TABLE 2 Genetic characterization of isolated *Candida glabrata* strains[b]

Strain (MIC [μg/ml] of MCFG)	Characterisitic(s) of gene:	
	FKS1	*FKS2*
NO1 (<0.015)	Wild type	Wild type
NO2 (<0.015)	Wild type	Wild type
NO3 (<0.015)	Wild type	F659Δ L1767Δ
NO4 (2)	Gene conversion[a]	F659Δ
NO5 (4)	Gene conversion[a]	F659Δ

[a] The gene with the insertion is predicted to encode an Fks1 protein with the following changes: M555T, V558I, L563V, V568I, T583S, H600Q, A620S, and Y623Δ.
[b] MCFG, micafungin. The sequence type for every strain was 22.

kg/day) on day 53. However, *C. glabrata* was still isolated from a blood culture taken on day 56 (NO5), and the patient died of septic shock on day 59. Following the patient's death, the NO3 strain was shown to be susceptible to micafungin (MIC, <0.015 μg/ml), while the NO4 and NO5 strains showed resistance to micafungin (based on CLSI document M27-S4), the MICs of which were 2 and 4 μg/ml, respectively (Table 1).

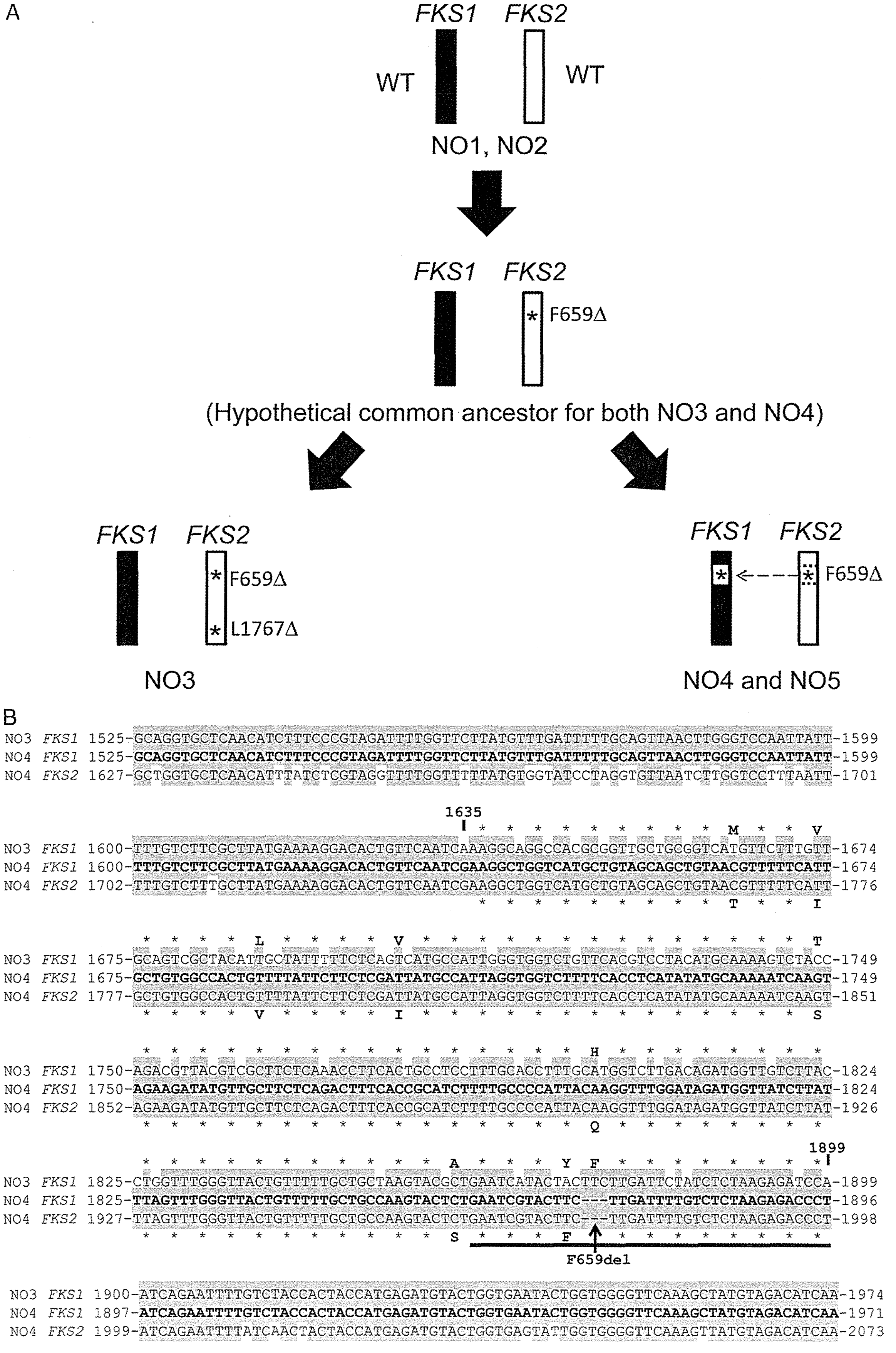
We performed morphological and genetic analyses for isolates NO1 to NO5. Interestingly, colonies observed in the isolation step for the NO1 and NO2 strains (Fig. S1A in the supplemental material) were of a size typical of *C. glabrata* ("normal"; they were light purple); in contrast, colonies for the NO3 and NO4 strains (Fig. S1B in the supplemental material) were heterogeneous, consisting of a mixture of small dark-purple and normal light-purple colonies, and the isolation step for NO5 (Fig. S1C in the supplemental material) yielded colonies of uniform size that were consistently small and darkly colored on CHROMagar *Candida* medium (Becton, Dickinson and Company). The change in colony size was thought to be due to mitochondrial deficiency (petite mutant) or *FKS* mutations, as described below. These results also suggest that the blood culture from which NO3 and NO4 were isolated contain