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Original article

Potent drugs that attenuate anti-*Candida albicans* activity of fluconazole and their possible mechanisms of action

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ABSTRACT

Fluconazole (FLCZ) is a first-line drug for treating *Candida albicans* infections, but clinical failure due to reduced sensitivity is a growing concern. Our previous study suggested that certain drug combinations pose a particular challenge in potently reducing FLCZ's anti-*C. albicans* activity, and cyclooxygenase inhibitors formed the major group of these attenuating drugs in combination with FLCZ. In this study, we examined the effects of diclofenac sodium (DFNa) and related compounds in combination with FLCZ against *C. albicans*, and investigated their possible mechanisms of interaction. DFNa, ibuprofen, and omeprazole elevated the minimum inhibitory concentration (MIC) of FLCZ by 8-, 4-, and 4-fold, respectively; however, loxoprofen sodium and celecoxib did not. An analogue of DFNa, 2,6-dichlorodiphenylamine, also elevated the MIC by 4-fold. Gene expression analysis revealed that diclofenac sodium induced *CDR1* efflux pump activity, but not *CDR2* activity. In addition, an efflux pump *CDR1* mutant, which was manipulated to not be induced by DFNa, showed less elevation of MIC compared to that shown by the wild type. Therefore, DFNa and related compounds are potent factors for reducing the sensitivity of *C. albicans* to FLCZ partly via induction of an efflux pump. Although it is not known whether such antagonism is relevant to the clinical treatment failure observed, further investigation of the molecular mechanisms underlying the reduction of FLCZ's anti-*C. albicans* activity is expected to promote safer and more effective use of the drug.

1. Introduction

Candida albicans is known as the primary cause of systemic candidiasis, which has a high mortality rate [1]. Fluconazole (FLCZ) is a first-line drug for *C. albicans* infections, but clinical failure due to reduced drug sensitivity is a growing concern [2]. In addition, alternative therapeutic options are limited, and the development of new drugs has been slow. Therefore, the identification of effective and safe conventional antifungal agents is necessary. For this purpose, we previously investigated the combinatorial effect of FLCZ with other drugs, and showed that certain stress response inhibitors could enhance the effects of azoles and echinocandins [3,4]. In another study in which we screened the combinatorial effects of FLCZ with 640 drugs approved by the Food and Drug Administration (FDA), we found that some drugs could attenuate the anti-*Candida* activity of FLCZ [5], suggesting that certain combinations have a tendency to potently reduce the antifungal activity. One major group of such attenuating drugs was identified as the cyclooxygenase (COX) inhibitors, which are also known as nonsteroidal anti-inflammatory drugs (NSAIDs) and are frequently used as antipyretics and analgesics. In contrast to our findings, other previous reports suggested that COX inhibitors could synergistically or additively enhance the anti-*C. albicans* and anti-*Candida* biofilm activity of FLCZ [6–15]. However, relatively high concentrations of these drugs were used in these studies. In order to estimate the actual interaction of these drugs, we examined their dose- and structure-dependent effects, and investigated the possible mechanisms underlying their combinatorial effects with FLCZ against *C. albicans*.

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2. Materials and methods

2.1. Chemicals

All general chemicals used in this study were purchased from Wako Chemicals (Tokyo, Japan) unless otherwise indicated, and were of the highest purity available. Ultra-pure water dispensed by a Milli-Q water system (Millipore; Bedford, MA, USA) was used for the preparation of buffers and solvents. FLCZ was purchased from Sigma Aldrich (St. Louis, MO, USA). Four COX inhibitors, diclofenac sodium (DFNa), ibuprofen (IBU), loxoprofen sodium (LOX), and celecoxib (CEL), a proton pump inhibitor, omeprazole (OPZ), and an analogue of DFNa, 2,6-dichlorodiphenylamine (2,6-DPA), were used as the combination drugs. Each drug was dissolved in dimethylsulfoxide (DMSO) at 2 mg/mL for stock solution and stored at −20 °C. Standard ergosterol was dissolved in methanol at 1 mg/mL.

2.2. Strains and growth conditions

The standard *C. albicans* strain SC5314 and an efflux pump *CDR1* mutant, TU202, were used in this study [16]. TU202 has an *ACT1* promoter-driven *CDR1* gene instead of the disrupted native *CDR1* gene (Δ*CDR1* with p*ACT1-CDR1*), which results in constant *CDR1* expression independent of exogenous stimulation. The strains used are listed in Supplemental Table S1. We also used two additional *C. albicans* strains ATCC10231 and ATCC10261.

