

# Ketorolac-fluconazole: A New Combination Reverting Resistance in *Candida albicans* from Acute Myeloid Leukemia Patients on Induction Chemotherapy: In vitro Study

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**Background and Objectives:** *Candida albicans* is a significant source of morbidity and mortality for patients with acute myeloid leukemia (AML). Prolonged use of fluconazole as empirical antifungal prophylaxis in AML patients leads to overexpression of efflux pump genes that resulted in the emergence of azole-resistant species. Consequently, the introduction of a new strategy to improve the management of *C. albicans* infections is an urgent need. Nonsteroidal anti-inflammatory drug (NSAID) ketorolac is associated with a reduction in cancer relapses. The present study was performed to investigate the use of ketorolac-fluconazole combination to reverse fluconazole resistance in *C. albicans* isolated from AML patients on induction chemotherapy.

**Patients and Methods:** One hundred and seventy AML patients were evaluated. Fifty *C. albicans* were isolated and subjected to disc diffusion assay and broth microdilution for fluconazole alone and combined with different concentrations of ketorolac. Efflux pump gene (*CDR1*, *CDR2*, and *MDR1*) expressions were quantified by real-time PCR.

**Results:** The tested ketorolac acted synergistically with fluconazole against resistant *C. albicans* with the minimum inhibitory concentration (MIC) of fluconazole decreased from >160 µg/mL to 0.3–1.25 µg/mL in (93.8%) of resistant isolates with fractional inhibitory concentration index (FICI) value of 0.25. The majority of the resistant isolates overexpressed *CDR1* (71.1%) and *MDR1* (60%).

**Conclusion:** Ketorolac-fluconazole in vitro combination would be a promising strategy for further clinical in vivo trials to overcome fluconazole resistance in AML patients on induction chemotherapy.

**Keywords:** ketorolac, fluconazole resistance, acute myeloid leukemia, *CDR1*, *MDR1*

## Introduction

Acute myeloid leukemia (AML) is a hematological disease caused by the clonal expansion of myeloblasts in the peripheral blood, bone marrow, or other tissues. It is characterized by various chromosomal abnormalities and gene mutations.<sup>1</sup> The typical clinical manifestations of AML are fever, fatigue, and bleeding caused by the expansion of blasts and decreased normal hematopoiesis in the bone marrow. Treatment of AML by combination chemotherapy results in persistent neutropenia, which further increases the risk of opportunistic infections.<sup>2</sup>

*Candida* spp. is an important opportunistic human pathogen that causes oropharyngeal candidiasis (OPC), vulvovaginitis, and invasive infections in AML

patients.<sup>3</sup> The of American Infectious Diseases Society has approved fluconazole as a primary drug of choice for prophylaxis and treatment of candidiasis.<sup>4</sup>

Fluconazole has several advantages over other antifungal drugs including the safety, oral bioavailability, cost, and ability to cross the blood–brain barrier.<sup>5</sup> Fluconazole inhibits the cytochrome P450 enzyme lanosterol demethylase, a critical enzyme in the synthesis of ergosterol which is encoded by the *ERG11* gene.<sup>6</sup> However, the extensive use of fluconazole as empirical therapy in cancer patients, especially in AML patients, has increased the incidence of resistance to the drug among different fungal strains, especially *Candida albicans*.<sup>7</sup> Fluconazole resistance has the potential to cross over to other azoles including voriconazole and itraconazole.<sup>8</sup>

There are multiple mechanisms for azole resistance, the major one is overexpression of plasma membrane efflux pumps.<sup>9</sup> The ATP-binding cassette (ABC) pumps and the major facilitator superfamily (MFS) transporters are the two main families of efflux proteins. They differ in the source of energy used for their activity and encoded by *Candida* drug resistance (*CDR1* and *CDR2*) and multidrug resistance (*MDR1*) genes. They are located in the plasma membrane and are responsible for pumping the drug out of the fungal cell decreasing its intracellular concentration which leads to treatment failure.<sup>10</sup>

