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Effect of alkylphospholipids on *Candida albicans* biofilm formation and maturation

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Objectives: The aim of this study was to evaluate miltefosine and four synthetic compounds (TCAN26, TC19, TC106 and TC117) for their *in vitro* inhibitory activity against *Candida albicans* planktonic and biofilm cells and investigate whether these compounds are able to inhibit the biofilm formation and to reduce the viability of mature *C. albicans* biofilm cells.

Methods: The XTT reduction assay and transmission and scanning electron microscopy were employed to determine the inhibitory effects of the test compounds in comparison with amphotericin B and fluconazole against both planktonic cells and sessile cells in biofilms.

Results: *C. albicans* planktonic cells were susceptible to miltefosine, TCAN26 and TC19, all alkylphospholipid compounds. Miltefosine and TCAN26 present a fungicidal activity with similar values of MIC and minimum fungicidal concentration (MFC), ranging from 2 to 8 mg/L. Cell treatment with sub-inhibitory concentrations of alkylphospholipids induced several ultrastructural alterations. In relation to biofilms, miltefosine reduced formation (38%–71%) and mature biofilms viability (32%–44%), at concentrations of 64 mg/L. TCAN26 also reduced biofilm formation (24%–30%) and mature biofilm viability (15%–20%), at concentrations of 64 mg/L. Although amphotericin B reduced biofilm formation similarly to miltefosine (51%–74%), its activity was lower on mature biofilms (24%–30%). Miltefosine antibiofilm activity was significantly higher than amphotericin B, on both formation and mature biofilms ($P<0.05$ and $P<0.0001$, respectively). Fluconazole was the least effective compound tested.

Conclusion: Promising antibiofilm activity was displayed by miltefosine and other alkylphosphocholine compounds, which could be considered a putative option for future treatment of candidaemia associated with biofilm formation, although further evaluation in *in vivo* systems is required.

Keywords: yeast, antifungal, miltefosine, synthetic analogues, catheter

Introduction

Candida spp. are pleomorphic fungi that can exist as both commensal and opportunistic pathogens, leading to the development of diseases that can range from superficial to life-threatening invasive infections. Invasive candidiasis has shown increasing importance in cases of nosocomial infections, and *Candida* spp. constitute the fourth most common pathogen isolated from hospital blood cultures.[1] Devices such as stents, shunts, prostheses, endotracheal tubes and vascular catheters, have been shown to support colonization and biofilm formation by *Candida*.[2] Among *Candida* spp., *Candida albicans* is still the major global pathogen, causing 50%–70% of invasive candidiasis cases.[1,3,4]

Even with current antifungal therapy, mortality of patients with candidaemia can be as high as 30%–40%.[1,5] One of the major contributions to *Candida* virulence is its versatility in

adapting to a variety of different habitats and to form biofilms over different surfaces.[6] Biofilms are heterogeneous microbial communities, composed of cells adhering to an abiotic or biotic surface, embedded in a polymeric extracellular matrix produced by themselves and with an altered phenotype compared with planktonic cells.[7]

Among the phenotypic alterations displayed by cells in biofilms, the most clinically relevant is their increased resistance to antifungal treatments.[7–9] This enhanced resistance contributes to fungus persistence in the patient despite antifungal therapy. It has been reported that *C. albicans* biofilms are resistant to a variety of clinical antifungal agents, including amphotericin B and fluconazole,[10,11] the major antifungal agents recommended for the treatment of candidaemia.[12] The expression of drug efflux pumps during the early phase of biofilm formation and alterations in sterol composition of the fungal membrane contribute to the resistance of biofilms to azoles.[11] Among the few available antifungal agents for the treatment of candidaemia, only the lipid formulations of amphotericin B and the echinocandins showed inhibitory activity against biofilms of *C. albicans*.[13]

Currently, new antifungal targets are emerging from studies with lysophospholipid analogues, which constitute a broad class of metabolically stable compounds.[14] Miltefosine (hexadecylphosphocholine) was initially developed as an antitumour agent, but its anticancer activity was shown to be limited. In parallel, alkylphosphocholines showed potent antiparasitic activity and selectivity, particularly against trypanosomatid parasites such as *Leishmania* spp. and *Trypanosoma cruzi*.[15–17] Miltefosine is effective against both visceral and cutaneous leishmaniasis, displays good bioavailability, and is registered as an oral drug for the treatment of the disease in India (since 2002) and Colombia (since 2005).[18]

Although many studies have been carried out with protozoa, little is known about the effects of phospholipid analogues in fungi. Recently, the inhibitory activity of miltefosine in several fungal species of medical importance, such as *C. albicans*, *Cryptococcus neoformans* and *Cryptococcus gattii*, *Aspergillus fumigatus*, *Fusarium solani*, *Scedosporium apiospermum*, *Scedosporium prolificans* and some zygomycetes, has been demonstrated.[14] However, the mechanisms of action related to this antifungal activity remain largely unknown.

Some structural analogues of miltefosine have been synthesized and tested for antileishmanial activity, and exhibited higher activity than miltefosine itself.[15–22] Thus, the aim of this work was to evaluate the antifungal activity of miltefosine and four synthetic analogues against both planktonic *C. albicans* cells and sessile *C. albicans* cells in biofilms and investigate whether these compounds are able to inhibit the biofilm formation and to reduce the viability of mature *C. albicans* biofilm cells.

Materials and methods

Strains

Antifungal assays were performed using a *C. albicans* standard strain (ATCC 10231) and a clinical isolate, *C. albicans* 44A, obtained from gastric cleavage at the Microbiology/Mycology Laboratory Hemorio, Rio de Janeiro, Brazil, and kindly donated by Dr Marcos Ribeiro Dornellas.[23] The isolates were maintained in Sabouraud dextrose agar plates at 4°C, and subcultures were used in each experiment. *Candida parapsilosis* ATCC 22019, a quality control strain, was also included in all planktonic susceptibility tests in order to validate our experiments (MIC values of 2 and 0.06 mg/L were obtained for fluconazole and amphotericin B, respectively).

Antifungal agents

Miltefosine (Cayman Chemical Company, MI, USA) and four synthetic analogues (Figure 1), were evaluated for antifungal activity. Compounds TCAN26 and TC19 were synthesized as previously described,[15,18] while the synthesis of compounds TC106 and TC117 will be reported elsewhere. Miltefosine was diluted in distilled water to obtain stock solutions of 1.56 mM (635.8 mg/L) and analogues were diluted in DMSO:ethanol (1:1) to obtain stock solutions of 50 mM (23.480 and 23.083 mg/L) for TCAN26 (23.48 mg/L) and TC19 (23.08 mg/L). The final concentration of DMSO after antifungal dilution was not higher than 0.14% in each test well. Fluconazole (Pfizer, São Paulo, Brazil) was diluted in water (2 mg/L) and amphotericin B (Sigma Chemical Co., MO, USA) in DMSO (1.6 mg/L) and they were used as reference antifungals. Antifungal agent dilutions were maintained at −70°C.

MICs

MICs of antifungal agents were determined for planktonic cells using the broth microdilution technique described in document M27-A3 published by the Clinical and Laboratory Standards Institute.[24] Briefly, serial 2-fold dilutions of the compounds were prepared in RPMI 1640 medium (Sigma Chemical Co.), buffered with 0.16 M MOPS, pH 7.0, in 96-well microtitre trays to obtain concentration ranges from 0.03 to 16 mg/L for amphotericin B, miltefosine and analogues, and from 0.125 to 64 mg/L for fluconazole. Yeasts were then added to each well at final concentrations of $0.5–2.5\times10^3$ cfu/mL. The microtitre trays were incubated at 35°C for 48 h in a dark, humid chamber. Minimum concentrations that inhibited 50% and 90% of the fungal yeast growth in relation to control (IC_{50} and IC_{90}, respectively) were determined by visual analysis and confirmed by spectrophotometry at 492 nm in a microtitre plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd, Sunnyvale, CA, USA). The percentage of inhibition was calculated with the following equation: $100-(A\times100/C)$, where A is the optical density (OD) of wells containing antifungal agent and C is the OD of control wells with fungi only. The document M27-A3 states that the IC_{50} should be considered as the MIC for all azoles and IC_{90} should be considered as the MIC for all polyenes.[24] Here, we also considered the IC_{90} value to be the MIC for the alkylphospholipids tested.

