EXPRESSION PATTERN OF DRUG-RESISTANCE GENES IN CANDIDA ALBICANS AT DIFFERENT FLUCONAZOLE CONCENTRATIONS.

A THESIS IN
Cell and Molecular Biology

Presented to the Faculty of the University of Missouri-Kansas City in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

By
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B.S., University of Iowa, 2011

Kansas City, Missouri
2015
Candida albicans is an opportunistic fungus that is part of the normal flora of our gastrointestinal and urinary tracts, but can cause infections in immune compromised individuals. Fluconazole (FLC), which is a fungistatic azole, is a common drug used for treatment of C. albicans infection by targeting lanosterol demethylase (ERG11). Some clinical isolates show resistance to FLC, which can be attributed to an over expression of ABC transporters (CDR1, CDR2), Major Facilitator Transporter (MDR1), and the azole target (ERG11). Commonly the expression pattern of these genes is compared across strains of susceptible and resistant strains in the absence of drugs. However, gene expression levels of each strain may be different in different drug doses. This study investigates the pattern of gene expression of MDR1, CDR1, CDR2, and ERG11 in different C. albicans matched isolates at different doses of FLC. Along with the wild-type strain SC5314, isolates from a single patient with varying susceptibility to FLC were selected. The Minimum Inhibitory Concentration (MIC80) to FLC for each strain was determined by microbroth dilution. The mRNA expression of the genes was analyzed.
using Quantitative Real-Time PCR (qRT-PCR) at FLC concentrations 4-fold lower, 4-fold higher, and at their respective MICs. Gene expression was analyzed with and without FLC induction and the MDR1/CDR1/CDR2/ERG11 gene expression levels of all the strains were normalized to their uninduced expression levels. The experiments show that in resistant strains, the genes *MDR1* and *ERG11* are over expressed even without FLC induction, consistent with previous work. Increasing the FLC concentration does not have any significant effect on gene expression in resistant strains. For the azole-susceptible strains, the gene expression of *CDR1* and *CDR2* increases at FLC concentration below its MIC<sub>80</sub> and declines at concentrations above the MIC<sub>80</sub>. Hence, the gene expressions of clinical isolates vary according to their MIC<sub>80</sub> values.
The faculty listed below, appointed by the Dean of the School of Biological Sciences, have examined a thesis titled “Expression Pattern of Drug-resistance Genes in Candida albicans at Different Fluconazole Concentrations,” presented by Aditi Khanna, candidate for the Master of Science degree, and certify that in their opinion it is worthy of acceptance.

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ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. Theodore C. White, Dr. Thomas Meness, and Dr. Michael Plamann for their advice through the work represented by this thesis. I would especially like to thank my committee chair and mentor, Dr. Theodore C. White, for being an outstanding teacher and advisor and guiding me throughout the whole process. I would also like to thank fellow members of the lab for their cooperation and assistance; Somanon Bhattacharya, Brooke Esquivel, and Emily McMichael for their constant help and support. School of Biological Sciences at University of Missouri-Kansas City funded this research.
CHAPTER 1

INTRODUCTION

*Candida albicans* is a pathogenic fungus that causes oral, vaginal and systemic infections in immune-compromised individuals such as HIV positive patients (White, Marr et al. 1998). Ergosterol is a fungal analog of cholesterol, which is important for cell membrane and fluidity. Lanosterol-14 α-demethylase, a cytochrome P450 enzyme is important for ergosterol biosynthesis and is encoded by *ERG11* gene. Many antifungal drugs have been designed to target enzymes in the ergosterol biosynthetic pathway from squalene to ergosterol (Sanglard, Ischer et al. 2003).

Previous studies have shown that long term administration of low-level azole antifungals result in azole resistant isolates of *Candida albicans* (White, Marr et al. 1998). The mechanisms behind the FLC resistance in *Candida albicans* are an important area of research. Several different factors have been found out to be important. ATP-binding cassette transporters (ABC transporters) and major facilitator transporters (MFS-transporter), which are drug efflux pumps, have been shown to be involved in FLC resistance (Sanglard, Ischer et al. 2003, Holmes, Keniya et al. 2012). Specifically, efflux pumps CDR1, CDR2 and the multidrug resistance transporter MDR1 have shown to be overexpressed in the resistant clinical isolates. Overexpression of ERG11 and point mutation within ERG11 has also been shown to be contributors to *C.albicans* resistance (White 1997).

A series of 17 clinical isolates taken from the same patient over the course of two years have been previously categorized according to their Minimum Inhibitory Concentrations (MIC$_{80}$)
(White, Pfaller et al. 1997) (Figure 1). The amount of drug given to the patient was increased which in turn increased the resistance of the isolates, with Isolate 17 being the most resistant. mRNA expression levels of CDRs, ERG11, and MDR1 identified the resistance mechanisms of these isolates, although the isolate that first exhibited the overexpression of each of the genes were different (White 1997, White, Marr et al. 1998) (Figure 1). CDR1 and CDR2 levels are overexpressed starting in isolate 16, MDR1 levels are overexpressed starting in isolate 2, and ERG11 levels are overexpressed starting in Isolate 13.