Several studies<sup>11,12</sup> have stated the ability of some drugs to reverse azole resistance in *C. albicans*. Generally, these drug concentrations required to reverse the azole resistance are above the therapeutic concentrations. Moreover, some of these drugs can result in serious side effects, such as those caused by cyclosporine and tacrolimus.<sup>13</sup> Remarkably, a few studies have reported the ability of some nonsteroidal anti-inflammatory drugs (NSAIDs) to act synergistically with different antifungals. For example, ibuprofen was found to exhibit synergistic activity with azoles against *Candida spp.*<sup>14</sup> NSAIDs have antipyretic, analgesic, and can be used alone or in combination with other drugs for the treatment of cancer. They also have direct and indirect antimicrobial effects.<sup>15</sup> The antifungal activities of NSAIDs against *Candida spp.* include the reduction of extracellular polysaccharide, hyphal, and biofilm formations.<sup>16</sup>

To repurpose drugs and explore new leads in the field of antifungal drug discovery; we explored another nonsteroidal anti-inflammatory drug “ketorolac” to reverse azole resistance in *C. albicans*. Ketorolac is a potent analgesic, antipyretic, and moderate anti-inflammatory drug used in

the treatment of severe cancer pain. The simple use of this safe and effective anti-inflammatory agent might eliminate most early cancer relapses.<sup>17</sup>

In the current study, we investigate a new strategy to improve the management of *C. albicans* infections through the use of in vitro ketorolac-fluconazole in combination to reverse fluconazole resistance in *C. albicans* isolated from AML patients and overexpressing efflux pumps genes (*CDR1*, *CDR2*, and *MDR1*) assessed by quantitative real-time qRT-PCR.

## Materials and Methods

### Ethical Statement

This study was approved by the Local Ethics Committee (no. 17100543), Faculty of Medicine, Assiut University in accordance with the provisions of the Declaration of Helsinki. Informed written consent was obtained from all patients before enrolment in the study.

### Patients

This study included 170 AML patients admitted from October 29, 2016, to March 11, 2020, to the Department of Internal Medicine (Hematology Unit), and South Egypt Cancer Institute (SECI) in Assiut University, Assiut, Egypt. All newly diagnosed non-M3 AML patients (aged 18–69 years) were enrolled in this study. The patients were diagnosed according to the WHO criteria for AML.<sup>18</sup> Standard induction chemotherapy for 170 non-M3 AML patients in this study was idarubicin 12 mg/m<sup>2</sup> per day for two to three days, and cytarabine 100 mg/m<sup>2</sup>/day for five to seven days. Patients received prophylactic treatment during the period of neutropenia following chemotherapy in the form of sulfamethoxazole (400 mg/trimethoprim 80 mg once or twice daily). Patients received empirical azole prophylaxis, fluconazole (400 mg PO/IV per day).<sup>19</sup> During induction chemotherapy, granulocyte colony-stimulating factor (G-CSF) was used in a few cases showing poor performance status and not applied routinely for all patients. The definition of treatment response generally followed the European Leukemia Network (ELN) 2010 recommendation.<sup>20</sup> Complete remission (CR) was defined as a blast count less than 5% in the bone marrow. Partial remission (PR) was defined as a decrease in bone marrow blasts by 50% but still remaining in a range of 5–25%. Resistant disease (RD) was referred to those who failed to achieve CR or PR in bone marrow examination after chemotherapy. The undefined response was referred to

those who had no available results of bone marrow examination after chemotherapy. Exclusion criteria including AML (M3), AML with antecedent hematologic malignancy, Patients with possible risk factors of candidiasis, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) positive, diabetes, and autoimmune diseases. Patients with contraindication to fluconazole therapy as abnormal heart rhythm, prolonged QT interval on EKG, abnormal liver function tests, pregnancy, and chronic kidney disease: moderate to severe stage. Data were collected and included the following parameters: age, gender, and, cytogenetic results at diagnosis, induction regimens, and treatment response, Table 1.

### Standard Control Strain

*C. albicans* ATCC 10231 was obtained from MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University, Egypt.

**Table 1** Clinical Data of 170 Patients with Newly Diagnosed Acute Myeloid Leukemia

Character	N (%)
<b>Gender</b>	72 F/98 M
<b>Median Age in years (range)</b>	49 (18–69)
<b>AML Diagnosis</b>	
<b>AML (M0)</b> Undifferentiated acute myeloblastic leukemia	11 (6.47)
<b>AML (M1)</b> Acute myeloblastic leukemia with minimal maturation	33 (19.41)
<b>AML (M2)</b> Acute myeloblastic leukemia with maturation	49 (28.82)
<b>AML (M4)</b> Myelomonocytic	51 (30)
<b>AML (M5)</b> Monocytic	19 (11.18)
<b>AML (M6)</b> Acute erythroid leukemia	4 (2.36)
<b>AML (M7)</b> Acute megakaryocytic leukemia	3 (1.76)
<b>Cytogenetic/Molecular Risk</b>	
Favorable risk	29 (17.05)
Intermediate risk	64 (37.67)
Unfavorable risk	31 (18.23)
Not available	46 (27.05)
<b>Neutropenic Fever</b>	116 (68.23)
<b>Response of Induction Chemotherapy</b>	
Complete remission (CR)	81 (47.64)
Partial remission (PR)	20 (11.76)
Resistant disease (RD)	46 (27.05)
Undefined response	23 (13.52)

