Antonie van Leeuwenhoek (2010) 97:201–205
DOI 10.1007/s10482-009-9394-8

SHORT COMMUNICATION

In vitro antifungal activity of the berberine and its synergism with fluconazole

Renata Sayuri Iwazaki · Eliana Harue Endo · Tânia Ueda-Nakamura · Celso Vataru Nakamura · Lourdes Botelho Garcia · Benedito Prado Dias Filho

Received: 19 June 2009 / Accepted: 21 October 2009 / Published online: 2 November 2009

Abstract Berberine with and without fluconazole was tested by an agar disk diffusion assay in which clinical isolates of *Candida albicans* were applied onto yeast extract-peptone-dextrose agar plate. Berberine, which had no intrinsic antifungal activity at the concentration tested, exerted a powerful antifungal activity in combination of fluzonazole. Combinations of berberine and fluconazole were also tested by the checkerboard assay to determine whether they had favorable or unfavorable antifungal interactions. The MIC of fluconazole was 1.9 μg/ml when the drug was tested alone and decreased to 0.48 μg/ml in the presence of berberine concentrations of 1.9 μg/ml. However, berberine at concentrations of >1.9 μg/ml combined with a fluconazole supra-MIC (i.e., >1.9 μg/ml) eliminated the residual turbidity in the incubation wells. This endpoint fitted to the definition of MIC-0 (optically clear wells) and reflected the absence of a trailing effect, which is the result of a residual growth at fluconazole concentrations greater than the MIC.

R. S. Iwazaki
Programa de Pós-graduação em Análises Clínicas, Universidade Estadual de Maringá, Maringá, Brazil

E. H. Endo · T. Ueda-Nakamura · C. V. Nakamura · L. B. Garcia · B. P. D. Filho (✉)
Departamento de Análises Clínicas, Universidade Estadual de Maringá, Av. Colombo, 5790, Maringá, PR 87020-900, Brazil
e-mail: bpdfilho@uem.br

Keywords Berberine · *Candida albicans* · Checkerboard · Fluconazole · Synergism

Candida species are now the fourth most common organism recovered from the blood of hospitalized patient (Gudlaugsson et al. 2003). Notwithstanding the increasing need for effective therapy, the range of antifungal agents available is limited, and some of the most effective agents are also toxic. In addition, while the azoles have been used successfully for the treatment of *Candida* infections, numerous reports of treatment failures are now appearing in the literature (Lipsitch et al. 2000; Perea and Patterson 2002).

Previous reports of synergistic combinations of antifungal compounds have not been extensive or encouraging. In a review of combination therapy in systemic mycosis, Polak (1990) discussed the combination of amphotericin B with flucytosine as one of the better-established synergistic combinations of antifungal agents that has been used clinically to treat candidiasis. The combination of an azole with a polyene resulted in conflicting outcomes, depending on the species and the strain tested, and specific antagonism was observed with *Candida albicans* (Polak 1990).

Berberine, a well-known alkaloid, was found in medicinal herbs such as *Coptis chinensis* Franch (Ranunculaceae) and *Hydrastis canadensis* L.

(Ranunculaceae). Currently, the predominant clinical uses of berberine preparations include the treatment of bacterial diarrhea, intestinal parasite infections and ocular trachoma infections (Birdsall 1997). Berberine was demonstrated to have weak activity against *C. albicans* and *C. glabrata* (Polak 1990; Park et al. 1999; Vollekova et al. 2003). Other pharmacological effects of berberine, such as antiarrhythmic (Zhao and Guo 1989), anti-inflammatory (Kuo et al. 2004), anticancer (Mitani et al. 2001) immunosuppressive (Marinova et al. 2000), vasorelaxant, and antiproliferative (Ko et al. 2000) have also been reported.

The main aim of the study described here was to investigate ways of enhancing the antifungal activities of the azoles. We have focused on combination of berberine with fluconazole, because fluconazole is one of the most popular drugs for treating candidiasis, especially in immunocompromised patients.

