, Germany). Each cDNA sample was analyzed in duplicate per biological replicate and normalized against the expression of the reference gene *CdACT1*. Assays for gene expression were optimized by adjusting the conditions for a comparable high-level efficiency for each assay. The composition of each PCR reaction and conditions were described previously [14]. Conditions for the reference gene *CdACT1* slightly varied. Primer concentrations were 100 nM (sense primer) and 200 nM (antisense primer) to ensure high amplification efficiency rates. *CdACT1* annealing temperature was 58°C. Primer and probes are listed in Table 2. No template controls were included in all runs. C_T values were calculated using the LightCycler® 480 Software (Roche) and relative quantification was performed applying the ΔΔC_T method by first normalising gene expression to the expression of the reference gene (ΔC_T) and relating results afterwards to results of the untreated control (ΔΔC_T).

All four *C. dubliniensis* isolates were checked for the presence of a common homozygous single nucleotide polymorphism (snp) in the *CdCDR1* gene that converts codon 756 (TAT, encoding tyrosine) to a TAG stop codon, resulting in the expression of a truncated, non-functional Cdr1p protein. The strains were tested for the loss of codon 756 (TAT) using a PCR assay as described previously [14]. The *C. dubliniensis* isolate Cd36 was included as positive control for a mutated *CdCDR1* gene.

**Results**

The checkerboard experiments revealed an antagonistic effect of DOX on the susceptibility of all four *C. dubliniensis*...
isolates to FLU. With isolate Cd1 the observed MIC\textsubscript{FLU} shift in the presence of DOX was two concentration levels above the MIC\textsubscript{FLU} determined without DOX. Two isolates (DSM13268, Cd3) showed a shift of three concentration levels and Cd2 a shift of four concentration levels above the MIC\textsubscript{FLU} determined without DOX (Fig. 1). Shifting of MIC\textsubscript{FLU} values was dependent on DOX concentration. The FIC index calculated for isolate DSM13268 ranged from 3–9, for Cd2 from 5–17, for Cd3 from 5–9 and the index for Cd1 was 5. Isolate Cd2 survived 40 μg/ml DOX during the chequerboard experiments whereas this concentration was lethal for the other three \textit{C. dubliniensis} isolates.

Moran and colleagues showed that approximately 50% of \textit{C. dubliniensis} isolates are homozygous for a mutated \textit{CdCDR1} allele containing a premature stop signal at codon 756, resulting in the translation of a truncated Cdr1p [14]. Isolates DSM13268 and Cd2 were found to be homozygous for this mutation within \textit{CdCDR1}, whereas Cd1 and Cd3 have the full length copy of the gene (data not shown).

The influence of DOX on \textit{C. dubliniensis} isolates was further investigated at the gene expression level. Several resistance genes are known for \textit{C. dubliniensis}, such as the efflux pumps \textit{CdCDR1}, \textit{CdCDR2} and \textit{CdMDR1}, as well as \textit{CdERG3} and \textit{CdERG11} [15–18]. The ATP-binding cassette (ABC) transporter \textit{CdCDR2} was upregulated in all DOX-treated isolates during short-term exposure (Fig. 2A).

Isolates showed a DOX-induced increase of \textit{CdCDR2} expression of between 2.3- (Cd2) and 5.2-fold (DSM13268) compared to the expression in untreated controls (Fig. 2). The expression of \textit{CdCDR1} was doubled in the presence of DOX in DSM13268 and Cd1, but Cd2 and Cd3 showed no increase in the expression of the gene. The results after long-term exposure to DOX confirmed, in part, the outcome of the short-term experiment. \textit{CdCDR2} expression was increased in all isolates during the complete test period of 10 days (Fig. 2B–E). Expression ranged from a 2.0- to 17.7-fold increase when compared to untreated controls. However, \textit{CdCDR1} expression was not increased in any of the four strains. Among the four isolates tested, the major facilitator gene \textit{CdMDR1}, as well as \textit{CdERG3} and \textit{CdERG11} were not overexpressed in the presence of DOX in both experiments.

**Discussion**

This present study addressed the question of whether the cytostatic drug DOX could affect FLU susceptibility of \textit{C. dubliniensis} isolates. We recently reported that the presence of 20 μg/ml DOX resulted in a MIC\textsubscript{FLU} shift in \textit{C. albicans} clinical isolates [3]. This shift of MIC\textsubscript{FLU} values was also observed in this study in all four of the tested \textit{C. dubliniensis} isolates. Furthermore, we showed that the increase of MIC\textsubscript{FLU} values was dependent
on DOX-concentration. FIC indices in the presence of 20 μg/ml DOX ranging from 5–17 indicated an antagonistic effect of DOX on FLU susceptibility.

Gene expression analyses of resistance-associated genes were conducted to find a possible explanation for the shifts of MIC<sub>FLU</sub> values. A concentration of 20 μg/ml DOX, which correlates to a therapeutic serum concentration in humans, induced efflux pump gene expression in <i>C. albicans</i> and was therefore also used in <i>C. dubliniensis</i> gene expression experiments [3]. The presented data showed a DOX-dependent induction of <i>CdCDR2</i> expression. Other tested efflux pumps showed only a slight (<i>CdCDR1</i>) or no (<i>CdMDR1</i>) overexpression. This outcome is consistent with the recently reported results for <i>C. albicans</i> demonstrating that <i>C. albicans</i> ABC transporter expression was induced by DOX whereas <i>CaMDR1</i>...
expression was not affected [3]. Interestingly, CdCDR2 expression is more pronounced than CdCDR1 expression in C. dubliniensis which is in contrast to an increased CaCDR1 expression after DOX exposure in C. albicans. Hence, up-regulation of CdCDR2 by DOX seems to play a major role in the increased MICFLU observed in C. dubliniensis. Moran et al. showed that C. dubliniensis CdCDR1 is not essential for FLU resistance and that some C. dubliniensis isolates only express a non-functional truncated Cdr1p [14]. Two isolates of this study have a full length CdCDR1 gene and two isolates are homozygous for a mutation within the CdCDR1 gene that results in the expression of a truncated CdCdr1p. Antagonism of fluconazole activity by DOX was still observed in isolates expressing the truncated protein, indicating that a functional CdcDr1p is not required for this antagonism. Furthermore, none of the isolates showed an upregulation of CdCDR1 after long-term exposure with DOX, indicating that CdCDR1 upregulation is not required for the decreased FLU susceptibility observed in response to DOX.

C. albicans CaTAC1, encoding the transcriptional activator of CaCDR1/CaCDR2, played a critical role in DOX-induced efflux pump expression. A tac1Δ/Δ null mutant showed no overexpression of CaCDR1 and CaCDR2 after DOX exposure [3]. This outcome is consistent with the critical role of CaTAC1 in the regulation of both genes. In the case of C. dubliniensis little is known about the regulation of ABC transporter pumps [19]. The C. dubliniensis ortholog, CdTAC1 shares 88% amino acid identity with CaTAC1 and very likely performs similar functions in C. dubliniensis [20].

Several studies exist showing influences of steroids, herbicides or antibiotic compounds on C. albicans efflux pump expression [2–5,21]. However, to the authors’ knowledge there are no studies of the manipulative influence of cytostatic agents on the efflux pump expression of C. dubliniensis or other non-C. albicans Candida species.

In summary, it may be speculated, that the use of DOX in cancer chemotherapy may have an impact on FLU susceptibility, as well as on the effective therapy of Candida infections in cancer patients.

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References


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