### Clinical Sample Collection and Processing

Samples collected from the patients according to their clinical presentation and different localizing symptoms. Vaginal swabs from patients with vaginal infection. Oropharyngeal swabs from patients with oral thrush were rubbed on the candidiasis lesion. Swabs were streaked on Sabouraud dextrose agar medium supplemented with chloramphenicol (0.5 g/L) (SDA, HiMedia, India) and incubated at 30°C for 48 h.<sup>21</sup> *Candida* spp. shows typical creamy white pasty colonies with characteristic yeasty odor. Each isolated *Candida* spp. was individually stored at –20°C with 20% glycerol.<sup>22</sup>

### Phenotypic Identification of *Candida albicans* Strains

- Germ tube test: by inoculating yeasts in small tubes containing 0.5 mL of human serum (Sigma-Aldrich, Germany) containing 0.5% glucose and incubated at 37°C for two to three hours. It is positive for *C. albicans* or *C. dubliniensis*.<sup>22</sup>
- Corn meal agar (CMA): by inoculating yeasts on CMA containing Tween 80 (Difco, USA), for four to seven days to ensure production of chlamydospores. It is positive for *C. albicans* or *C. dubliniensis*.<sup>22</sup>
- CHROMagar<sup>®</sup> *Candida* medium: (CHROMagar, Paris, France) which allows selective isolation and identification of *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. krusei* by morphology and color reaction. The strains were identified as *C. albicans* or *C. dubliniensis* for green colonies, *C. tropicalis* for steel blue colonies, *C. krusei* colonies showing rose color.<sup>21,22</sup>
- Growth at 45°C: Growth at 45°C has been considered a useful test for the differentiation of *C. dubliniensis* (no growth) from *C. albicans* (growth).<sup>21,22</sup>
- Sugar assimilation test: using API 20C AUX (bioMérieux SA, France) according to the manufacturer's instructions.<sup>23</sup>

### Genotypic Identification of *Candida albicans* Strains by Polymerase Chain Reaction (PCR)

The species specific primer pair sequence for the amplification of the 25S rRNA gene was described by Mannarelli and Kurtzman,<sup>24</sup> (5'TGTTGCTCTCTCGGGGGCGGCC G3' and 5' AGATCATTATGCCAACATCCTAGGTAA

A 3'). It is specific for *C. albicans* and amplifies a 175-bp DNA fragment. DNA extraction was done by a commercial QIAamp DNA Mini Kit (Qiagen, Germany). DNA amplification performed using the Thermocycler T100 gradient system (BioRadT100, USA). PCR reaction mixture and PCR amplification conditions were performed according to the method described by Marinho et al.<sup>22</sup> Amplification product visualized by electrophoresis on 2% agarose gel using a 100-bp ladder molecular weight ladder (Gen Ruler 100bp DNA ladder plus).

## Antifungal Susceptibility Test

### Disk Diffusion Method

Adopted by the Clinical and Laboratory Standards Institute (CLSI), the M44-A2 protocol<sup>25</sup> was used to evaluate the degree of fungal sensitivity for four common azoles. Antifungal discs were obtained from (HiMedia, India). The response to the antifungal agents was determined via the interpretive breakpoints<sup>26</sup> described in Table 2.

