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Antifungal effect with apoptotic mechanism(s) of Styraxjaponoside C

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ABSTRACT

The antifungal effects and mechanisms of Styraxjaponoside C were investigated. Styraxjaponoside C was active against several human pathogens, including *Candida albicans*. Styraxjaponoside C induced a series of cellular changes characteristic of apoptosis in *C. albicans*, including increased reactive oxygen species (ROS) production, measured by DHR-123 staining; phosphatidylserine externalization, visualized by Annexin V staining; DNA fragmentation, as seen by TUNEL; and plasma membrane depolarization, observed by $DiBAC_4(3)$ staining. The plasma membrane depolarization is likely to be associated with production of ROS. The current study suggests that Styraxjaponoside C exerts an antifungal effect by promoting apoptosis.

Introduction

Antibiotic resistance impacts the efficacy of all licensed antibacterial and antifungal agents. Resistance limits therapeutic options and requires the use of more expensive agents, and multi-antibiotic resistance may result in a complete lack of treatment options [1,2]. To combat this serious clinical situation, and to reduce the morbidity and mortality associated with infection, extensive ongoing research is focusing on novel alternative strategies. Current research areas include the use of antisense RNAs and antigenes to inhibit resistance mechanisms at the nucleic acid level [3], increasing the efficiency of existing agents by modification or production of synthetic derivatives such as methicillin and related beta-latams [4], and investigation of natural products that may be templates for novel antimicrobial targets [5]. In this article, a possible novel template for an antimicrobial compound, along with its mechanism of action, was investigated.

Styraxjaponoside C is a novel lignan glycoside derivative that is isolated from the stem bark of *Styrax japonica* S. et Z [6]. Lignans are a class of secondary plant metabolites produced by oxidative dimerization of two phenylpropanoid units that are found in roots, thizomes, stems, leaves and fruits [7,8]. In spite of this extensive distribution, the biological functions of lignans are not clear. Styraxjaponoside C is a structurally unique lignan, so it was tested as a novel compound for intrinsic antifungal activity. This paper presents the first investigation of the *in vitro* antifungal activity of Styraxjaponoside C, and its mechanism of action.

Materials and methods

Extraction and isolation of compound from Styrax japonica. The stem bark of *S. japonica* was collected from Jogyesan, Suncheon, Chonnam, Korea, in September 2002. Voucher specimens were deposited in the Herbarium of the College of Pharmacy, Chosun University, Korea (CSU-964-17). Air-dried stem bark (654 g) was cut and extracted with MeOH at 80 °C for 4 h before 120.32 g of the extract was resuspended in water and partitioned sequentially with equal volumes of dichloromethane, ethyl acetate, and *n*-butanol. Each fraction was evaporated *in vaccuo* to yield residues designated CH_2Cl_2 (13.98 g), EtOAc (12.16 g), *n*-BuOH (75.88 g), and water (2.90 g). The *n*-BuOH extract (10 g) was purified by column chromatography on a silica gel, eluting with a $CHCl_3$–MeOH (50:1–0:100) in a step-wise system. The fractions were pooled into seven subfractions (Bu1: 1.26 g, Bu2: 2.47 g, Bu3: 3.34 g, Bu4: 0.85 g, Bu5: 1.55 g, Bu6: 0.27 g) based on TLC profiles. The subfraction, Bu3 (3.34 g) was purified by column chromatography on a silica gel ($CHCl_3$–MeOH, 50:1–0:100), followed by gel-filtration column chromatography (Sephadex LH-20, MeOH–H_2O, 35:65) and preparative TLC (RP-18 F_{254}s, 1.0 mm, MeOH–H_2O, 1:1), yielding Styraxjaponoside C (28 mg) [6].

Antifungal activity assay. Candida albicans (ATCC 90028) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) (Daejeon, Korea).

Abbreviations: MTT, (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide); $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PI, propidium iodide; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; ROS, reactive oxygen species; DHR, dihydrorhodamine; NAC, *N*-Acetyl-L-cysteine.

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Log-phase fungal cells (2×10^4/ml) were inoculated into YPD broth and aliquoted into 0.1 ml/well microtiter plates. MICs were determined by twofold serial dilution of compound, following a micro-dilution method, and by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [9,10]. After 48 h at 28 °C, the minimal compound concentration that prevented the growth of a given test organism was determined, and defined as the MIC. Growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, California, USA) at 580 nm. MIC values were determined from three independent assays.

Preparation and microscopic observation of GUVs. Giant unilamellar vesicles (GUVs) were prepared by the electroformation method of Angelova and Dimitrov [11,12]. A lipid mixture solution (3.75 mg/mL) of PC/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (18:1 Liss Rhod PE)/PI/ergosterol (5:4:1:2, w/w/w/w) dissolved in chloroform was spread onto a indium tin oxide-coated glass, which was evaporated under vacuum for 2 h. Both lipid-coated and uncoated glass were held at a distance of 2 mm by a thin Teflon spacer. After filling the chamber with 10 mM Hepes buffer (pH 7.4), a sinusoidal 1.7 Vpp (peak-to-peak) and 10 Hz frequency was applied for 2 h at room temperature. To detach the vesicles from the plate, a sinusoidal 4 Vpp and 4 Hz frequency was applied for 10 min. Ten microliters of GUV were place on an inverted fluorescence phase-contrast microscope (Leica DFC 420C), and compound solutions were added after the selection of a single GUV.