several *C. glabrata* clones with heterogeneous growth rates. To examine whether the five clinical isolates originated from a single strain, randomly amplified polymorphic DNA (RAPD) and multilocus sequence typing (MLST) analyses were performed. Briefly, the template genomic DNA was extracted from *C. glabrata* cells, and a series of PCR and DNA sequencing reactions were performed using the primers indicated in Table S1 in the supplemental material. In RAPD assays (performed per previously reported methods [2]), all five of the tested strains yielded identical amplification patterns (data not shown). Furthermore, MLST analysis revealed that the five strains demonstrate a shared sequence type, ST22 (Table 2) (http://cglabrata.mlst.net/). These results suggest that the strains were probably derived from a single parental strain; however, more-detailed genetic analyses would be necessary for identification of the source of the strains.

Cases harboring *Candida* spp. with reduced susceptibility to echinocandins are still uncommon, and strains of *C. glabrata* ranked as nonsusceptible to echinocandin have been reported only rarely in Japan (3, 4, 5). In previous reports, reduced susceptibility to echinocandins was related primarily to single point mutations within the *FKS* genes, which code for β-1,3-glucan-synthases. A total of 48 distinct mutations have been reported in 5 different yeast species, with 44 lesions occurring in hot spot 1 and 4 lesions occurring in hot spot 2 (6, 7, 8). The majority of the *FKS1* mutations are predicted to result in an amino acid substitution at S629P (9), Phe625 (F625S or F625I), or Asp632 (D632G, D632E, or D632Y); *FKS2* mutations are predicted to result in a substitution (F659V, F659S, or F659Y) (10) or a deletion (a deletion of F659 [F659del]) (11) in Phe659, as well as multiple mutations in residues 662 to 667, especially S663P (12, 13).