Minimum fungicidal concentration (MFC)

The MFC is defined as the lowest concentration of compound that produces no fungal growth and was determined by transferring an aliquot (5 μL) of each sample treated with concentrations higher than the MIC into an antifungal agent-free Sabouraud dextrose agar plate and incubated at 35°C for 48 h. A fungicidal effect was considered significant when the MFC value was ≤4-fold higher than the MIC value. Above this value, the antifungal effect was considered fungistatic.[25]

Transmission electron microscopy (TEM)

C. albicans planktonic cells (isolate 44A) were treated with subinhibitory concentrations (0.25×MIC) of alkylphospholipids and incubated at 35°C for 48 h. Yeasts were washed in PBS, pH 7.2, and fixed in a solution of 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature. Yeasts were then post-fixed for 2 h in 1% osmium tetroxide containing 1.25% potassium ferrocyanide and 5 mM $CaCl_2$ in cacodylate buffer, pH 7.2, washed in the same buffer,

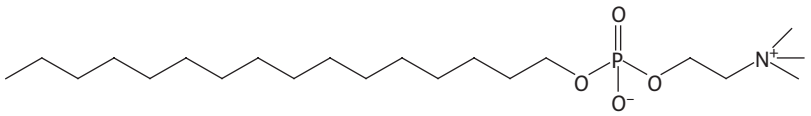
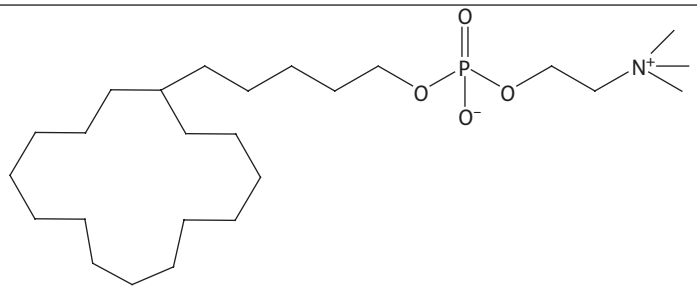
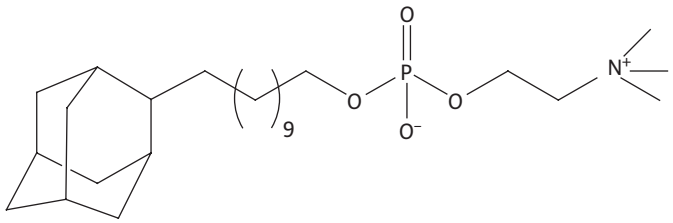
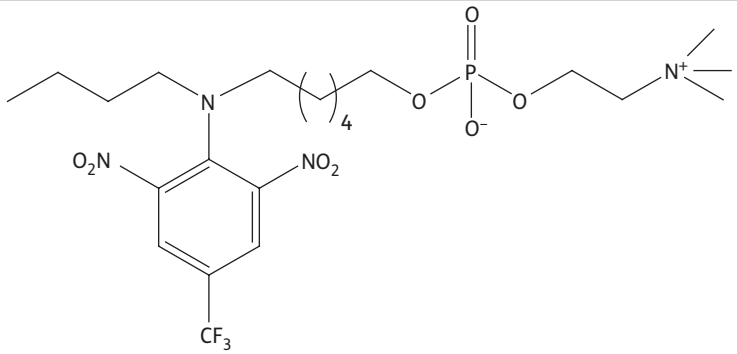
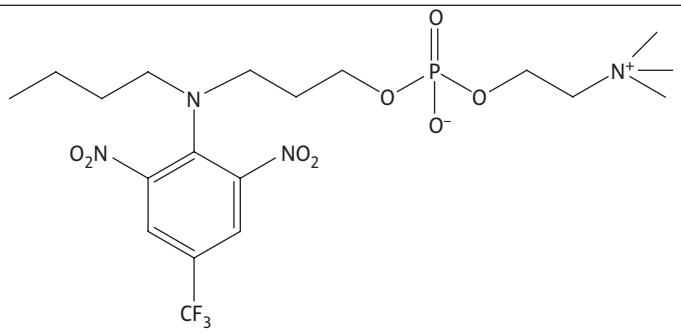
Compound class	Code	Structure
Alkylphospholipids	MLT	
	TC19	
	TCAN26	
Alkylphospholipid-dinitroaniline hybrids	TC106	
	TC117	

Figure 1. Molecular structures and names of all synthetic alkylphospholipid analogues used in this work.

dehydrated in increasing ethanol concentrations (30%, 50%, 70%, 90%, 100% and ultra-dry ethanol) for 30 min at each concentration, and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and images were obtained in a Zeiss 900 electron microscope equipped with a CCD camera (MegaView III; Soft Image System, Germany). Images were processed with iTEM software (Soft Image System, Germany).

Biofilm formation

C. albicans biofilms were formed as described by Jin and co-workers with some modifications.[26] Briefly, biofilms were grown in commercially available pre-sterilized, polystyrene, flat-bottomed 96-well microtitre plates. Aliquots of 100 μL of standard cell suspensions of yeasts (10^7 cfu/mL) were transferred into each well and incubated for 1.5 h (adhesion

phase) under constant agitation. After the adhesion phase, cell suspensions were gently aspirated and each well was washed twice with PBS to remove any remaining non-adhering cells. In order to allow the growth of biofilm (biofilm formation phase), 100 μL of freshly prepared RPMI 1640 supplemented with 2% glucose and 20% fetal bovine serum (FBS) (Fetal Bovine Serum; Gibco) was added to each well. The plates were incubated for 48 h at 35°C under constant agitation. Biofilms formed were quantified using the tetrazolium (XTT) reduction assay. All assays were repeated on three separate occasions.

XTT reduction assay

Biofilm formation was quantified using the XTT reduction assay, as described elsewhere.[27] Biofilms were first washed with 200 μL of PBS, and then 150 μL of an XTT–menadione solution [0.1 mg/mL XTT, 10 μM menadione (both from Sigma Chemical Co.)] was added to each well, and the microtitre plates were incubated in the dark for 2 h at 35°C. Next, 100 μL of solution was transferred to a new microtitre plate and the colour change in the solution was measured with spectrophotometric readings at 490 nm with a microtitre plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd). The absorbance values for the controls were then subtracted from the values for the test wells to eliminate spurious results due to background interference.

Effects of antifungals on biofilm formation

Antifungal susceptibility tests on biofilms during the developmental phase were performed based on a protocol previously described by Braga and co-workers, with some modifications.[28] First, 100 μL of standardized fungal suspension (1×10^7 cfu/mL) was dispensed in wells of a 96-well microtitre plate, and the plate was incubated at 35°C for 1.5 h under constant agitation. After this adhesion period, plates were washed with sterile 0.01 M PBS and 100 μL of different antifungal agent concentrations, diluted in RPMI 1640 buffered with MOPS and supplemented with 2% glucose and 20% FBS, was added to the wells. Three antifungal agent concentrations were used based on MIC values obtained for planktonic cells (MIC, 4×MIC and 16×MIC). The plates were incubated in the presence of compounds for 48 h at 35°C under agitation to allow biofilm formation. The supernatant was discarded and the biofilm was washed in PBS. Biofilm quantification was performed using the XTT reduction assay, as described above. This protocol was used to investigate the presence of inhibitory activity on initial stages of biofilm formation. The inhibition percentage was calculated as described above.