C. albicans ATCC 10231 and clinical isolates from vaginal fluid were tested by disk diffusion test (Marchetti et al. 2000). A 10^6 CFU/ml yeast suspension was applied onto the yeast extract-peptone-dextrose (YEPD) agar plate with and without 6 μg/ml of fluconazole using cotton swabs. Then, for *C. albicans* ATCC 10231, paper disks impregnated with 0, 1, 10, 100, and 500 μg of berberine were placed onto agar plates. For isolates, berberine was tested at 0, 10 and 100 μg/disk. Diameters of growth inhibition zones were measured after incubation at 37°C for 24 h.

Checkerboard tests were performed by broth microdilution reference procedure at a final inoculum of 0.5–2.5 × 10^3 CFU/ml, using RPMI 1640 medium buffered with 0.165 M MOPS (CLSI 2004). The final concentrations ranged from 0.06 to 62.5 μg/ml for fluconazole and 1.9 to 125 μg/ml for berberine. Plates were incubated at 37°C for 48 h and absorbance was read in a ELISA plate reader at 492 nm. The fractional inhibitory concentration (FIC) index is the sum of the MIC of each drug in combination divided by the MIC of the drug used alone. An FIC index ≤0.5 is considered synergism; FIC index >4 is antagonism and a result >0.5 but ≤4 is indifferent (Odds 2003).

In order to investigate the effect of the berberine and fluconazole alone and in combination on morphology of *C. albicans*, treated and control cells were examined by scanning electron microscopy. Treated and untreated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 and small drops of the fixed cells were placed on specimen support with poly-L-lysine for 1 h at room temperature. Subsequently, the samples were dehydrated in graded ethanol, critical-point dried in CO_2, coated with gold and examined in a Shimadzu SS-550 scanning electron microscope.

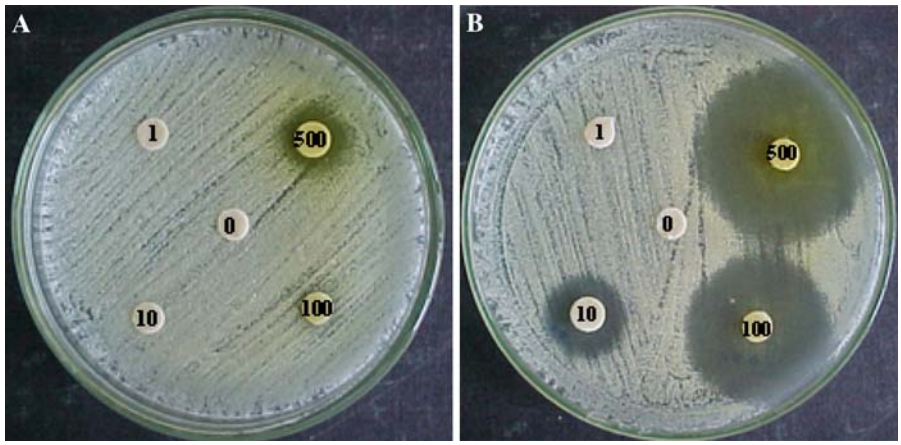

Fig. 1 Disk diffusion halo assays demonstrate enhanced inhibition of *C. albicans* ATCC 10231 when berberine is combined with fluconazole. A 10^6 CFU/ml yeast suspension was applied onto the yeast extract-peptone-dextrose (YEPD) agar plate without (**a**) and with (**b**) 6 μg/ml of fluconazole using cotton swabs. Paper disks impregnated with 0, 1, 10, 100 and 500 μg of berberine were placed onto agar plates. Diameters of growth inhibition zones were measured after incubation at 37°C for 24 h

The alkaloid berberine is a common component of a variety of plant species, particularly in the family *Berberidaceae*. Berberine exhibits relatively weak antibiotic properties, apparently because of its efflux by multidrug resistance pumps (Stermitz et al. 2001).

In the present study, combinations of berberine and fluconazole were tested by an agar disk diffusion assay in which clinical isolates of *C. albicans* were applied onto YEPD agar plate. Agar diffusion tests visualized a synergistic interaction. Berberine had no antifungal activity in smaller amounts and showed a weak antifungal activity at 500 μg (Fig 1a). Fluconazole at 6 μg/ml had no antifungal activity. In contrast, berberine showed a powerful fungistatic effect on the agar plate containing 6 μg/ml fluconazole (Fig. 1b).