### Determination of MICs by Broth Microdilution

The MICs of fluconazole (Sigma, USA) and ketorolac (Sigma, USA) separately were identified by the protocol recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).<sup>27</sup> The test was conducted in 96-well microtiter plates with yeast ( $2.5 \times 10^5$  CFU/mL) in RPMI-1640 medium (PH 7.0) buffered with MOPS (Sigma, USA) and supplemented with glucose to a final concentration of 2%. RPMI-1640 containing wells were considered negative controls, and a drug-free well was set as growth control. After 24 h of incubation at 35°C, MIC values were determined with a spectrophotometer (wavelength of 450 nm; ETI System Fast Reader ELX, BioTek, US) as the lowest concentration of drug that resulted in >50% inhibition of growth relative

to that of the growth control. MIC interpretive criteria of fluconazole for *C. albicans* were those described in the document E.DEF 7.3.1 [available on the EUCAST website: <http://www.eucast.org>]. MIC  $\leq 2.0$  mg/L was considered to be sensitive, MIC between 2.0 and 4.0 mg/L was considered to be intermediate and that >4.0 mg/L was considered resistant.

### Fractional Inhibitory Concentration Index (FICI)

The intensity of interaction between ketorolac and fluconazole antifungals against azole-resistant *C. albicans* clinical isolates was determined briefly as follow, drugs were serially diluted 2-fold in RPMI-1640 medium, and 0.31–160  $\mu\text{g/mL}$  fluconazole and 0.16–10  $\mu\text{g/mL}$  ketorolac were added to the wells. Subsequently, yeast at a final concentration of  $2.5 \times 10^5$  CFU/mL was added to each well. Wells containing only RPMI-1640 medium served as negative controls, and a drug-free well was set as a growth control. After 24 h of incubation at 35°C, MICs were determined as described above. To evaluate the intensity of the drug interactions, the fractional inhibitory concentration index (FICI) model was used to analyze the obtained data. The FICI model is based on the Loewe additivity theory<sup>28</sup> and is expressed as  $FICI = FIC_A + FIC_B = (A/MIC_A) + (B/MIC_B)$  where A and B are the MIC of each drug in combination (in a single well), and  $MIC_A$  and  $MIC_B$  are the MIC of each drug individually. The drug interaction is interpreted as synergistic when  $FICI \leq 0.5$ , indifferent when  $FICI > 0.5-4.0$ , and antagonistic when  $FICI > 4.0$ .<sup>29</sup>

### Efflux Pump Genes Expression Analysis by qRT-PCR

#### Reverse Transcriptase (RT)-PCR

The reverse transcription was performed using HiSenScript™ RH(-) cDNA Synthesis Kit (iNtRON, Cat. no. 25014) according to the manufacturer's instructions.

**Table 2** Interpretative Breakpoints of Disk Diffusion Method for Fluconazole, Itraconazole, Miconazole, and Voriconazole<sup>26</sup>

Antifungal	Abbr.	Conc./Disc	Zone Diameter in mm		
			S	I	R
Fluconazole	FU	25 $\mu\text{g}$	$\geq 19$	15–18	$\leq 14$
Itraconazole	IT	10 $\mu\text{g}$	$\geq 23$	14–22	$\leq 13$
Miconazole	MIC	10 $\mu\text{g}$	$\geq 20$	12–19	$\leq 11$
Voriconazole	VRC	1 $\mu\text{g}$	$\geq 17$	14–16	$\leq 13$

**Abbreviations:** S, susceptible; I, intermediate; R, resistant.

Reverse transcription was performed at 45°C for 60 min, followed by 80°C for 10 min. The cDNA products were stored at -20°C for later use as templates for quantitative real-time PCR (qPCR).

## Real-Time PCR

The procedures of qRT-PCR analysis were described in a previous study.<sup>30</sup> Expression levels of the target genes (*CDR1*, *CDR2*, and *MDR1*) and the housekeeping gene (*ACT1*, used as a normalizing gene) were assessed by quantitative real-time RT-PCR (qRT-PCR). The primers (analysis) used tested through the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) and listed in Table 3.<sup>31</sup> The expression of target genes was carried out using SensiFAST SYBR<sup>®</sup> No-ROX (Bioline, USA) using a 7500 Fast Real-Time PCR System (Applied Biosystems). The fold change in expression of these genes was calculated according to the  $2^{-\Delta\Delta CT}$  method using *ACT1* as a control gene to normalize cDNA levels.<sup>32</sup> Fluconazole-susceptible *C. albicans* clinical isolate (MIC, 0.5 mg/L) was used as a reference isolate for gene expression analysis. Real-time PCR reaction mix for (*CDR1*, *CDR2*, *MDR1* and *ACT1*) contained 0.5 µL of cDNA, 1.5 µL of 2 µM primer and 7.5 µL of 2×SYBR Green Master Mix in a final volume of 15 µL and qRT-PCR was performed using the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 seconds, 50°C for 15 seconds for *CDR1*, *CDR2*, and *ACT1*, or 55°C for 15 seconds for *MDR1*, and 60°C for 30 seconds.

