Antifungal Activity of Oleylphosphocholine on *In Vitro* and *In Vivo* *Candida albicans* Biofilms

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ABSTRACT In this study, we investigated the potential antifungal activity of the alkylphospholipid oleylphosphocholine (OIPC), a structural analogue of miltefosine, on *in vitro* and *in vivo* *Candida albicans* biofilm formation. The effect of OIPC on *in vitro* and *in vivo* *C. albicans* biofilms inside triple-lumen polyurethane catheters was studied. *In vivo* biofilms were developed subcutaneously after catheter implantation on the lower back of Sprague-Dawley rats. Animals were treated orally with OIPC (20 mg/kg of body weight/day) for 7 days. The effect of OIPC on biofilms that developed on the mucosal surface was studied in an *ex vivo* model of oral candidiasis. The role of OIPC in *C. albicans* morphogenesis was investigated by using hypha-inducing media, namely, Lee, Spider, and RPMI 1640 media. OIPC displayed activity against both planktonic cells and *in vitro* *C. albicans* biofilms. To completely abolish preformed, 24-h-old biofilms, higher concentrations (8, 10, and 13 mg/liter) were needed. Moreover, OIPC was able to reduce *C. albicans* biofilms formed by caspofungin-resistant clinical isolates and acted synergistically when combined with caspofungin. The daily oral administration of OIPC significantly reduced *in vivo* *C. albicans* biofilms that developed subcutaneously. In addition, OIPC decreased biofilm formation on mucosal surfaces. Interestingly, the application of subinhibitory concentrations of OIPC already inhibited the yeast-to-hypha transition, a crucial virulence factor of *C. albicans*. We document, for the first time, the effects of OIPC on *C. albicans* cells and suggest the potential use of OIPC for the treatment of *C. albicans* biofilm-associated infections.

KEYWORDS oleylphosphocholine, OIPC, *Candida albicans*, *in vitro* biofilm, *in vivo* biofilm, caspofungin

In the past decade, we have been dealing with a growing population at risk of invasive fungal infections. The persistence of these infections is a problem, especially when fungal pathogens form biofilms on implantable medical devices. Since the employment of a device is often compulsory for the survival of patients with an impaired immune system, the ability of *Candida albicans* to colonize and develop biofilms on artificial substrates, as well as on mucosal surfaces, has a significant impact on the mortality and morbidity of this patient group. In comparison with their planktonic counterparts, *Candida* cells associated with biofilms withstand high concentrations of antifungal drugs (1). So far, only echinocandins and liposomal formulations of amphotericin B have displayed significant activity against fungal biofilms (2). The increased use of echinocandins as therapy against invasive candidiasis may lead to increasing rates of

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TABLE 1 MICs and MFCs of OIPC against planktonic *Candida albicans* cells

Strain or clinical isolate	MIC ₅₀ (mg/liter)	MFC (mg/liter)
<i>C. albicans</i> SC5314	2	2
<i>C. albicans</i> U-0503-18	2	2
<i>C. albicans</i> U-0503-12	1	2
<i>C. albicans</i> U-0203-29	1	2
<i>C. albicans</i> U-0203-15	1	2
<i>C. albicans</i> U-04030-255	1	2
<i>C. albicans</i> HC2601-30	2	2
<i>C. albicans</i> HC0202-25	2	2
<i>C. albicans</i> HC2102-19	2	2
<i>C. albicans</i> HC1602-5	2	2
<i>C. albicans</i> HC0702-14	2	2
<i>C. albicans</i> M89	4	4
<i>C. albicans</i> M177	2	2
<i>C. albicans</i> M205	4	4

acquired resistance against these compounds, which is associated with amino acid changes in “hot spot” regions of the cellular targets echinocandins, the Fks subunits (3). Due to the limited number of drugs that are active against biofilms, it is crucial to search for novel strategies to prevent adhesion, which is the first step of biofilm development, or to obviate biofilm formation once the cells are attached to a substrate. Recently, miltefosine, an alkylphospholipid, was shown to have very good activity against *C. albicans* biofilms (4). It was also effective against oral candidiasis that developed on murine tongues (5). However, side effects and teratogenicity narrow the therapeutic window and limit its potential use (6, 7). A variant of miltefosine, oleylphosphocholine (OIPC), exhibited fewer side effects when tested *in vivo* and had good oral bioavailability. Moreover, it demonstrated very good antineoplastic and antiprotozoal activities, with a large safety margin (8). It was previously shown that OIPC was effective against infections caused by *Leishmania infantum* in a hamster model (8) and in naturally infected dogs (9) and furthermore was effective against cutaneous leishmaniasis caused by *Leishmania major* that developed in a mouse model (10). Interestingly, OIPC was also active against *Aspergillus* spp. (11) and *Cryptosporidium parvum* (12). Because of these findings, we assumed that OIPC could be a good candidate to treat device-associated *C. albicans* infections. Therefore, we investigated the potential effect of OIPC on *C. albicans* planktonic cells and on *in vitro* biofilms that developed on polyurethane catheters. Moreover, the activity of OIPC on *in vivo* *C. albicans* biofilms that developed on foreign bodies in a subcutaneous rat model is documented here for the first time. Additionally, we assessed the potency of OIPC against biofilm formation on mucosal tissue using an *ex vivo* tongue assay as a model. We were also able to demonstrate the activity of OIPC against *C. albicans* strains with increased tolerance to the echinocandin caspofungin and a possible mode of action for this molecule based on the inhibition of the yeast-to-hypha transition.