Effects of antifungals on mature biofilms

Antifungal susceptibility tests of mature biofilms were performed based on a protocol previously described, with some modifications.[28] Biofilms were formed for 48 h at 35°C on the surfaces of the wells of microtitre plates using the protocol described above, without the antifungal agent present. Then, different antifungal agent concentrations (the same concentrations as those used in the assay described above) were added to the wells and the plates were incubated for another 48 h at 35°C, under constant agitation, completing the total development time of 96 h. The effect of compounds on pre-formed biofilms was estimated using the XTT reduction assay as described above.

Scanning electron microscopy

These experiments were done using central venous catheters (CVCs) (Intracath Vialon™; BD). For this purpose, CVC sections of 5 mm were incubated with *C. albicans* planktonic cells treated or not with alkylphospholipids at concentrations 16 times higher than the respective MIC obtained for the planktonic cells. Biofilm treatments following the protocol described above were performed during biofilm formation or after biofilm maturation. Then, the catheters containing biofilms were washed in 0.01 M PBS, pH 7.2, fixed in 2.5% glutaraldehyde and 4% formaldehyde, in 0.1 M cacodylate buffer, for 1 h at room temperature. Subsequently, the catheters were washed in the same buffer and post-fixed in 1% osmium tetroxide and 1.25% potassium ferrocyanide for 2 h and then dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70%, 90%, 100% and ultra-dry ethanol) for 30 min at each concentration. The samples were critical-point-dried in CO_2, coated with gold and observed under a scanning electron microscope. An FEI Quanta 250 scanning electron microscope was used to visualize the samples and evaluate the effect of antifungal agents on the formation of biofilms and on mature biofilms, compared with control biofilms.

Statistical analysis

For all quantitative assays, statistical analyses were performed with Dunnett's test (one-way analysis of variance) and statistical significance was accepted at $P<0.05$.

Results

Antifungal activity

Amphotericin B showed the lowest values of MIC for planktonic cells of both strains (ATCC 10231 and 44A), but interestingly it did not have fungicidal activity for these two strains. The group of alkylphospholipids had similar MIC values to fluconazole. In this group, miltefosine was the most effective, followed by its synthetic analogues TCAN26 and TC19. The alkylphospholipid compounds showed MIC values of 2 and 4 mg/L for miltefosine and 4 mg/L for TCAN26 and TC19. Interestingly, the concentration that inhibited 50% of cell growth was close to the concentration that inhibited 90% of cell growth and the MFC (Table 1). These data suggested fungicidal activity of miltefosine, TCAN26 and TC19 for both *C. albicans* strains (Table 1).

Effect on C. albicans planktonic cell ultrastructure

The general morphology of both untreated and treated *C. albicans* 44A planktonic cells was observed using TEM (Figure 2). Planktonic cells of *C. albicans* 44A were treated for 24 h with subinhibitory concentrations of miltefosine (1 mg/L), TCAN26 (1 mg/L) and TC19 (1 mg/L). TEM images of control cells (Figure 2a and b) revealed the presence of cells with a well-defined shape and a homogeneous and electron-dense cytoplasm (Figure 2a), a regular cell wall (CW) with a compact outermost fibrillar layer (F) juxtaposed to the cell membrane (CM) (Figure 2b). In contrast, cell walls of cells treated with alkylphospholipids (Figure 2c–h) showed a thickening of the cell wall and an increase in the external fibrillar layer (Figure 2d, f and h). In addition, cell budding seemed to be drastically affected by treatment with these compounds, leading to the appearance of strongly altered buds (Figure 2c and d). After treatment, electron-dense vacuole (DV) accumulation (Figure 2g and h) and electron-lucent vacuoles (LV) (Figure 2g and h) were also observed in these cells.

Effect on C. albicans biofilm formation

Fluconazole was the least effective antibiofilm agent and at concentrations ≤16 times the MIC for planktonic cells did not

Table 1. Susceptibility of *C. albicans* strains to standard antifungal amphotericin B (AMB) and fluconazole (FLC), miltefosine (MLT) and four other synthetic analogues by the CLSI reference broth microdilution method

		C. albicans 44A		*C. albicans* ATCC 10231	
Compounds		MIC	MFC	MIC	MFC
Standard antifungal	AMB	0.12	8	0.25	8
	FLC	1	>64	1	>64
Alkylphospholipids	MLT	4	4	2	2
	TCAN26	4	8	4	8
	TC19	4	>16	4	8
Alkylphospholipid-dinitroaniline hybrids	TC106	>16	>16	>16	>16
	TC117	>16	>16	>16	>16

Antifungal concentrations are expressed in mg/L.

significantly reduce biofilm formation when compared with biofilms formed in the absence of antifungal agents (Table 2). In contrast, amphotericin B significantly reduced biofilm formation ($P<0.001$) at concentrations equal to the MIC for planktonic cells (0.12 mg/L). Furthermore, concentrations of 0.5 and 2 mg/L amphotericin B (4× and 16×MIC, respectively) induced an inhibition in biofilm growth of around 70% ($P<0.0001$) (Table 2). Among the phospholipid analogues, only miltefosine, TCAN26 and TC19 at concentrations up to 16× MIC were able to reduce ATCC 10231 biofilm formation significantly ($P<0.01$) (Table 2). The amounts of inhibition caused by miltefosine and TC19 were concentration-dependent and were greater than that caused by TCAN26 (Table 2).

The effects of the antifungal agents on biofilm formation of the clinical isolate 44A were different from those observed on ATCC 10231 biofilm (Table 2). The addition of amphotericin B at concentrations from the MIC up to 16×MIC significantly reduced the biofilm formation ability of the clinical isolate ($P<0.001$) (Table 2). Interestingly, amphotericin B at its MIC showed almost 50% inhibition of biofilm formation, similar to the results obtained with 4× and 16×MIC (Table 2).

The phospholipid analogues were also able to inhibit biofilm formation by the clinical isolate of *C. albicans* (44A). Miltefosine at 16×MIC resulted in a significant reduction ($P<0.0001$) of 71% of the biofilm formed at the end of 48 h (Table 2). Likewise, a slight reduction was observed when TCAN26 was used at 16×MIC (Table 2); therefore, reducing biofilm formation significantly more than amphotericin B ($P<0.05$).

Effect on C. albicans mature biofilms

The addition of fluconazole at all concentrations to mature biofilms of *C. albicans* ATCC 10231 and 44A failed to alter the mature biofilm susceptibility profile (Table 3). However, amphotericin B at concentrations of 0.5 and 2 mg/L (4× and 16×MIC, respectively) led to a significant reduction (around 30%) in the metabolic activity of the cells composing the ATCC biofilm ($P<0.05$) but had no effect on the clinical 44A isolate biofilm (Table 3). Curiously, only a concentration of 16×MIC of miltefosine was able to significantly reduce the metabolic activity of mature biofilm cells formed by both ATCC and the clinical isolate 44A, by 32% and 44%, respectively ($P<0.05$) (Table 3). Remarkably, miltefosine showed a better activity against mature biofilms than amphotericin B ($P<0.0001$).

Morphological changes in biofilm formation on central venous catheters

The antifungal effect on biofilms was evaluated qualitatively by visual analysis of the images obtained by scanning electron microscopy. Biofilm density and the cell morphology of control biofilms were compared with values for biofilms treated with antifungal agents, in the different developmental stages (during biofilm formation and mature biofilms). To confirm the frequency of the cell alterations described in the text, extremely careful observation of the entire area of the biofilm was made in each sample and images were acquired from different locations in the material.

