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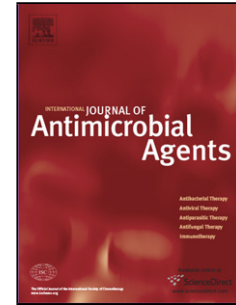
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## **Possible role of azole and echinocandin lock solutions in the control of *Candida* biofilms associated with silicone**

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## ABSTRACT

Until now, management of candidiasis related to implanted devices has remained problematic. The aim of this study was to investigate antifungal lock strategies against *Candida albicans* and *Candida glabrata* biofilms in vitro. Three antifungal agents were used against eight *C. albicans* and six *C. glabrata* clinical strains isolated from infected catheters. Caspofungin and micafungin, both echinocandins, as well as the azole posaconazole were tested. An in vitro model of *Candida* biofilm on 100% silicone catheters was used. Efficacy of the antifungal lock was tested against biofilms aged 12 h and 5 days following exposure to caspofungin (5 mg/L and 25 mg/L), micafungin (5 mg/L and 15 mg/L) and posaconazole (10 mg/L) for 12 h. Persistence of antibiofilm activity was investigated 1–3 days following drug elimination. Antifungal lock was considered effective in the event of a significant decrease ( $P < 0.001$ ) in the metabolic activity of the biofilm yeast. The results showed that micafungin had significant inhibitory effectiveness against young and mature *C. albicans* and *C. glabrata* biofilms. Moreover, this activity appeared to persist for up to 3 days. Caspofungin displayed similar activity against all *C. albicans* biofilms, but the activity was less persistent for *C. glabrata* biofilms. Posaconazole was less effective against *C. albicans* biofilms, but its activity was sustained. Echinocandin lock therapy could significantly enhance the management of candidiasis in patients with indwelling catheters by combating biofilms and enabling device maintenance in situ.

## 1. Introduction

*Candida* spp. are one of the most common causes of hospital-acquired infections. Candidaemia is frequently associated with intravascular catheter-related infections, which is usually correlated with the formation of a biofilm. The biofilm alters the yeast's susceptibility to antifungal agents [1,2].

The French Society for Hospital Hygiene recommends that peripheral venous catheters be maintained for no more than 96 h (<http://www.sfhh.net>). Central venous catheters are to be left in situ until colonised. When candidiasis has been documented, it is currently recommended that the central venous catheter should be removed [3]. However, in many patients with catheter-related *Candida* infection, removal of an infected device requires its replacement by a new one. In addition, this procedure generates additional costs. The antibiotic lock technique may constitute an alternative with regard to the prevention and treatment of intravascular catheter-related infections, particularly insofar as the technique would enable catheter maintenance in situ. The lock technique involves instillation of high concentrations of an antimicrobial agent directly into the catheter in order to 'lock' it for several hours or days. The antimicrobial solution fills the lumen without spilling into the circulation [4]. In 2005, Lepape [5] showed that the antibiotic concentrations used for lock therapy and effective on biofilm microorganisms were approximately 100–1000-fold the minimum inhibitory concentration (MIC).

The role of antifungal lock solutions is not well defined [3] as few reports on their use in clinical practice are available in the literature [6,7].

The activity of echinocandins on biofilms has already been demonstrated in vitro [2,8]. These agents are known to have potent and lasting antifungal and antibiofilm activity [9]. Used as lock solutions, echinocandins could be particularly useful against catheter infections.

The in vitro efficacy of echinocandin lock solutions with respect to *Candida albicans* biofilm growth was investigated in a preliminary study (two strains). The results suggested that micafungin and caspofungin indeed reduced the metabolic activity of *C. albicans* growing as biofilm. Moreover, this reduction was maintained for up to 72 h post lock, suggesting a persistent effect [10].

A few recent articles suggest that posaconazole treatment ( $\geq 24$  h) does not reduce the metabolic activity of *C. albicans* in biofilms by  $>50\%$  [9,11]. Posaconazole was included in the current study to investigate its antibiofilm activity as a short-term treatment (12 h).

The aim of this study was to confirm the preliminary results obtained with micafungin and caspofungin lock solutions against *C. albicans* biofilms using eight additional strains isolated from infected catheters (Table 1). The study was then extended to include *Candida glabrata* species, including six strains isolated from infected catheters (Table 1). Subsequently, the antibiofilm activity of posaconazole lock solution was investigated using the same strains of *C. albicans* and *C. glabrata*.