## Statistical Analysis

Data entry and data analysis get done using SPSS (Statistical Package for Social Science) version 19. Data were presented as a number, percentage, mean, median, standard deviation, and standard error. Chi-squared test

and Fisher's exact test were used for comparing qualitative variables. The Mann-Whitney *U*-test was used to compare nonparametric tests. *P*-value considered statistically significant when *P*<0.05.

## Results

### Patient Population

The demographic and clinical characteristics of 170 non-M3-AML patients who received induction chemotherapy admitted to Clinical Hematology Unit, Internal Medicine Department, Assiut University Hospital, and South Egypt Cancer Institute (SECI) presented in Table 1. The median age was 49 years (range: 18–69 years). Female patients' were 72 (42.35%) while male patients were 98 (57.65%). The diagnosis was performed according to the WHO criteria for AML. There were mainly AML M4, AML M2, AML M1 and AML M5 (51 (30%), 49 (28.82%), 33 (19.41%) and 19 (11.17%) respectively. A total of 39 patients (22.9%) had favorable cytogenetic risk, 74 (43.52%) intermediate cytogenetic risk, and 41 (24.11%) unfavorable risk.

The collected samples were 76 oropharyngeal swabs, 42 vaginal swabs. Seventy-five samples were diagnosed as yeast infection from which 50 isolates were diagnosed as *Candida albicans* by phenotypic tests and PCR.

### Antifungal Susceptibility Test Disk Diffusion Method

The pattern of antifungal resistance to tested azoles for *Candida albicans* isolates was relatively high 94% for both fluconazole and voriconazole, 74% for miconazole and itraconazole as shown in Table 4.

### Ketorolac Acted Synergistically with Fluconazole Against Resistant *C. albicans* in vitro

The minimal inhibitory concentrations (MICs) of ketorolac and fluconazole against resistant *C. albicans* are listed in Table 5. The MIC of fluconazole was all >160 µg/mL for 94% of tested *C. albicans* isolates, indicating strong resistance of these *C. albicans* isolates. The MIC of ketorolac was >10 µg/mL. However, when used in combination with fluconazole, ketorolac could significantly decrease the MICs of fluconazole from >160 µg/mL to 0.3–1.25 µg/mL, indicating a significantly increased sensitivity of resistant *C. albicans* to fluconazole caused by ketorolac. When the MIC of FLC was decreased to <2 µg/mL, the

**Table 3** Gene-specific Primers Used for RT- qPCR<sup>31</sup>

Gene	Primer Sequence
<i>CDR1</i>	F: 5'- TGCCAAACAATCCAACAA-3' R: 5'-CGACGGATCACCTTTCATACGA-3'
<i>CDR2</i>	F: 5'- AAGGTTT TGATGCTACTGC-3' R: 5'-GTCGGACATGTGGCTCAAA-3'
<i>MDR1</i>	F: 5'-GTGTTGGCCATTGGTTTTTCAGTC-3' R: 5'-CCAAGCA GTGGGGATTGTAG-3'
<i>ACT1</i>	F: 5'-AAGAATTGATTTGGCTGGTAGAGA-3' R: 5'-TGGCAGAAGATTGAGAAGAAGTTT-3'



**Table 4** Resistance Patterns of 50 *C. albicans* Using a Kirby–Bauer Disk Diffusion Method for Azoles

Total (n= 50)	Fluconazole (25 µg/Disc) N (%)	Miconazole (10 µg/Disc) N (%)	Voriconazole (1 µg/Disc) N (%)	Itraconazole (10 µg/Disc) N (%)
Sensitive (S)	3 (6)	3 (6)	3 (6)	3 (6)
Intermediate (I)	0 (0)	10 (20)	0 (0)	10 (20)
Resistant (R)	47 (94)	37 (74)	47 (94)	37 (74)

**Note:** Data are presented as number (%) of isolates resistant to antimicrobial indicated.

**Table 5** In vitro Interaction of Ketorolac with Fluconazole Against Resistant *C. albicans*

Mean MIC (µg/mL)		Mean MIC (µg/mL)		Mean FICI	IN
Alone		Combination			
Ketorolac	Fluconazole	Ketorolac	Fluconazole		
>10	>160	2.5	0.62	0.25	SYN

**Abbreviations:** IN, interpretation; SYN, synergism.

concentration of ketorolac required was 2.5 µg/mL. Moreover, the FICI values obtained from the FICI model were <0.5, showing a strong synergism induced by ketorolac plus fluconazole.

