Biochimica et Biophysica Acta 1818 (2012) 1648–1655

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Antifungal property of dihydrodehydrodiconiferyl alcohol 9′-O-β-D-glucoside and its pore-forming action in plasma membrane of *Candida albicans*

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ARTICLE INFO

Article history:
Received 5 November 2011
Received in revised form 10 February 2012
Accepted 22 February 2012
Available online 1 March 2012

Keywords:
DDDC9G
Antifungal effect
Plasma membrane
Antifungal mechanism
Antifungal compound

ABSTRACT

The aims of this study were to investigate the antifungal activity as a bioactive property of dihydrodehydrodiconiferyl alcohol 9′-O-β-D-glucoside (DDDC9G) and the mode of action(s) involved in its effect. Antifungal susceptibility testing showed that DDDC9G possessed potent antifungal activities toward various fungal strains with almost no hemolytic effect. To understand the antifungal mechanism(s) of DDDC9G, we conducted the following experiments in this study using *Candida albicans*. Fluorescence experiments using the probes, 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and propidium iodide suggested that DDDC9G perturbed the fungal plasma membrane. Consecutively, the analysis of the transmembrane electrical potential ($\Delta\Psi$) with 3, 3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] and bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] indicated that DDDC9G induced membrane-depolarization. Furthermore, model membrane studies were performed with rhodamine-labeled giant unilamellar vesicles (GUVs), calcein encapsulating large unilamellar vesicles (LUVs), and FITC-dextran (FD) loaded LUVs. These results demonstrated that the antifungal effects of DDDC9G upon the fungal plasma membrane were through the formation of pores with the radii between 0.74 nm and 1.4 nm. Finally, in three dimensional (3D) flow cytometric contour plots, a reduced cell size was observed as a result of osmolarity changes from DDDC9G-induced structural and functional membrane damages. Therefore, the present study suggests that DDDC9G exerts its antifungal effect by damaging the membrane through pore formation in the fungal plasma membrane.

1. Introduction

Over the past few decades, the prevalence of fungal infections has been progressively increasing, for instance, systemic infections occurring in patients who received solid organ, and allogenic bone marrow transplantation, and cancer chemotherapy [1]. The morbidity and mortality resulting from these invasive fungal infections caused by opportunistic yeasts and filamentous fungi remain at unacceptably high levels [2]. Hence, fungal infections are a challenging clinical problem and intensive efforts have been made to develop clinically useful antifungal agents. However, fungal infections are difficult to treat since the majority of antifungal drugs are also toxic to human cells, which have similar mechanisms for DNA, RNA, and protein synthesis [3], and resistant organisms have emerged due to the limited number of available agents used to cure these fungal infections [4].

Plants, unlike mammals that possess a circulating immune system such as immunoglobulin molecules and phagocytes, lack a somatic adaptive immunity [5]. Instead, they rely on innate immunity as self-defense mechanisms including structural barriers that prevent pathogens from gaining entrance and spreading throughout the plant as well as chemical compounds that inhibit the growth of pathogens. [6]. Inhibitory compounds in plants contain various antimicrobial peptides, proteins, and nonproteinaceous secondary metabolites [7]. Among them, phytochemicals as nonproteinaceous secondary metabolites are non-nutritive bioactive compounds that act as protective agents against external stress and pathogenic stress [8]. The phytochemicals, including saponin, helihumulone, 3,5,7-trihydroxyflavone, anolignan B, and so on, have been known to have antimicrobial effect [9]. In addition, natural phytochemicals with bioactive properties have less human toxicity [10] and are more cost effective [11]. These properties have been applied in the search for new, safer, and more efficacious medicinal plants as a source of bioactive compounds to combat serious fungal infections.

Dihydrodehydrodiconiferyl alcohol 9′-O-β-D-glucoside (DDDC9G) is a neolignan glucoside, a secondary metabolite discovered in *Trachelospermum asiaticum*, *Styrax japonica* S. *et* Z, and so on [12,13]. Several

Abbreviations: DDDC9G, dihydrodehydrodiconiferyl alcohol 9′-O-β-D-glucoside; MeOH, methanol; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; DPH, 1,6-diphenyl-1,3,5-hexatriene; $diSC_3(5)$, 3,3′-dipropylthiacarbocyanine; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; GUV, giant unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; ITO, indium tin oxide; LUVs, large unilamellar vesicles; FITC, fluorescein isothiocyanate; FD, FITC-dextran; FS, forward scatter; SS, side scatter

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doi:10.1016/j.bbamem.2012.02.026

studies have been done to investigate the biological effects of DDDC9G on the matrix metalloproteinase-1 inhibitory activity [13], human immunodeficiency virus (HIV)–host cell fusion inhibitory activity [14], and estrogenic activity [15]; however no useful effects were demonstrated. The structure of DDDC9G consists of two fragments, the coniferyl alcohols as the aglycone moiety and a glucoside as the glycoside moiety, which are linked by a rotatable β-glycosidic bond [12]. The coniferyl alcohol, a component of DDDC9G, is associated with the defense mechanisms of trees and acts as an inhibitor of fungal growth [16]. Some cases of fungal inhibition by various glycosides and aglycones have been reported [17]. This structure shows potential for clinical applications and also, influences the pharmacokinetic properties [18].

In this study, we investigated the antifungal effect of DDDC9G and determined its mode of action.

