The Nonsteroidal Antiinflammatory Drug Diclofenac Potentiates the In Vivo Activity of Caspofungin Against Candida albicans Biofilms

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In this study, we demonstrated that in vitro Candida albicans biofilms grown in the presence of diclofenac showed increased susceptibility to caspofungin. These findings were further confirmed using a catheter-associated biofilm model in rats. C. albicans–inoculated catheters retrieved from rats that were treated with both diclofenac and caspofungin contained significantly fewer biofilm cells and showed no visible biofilms inside the catheter lumens, as documented by scanning electron microscopy, as compared to catheters retrieved from rats receiving only caspofungin or diclofenac. This report indicates that diclofenac could be useful in combination therapy with caspofungin to treat C. albicans biofilm–associated infections.

Upon contact with various surfaces, the fungal pathogen Candida albicans can form biofilms, which are a cause of infections associated with medical devices, such as catheters. Such biofilm-associated Candida cells are resistant to a wide spectrum of antifungal drugs [1]. Among the current antifungals in clinical use, only the liposomal formula of amphotericin B and echinocandins have shown consistent in vitro and in vivo activity against C. albicans biofilms [2, 3]. The echinocandin caspofungin disturbs the integrity of the fungal cell wall by inhibiting β-(1,3)-D-glucan synthase and lyses sessile cells within C. albicans biofilms [4]. Caspofungin doses that are effective against planktonic cells (ie, 0.05–0.5 µM) cannot decrease the metabolic activity of C. albicans biofilm cells. However, the use of a therapeutic concentration of caspofungin (ie, 2–4 µM) significantly decreased metabolic activity of C. albicans biofilm cells [5, 6]. These findings make caspofungin (and possibly other echinocandins) an attractive antifungal, preferentially in combination with compounds that improve its antibiofilm activity and concomitantly result in a reduction of its minimal inhibitory concentration (MIC) against C. albicans biofilms.

In this study, we aimed to identify compounds that increase the activity of caspofungin against C. albicans biofilms. The nonsteroidal antiinflammatory drug diclofenac was previously shown to reduce the expression level of different key genes involved in the cAMP-EFG1 pathway, thereby affecting the yeast-to-hyphae transition, which plays an important role in biofilm formation and development [7, 8]. Diclofenac and other nonsteroidal antiinflammatory drugs are widely used therapeutics, primarily for the treatment of pain and inflammation. These drugs inhibit the cyclooxygenase isoenzymes COX-1 and COX-2, which are involved in the biosynthesis of mammalian prostaglandins. Prostaglandins are small lipid molecules with diverse biological functions [9]. They are also known to be produced by both C. albicans planktonic and biofilm cells. However, their role in fungal biology is not yet known [10]. Erb-Downward and Noverr [11] suggested that prostaglandins can be viewed as regulators of C. albicans virulence because the eicosanoids pathway in C. albicans plays a central role in the...
control of morphogenesis and biofilm formation. As a consequence, they further suggested that the development of drugs that specifically target the fungal prostaglandins pathways may be one strategy to combat fungal colonization and infection [11]. In addition, Alem and Douglas demonstrated that diclofenac could decrease the biofilm production and hyphal formation by C. albicans, possibly through inhibition of prostaglandin biosynthesis [8, 10]. These previously reported results suggest that diclofenac may inhibit hyphal morphogenesis (and, consequently, biofilm formation) and could contribute to enhancing the antifungal activity of caspofungin toward biofilms. Therefore, we investigated a potential enhancing effect of diclofenac on the activity of caspofungin against C. albicans biofilms.

MATERIALS AND METHODS

Strains and Growth Conditions
C. albicans CAF2 [12], C. albicans clinical isolate 4.19 (K. Lagrou, UZ Leuven), and C. albicans caspofungin-resistant mutant C. albicans M89 [13] were grown routinely on YPD (1% yeast extract, 2% peptone, and 2% glucose) agar plates at 37°C. Stock solutions of caspofungin (Cancidas; Merck, Beeston Nottingham, United Kingdom) and diclofenac (Sigma, St. Louis, MO) were prepared in sterile water. Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine and without bicarbonate (pH 7.0) was purchased from Sigma.