We determined the entire coding sequences of *FKS1* and *FKS2* in the five isolates (NO1 to NO5). Briefly, the entire *FKS* genes (7,332 bp and 7,941 bp for *FKS1* and *FKS2*, respectively) were amplified by PCR from genomic DNA extracted from each strain, and DNA sequences of the PCR fragments were determined as described elsewhere but with different set of primers (see the primer list in Table S1 in the supplemental material) (5). The *FKS* sequences of the *C. glabrata* strains were reconfirmed with an independent analysis. In comparison to database sequences (the GenBank [https://www.ncbi.nlm.nih.gov/genbank/] accession numbers for *FKS1* and *FKS2* are HM366440 and HM366442, respectively), both NO1 and NO2 do not have any mutations which cause amino acid substitutions in both *FKS1* and *FKS2*. The subsequently isolated strain NO3 harbored two deletion mutations (F659del and L1767del) in *FKS2* (Table 2 and Fig. 1A). It was curious that NO3 was still susceptible to micafungin (MIC <0.015 μg/ml); however, F659del in *FKS2* was thought to confer echinocandin resistance, as mentioned above (11, 12). It was also demonstrated that the laboratory-constructed *C. glabrata* mutant which had F659del in *FKS2* was susceptible to echinocandins (8). These observations all together suggest that F659del in *FKS2* alone does not necessarily confer echinocandin resistance to *C. glabrata*. It is also conceivable that L1767del in *FKS2* or an unknown genetic modification(s) other than those in *FKS* genes suppress the effect of F659del; however, we have no evidence supporting these hypotheses.

In micafungin-resistant NO4 and NO5, bp 262 of the *FKS2* sequence (containing F659del) was substituted for the homolo-

FIG 1 (A) Proposed model for the introduction of mutations into *FKS* genes. The flow chart of *FKS1* and *FKS2* modification is schematically rendered. The *FKS1*-type sequence and *FKS2*-type sequence are black and white, respectively. The representative point mutations which resulted in an amino acid substitution are designated with asterisks. *FKS2* in NO3 carries two mutations, resulting in a predicted protein harboring F659del and L1767del (bottom left). In contrast, *FKS2* in strains NO4 and NO5, both isolated from the patient subsequent to NO3's isolation, carries the F659del-encoding mutation alone (bottom right). This discrepancy suggests that NO3 and NO4 originated from a common ancestor (center middle) that was presumably susceptible to micafungin. The blood culture from which NO3 and NO4 were isolated also may have contained this hypothetical strain. (B) Alignment of flanking sequences around the region of the *FKS* gene substitution. *FKS1* in NO3 (top), *FKS1* in NO4 (middle, bold), and *FKS2* in NO4 (bottom) are aligned for comparison. The numbering represents positions within the respective open reading frame, and the conserved nucleotides are highlighted by gray shading. The *FKS1* sequence in NO4 is identical to a homologous *FKS2* sequence in NO4 from bp 1635 to 1899 (numbering for *FKS1* is above the alignment), whereas the *FKS1* sequence in NO4 is identical to parental *FKS1* in NO3 both upstream of bp 1635 and downstream of bp 1899. The F659del-encoding mutation in *FKS2* also was inserted into *FKS1* in NO4 (arrow). The resulting eight amino acid substitutions from the gene conversion are designated with single letters above and below the alignment (above for parental Fks1p amino acid residues and below for Fks2p; asterisks indicate amino acid residues conserved between Fks1p and Fks2p). The hot spot region related to echinocandin resistance is underlined. WT, wild type.