RESULTS

Planktonic cells of *C. albicans* display susceptibility to OIPC. First, we evaluated the MICs of OIPC that inhibited the growth of planktonic *C. albicans* cells *in vitro* by 50% (MIC₅₀). These results are presented in Table 1. The MIC₅₀ indicated that a concentration range between 1 mg/liter and 4 mg/liter of OIPC inhibited the growth of *C. albicans*. Moreover, we found that concentrations starting from 2 mg/liter of OIPC had a fungicidal effect on planktonic growth, documented as the minimal fungicidal concentration (MFC).

OIPC alters *in vitro* *C. albicans* biofilm development and preformed biofilms, whereas adhesion remains unaffected. Prior to biofilm susceptibility testing, we evaluated the abilities of *C. albicans* SC5314 and all clinical isolates to adhere to and form biofilms on polyurethane devices (see Fig. S1 in the supplemental material). The *Candida* wild-type (WT) strain and all clinical isolates exhibited comparable abilities to attach to and form biofilms on catheters.

Next, we assessed whether OIPC would influence the process of biofilm formation. Therefore, we investigated the influence of OIPC on three different developmental stages in biofilm formation, including adhesion, biofilm development, and on preformed biofilms. To know the effect of OIPC on *C. albicans* SC5314 adhesion, catheters preincubated with serum were submerged in a mixture containing *C. albicans* cells and different concentrations of OIPC for a period of 90 min at 37°C. The results indicated that OIPC had no effect on *C. albicans* adhesion (Fig. 1A). Next, we investigated the activity of OIPC on biofilm formation by treatment of the biofilms after adhesion for 24 h. The results show that biofilm development of the *Candida* WT strain (Fig. 1B) and clinical isolates (Fig. 2A and B) was significantly reduced at 2 mg/liter and 4 mg/liter of OIPC, respectively ($P < 0.05$). Strikingly, higher concentrations (8, 10, and 13 mg/liter) completely abolished biofilm development. The biofilm architecture of nontreated WT cells showed the formation of a thick layer of hyphal cells embedded in material resembling an extracellular matrix (ECM) (Fig. 1D). *Candida* cells treated with 1 and 2 mg/liter of OIPC displayed a rudimentary biofilm composed of hyphal cells attached to a substrate. Strikingly, only yeast cells were observed on the surface of catheters when treated with 4 mg/liter of OIPC. Scanning electron microscopy (SEM) images were in agreement with the CFU counts, displaying that no biofilms were observed on substrates treated with 8, 10, and 13 mg/liter of OIPC (data not shown).

These data demonstrated the activity of OIPC against biofilm development; however, preformed biofilms represent a major clinical problem in hospitalized patients. Therefore, we examined the activity of this compound on preformed (24-h-old) biofilms of *C. albicans* SC5314 (Fig. 1C) and clinical isolates (Fig. 2C and D). Concentrations starting from 2 mg/liter and 8 mg/liter of OIPC significantly reduced the biofilms of clinical isolates and the WT, respectively ($P < 0.05$). No cells were recovered from WT biofilms treated with 10 and 13 mg/liter of OIPC. Only a few *Candida* cells were retrieved from biofilms formed by clinical isolates U-0203-15 and U-0403-255 after treatment with 10 and 13 mg/liter of OIPC.

Oral administration of OIPC significantly decreases *C. albicans* biofilm development in a rat subcutaneous model. We investigated the activity of OIPC on *in vivo* *C. albicans* biofilm development in a rat subcutaneous biofilm model. Figures 3A and B demonstrate that the daily oral administration of OIPC (20 mg/kg of body weight/day for 7 days) to Sprague-Dawley rats resulted in a significant inhibition of *C. albicans* biofilms ($P < 0.05$) (CFU determined on day 9). Confocal laser scanning microscopy (CLSM) images of biofilms retrieved from the control group of animals displayed a thick network ($\sim 135 \mu\text{m}$) of hyphal cells characteristic of *Candida* biofilm architecture (Fig. 3C; see also Movie S1 in the supplemental material). Strikingly, the examination of biofilms from OIPC-treated animals showed decreased fungal loads with scattered yeast and hyphal cells alongside the lumen. The thickness of such biofilms was $\sim 67 \mu\text{m}$ (Fig. 3C). Taken together, these results demonstrate the potency of OIPC as a candidate to treat device-associated infections.