### Efflux Pump Genes Expression Analysis

*CDR1*, *CDR2*, and *MDR1* gene expression levels were quantified for all 50 isolates and normalized relative to the housekeeping gene, *ACT1*. This study found that the *MDR1* gene showed the most gene overexpression (88%) followed by *CDR1* gene (84%), and finally *CDR2* gene (12%). When comparing the relationship between fluconazole MICs and the expression of efflux-related genes (Figure 1), it was clear that isolates with higher fluconazole MIC (>4.0 mg/L) showed higher expression levels of *CDR1* and *MDR1*, Table 6. These results confirm those expression levels of efflux-related genes *CDR1* and *MDR1* agree with fluconazole MICs in the *C. albicans* isolates.

### Discussion

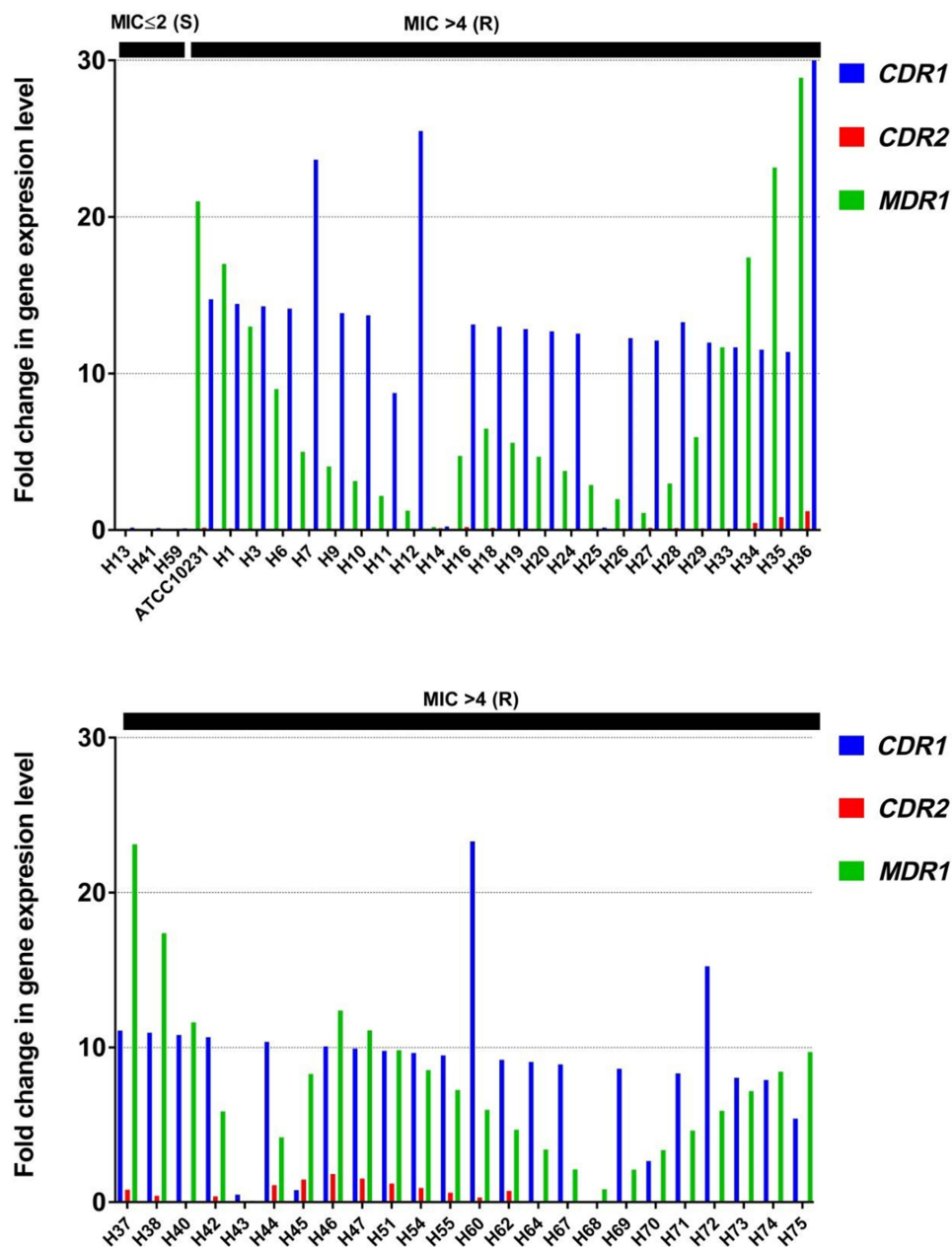
In Egypt, leukemia comprises (10%) all malignancies, with AML representing (16.9%).<sup>33</sup> This study included 170, non-M3 (acute promyelocytic) AML patients. The ages ranged from 18 to 69 years, with a mean of 49 years. A slight male predominance was noted (female to male ratio was 1:1.36). The commonest FAB subgroup was M4 followed by M2. Another study at National Cancer Institute (NCI)-Egypt on 82 adult AML patients showed epidemiological characteristics of AML Egyptian patients, slightly similar to our data. Their ages ranged