2. Materials and methods

2.1. Extraction and isolation of DDDC9G from S. japonica

The stem bark of *S. japonica* was collected from Jogyesan, Suncheon, Chonnam, Korea. A voucher specimen was deposited at the Herbarium of the College of Pharmacy, Chosun University, Korea (CSU-964-17). The air-dried stem bark of *S. japonica* (654 g) was cut and extracted with methanol (MeOH) at 80 °C for 4 h. The MeOH extract (120.32 g) was suspended in water and then partitioned sequentially with equal volumes of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). The EtOAc fraction (4 g) was subjected to column chromatography over a silica gel by eluting it with an EtOAc:*i*-propanol:H_2O (20:1:0.5). The groups were then divided into 7 separate groups based on their TLC profiles. The subgroup, E5 (830.0 mg) was then purified by column chromatography on a silica gel eluting it with a $CHCl_3$:MeOH:Me_2CO:H_2O (50:4:2:0.3) and was followed by a gel filtration column chromatography (Sephadex LH-20, MeOH:H_2O = 1:1) to yield DDDC9G (40 mg). The physico-chemical data including the ^1H NMR, ^{13}C NMR, and HSQC spectra of DDDC9G were identical with those reported in the literature [13].

2.2. Fungal strains and antifungal activity assay

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Trichosporon beigelii* (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Daejeon, Korea).

The fungal strains were cultured in YPD broth (Difco) with aeration at 28 °C, and the *M. furfur* was cultured at 32 °C in a modified YM broth (Difco) containing 1% olive oil. The cell suspensions were adjusted to obtain standardized populations by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, U.S.A.). Fungal cells at the log phase (2×10^6/ml) were inoculated into 0.1 ml/wells of YPD or YM broth, and then, dispensed to microtiter plates. Minimum inhibitory concentrations (MICs) were determined with a two-fold serial dilution of the test compounds, based on the Clinical and Laboratory Standards Institute (CLSI) method and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [19]. After 48 h of incubation, the minimal concentration of compounds required to prevent the growth of the microorganisms was determined, and defined as the MIC. The growth was measured with a microtiter ELISA Reader (Molecular Devices Emax, CA, USA) by monitoring the absorption at 580 nm. The MIC values were determined by three independent assays [20].

2.3. Hemolytic activity assay

The hemolytic activity of the compounds was evaluated by determining the release of hemoglobin from a 4% suspension of human erythrocytes at 414 nm with an ELISA reader. Hemolytic levels of zero and 100% were determined in a phosphate buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4) alone and with 0.1% Triton X-100, respectively. The hemolysis percentage was calculated with the following equation: hemolysis (%) = [(Abs_{414nm} in the compound solution − Abs_{414nm} in PBS)/(Abs_{414nm} in 0.1% Triton X-100 − Abs_{414nm} in PBS)] × 100 [21].

2.4. Membrane fluorescence intensity measurements

The fluorescence intensity from the plasma membrane of *C. albicans* cells labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular probes, Eugene, Oregon, U.S.A.) was used to monitor changes in the membrane dynamics. Fungal cells (2×10^6/ml YPD) treated with the compounds were incubated for 2 h at 28 °C. Control cells were incubated without the compounds. Samples of the fungal cultures were fixed with 0.37% formaldehyde for 30 min and washed with PBS. The pellets were frozen with liquid nitrogen. For labeling, the cells were thawed and resuspended in PBS. The suspension was incubated with 0.6 mM DPH (dissolved in *N*,*N*-dimethylformamide at 6 mM) for 45 min at 28 °C and washed with PBS buffer [22]. To homogenize the cells, the samples on ice were sonicated (VC 130, Sonics & Materials, Inc., USA), and then, separated by centrifugation. The supernatant including DPH-labeled lipid matrix was transferred to a cuvette and the fluorescence intensity of DPH was measured by a Spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Kyoto, Japan) at 350 nm excitation and 425 nm emission wavelengths.

2.5. Propidium iodide influx assay

C. albicans cells in the log phase (2×10^6/ml YPD), resuspended in PBS, were treated with 50.0 μM of the compounds and incubated for 2 h at 28 °C. Cells were then harvested by centrifugation and suspended in PBS. Subsequently, the cells were treated with 9 μM propidium iodide and incubated for 5 min at room temperature [23]. The cells were analyzed using fluorescence microscopy (Microphot FXA photomicroscope, Nikon, Japan).

2.6. Analysis of the changes in the transmembrane electrical potential ($\Delta\Psi$)

2.6.1. Real-time kinetics: changes in the $\Delta\Psi$

C. albicans (2×10^6/ml) were cultured aerobically at 35 °C for 24 h in RPMI 1640 (165 mM MOPS, pH 7.0, with L-glutamine and $NaHCO_3$). The cells were washed with Ca^{2+} and Mg^{2+} free PBS and changes in the $\Delta\Psi$ were measured with a membrane potential sensitive probe, 3,3′-dipropylthiacarbocyanine [$diSC_3(5)$]. Changes in the fluorescence due to the collapse of the $\Delta\Psi$ by 50.0 μM of the compounds were continuously monitored using a Spectrofluorophotometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The experiment was repeated three times under each condition to ensure reproducibility [24].

2.6.2. The changes in the $\Delta\Psi$ after incubation for 2 h

The log-phased cells of *C. albicans* (2×10^6/ml YPD) were harvested and resuspended in PBS buffer, containing 50.0 μM of the compounds. After incubation for 2 h, the cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were treated with 50 μg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Molecular Probes, Eugene, OR, USA) as the indicator of membrane potential. Flow cytometric analysis was done with a FACSCalibur flow cytometer (Becton Dickinson, San Jose,