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Research paper

(+)-Medioresinol leads to intracellular ROS accumulation and mitochondria-mediated apoptotic cell death in *Candida albicans*

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ABSTRACT

The phytochemical (+)-Medioresinol, a furofuran type lignan identification and isolation on the stem bark of *Sambucus williamsii*, which is a folk medicinal plant used in traditional medicine. (+)-Medioresinol is known to possess a lesishmanicidal activity and cardiovascular disease risk reduction but its antifungal effects have not yet been identified. In this study, to confirm (+)-Medioresinol`s antifungal properties and mode of action, we observed morphological and physiological change in *Candida albicans*. In cells exposed to (+)-Medioresinol, arrested the cell cycle and intracellular reactive oxygen species (ROS) which is a major cause of apoptosis were increased. The increase of ROS induced oxidative stress and the mitochondria dysfunction which causes release of pro-apoptotic factors. We investigated a series of characteristic cellular changes of apoptosis by using various apoptosis detection methods. We report here for the first time that (+)-Medioresinol has effects on mitochondria and induced the accumulation of ROS in *C. albicans* cells. We demonstrated that one of the important features of apoptosis, mitochondrial membrane depolarization is caused by ROS. Substantially, we investigated the release of cytochrome *c*, which is one of the factors of metacaspase activity. We also show that the effects of (+)-Medioresinol are mediated at an early stage in apoptosis acting on the plasma membrane phosphatidylserine externalization. In addition, (+)-Medioresinol induced apoptotic morphological changes, showing the reduced cell size (low FSC) and enhanced intracellular density (high SSC). In late stage of confirmation of diagnostic markers in yeast apoptosis include the effects of nucleus morphological change, DNA fragmentation and condensation by influence of oxidative stress. These apoptotic phenomena represent that oxidative stress and mitochondria dysfunctions by inducing the phytochemical (+)-Medioresinol must be an important factors of the apoptotic process in *C. albicans*. These results support the elucidation of the underlying antifungal mechanisms of (+)-Medioresinol.

1. Introduction

Infections caused by *Candida* spp. and other fungi continue to represent a significant health burden. Some cases are highly resistant to traditional antifungal agents, such as azoles and polyenes. This is especially true in the case of *Candida albicans*, a human pathogen that causes a range of opportunistic superficial infection and life-threatening systemic infections in immunocompromised patients [1,2]. *C. albicans* is also a well-known yeast model system for studying programmed cell death in higher eukaryotes [3], as well as being the most prevalent systemic fungal pathogen, causing candidiasis with mortality rates as high as 47% [4]. The clinical significance and the severity of drug resistance in candidiasis pose an urgent need to search for antifungal agents with novel mechanisms of action [5]. Recently, studies have discovered new antibiotics such as synthetic chemicals [6,7], peptides [8,9], and phytochemicals [10]. Among the new antibiotics, phytochemicals are chemical compounds which are secondary plants metabolites, that possess various biological activities [10,11].

Phytochemical is a term generally used to refer to those chemicals that may affect health without human toxicity and are acquired at a low price, but are not established as essential nutrients. One of these phytochemicals, lignan is abundant in the natural world with a variety of structures and contained in many common

Abbreviations: H_2O_2, Hydrogen Peroxide; PI, propidium iodide; DHR-123, dihydrorhodamine-123; Cyt *c*, Cytochrome *c*; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-imidacarbocyanine iodide; DAPI, 4′, 6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ROS, Reactive Oxygen species.

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plant foods and resource [12]. (+)-Medioresinol is a furofuran type lignan derivative from the stem bark of *Sambucus williamsii*, which is a plant used in folk medicinal for its therapeutic properties. The genus *Sambucus*, widely distributed in Europe, Asia and North Africa, has been used in traditional medicine as an analgesic, antivirus [13], anti-inflammatory [14], homoeostatic, and diuretic drug which acts as drug used to treat bruises, fractures, and edema [15]. Furofuran lignans have also been reported to exhibit various biological activities [16], including antifungal, anti-inflammatory, antimalarial activities, the inhibition of cyclic AMP phosphodiesterase, and the DNA cleavage effect. (+)-Medioresinol has already been known to leishmanicidal activity [17] and cardiovascular disease risk reductions, it was derived from lemon and rye, sesame seeds, dilleniaceae [18]. Nevertheless, (+)-Medioresinol has not yet been specifically identified to have antifungal effects. Therefore, to verified antifungal effect and mode of action in *C. albicans*. Recently, the studies of cellular death mechanisms that reactive oxygen species (ROS) generate and the inhibition of cellular function induced by antibiotics have been analyzed [19]. ROS can damage almost every essential cellular component including DNA, proteins, lipids and the cytoskeleton [20]. In general, the generation of ROS is an early event in apoptotic cell death [21]. Similar to mammalian apoptosis, ROS or low concentrations of H_2O_2 are key regulators of apoptotic process in yeast. Mitochondria have a central role in apoptosis, are the richest source of ROS in the cell, the inhibition of the mitochondria electron transport chain, which results in the subsequent release of ROS. Many important aspects of the apoptotic process converge in mitochondria [22,23].