## 2. Materials and methods

All the strains studied are referenced in Table 1. MIC values were determined using Etest strips in accordance to the manufacturer's instructions (AB BIODISK, Solna, Sweden).

Strains were identified using conventional methods such as the germ tube test in serum, agglutination (Bichrolatex; Fumouze, Levallois-Perret, France) and metabolic properties (API20C; bioMérieux, Marcy-l'Étoile, France).

Yeasts were first cultured on Sabouraud agar slants (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) at 37 °C for 48 h. A loopful of the culture was then transferred to 25 mL of Yeast Nitrogen Base medium (Difco, Detroit, MI) supplemented with 30 mM of glucose (Sigma, St Louis, MO) (YNB-Glc) and was incubated without shaking at 37 °C for 16 h.

Blastospores were then harvested, washed twice in 0.1 M phosphate-buffered saline (PBS) (pH 7.2) (bioMérieux) and adjusted to a concentration of  $5 \times 10^6$  blastospores/mL in YNB-Glc for use in the biofilm experiments.

*Candida* biofilms aged 12 h (young biofilm) and 5 days (mature biofilm) were formed in untreated microtitre plates (Corning Inc., Corning, NY) as previously described using 5 mm sections of 100% silicone catheters (A-M Systems, Carlsberg, WA) [10]. These maturation stages were chosen arbitrarily. For the 5-day incubation period, media were replenished every 2 days with up to 300  $\mu$ L of YNB-Glc to compensate

for evaporation. Prior to biofilm production, catheter sections were autoclaved, incubated overnight in neat fetal bovine serum and washed with YNB-Glc.

Stock solutions were prepared as follows: 2 mg/mL caspofungin (Merck Research Laboratories, Rahway, NJ) stock solution in sterile distilled water; 2 mg/mL micafungin (Astellas Pharma Inc., Tokyo, Japan) stock solution in sterile distilled water; and 1 mg/mL posaconazole (Schering-Plough, Kenilworth, NJ) stock solution in dimethyl sulphoxide (DMSO). Antifungal stock solutions were then diluted in YNB-Glc. Caspofungin, micafungin and posaconazole concentrations tested were approximately 100–500× MIC and were thus compatible with the lock therapy strategy [4]. Subsequently, 300 µL of 5 mg/L or 25 mg/L caspofungin, 5 mg/L or 15 mg/L micafungin, or 10 mg/L posaconazole solution were added to pre-formed biofilms and the plates were incubated at 37 °C for 12 h. Controls consisted of biofilms incubated with 300 µL of YNB-Glc for 12 h. All the silicone sections were then moved to new wells and were incubated with YNB-Glc without an antifungal agent at 37 °C for 24, 48 or 72 h for young biofilms and for 24 h or 48 h for mature biofilms in order to evaluate the persistence of antibiofilm activity.

Antibiofilm effects were monitored using a previously described metabolic assay based on the reduction of a tetrazolium salt (XTT) [12]. Briefly, each catheter section coated with biofilm and either exposed or not exposed to antifungal solution was incubated with XTT (300 mg/L) and menadione (0.13 mM) in 150 µL of PBS at 37 °C for 3 h. Absorbance was then measured at 492 nm ( $A_{492}$ ) in a microplate reader (LP400; Sanofi Diagnostics Pasteur). Absorbance indicates the metabolic activity of the yeast in the biofilm. All experiments were performed twice with eight replicates.



Analysis of variance (ANOVA) and Scheffe's test were performed to determine whether the between-group differences were statistically significant.

To facilitate analysis of the results, the activity inhibition percentages were calculated as follows: inhibition (%) =  $100 \times [1 - (A_{492} \text{ lock-treated strain}/A_{492} \text{ untreated strain})]$ .

### 3. Results and discussion

Young biofilms were used as an in vitro model of peripheral or central venous catheters, which may be colonised within a few hours of insertion. Persistence of lock activity was studied up to 72 h post treatment because catheters may be left in situ for up to 96 h.

The results obtained against *C. albicans* young biofilms 24 h post echinocandin lock are shown in Table 2. For all the strains, the mean decrease in metabolic activity of the biofilm yeasts due to the antifungal lock was expressed as a function of the antifungal drug and test concentration.

Inhibition of biofilm yeast growth ranged from 46% (5 mg/L caspofungin, strain 1119) to 89% (5 mg/L caspofungin, strain 1137), depending on the strain, antifungal agent and test concentration.