C. albicans Planktonic Cells Susceptibility Testing
The susceptibility testing of C. albicans planktonic cells to caspofungin in the presence or absence of diclofenac was performed according to Clinical and Laboratory Standards Institute protocol M27-A3 [14]. Data were determined as MICs of the drug that inhibit fungal growth by 50% (MIC50).

In Vitro Drug Susceptibility Testing of C. albicans Biofilms Grown on 96-Well Microtiter Plates
In vitro C. albicans biofilms were grown on 96-well polystyrene microtiter plates in RPMI 1640 medium. Candida cells (1 × 10⁶ cells/mL) were allowed to adhere to the substrate for 90 minutes, whereafter biofilm formation occurred over a 16-hour period in RPMI 1640 medium. In case of diclofenac pretreatment, diclofenac (2 mM) was administered during adhesion and throughout the period of biofilm formation. Catheter fragments containing diclofenac-pretreated mature biofilms were washed and subsequently incubated with caspofungin (125 μM) or water and diclofenac for 24 hours, whereas mature biofilms without diclofenac pretreatment were washed and subsequently incubated with caspofungin (125 μM) or water for 24 hours. Biofilms were washed and quantified by determination of colony-forming units (CFU), as previously described [17].

For in vivo drug susceptibility assays involving C. albicans biofilms grown on diclofenac-soaked or unsoaked catheter fragments, the catheter fragments were soaked overnight at 37°C in serum in the presence or absence of 2 mM diclofenac, respectively. Either diclofenac-soaked or unsoaked catheters were inoculated with C. albicans cells, and caspofungin susceptibility tests were performed as described above.

In Vivo Biofilm Drug Susceptibility Testing, Using a Rat Subcutaneous Biofilm Model
Animals were maintained in accordance with the Katholieke Universiteit Leuven animal care guidelines, and animal experiments were approved by the ethical committee of the Katholieke Universiteit Leuven (project P125/2011). A rat subcutaneous catheter infection model was used for in vivo biofilm susceptibility studies [17], in which 6 rats were pretreated (for 2 days starting immediately after catheter implant) and further treated with sterile saline (control, 40 catheters), 6 rats were pretreated with diclofenac and treated with diclofenac (48 catheters), 5 rats were pretreated with sterile saline and treated with caspofungin (40 catheters), and 6 rats were
pretreated with diclofenac and further treated with a combination of diclofenac and caspofungin (45 catheters). Diclofenac and caspofungin solutions for intravenous injection were prepared in sterile physiological solution. For diclofenac pretreatment, the administration of diclofenac (3 mg/kg/day) started immediately after the catheter implant, daily, for 2 days. Subsequently, rats pretreated with diclofenac received 3 mg/kg/day caspofungin or sterile saline daily for 7 days; rats without diclofenac pretreatment received 3 mg/kg/day caspofungin or sterile saline daily for 7 days. Further analysis and quantification of the number of cells per individual biofilm was performed as previously described [17]. Six independent catheters retrieved from each test group were used for scanning electron microscopy (XL30, FEG) analyses. Before microscopy, each catheter fragment was cut longitudinally through the lumen and subsequently prepared as previously described [17].

Membrane-Permeability Assay
Membrane-disruptive activity of diclofenac on in vitro–grown C. albicans biofilm cells was determined by measuring the fluorescence enhancement of propidium iodide (Sigma). To this end, C. albicans biofilms were grown in 96-well microtiter plates in RPMI 1640 medium in the presence or absence of diclofenac (concentration range, 0.25–4 mM) for 24 hours. The biofilm cells were washed and incubated with 3% propidium iodide for 20 minutes at room temperature in the dark. Membrane permeability was quantified by measurement of propidium iodide fluorescence (MultiMode Microplate Reader, Synergy MX, BioTek; excitation at 535 nm, emission at 617 nm). Fluorescence values presented are corrected with those obtained from untreated biofilms.

Statistical Analysis
Statistical analysis was performed using an unpaired t test. P values of <.05 were considered indicative of statistically significant differences.

