SPANDIDOS PUBLICATIONS

BIOMEDICAL REPORTS 4: 449-452, 2016

Sensitization of *Candida albicans* to terbinafine by berberine and berberrubine

PIKLING LAM[1], STANTON HON LUNG KOK[2], KENNETH KA HO LEE[2], KIM HUNG LAM[1], DESMOND KWOK PO HAU[3], WAI YEUNG WONG[4], ZHAOXIANG BIAN[3], ROBERTO GAMBARI[5] and CHUNG HIN CHUI[1,3]

[1]State Key Laboratory of Chirosciences, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University; [2]School of Biomedical Sciences, Chinese University of Hong Kong; [3]Clinical Division, School of Chinese Medicine, [4]Department of Chemistry, Hong Kong Baptist University, Hong Kong, SAR, P.R. China; [5]Department of Life Sciences and Biotechnology, Section of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy

Received September 22, 2015; Accepted November 27, 2015

DOI: 10.3892/br.2016.608

Abstract. *Candida albicans* (*C. albicans*) is an opportunistic fungal pathogen, particularly observed in immunocompromised patients. *C. albicans* accounts for 50-70% of cases of invasive candidiasis in the majority of clinical settings. Terbinafine, an allylamine antifungal drug, has been used to treat fungal infections previously. It has fungistatic activity against *C. albicans*. Traditional Chinese medicines can be used as complementary medicines to conventional drugs to treat a variety of ailments and diseases. Berberine is a quaternary alkaloid isolated from the traditional Chinese herb, *Coptidis Rhizoma*, while berberrubine is isolated from the medicinal plant *Berberis vulgaris*, but is also readily derived from berberine by pyrolysis. The present study demonstrates the possible complementary use of berberine and berberrubine with terbinafine against *C. albicans*. The experimental findings assume that the potential application of these alkaloids together with reduced dosage of the standard drug would enhance the resulting antifungal potency.

Correspondence to: Professor Roberto Gambari, Department of Life Sciences and Biotechnology, Section of Biochemistry and Molecular Biology, University of Ferrara, Via Fossato di Mortara 74, I-44121 Ferrara, Italy
E-mail: gam@unife.it

Dr Chung Hin Chui, Clinical Division, School of Chinese Medicine, Hong Kong Baptist University, Jockey Club School of Chinese Medicine Building, 7 Baptist University Road, Hong Kong, SAR, P.R. China
E-mail: chchui@hkbu.edu.hk

Key words: antifungal, berberine, berberrubine, *C. albicans*, terbinafine

Introduction

The incidence of Candida infections has increased in patients who receive immunosuppressive therapy, cancer chemotherapy and transplantation. Among the *Candida* species, *Candida albicans* (*C. albicans*) is responsible for 50-70% of cases of invasive candidiasis in the majority of clinical settings (1,2). *C. albicans*, an opportunistic fungal pathogen that normally inhabits the mucous membranes of the gastrointestinal and female genital tracts (3), is able to attack the immunocompromised patients (4). When the normal microbial barrier is disrupted, *C. albicans* can invade the intestinal and female genital mucosal barriers and causes candidiasis and candidemia (5,6). A cross-sectional study has reported that *Candida* species isolation from the vaginal mucosa was more frequent in human immunodeficiency virus (HIV)-infected patients (29.7%) when compared with uninfected women (14.5%). *C. albicans* was the most prevalent pathogen isolated in HIV-infected (52.9%) and uninfected women (85.7%) (7). A previous study investigated 128 *Candida* isolates from South Africa and 126 Cameroonian *Candida* isolates. Of those, *C. albicans* was responsible for the highest percentage of them, with 82.8% of South African isolates and 73.0% of the Cameroonian isolates (8). In another study, of the 103 *Candida* species-infected oral mucosal isolates identified in HIV patients, *C. albicans* accounted for the majority of *Candida* species (77.7%) (9). A prospective observational study also examined the occurrence of oral Candida colonization among cancer patients in China between October 2012 and March 2013. *C. albicans* was the most common species isolated from patients, accounting for 30.8% in patients with pulmonary cancer (n=78), 33.7% in patients with digestive tract malignant tumor (n=101), and 12.7% in patients with hematopoietic system tumor (n=79). Cancer patients are a high-risk population for Candida colonization (10).