Figure 1: Series of clinical isolates and their fluconazole susceptibility: The figure shows where the increase in expression of the CDRs, MDR1, and ERG11 is seen and its relation to the increasing MIC\textsubscript{80} values (White, 1997).
In this paper we look at how the mRNA expression of CDR1, CDR2, MDR1, and ERG11 changes in these four clinical isolates (1, 4, 13, and 17) when grown under eight different FLC concentrations. The drug concentrations were determined according to each isolate’s MIC$_{80}$. A previous study that looked into the gene expressions of ERG11, CDR1, and MDR1 at different concentrations of unrelated clinical isolates showed that the molecular mechanism of resistance was similar in all of the isolates but are regulated differently (Lee, Williams et al. 2004). This paper looks at a larger range of drug concentrations, which were chosen according to the MIC of the isolates.
MATERIALS AND METHODS

Isolates and Growth of Culture

The isolates chosen for this study were selected from a series of 17 clinical isolates taken from the same patient (Table 1) (White, Pfaller et al. 1997). Four isolates from the series were chosen for comparison. The overnight cultures were inoculated from single colonies grown on CSM-complete with 2% glucose plates and grown in CSM-complete broths with 2% glucose for each experiment.

Table 1: Clinical isolates used in this study

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>TW number</th>
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<tr>
<td>Isolate 1</td>
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<td>2-76</td>
</tr>
<tr>
<td>Isolate 4</td>
<td>TW 072-31</td>
<td>2-81</td>
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<td>Isolate 13</td>
<td>TW 072-39</td>
<td>8-44</td>
</tr>
<tr>
<td>Isolate 17</td>
<td>TW 072-43</td>
<td>12-99</td>
</tr>
</tbody>
</table>

Minimum Inhibitory Concentration

The four clinical isolates were tested for susceptibility towards FLC. MICs of the isolates were determined using the CLSI-approved microbroth microdilution protocol that determines MICs as the concentration of drug that inhibits 80% growth of the organism (Bertout, Dunyach et al.). Cells were grown in 96 well plates containing a gradient of drug in two fold serial dilutions. Plates were incubated for 48 hours in 30°C. CSM Complete media with 2% glucose was used for
the cell growth. One row of wells lacking any drug was used as positive control (100% growth) and cell growth in the wells containing drugs were standardized to the positive control. The negative control, which checks for contamination, is a row containing media alone.

**Growth Curve Analysis**

Overnight cultures in CSM-complete were used to inoculate 100 ul CSM-complete with different FLC concentrations at an OD600 of 0.2. Cultures were grown at 30°C with shaking and OD measurements were taken every half hour for 48 hours using a microplate reader (Biotek, Winooska, VT). The growth curves were plotted using Prism.

**Quantitative Real Time PCR analysis**

The mRNA expression of the genes *CDR1*, *CDR2*, *MDR1* and *ERG11* were measured using qRT-PCR. Starting with a single colony from the four clinical isolates, the cells were grown to the exponential phase and RNA was extracted using Qiagen RNeasy mini purification kit [at eight difference concentrations of FLC (0, 0.25, 1, 2, 4, 8, 32, and 128 ug/ml)]. The concentrations of the mRNAs were measured using a multimode platereader (Biotek, Winooski, VT). The A260/A280 ratio was assessed to determine the purity of RNAs and the ratio above 2 was considered satisfactory. To assess degradation, 1.2% agarose gels were run, and the appearance of two distinct ribosomal RNA bands was taken to indicate that the RNA was intact. Superscript III First Strand kit (Invitrogen, Waltham, MA) was used to prepare cDNA from 425ng of RNA each. The manufacturers protocol for iTaq™ SYBR® GREEN supermix (BIO-RAD, Hercules, CA) was followed to perform qRT-PCR in 7500 Real-Time PCR system (Applied Biosystems). Primers that were used to amplify the specified genes are listed in Table
2. These primers were previously used to determine the mRNA expression levels of drug resistance genes in *C. albicans* (Livak and Schmittgen 2001, Oliver, Silver et al. 2008). The data obtained was normalized to α-actin. The expression levels of each isolate grown without FLC were compared to expression levels of cells in the presence of drug. All qRT-PCR experiments were performed in biological triplicates. After qRT-PCR, a dissociation step was performed for each reaction to determine the specificity of the primers. Fold changes were analyzed as $2^{\Delta \Delta Ct}$ and error bars were calculated as previously described (Livak and Schmittgen 2001, Flowers, Barker et al. 2012). Fold changes greater 2 fold above or 2 fold below the no drug controls were considered to be significant and statistical analysis was done using unpaired t-test using Prism.