To quantify the antifungal activities obtained with berberine alone and in combination with fluconazole, antifungal indices were calculated. An increase of the antifungal index reflected a synergistic effect. Table 1 summarizes the in vitro susceptibilities of 25 isolates of *C. albicans* as determined by disk diffusion testing. Berberine alone had in this experimental setting a low intrinsic antifungal activity (median 0.04; range 0–0.2), and its combination with fluconazole had a powerful antifungal activity with a median index of 1.26 (range 0–2.1).

Berberine and fluconazole were also tested by the checkerboard assay to determine whether they had favorable or unfavorable antifungal interactions. The MIC of fluconazole was 1.9 μg/ml when the drug was tested alone and decreased to 0.48 μg/ml in the presence of berberine concentrations of 1.9 μg/ml. However, berberine at concentrations of >1.9 μg/ml combined with a fluconazole supra-MIC (i.e., >1.9 μg/ml) eliminated the residual turbidity (trailing) in the incubation wells. This observation made it possible to use a different endpoint, the MIC-0. The MICs-0 for yeast were graphed in isobolograms and yielded highly concave curve, characteristic of strong drug synergism (data not shown).

Electron micrographs were taken to determine if any structural changes took place in *C. albicans* after treatment with berberine alone and in combination with fluconazole (Fig. 2). The control cells of *C. albicans* presented a smooth surface and sub-spherical or oval shape, with a great capacity for producing blastospores (Fig. 2a). After exposure to berberine alone, the general aspect of the cells was not modified; although, some of the yeast cells began to cluster (Fig 2b). Cells exposed to fluconazole alone were rounder and showed a tendency to agglutinate; lemon-shaped yeasts with small buds were still present, but we also observed a great capacity for producing pseudohyphae (Fig. 2c). Cells exposed to berberine in combination with fluconazole were collapsed and showed deep surface folds. Some of them also showed clear degradations and appeared surrounded by cellular debris (Fig. 2d).

Table 1 Agar disk testing: antifungal indices of Berberine tested alone and in combination with fluconazole (FLC) against clinical isolates of *Candida albicans*

Candida albicans strains	Antifungal index (mm/μg)[a]	
	Berberine alone	Berberine plus FLC
ATCC	0	1.0
21	0.0	1.3
52	0.1	2.1
55	0.1	2.0
56	0.1	2.5
62	0.0	1.3
111	0.2	1.7
174	0.0	2.0
197	0.0	0.8
250	0	2.0
299	0	0.8
306	0.2	1.0
367	0.0	1.3
372	0	0
385	0	1.0
393	0.0	1.2
396	0.0	1.3
405	0.1	1.9
410	0	1.2
427	0.1	0.7
443	0.1	0
454	0	0
465	0	1.5
504	0	1.5
505	0.0	1.5
Median	0.04	1.26
Range	0–0.2	0–2.1

[a] Calculated as the mean diameter of the corresponding growth inhibition zone (in mm)/the minimal drug amount resulting in growth inhibition (in μg). Similar results were obtained in the different analyses. Standard errors were less than 10% of means