RESULTS
Diclofenac Increases the Efficacy of Caspofungin Against C. albicans Biofilms Grown In Vitro
First, we determined the MIC50 of caspofungin and/or diclofenac against planktonic cultures of C. albicans CAF2-1, clinical isolate 4.19, and caspofungin-resistant mutant M89 in RPMI 1640 medium (Table 1). We observed no increased activity of caspofungin in combination with diclofenac against planktonic cultures. Moreover, we observed no inhibitory activity of diclofenac concentrations of up to 2 mM against planktonic cells nor an effect of diclofenac on the morphology of the planktonic cells, as analyzed by fluorescence microscopy (data not shown). However, when incubating the planktonic cells in YPD medium supplemented with 10% serum at 37°C for 2 hours, 2 mM diclofenac inhibited yeast-to-hyphae transition, which is in line with previous reports [7, 8]. Apparently, depending on the medium, diclofenac might have an effect on morphology of the yeast cells.

Next, we determined the MIC50 of caspofungin against biofilm cells grown on 96-well microtiter plates in RPMI 1640 medium in the presence or absence of diclofenac (Table 1). The MIC50 of caspofungin against biofilms of C. albicans CAF2-1 was 6 µM, which was at least 100 times higher than the MIC50 of caspofungin against planktonic cultures (MIC50 < 0.05 µM), documenting the increased resistance of C. albicans biofilms against caspofungin. Diclofenac was either added during adhesion and biofilm maturation (“pretreated biofilms”) and subsequent eradication by caspofungin or, alternatively, during biofilm eradication together with caspofungin (“mature biofilms”). Diclofenac alone had a moderate antibiofilm effect (MIC50 = 6 mM when added as pretreatment or MIC50 = 10 mM when added against mature biofilms). However, the morphology of biofilm cells grown on 96-well microtiter plates pretreated with diclofenac alone (concentration range, 0.5–3 mM) was not altered (Supplementary Figure 1). The MIC50 of caspofungin in combination with 2 mM diclofenac pretreatment against C. albicans CAF2-1 biofilms was 0.75 µM.

Table 1. Minimum Inhibitory Concentrations of Caspofungin (CAS) and Diclofenac (DF), Alone or in Combination, That Inhibit Growth by 50% of Planktonic Cells or Biofilm Cells (µM) of C. albicans CAF2-1, Clinical Isolate C. albicans 4.19, and CAS-Resistant Mutant C. albicans M89

<table>
<thead>
<tr>
<th>C. albicans</th>
<th>Planktonic Cells</th>
<th>Pretreated Biofilms</th>
<th>Mature Biofilms</th>
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<tr>
<td></td>
<td>CAS</td>
<td>DF</td>
<td>CAS + DF</td>
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<tr>
<td>CAF2-1</td>
<td>&lt;0.05</td>
<td>&gt;2000</td>
<td>0.23</td>
</tr>
<tr>
<td>Clinical isolate 4.19</td>
<td>0.5</td>
<td>&gt;2000</td>
<td>1</td>
</tr>
<tr>
<td>CAS-resistant strain M89</td>
<td>8</td>
<td>&gt;2000</td>
<td>16</td>
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Data are the mean of at least 2 independent biological repeats, each consisting of at least 4 technical repeats. Pretreated biofilms received 2 mM DF during adhesion and further biofilm formation (16 hours) before the treatment with either CAS or DF or a combination of both for an additional 24 hours. Mature biofilms were not pretreated with DF.

