



Downregulation of fungal cytochrome *c* peroxidase expression by antifungal quinonemethide triterpenoids

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Abstract To handle the development of antifungal drug resistance, the development of new structural modules and new modes of action for antifungals have been highlighted recently. Here, the antifungal activity of quinonemethide triterpenoids such as celastrol, dihydrocelastrol, iguestein, pristimerin, and tingenone isolated from *Tripterygium regelii* were identified (MIC 0.269–19.02 μ M). *C. glabrata* was the most susceptible to quinonemethide among the tested fungi. Furthermore, quinonemethide suppressed cytochrome *c* peroxidase expression dramatically, decreasing fungal viability caused by the accumulation of hydrogen peroxide. Thus, cytochrome *c* peroxidase downregulation of quinonemethide may be a key mode of action for antifungals.

Keywords Anti-fungal · Antioxidant · Cytochrome *c* peroxidase · Quinonemethide triterpenoid

Introduction

Tripterygium regelii is wide spread in North-east Asia and is used as an herbal medicine to treat swelling, chills and inflammation (Brinker et al. 2007). Quinonemethide triterpenoids isolated from *T. regelii* such as celastrol, iguestein, pristimerin, and tingenone

are reported to possess several biological activities such as anticancer, anti-inflammatory, antimalarial, antioxidant, antiviral, and insecticidal activities (Luo et al. 2005; Brinker et al. 2007; Gao et al. 2007; Su et al. 2009; Ryu et al. 2010) as well as antifungal activity (Dos Santos et al. 2010). Furthermore, these triterpenoids induced an increase in intracellular reactive oxygen species (ROS) and mitochondrial dysfunction (Byun et al. 2009), and the quinonemethide moiety with electrochemical potential is regarded as a key component of the triterpenoid (Ryu et al. 2010). Despite the many biological activities of quinonemethide triterpenoid, the structure-based antifungal activity with quinonemethide triterpenoid derivatives and the antifungal mechanism has not been well studied.

Over 20 species of *Candida* cause candidiasis in humans such as thrush, vaginal yeast infection, and invasive candidiasis. Another pathogenic yeast, *Cryptococcus neoformans*, is an ubiquitous human pathogen and causes life-threatening meningoencephalitis (Heitman and Lin 2006). Approximately one million cases of cryptococcosis in immunocompromised patients are reported globally each year, with over 60 % mortality. To treat the fungal disease, the therapeutics used currently are amphotericin B, flucytosine, and fluconazole, which were developed from antifungal screening on natural extracts and structure-based semisynthesis from a key module (Estevinho et al. 2011; Pasqua et al. 2011; Kim et al. 2014). Even though therapeutic drugs were successfully developed, new antifungal development research is ongoing due to adverse effects and the increasing resistance of strains to the therapeutics. Thus, development studies for new structure modules or mode of action (MOA) candidates is highlighted currently. Herein, we studied the antifungal activity of the quinonemethide from *Celastraceae* against several pathogenic fungi such as *Candida albicans* (SC5314), *Candida tropicalis* (KCTC7212), *Candida parapsilosis* (KCTC7214), *Candida glabrata* (KCTC7219), and *Cryptococcus neoformans* (H99) as well as the antifungal mechanism based on reactive oxygen species (ROS) dependent gene expression on *C. neoformans*.

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Materials and Methods

Preparation of quinonemethide

Four quinonemethide triterpenoids (celestrol **1**, iguestein **3**, pristimerin **4** and tingenone **5**) were isolated from *Tripterygium regelii* and dihydrocelestrol **2** was prepared from isolated celestrol by the reported method (Seo et al. 2011). Briefly, the stem bark of *Tripterygium regelii* was collected at Jiri Mountain (Korea). The stem root (5 kg) of *T. regelii* was air-dried, chopped and extracted three times with 95 % MeOH (3×10 L) for 7 days at room temperature. The combined extract was concentrated, and the dark residue (453 g) was partitioned between water and chloroform (1 L:1 L). The organic layer was concentrated to give a dark brown residue (310 g) and the CHCl₃ extract was subjected to column chromatography using silica gel with hexane-acetone gradient and hexane-EtOAc gradient. Fifteen pooled fractions (Fr.1-Fr.30) were obtained after combining fractions with similar TLC profiles from this initial column chromatography. The column was eluted with solvents of increasing polarity (CHCl₃-acetone) to give 30 fractions. Fraction 5 (14.3 g) was chromatographed on a silica gel column with hexane-acetone to give compounds **1** (celestrol, 280 mg) [*R_f* 0.53 (hexane/acetone=4/1)], and compound **3** (iguesterin, 100 mg) [*R_f* 0.51 (hexane/EtOAc=2/1)], respectively. Fraction 10 (21.6 g) was chromatographed on a silica gel column and eluted with hexane-EtOAc to give compound **4** (pristimerine, 120 mg) [*R_f* 0.27 (hexane/Acetone=3/1)], and compound **5** (tingenone, 78 mg) [*R_f* 0.27 (hexane/Acetone=2/1)]. Dihydrocelestrol **2** was prepared through the hydrogenation of celestrol with 10% Pd/C in ethanol. The detailed preparation information and identification data are available in supporting information.