OIPC decreases *Candida albicans* biofilm formation on the mucosal surface in an *ex vivo* model of candidiasis. The emergence of biofilm-related infections on mucosal surfaces represents a major medical problem. Oral thrush, found in the oral cavity of immunocompromised patients, is one of the most common forms of biofilms (13). Therefore, we employed an *ex vivo* model for biofilm formation on mucosal surfaces (tongues extracted from sacrificed mice) to assess the effect of OIPC on *C. albicans* biofilm development on soft tissues. *Candida* cells were allowed to attach to the tissue during the adhesion phase and were subsequently grown for 24 h (see Fig. S2 in the supplemental material) in the presence of OIPC. Histopathology images of nontreated tongues and tongues treated with 1 and 2 mg/liter of OIPC exhibited intense yeast-to-hypha switching and massive dissemination of *Candida* hyphae inside the tissues (Fig. 4). However, scattered amounts of hyphae and a large majority of yeast cells were displayed on tissues treated with 4 mg/liter of OIPC. Strikingly, *Candida* cells treated with 8 and 10 mg/liter of OIPC remained solely in the yeast form, with no signs of dissemination. Importantly, no alterations in the structures of the tissues between

FIG 2 Efficacy of OIPC against *Candida albicans* biofilms formed by clinical isolates isolated from urinary catheters and blood culture. (A and B) Effect of different concentrations of OIPC (0, 0.5, 1, 2, 4, 8, 10, and 13 mg/liter) on biofilm development of *C. albicans* clinical isolates obtained from urinary catheters (A) and blood culture (B). OIPC was administered to *Candida* cells after the period of adhesion for the next 24 h. (C and D) Activity of OIPC on preformed *C. albicans* biofilms formed by clinical isolates from urinary catheters (C) and blood culture (D). In this assay, biofilms were allowed to develop in drug-free medium. OIPC was added subsequently for the next 24 h. Data are presented as the mean $\log_{10}$ CFU $\pm$ standard errors of the means for catheters from 2 independent experiments.

noninfected tongues and samples treated with the highest concentration of OIPC (13 mg/liter) were observed (Fig. S3). These data suggest that OIPC has the potential to treat not only catheter-associated infections but also biofilm development on mucosal surfaces.

OIPC is active against *C. albicans* biofilm development by clinical isolates resistant to caspofungin; moreover, OIPC synergizes with caspofungin to induce cell death of caspofungin-resistant isolates. The echinocandins form a unique class of drugs that possess activity against *in vitro* and *in vivo* biofilms (14, 15). However, by its excessive use, there is an increasing incidence of clinical isolates of *C. albicans* that show resistance against them. This raised the question of whether OIPC would be an effective alternative against *C. albicans* isolates M177, M205, and M89 (Table 2), with elevated MICs against caspofungin (16). OIPC at 2 mg/liter and 4 mg/liter inhibited fungal growth by 50% (MIC_{50}). Additionally, 2 mg/liter of OIPC caused a fungicidal

FIG 1 Antifungal activity of OIPC against different stages of *Candida albicans* SC5314 biofilm formation. (A to C) Different concentrations of OIPC (0, 0.5, 1, 2, 4, 8, 10, and 13 mg/liter) were introduced to *C. albicans* cells during the period of adhesion (90 min) (A) and after adherence for the next 24 h of biofilm development (B) and on preformed 24-h-old biofilms (C). Experiments with adhesion and biofilm formation were performed on serum-coated polyurethane catheter pieces. After the desired period, catheters were washed, sonicated, and plated onto YPD plates to enumerate the amounts of adhered and biofilm-forming cells. Each column represents the mean $\log_{10}$ CFU $\pm$ standard errors of the means retrieved from catheters from 3 independent experiments. The asterisk indicates a statistically significant difference between the nontreated group and the OIPC-treated groups (*, $P \leq 0.05$). (D) Scanning electron microscopy of catheters treated with OIPC during biofilm development for 24 h. Samples were coated with Au-Pd prior to microscopy. Arrows indicate the presence of scattered hyphal and yeast cells on the catheter surface.

FIG 3 OIPC is active against *in vivo* *Candida albicans* SC5314 biofilms. (A) Effect of oral administration of OIPC on *C. albicans* SC5314 biofilms developed in the subcutaneous rat model. Animals were treated with OIPC (20 mg/kg of body weight/day) daily for 7 days. Data are presented as log₁₀ numbers of CFU retrieved from each catheter piece. (B) Mean log₁₀ CFU ± standard errors of the means from independent catheters/rats. The horizontal lines indicate the mean log₁₀ CFU obtained per device or per rat. The asterisk indicates a statistically significant difference between the control group (nontreated) and the OIPC-treated group (*, $P \leq 0.05$). (C) Confocal laser scanning microscopy of biofilm structures that developed inside polyurethane catheters of control and OIPC-treated animals. After catheter removal, devices were cut longitudinally and stained with concanavalin A (50 mg/liter) (green fluorescence) at 37°C for 30 min. Arrows demonstrate rudimentary biofilms retrieved from catheters from OIPC-treated animals.

effect on *C. albicans* M177 planktonic cells, while a dose of 4 mg/liter was sufficient to abolish the growth of *C. albicans* M89 and M205 (Table 1).

