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Effect of Usnic Acid on *Candida orthopsilosis* and *C. parapsilosis*

Regina Helena Pires,[a] Rodrigo Lucarini,[b] and Maria Jose Soares Mendes-Giannini[a]

Universidade Estadual Paulista, UNESP, Araraquara, SP, Brazil,[a] and Universidade de Franca, Franca, SP, Brazil[b]

The activity of usnic acid against *Candida orthopsilosis* and *Candida parapsilosis* on planktonic and biofilm conditions was investigated by using a broth microdilution and microplate methods. Potent *in vitro* activities against different *Candida* species were obtained. The metabolic activity of sessile cells of *C. parapsilosis* complex was reduced by 80% at four times the 80% inhibitory concentration. The *in vitro* studies support further efforts to determine whether usnic acid can be used clinically to cure patients with *Candida* infections.

Among the *Candida* strains reported to cause human diseases, more than 17 different species have been identified (9, 19). Many of these species have been observed to occur in the hemodialysis setting and/or to exhibit innate or acquired resistance to one or more established antifungal agents (4, 12, 13, 16, 21). In addition, the use of molecular identification methods has resulted in the identification of new species within larger species complex such as *Candida orthopsilosis* and *Candida metapsilosis* within the *Candida parapsilosis* complex (23). In particular, the percentage of isolates of *C. orthopsilosis* has been much higher in the *C. parapsilosis* complex isolates in Latin America (12.7%) (11).

The small number of drugs available for fungal treatment encourages the search for new chemotherapeutic agents. Usnic acid (2,6-diacetyl-1,2,3,9b-tetrahydro-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3-dione), a secondary lichen metabolite, is known to possesses antimicrobial properties in addition to antiviral, antiprotozoal, antiproliferative, anti-inflammatory, and analgesic activity (10).

With respect to antimicrobial properties, usnic acid has activity against a number of planktonic Gram-positive bacteria and also has the capacity to control biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (8). Indeed, the mechanism of action expressed by usnic acid is still unknown. According to the same study (8), usnic acid could inhibit quorum sensing in *P. aeruginosa* biofilms.

Usnic acid has been tested against some *Candida* species, but there is no published data concerning its activity against the newly described species within the *C. parapsilosis* complex. Thus, we tested usnic acid in both planktonic or biofilm modes of growth in *C. orthopsilosis* and *C. parapsilosis*.

Six isolates of *C. parapsilosis* complex able to form biofilms were collected from a hemodialysis unit located in the state of São Paulo, Brazil, between March 2006 and March 2007 obtained from 110 samples of water that were selected for testing. The identification of *C. parapsilosis* and *C. orthopsilosis* was confirmed by molecular methods as described previously (23). The MIC for usnic acid of planktonic *C. parapsilosis* complex cultures was determined in RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) (both from Sigma Chemical Co., St. Louis, MO) using a broth microdilution method adapted from the CLSI approved standard (M27-A3) (6). Briefly, the usnic acid (Sigma) was dissolved in 5% (vol/vol) in dimethyl sulfoxide (DMSO) (Sigma) that had been filter sterilized through a 0.22-μm-pore-size filter (Millipore, Billerica, MA) and added to the growth medium to obtain final concentrations ranging from 0.48 μg/ml at 1,000 μg/ml. Yeast cells were then added to each well at the final concentration of 1×10^6 cells/ml (2, 5, 15). The microtiter trays were incubated at 35°C for 48 h. Afterwards, IC_{50} and IC_{80} values (the lowest concentrations that inhibit 50% and 80% of the yeast growth in comparison to untreated control, respectively), were determined using a spectrophotometer at 492 nm. Controls containing antimicrobial agents in broth without fungal inocula were included. Amphotericin B (final concentration of 0.0156 to 16 μg/ml) and the *C. parapsilosis* ATCC 90018 strain were used as quality controls.

After the MIC assay, minimal fungicidal concentrations (MFCs) were determined by plating 10 μl of the material in each of the clear wells onto Sabouraud dextrose agar (SDA) (Difco) plates. The MFC was defined as the lowest concentration yielding no growth following incubation at 37°C for 48 h.