Measuring of minimal inhibitory concentration

To determine the MIC of the compounds, the microdilution method was used according to the recommendations of the National Committee for Clinical Laboratory Standards (reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard NCCLS Document M27-A2). Briefly, cells were grown in YPD medium at 30 °C overnight and were diluted with RPMI-1640 medium. The cell suspension containing 2.5×10³ cells was added to the wells of a flat-bottom 96 well plate. Two-fold serial dilutions of compounds were added to the wells, and the plates were incubated at 34 °C for 72 h. The MIC was defined as the lowest concentration that did not allow the detection of any fungal growth and was determined visually by comparing with the growth in the drug-free well (Kim et al. 2014).

Real-time PCR analysis

The overnight cultures of the *C. neoformans* (H99) were diluted to 1×10⁸ cells in 5 mL of RPMI-1640 medium (pH7.2) containing 5 µg/mL of quinonemethide and incubated at 30 °C for 6 h. Total RNA was extracted using QIAzol lysis agent (QIAGEN, German),

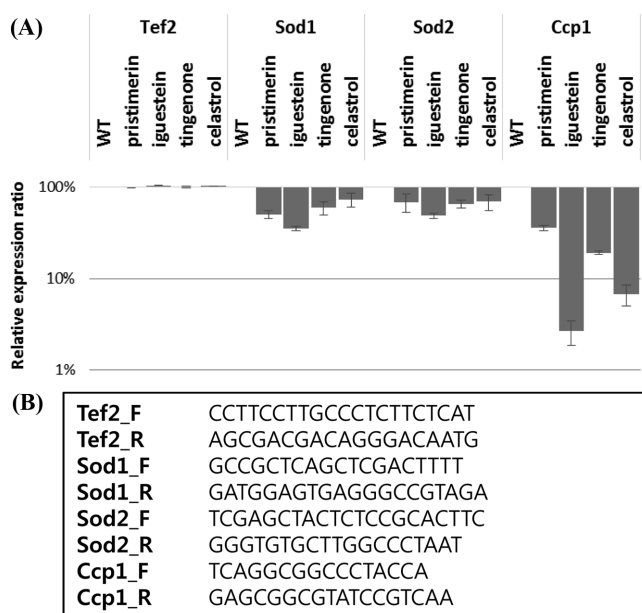


Fig. 1 ROS dependent gene (*tef2*, *sod1*, *sod2* and *ccp1*) expression ratio after treatment of natural quinonemethide (A) and list of primers used for quantitative RT-PCR analysis (B)

and cDNA was synthesized with the RevertAid™ First Strand cDNA synthesis Kit (Thermo Fisher Scientific, Canada) following the manufacturer's instructions. The relative gene expression experiments were performed by quantitative real-time PCR using a 7500 system of Applied Biosystems (Carlsbad, CA, USA) based on the 2^{-ΔΔC_T} method. The primers for real-time PCR were designed using Primer Express software 3.0 (Applied Biosystems) (Fig. 1). The transcript levels of superoxide dismutase 1 (*sod1*), superoxide dismutase 2 (*sod2*), and cytochrome *c* peroxidase (*ccp1*) were monitored, and translation elongation factor 2 (*tef2*) was used as the internal control.

Construction of strain

The sequence of the *ccp1* (CNAG_01138) gene was obtained from the *C. neoformans* var. *grubii* serotype A genome database (http://www.broadinstitute.org/annotation/genome/CRYPTOCOCCUS_neoformans). The strain expressing the Ccp1-FLAG fusion protein was constructed using the primers listed in supporting information. To construct the strain, the *ccp1* gene was amplified with primers Ccp1(FLAG)_F and Ccp1(FLAG)_R using wild-type genomic DNA as the template. The amplified 2.6 kb DNA fragment was digested by *Hind* III and *Bam*HI and then was ligated with the plasmid pWH181 containing the 3×FLAG epitope tag (DYKDDDDK), the GAL7 terminator and the nourseothricin resistance (NAT) gene. The ligated plasmid, named pWH204, was linearized by *Sal* I and introduced into the wild-type *C. neoformans* by biolistic transformation (Yu et al. 2004). Positive transformants including the recombinant gene at the *ccp1* locus were confirmed by PCR using Ccp1(FLAG)_F and NATterm2F

and western blot analysis.

