CLINICAL MICROBIOLOGY - RESEARCH PAPER

Incorporation of 2-amino-thiophene derivative in nanoparticles: enhancement of antifungal activity

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Abstract

The objective of this study was to evaluate the effects of nanoparticles (nanospheres and nanocapsules) of the promising antifungal 2-amino-thiophene (6CN10) and 6CN10 complexed with 2-hydroxypropyl-β-cyclodextrin (6CN10:HP-β-CD) in vitro and compared with free drug against Candida and Cryptococcus, using a microdilution method to measure susceptibility. The Candida and Cryptococcus clinical strains were identified using phenotypic methods and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). To measure in vitro antifungal susceptibility, we used microdilution trials. Serial drug or nanoparticle dilutions were prepared according to the CLSI M27-A3 guidelines. Anti-biofilm activity was verified for Cryptococcus neoformans. All Candida isolates were sensitive to the free drug (MIC = 41.66–333.33 μg/mL) and were able to grow even at the higher concentration tested for all 6CN10 nanoparticles. However, the Cryptococcus neoformans strains presented MIC values of 0.32–83.33 μg/mL for 6CN10 nanoparticles, and MIC values of 0.1–0.2 μg/mL for 6CN10:HP-β-CD nanoparticles, i.e., 3333 times more active than the free drug (MIC values 166.66–333.33 μg/mL), and presenting activity greater than that of the reference drug amphotericin B (MIC = 0.5–0.125 μg/mL). 6CN10:HP-β-CD nanosphere also showed high anti-biofilm potential. The in vitro study showed that the nanoparticles allowed better drug efficiency against Cryptococcus than did the free drug. These results suggest that 6CN10-loaded nanoparticles may become a future alternative for cryptococcosis and candidiasis therapy. In vivo experiments are essential prior to clinical use.

Keywords Antifungal susceptibility · 2-Amino-thiophene · Cryptococcus neoformans · Nanospheres · Nanocapsules · Biofilm

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Introduction

The incidence of fungal infections has been increasing since 1970, primarily because of yeasts that breach primary immune barriers via medical devices such as catheters. In immunosuppressed patients, fungal infections remain a significant cause of morbidity and mortality, mainly caused by Candida and Cryptococcus species [1–3].

Cryptococcosis is a systemic opportunistic mycosis with an estimated worldwide annual incidence of 223,100 and a mortality rate of about 81%, predominantly in immunocompromised patients. The primary organ affected is the central nervous system (CNS) [4, 5].

Candidiasis (i.e., infections caused by Candida spp.) is an important fungal infection intrinsically related to health. The prevalence of these organisms varies with geographic location and specific anatomical site, with C. albicans being the most prevalent, and an associated mortality of over 70% in cases of candidemia [6].

The initial treatment recommended by the American Infectious Diseases Society for cases of disseminated candidiasis and cryptococcosis includes azole derivatives, caspofungin, amphotericin B (AmpB), or a combination of fluconazole and AmpB for relapses. However, because of the increasing appearance of resistance to all commercially available antifungals [7, 8] and because of lack of more effective, less toxic alternatives, several researchers worldwide have been seeking alternatives for the treatment of fungal infections. For example, our group suggested the use of ciclopirox olamine for systemic cryptococcosis therapy [9], and Magalhães et al. [10] reported the effectiveness of hydroxyaldimines for inhibition of growth of *Cryptococcus* spp.

In recent years, our research group demonstrated the potential of 2-amino-thiophene derivatives to inhibit fungal growth [11–15]. In 2011 [11], an analysis of 44 derivatives against clinical isolates of *Candida* and *Cryptococcus* strains showed that (i) 2-amino-thiophenes have fungicidal activity; (ii) *C. neoformans* strains have greater sensitivity to the compounds, and the most promising compound was 2-[(4-nitrobenzylidene)-amino]-4,5,6,7-tetrahydro-4*H*-benzo[*b*]thiophene-3-carbonitrile, called 6CN10 (Fig. 1), with MIC values of 100 μg/mL. In 2012 [12], computer-aided drug design (CADD) studies were performed with more than 50 2-amino-thiophene derivatives, including 6CN10; the authors observed that the main factor limiting the antifungal activity of these compounds was their low aqueous solubility. In order to overcome this undesirable characteristic, in 2017 [15], we generated complexes of 6CN10 with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and observed an increase in anti-*Cryptococcus* activity of about 3.5 to 7 times, demonstrating improvements in water solubility, and promoting an increase in the antifungal potential of 2-amino-thiophene derivatives.