Biofilms of *C. albicans* clinical isolate 44A formed in the absence of antifungal agents were highly filamented with a large degree of colonization of cells adhering to almost the entire catheter surface (Figure 3a–c). Interestingly, biofilm formed by *C. albicans* ATCC 10231 showed lower cell density than biofilms formed by the clinically isolated strain of *C. albicans*, 44A (data not shown). Analysing the effects of standard antifungal agents on the biofilm formation, the addition of fluconazole (16×MIC) (data not shown) or amphotericin B (16×MIC) (Figure 3d–f) reduced the final mass of adhering cells and inhibited the formation of hyphae in *C. albicans* 44A. Similar results were observed when fluconazole (16×MIC) or amphotericin B (16×MIC) was added to the catheter during biofilm development in the strain ATCC 10231; there was a reduction in the number of cells adhering to the device, with most of the cells in yeast form (data not shown). The addition of miltefosine (Figure 3g–i) or TCAN26 (Figure 3j–l) (16×MIC) to the catheter during the formation of biofilms of the clinical isolate 44A resulted in a reduction of cells adhering, with the elimination of almost all cells on miltefosine-treated catheters (Figure 3g–i) and reduction of filamentation by TCAN26, the cells appearing mostly as yeasts and pseudohyphae (Figure 3j–l).

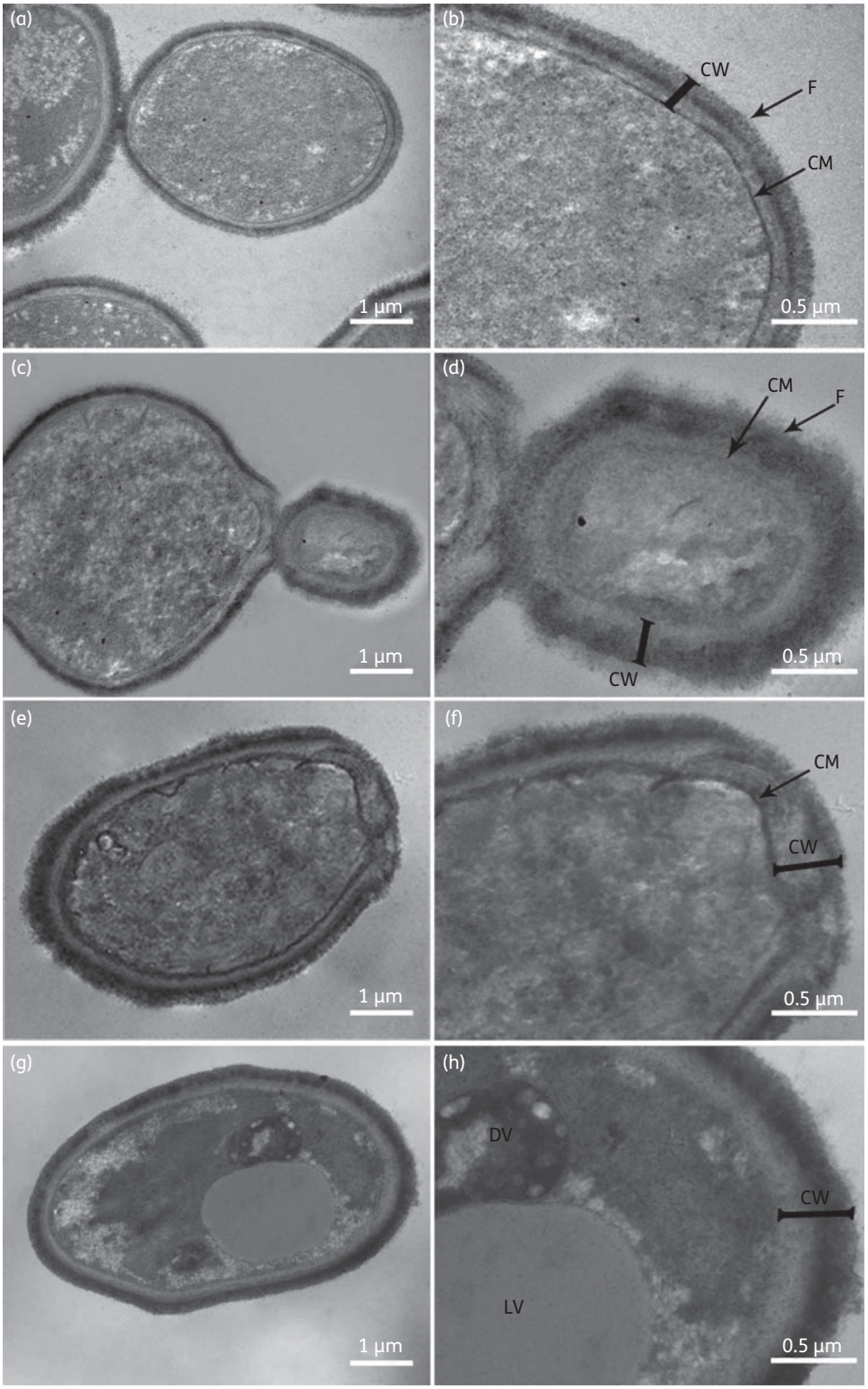


Figure 2. Transmission electron micrographs of *C. albicans* 44A control (a and b) and 44A treated with 1 mg/L miltefosine (c and d), 1 mg/L TCAN26 (e and f) or 1 mg/L TC19 (g and h). Treatment with alkylphospholipids led to an increase in the external fibrillar layer (F), thickening of the cell wall (CW) and disruption of the cell membrane (CM). Treatment also led to the appearance of strongly altered buds and electron-dense vacuoles (DV) and electron-lucent vacuoles (LV).

Table 2. Evaluation of the effect of antifungal agents during biofilm formation by the XTT reduction method

		Absorbance: mean ± SD (% inhibition)			
			Antifungal concentrations (mg/L)		
Strains	Antifungal	Control	MIC	4 × MIC	16 × MIC
C. albicans ATCC 10231	AMB	0.52 ± 0.1	0.27 ± 0.08** (49.5)	0.15 ± 0.09*** (71.0)	0.14 ± 0.19*** (74.2)
	FLC	0.52 ± 0.1	0.6 ± 0.18 (0)	0.51 ± 0.11 (4.1)	0.48 ± 0.03 (8.9)
	MLT	0.52 ± 0.1	0.64 ± 0.17 (0)	0.41 ± 0.12 (3.8)	0.35 ± 0.11** (38.2)
	TCAN26	0.52 ± 0.1	0.6 ± 0.12 (0)	0.48 ± 0.08 (18.4)	0.43 ± 0.03* (29.5)
	TC19	0.52 ± 0.1	0.47 ± 0.15 (11.2)	0.42 ± 0.13 (20.6)	0.31 ± 0.09** (41.2)
C. albicans 44A	AMB	0.46 ± 0.1	0.23 ± 0.02** (49.7)	0.21 ± 0.05** (55.0)	0.23 ± 0.06** (51.1)
	FLC	0.46 ± 0.1	0.43 ± 0.08 (7.2)	0.4 ± 0.1 (12.7)	0.38 ± 0.09 (18.5)
	MLT	0.46 ± 0.1	0.35 ± 0.1 (23.1)	0.31 ± 0.11* (32.3)	0.13 ± 0.14*** (71.2)
	TCAN26	0.46 ± 0.1	0.44 ± 0.07 (4.3)	0.39 ± 0.06 (15.8)	0.35 ± 0.06 (24.3)
	TC19	0.46 ± 0.1	0.51 ± 0.17 (0)	0.53 ± 0.28 (0)	0.44 ± 0.14 (5.0)

AMB, amphotericin B; FLC, fluconazole; MLT, miltefosine; MIC, minimum concentration that inhibits 50% (FLC) or 90% (AMB, MLT, TCAN26 and TC19) of planktonic cell growth.
*$P < 0.05$.
**$P < 0.001$.
***$P < 0.0001$.