Table 2: Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>ACT1</td>
<td>ACGGTGAAGTTGCTGCTTT AGTT</td>
<td>CGTCGTCACCGGCAAAA</td>
<td>Oliver, Silver 2008</td>
</tr>
<tr>
<td>CDR1</td>
<td>AAGATGTCGTCGCAAGAT GAATC</td>
<td>GAGTGAAAGTTCTGGCTA AATTCTGA</td>
<td>Oliver, Silver 2008</td>
</tr>
<tr>
<td>CDR2</td>
<td>TTGAGCCACATGTCCGACA AT</td>
<td>GGAATCTGGGTCTAATTG TTCATGA</td>
<td>Oliver, Silver 2008</td>
</tr>
<tr>
<td>ERG11</td>
<td>CCCCTATTAATTTTGTTTTC CCTAATTTAC</td>
<td>CACGTTCTCTTCTCAGTTT AATTTCTTT</td>
<td>Oliver, Silver 2008</td>
</tr>
<tr>
<td>MDR1</td>
<td>ATCACCGGTAAACGACAGA ATCA</td>
<td>TCTAATGGTCTCCATAAT GTATCAATGA</td>
<td>Oliver, Silver 2008</td>
</tr>
</tbody>
</table>
RESULTS

Minimum Inhibitory Concentration and Susceptibility Testing

MICs were determined for all four clinical isolates from a single patient for FLC. The MIC breakpoint for strains to be resistant to FLC is ≥8 µg/ml (Danby, Boïkov et al., Sobel, Zervos et al. 2003, Suarez and Fernandez Andreu 2003, Hamza, Matee et al. 2008). Out of the 4 clinical isolates, 1 was very susceptible (1 µg/ml), 4 was a little more resistant than 1 (8 µg/ml respectively), and 2 isolates, 13 and 17 showed high resistance to FLC (32 µg/ml and >256 µg/ml respectively) (Figure 2), consistent with previous reports (White 1997).

![Figure 2: MIC80 of the isolates](image)

**Figure 2: MIC80 of the isolates:** The growth of isolates 1, 4, 13, and 17 up to 48h in CSM-complete. Minimum Inhibitory concentration of Isolates 1, 4, 13, and 17 can be calculated as the amount of drug concentration that inhibits 80% of the cellular growth.
Growth Curve Analysis

Growth curve analysis showed that the susceptible isolates 1 and 4 exhibit a decline in the cell multiplication rate starting from 1 ug/ml of FLC (Figure 3A and 3B). The growth was further reduced as the drug concentration was increased with the slowest rate being at the highest concentration (128 ug/ml). The growth rate for resistant isolates 13 and 17 were unchanged with all amount of FLC addition when compared to uninduced conditions (Figure 3C and 3D).

Figure 3: Growth curve analysis of a) Isolate 1, b) Isolate 4, c) Isolate 13, d) Isolate 17 at different drug concentrations is shown. The cells were started at a culture of 0.2OD at 600nm and grown in CSM-complete for 48 hours.
mRNA Expression of Genes Encoding the Efflux Pumps and Lanosterol 14α-demethylase

The mRNA expressions for CDR1, CDR2, MDR1, and ERG11 without FLC were significantly higher in resistant isolates when compared to the susceptible isolate 1 (Table 3) (White 1997). Only MDR1 was overexpressed for isolate 4, whereas Isolate 17 showed overexpression for all four genes in the absence of drug. Isolate 13 showed overexpression of all but CDR2 genes, and a trend of overexpression of CDR2 without any FLC (Table 3).
Table 3: Gene expression comparison without FLC.