A
FKS1 FKS2
WT
WT
NO1, NO2
FKS1 FKS2
F659Δ
(Hypothetical common ancestor for both NO3 and NO4)
FKS1 FKS2
F659Δ
L1767Δ
NO3
FKS1 FKS2
F659Δ
NO4 and NO5
B
NO3 FKS1 1525-GCAGGTGCTCAACATCTTTCCCGTAGATTTTGGTTCTTATGTTTGATTTTTGCAGTTAACTTGGGTCCAATTATT-1599
NO4 FKS1 1525-GCAGGTGCTCAACATCTTTCCCGTAGATTTTGGTTCTTATGTTTGATTTTTGCAGTTAACTTGGGTCCAATTATT-1599
NO4 FKS2 1627-GCTGGTGCTCAACATTTATCTCGTAGGTTTTGGTTTTTATGTGGTATCCTAGGTGTTAATCTTGGTCCTTTAATT-1701
1635
* * * * * * * * * M * * V
NO3 FKS1 1600-TTTGTCTTCGCTTATGAAAAGGACACTGTTCAATCAAAGGCAGGCCACGCGGTTGCTGCGGTCATGTTCTTTGTT-1674
NO4 FKS1 1600-TTTGTCTTCGCTTATGAAAAGGACACTGTTCAATCGAAGGCTGGTCATGCTGTAGCAGCTGTAACGTTTTTCATT-1674
NO4 FKS2 1702-TTTGTCTTTGCTTATGAAAAGGACACTGTTCAATCGAAGGCTGGTCATGCTGTAGCAGCTGTAACGTTTTTCATT-1776
* * * * * * * * * T * * I
* * * * L * * * * V * * * * * * * * * * * * * * T
NO3 FKS1 1675-GCAGTCGCTACATTGCTATTTTTCTCAGTCATGCCATTGGGTGGTCTGTTCACGTCCTACATGCAAAAGTCTACC-1749
NO4 FKS1 1675-GCTGTGGCCACTGTTTTATTCTTCTCGATTATGCCATTAGGTGGTCTTTTCACCTCATATATGCAAAAATCAAGT-1749
NO4 FKS2 1777-GCTGTGGCCACTGTTTTATTCTTCTCGATTATGCCATTAGGTGGTCTTTTCACCTCATATATGCAAAAATCAAGT-1851
* * * * V * * * * I * * * * * * * * * * * * * * S
* * * * * * * * * * * * * * * * H * * * * * * * *
NO3 FKS1 1750-AGACGTTACGTCGCTTCTCAAACCTTCACTGCCTCCTTTGCACCTTTGCATGGTCTTGACAGATGGTTGTCTTAC-1824
NO4 FKS1 1750-AGAAGATATGTTGCTTCTCAGACTTTCACCGCATCTTTTGCCCCATTACAAGGTTTGGATAGATGGTTATCTTAT-1824
NO4 FKS2 1852-AGAAGATATGTTGCTTCTCAGACTTTCACCGCATCTTTTGCCCCATTACAAGGTTTGGATAGATGGTTATCTTAT-1926
* * * * * * * * * * * * * * * * Q * * * * * * * *
1899
* * * * * * * * * * * A * * * Y F * * * * * * * *
NO3 FKS1 1825-CTGGTTTGGGTTACTGTTTTTGCTGCTAAGTACGCTGAATCATACTACTTCTTGATTCTATCTCTAAGAGATCCA-1899
NO4 FKS1 1825-TTAGTTTGGGTTACTGTTTTTGCTGCCAAGTACTCTGAATCGTACTTC---TTGATTTTGTCTCTAAGAGACCCT-1896
NO4 FKS2 1927-TTAGTTTGGGTTACTGTTTTTGCTGCCAAGTACTCTGAATCGTACTTC---TTGATTTTGTCTCTAAGAGACCCT-1998
* * * * * * * * * * * S * * * F * * * * * * * *
F659del
NO3 FKS1 1900-ATCAGAATTTTGTCTACCACTACCATGAGATGTACTGGTGAATACTGGTGGGGTTCAAAGCTATGTAGACATCAA-1974
NO4 FKS1 1897-ATCAGAATTTTGTCTACCACTACCATGAGATGTACTGGTGAATACTGGTGGGGTTCAAAGCTATGTAGACATCAA-1971
NO4 FKS2 1999-ATCAGAATTTTATCAACTACTACCATGAGATGTACTGGTGAGTATTGGTGGGGTTCAAAGTTATGTAGACATCAA-2073