Western blot analysis

The strains expressing Ccp1-FLAG fusion protein were cultured in yeast extract-Bacto peptone medium containing 2.0 % glucose (YPD) in 30 °C for 16 h, and transferred into RPMI-1640 medium (pH 7.2) containing 5 µg/mL quinone-methide. The cells were incubated at 30 °C for 16 h prior to harvesting, and the pellets were resuspended in protein extraction buffer including 1 % Triton X-100, 50 mM HEPES KOH at pH 7.5, 1 mM EDTA, 140 mM NaCl, 0.1 % Na-deoxycholate, 1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich Co. MO, USA) (Do et al. 2015). The cells were disrupted with acid-washed glass-beads (0.4 mm; Sigma-Aldrich Co. MO, USA) using a bead-beater (BioSpec, Bartlesville, OK, USA). Total protein concentration was determined with the Bradford assay (Bradford 1976). Western blot analysis was performed using an anti-DDDDK polyclonal rabbit antibody (Abcam, UK) as the primary antibody and a goat anti-rabbit IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, TX, USA) as the secondary antibody, followed by visualization using chemiluminescence (Do et al. 2015). The band intensities were measured with ImageJ 1.50i (Windows version of NIH Image, <http://imagej.nih.gov/>) (Gassmann et al. 2009).

Results and Discussion

First, all the triterpenoids except dihydrocelastrol showed strong inhibitory potentials at sub-micromolar concentrations (MIC 0.269–0.555 µM) on *C. glabrata* and at micromolar concentrations (MIC 0.552–8.877 µM) on *C. albicans*, *C. tropicalis*, and *C. neoformans* (Table 1). Their inhibition activities were comparable to fluconazole (MIC 0.816–6.532 µM) as the positive control, and *C. glabrata* was more susceptible than other candida fungi to the triterpene. However, no clue to the key structure module for the biological activity could be found from the constitutional derivation of the triterpene.

To understand the antifungal activity of the quinonemethide, the ROS dependent gene expressions in *C. neoformans* were measured owing to their known antioxidative activity (Allison et al. 2001, Wang et al. 2014). Because terpenoids are known to induce an increase in intracellular ROS, we selected genes encoding anti-oxidant proteins, *sod1* and *sod2*, for the superoxide dismutases that convert superoxide radical to hydrogen peroxide, and *ccp1*,

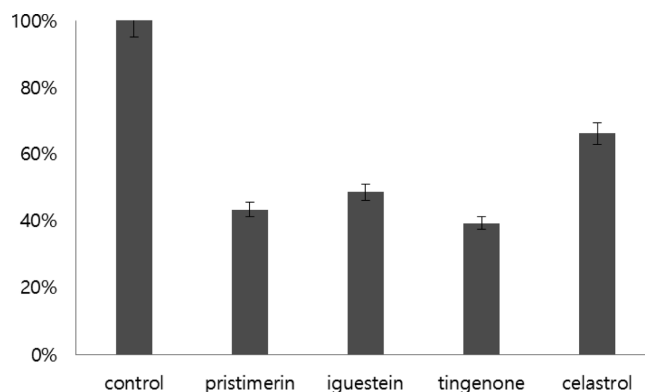


Fig. 2 Relative quantification of cytochrome *c* peroxidase expression by Western blot

for the cytochrome *c* peroxidase that detoxifies hydrogen peroxide (Cox et al. 2003; Narasipura et al. 2003; Giles et al. 2005; Narasipura et al. 2005). In the gene expression experiment, the tendency of the anti-oxidant gene expression decreased when the cells were exposed to quinonemethide. Especially, the *ccp1* expression significantly decreased 3–38 folds, indicating that the antifungal quinonemethide triterpenoids inhibits protection against oxidative stress in *C. neoformans* (Fig. 1). Considering the role of *ccp1*, exerting resistance against oxidative stress and functioning as a sensor of antioxidants, the strong inhibition of *ccp1* results in the inhibition of growth by the quinonemethide (Giles et al. 2005; Martins et al. 2013). We also constructed a strain expressing the Ccp1-FLAG fusion protein and evaluated the protein levels in the cells treated with each compound (1, 3, 4, 5). The results in Fig. 2 showed that treatment of the compound reduced the protein levels by over 40 % and were in agreement with our quantitative real-time PCR data, although no exact correlation was observed between transcript and protein levels.

In summary, the quinonemethide triterpenoids celastrol, dihydrocelastrol, iguestein, pristimerin, and tingenone showed strong antifungal activity against human pathogenic fungi, and *C. glabrata* was more susceptible to the quinonemethide than the other tested fungi. After treatment with quinonemethide, cytochrome *c* peroxidase expression was significantly reduced, resulting in the accumulation of hydrogen peroxide in the fungi. Thus, this cytochrome *c* peroxidase downregulation is an important MOA for anti-fungal activity and could be used for screening among antioxidants to find valid anti-fungal agents.

Table 1 Minimal inhibitory concentration (mM) of the quinonemethide triterpenoids on pathogenic fungi

	Fluconazole	Celastrol	Dihydrocelastrol	Iguestein	Pristimerin	Tingenone
<i>C. albicans</i>	0.816	4.439	4.419	1.236	1.076	4.756
<i>C. tropicalis</i>	>418	8.877	17.67	2.472	4.305	2.378
<i>C. parapsilosis</i>	3.266	17.754	17.67	4.944	17.21	19.02
<i>C. glabrata</i>	1.633	0.555	0.276	0.309	0.269	0.297
<i>C. neoformans</i>	6.532	4.439	0.552	1.236	1.076	1.189

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