Traditional Chinese medicines (TCMs) remain a fundamental role in the treatment of various diseases due to their long history of clinical practice and reliable therapeutic efficacy. Berberine (Fig. 1A) is an isoquinoline quaternary alkaloid

isolated from the traditional Chinese herb, *Coptidis Rhizoma* (also known as Huanglian), which includes several species: *Coptis chinensis* French, *Coptis deltoidea* and *Coptis teetoides* (11,12). Berberrubine (Fig. 1B) is isolated from the medicinal plant *Berberis vulgaris* (13), but is also readily derived from berberine by pyrolysis (14). Kim *et al* (15) studied the antimicrobial activity of berberine, berberrubine, 9-*O*-acylberberrubines and 9-*O*-alkylberberrubines. Berberrubine exhibited a relatively weaker anti-*C. albicans* activity [minimum inhibitory concentration (MIC) >128 μg/ml] than berberine (MIC = 128 μg/ml), while some of the acyl derivatives (decanoyl and lauroyl berberrubine chlorides) and the alkyl derivatives (heptyl, octyl, nonyl, decyl and undecyl) showed much stronger growth inhibition against *C. albicans*, with the MICs ranging from 1-4 μg/ml. Park *et al* (16) reported the antifungal activity of 13-(substituted benzyl) berberine and berberrubine derivatives. Among them, 13-(4-tertbutylbenzyl) and 13-(4-isopropyl benzyl) berberine derivatives exhibited the strongest antifungal activity against *C. albicans* (MICs = 4 μg/ml) when compared with berberine (MIC = 128 μg/ml). The 13-(4-tertbutylbenzyl) and 13-(4-isopropyl benzyl) berberrubines showed a better anti-*C. albicans* activity (MICs = 16 μg/ml) when compared with berberrubine (MIC >128 μg/ml).

TCMs can be used as complementary medicines to conventional drugs for a variety of disease treatments. Our previous study reported the complementary use of corilagin, a gallotannin identified in numerous plants, including *Phyllanthus urinaria*, with two chemotherapeutic drugs, cisplatin and doxorubicin, in order to lower the working concentration of these two agents and to obtain an increment in the anticancer effect. The IC_{50} values were ~3-fold reduced for cisplatin and ~4-fold decreased for doxorubicin with the Hep3B hepatoma cells in the presence of corilagin (17). The efficacy of standard antibiotics against bacterial strains was improved by the use of plant materials (18-21). TCMs as a complementary therapy with the standard antifungal drugs against fungal pathogens have emerged as a new choice for the treatment of infectious diseases. The present study utilizes the TCMs, berberine and berberrubine, as complementary agents with the standard antifungal drug, terbinafine, against the most common *Candida* species, *C. albicans*, responsible for the increased incidence of fungal infections.

Materials and methods

Chemical analysis. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker/Varian 500 MHz Fourier transform spectrometer (Agilent Technologies, Santa Clara, CA, USA). ^{1}H and ^{13}C-NMR spectra were recorded relative to residual protiated solvent; a positive value of the chemical shift denoted a resonance downfield from tetramethylsilane (TMS, internal standard). *J*-values were in Hz. All the chemicals were purchased from commercial suppliers and used without further purification. All the reactions were monitored by analytical thin-layer chromatography (TLC) on Merck aluminum-precoated plates of silica gel 60 F254 with detection by spraying with 5% (w/v) dodecamolybdophosphoric acid in ethanol or 5% (w/v) ninhydrin in ethanol and subsequent heating.

Synthesis of natural berberrubine. Natural berberrubine was synthesized according to the reported procedure (22). Berberine

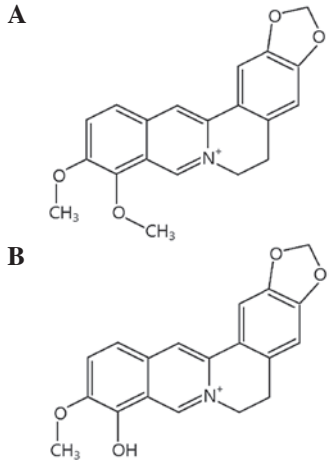


Figure 1. Chemical structure of (A) berberine and (B) berberrubine.

chloride (2.01 g, 5.4 mmol) was heated at 190˚C under reduced pressure for 2 h and the crude product was recrystallized from chloroform and hexane to obtain the title berberrubine in 83% yield as a dark red precipitate: R*f*=0.29 (dichloromethane-methanol, 15:1); ^{1}H-NMR ($CDCl_3$) d9.20 (s, 1H), 7.58 (s, 1H), 7.30-7.24 (m, 2H), 6.75 (s, 1H), 6.51-6.49 (m, 1H), 6.06 (s, 2H), 4.42 (s, 2H), 3.89 (s, 3H), 3.09 (s, 2H); ^{13}C-NMR ($CDCl_3$) d167.7, 150.3, 149.1, 148.2, 145.9, 133.1, 131.5, 128.3, 122.2, 120.7, 120.2, 117.7, 108.4, 104.6, 103.3, 101.9, 56.2, 53.4, 28.6.