Furthermore, we investigated the activity of OIPC against biofilms formed by WT strain SC5314 (Fig. 5A) and caspofungin-resistant isolates (Fig. 5B to D). OIPC (4 mg/liter) significantly diminished preformed *C. albicans* M177 and M205 biofilms, whereas 8 mg/liter significantly decreased *C. albicans* M89 biofilms ($P < 0.05$). As expected, caspofungin alone was shown to be ineffective. Strikingly, no fungal cells were recovered from *C. albicans* M89 and M177 biofilms treated with 8, 10, and 13 mg/liter of OIPC ($P < 0.05$). A similar phenomenon was observed for *C. albicans* M205 biofilms treated with 10 and 13 mg/liter of OIPC ($P < 0.05$). These results strongly emphasize that OIPC may be an important component in the treatment of planktonic cells and biofilms formed by clinical isolates that are resistant to caspofungin.

In a next step, we explored a novel therapeutic approach, based on the combination of caspofungin and OIPC. This may result in the inhibition of *C. albicans* clinical isolates M177, M205, and M89, which are resistant to caspofungin. Overall, the combination of both drugs led to an improvement of MIC₅₀ values against all strains tested (Table 3). The combination of both drugs against the growth of *C. albicans* SC5314 resulted in a fractional inhibitory concentration index (FICI) value of 0.73, indicating no interaction. Strikingly, when this strategy was used on caspofungin-resistant isolates, a synergistic effect was observed, suggesting that this combination may represent an alternative way to treat infections caused by isolates that are resistant to caspofungin (Table 3).

FIG 4 Effect of OIPC on *Candida albicans* SC5314 biofilm formation on mucosal surfaces in an *ex vivo* model of oral infection. Shown are data from histopathology analyses of mouse tongues infected with *C. albicans* in the presence and absence of OIPC. Representative microscopic images from periodic acid-Schiff-stained tissues display significantly decreased adherence and invasion of tongue tissue treated with 4, 8, and 10 mg/liter of OIPC. *Candida* cells treated with 8 and 10 mg/liter of OIPC did not undergo a yeast-to-hypha transition and were found solely as yeasts. Magnification, $\times 40$.

OIPC strongly inhibits the yeast-to-hypha transition, a crucial virulence factor for *C. albicans* pathogenesis. In this study, we noticed an increased amount of yeast cells and reduced hypha formation in OIPC-treated biofilms (Fig. 1D) compared to the control. Therefore, we examined the morphological changes of planktonic cells in hypha-inducing media, namely, RPMI 1640, Spider, and Lee media, upon treatment with OIPC (0.5, 1, and 2 mg/liter) at 37°C. The pictures shown in Fig. 6 were taken after 4 h and 24 h. Cells grown in RPMI 1640 and Spider media for 4 h and treated with 0.5 mg/liter of OIPC were able to undergo morphogenesis. Cells treated with higher concentrations (1 and 2 mg/liter) and grown in RPMI 1640 and Spider media for 4 h remained in the yeast form. Cells cultured in Lee medium and treated with even the lowest concentration of OIPC (0.5 mg/liter) were found solely as yeasts. After 24 h of growth, nontreated cells and *Candida* cells treated with 0.5 mg/liter of OIPC created typical clusters of hyphae and pseudohyphae. OIPC-treated (1 and 2 mg/ml) *Candida* cells appeared individually as yeasts or in groups composed of aberrant hyphae. These

TABLE 2 Strains and clinical isolates used in this study

Strain or clinical isolate (reference)	General characteristic	Origin
<i>C. albicans</i> SC5314 (24)	Wild type	Disseminated candidiasis
<i>C. albicans</i> U-0503-18	Clinical isolate	Urinary catheter
<i>C. albicans</i> U-0503-12	Clinical isolate	Urinary catheter
<i>C. albicans</i> U-0203-29	Clinical isolate	Urinary catheter
<i>C. albicans</i> U-0203-15	Clinical isolate	Urinary catheter
<i>C. albicans</i> U-04030-255	Clinical isolate	Urinary catheter
<i>C. albicans</i> HC2601-30	Clinical isolate	Blood culture
<i>C. albicans</i> HC0202-25	Clinical isolate	Blood culture
<i>C. albicans</i> HC2102-19	Clinical isolate	Blood culture
<i>C. albicans</i> HC1602-6	Clinical isolate	Blood culture
<i>C. albicans</i> HC0702-14	Clinical isolate	Blood culture
<i>C. albicans</i> M89 (16)	Clinical isolate	
<i>C. albicans</i> M177 (16)	Clinical isolate	
<i>C. albicans</i> M205 (16)	Clinical isolate

