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Antifungal effect and mode of action of glochidioboside against *Candida albicans* membranes

Heejeong Lee [a], Hyemin Choi [a], Hae Ju Ko [b], Eun-Rhan Woo [b], Dong Gun Lee [a,*]

[a] School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daehak-ro 80, Buk-gu, Daegu 702-701, Republic of Korea
[b] College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, Republic of Korea

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ABSTRACT

Glochidioboside was obtained from *Sambucus williamsii* and its biological effect has not been reported. Its antifungal activity against pathogenic fungi and the mode of action involved in its effect were examined. Glochidioboside exerted antifungal effect with almost no hemolytic effect against human erythrocytes. To understand its antifungal mechanisms, membrane studies were done. Using two dyes, 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$] and propidium iodide, membrane depolarization and permeabilization by glochidioboside were confirmed. Furthermore, the membrane-active mechanism was proven by synthesizing a model membrane, calcein-encapsulating large unilamellar vesicles (LUVs), and also by observing the influx of different sized fluorescent dyes, such as calcein, FD4 and FD10, into the fungal cells. The membrane-active action was pore-forming action with radii between 1.4 and 2.3 nm. Finally, three dimensional (3D) flow cytometric analysis showed the shrinkage of the fungal cells from the membrane damage. In conclusion, this study suggests that glochidioboside exerts an antifungal activity through a membrane-disruptive mechanism.

1. Introduction

The importance of antifungal agents is continually highlighted by increasing invasive fungal diseases. However, the discovery and development of antifungal drugs is extremely slow [1,2]. To date, only a few groups of antifungal drugs such as polyenes, azoles and echinocandins have been used. Among them, azoles and polyenes, the largest group of antifungal drugs, act on ergosterol in the fungal membrane, but each has a different mechanism. Azoles inhibit ergosterol biosynthesis and polyenes disrupt the fungal membrane by binding to ergosterol. Additionally, echinocandins inhibit the synthesis of glucan in the fungal cell wall [2].

However, incurable fungi, such as azole-resistant *Candida* spp. seem to be common nowdays. Even amphotericin B-resistant *Candida* and *Cryptococcus* strains have been reported [3]. Partients infected by antibiotics-resistant organisms continually fight the infection but it is difficult to treat fungal infections due to delays in diagnosis, restrictions in the route of administration and cytotoxicity of the drugs [1,4,5]. In view of this situation, the discovery of novel antifungal agents that are safer is necessary [2,5].

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, lectins [6–8] and lignans [9], which have been found *in vitro* to have antimicrobial properties [10]. These compounds called phytochemicals serve as the plant immune system against microorganisms, insects, and herbivores [11]. Phytochemicals are an important natural resource for the discovery of antifungal agents because of their long-term use without human toxicity [12]. The herb *Sambucus williamsii* is a folk plant with medicinal properties. The genus *Sambucus*, widely found in Europe, Asia and North Africa, has been used in pharmaceutical products as an analgesic, antivirus, anti-inflammatory, homoeostatic, and diuretic drugs, which act on bruises, fractures, and edema by traditional users [12,13]. In this study, glochidioboside, a neolignan glucoside, was derived from *S. williamsii*. To date, glochidioboside has been extracted from *Acer truncatum* and *Glochidion obovatum* [14,15]. However, its bioactive property has not been reported. Therefore, its antifungal effect and mode of action(s) were investigated.

Abbreviations: MeOH, methanol; CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; *n*-BuOH, *n*-butanol; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; KRIBB, Korea Research Institute of Bioscience and Biotechnology; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate buffered saline; $DiSC_3(5)$, 3,3′-dipropylthiacarbocyanine iodide; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; 3D, three dimensional; FSC, forward scatter; SSC, side scatter.

* Corresponding author. Fax: +82 53 955 5522.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

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2. Materials and methods

2.1. Extraction and isolation of glochidioboside

The air-dried stem bark of *S. williamsii* (840 g) was cut and extracted with methanol (MeOH) at 80 °C for 4 h. The MeOH extract (57.1 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 (4.2 g) was subjected to column chromatography over a HP-20 column by eluting it with a MeOH:H_2O (5:95 → 25:75 → 40:60 → 100:0) gradient system. Based on their TLC pattern, the fractions were combined to yield subfractions, which were designated as E1–E4. The subfraction E1 (1.49 g) was then purified by column chromatography over a HP-20 column with a MeOH:H_2O (0:100 → 50:50 → 15:85 → 100:0) gradient system to yield four subfractions (E1-1–E1-4). The subfraction E1-1 was subjected to repeated column chromatography (Silica gel, $CHCl_3$:MeOH:H_2O, 10:1:0.1 → 1:1:0.1) to yield glochidioboside (2.3 mg). The physico-chemical data including ^{1}H NMR, ^{13}C NMR, and HSQC of the glochidioboside were identical with those reported in the literature [14,16].