between 18 and 68 years with a median of 34 years. The male to female ratio was 1.05:1, and the commonest FAB subgroups reported were M1 and M2.<sup>34</sup>

Another Egyptian study included ninety AML patients. The ages ranged from 18 to 76 years, with a mean of 37.8 years. A slight female predominance was noted (male to female ratio was 1:1.3). The commonest FAB subgroup was M2 followed by M5.<sup>35</sup>

Response of induction chemotherapy in the current study, were complete remission (CR), partial remission, resistant disease and undefined response: 81 (47.64%), 20 (11.76%), 46 (27.05%) and 23 (13.52%), respectively. The time elapsed between presentation and start of treatment ranged from 4 to 85 days, with a mean of 13 days. The treatment delay in most patients of our study was mainly due to uncontrolled infection. An Egyptian study showed CR achievement in (57.5%) of AML patients, which is a slightly higher rate than that of our study.<sup>35</sup> It can be explained that we adopted treatment strategy interruption with neutropenic fever onset, which reduced treatment-related mortality at the expense of response rate. Also, slightly longer duration from onset of presentation to treatment beginning when compared with such study.

A retrospective study on 1317 AML patients had time elapsed between presentation and start of treatment with a median of four days, and a range of 1–78 days. The longer time was associated with worse CR in patients younger than 60 years, and this effect was more pronounced with duration of five days or more.<sup>36</sup> It is crucial that public health systems in developing countries (DC), including



**Figure 1** Efflux pump gene expression of *C. albicans* isolates in relation to the MIC of fluconazole.

Egypt, turn to larger epidemiological studies to better understand how the disease characteristics interact with socioeconomic factors. An appropriate health system must shorten the time from diagnosis to treatment to ensure a better outcome of induction therapy and more successful result in AML.

*C. albicans* is an important cause of morbidity and mortality for AML patients. The rise of multidrug resistant organisms causes a challenge in the treatment of infective diseases<sup>37</sup> Resistance among *C. albicans* represents a serious therapeutic problem that is mainly attributed to the

overexpression of efflux pump genes encoded by *CDR1*, *CDR2* (related to azole cross-resistance), and *MDR1* genes (confined to selective resistance to fluconazole).<sup>38</sup>

In our study, in vitro susceptibility testing for *C. albicans* isolated from AML patients to four azoles using disc diffusion method showed a high level of resistance pattern that was 94% for both fluconazole and voriconazole, 74% for miconazole and itraconazole. Also, the MIC of fluconazole was all >160 µg/mL for 94% of tested *C. albicans* isolates, indicating a very high resistance of these *C. albicans* isolates, which is consistent with 86.2% and

**Table 6** The Level of the Efflux Pumps Gene Expression in Resistant and Sensitive Strains in the Human Study Group

Gene	Mean $\pm$ SE	Median	P-value
<b>CDR1</b>			
Resistant strains	11.16 $\pm$ 0.9	11.02	0.006**
Sensitive strains	0.13 $\pm$ 0.12	0.13	
<b>CDR2</b>			
Resistant strains	0.33 $\pm$ 0.07	0.1	0.078*
Sensitive strains	0.02 $\pm$ 0.003	0.02	
<b>MDR1</b>			
Resistant strains	7.81 $\pm$ 0.96	5.88	0.004***
Sensitive strains	0.37 $\pm$ 0.003	0.04	

Notes: \*Nonsignificant ( $P > 0.05$ ), \*\*significant ( $P < 0.05$ ), \*\*\*highly significant ( $P < 0.005$ ).