FIG 5 OIPC displays antifungal activity against *Candida albicans* biofilms formed by caspofungin-resistant clinical isolates (*C. albicans* M89, M177, and M205). *C. albicans* SC5314 biofilms were susceptible to caspofungin (A), whereas fungal cells within biofilms formed by three caspofungin-resistant isolates were tolerant to the highest concentration of this drug (B to D). OIPC significantly decreased the development of preformed biofilms by *C. albicans* M89, M177, and M205 (*, $P < 0.05$). Data are presented as the mean log₁₀ CFU $\pm$ standard errors of the means from catheters from 3 independent experiments. Black columns represent caspofungin-treated samples, whereas white bars show samples treated with OIPC.

findings demonstrate a strong effect of OIPC on *C. albicans* morphogenesis, which can be considered a potential mode of action for this molecule.

DISCUSSION

In the present work, we proved that OIPC has antifungal activity against planktonic and biofilm-forming *C. albicans* cells. The present study is the first to deal with the antifungal effects of OIPC on *C. albicans*. Used against planktonically grown cells, OIPC showed high activity against all clinical isolates tested, with MIC values ranging between 1 and 4 mg/liter. Moreover, fungicidal activity of OIPC against *C. albicans* cells was reported for concentrations ranging between 2 and 4 mg/liter, which are identical

TABLE 3 Potential combination of caspofungin and OIPC against planktonic cells of caspofungin-susceptible (*Candida albicans* SC5314) and caspofungin-resistant (*Candida albicans* M177, M205, and M89) clinical isolates^a

Strain	MIC _{CAS[alone]} (mg/liter)	MIC _{CAS[combined]} (mg/liter)	MIC _{OIPC[alone]} (mg/liter)	MIC _{OIPC[combined]} (mg/liter)	FICI
SC5314	0.125	0.06	2	0.5	0.73
M177	8	1	2	0.5	0.38
M205	16	4	4	1	0.50
M89	8	0.5	4	1	0.31

^aInterpretation of drug combination interactions was based on the FICI (26) {FICI = (MICA_[combined]/MICA_[alone]) + (MICB_[combined]/MICB_[alone])}, where an FICI of ≤ 0.5 indicates a synergistic interaction, an FICI of 0.5 to 4 indicates no interaction, and an FICI of >4.0 indicates an antagonistic interaction.

FIG 6 OIPC inhibits *Candida albicans* SC5314 yeast-to-hypha transition. Shown are light microscopy images of the *C. albicans* yeast-to-hypha transition in hypha-inducing media, such as RPMI 1640, Spider, and Lee media, at 37°C. Samples were taken 4 h and 24 h after the administration of different concentrations of OIPC (0.5, 1, and 2 mg/liter) to fungal cells. Control (no drug) and OIPC-treated (0.5 mg/liter) cells were able to undergo the switch after 4 h of growth in RPMI 1640 and Spider media. Cells treated with 0.5 mg/liter OIPC in Lee medium remained in the yeast form. At higher concentrations tested (1 and 2 mg/liter), *Candida* cells did not switch from yeast to hyphae. The formation of hyphae and pseudohyphae was noticed after 24 h of growth under control conditions (no drug) and with 0.5 mg/liter OIPC. At 1 and 2 mg/liter OIPC, cells were present as pseudohyphae, and the majority of cells remained in the yeast form. MOPS, morpholinepropane-sulfonic acid. Bar, 10 μ m. Magnification, $\times 60$.