Table 3. Evaluation of the effect of antifungal agents on mature biofilms by the XTT reduction method

		Absorbance: mean ± SD (% inhibition)			
			Antifungal concentrations (mg/L)		
Strains	Antifungal	Control	MIC	4 × MIC	16 × MIC
C. albicans ATCC 10231	AMB	0.43 ± 0.08	0.37 ± 0.11 (13.5)	0.30 ± 0.09** (30.4)	0.33 ± 0.07** (24.3)
	FLC	0.43 ± 0.08	0.50 ± 0.05 (0)	0.50 ± 0.10 (0)	0.46 ± 0.07 (0)
	MLT	0.43 ± 0.08	0.39 ± 0.03 (9.1)	0.45 ± 0.10 (0)	0.29 ± 0.07* (32.4)
	TCAN26	0.43 ± 0.08	0.36 ± 0.02 (15.8)	0.37 ± 0.02 (12.9)	0.37 ± 0.03 (14.7)
	TC19	0.43 ± 0.08	0.37 ± 0.21 (14.7)	0.37 ± 0.19 (14.3)	0.41 ± 0.09 (5.01)
C. albicans 44A	AMB	0.3 ± 0.07	0.27 ± 0.06 (10.1)	0.28 ± 0.07 (3.7)	0.27 ± 0.07 (7.9)
	FLC	0.3 ± 0.07	0.30 ± 0.07 (0)	0.30 ± 0.06 (0)	0.29 ± 0.06 (0.7)
	MLT	0.3 ± 0.07	0.3 ± 0.07 (0)	0.28 ± 0.07 (5.5)	0.16 ± 0.01*** (44.3)
	TCAN26	0.3 ± 0.07	0.35 ± 0.008 (0)	0.28 ± 0.05 (5.8)	0.24 ± 0.04 (19.5)
	TC19	0.3 ± 0.07	0.28 ± 0.07 (3.8)	0.25 ± 0.07 (15.5)	0.25 ± 0.05 (15.4)

AMB, amphotericin B; FLC, fluconazole; MLT, miltefosine; MIC, minimal inhibitory concentration that inhibits 50% (FLC) or 90% (AMB, MLT, TCAN26 and TC19) of planktonic growth.
*$P < 0.05$.
**$P < 0.001$.
***$P < 0.0001$.

However, the addition of 64 mg/L TC19 (16×MIC) did not affect 44A biofilm formation (Figure 3m–o).

Morphological changes after adding antifungal agents to mature biofilm

Control biofilms of both *C. albicans* strains, ATCC 10231 and clinical isolate 44A, after a total of 96 h of incubation in rich medium supplemented with glucose (2%) and FBS (20%) were highly dense and had long hyphae and abundant branches and buds over the entire surface of the CVC with which they were in contact (Figure 4a–c; clinical isolate 44A). When the reference antifungal agents (amphotericin B and fluconazole) were added to mature biofilms (after the first 48 h of formation) they did not affect their density or filamentation profile (Figure 4d–f for amphotericin B; data not shown for fluconazole).

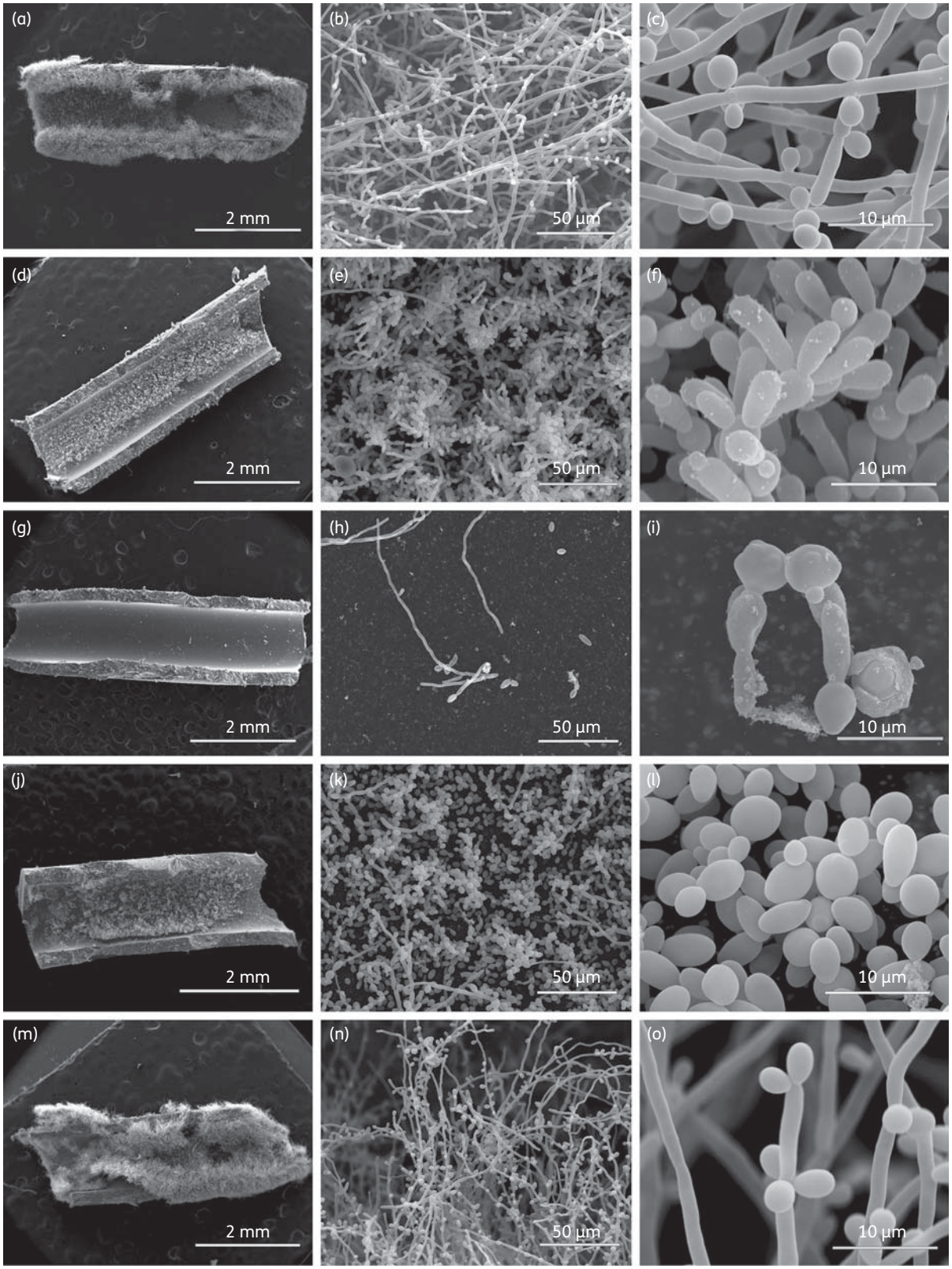


Figure 3. Scanning electron micrographs of *C. albicans* 44A control biofilms (a–c) and biofilms formed in the presence of 2 mg/L amphotericin B (d–f), 64 mg/L miltefosine (g–i), TCAN26 (j–l) and TC19 (m–o), for 48 h at 35°C. A reduction in cells adhering to the surface of the catheter and inhibition of filamentation in biofilms formed in the presence of miltefosine and TCAN26 were observed.