75.9 fluconazole resistance among *C. albicans* isolated in two recent local studies in Egypt, respectively.<sup>39,40</sup>

All the MICs obtained to support the antibiogram of isolates to fluconazole, which indicates that disk diffusion has a good correlation with MIC. Pfaller et al showed similar results.<sup>41</sup>

Many researchers indicated that prolonged therapy and increased use of antifungals for prophylaxis or treatment of recurrent candidiasis are the most common risk factors to azole resistance.<sup>42</sup> To overcome fungal resistance, research on antifungal sensitizers has attracted considerable attention.<sup>43</sup> The need for novel antifungal regimens to overcome resistance prompted us to study the activity of ketorolac, which has a superior effect on pain control in cancer patients that suffer from frequent and recurrent *Candida* infections which showed a high level of resistance to the most common group of antifungals, the azoles, especially fluconazole.

In vitro, we found that ketorolac acted synergistically with fluconazole against tested *C. albicans* isolates, as interpreted by the FICI as it decreased the MIC of fluconazole by  $>4$  folds against 93.6% of resistant isolates (new finding). This is superior to the recently reported 60.9% reversal of fluconazole resistance using ibuprofen by Sharma et al.<sup>44</sup>

The MIC of ketorolac was  $>10$   $\mu\text{g/mL}$ . However, when used in combination with fluconazole, ketorolac could significantly decrease the MICs of fluconazole from  $>160$   $\mu\text{g/mL}$  to 0.3–1.25  $\mu\text{g/mL}$ , indicating a significantly increased sensitivity of resistant *C. albicans* to fluconazole caused by ketorolac. When the MIC of FLC was decreased to  $<2$   $\mu\text{g/mL}$ , the concentration of ketorolac required was 2.5  $\mu\text{g/mL}$ . Similar study on the combination of ibuprofen with fluconazole showed synergic activity in 8/12 of studied *Candida spp.* including four of the five fluconazole-resistant strains. The MICs of fluconazole in fluconazole-resistant *Candida spp.*

decreased 2 to 128-fold when the drug was associated with ibuprofen. Also the MICs for ibuprofen decreased 64-fold for the 12 studied *Candida spp.* They reported the practicability of using ibuprofen in combination with fluconazole in the treatment of *Candida* infections.<sup>14</sup>

The continuous emergence of resistance to conventional drugs through efflux pumps leads to increasing efforts directed toward discovering efflux inhibitory molecules.<sup>45</sup> This study found that the MDR1 gene was the gene that showed the most overexpression (88%) followed by *CDR1* gene (84%), and finally *CDR2* gene (12%). This finding is suggesting that *CDR1* protein contributes more than *CDR2* protein in resistance by ABC family of efflux pump which agreed with the finding of Tsao et al,<sup>46</sup> and Holmes et al<sup>47</sup> who conclude that in *C. albicans* Cdr1p efflux activity makes a greater contribution than Cdr2p to resistance to fluconazole and Cdr1p was present in greater amounts (2 to 20-fold) than Cdr2p. This result was contradictory with Chau et al<sup>30</sup> who found that *CDR2* was overexpressed in the majority of the patient isolates. This can be explained by that reported by Niimi et al<sup>48</sup> who found that the strains hyperexpression *CDR2* showed decreased susceptibility to caspofungin in agar plate drug resistance assays because ABC transporters confer resistance to a wide range of structurally unrelated xenobiotics so *CDR2* may be related to other antifungal resistance.

In three *C. albicans* isolates, although their resistance profile to fluconazole, they exhibited downregulation of genes of efflux pump which suggested different azole resistance mechanisms as those belonging to *ERG11* which was described.<sup>49</sup>

## Conclusion

To our knowledge, the current study is the first in vitro report on the use of ketorolac in reverting fluconazole resistance in



*C. albicans* isolated from AML patients. Resistance of *C. albicans* to azole antifungals is associated with overexpression of efflux pump genes especially *CDR1* and *MDR1*. Ketorolac concentration as low as (2.5 µg/mL) was able to revert resistance in 93.8% of tested strains, so the current study recommends for the next step to run clinical studies based on the in vivo ketorolac-fluconazole combination therapy for AML patients.

## Data Sharing Statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

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