to the MICs of almost all isolates tested. Interestingly, similar concentrations of OIPC were able to reduce or to completely abolish biofilm development, reminiscent of the results that were previously obtained with miltefosine (5, 11). Additionally, we show that highly dense preformed biofilms can also be inactivated with OIPC at concentrations only 2 to 4 times higher than the MIC for planktonic cells. Existing data show that, *in vitro*, all azoles, including the “gold standard” fluconazole and the newer molecule voriconazole, require concentrations of >1,000 and 256 mg/liter, respectively, to inactivate cells present in a biofilm. Moreover, *Candida* biofilms displayed decreased sensitivity to almost all available antifungals, including amphotericin B, flucytosine, nystatin, and terbinafine, as reviewed by Taff et al. (17). Next, we employed a subcutaneous *C. albicans* biofilm rat model to determine whether the *in vitro* activity of OIPC against *Candida* biofilms could be translated *in vivo*. In this particular model, *C. albicans* biofilms develop inside the lumen of a catheter piece, and cells dispersing from biofilms spread only into the tissue surrounding the device, without affecting vital organs (18). This model has already successfully been used to study the effects of fluconazole and echinocandins on preformed *C. albicans* biofilms (14, 15). Fluconazole (125 mg/kg of body weight/day) failed to be active against *C. albicans* biofilms, whereas caspofungin (5 mg/kg of body weight/day), anidulafungin (10 mg/kg of body weight/day), and micafungin (20 mg/kg of body weight/day) remained effective (14, 15). Despite the effectiveness of echinocandins against *C. albicans* biofilms, the intravenous route of administration of these drugs is a major disadvantage. OIPC is an orally bioavailable drug, which represents a great practical advantage for a patient. In our study, rats were given OIPC (20 mg/kg of body weight/day) orally, daily for 7 days. This treatment resulted in a significant reduction but not a complete eradication of biofilm-forming cells retrieved from catheters, accompanied by the rudimentary biofilm architecture. In comparison with *in vitro* data, where the action of drugs on *C. albicans* catheter-associated cells is more direct, we could not achieve a complete inhibition of biofilms *in vivo*. In fact, pharmacodynamics (PD), pharmacokinetics (PK), bioavailability, and drug interactions represent major factors that may influence the effect of drugs on pathogens *in vivo*. Hence, it is crucial to understand the limitations of the *C. albicans in vitro* model to mimic the situation *in vivo*. As mentioned above, we tested only one regimen of OIPC (20 mg/kg of body weight/day for 7 days) on *C. albicans* biofilms *in vivo* and looked only at one time point after treatment (day 8), which can be considered limitations of this study. Although PK/PD analyses of OIPC were not performed in this study, pharmacokinetic analyses have been established by using mice (10), hamsters (8), and dogs (9), documenting its good oral bioavailability and extensive tissue distribution. Moreover, based on the long half-life of OIPC (~50 h in rodents), 7-day treatment is most likely to lead to an accumulation of the drug, which will prolong the therapeutic effect beyond the end of the treatment period (8). In the future, we suggest that other total doses of OIPC should be administered and that the evolution of the treatment effect should be monitored for a longer period of time, for example, by bioluminescence imaging.

Besides the activity of OIPC on biofilms formed on abiotic surfaces, we have also demonstrated that OIPC can be a good candidate to treat candidiasis that develops on mucosal surfaces. Quite recently, the topical administration of miltefosine (50 mg/kg twice a day) was successfully used to treat oral candidiasis in immunocompromised mice (5). Histopathology images (Fig. 4A) provide great evidence that tongues treated with 8 and 10 mg/liter of OIPC displayed lower fungal burdens. In order to support these promising findings, future experiments dedicated to OIPC treatment of oropharyngeal candidiasis in an animal model are essential.

Although echinocandins remain a favorable class of drugs for the treatment of invasive candidiasis, its widespread usage is one of the main reasons for drug resistance among clinical *Candida* isolates (19). Echinocandins proved to be some of the most active agents in the treatment of catheter-associated infections caused by wild-type *C. albicans* strain SC5314 in a subcutaneous rat model (15). In this study, we show that *in vitro* biofilms formed by *C. albicans* caspofungin-resistant clinical isolates did not

respond to echinocandin therapy, whereas OIPC was highly active against device-associated infections by these strains. Besides biofilms, planktonic cells responded to OIPC therapy as well. The concentration for the fungicidal activity of OIPC against free-living cells of these clinical isolates ranged between 2 and 4 mg/liter. These findings demonstrate that OIPC can be a valuable option for the treatment of catheter-associated infections caused by *C. albicans* clinical isolates that are resistant to echinocandins. As an alternative approach, we performed a checkerboard assay to demonstrate potential synergism when both compounds are combined to fight infections caused by caspofungin-resistant isolates. Interestingly, all three caspofungin-resistant clinical isolates responded to the combinatorial therapy, and both drugs behaved synergistically (Table 3). In comparison with other compounds and existing drugs on the market, OIPC seems to be cleared rapidly, which may be beneficial with regard to the potential for the selection of resistance (10). Our findings demonstrate the potential of the combination of echinocandins with OIPC, which can reduce the growth of planktonic cells that are resistant to caspofungin.

In this study, we have revealed that *C. albicans* cells treated with 1 and 2 mg/liter of OIPC did not undergo a yeast-to-hypha transition, even in hypha-inducing media. After 24 h of treatment, only yeast cells and pseudohyphae were present in treated cultures (1 and 2 mg/liter of OIPC) compared to the control. Additionally, *C. albicans* cells found on the surface of catheters after treatment with 4 mg/liter of OIPC remained in the yeast form (Fig. 1). Although the exact mode of action of OIPC on *C. albicans* is not known, we observed that the application of a sublethal dose of OIPC (1 and 2 mg/liter of OIPC) already inhibited the *C. albicans* yeast-to-hypha transition, even in hypha-inducing media. After 24 h of treatment, only yeast cells and pseudohyphae were present in treated cultures compared to the control. Additionally, *C. albicans* cells found on the surface of catheters after treatment with 4 mg/liter of OIPC remained in the yeast form. Future analyses of the effect of OIPC on known transcriptional regulators involved in the control of the yeast-to-hypha transition should be able to identify the transcription factors and signaling pathway(s) involved in the OIPC response. The extent to which these pathways are also responsible for clearing mature biofilms and/or the fungicidal effect of OIPC will need to be investigated. Interestingly, miltefosine has been shown to impact mitochondrial functioning, thereby leading to enhanced oxidative stress and apoptotic-like cell death (20–22). As mitochondrial function was recently linked to the regulation of virulence pathways in *C. albicans*, including the morphogenetic switch to hyphae (23), mitochondria are an attractive potential target for OIPC, although this remains to be verified.