Persistence of activity was evaluated 48 h and 72 h after removal of the antifungal solutions. The inhibitory activities of caspofungin and micafungin lock solutions against young *C. albicans* biofilms were sustained 48 h and 72 h post lock,

irrespective of the strain concerned. Inhibition ranged from 32% (5 mg/L micafungin, strain 1119) to 92% (15 mg/L micafungin, strain 1160) at 48 h post lock. The young biofilms of strains 1126 and 1163 exhibited >80% inhibition up to 3 days post lock.

Despite some differences between *C. albicans* strains, inhibition of metabolic activity persisted for up to 72 h post lock. There was no significant difference between the activities of caspofungin and micafungin.

Results suggested that, irrespective of the antifungal concentration and test strain concerned, caspofungin and micafungin locks exerted a significant ( $P < 0.001$ ) inhibitory activity (>70%) against young *C. albicans* biofilms.

Interestingly, the highest echinocandin concentrations (500× MIC) did not have a significantly greater antibiofilm activity than the lowest concentrations (100–200× MIC). A paradoxical effect could explain this phenomenon [13]. However, the highest concentration tested in the current study corresponds to one of the lowest concentrations involved in the paradoxical effect, so it was not possible to establish this explanation. These results need to be confirmed in clinical practice but suggest that it may not be necessary to use the highest concentrations. This would also be a more cost-effective approach.

The activities of caspofungin and micafungin lock solutions were then tested against mature *C. albicans* biofilm. Persistence of activity was monitored over a 48-h period post lock (Table 3). In clinical practice, mature biofilms only occur with central venous catheters, since peripheral venous catheters are changed every 96 h.

With all the strains under study, 24 h after the echinocandin lock, inhibition of mature *C. albicans* biofilms ranged from 44% (5 mg/L micafungin, strain 1119) to 90% (25 mg/L caspofungin, strain 1160).

Both echinocandins exerted a significant inhibitory activity ( $P < 0.001$ ) on mature biofilms for 48 h post lock. This inhibition ranged from 28% (25 mg/L caspofungin, strain 1119) to 92% (25 mg/L caspofungin, strain 1163).

The weakest inhibitory activity was observed with young and mature biofilms of *C. albicans* strain 1119, even though the MIC of caspofungin was 0.064 mg/L, indicating strain susceptibility (Table 1).

These results showed that, irrespective of biofilm age, both caspofungin and micafungin locks induced an inhibition of *C. albicans* biofilm growth. This suggests that lock approaches may be useful when used in combination with systemic antifungal therapy to eradicate *C. albicans* biofilms on catheters, as has already been demonstrated in vivo by Shuford et al. [14]. However, these authors' studies were based on 8-h or 7-day locks using amphotericin B or caspofungin. They reported that lock therapy was of particular value in the treatment of intraluminal biofilms but had to be combined with treatment of the extraluminal biofilm.

Lock activity of echinocandins was also tested against *C. glabrata* biofilms. However, since the highest concentrations had not been shown to be more effective against *C.*

*albicans* biofilms, the study was only performed at an echinocandin concentration of 5 mg/L.

The results for young biofilms at 24 h post caspofungin or micafungin lock are shown in Table 2. For all the strains, the metabolic activity of biofilm yeasts was significantly reduced post lock ( $\geq 62\%$ ). For strains 1, 767 and 788, inhibition was significantly greater ( $P < 0.001$ ) following micafungin lock than following caspofungin lock. Mean inhibition reached 92% with micafungin versus 77% with caspofungin.

Moreover, the levels of inhibition induced by micafungin lock (ca. 93%) were similar for all of the strains. Statistical analysis showed that the inhibition induced by caspofungin lock were also similar with all of the strains (range 62–89%; differences not significant).

Inhibitory activity was shown to persist for 48 h and 72 h post lock. Micafungin induced a stronger inhibition ( $P < 0.01$ ) of the metabolic activity of *C. glabrata* biofilms than caspofungin: at 72 h post lock, micafungin induced a mean level of inhibition  $>90\%$  versus 38% with caspofungin.

The antibiofilm activity of micafungin on young *C. glabrata* biofilms persisted for 3 days, whilst that of caspofungin did not.

The results obtained using echinocandin lock on mature *C. glabrata* biofilms at 24 h and 48 h post lock showed that, irrespective of echinocandin, the metabolic activity of the yeasts included in mature *C. glabrata* biofilms was significantly reduced ( $P <$

0.001) (caspofungin, 58–87% inhibition; micafungin, 90–95% inhibition) at 24 h post lock. The inhibition induced by micafungin persisted for 48 h after drug removal (mean inhibition 90%) and was stronger than that achieved with caspofungin (mean inhibition 44%) ( $P < 0.01$ ).