gous region of *FKS1*, resulting in a predicted protein harboring multiple amino acid mutations (M555T, V558I, L563V, V568I, T583S, H600Q, A620S, Y623del) compared to the parental sequence (Table 2 and Fig. 1B). One possible cause for the micafungin resistance in NO4 and NO5 is the multiple mutations in *FKS1* resulting from the genetic substitution from *FKS2* to *FKS1*; however, both NO4 and NO5 still keep F659del in *FKS2*, presumably related to echinocandin resistance (Fig. 1A and B). It is ambiguous which *FKS* mutation(s) conferred micafungin resistance to NO4 or NO5; therefore, each *FKS* gene in NO4 and NO5 should be separately expressed in a *C. glabrata* laboratory strain and functionally characterized in future work.

Also of note was the fact that the mutation encoding L1767del in *FKS2* (observed in NO3) was not observed in NO4 and NO5. The loss of this mutation indicates that NO3 and NO4 developed from a common ancestor whose *FKS2* gene harbored the F659del-encoding mutation alone (Fig. 1A). Thus, the heterogeneous colony sizes observed in NO3 and NO4 may reflect the existence of a population of *C. glabrata* organisms carrying heterogeneous *FKS* gene sequences in NO3 and NO4.

To our knowledge, this is the first report suggesting that a genetic addition from *FKS2* to *FKS1* can mediate micafungin resistance in *C. glabrata*. This case also suggests that morphological colony phenotypes may be associated with changes in micafungin susceptibility in *C. glabrata* isolates.

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We have no conflicts of interest to declare.

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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.

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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).

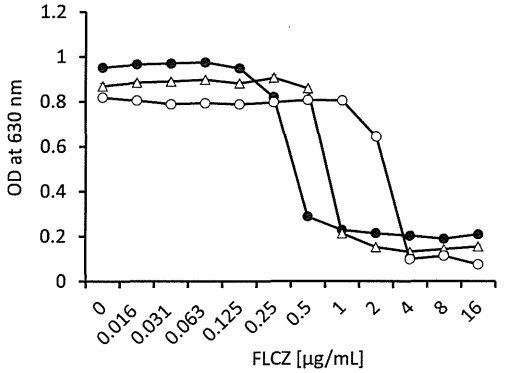

Fig. 1. Effect of DFNa on the antifungal activity of FLCZ against *C. albicans* SC5314. Closed circles, FLCZ alone; open triangles, DFNa (2.5 μM); open circles, DFNa (25 μM). The MIC was 0.5 μg/mL with FLCZ alone, but shifted to 1 μg/mL and 4 μg/mL when 2.5 μM and 25 μM of DFNa were added, respectively.

We next evaluated the expression of genes that are related to ergosterol synthesis and efflux pumps. FLCZ induced *ERG3*, *ERG9*, and *ERG11* gene expressions, which were markedly reduced following the addition of DFNa (Fig. 3a, DFNa + FLCZ), and were not induced by DFNa alone (Fig. 3a, DFNa). These findings suggested that DFNa did not directly affect ergosterol synthesis, and that the reduced anti-*C. albicans* activity is independent of alterations in ergosterol synthesis. *CDR1* was significantly induced by DFNa with or without FLCZ (Fig. 3b, FLCZ + DFNa, DFNa) but not by FLCZ alone (Fig. 3b, FLCZ), whereas *CDR2* was not induced by any drug or drug combination. IBU, 2,6-DPA and OPZ also induced *CDR1* expression, but LOX and CEL did not (Supplemental Fig. S2).