Therefore, in the present study, we compared the in vitro antifungal activity of nanoparticles containing 6CN10 and 6CN10:HP-β-CD complex with that of free drug and amphotericin B, using minimal inhibitory concentrations (MICs) against species of pathogenic yeasts (*Candida* and *Cryptococcus*), in addition to measuring the compounds' ability to eradicate *Cryptococcus* in biofilms.
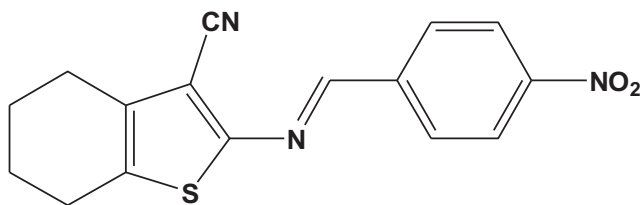

Fig. 1 Chemical structure of 6CN10

Methods

Reagents

Compound 6CN10 was synthesized according to previously published procedures [11], and its chemical structure was confirmed by comparing its ^{1}H NMR spectrum. 6CN10:HP-β-CD complex was prepared according to the procedure describe in Eleamen et al. [15]. Amphotericin B, poly-ε-caprolactone, 2-hydroxypropyl-β-cyclodextrin, Tween 80, Mygliol 812, chloramphenicol, and DMSO were purchased from Sigma-Aldrich (Brazil).

Nanoparticle preparation

Six formulations (Table 1) were prepared using the method of nanoprecipitation described by Fessi et al. [16]. For production of the nanospheres, pure 6CN10 or 6CN10:HP-β-CD complex was poured into acetone containing the polymer poly-ε-caprolactone (PCL). Then, the organic phase was added dropwise to the aqueous phase containing surfactant (Tween 80) under moderate magnetic stirring at 25 °C. The organic solvent was removed by evaporation under reduced pressure, and the final volume of the suspensions was adjusted to a final concentration of 100 μg/mL of 6CN10 to 6CN10:HP-β-CD nanocapsules and 6CN10:HP-β-CD nanospheres and of 250 μg/mL to 6CN10 nanocapsules and 6CN10 nanospheres (see Table 1). To produce the nanocapsules, Mygliol 812 was added to the organic phase. The nanoparticles were stored at 4 °C until use.

Nanoparticle characterization

The nanoparticle size distribution and surface charge (zeta potential) were determined using a Zetatrac Legacy (Microtrac®, USA), which was controlled by Microtrac Flex Version 10.5.0 software. The amount of the 6CN10 in the formulations was assayed spectrophotometrically (Genesys 10S, Thermo Scientific, USA) at 280 nm after solubilizing the nanoparticles in acetone.

Strains and growth cultures

Fifteen isolates of *Candida* obtained from patients with candidemia, four isolates of *Cryptococcus* obtained from cerebrospinal fluid (CSF) of immunocompromised patients, and the reference strain *Candida parapsilosis* ATCC 22019 were evaluated for the antifungal potential of pure 6CN10, 6CN10:HP-β-CD complex, and nanoparticles containing 6CN10. The clinical isolates were obtained from two tertiary referral public hospitals in Recife, Brazil. All *Cryptococcus* isolates evaluated in this work were stored under mineral oil [17] in the URM Culture Collection, UFPE. This collection is

Table 1 Nanoparticle characterization

Sample	Mean diameter (nm)	PDI	Zeta potential (mV)	6CN10 amount (µg/mL)
Blank nanocapsules	130 ± 2	0.23	− 2.85	-
6CN10 nanocapsules	126 ± 12	0.45	− 2.38	250
6CN10:HP-β-CD nanocapsules	131 ± 4	0.22	− 4.4	100
Blank nanospheres	125 ± 2	0.18	− 2.4	-
6CN10 nanospheres	140 ± 6	0.22	− 6.69	250
6CN10:HP-β-CD nanospheres	147 ± 5	0.44	− 4.52	100

PDI polydispersity index

registered in the WDCM of the WFCC as "604" under the acronym URM (University Recife Mycology).

Blood samples from patients with candidemia and CSF from immunocompromised patients were processed for mycological diagnosis using standard methods (direct examination and isolation in culture) at the Medical Mycology Laboratory, Federal University of Pernambuco, Recife, Brazil. Direct examination was performed without staining or clarification of blood samples or with India ink staining of CSF. Cultures were prepared using Sabouraud dextrose agar (SDA) (Difco) with chloramphenicol (50 mg/mL) and incubated at 30 ± 5 °C in an aerobic atmosphere for 5 days. Pure cultures were transferred onto the surface of SDA for species identification.