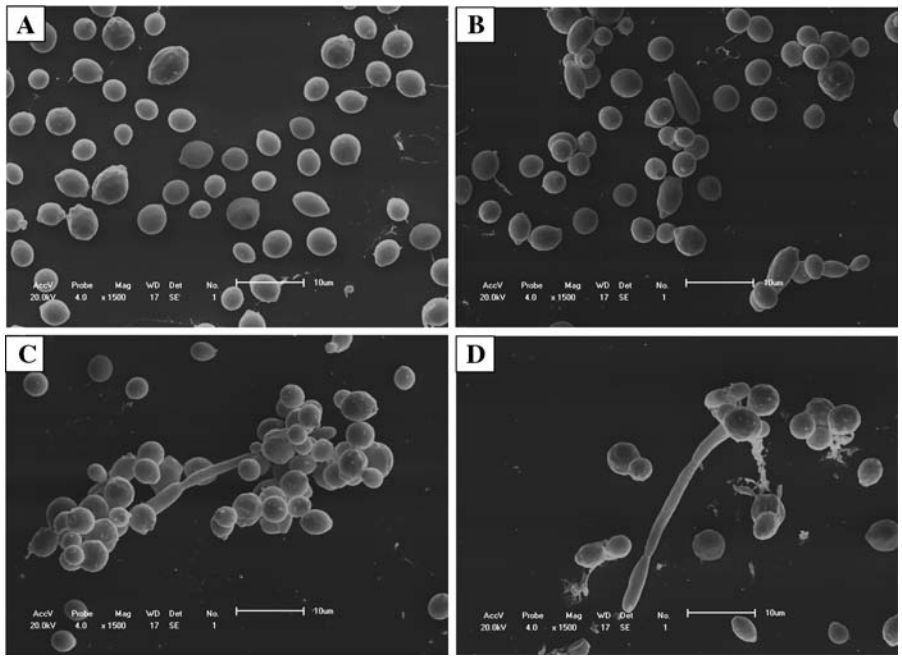

Fig. 2 Scanning electron microscopy photographs of *C. albicans* ATCC 10231 treated with berberine alone and in combination with fluconazole. **a** Control; **b** berberine 10 μg/ml; **c** fluconazole 6 μg/ml; and **d** berberine plus fluconazole. *Bar* = 10 μm

The results presented here indicate a strong synergistic interaction between berberine and fluconazole in inhibiting the growth of *C. albicans*. These drugs had no intrinsic antifungal activity at the concentration tested. By checkerboard microtiter testing it was observed that supra-MIC concentrations of fluconazole had only a weak fungistatic effect, and this finding correlated well with the measured residual turbidity, the so-called trailing. However, above a given minimal concentration, berberine eliminated completely the trailing effect when combined with supra-MIC of fluconazole. The term trailing has been used to describe the reduced but persistent growth that some isolates of *Candida* sp. exhibit over an extended range of drug concentrations. For some isolates, however, trailing growth is so significant that the MICs for these isolates will appear to be low after 24 h but much higher after 48 h. These MICs are so discordant that they place the isolate into different MIC interpretive categories at the two time points. The reduction of trailing observed in the present study was explained by a strain-dependent potent fungistatic or fungicidal effect resulting from the combination of berberine with fluconazole. Since this effect decreased the viable counts by >99.9% compared to those of the growth control, a different endpoint, the MIC-0, was chosen.

Prolonged administration of fluconazole in immunocompromised patients can result in the appearance of azole-resistant clinical isolates over expressing multi-drug efflux transporters including ABC transporters or major facilitator super family (MFS) transporters (White et al. 1998). Suppressing the activity of these fungal ABC transporters with small molecule multi-drug efflux pump inhibitors could reduce the drug resistance of these pathogenic fungi and therefore help to increase the efficacy of antifungal chemotherapy with triazoles. Berberine is a weak antimicrobial produced by a wide variety of plant species. It is an amphipathic cation that resembles quaternary ammonium antiseptics in its chemical properties and possibly in its mechanism of action as well. The likely targets of berberine are the cytoplasmic membrane and DNA, into which it intercalates (Jennings and Ridler 1983). Amphipathic cations are the preferred substrates of most multidrug resistance pumps (Lewis 2001) Several studies have shown that plants of the genus *Berberis* (*Berberis repens*, *B. aquifolium* and *B. fremontii*) producing berberine synthesise two substances, the flavonolignan 5′-MHC-D and the porphyrin pheophorbide *a*,

which have no antibacterial activity but have an inhibiting property against MDR efflux pumps found so far in *Staphylococcus aureus* (allowing berberine to carry out its activity) (Stermitz et al. 2000a, b, 2001). In the absence of efflux, berberine, a hydrophobic cation, accumulates in the cells of microbial pathogens, and the accumulation is driven by the membrane potential (Severina et al. 2001).

Since the berberine concentration effective in vitro is achievable in vivo, the combination of this agent with fluconazole represents an attractive perspective for the development of new management strategies for candidiasis.