The analogues of phospholipids showed different behaviour with respect to mature biofilms of *C. albicans* 44A. While miltefosine only slightly reduced the extent of catheter colonization by yeasts (Figure 4g–i), TCAN26 led to greater reduction in both the extent and the thickness of the biofilm on the catheter (Figure 4j–l). Although the addition of miltefosine to mature biofilm of *C. albicans* 44A did not reverse the density of already formed biofilm, it induced alterations in conidia cell shape profile (arrows in Figure 4i). TCAN26-treated cells remained adherent to the catheter but showed an exclusively yeast form with many elongated buds and a minor number of pseudohyphae (arrows and arrowheads in Figure 4k and l), demonstrating complete inhibition of filamentation of these cells. Mature biofilms treated with all the TC19 concentrations tested did not induce alterations in the biofilm profile (Figure 4m–o).

Discussion

The *Candida* biofilm lifestyle results in resistance to antifungal agents and protection of the fungus from host defences, which have important clinical repercussions.[29,30] Device-related infections are difficult to treat and affected devices often need to be removed, which can be hazardous for some patients.[31,32] CVCs appear to be the most common risk factor for candidaemia development in patients without neutropenia or major immunodeficiencies.[33]

In the present work, five phospholipid analogues, including miltefosine, were tested to determine whether they had inhibitory activity against planktonic and biofilm cells of *C. albicans*. Planktonic cells of *C. albicans* were susceptible to treatment with miltefosine, TCAN26 and TC19, all alkylphospholipid compounds. Although it has been described that both TCAN26 and TC19 show inhibitory activity against *Leishmania donovani* and *Leishmania infantum* cells,[15,18] the present study is the first to deal with the antifungal effects of TCAN26 and TC19 on *C. albicans*. The MIC values of TCAN26 and TC19 obtained for *C. albicans* planktonic cells (2–4 mg/L or 5–10 μM) were quite similar to those obtained for *L. donovani* and *L. infantum*.[15,18] Some differences in susceptibility occurred, however, as both alkylphospholipid analogues were more potent than miltefosine for both *Leishmania* spp., while in the present study they were equal or less potent than miltefosine in *C. albicans*. The antifungal activity of miltefosine against *C. albicans* has been demonstrated previously, the MIC values being similar to those obtained in our work.[14,34] Besides, only miltefosine and TCAN26 presented fungicidal effects against *C. albicans*. The alkylphospholipid-dinitroaniline hybrid compounds were also tested and present no effect on *C. albicans* planktonic cells.

Alkylphosphocholines have structural similarity to the natural substrates of fungal phospholipase B1 (PLB1), phosphatidylcholine and lysophosphatidylcholine. Miltefosine treatment in *Cryptococcus* spp. led to concentration-dependent inhibition of lysophospholipase transacylase (LPTA) and PLB1 activity.[14] PLB1 appears to be required for *Cryptococcus* adherence to lung epithelial cells and for its haematogenous dissemination, while LPTA seems to be associated with membrane synthesis, remodelling and repair. Therefore, miltefosine may exert an antifungal effect by interfering in the biochemistry of the cell wall and cell membrane.[14] Little is known about the effects of alkylphospholipids on fungal growth and the mechanisms involved in this process. In trypanosomatids, previous studies in *Leishmania* spp. showed that treatment with miltefosine induced changes in lipid metabolism, such as the inhibition of phosphatidylcholine and glycosylphosphatidylinositol anchor biosynthesis and choline uptake.[21,35,36]

Due to their chemical structure, alkylphospholipids are also easily inserted into lipid membranes and resist catabolic degradation. The level of partitioning into lipid bilayers depends on the degree of unsaturation of the phospholipid alkyl chains and the amount of cholesterol. Miltefosine interacts with the cell membrane and rapidly reaches other subcellular membranes, thus being able to affect cell metabolism at different levels.[37] Experiments with model membranes showed that TCAN26 interacts with both the polar and the hydrophobic regions of membrane bilayers, and it was proposed that this compound remains embedded in the membrane bilayers, causing significant perturbation.[15] This behaviour probably also occurs in fungal cells. Insertion of the molecule into the membrane can cause changes in the composition of the membrane, altering its fluidity and permeability, and can interfere with the functioning of membrane proteins, affecting proteins involved in cell wall synthesis, such as β-1,3 glucan synthase. These alkylphospholipids also appear to exert their effects by interfering directly with the cell membrane constitution and/or inhibiting biosynthetic pathways of membrane lipids within cells.

Thus, it is possible that the changes observed by us in cell shape, the cell wall and the plasma membrane and the increase in electron-dense vacuoles in *C. albicans* strain 44A treated with subinhibitory concentrations of alkylphospholipids may be related to three mechanisms: (i) imbalance of the biosynthetic pathways of membrane phospholipids, altering cellular lipid metabolism; (ii) phospholipase inhibition; and/or (iii) insertion of the alkylphospholipid compounds into the fungal cytoplasmic membrane.

To study the effect of the alkylphospholipids on *C. albicans* biofilm, we used a dual approach, employing two stages of biofilm development, seeking to correlate their effects on the initial and mature biofilm phases in comparison with amphotericin B and fluconazole. Previous studies have shown that the resistance of the biofilm to polyenes and azoles changes during its maturation,[10,11] and these data are consistent with the findings of our study, in which both *C. albicans* strains were highly susceptible to amphotericin B during the process of biofilm formation and reduced activity was observed in mature *C. albicans* biofilms, despite the good antifungal activity on planktonic cells of *C. albicans* exhibited by fluconazole and amphotericin B. Among the alkylphospholipids compounds tested in this study, only miltefosine was able to reduce biofilm formation and to reduce the viability of mature biofilm *C. albicans* strains, by 32% for the ATCC strain and 44% for the 44A clinical isolate (at 16×MIC), showing higher activity in the earliest stages of biofilm development than in mature biofilms. TCAN26 was also effective against biofilm formation and mature biofilm but to a lesser extent than miltefosine.

The ability to penetrate the *C. albicans* biofilm extracellular matrix (ECM) is one of the existing explanations for the greater efficacy of lipid formulations of amphotericin B in biofilms compared with the traditional formulation of amphotericin B deoxycholate and fluconazole.[13] Interestingly, the higher activity of