Taken together, data from this work confirmed that low concentrations of OIPC are efficacious in inhibiting *C. albicans* planktonic cells and biofilm formation *in vitro* and that preformed biofilms are also susceptible to this drug. Its activity against caspofungin-resistant clinical isolates highlights the importance of expanding studies on its antifungal properties. Finally, the demonstration of the oral activity of OIPC *in vivo* in a subcutaneous biofilm model of catheter-associated candidiasis supports the potential of this molecule as a promising antifungal candidate for the treatment of deep-seated *Candida* infections.

MATERIALS AND METHODS

Ethics. All animal experimental procedures were performed in accordance with KU Leuven animal care guidelines and were approved by the Ethical Committee of KU Leuven (project number P090/2013).

Strains and clinical isolates. *C. albicans* standard strain SC5314 (24) and clinical isolates obtained from urinary catheters and blood cultures (UZ Gasthuisberg, Belgium) were used. All clinical isolates used in this study are described in Table 2. All strains and clinical isolates were grown on YPD medium (1% yeast extract, 2% Bacto peptone, and 2% D-glucose, supplemented with 2% agar) at 37°C.

OIPC and caspofungin. OIPC in a crystalline form was kindly provided by Dafra Pharma R&D (Turnhout, Belgium). The stock solution of OIPC was prepared in sterile water. For all experiments, different concentrations of OIPC (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 10, and 13 mg/liter) were tested, unless stated otherwise. Because of the fact that OIPC is an alkylphospholipid with the tendency to foam, the highest concentration that did not cause this phenomenon was 13 mg/liter. Caspofungin (Cancidas; MSD) was prepared in dimethyl sulfoxide (DMSO) and further diluted in RPMI 1640 before use. Multiple concentrations of caspofungin, namely, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 10, and 13 mg/liter, were tested.

MIC and MFC determinations. The antimicrobial activity of OIPC against planktonic growth was assessed according to the CLSI M27-A3 protocol (25). Data were determined as MIC₅₀ values. Next, 100 μ l of a *C. albicans* suspension, treated with OIPC, was plated onto YPD plates, which were further incubated at 37°C overnight. The concentration of OIPC that completely abolished the growth of *C. albicans* was considered the MFC.

Checkerboard assay. A checkerboard assay was performed according to methods described previously by Tobudic et al. (4). *C. albicans* planktonic cultures were inoculated into round-bottomed 96-well polystyrene plates and subsequently supplemented with either OIPC, caspofungin, or both. Control wells contained only RPMI 1640 medium. Plates were incubated for 48 h at 37°C. Here, plates were read visually, and the content of each well was plated onto YPD plates and incubated at 37°C overnight. Interpretation of the interactions of the drug combination against *C. albicans* planktonic cell growth was done based on the FICI (26). The FICI was calculated by the formula $FICI = (MIC_{A(combined)} / MIC_{A(alone)}) + (MIC_{B(combined)} / MIC_{B(alone)})$, in which $MIC_{A(combined)}$ and $MIC_{B(combined)}$ are the MICs of agents in combination and $MIC_{A(alone)}$ and $MIC_{B(alone)}$ are the MICs of drugs A and B alone, respectively. An FICI of ≤ 0.5 was defined as a synergistic interaction, an FICI of 0.5 to 4 was defined as no interaction, and an FICI of > 4.0 was defined as an antagonistic interaction.

In vitro biofilm susceptibility testing. *In vitro C. albicans* biofilm drug susceptibility testing was performed on serum-coated polyurethane catheter fragments. The effect of OIPC on biofilms was tested in three setups differing in the developmental stages, namely, during the periods of adhesion and biofilm development and on preformed 24-h-old biofilms. For studies of the effect of OIPC on *C. albicans* adhesion (90 min at 37°C), the polyurethane devices were submerged in 1 ml *Candida* cells (5×10^4 cells/ml) dissolved in medium (control sample) or in the presence of different concentrations of OIPC. After this, nonattached cells were removed by washing steps. The amount of attached cells per catheter piece was quantified by CFU counting as described previously by Řičicová et al. (18). For assessment of the efficacy of OIPC against biofilm development, the adhesion period was induced in drug-free medium. After 90 min, catheters were washed with phosphate-buffered saline (PBS) and submerged in fresh RPMI 1640 medium (control sample) or supplemented with OIPC. Biofilms were allowed to develop for 24 h at 37°C, after which the catheters were washed twice with PBS, and the number of biofilm-forming cells was quantified as CFU. For assessment of the effect of OIPC on preformed biofilms, *Candida* cells were allowed to form 24-h-old biofilms in RPMI 1640 medium only. After 24 h, biofilms were washed twice with PBS and submerged in fresh RPMI 1640 medium (control sample) or in medium containing different concentrations of OIPC. Catheters were further incubated for 24 h at 37°C. Afterwards, catheters were washed and transferred to microcentrifuge tubes containing 1 ml of PBS. The efficacy of OIPC against preformed biofilms was documented by CFU determination.