<table>
<thead>
<tr>
<th>FLC conc (µg/ml)/Strain</th>
<th>0</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CDR1</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>4</td>
<td>0.90</td>
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<tr>
<td>13</td>
<td>8.64</td>
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<td></td>
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<td>CDR2</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>13</td>
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<tr>
<td>17</td>
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<td></td>
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<tr>
<td></td>
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<td>13</td>
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<tr>
<td>17</td>
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</tr>
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<td></td>
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<td>ERG11</td>
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<tr>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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In the presence of drug, the expression levels changed. For Isolate 1, the levels of CDR1, MDR1, and ERG11 were the highest at 4 µg/ml while CDR2 peaked at 2 µg/ml, its MIC80 (Figure 4 and Table 4, the MIC80 are boxed and the significant over expressers are bolded). Above these concentrations, all expression levels declined as the amount of drug increases. For Isolate 4, the levels of CDR1, CDR2, and ERG11 peak at 8 µg/ml, the MIC80, but MDR1 shows significant increase at 4 µg/ml (Figure 4B). For Isolate 13, the levels of all four genes, CDR1, CDR2, MDR1, and ERG11 peak at 32 µg/ml, the MIC80 (Figure 4C). For Isolate 17, all four genes were highly overexpressed without any FLC, which is the cause of its high MIC80 (>256 µg/ml). Because of Isolate 17’s high MIC80, testing gene expression at or above the MIC80 is not possible (Figure 4D and Table 4). Only CDR2 expression at 8 µg/ml and ERG11 expression at 128 µg/ml showed a significant increase. The data was summarized in Table 4 and the expression levels were color coded with blue being the lower levels of gene expression and orange being the higher levels.
Figure 4
**Figure 4: mRNA expression levels** is shown for CDR1, CDR2, MDR1, and ERG11 genes of a) Isolate 1, b) Isolate 4, c) Isolate 13, d) Isolate 17. The data was normalized to each isolate in the absence of FLC. ACT1 expression was used as a control. The experiment was done in biological triplicates. * - p<0.05, ** - p=0.05-0.001, *** - p<0.001.
Table 4: Summary of qRT-PCR under various FLC concentrations

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</table>
DISCUSSION

The experiments described above were done in order to determine if altering FLC doses alters the gene induction response in *Candida albicans*. While previous works have shown that there are genes that overexpressed in isolates 4, 13, and 17 when compared to isolate 1, none of studies showed how this expression varied at FLC concentrations below or above their MIC₈₀. In the absence of fluconazole Isolate 1 has the lowest expression of CDR1, CDR2, MDR1, and ERG11, whereas Isolate 17 overexpress all four genes. The levels for isolates 4 and 13 are intermediate (Table 3).

CDR1 and CDR2 showed maximum induction for isolates 1, 4, and 13 at or near their respective MIC₈₀ values. At higher concentrations expression levels decline. This could be because of FLC’s fungistatic nature that leads to a lower mRNA production because of cell death or stasis. At a very high concentration, the cells can switch on other anti-stress mechanisms, which are not identified yet, that increase their resistance. Isolate 1 shows a similar peaking pattern for its induction of MDR1 and ERG11. MIC₈₀ correlated with the decline in the expression levels, as *C.albicans* cells may have started to die or undergo stasis at high concentrations of FLC. This is likely the reason for the decline in the growth for Isolates 1 and 4 at increasing drug concentrations (Figure 3A and 3B). MDR1 starts its over expression at isolate 4 in the series; hence Isolate 4 does not show significant overexpression with increasing drug concentrations. There is very little statistically significant expression for Isolate 17 for all four genes because it over expresses the genes in the absence of drug, making it resistant to FLC. Hence, at
concentrations below its MIC₈₀ (<256 µg/ml), FLC did not have a significant effect on gene induction.

Whether this expression pattern of the genes is due to similar changes in their regulators still needs to be seen. MRR1, the upregulator of MDR1, TAC1, the activator of CDR1/2, and UPC2, the transcriptional regulator of ERG11 (Morio, Pagniez et al. 2013) could be analyzed in a similar fashion as the genes in this study. A recent study has shown that neither TAC1 nor MRR1 gain of function mutation changes the virulence of Candida, while the hyperactivity of UCP2 reduced the virulence (Lohberger, Coste et al. 2014). This can be further cemented by a study that can show transcription factor induction in relation to FLC dosage in the susceptible and resistant clinical isolates. Other transcription factors may also be researched, e.g. Cas5 which is part of the resistance fluconazole resistance mechanism (Vasicek, Berkow et al. 2014).

Since mRNA expression does not necessarily correlate with the protein expression, an analysis similar to that described in this paper can be performed with the protein levels of CDR1, CDR2, MDR1, and ERG11 for the four isolates. Immunoblotting may be used to see the levels of the four proteins found at different drug concentrations. A similar result would solidify our findings of the expression patterns and show that FLC concentrations in relation to the isolates’ MIC₈₀ can affect the gene at the transcriptional and translational levels. A comprehensive study like this can be useful for a future drug assay or experiment, noting that the expression levels of genes are not the same at FLC concentrations, and the variation is dependent on the strain and its MIC₈₀.


VITA

Aditi Khanna was born on March 24, 1989, in Lucknow, U.P, India. She graduated from Springdales School in 2006. She received an academic scholarship for International Students to The University of Iowa, Iowa City, Iowa where she received a Bachelor of Science in Biochemistry with a minor in Biology and Chemistry in 2011. Throughout her undergraduate career she worked as an undergraduate student assistant at University of Iowa Hospitals and Clinics, and finished an undergraduate thesis under Dr. Bryce V. Plapp. She worked full-time as a Research Assistant at the department of Ophthalmology at The University of Iowa between 2011-2013. In 2013 she enrolled in the Master of Science program at the School of Biological Sciences at the University of Missouri-Kansas City.