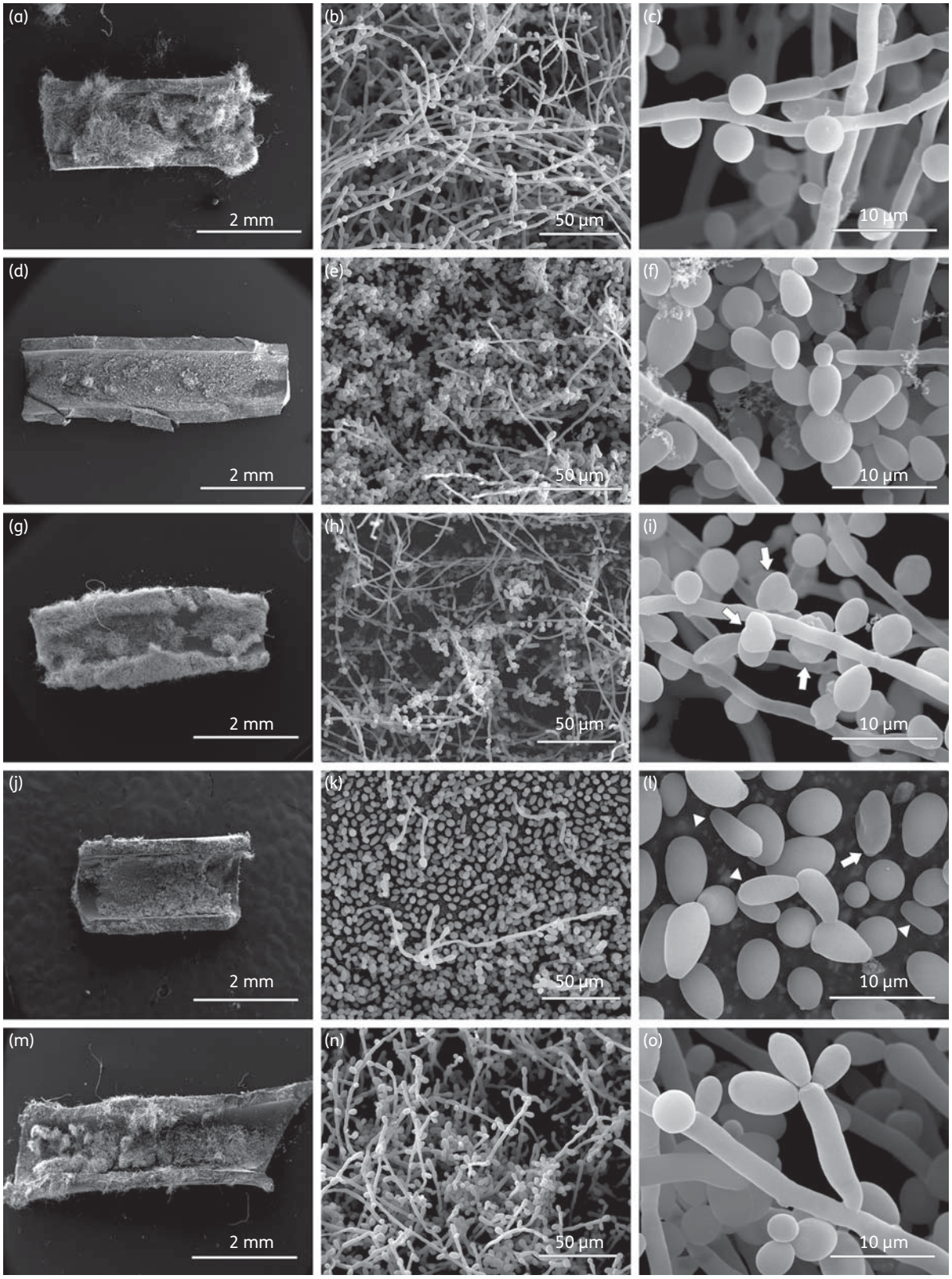


Figure 4. Scanning electron micrographs of mature biofilms of *C. albicans* 44A. Control biofilms (a–c) and mature biofilms treated with 2 mg/L amphotericin B (d–f), 64 mg/L miltefosine (g–i), TCAN26 (j–l) and TC19 (m–o) for 48 h at 35°C. Treatment with miltefosine and TCAN26 led to changes in the shape of budding cells (i and l, white arrows and arrowheads) and TCAN26 also inhibited cell filamentation (l).

alkylphospholipid analogues on biofilms compared with other antifungal agents tested in this work can be related to the phospholipid nature of these compounds, which must also penetrate through the ECM of the biofilm to reach the fungal cell.

In the present study, biofilms formed on CVCs were used to compare the ultrastructural effects of treatment with 16×MIC of amphotericin B and the alkylphospholipids. Miltefosine exhibit the most drastic effect on biofilm formation, inducing the detachment of almost all cells. Although mature biofilms are less sensitive to miltefosine than forming biofilms, their cells exhibit a peculiar profile of quite altered cells and deformed buds. When we analysed the morphological aspects of *C. albicans* biofilms, amphotericin B and TCAN26 were the most active compounds. Despite having shown a low inhibition profile (around 20%) in biofilms formed on polystyrene plates, TCAN26 was extremely active on biofilms in CVCs. However, even when this compound was added to pre-formed mature biofilms, there was almost complete inhibition of cell filamentation at the end of the incubation time. This result shows that TCAN26 can probably act either on cells that are already filamented or by inhibiting filamentation in yeast. Inhibition of morphogenesis may be important since the lesser complexity of the biofilm may be related to lower virulence of the cells involved.

The ability of *C. albicans* to form biofilms can be modified by the surface properties of the biomaterial over which the biofilm will be formed.[38] Therefore, the difference between the biofilm-forming abilities of cells on polystyrene and CVCs can be explained by differences in chemical composition between the surfaces, which may influence the adherence of *C. albicans* and consequently biofilm development.[38]

The antibiofilm activity of alkylphospholipids is an important finding as these compounds seem to be active on both planktonic and biofilm cells on two different medically important surfaces (polystyrene and catheters) and may be considered as a putative alternative for candidaemia treatment.

The pharmacokinetics, absorption, distribution and metabolism of miltefosine have been studied in rats and mice.[39–41] Oral administration of miltefosine for 5 days following infection increased survival and reduced brain and lung cryptococcal burdens. This was achieved with relatively low doses of 7.2 and 3.6 mg/kg/day of miltefosine and confirmed the potential of this antifungal agent for the treatment of invasive mycoses, including intracerebral infections.[14] Serum concentrations of 110 μM (44.83 mg/L) were achieved in rats after 2 weeks of daily dosing with 10 mg/kg.[14] This concentration is 10–20 times the MIC for miltefosine against fungi causing invasive mycoses,[14] including the *C. albicans* strains used in this work. Despite being the most potent antifungal agent tested, miltefosine has a long half-life (100–200 h) in humans and a low therapeutic ratio, characteristics that could encourage the development of resistance.[42] In addition, treatment of pregnant women is contraindicated because of its teratogenic properties in animals.[42] Miltefosine is an orally active fungicidal compound and although it has significant side effects it is still less toxic than amphotericin B.[14]

Previous experiments have shown that TCAN26 and TC19 are less cytotoxic to the human monocytic cell line THP1 than miltefosine and may be considered as safer alternatives in cases in which miltefosine is not recommended.[15,18] Miltefosine has haemolytic activity with an HC_{50} (concentration that produces 50% of haemolysis) of 38.6 μM (15.7 mg/L), a feature that prevents its use in injectable form. However, TCAN26 and TC19 are much less haemolytic than miltefosine, showing HC_{50} values of >60 μM (24.5 mg/L) and >100 μM (40.8 mg/L), respectively. This indicates that these compounds could be administered intravenously, and thus they may show reduced gastrointestinal toxicity and higher plasma concentrations *in vivo*.[18,43]

Alkylphospholipids are phospholipid derivatives substituted with an alkyl chain in the lipid portion. Miltefosine possesses a 16-carbon saturated aliphatic chain while TCAN26 and TC19 contain cycloalkane rings—adamantane and cyclohexadecane, respectively—in the lipid portion. These chemical differences may be related to the differences observed in cytotoxicity and haemolytic activity. Studies with a series of ring-substituted ether phospholipid derivatives showed that, with regard to TCAN26, the presence or absence of a double bond on the alkyl chain does not affect the antileishmanial activity of the molecule.[15] On the other hand, studies with a series of cycloalkylidene-substituted ether phospholipids showed that for TC19 the absence of the double bond increased antileishmanial activity and reduced the cytotoxicity of the molecule when compared with its unsaturated counterpart.[18] The length of the alkyl chain of the most active ring-substituted compounds varies from 5 to 14 carbon atoms, which could be advantageous for the solubility and/or toxicity of the new compounds, and for their metabolic clearance.[15,18] Further experiments are therefore required in order to gain a better understanding of the structural features that impart enhanced activity and minimal cytotoxicity for this class of compounds. However, in general these data indicate that ring-substituted ether phospholipids are less cytotoxic to human monocytes and more potent than miltefosine against *L. infantum* and *L. donovani*,[15,18] showing that modifications of the alkyl chain of alkylphospholipids are effective in reducing cytotoxicity. Conversely, a hydrophobic chain at least 16 carbon atoms long seems to be important for the maintenance of antifungal activity.

Conclusions

Taken together, our data demonstrate that alkylphospholipids such as miltefosine and TCAN26 are active on both planktonic and biofilm cells of *C. albicans*. Miltefosine was the most effective compound tested by being active against *C. albicans* biofilms, making this compound particularly interesting. Although TCAN26 was less effective than miltefosine in our model, it is considered a less toxic compound than miltefosine by workers in this field of study. Therefore, TCAN26 effectiveness as a treatment for biofilms should also be considered in the future, especially since it was extremely active on biofilms in CVCs.