The effect of caspofungin on the formation of preformed *C. albicans* biofilms on polyurethane fragments was also studied. Twenty-four-hour-old biofilms were formed in drug-free RPMI 1640 medium as described above. After washing, catheters were supplemented with clean RPMI 1640 medium (control sample) or in medium containing caspofungin for the next 24 h at 37°C. The effect of caspofungin on *Candida* biofilms was also assessed by CFU determination.

In vivo biofilm drug susceptibility testing using a rat subcutaneous biofilm model. *In vivo C. albicans* SC5314 biofilms were formed inside serum-coated polyurethane catheter pieces in immunosuppressed female Sprague-Dawley rats as described previously by Řičicová et al. (18). *In vivo C. albicans* biofilm treatment was initiated after 24 h of biofilm formation. OIPC was administered orally, once daily, at a concentration of 20 mg/kg of body weight/day for 7 days. Sterile water was administered orally to the control group of animals. On day 9, catheters were explanted from euthanized animals, and the number of cells per individual biofilm was determined as CFU (18).

Ex vivo model of infection. Experiments using an *ex vivo* model of infection were performed according to methods described previously by Peters et al. (27), with small modifications. Tongues excised from sacrificed 8-week-old female BALB/c mice (Janvier, France) were placed into a 24-well tissue culture plate and infected with *C. albicans* SC5314 (1×10^7 cells/ml) during 30 min of incubation at 37°C with gentle shaking (120 rpm). Afterwards, tongues were supplemented with fresh RPMI 1640 medium (control) or with medium containing OIPC, and they were further incubated for 24 h at 37°C with gentle shaking (120 rpm). Following washing with PBS, the tongues were transferred to tubes containing PBS. Half of the harvested tongues were fixed and processed for histopathology, whereas the other half were homogenized and sonicated for 10 min in a water bath sonicator. Series of dilutions were plated onto YPD agar plates, and the CFU were determined.

Yeast-to-hypha transition. Cultures of *Candida* cells grown overnight were washed twice with water and adjusted to a final concentration of 1×10^5 cells/ml in either RPMI 1640 medium (pH 7.0), Spider medium (28), or Lee medium (29) with 0.5, 1, or 2 mg/liter of OIPC. Samples supplemented with water were considered controls. Cultures were incubated for 4 h and 24 h at 37°C. The proportion of true hyphae versus budding yeasts was determined by light microscopy (Axiostar plus; Carl Zeiss, Germany) at a magnification of $\times 60$.

Scanning electron microscopy. A qualitative analysis of the biofilm architecture was performed by SEM (XL30-FEG; FEI). Prior to SEM, samples were coated with Au-Pd by using a sputtering device (Edwards S150). The scanning electron microscope was operated with standard high-vacuum settings using a 10-mm working distance and a 20-keV accelerating voltage.

Confocal laser scanning microscopy. *In vivo C. albicans* biofilms were analyzed by CSLM (LSM510/ConfoCor2 system; Carl Zeiss, Jena, Germany). Catheters were stained with concanavalin A (50 mg/liter) (Molecular Probes, Eugene, OR, USA) at 37°C for 1 h (green fluorescence). Before biofilm thickness was assessed, three-dimensional images of biofilms were captured. Approximately 150 sections were made

through the whole biofilm architecture. Next, biofilm thickness was estimated from the outer edges of the area where the fluorescent signal gain showed maximum intensity to the area where no fluorescent signal could be detected.

Statistical analyses and reproducibility of results. Statistical analyses were performed by using Student's *t* test (GraphPad Prism software). Differences were considered significant at a *P* value of ≤ 0.05 . All *in vitro* experiments were performed at least three times, always using three devices per concentration. The experiment with the *ex vivo* model of infection was repeated twice, including at least 3 tongues/concentration tested. *In vivo* experiments were performed three times (in total, 8 animals/group). Experiments related to the yeast-to-hypha transition were repeated three times.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01767-17>.

SUPPLEMENTAL FILE 1, PDF file, 4.5 MB.

SUPPLEMENTAL FILE 2, AVI file, 2.5 MB.

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We have no conflicts of interest.

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