Gene expression analysis suggested that *CDR1* induction was responsible for the reduced anti-*C. albicans* activity of FLCZ. Therefore, we evaluated whether the induction of *CDR1* expression was necessary for the effect. The TU202 mutant has an *ACT1* promoter-driven *CDR1* gene instead of the disrupted native *CDR1* gene (Δ*CDR1* with p*ACT1-CDR1*), which results in the constant expression of *CDR1* independent of exogenous stimulation. The MIC curves of the wild type and TU202 were comparable without DFNa; however, the shift of the curve induced by DFNa in the wild type

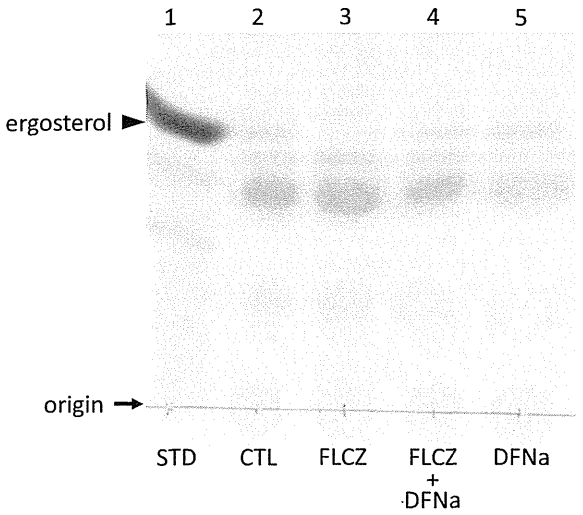

Fig. 2. Profiles of cellular sterols separated by thin layer chromatography. Lane 1, ergosterol standard (STD); lane 2, untreated control (CTL); lane 3, FLCZ-treated; lane 4, FLCZ and DFNa-treated; lane 5, DFNa-treated. The ergosterol spot disappeared when FLCZ was added alone (lane 3), but was restored following the addition of DFNa (lane 4).

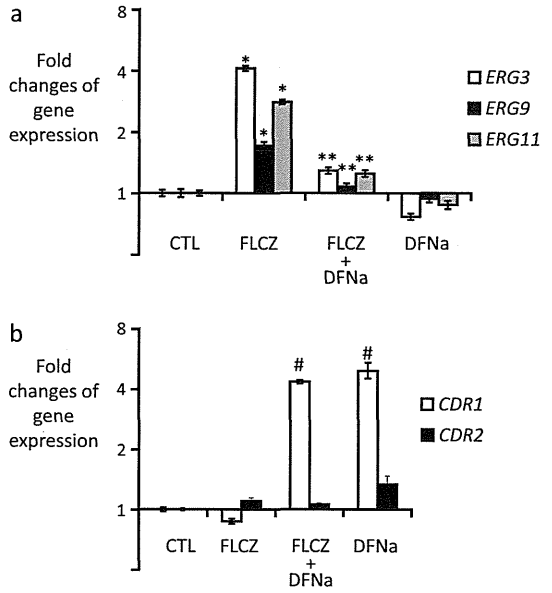

Fig. 3. Expression levels of representative genes related to the FLCZ resistance of *C. albicans*. a, Ergosterol synthesis. White, black, and grey bars indicate the expression of *ERG3*, *ERG9*, and *ERG11*, respectively; FLCZ significantly induced these genes but DFNa did not. b, Efflux pumps. White and black bars indicate *CDR1* and *CDR2* expression, respectively; DFNa significantly induced *CDR1*, with or without FLCZ, but not *CDR2*. CTL, control; FLCZ, fluconazole alone; FLCZ + DFNa, fluconazole with diclofenac sodium; DFNa, diclofenac sodium alone. **P* < 0.001 compared to CTL; ***P* < 0.005 compared to FLCZ; #*P* < 0.001 compared to CTL and FLCZ.

was partly diminished in TU202, suggesting that induced *CDR1* partly contributed to the attenuated anti-*C. albicans* activity of FLCZ by DFNa (Fig. 4).