The collection of the clinical samples from patients was approved by the Ethics Committee of the Centre of Health Sciences of the Federal University of Pernambuco under protocol 01847812.0.0000.5208.

Classical phenotypic identification

The isolates were identified by macro- and micromorphology. The color, shape, and topology of each colony and cell morphology were evaluated using SDA medium with 2.5% yeast extract (Difco, USA). We induced sexual reproduction structures using Gorodkowa agar (Difco, USA). Chlamydospores were induced on bile agar (Difco, USA) and scored as present or absent after 3 days at 25 °C. The isolates were tested biochemically using carbohydrate assimilation and fermentation assays. The production of urease and acetic acid was assessed using urea and calcium carbonate media (Difco, USA), respectively. The maximum temperature of growth for each isolate was determined. All classical phenotypic parameters were analyzed according to Barnett et al. [18] and Hoog et al. [19].

MALDI-TOF MS identification

Homogeneous inocula of yeast cells were grown and maintained on yeast extract peptone dextrose agar medium (YEPD). Incubations were standardized at 20 h and strains were grown aerobically at 37 °C. To avoid changes in protein expression patterns, culture conditions and growth times were standardized as described above. All cultures were checked for purity prior to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis.

One single colony was directly deposited onto a 196-position target plate (Bruker Daltonik GmbH), and two such deposits were made for each isolate. Aliquots of 1 µL of 70% formic acid were added and mixed gently with the yeasts. When the liquid medium was almost evaporated, the preparation was overlaid with 1 µL of saturated matrix solution (75 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA) in ethanol/water/acetonitrile [1:1:1] with 0.03% trifluoroacetic acid (TFA)). A total of 20 isolates (2 × 20 spots) were deposited per plate, and the matrix samples were crystallized by air-drying at room temperature for 5 min [9].

We used a MALDI TOF Autoflex III Mass Spectrometer (Bruker Daltonics Inc., USA/Germany) equipped with a neodymium-doped yttrium aluminum garnet ($Nd:Y_3Al_5O_{12}$) laser of 1064 nm, set to 66% power. The mass range from 2000 to 20,000 Da was recorded using a linear mode with a delay of 104 ns and an acceleration voltage of + 20 kV. The resulting peak lists were exported to the software package MALDI Biotyper™ 3.0 (Bruker Daltonics, Bremem, Germany) where the final identifications were achieved.

In vitro antifungal susceptibility

Reference microdilution trials containing serial drug or nanoparticle dilutions were prepared by following the CLSI M27-A3 guidelines [20]. Dispersion of nanospheres and nanocapsules of 6CN10 (containing 250 µg/mL of 6CN10) and 6CN10:HP-β-CD complex (containing 100 µg/mL of 6CN10) was used.

Pure 6CN10 (10.0 mg) and 6CN10:HP-β-CD complex containing 14% of 6CN10 (10.0 mg) were dissolved in 1.0 mL of dimethylsulfoxide (DMSO), and then diluted in 9.0 mL of standard RPMI 1640 medium (Sigma Chemical Co., St., Louis, MO) buffered to pH 7.0 with 0.165 M of morpholinopropanesulfonic acid (MOPS; Sigma, Brazil)

resulting in solutions with concentrations of 1.0 mg/mL and 140 μg/mL, respectively. The concentrations tested ranged from 0.32 to 333.33 μg/mL for pure 6CN10; from 0.045 to 46.66 μg/mL for 6CN10:HP-β-CD complex; from 0.08 to 83.33 μg/mL for nanosphere and nanocapsule of 6CN10; and from 0.003 to 3.33 μg/mL for nanosphere and nanocapsule of 6CN10:HP-β-CD complex. AmpB was used as the reference drug at concentrations ranging from 0.015 to 8.0 μg/mL.

To obtain a yeast inoculum containing 1.0 to 5.0×10^6 CFU/mL, each strain was cultured in tubes containing 20 mL of 4% SDA (Difco) plus yeast extract at 35 °C for 2 days. Subsequently, yeast suspensions were prepared in sterile physiological solution (0.85%) and maintained at 28 ± 2 °C, then adjusted to 90% transmittance at 530 nm. Two serial dilutions from 1:100 and 1:20 were made to obtain a final inoculum containing 1.0 to 5.0×10^3 CFU/mL.