The MICs for sessile (biofilm) cells (SMICs) were determined by a microtiter plate assay as described previously (20). Briefly, each well on a 96-well microtiter plate was filled with 100 μl of RPMI 1640 containing 10^6 cells of an overnight culture. After 24 h of incubation at 37°C, the biofilms were washed three times with sterile phosphate-buffered saline (PBS) (10 mM potassium phosphate, 0.15 M NaCl [pH 7.0]). The biofilms were exposed to 100 μl of antimicrobial agent, and the plates were incubated for 48 h at 37°C, after which the usnic acid was removed by washing each well twice with 100 μl PBS. Fungal viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) as described previously (17). Briefly, 200 μl prewarmed MTT solution (0.5 mg/ml) in PBS containing 0.1% glucose and 10 μl of 10 μM menadione was added to each well. The plates were incubated at 37°C for 30 min, and the MTT solution was removed. The yeast cells were washed once with PBS and resuspended in acid isopropanol (5% [vol/vol] 1 M HCl in isopropanol). Finally, the absorbance at 540 nm (A_{540}) was measured using a microtiter plate reader (ASYS, Eugendorf, Salzburg, Austria). The BEC_{50} and BEC_{80} for *Candida* biofilms were defined as the lowest drug concentration with a 50% and 80% reduction, respectively, in the

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Address correspondence to Regina Helena Pires, rehepi@gmail.com.
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TABLE 1 Activities of usnic acid against *C. orthopsilosis* and *C. parapsilosis* isolates

Candida species	Antifungal activity (μg/ml)[a]					
	Planktonic growth			Biofilm growth		
	IC_{50}	IC_{80}	MFC	BEC_{50}	BEC_{80}	MBFC
C. orthopsilosis	1.95	7.8	125	3.9	31.2	125
C. parapsilosis	1.95	15.6	250	3.9	62.5	250

[a] IC_{50} and IC_{80}, concentration required for 50% and 80% inhibition in cell growth compared with the untreated controls, respectively; MFC, minimal fungicidal concentration; BEC_{50} and BEC_{80}, 50% and 80% reduction in the metabolic activity of the biofilms compared to untreated controls, respectively; MBFC, minimum biofilm fungicidal concentration.

metabolic activity of the biofilms compared to controls (drug free). The minimum biofilm fungicidal concentration (MBFC) was defined as the concentration with A_{540} values below or equal to the background level (acid isopropanol). The antimicrobial activity of 5% (vol/vol) DMSO was also studied on a separate plate alongside the assay plate. *C. albicans* SC5314 was used as the biofilm control strain (20, 22). All experiments were performed in triplicate on three different days.

As shown in Table 1, usnic acid exhibited an anti-*Candida* effect, with IC_{50} of 1.95 μg/ml and IC_{80}s of 7.8 and 15.6 μg/ml. Reduction of 50% in the metabolic activities of biofilms of both *C. parapsilosis* strains (BEC_{50}) was achieved at a concentration of 3.9 μg/ml; 31.2 and 62.5 μg/ml reduced the growth of the cells in 80% (BEC_{80}) (Table 1). In contrast, the MBFCs of usnic acid were comparable to the MFCs estimated at 125 and 250 for *C. orthopsilosis* and *C. parapsilosis*, respectively (Table 1). For these environmental isolates, *C. orthopsilosis* was more susceptible to usnic acid than the *C. parapsilosis*. The MIC values for the reference strain that was used as a positive control for amphotericin B were within the established values for the CLSI M27-A3 protocol. DMSO (5% [vol/vol]), which was used as a cosolvent in the drug suspensions, did not show anticandidal activity against *C. parapsilosis* complex grown in suspension or as a biofilm.

Based on the existing literature, usnic acid seems to be produced only in lichens and appears to be effective against a wide variety of bacterial strains, and the antifungal properties previously reported by Cardarelli et al. (3) were confirmed by the results of this study. Because of their antimicrobial activity, usnic acid has been the target of many studies for the purpose of developing phytotherapeutic options for treatment of infections (14). According to Francolini et al. (8), there was no evidence of a toxic effect of usnic acid in pharmacokinetics studies and after oral administration.

According to the values of EC_{50}, EC_{80}, BEC_{50}, and BEC_{80} obtained for the isolates of *C. orthopsilosis* and *C. parapsilosis*, usnic acid demonstrated potent inhibitory activity against both planktonic and biofilm cells (Table 1). Furthermore, Yilmaz et al. (24) showed that usnic acid with quite low MIC values (0.15 μg per disk) was effective against 10^7 cells/ml of *Candida albicans* or *Candida glabrata*. Similarly, lower MICs (ranging from 1 to 26 μg/ml) determined by the disk diffusion method have been observed against *Bacillus subtilis* (18). One factor that may explain our results include the absence of standardized anticandidal assays for natural products. Results can be profoundly influenced by the testing method (7). Previous studies have shown that antimicrobial activity can be more effectively evaluated in liquid media than in solid media, since in the latter, the diffusion of drug may not be appropriate (1).

The results presented in this study are the first report of usnic acid showing *in vitro* inhibitory and fungicidal activity against environmental isolates of *C. orthopsilosis* and *C. parapsilosis*. Considering also the absence of cytotoxicity and low concentrations obtained, usnic acid represents a promising area of future research. The mechanisms of its antifungal activity should be studied in order to validate the use of usnic acid as a natural antifungal product.

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