Abbreviation: FICI, fractional inhibitory concentration index.
 whereas co-incubation of mature biofilms with caspofungin and 2 mM diclofenac resulted in an MIC\textsubscript{50} of 1.5 µM. FICI values for the diclofenac pretreatment/caspofungin combination and the diclofenac coinubcation/caspofungin combination against \textit{C. albicans} biofilms were 0.458 and 0.450, respectively. These FICI values, both <0.5, point to the synergistic interaction between caspofungin and diclofenac against biofilms [16]. Similar data were obtained using biofilms of \textit{C. albicans} clinical isolate 4.19 and caspofungin-resistant strain M89 (Table 1). FICI values for the diclofenac pretreatment/caspofungin combinations against \textit{C. albicans} biofilms of the clinical isolate and of the caspofungin-resistant strain were 0.463 and 0.453, respectively. The MIC\textsubscript{50} of caspofungin in combination with 2 mM diclofenac against mature \textit{C. albicans} 4.19 biofilms was 4 µM, resulting in a FICI of 1.1 and indicating no interaction between diclofenac and caspofungin against this isolate. The MIC\textsubscript{50} of caspofungin in combination with 2 mM diclofenac against mature biofilms of \textit{C. albicans} caspofungin-resistant mutant M89 was 4 µM, which points to a reduction of at least 38-fold in mature biofilm development as compared to biofilms treated with caspofungin only (MIC\textsubscript{50} > 150 µM). The corresponding FICI is 0.160, which characterizes synergism. All these data point to the general synergistic interaction between caspofungin and diclofenac, when added during the period of adhesion and throughout mature biofilm development, against biofilms of various \textit{C. albicans} isolates.

Prior to the in vivo biofilm experiments, in which polyurethane catheters were used as a substrate, we investigated the effect of diclofenac pretreatment on the activity of caspofungin against in vitro-grown \textit{C. albicans} CAF2-1 biofilms on catheter fragments. To this end, we used a concentration of caspofungin that only had a moderate effect on the viability of \textit{C. albicans} biofilm cells grown on catheters. Treatment of \textit{C. albicans} biofilms grown on catheters with 125 µM caspofungin did not result in a statistically significant reduction of the number of biofilm cells as compared to control treatment (Figure 1). We used this caspofungin concentration to further investigate the potential effect of diclofenac on the antibiofilm activity of caspofungin against in vitro-grown \textit{C. albicans} biofilms. As pretreatment of biofilms grown on microtiter plates with 2 mM diclofenac most effectively increased the caspofungin antibiofilm activity, we used this diclofenac concentration also in the catheter biofilm in vitro setup (Figure 1). Treatment of \textit{C. albicans}-inoculated catheters with 2 mM diclofenac during biofilm growth and subsequent treatment with 125 µM caspofungin resulted in a significant reduction of at

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**Figure 1.** Susceptibility of diclofenac-pretreated and untreated \textit{Candida albicans} CAF2-1 biofilms to caspofungin in vitro. Biofilms of \textit{C. albicans} CAF2-1 were grown on catheter fragments in Roswell Park Memorial Institute 1640 medium (pH 7.0). In case of diclofenac pretreatment, diclofenac (2 mM) was administrated during adhesion and throughout the period of biofilm formation. Diclofenac-pretreated mature biofilms were washed and subsequently incubated with caspofungin (125 µM) or diclofenac for 24 hours, whereas mature biofilms without diclofenac pretreatment were washed and subsequently incubated with caspofungin (125 µM) or water for 24 hours. Biofilms were washed and quantified by determination of colony-forming units. Data presented are the mean and standard errors of the mean of 5 independent experiments, using at least 2 catheters per tested group. Statistical analysis was performed using an unpaired t test. **P < .01.

**Figure 2.** Susceptibility of \textit{Candida albicans} CAF2-1 biofilms grown on diclofenac-soaked or unsoaked catheters to caspofungin in vitro. Catheter fragments were soaked overnight at 37°C in serum in the presence or absence of 2 mM diclofenac. Diclofenac-soaked and unsoaked catheters were inoculated with \textit{C. albicans} CAF2-1 cells and were or were not treated with 125 µM caspofungin. After incubation for 24 hours, biofilms were washed, and biofilm cells were quantified by determination of colony-forming units. Data presented are the means and standard errors of the mean of 2 independent experiments, using at least 2 catheters per test group. Statistical analysis was performed using an unpaired t test. *P < .05.
least 12-fold in the number of in vitro biofilm cells as compared to untreated biofilms ($P < .01$) but not a significant reduction in cell numbers as compared to caspofungin treatment alone. These results show that a moderate caspofungin dose (125 µM) can be potentiated by 2 mM diclofenac, resulting in a significant reduction in the CFU of treated biofilms as compared to control treatment. There was no significant difference in the number of biofilm cells that were retrieved from catheters upon diclofenac pretreatment alone as compared to untreated catheters, as shown in Figure 1. Apparently, depending on the experimental setup, 2 mM diclofenac can have an inhibitory effect on biofilm formation: the MIC$_{50}$ of diclofenac against biofilms grown in 96-well polystyrene microtiter plates was 2 mM, whereas this diclofenac concentration did not affect biofilm growth on polyurethane catheter fragments.