In this study, we investigated the antifungal effects of (+)-Medioresinol using metabolic, morphological, and molecular assays to confirm its mode of action in *C. albicans* cells, we measured both the cell proliferation and the production of intracellular ROS. Reactive oxygen species must be an important in apoptotic cell death. Furthermore, we observed apoptotic features caused by (+)-Medioesinol, including depolarization of mitochondrial membrane, cytochrome *c* release, metacaspase activation, phosphatidylserine externalization, morphological change, DNA fragmentation, nuclear fragmentation and condensation.

2. Materials and methods

2.1. Extraction and isolation of compounds from Sambucus williamsii

The air-dried stem bark of *S. williamsii* (840 g) was cut and extracted with MeOH at 80 °C for 4 h. The MeOH extract (57.10 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 CH_2Cl_2 fraction (4.2 g) was subjected to column chromatography over a silica gel by eluting it with a Hex:EtOAc (100:1 → 80:1 → 50:1 → 20:1 → 10:1 → 1:1) and $CHCl_3$:MeOH:H_2O (30:10:1 → 1:1:0.1 → MeOH only) gradient system. Based on their TLC pattern, the fractions were combined to yield subfractions, which were designated D1-D8. The subgroup, D6 (1.27 g) was then purified by column chromatography on a silica gel eluting it with a $CHCl_3$:MeOH:Me_2-CO:H_2O (50:4:2:0.3) and was followed with a repeated gel filtration column chromatography (MCI gel, MeOH:H_2O = 3:8) to make (+)-Medioresinol (7.8 mg). The physico-chemical data including ^1H NMR, ^{13}C NMR, and HSQC of the (+)-Medioresinol were identical to those reported in the literature [24,25].

2.2. Antifungal activity test

Trichosporon beigelii (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Culture (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. *C. albicans* (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *T. beigelii*, *C. albicans* and *C. parapsilosis* were cultured in a YPD broth (Difco) with aeration at 28 °C and *M. furfur* was cultured at 32 °C in a modified Bacto yeast extract/malt extract (YM) broth (Difco) and 1% olive oil. Fungal cells (2×10^9/ml) were inoculated into a YPD or YM broth and 0.1 ml/well was dispensed into 96-well microtiter plates. The two-fold diluted (+)-Medioresinol and H_2O_2 were added to each fungal cell [26]. After 12 h of incubation at either 28 °C or 32 °C, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution of 5 mg/ml MTT in phosphate-buffered saline (PBS, pH 7.4) was added to each well. Growth was assayed with a microtiter ELISA reader (Molecular Devices Emax, CA, USA) by monitoring absorption at 580 nm. MIC values were determined by three independent assays [27].

2.3. Flow cytometric analysis for fungal cell cycle

The DNA content was quantified by the flow cytometry of the cells stained with the DNA-specific fluorescent dye propidium iodide (PI) [28]. The exponential phased cells of *C. albicans* (2×10^9/ml) cultured in YPD medium, were harvested and treated with 3.125 μg/ml of (+)-Medioresinol. After incubation for 4 h, the cells were washed with a PBS and fixed with 70% ethanol overnight at 4 °C. The cells were treated with 200 μg/ml of RNase A and the mixture was left to react for 2 h at 37 °C. For the DNA staining, 50 μg/ml of PI was added and incubated for 1 h at 4 °C in the dark. Flow cytometric analysis was performed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The values represent the average of the measurements conducted in triplicate for three independent assays.

2.4. Intracellular ROS accumulation

The intracellular ROS production was measured using a fluorescent dye DHR-123. The H_2O_2, in MIC values of 25 μg/ml, but we suggested (+)-Medioresinol affect apoptosis in *C. albicans*. Therefore, we used the most suitable concentration to be apoptosis was 17 μg/ml of H_2O_2. *C. albicans* cells in the exponential phase (2×10^9/ml) were treated with 3.125 μg/ml of (+)-Medioresinol and 17 μg/ml of H_2O_2 for 2 h at 28 °C. After incubation, the cells were washed with PBS (pH 7.4) before be stained with 5 μg/ml of DHR-123. The cells were quantitatively analyzed by a FACSCalibur flow cytometer [29]. Experiments were performed in triplicate, the data was then averaged and the S.D. was calculated.

2.5. Measurement of mitochondrial membrane potential (MMP)

The change of the MMP in *C. albicans* after treating with (+)-Medioresinol was analyzed by using 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-imidacarbocyanine iodide (JC-1) (Molecular Probes) as previously described [31]. Briefly, the *C. albicans* cells in exponential phase (2×10^9/ml) were treated with 3.125 μg/ml of (+)-Medioresinol and 17 μg/ml of H_2O_2 for 2 h at 28 °C. The fungal cells were washed three times and then resuspended in PBS (pH 7.4). JC-1 was added to the final concentration of 0.217 μg/ml and the mixture was incubated at 35 °C for 15 min. The fluorescence intensities at FL-1 (green fluorescence, 525 nm) and FL-2 (red fluorescence, 595 nm) were recorded by a FACSCalibur flow cytometer. The ratio of JC-1 aggregate (FL-2, green) to monomer (FL-1, red) intensity was calculated. An increase in this ratio was interpreted as a decrease of the MMP. Values represent the mean ± S.D. from three independent experiments.