Thus, the efficacy of caspofungin locks both on young and mature *C. glabrata* biofilms was of shorter duration than that of micafungin. To our knowledge, this is the first report of a difference between the activities of caspofungin and micafungin in this context. No obvious hypothesis can explain this difference.

In conclusion, this study thus showed that the antibiofilm potential of caspofungin and micafungin locks with respect both to young and mature *C. albicans* biofilms was maintained for *C. glabrata* biofilms, although micafungin showed greater sustained efficacy than caspofungin both against young and mature *C. glabrata* biofilms. These findings are of interest because of the lack of susceptibility of *C. glabrata* to current azoles [1]. In 2006, Seidler et al. [8] reported that 48-h micafungin treatments (0.5–16 mg/L) were active against *C. albicans* and *C. glabrata* biofilms on polystyrene and silicone elastomers in vitro. The present study has shown that 5 mg/L micafungin was effective against biofilms of the same species on 100% silicone even after a shorter exposure time (12 h) than that usually used in lock strategies.

Under the same experimental conditions, the activity of 10 mg/L posaconazole solutions was tested on *C. albicans* biofilms. The antifungal concentration range was again 100–200× MIC (Table 1).

Despite some differences between strains, young and mature biofilm growth inhibition reached ca. 50% at 24 h post lock (Tables 2 and 3). The results concur with those reported by Tobudic et al. [11] and Katragkou et al. [9] Irrespective of whether the biofilm was young or mature, antibiofilm activity remained significant ( $P < 0.001$ ) at 48 h and 72 h post lock (41–49%) (Tables 2 and 3), suggesting a persistence of antibiofilm activity.

Curiously, the inhibition observed for strain 66396 was still ca. 80% (data not shown). This strain did not have higher posaconazole susceptibility (MIC = 0.023 mg/L) than the other strains of *C. albicans* in planktonic culture (Table 1), but as a biofilm appeared to be more susceptible to the drug.

*Candida albicans* biofilms are known to be resistant to azoles [1]. The present results were therefore unexpected. However, the inhibition of metabolic activity was significantly weaker than that observed following echinocandin locks.

Investigation of the efficacy of posaconazole solution against *C. glabrata* biofilms generated similar results (Tables 2 and 3) both for young and mature biofilms. Posaconazole was devoid of inhibitory activity on *C. glabrata* biofilms irrespective of the incubation time post drug elimination. However, the strains of *C. glabrata* under study were all considered resistant to posaconazole, as indicated by their MIC values (Table 1). The results confirmed that the strains remained resistant to posaconazole when cultured as biofilms and overall were consistent with published data. Pfaller et al. [15] had previously demonstrated that planktonic *C. glabrata* are resistant to fluconazole [MIC for 90% of the organisms (MIC<sub>90</sub>) = 32 mg/L] and display weak

susceptibility to posaconazole ( $MIC_{90} = 2 \text{ mg/L}$ ). Furthermore, *Candida* strains embedded in biofilms are known to be less susceptible to azole antifungal agents [1,12].

#### 4. Conclusion

These findings confirm the potential value of lock strategies in the prevention of silicone-related *C. albicans* biofilms. Although it was not possible to achieve 100% biofilm inhibition under the current experimental conditions, use of caspofungin or micafungin could be of value in lock solution management of *C. albicans* infections in combination with systemic antifungal therapy. Similarly, micafungin lock solutions displayed efficacy against *C. glabrata* biofilms. However, caution is required with regard to caspofungin locks. Because their activity is less persistent, it may be necessary to repeat locks more frequently or to use higher antifungal concentrations to ensure effective prevention. Interestingly, posaconazole locks may be partially effective on *C. albicans* biofilms so long as they are used in combination with systemic antifungal therapy.

In conclusion, if used in combination with systemic medication, echinocandin lock therapy may contribute to controlling candidiasis in catheterised patients.

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None declared.