For susceptibility tests, 100 μL of each tested solution (6CN10 and 6CN10:HP-β-CD complex solutions, nanoparticles containing 6CN10 and 6CN10:HP-β-CD complex, drug-free nanoparticles and reference drug) and of 10 serial dilutions was added in microdilution wells containing 100 μL of standard RPMI 1640 medium buffered to pH 7.0 with 0.165 M of MOPS; then, wells were inoculated with 100 μL of the previously obtained inocula. The microplates were incubated at 35 °C in a non-CO₂ incubator and were visually evaluated 48 h after the incubation to *Candida* isolates and 72 h after incubation to *Cryptococcus* isolates. MICs corresponded to the lowest drug dilution that showed 100% growth inhibition compared with untreated yeasts. All tests were performed in duplicate.

Formation and treatment of the biofilm on silicone discs

Biofilm formation was carried out according to a modification of Vandenbosch et al. [21]. *Cryptococcus* biofilms were obtained on sterile silicone discs in 24-well microtiter plates (TPP, Trasadingen, Switzerland). The discs were washed in MilliQ water (Millipore, Billerica, MA, USA) and autoclaved. Yeast cells were cultured on SDA for 48 h at 37 °C; then, 3–5 colonies were suspended in saline solution 0.9% (w/v) and centrifuged for 5 min at 1000 *g*. Subsequently, supernatants were removed and cells were washed and resuspended three times in 1 mL of saline solution 0.9% (w/v). These inocula were further diluted in yeast nitrogen base 0.1× (YNB, BD, Franklin Lakes, USA) supplemented with 5 mM glucose (Sigma-Aldrich) to yield an optical density of 0.07 at a wavelength of 600 nm. Then, 1 mL of a 1:100 dilution of the inoculum in YNB 0.1 was added to each well containing a silicone disc with three 6CN10 formulations (6CN10 nanosphere, 6CN10:HP-β-CD nanosphere, and 6CN10:HP-β-CD nanocapsule at final concentrations of 83.33 μg/mL, 3.33 μg/

mL, and 3.33 μg/mL, respectively). Appropriate controls were also included. The 24-well microtiter plates were incubated for 48 h at 37 °C.

Numbers of CFUs on each silicone disc were determined by pour plating, as follows: the silicone discs with biofilms were transferred to 10 mL of SDB, and biofilm cells were removed from the silicone using three cycles of 30-s sonication and 30-s vortex mixing. Using this procedure, all sessile cells were removed from the silicone discs and clumps of cells were broken apart. Serial 10-fold dilutions of the resulting cell suspensions were made, and 1 mL of each dilution was plated and SDA was added. Plates were incubated for 48 h at 37 °C, after the numbers of CFUs per disc were counted. For each strain and treatment, biofilms forming on at least three silicone discs in at least three independent experiments were included, and the results were expressed as arithmetic means.

Statistical analysis

Various anti-biofilm treatments were analyzed using one-way analysis of variance (ANOVA) with significance level defined as $p < 0.05$. The multiple comparisons test (Tukey) was used after ANOVA to compare the means one-by-one. All statistical analyses were performed using GraphPad Prism version 5.01.

Results

Nanoparticle preparation and characterization

The mean diameter, PDI, zeta potential, and amount of 6CN10 were measured (Table 1). The mean diameter of nanocapsule formulations was 130 nm. The blank nanospheres had a similar mean diameter, with a slight increase in the mean diameter size for the drug-loaded nanospheres. All formulations showed moderate-to-good polydispersity index (0.18–0.45). The zeta potential for the samples varied from − 2 to − 7 mV.

Strain identifications

The yeasts were identified using the phenotypic approach as five *Candida albicans*, one *C. famata*, one *C. glabrata*, one *C. guilliermondii*, one *C. krusei*, five *C. parapsilosis*, one *C. tropicalis*, and four *Cryptococcus neoformans*. MALDI-TOF identified the *C. parapsilosis* complex isolates as *C. parapsilosis* sensu stricto and *Cryptococcus neoformans* as *Cryptococcus neoformans* var. *grubii*, all with scores above 2.0.

In vitro antifungal susceptibility

For each antifungal susceptibility experiment, the inoculum controls showed clearly detectable growth after the incubation period, indicating that all isolates were viable and that the conditions were suitable for fungal growth. The MIC of the reference strain was 0.5 μg/mL to AmpB, confirming the reproducibility of the test. Drug-free nanoparticles (blank) were also used as controls and showed no activity against any fungal pathogens.