Determination of MIC and minimum fungicidal concentration (MFC). C. albicans was obtained from American Type of Culture Collection (Manassas, VA, USA). The MIC values of berberine, berberrubine (kindly provided by Professor K.K.H. Lee) and terbinafine (both from Sigma-Aldrich, St. Louis, MO, USA) on *C. albicans* were determined by the broth dilution method. Briefly, different concentrations of berberine, berberrubine and terbinafine were loaded from a starting concentration of 100 μg/ml containing 1% dimethylsulfoxide (DMSO) as the vehicle and they were diluted serially. DMSO (1%) was used as a vehicle control. The fungal samples were subsequently incubated at 35˚C for 48 h. The minimum concentrations of berberine, berberrubine and terbinafine, which induced a complete growth inhibition would be recorded as their MIC values (16,23). For the determination of MFC, 10 μl of the 48 h incubated medium was removed and plated. MFC was recorded at a concentration where no colony of fungal growth was observed. When MFC/MIC <4, the compound or combination would be considered as fungicidal, while when MFC/MIC ≥4 the compound or combination would be considered as fungistatic. In each case, three independent experiments were conducted and each experiment was carried out in triplicates.

Sensitization test. For the sensitization investigation experiment, the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay was employed (24). Briefly, *C. albican* cells were seeded at day 0 in the 96-well microplate. Terbinafine was added at 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.2 μg/ml, respectively, while berberine and berberrubine were added at 100 μg/ml together with terbinafine. After 48 h of incubation, MTS (Promega, Madison, WI, USA)/phenazine methosulfate (PMS) as

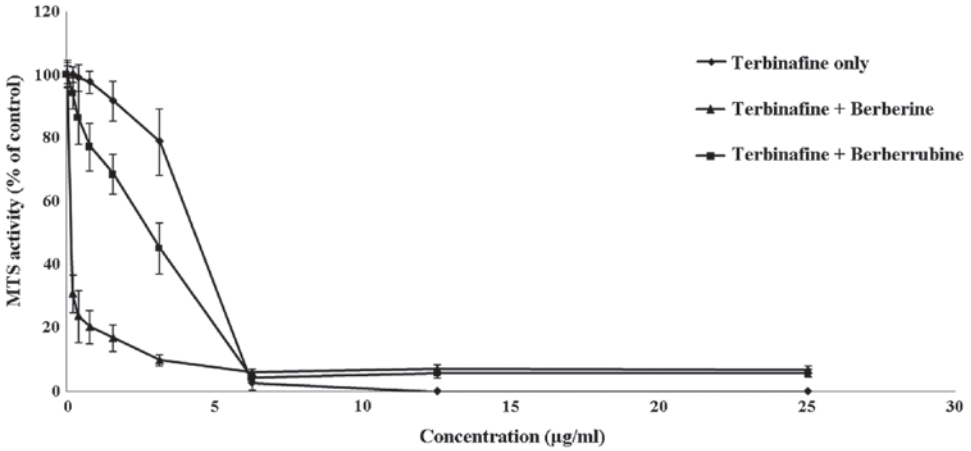


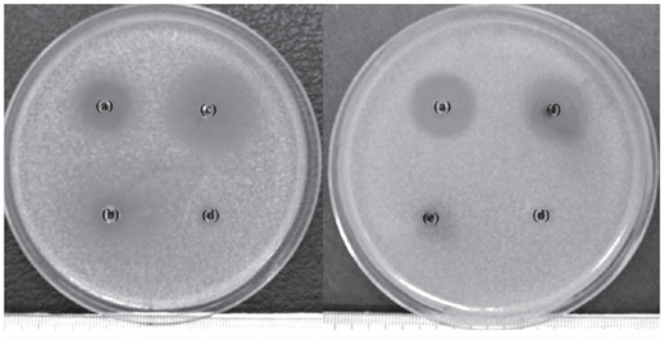
Figure 2. Sensitization of *Candida albicans* to terbinafine by berberine and berberrubine. Berberine and berberrubine could assist the antifungal action of the standard antifungal drug, terbinafine. The complementary activity of berebrine with terbinafine was much stronger than that of berberrubine. In each case, three independent experiments were conducted and a mean value was obtained. Each experiment was carried out in triplicates. Results are shown as mean ± standard deviation from three independent experiments.

electron coupling agent mixed solution was added. Lastly, optical absorbance was determined at 490 nm using a micro-plate reader (Perkin Elmer Victor V) according to the manufacturer's protocol. To determine MIC and MFC values from the sensitization test, no MTS/PMS was added and the experimental procedures were conducted as mentioned before. In each case, three independent experiments were conducted and each experiment was carried out in triplicates.