4. Discussion

High doses of COX inhibitors have previously been reported to enhance azoles' anti-*Candida* activity [6–15]. Our study is the first to show that DFNa and IBU are potent factors contributing to the attenuation of this activity at lower doses though Arai R. et al. showed synergism of FLCZ and IBU. When used at therapeutic doses, the maximum blood concentrations of DFNa and IBU were

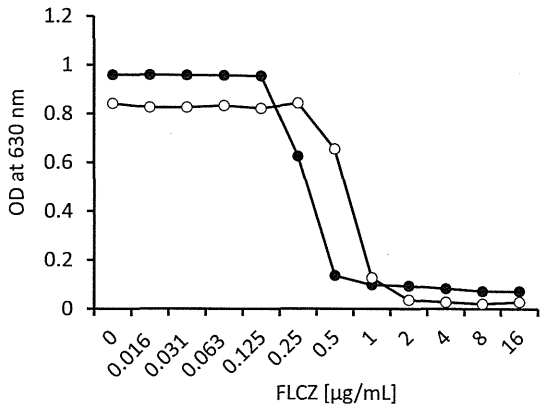

Fig. 4. Effect of DFNa on the antifungal activity of FLCZ in a *CDR1* mutant, TU202. TU202 has an *ACT1* promoter-driven *CDR1* gene instead of the disrupted native *CDR1* gene (Δ*CDR1* with p*ACT1-CDR1*), which results in the constant expression of *CDR1* independent of exogenous stimulation. Closed circles, FLCZ alone; open circles, DFNa (25 μM). The dose–response curves of TU202 were comparable to those of the wild type without the addition of DFNa. DFNa shifted the dose response curve in TU202 less than it did in the wild type.

reported to be 9 μM and 15–50 μM, respectively [19,20]. Therefore, the concentrations we used in this study were closer to the clinically available concentrations than those tested in most previous reports [6–15], with the exception of the study by Arai et al. [14] showing that IBU did not affect FLCZ activity against other strains at a concentration close to that used in the present study. Interestingly, the anti-*Candida* activity of FLCZ was not altered by either LOX or CEL. LOX is frequently used in Japan but not in the US, and CEL is a selective COX2 inhibitor that is dissimilar to traditional COX inhibitors. Therefore, our findings and those of previous reports suggest that the combinatorial effect of COX inhibitors could be dependent on the tested strains and conditions, as well as the specific type of inhibitors used.

We also identified possible mechanisms underlying FLCZ resistance to *C. albicans*. Our data could not completely explain the mechanisms by which DFNa attenuated FLCZ activity, but suggested that induced *CDR1* pumps, which are most frequently related to FLCZ resistance of *C. albicans*, might be at least partially involved in the effect. IBU and OPZ may be also able to disturb the anti-*Candida* activity of FLCZ, possibly via the same mechanism. In contrast to our results, IBU has been reported to reverse the antifungal resistance of *C. albicans* overexpressing *CDR* genes (12). Therefore, the effect of these drugs appears to fluctuate, and it is difficult to accurately predict how the drugs will interact with antifungal agents. We also showed that the mechanism might be independent of COX activity inhibition, as LOX and CEL did not show the reduced anti-*Candida* activity effect. In addition, an analogue of DFNa did show the effect, suggesting that the mechanism might be more related to the structure of the compounds rather than to the inhibition of COX function; however, the structure–activity relationship could not be clearly determined.

In conclusion, several COX inhibitors and proton pump inhibitors show potent effects on reducing the sensitivity of *C. albicans* to FLCZ at clinically realistic concentrations, possibly via inducing an efflux pump. Although it is not known whether this antagonistic effect is the main cause of clinical treatment failure, further investigation of the molecular mechanisms underlying the effect of the reduction on FLCZ's anti-*Candida* activity is expected to promote safer and more effective use of these drugs.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jiac.2014.06.004.

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