Table 2 shows the minimal inhibitory concentrations (MICs) of 6CN10 (free drug), 6CN10 complexed with 2-hydroxypropyl-β-cyclodextrin (6CN10:HP-β-CD), and the four nanoparticles (6CN10 nanocapsule, 6CN10:HP-β-CD nanocapsule, 6CN10 nanosphere, and 6CN10:HP-β-CD nanosphere) compared with amphotericin B (AmpB) against isolates of *Cryptococcus neoformans*, *Candida albicans*, *C. famata*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* (including the reference strain *C. parapsilosis* ATCC 22019).

As can be seen in Table 2, the 6CN10 free drug inhibited the growth of all strains at various concentrations. The

Candida strains were slightly more susceptible, showing MIC values ranging between 41.66 and 333.33 μg/mL, while the *Cryptococcus* strains presented MIC values of 166.66 and 333.33 μg/mL.

6CN10:HP-β-CD complex was completely ineffective for all *Candida* strains (all strains grew (G) at the highest concentration tested of 46.66 μg/mL); however, it showed MIC values of 46.66 μg/mL against all *Cryptococcus* strains, being four times more active than the 6CN10 free drug.

We also observed this difference in sensitivity between the two species (*Candida* and *Cryptococcus*) for all nanoparticles evaluated, where all isolates of *Candida* grew (at the highest concentrations tested), while *Cryptococcus* strains showed MIC values ranging from 0.1 to 83.33 μg/mL, therefore more active than the 6CN10 free drug, and with MIC values comparable or better to that of the reference drug AmpB.

The nanocapsules of 6CN10 and 6CN10:HP-β-CD complex inhibited the growth of *Cryptococcus* strains with MIC values of 83.33 μg/mL and 0.1–0.2 μg/mL, respectively. 6CN10 nanocapsules were 2 to 4 times more effective than the 6CN10 free drug. 6CN10:HP-β-CD nanocapsules were 233 to 466 times more active than the 6CN10:HP-β-CD

Table 2 Antifungal activity of free drug (6CN10), 6CN10:HP-β-CD complex, 6CN10 nanoparticles, and amphotericin B against *Candida* and *Cryptococcus* clinical isolates, obtained from patients with candidemia and neurocryptococcosis, respectively, in two tertiary referral public hospitals in Recife, Brazil, from July 2012 to June 2013

Microorganism	Strain or patient number	Compounds and formulation—MIC values in μg/mL						
		6CN10 nanocapsule	6CN10:HP-β-CD nanocapsule	6CN10 nanosphere	6CN10:HP-β-CD nanosphere	6CN10:HP-β-CD complex	6CN10 free drug	AmpB
C. albicans	99	G	G	G	G	G	41.66	0.25
C. albicans	122	G	G	G	G	G	41.66	0.25
C. albicans	4451	G	G	G	G	G	41.66	1
C. albicans	8299	G	G	G	G	G	166.66	2
C. albicans	9925	G	G	G	G	G	166.66	2
C. famata	5623	G	G	G	G	G	333.33	2
C. glabrata	8340	G	G	G	G	G	166.66	0.5
C. guilliermondii	632	G	G	G	G	G	83.33	0.5
C. krusei	14,206	G	G	G	G	G	333.33	1
C. tropicalis	7866	G	G	G	G	G	83.33	1
C. parapsilosis	290	G	G	G	G	G	83.33	1
C. parapsilosis	7736	G	G	G	G	G	83.33	1
C. parapsilosis	9968	G	G	G	G	G	333.33	2
C. parapsilosis	13,531	G	G	G	G	G	83.33	0.25
C. parapsilosis	451	G	G	G	G	G	83.33	1
C. parapsilosis	ATCC 22019	G	G	G	G	G	83.33	0.5
C. neoformans	URM 6895	83.33	0.2	0.65	0.1	46.66	333.33	0.5
C. neoformans	URM 6901	83.33	0.1	0.32	0.1	46.66	333.33	0.25
C. neoformans	URM 6907	83.33	0.1	41.66	0.2	46.66	166.66	0.125
C. neoformans	URM 6909	83.33	0.1	41.66	0.2	46.66	166.66	0.125

MIC minimal inhibitory concentration, *AmpB* amphotericin B, *G* growth at the highest concentration tested, *URM* URM Culture Collection, UFPE, Brazil

complex, and 3333 times more active than the 6CN10 free drug, as well as being more efficient than the reference drug (AmpB) for all *Cryptococcus* strains.

The best results of inhibition were observed for the nanospheres. 6CN10 nanospheres presented MIC values of 41.66 to 0.32 μg/mL, 4 to 1040 times more active than the 6CN10 free drug. 6CN10:HP-β-CD nanospheres showed MIC values of 0.2 to 0.1 mg/mL, 233 to 466 times more active than the 6CN10:HP-β-CD complex, and up to 3333 times more active than the 6CN10 free drug, and 2.5 to 5 times more active than AmpB for *C. neoformans* URM 6895 and URM 6901.