We used yeast nitrogen base medium (YNB; Difco Laboratories; USA) with 2% dextrose (YNB2D) instead of RPMI medium, which is recommended as standard medium by the Clinical and Laboratory Standards Institute (CLSI). YNB2D was used because growth was slow in RPMI, which made it difficult to detect the combinatorial effect. A single colony was inoculated in the medium, and cells were grown in YNB2D at 37 °C with agitation. For microdilution, after the cell density of the overnight culture was measured, the cell suspension was diluted with YNB2D to inoculate approximately 1×10^4 colony-forming units (cfu)/mL of cells for the subsequent experiments. For cellular sterol and gene expression analysis, mid-log phase yeast was incubated with or without DFNa (25 μM) and/or FLCZ (0.5 μg/mL) in 5 mL of medium at 37 °C with agitation, and the cells were pelleted 4 h after treatment.

DFNa is an FDA-approved drug, and we previously reported that it attenuated FLCZ activity against *C. albicans* [5]. We tested the dose-dependent effect of DFNa on the anti-*C. albicans* activity of FLCZ. High concentration of DFNa (>250 μM) alone remarkably inhibited the growth of *C. albicans*; therefore, the combinatorial effect was tested at lower concentrations.

2.3. Microdilution methods for evaluation of combinatorial effects

The cells were seeded in 96-well plates in the presence of the combination drugs and FLCZ and incubated at 37 °C without agitation. FLCZ was serially diluted in the plates and the other drugs were used at the doses indicated in Table 1. After 24 h, cell growth was monitored by measuring the optical density at 630 nm (OD_{630}) by using a microplate reader.

Minimum inhibitory concentration (MIC) was defined as 50% or more growth inhibition compared to the growth without FLCZ treatment, and the growth inhibition was evaluated by the reduction of the OD_{630} as measured above.

2.4. Analysis of cellular sterols

C. albicans is known to have several resistance mechanisms: 1) alternative sterol synthesis, 2) overexpression of ergosterol synthesis, and 3) activated or overexpressed efflux pumps. We first investigated whether DFNa altered ergosterol synthesis by using TLC analysis. Cell pellets were suspended in 4 mL of 1% NaCl solution and lipids were extracted by using Bligh-Dyer methods [17]. The extracted lipids were dried and resolved in chloroform/methanol (2:1). Standard ergosterol (5 μg) and 5 μL of the solution were spotted on a thin layer chromatography (TLC) plate, and lipids were separated in hexan/diethylether/acetate (25:25:1). Spots were visualized after spraying $FeCl_3/CH_3COOH/H_2SO_4$ solution on the plate, and the image was obtained using a scanner.

Table 1
Effects of COX inhibitors and a proton pump inhibitor on the antifungal activity of FLCZ against *C. albicans* SC5314 and TU202.

Strains and drugs		MIC (μg/mL)
SC5314 (wild type)		
FLCZ only		0.5
FLCZ + COX inhibitor	DFNa (25 μM)	4
	IBU (20 μM)	2
	LOX (20 μM)	0.5
	LOX (160 μM)	0.5
	CEL (25 μM)	0.5
	CEL (50 μM)	0.5
FLCZ + analogue of DFNa	2,6-DPA (25 μM)	2
FLCZ + proton pump inhibitor	OPZ (20 μM)	2
TU202 (Δ*CDR1* with p*ACT1-CDR1*)		
FLCZ only		0.5
FLCZ + DFNa (25 μM)		1

2.5. Analysis of gene expression

RNA extraction from the pelleted cells and the subsequent real-time polymerase chain reaction (RT-PCR) were performed as previously reported [3,18]. Briefly, approximately 800 ng of total RNA was used as a template to synthesize the cDNA (final volume, 20 μL) and cDNA equivalent to approximately 40 ng of total RNA was used as a template for RT-PCR. The actin gene, *ACT1*, was used as the internal control, and all expression values were normalized against *ACT1* expression. The sequences of the RT-PCR primers are listed in Supplemental Table S1. The data were analysed using Student's *t*-tests. The data are presented as fold changes in comparison to the control (untreated) and the mean ± standard error (SE) of replicates ($n = 4$). The data are representative of 3 or more individual experiments.

3. Results

3.1. DFNa and related compounds reduce the anti-C. albicans activity of FLCZ

At 2.5 μM and 25 μM, DFNa shifted the dose–response curves, indicating a reduction in the anti-*C. albicans* activity of FLCZ (Fig. 1). DFNa also shifted the dose–response curves in the two additional *C. albicans* strains ATCC10231 and ATCC10261 (Supplemental Fig. S1).

To evaluate whether the COX structure or inhibiting function affect the anti-*C. albicans* activity of FLCZ, the COX-related compounds were tested (Table 1). IBU also elevated the MICs by 4-fold; however, LOX and CEL did not alter the MICs, even at doses of 160 μM and 50 μM, respectively. We also tested an analogue of DFNa, 2,6-DPA, which similarly elevated the MICs by 4-fold. OPZ also elevated the MICs by 4-fold.

3.2. Attenuated anti-C. albicans activity of FLCZ partly depends on induced CDR1 expression

FLCZ quenched ergosterol synthesis (Fig. 2, lane 3), but ergosterol reappeared when DFNa was added (Fig. 2, lane 4), and DFNa alone did not alter the ergosterol spot pattern (Fig. 2, lane 5).