Finally, we assessed the antibiofilm effect of caspofungin against mature biofilms, which were formed on diclofenac-soaked catheter fragments, versus those formed on unsoaked catheter fragments. A caspofungin dose (125 µM) that did not display significant activity against mature biofilms grown on unsoaked catheter fragments could significantly reduce the number of biofilm cells recovered from catheters upon caspofungin treatment alone as compared to untreated catheters. As shown in Figure 1, apparently, depending on the experimental setup, 2 mM diclofenac can have an inhibitory effect on biofilm formation: the MIC$_{50}$ of diclofenac against biofilms grown in 96-well polystyrene microtiter plates was 2 mM, whereas this diclofenac concentration did not affect biofilm growth on polyurethane catheter fragments.

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number of cells of biofilms grown on diclofenac-soaked catheters by at least 17-fold as compared to control treatment (P < .05) (Figure 2).

**Diclofenac Increases the Efficacy of Caspofungin Against C. albicans Biofilms Grown In Vivo**

To assess the increased susceptibility of diclofenac-pretreated C. albicans biofilms to caspofungin under in vivo conditions, we used a recently developed subcutaneous biofilm model system in rats [17]. This model has already been used to determine the antibiofilm activity of the echinocandin anidulafungin against C. albicans biofilms [18]. The numbers of sessile C. albicans cells recovered from the implanted catheters after various treatments are represented in Figure 3A. There was no significant difference in the number of biofilm cells on catheters retrieved from rats receiving no drugs (control treatment) and those receiving 48 hours of diclofenac pretreatment and further diclofenac treatment (3 mg/kg/day) for up to 7 days, indicating that diclofenac treatment alone does not affect in vivo biofilm formation. Caspofungin treatment alone at 3 mg/kg by daily injections for 7 days without diclofenac pretreatment of the rats, resulted in a >10-fold significant reduction in the number of surviving C. albicans biofilm cells on the implanted catheters as compared to control treatment (P < .001). However, daily caspofungin treatment for 7 days in combination with 48 hours of diclofenac pretreatment and further diclofenac treatment for up to 7 days resulted in a >15-fold significant reduction in biofilm cell numbers as compared to control treatment and in a >5-fold significant reduction in cell numbers as compared to caspofungin treatment alone (P < .001 and P < .05, respectively). These observations are reflected in the number of sterile catheter fragments retrieved from the different treatment groups: 3 catheters (7.5%) retrieved from animals treated with caspofungin alone were sterile, whereas 13 sterile catheters (29%) were retrieved from animals treated with both caspofungin and diclofenac.

Next, we analyzed the C. albicans biofilm structure and morphology of various catheters per animal, using scanning electron microscopy (Figure 3B). Catheters retrieved from untreated and diclofenac-pretreated rats showed dense patches of typical C. albicans biofilm architecture alongside the catheter lumen, composed of a hyphal network and covered with a layer that can be considered as extracellular matrix. Catheters retrieved from rats treated with caspofungin without diclofenac pretreatment showed C. albicans biofilm cells formed in minor clusters, still revealing biofilm structure similar to untreated biofilms with signs of extracellular polymeric material present on top. However, only scattered yeast cells and hyphae were observed after treatment of animals with diclofenac and caspofungin together, confirming the data based on CFU determination.