Treatment of the biofilm

The effects of the three most bioactive 6CN10 formulations were determined on *Cryptococcus* biofilms formed on silicone discs using four different strains (Table 3). Blank and control groups did not present statistically significant difference for any of the evaluated strains, suggesting that the nanoparticle components did not present fungicidal activity against the biofilms. The most efficient nanoparticle was 6CN10:HP-β-CD nanosphere, which was able to reduce CFU number recovered from the discs for all strains investigated, with reduction percentages ranging from 90.9 to 100% ($p < 0.05$) compared with control. The highest reduction was observed for *C. neoformans* URM 6907 (100%, $p < 0.05$).

6CN10 nanosphere was the second-best nanoparticle, promoting a statistically significant reduction ($p < 0.05$) in CFU for 50% of the strains (URM 6895 and URM 6909) when compared with the control group. For the strain URM 6909, the reduction values of the groups treated with 6CN10 nanosphere and 6CN10:HP-β-CD nanosphere did not show significant differences.

The lowest treatment effect occurred with 6CN10:HP-β-CD nanocapsule, giving a small reduction in CFU for strains URM 6895 and URM 6906 (around 50%; $p < 0.05$).

Our results showed only a fungistatic effect for 6CN10 nanosphere and 6CN10:HP-β-CD nanocapsule, whereas 6CN10:HP-β-CD nanosphere showed fungicidal activity against *Cryptococcus* biofilms.

We used catheter discs for biofilm formation in accordance with Vandenboch et al. [21]. Figure 2 shows a scanning electron microscopy of discs with biofilm formation. Figure 2a and b represent the positive controls demonstrating growth of *C. neoformans* URM 6906 and *C. neoformans* URM 6907. No drugs were used for control (YNB media only) that showed the expected growth. Figure 2c and d show growth of *C. neoformans* URM 6906 and *C. neoformans* URM 6907 using blank (nanoparticle without 6CN10). Figure 2e and f represent the use of 6CN10:HP-β-CD nanosphere against *C. neoformans* URM 6907 and *C. neoformans* URM 6909 respectively, where there is no growth of the *Cryptococcus* strains in the biofilm.

Discussion

Six formulations were prepared to improve the aqueous solubility and bioavailability of 6CN10. There were three matricial-type nanoparticles: blank nanospheres (drug-free); nanospheres containing 6CN10; nanospheres containing inclusion complexes of 6CN10

with HP-β-CD; and three reservoir-type nanoparticles: blank nanocapsules (drug-free); nanocapsules containing 6CN10; and nanocapsules containing inclusion complex of 6CN10 with HP-β-CD.

The method of nanoprecipitation produced nanoparticles with very reduced particle sizes as well as a slight variation in the particles' diameters, confirming the feasibility and robustness of the technique [22]. Concerning size distribution, four formulations showed good homogeneity, with PDI values less than 0.3. Furthermore, the formulations of 6CN10 nanocapsules and 6CN10:HP-β-CD nanospheres showed moderate homogeneity (0.3 < PDI value < 0.5) [23]. The negative zeta potential of the nanoparticles could be attributed to the presence of terminal carboxylic groups of the PCL. Zeta

Table 3 Number of CFUs of *Cryptococcus neoformans* biofilms on silicone discs after 6CN10 nanoparticle treatment

Cryptococcus strain	6CN10 nanosphere	6CN10:HP-β-CD nanosphere	6CN10:HP-β-CD nanocapsule	Blank[†]	Control[‡]
URM 6895	12.33 ± 4.16	3.00 ± 1.00	17.66 ± 2.08	43.00 ± 6.08	47.00 ± 4.00
URM 6906	30.66 ± 4.72	3.33 ± 1.52	18.33 ± 2.08	36.00 ± 4.58	36.66 ± 3.51
URM 6907	8.66 ± 1.52	0.66 ± 0.57	7.66 ± 1.52	9.66 ± .2.51	9.33 ± 3.78
URM 6909	2.33 ± 1.52	1.66 ± 0.57	26.00 ± 3.00	6.66 ± 2.51	15.00 ± 6.08

[†] Blank—yeasts and silicone discs in compounds of the nanoparticles without 6CN10

[‡] Control—yeasts and silicone discs in culture media only (YNB supplemented with 5 mM glucose)

URM URM Culture Collection, UFPE, Brazil

Plate counts corresponded to the mean ± standard error of mean of three independent experiments on three silicone discs