Zone of inhibition study. C. albicans was used to study the effectiveness of berberine alone (100 μg), berberine (100 μg) plus terbinafine (6 μg), berberrubine alone (100 μg), and berberrubine (100 μg) plus terbinafine (6 μg) against its growth in culture. *C. albicans* was diluted with yeast mold broth and plated on the yeast mold agar plate, and the holes were created on the agar using a sterile transfer pipette. For the tested samples, 1% DMSO (as negative control) and 6 μg terbinafine (as positive control) were placed in the holes of agar. The plates were subsequently incubated at 35˚C for 48 h and the inhibition zones (mm, in terms of diameter) of fungi on the agar plates were recorded (23,25,26).

Results and Discussion

Determination of MIC, MFC and sensitization assay. Terbinafine is an allylamine agent with a broad spectrum of antifungal activity. It interferes with the biosynthesis of ergosterol, an essential component of fungal cell membranes, via inhibition of the fungal enzyme squalene epoxidase. In the *in vitro* susceptibility tests, terbinafine have been shown to possess primarily fungicidal activity against dermatophytes, moulds and certain dimorphic fungi, but only fungistatic activity against *C. albicans* (27). The cell death mechanisms of berberine against *C. albicans* have been fully addressed in the previous studies. They involved the ability to impair mitochondrial function, generation of reactive oxygen species, targeting cell wall integrity pathway and also affecting heat shock transcription factor, HSF1 (28). The MIC and MFC values of terbinafine against *C. albicans* were determined as



Inhibition zone (mm, in terms of diameter, mean ± SD)

(a) Terbinafine	(b) Berberine	(c) Berberine+terbinafine	(d) DMSO	(e) Berberrubine	(f) Berberrubine+terbinafine
14.67 ± 3.06	6.83 ± 1.26	25.67 ± 2.52	Nil	Nil	15.83 ± 2.25

Figure 3. Inhibition zone (mm, in terms of mean diameter ± standard deviation).

6 and 24 μg/ml, respectively. As MFC/MIC was equal to 4, it was considered to be fungistatic. For berberine and berberrubine, their MIC values were >100 μg/ml. No MFC value was determined. These findings were consistent with the results reported previously (16). However, berberine and berberrubine at 100 μg/ml could not improve the MFC, and the MFC of terbinafine could significantly potentiate the antifungal activity of terbinafine on *C. albicans* as determined by the MTS/PMS assay. Berberine and berberrubine at 100 μg/ml could effectively assist the antifungal potential of terbinafine (Fig. 2). Notably, the complementary activity of berebrine with terbinafine was much stronger than that of berberrubine when terbinafine was loaded from 3.13, 1.56, 0.78, 0.39 and 0.2 μg/ml. Therefore, berberine was more effective in amplifying the antifungal action of terbinafine when compared with its analogue, berberrubine.

Zone of inhibition study. Previous studies have reported that berberine in combination with fluconazole or miconazole showed a synergistic antifungal activity against *C. albicans* with larger inhibition zones in the agar diffusion tests (29-31). In order to evaluate the sensitivity of *C. albicans* to the combination of terbinafine and berberine or berberrubine, agar diffusion assays were conducted to determine their inhibition zones on the agar

plates. Terbinafine alone at 6 μg possessed certain fungistatic effect against *C. albicans* (inhibition zone, 14.67±3.06 mm), whereas berberine or berberrubine alone showed small or no level of growth inhibition of the fungi (Fig. 3). The combination of 100 μg berberine and 6 μg terbinafine showed an increased inhibition zones in size (inhibition zone, 25.67±2.52 mm) when compared with terbinafine alone. The result may suggest that a synergistic fungistatic activity against *C. albicans* may occur between the combined drugs even if the MIC and MFC of this combination were not improved. However, the combination of 100 μg berberrubine and 6 μg terbinafine did not enhance the fungal sensitivity, with no significant enlargement in size of inhibition zone. No growth inhibition of *C. albicans* could be found in 1% DMSO (vehicle control).

In conclusion, the present study reports the complementary application of berberine and berberrubine with terbinafine against the opportunistic fungal pathogen, *C. albicans*. A recent study has demonstrated that berebrine can assist fluconazole to kill fluconazole-resistant *C. albicans* (32). The present results further indicate the possible sensitization of various pathogenic fungi to other standard drugs by berberine and berberrubine, with the purpose of obtaining an increment in the antifungal potency.

Acknowledgements

The present study was supported by the Innovation Technology Commission to ABCT and HKPU [with the grant codes FRG/14-15/021, 30-14-121 and 38-40-116 (Dr C.H.C.)], as well as grant no. 03-16-176 (Professor Z.X.B.). Professor R.G. received a grant from AIRC (contract no. IG 13575).

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