Candida albicans apoptosis assay. Annexin V-FITC labeling was performed following a modified method of Madeo et al. [13]. *C. albicans* cells were harvested by centrifugation, washed in 0.1 M potassium phosphate buffer (PPB, pH 6.0), resuspended in PPB containing 1 M sorbitol, and digested with lysing enzyme (20 mg/ml). Cells were washed with 0.1 M PPB containing 1 M sorbitol, treated with compounds for 2 h and washed and resuspended in an annexin binding buffer (1 M sorbitol) [14]. Cells were incubated with 5 μl of annexin V-FITC/ml and incubated for 20 min prior to the addition of 5 μl of PI.

DNA fragmentation was analyzed by the TUNEL method [13]. Cells treated for 160 min with compounds were washed in PBS containing 1 M sorbitol. DNA ends were labeled with an *in situ* cell death detection kit. Cells were examined by fluorescence microscopy (Axio Imager A1 and Axio CamMR5, Carl Zeiss).

Intracellular ROS accumulation was determined by incubation of cells with 5 μg/ml of DHR-123. Cells were analyzed by flow cytometry [15].

Flow cytometry for plasma membrane potential. Log-phase *C. albicans* (2×10^7 cells/ml, YPD) were treated with Styraxjaponoside C or 10 μM CCCP. After incubation for 3 h, the cells were washed with, and suspended in PBS, before staining with 10 μg of $DiBAC_4(3)$ for 30 min at 4 °C in the dark [16]. Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Results and discussion

Antifungal activity of Styraxjaponoside C

Styraxjaponoside C, a novel glycoside derivative of lignans, was isolated from the stem bark of *Styrax japonica* S. et Z. NMR showed that its structure is (−)-fargesin-4-*O*-β-D-glucopyranoside [6]. In this study, the antifungal activity and mechanisms of action of Styraxjaponoside C was investigated.

The antifungal activities of Styraxjaponoside C against the human pathogens *C. albicans, M. furfur*, and *T. beigelii* were determined by the micro-dilution method [9] and MTT assay [10]. Amphotericin B [17] was used as a positive control. All fungal strains tested were susceptible to Styraxjaponoside C with MIC value of 40 μg/ml (Table 1). Significantly, Styraxjaponoside C was active against *C. albicans*, a well-known yeast model system for studying programmed cell death in higher eukaryotes [18], as well as the most prevalent systemic fungal pathogen, causing candidiasis with mortality rates as high as 47% [19]. Because of these indications, *C. albicans* was selected used as the test organism for subsequent experiments.

Membrane-active activity assay

To understand the mode of the antifungal action of Styraxjaponoside C, a membrane-active antifungal activity assay using fungal membrane mimetic liposomes was conducted. Amphotericin B, a polyene antibiotic, associates with ergosterol and is thought to form membrane-spanning channels with hydrophilic interiors that allow leakage of essential components, ultimately resulting in fungal cell death [16]. Therefore, artificial lipid bilayers of PC:PE:PI:ergosterol, 5:4:1:2 (w/w/w/w), which resembles the phospholipid composition of *C. albicans* membranes, were used to make giant unilamellar vesicles (GUV) [20]. A single GUV was selected for observation and its morphological changes in reaction to the compounds were analyzed for a maximum of 5 min. Styraxjaponoside C (Fig. 1A) did not cause morphological changes to GUVs, which retained their shape, whereas amphotericin B induced a decrease in liposome size over time (Fig. 1B). This result suggested that, unlike the membrane-active mechanism of amphotericin B, Styraxjaponoside C does not target the membrane, and does not have a physical interaction with it. Exposure of *C. albicans* to Styraxjaponoside C might cause environmental stress [21,22], or Styraxjaponoside C might penetrate cell membranes and affect intracellular organelles. These factors could induce physiological cellular responses, such as programmed cell death. Accordingly, we investigated the effect of Styraxjaponoside C on programmed cell death markers.

Candida albicans apoptosis assay

The induction of programmed cell death in *C. albicans* by Styraxjaponoside C was evaluated by co-labeling with annexin V-FITC and PI. As illustrated in Fig. 2A, *C. albicans* cells exposed to Styraxjaponoside C stained annexin V-positive and PI-negative, which is similar to the response to H_2O_2, an inducer of apoptosis in yeast cells [13]. The phosphatidylserine externalization to the outer leaflet of the cytoplasmic membrane is an early marker of apoptosis in mammalian and yeast cells [15], so this result indicated an early event of apoptosis in *C. albicans* induced by Styraxjaponoside C.

The TUNEL assay was performed in order to detect apoptotic DNA fragmentation by labeling 3′-OH termini with modified nucleotides catalyzed by terminal deoxynucleotidyltransferase. The labeling of DNA breaks by TUNEL is one of the most reliable methods for identifying apoptotic cells, and visualizing the apoptotic phenotype [13]. TUNEL-positive cells were observed in the population treated with Styraxjaponoside C (Fig. 2B).

Table 1
Antifungal activity of Styraxjaponoside C.

Fungal strains	MIC (μg/ml)	
	Styraxjaponoside C	Amphotericin B
C. albicans	40	5
T. beigelii	40	5
M. furfur	40	10