Values are expressed as mean ± SEM of three independent experiments

Fig. 2 Scanning electron micrographs after 48-h biofilm formation of *Cryptococcus neoformans* on silicone discs. URM 6906 (**a**) and URM 6907 (**b**) in YNB media only (control); URM 6906 (**c**) and URM 6907 (**d**) treated with blank (nanoformulations without 6CN10); URM 6907 (**e**) and URM 6909 (**f**) treated with 6CN10:HP-β-CD nanosphere
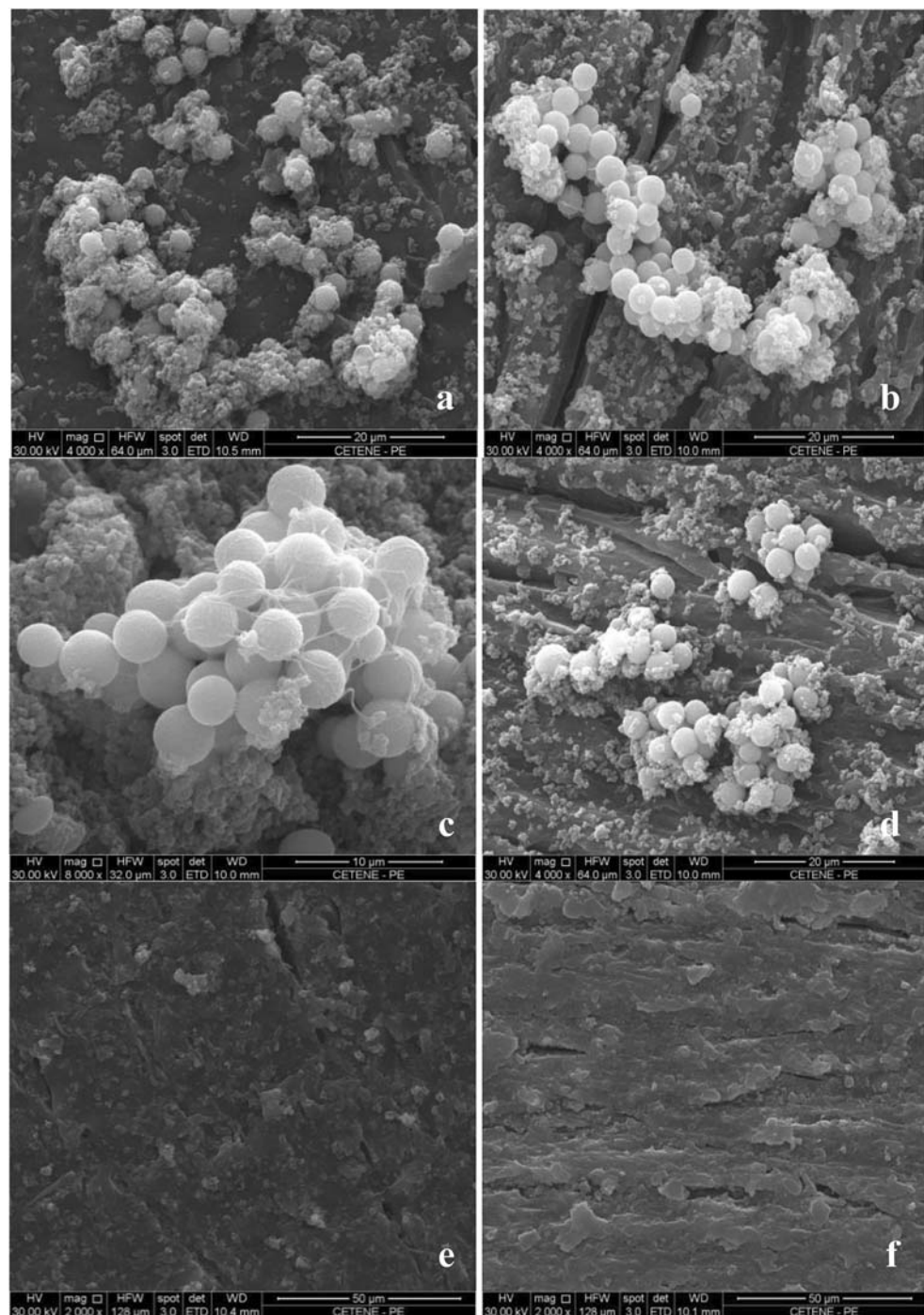
potential values between [20] and [35] are thought to favor the best stability due to repulsive forces preventing aggregation upon aging. By contrast, zeta potential values close to 0 are related to the biocompatibility of the nanoparticles [24]. The addition of 6CN10 (free or complex) did not induce any drastic modification in the size and zeta potential of the nanoparticles (Table 1).