Acknowledgments This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Capacitação e Aperfeiçoamento de Pessoal de Nível Superior, (Capes), Fundação Araucária, and Programa de Pós-graduação em Análises Clínicas da Universidade Estadual de Maringá. Part of the experiments were carried out at the Complexo de Central de Apoio a Pesquisa (COMCAP) MCT/FINEP/UEM.

References

Birdsall TC (1997) Berberine: therapeutic potential of an alkaloid found in several medicinal plants. Altern Med Rev 2(2):94–103

Clinical and Laboratory Standards Institute (CLSI) (2004) Method for antifungal disk diffusion susceptibility testing of yeasts: NCCLS document M44-A. National Committee for Clinical Laboratory Standards, Wayne, Pa, pp 1–36

Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, Herwaldt L, Pfaller M, Diekema D (2003) Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis 37:1172–1177

Jennings BR, Ridler PJ (1983) Interaction of chromosomal stains with DNA, an electrofluorescence study. Biophys Struct Mech 10:71–79

Ko W, Yao X, Lau C, Law W, Chen Z, Kwok W, Ho K, Huang Y (2000) Vasorelaxant and antiproliferative effects of berberine. Eur J Pharmacol 399:187–196

Kuo C, Chi C, Liu T (2004) The anti-inflammatory potential of berberine in vitro and in vivo. Cancer Lett 203:127–137

Lewis K (2001) In search of natural substrates and inhibitors of MDR pumps. J Mol Microbiol Biotechnol 3:247–254

Lipsitch M, Bergstrom CT, Levin BR (2000) The epidemiology of antibiotic resistance in hospitals: paradoxes and prescriptions. Proc Natl Acad Sci USA 97:1938–1943

Marchetti O, Moreillon P, Glauser MP, Bille J, Sanglard D (2000) Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. Antimicrob Agents Chemother 44:2373–2381

Marinova E, Nikolova D, Popova D, Gallacher G, Ivanovska N (2000) Suppression of experimental autoimmune tubulointerstitial nephritis in BALB/c mice by berberine. Immunopharmacology 48:9–16

Mitani N, Murakami K, Yamaura T, Ikeda T, Saiki I (2001) Inhibitory effect of berberine on the mediastinal lymph node metastasis produced by orthotopic implantation of Lewis lung carcinoma. Cancer Lett 165:35–42

Odds F (2003) Synergy, antagonism, and what the checkerboard puts between them. J Antim Chemother 52:1

Park KS, Kang KC, Kim JH, Adams DJ, Johng TN, Paik YK (1999) Differential inhibitory effects of protoberberines on sterol and chitin biosyntheses in *Candida albicans*. J Antimicrob Chemother 43:667–674

Perea S, Patterson TF (2002) Antifungal resistance in pathogenic fungi. Clin Infect Dis 35:1073–1080

Polak A (1990) Combination therapy in systemic mycosis. J Chemother 2:211–217

Severina II, Muntyan MS, Lewis K, Skulachev VP (2001) Transfer of cationic antibacterial agents beerberine, palmatine and benzalkonium through bimolecular planar phospholipid film and *Staphylococcus aureus* membrane. IUBMB-Life Sci 52:321–324

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, Lewis K (2000a) Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci 97:1433–1437

Stermitz FR, Tawara-Matsuda J, Lorenz P, Mueller P, Zenewicz L, Lewis K (2000b) 5′-Methoxyhydnocarpin-D and pheophorbide A: Berberis species components that potentiate berberine growth inhibition of resistant *Staphylococcus aureus*. J Nat Prod 63:1146–1149

Stermitz FR, Beeson TD, Mueller PJ, Hsiang JF, Lewis K (2001) *Staphylococcus aureus* MDR efflux pump inhibitors from a Berberis and a Mahonia (sensu strictu) species. Biochem Syst Ecol 29:793–798

Vollekova A, Kost'alova D, Kettmann V, Toth J (2003) Antifungal activity of Mahonia aquifolium extract and its major protoberberine alkaloids. Phytother Res 17:834–837

White TC, Marr KA, Bowden RA (1998) Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev 11(2):382–402

Zhao XZ, Guo X (1989) Antiarrhythmic effect and electrophysiologic study of berberine. Zhong Hua Xin Xue Guan Bing Za Zhi 17:159–161