2.2. 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 *M. furfur* was cultured at 32 °C in a modified YM broth (Difco) containing 1% olive oil. Fungal cell suspensions were adjusted to obtain standardized populations by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, USA). Fungal cells (1×10^6 cells/ml) were incubated in 0.1 ml/wells containing YPD or YM broth. The minimum inhibitory concentrations (MICs) were determined with a twofold serial dilution of the test compounds, based on the Clinical and Laboratory Standards Institute (CLSI) method [17] and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [18]. After 48 h of incubation, the minimal concentration of the 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 [19].

2.3. Hemolytic activity assay

Fresh human erythrocytes were centrifuged at 2000 rpm for 10 min and washed three times with phosphate buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4). The final concentration of the erythrocytes was 4%. The erythrocyte suspension was transferred to sterilized 96-well plates and twofold serial dilutions of the compound were added to the wells of a 96-well plate. The samples were then incubated with the compounds at 37 °C for 1 h and the plate was centrifuged at 1500 rpm for 10 min. An aliquot of the supernatant was taken, and then, the hemolytic activity of the compounds was evaluated by measuring 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 PBS alone and with 0.1 Triton X-100, respectively. The hemolysis percentage was calculated with the following equation: hemolysis (%) = [(Abs_{414nm} in the peptide solution − Abs_{414nm} in PBS)/(Abs_{414nm} in 0.1% Triton X-100 − Abs_{414nm} in PBS)] × 100 [20].

2.4. Detection of changes in membrane electrical potential

C. albicans cells (1×10^6 cells/ml) were washed with Ca^{2+} and Mg^{2+} free PBS and changes in the membrane potential were measured with membrane potential sensitive probe 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$]. Changes in fluorescence due to the collapse of the cytoplasmic membrane potential by 6.5 μg/ml of compounds were continuously monitored using a spectrofluorophotometer (Shimadzu, RF-5301PC, Shimadzu, Kyoto, Japan) 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 [21].

2.5. Propidium iodide influx assay

To analyze fungal membrane permeabilization after treatment with the compound, *C. albicans* cells (1×10^6 cells/ml), suspended in PBS, were treated with 6.5 μg/ml of the compounds and incubated for 4 h at 28 °C. After incubation, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 6 μM of propidium iodide and incubated for 5 min at room temperature. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [22].

2.6. Calcein leakage measurement

Large unilamellar vesicles (LUVs) encapsulating calcein, composed of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w), were prepared by vortexing dried lipids in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]). The suspension was freeze-thawed in liquid nitrogen 13 times and extruded through polycarbonate filters (two stacked 200 nm pore size filters) with a LiposoFast extruder (Avestin Inc., Ottawa, Canada). Untrapped calcein was removed using a gel filtration process on a Sephadex G-50 column. For the assay, a suspension of liposomes including calcein was treated with the compounds. The mixture (1 ml, final volume) was stirred for 10 min in the dark and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred, and the release of calcein from the LUVs was monitored at 25 °C by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm with a spectrofluorophotometer (Shimadzu, RF-5301PC, Shimadzu, Kyoto, Japan). Twenty microliters of 10% Triton X-100 was added to the vesicles to determine 100% calcein leakage. The percentage of calcein leakage caused by the compounds was calculated as follows: calcein leakage (%) = $100 \times (F - F_0)/(F_t - F_0)$, where F represents the fluorescence intensity achieved after addition of the compounds and F_0 and F_t represent the fluorescence intensities without the compounds and with Triton X-100, respectively [23].

2.7. Estimation of the pore size in C. albicans membrane

C. albicans cells (1×10^6 cells/ml) were suspended in PBS, treated with 6.5 μg/ml of the compound and incubated for 4 h at 28 °C. After incubation, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the soluble fluorescent molecules calcein, FD4 and FD10, were added to the *C. albicans* cells to a final concentration of 2 μM, 0.1, and 0.1 mg/ml, respectively [24]. All FDs were purchased from Sigma Chemical Co. (USA). The