According to Abreu et al. [25] and Mohareb et al. [26], compounds containing thiophene rings possess selective and significant antifungal activity against *C. albicans*. In accordance with Ram et al. [27], strains of *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Candida albicans*, *Trichophyton mentagrophytes*, and *Sporothrix schenckii* were sensitive to the analogous thiophene evaluated in the present work. Experiments carried out by Ryu et al. [28] demonstrated that synthetic thiophenes possess good activity against *A. niger*, *C. albicans*, *C. krusei*, and *C. tropicalis*, differing in part of our findings that showed moderate-to-weak antifungal effects

against *Candida* strains. However, Lima et al. [29] found growth reduction of dermatophytes, including *Trichophyton rubrum*, corroborating our results.

Analogous or homologous structural variations can be created to provide new physical properties and to modify the reactivity of molecules, causing changes in distribution in cells and tissues, as well as increased access to the active sites of enzymes and receptors. Heterocyclic compounds such as thiophene derivatives possess broad biological activity; however, they have not been extensively studied as antifungals. Our group tested the in vitro antifungal activity of various thiophene derivatives against pathogenic fungi; we observed *Cryptococcus neoformans* were more sensitive to 2-amino-thiophene derivatives than were *Candida* [11–13].

There are several factors involved in *C. neoformans* virulence, including the ability to grow at 37 °C, the secretion of extracellular proteases and phospholipases, and the production of mannitol, urease, lactase, and melanin that protect against oxidizing agents, the immune system, and damage caused by heat, cold, and ultraviolet radiation. Furthermore, the presence of a glucuronoxylomannan-polysaccharide capsule has been implicated in modulating the *Cryptococcus* blood-brain barrier interaction [30]. The capsule is a major virulence factor; however, its role in the central nervous system invasion remains unclear. Capsule-associated structural changes such as phenotypic switching have been reported to enhance crossing of the blood-brain barrier [31, 32]. We believe that the capsule interacts in vitro with the nanoparticle structures, improving the access of the 6CN10 to the yeast, resulting in lower MIC values. This interaction may be mediated by oxylipins, 3-OH fatty acids that are released in the capsule surface as described by Sebolai et al. [33]. However, complementary ex vivo and in vivo studies are necessary to determine the mechanisms of the antifungal activity of these nanoparticles containing thiophene.

In previous studies, our group observed that incorporation of thiophene derivatives in pharmaceutical preparations permitted increased antifungal activity of the compounds, as also observed by Guimarães et al. [14], who found that a 2-amino-thiophene derivative called 5CN05 embedded in a microemulsion system gave 7.7-fold increased activity against *C. neoformans* (reducing MIC to 17.0 for 2.2 μg/mL). Eleamen et al. [15] observed a 3.5–7-fold increase in anti-*Cryptococcus* activity when compound 6CN10 was complexed with HP-β-CD. These results help to validate the study published by Scotti et al. [12] demonstrating that pharmacotechnical procedures (that help to mask the intrinsic low aqueous solubility of 2-amino-thiophene derivatives) contributed to increased antifungal potential.

The present study confirmed the promising potential of 2-amino-thiophene derivatives as a new class of antifungal agents that would be especially useful for infections caused by *Cryptococcus*. Incorporating 6CN10, and more clearly

6CN10 complexed with 2-hydroxypropyl-β-cyclodextrin (6CN10:HP-β-CD), in nanosystems such as nanospheres and nanoparticles proved to be an excellent way to minimize inherent unwanted drug physicochemical properties, in particular very low water solubility. Previously, these characteristics made the compounds less amenable to in vivo studies because of this substantial pharmacokinetics barrier, thus allowing us to perform in vivo studies. The complexation of the drug with HP-β-CD improved the water solubility and incorporation in nanoparticles conferred high bioavailability to the drug (6CN10) without changing the drug's chemical structure. Therefore, a smaller amount of drug was necessary to ensure the antifungal activity, possibly minimizing the possibility of the occurrence of side effects when performing in vivo studies. These facts associated with the significant increase in anti-cryptococcal activity (more than 3000 times compared with the free drug), with MIC values lower than the reference drug AmpB (up to 5 times more active), lead us to believe that after conducting more studies to ensure the efficacy, the mode of action at molecular level, and the safety, some of these nano-systems containing 6CN10 may be used clinically to treat systemic cryptococcosis infections.

Compliance with ethical standards

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