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Transparency declarations

None to declare. This article was edited for proper English language by American Journal Experts.

References

1 Nucci M, Queiroz Telles F, Tobón AM *et al*. Epidemiology of opportunistic fungal infections in Latin America. *Clin Infect Dis* 2010; **51**: 561–70.

2 Chandra J, Kuhn DM, Mukherjee PK *et al*. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* 2001; **183**: 5385–94.

3 Sifuentes-Osornio J, Corzo-León DE, Ponce-de-León LA. Epidemiology of invasive fungal infections in Latin America. *Curr Fungal Infect Rep* 2012; **6**: 23–34.

4 Bassetti M, Taramasso L, Nicco E *et al*. Epidemiology, species distribution, antifungal susceptibility and outcome of nosocomial candidemia in a tertiary care hospital in Italy. *Plos One* 2011; **6**: e24198.

5 Colombo AL, Guimaraes T. Epidemiologia das infecções hematogênicas por *Candida* spp. *Rev Soc Bras Med Trop* 2003; **5**: 599–607.

6 Ramage G, VandeWalle K, Wickes BL *et al*. Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol* 2001; **18**: 163–70.

7 Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; **15**: 167–93.

8 Hawser SP, Douglas LJ. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimicrob Agents Chemother* 1995; **39**: 2128–31.

9 Mukherjee PK, Chandra J. *Candida* biofilm resistance. *Drug Resist Updat* 2004; **7**: 301–9.

10 Ramage G, Bachmann S, Patterson TF *et al*. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother* 2002; **49**: 973–80.

11 Mukherjee PK, Chandra J, Kuhn DM *et al*. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* 2003; **71**: 4333–40.

12 Pappas PG, Kauffman CA, Andes D *et al*. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2009; **48**: 503–35.

13 Kuhn DM, George T, Chandra J *et al*. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother* 2002; **46**: 1773–80.

14 Widmer F, Wright LC, Obando D *et al*. Hexadecylphosphocholine (miltefosine) has broad-spectrum fungicidal activity and is efficacious in a mouse model of cryptococcosis. *Antimicrob Agents Chemother* 2006; **50**: 414–21.

15 Avlonitis N, Lekka E, Detsi A *et al*. Antileishmanial ring-substituted ether phospholipids. *J Med Chem* 2003; **46**: 755–67.

16 Urbina JA. Mechanisms of action of lysophospholipid analogues against trypanosomatid parasites. *Trans R Soc Trop Med Hyg* 2006; **100**: 9–16.

17 Kapou A, Benetis NP, Avlonitis N *et al*. 3D-Quantitative structure–activity relationships of synthetic antileishmanial ring-substituted ether phospholipids. *Bioorg Med Chem* 2007; **15**: 1252–65.

18 Calogeropoulou T, Angelou P, Detsi A *et al*. Design and synthesis of potent antileishmanial cycloalkylidene-substituted ether phospholipid derivatives. *J Med Chem* 2008; **51**: 897–908.

19 Chan JH, Neal RA, Pendergast W. The activity of alkyl phosphorylcholines and related derivatives against *Leishmania donovani*. *Biochem Pharmacol* 1987; **36**: 2633–6.

20 Unger C, Maniera T, Kaufmann-Kolle P *et al*. In vivo antileishmanial activity of hexadecylphosphocholine and other alkylphosphocholines. *Drugs Today* 1998; **34**: 133–40.

21 Croft SL, Seifert K, Duchêne M. Antiprotozoal activities of phospholipid analogues. *Mol Biochem Parasitol* 2003; **126**: 165–72.

22 Croft SL, Barrett MP, Urbina JA. Chemotherapy of trypanosomiases and leishmaniasis. *Trends Parasitol* 2005; **21**: 508–12.

23 Braga-Silva LA, Mesquita DG, Ribeiro DM *et al*. Trailing end-point phenotype antibiotic-sensitive strains of *Candida albicans* produce different amounts of aspartyl peptidases. *Braz J Med Biol Res* 2009; **42**: 765–70.

24 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts—Third Edition: Approved Standard M27-A3*. CLSI, Wayne, PA, USA, 2008.

25 Pfaller MA, Sheehan DJ, Rex JH. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. *Clin Microbiol Rev* 2004; **17**: 268–80.

26 Jin Y, Yip HK, Samaranayake YH *et al*. Biofilm-forming ability of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. *J Clin Microbiol* 2003; **41**: 2961–7.

27 Ramage G, VandeWalle K, Wickes BL *et al*. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 2001; **45**: 2475–9.

28 Braga PC, Culici M, Alfieri M *et al*. Thymol inhibits *Candida albicans* biofilm formation and mature biofilm. *Int J Antimicrob Agents* 2008; **31**: 472–7.

29 Kumamoto CA. *Candida* biofilms. *Curr Opin Microbiol* 2002; **5**: 608–11.

30 Ramage G, Saville SP, Thomas DP *et al*. *Candida* biofilms: an update. *Eukaryot Cell* 2005; **4**: 633–8.

31 Mermel LA, Farr BM, Sherertz RJ *et al*. Guidelines for the management of intravascular catheter-related infections. *Clin Infect Dis* 2001; **32**: 1249–72.

32 Walsh TJ, Rex JH. All catheter-related candidemia is not the same: assessment of the balance between the risks and benefits of removal of vascular catheters. *Clin Infect Dis* 2002; **32**: 600–2.

33 Tumbarello M, Posteraro B, Trecarichi EM *et al*. Biofilm production by *Candida* species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. *J Clin Microbiol* 2007; **45**: 1843–50.

34 Obando D, Widmer F, Wright LC *et al*. Synthesis, antifungal and antimicrobial activity of alkylphospholipids. *Bioorg Med Chem* 2007; **15**: 5158–65.

35 Lux H, Hart DT, Parker PJ *et al*. Ether lipid metabolism, GPI anchor biosynthesis, and signal transduction are putative targets for anti-leishmanial alkylphospholipid analogues. *Adv Exp Med Biol* 1996; **416**: 201–11.

36 Zufferey R, Mamoun CB. Choline transport in *Leishmania major* promastigotes and its inhibition by choline and phosphocholine analogs. *Mol Biochem Parasitol* 2002; **125**: 127–34.

37 Jiménez-López JM, Ríos-Marco P, Marco C *et al*. Alterations in the homeostasis of phospholipids and cholesterol by antitumor alkylphospholipids. *Lipids Health Dis* 2010; **9**: 33.

38 Chandra J, Patel JD, Li J *et al*. Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. *Appl Environ Microbiol* 2005; **71**: 8795–801.

39 Breiser A, Kim DJ, Fleer E *et al*. Distribution and metabolism of hexadecylphosphocholine in mice. *Lipids* 1987; **22**: 925–6.

40 Marschner N, Kötting J, Eibl H *et al*. Distribution of hexadecylphosphocholine and octadecyl-methyl-glycero-3-phosphocholine in rat tissues during steady-state treatment. *Cancer Chemother Pharmacol* 1992; **31**: 18–22.

41 Unger C, Fleer E, Damenz E *et al*. Hexadecylphosphocholine: determination of serum concentrations in rats. *J Lipid Mediat* 1991; **3**: 71–8.

42 Soto J, Soto P. Miltefosine: oral treatment of leishmaniasis. *Expert Rev Anti-Infect Ther* 2006; **4**: 177–85.

43 Papanastasiou I, Prousis KS, Georgikopoulou K *et al*. Design and synthesis of new adamantyl-substituted antileishmanial ether phospholipids. *Bioorg Med Chem Lett* 2010; **20**: 5484–7.