**DISCUSSION**

The spectrum of antifungal drugs currently available on the market that are effective against Candida biofilms is very limited. It is therefore necessary to search for new approaches for the treatment of such infections. The data demonstrated in this study reveal successful reduction of in vitro and in vivo C. albicans biofilms on catheters by combination therapy with
the antiinflammatory drug diclofenac and the antymycotic caspofungin. Caspofungin used as a lock solution during treatment of C. albicans biofilms developed in central venous catheter models has been shown to successfully reduce the amount of biofilm cells as compared to untreated control [3]. Unfortunately, the concentration of antifungals that eliminate Candida biofilms is usually extremely high. In this study, we demonstrated that biofilms of various C. albicans isolates and strains, grown in the presence of diclofenac, showed increased susceptibility to caspofungin. Even the activity of caspofungin against biofilms of a caspofungin-resistant isolate could be increased by diclofenac pretreatment. Echinocandin resistance is uncommon, but it has been associated with amino acid substitutions in 2 conserved regions of the glucan synthase Fks1 [10]. The caspofungin resistance of the strain used in this study was based on a mutation in hotspot 1 of FKS1 and was previously shown to have a prominent decrease in caspofungin susceptibility [13]. The increased antibiofilm activity of caspofungin upon diclofenac pretreatment of biofilm cells may result from a diclofenac-induced increase of the membrane permeability of the C. albicans biofilm cells. In addition, the membrane-disruptive activity of diclofenac may be linked to the potential involvement of diclofenac in the biosynthesis of fungal prostaglandins, as previously reported by Alem and Douglas [10].

Previous studies have documented the effect of diclofenac on C. albicans biofilms [8] or on cells grown on solid media [7]. The latter study showed inhibition of the yeast-to-hyphae transition by diclofenac on solid YPD media [7]. The first study demonstrated a significant reduction of biofilm formation in YPD on small polyvinyl chloride disks by diclofenac when added during the period of adhesion and biofilm formation, quantified by XTT measurements [8]. Our results clearly show that the diclofenac dose used in our setup (2 mM) has no effect on the morphology of either planktonic or biofilm C. albicans cells grown in RPMI 1640 medium (Supplementary Figure 1). However, diclofenac was moderately inhibitory against biofilms grown in polystyrene microtitre plates (MIC50 = 5–6 mM, Table 1) but was not inhibitory against biofilms grown on polystyrene catheter fragments. Apparently, on the basis of our data and those of Ghalehnoo et al [7] and Alem and Douglas [8], diclofenac can have activity against C. albicans biofilm cells, depending on the experimental setup. Such interstudy variations highlight the differences in behavior/physiology of Candida biofilms, which can be strongly influenced by different setups, including the type of device, composition and pH of the medium [20], and methods of quantification and susceptibility testing [21].

Recently, Gregori and coworkers [22] reported a novel caspofungin-induced flocculation phenotype of C. albicans triggered by Efg1-dependent expression of the adhesion ALS1. Cells lacking Efg1 show strongly diminished caspofungin-induced flocculation and marked caspofungin hypersusceptibility. These findings are in line with our finding that diclofenac treatment of C. albicans cells, known to reduce the expression of EFG1 [7], increases the antibiofilm activity of caspofungin against C. albicans.

Furthermore, we confirmed the in vitro observations of increased caspofungin susceptibility of diclofenac-pretreated C. albicans biofilms, using a rat model for in vivo biofilm analysis. Hence, this study provides clear evidence that diclofenac is useful in combination therapy with antifungals like caspofungin to treat C. albicans biofilm–associated infections. More specifically, we anticipate that coating of medical devices such as implants and plastics with diclofenac can, in combination with conventional antifungal therapy, be envisaged for the eradication of C. albicans biofilms. A possible concern that can be raised in this context is whether diclofenac concentrations requested for efficient treatment can be achieved in coatings or controlled release formulas on medical devices. In our experiments, we demonstrated a significantly increased antibiofilm activity of caspofungin against C. albicans biofilms grown on diclofenac-pretreated catheters (soaked in 2 mM diclofenac prior to cell adhesion) as compared to untreated catheters. These data at least point to the feasibility of achieving a sufficiently high diclofenac concentration on catheters in order to affect biofilm susceptibility for caspofungin.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

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