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**Table 1**

Susceptibility of *Candida* isolates from infected catheters to caspofungin, micafungin and posaconazole

Strain	Species	Source	MIC (mg/L)		
			Caspofungin	Micafungin	Posaconazole
3153	<i>C. albicans</i>	ATCC	0.19	0.012	0.032
66396	<i>C. albicans</i>	ATCC	0.064	0.004	0.023
1119	<i>C. albicans</i>	Poitiers Hospital	0.064	0.006	0.047
1126	<i>C. albicans</i>	Poitiers Hospital	0.064	0.006	0.023
1137	<i>C. albicans</i>	Poitiers Hospital	0.094	0.016	0.064
1150	<i>C. albicans</i>	Poitiers Hospital	0.047	0.012	0.064
1151	<i>C. albicans</i>	Poitiers Hospital	0.094	0.008	0.047
1156	<i>C. albicans</i>	Poitiers Hospital	0.064	0.008	0.047
1160	<i>C. albicans</i>	Poitiers Hospital	0.064	0.012	0.064
1163	<i>C. albicans</i>	Poitiers Hospital	0.094	0.012	0.032
1	<i>C. glabrata</i>	Versailles Hospital	0.19	0.008	2
767	<i>C. glabrata</i>	Poitiers Hospital	0.125	0.008	2
788	<i>C. glabrata</i>	Poitiers Hospital	0.19	0.008	1.5
924	<i>C. glabrata</i>	Poitiers Hospital	0.19	0.008	2
961	<i>C. glabrata</i>	Poitiers Hospital	0.125	0.008	2
1141	<i>C. glabrata</i>	Poitiers Hospital	0.125	0.008	8

MIC, minimum inhibitory concentration; ATCC, American Type Culture Collection (Manassas, VA).

**Table 2**

Reductions in the metabolic activity of young *Candida* biofilm yeasts induced by antifungal lock solutions

<i>Candida</i> spp.	Post-lock interval (h)	Decrease in metabolic activity (%) <sup>a</sup>				
		Caspofungin		Micafungin		Posaconazole
		5 mg/L	25 mg/L	5 mg/L	15 mg/L	10 mg/L
<i>C. albicans</i>	24	81.3 ± 8.8	76.9 ± 12	75.1 ± 14.1	70.7 ± 11.3	54.2 ± 6
		76.8 ± 12.4	78.6 ± 11.1	77.7 ± 14.4	65.4 ± 18.3	49.7 ± 12
	48	78.6 ± 17.4	79.5 ± 8.3	81.2 ± 9.3	74.8 ± 13.3	41.3 ± 7.9
		76.7 ± 10.1	N/S	92.7 ± 0.4	N/S	None <sup>b</sup>
	72	55.8 ± 16.7	N/S	91.6 ± 20.7	N/S	None
		42.8 ± 19.1	N/S	92.4 ± 0.8	N/S	None

N/S, not studied.

<sup>a</sup> Decreases are calculated as the mean reduction for each species (10 strains of *C. albicans* and 6 of *C. glabrata*; Table 1); mean reductions are presented for each antifungal concentration tested (5 mg/L and 25 mg/L caspofungin, 5 mg/L and 15 mg/L micafungin and 10 mg/L posaconazole) and for each post-lock interval studied (24, 48 or 72 h).

<sup>b</sup> None indicates that no inhibition was measured.

**Table 3**

Reductions in the metabolic activity of mature *Candida* biofilm yeasts induced by antifungal lock solutions

<i>Candida</i> spp.	Post-lock interval (h)	Decrease in metabolic activity (%) <sup>a</sup>				
		Caspofungin		Micafungin		Posaconazole
		5 mg/L	25 mg/L	5 mg/L	15 mg/L	10 mg/L
<i>C. albicans</i>	24	84.1 ± 7	75.2 ±	71.7 ±	66.4 ±	57.6 ± 3.5
	48		15.3	15.3	13.3	
<i>C. glabrata</i>	24	80.4 ±	81.5 ±	78.7 ±	75.9 ±	48.4 ± 6.8
	48		11.1	8.5	16.1	18.3
<i>C. glabrata</i>	24	77.7 ±	N/S	87.5 ±	NS	None <sup>b</sup>
	48		10.6	11.9		
		44.4 ±	N/S	90.3 ±	NS	None
			18.5	4.3		

N/S, not studied.

<sup>a</sup> Decreases are calculated as the mean reduction for each species (10 strains of *C. albicans* and 6 of *C. glabrata*; Table 1); mean reductions are presented for each antifungal concentration tested (5 mg/L and 25 mg/L caspofungin, 5 mg/L and 15 mg/L micafungin and 10 mg/L posaconazole) and for each post-lock interval studied (24 h or 48 h).

<sup>b</sup> None indicates that no inhibition was measured.