Biochimica et Biophysica Acta 1828 (2013) 1153–1158

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

The antifungal activity and membrane-disruptive action of dioscin extracted from *Dioscorea nipponica*

Jaeyong Cho [a], Hyemin Choi [a], Juneyoung Lee [a], Mi-Sun Kim [b], Ho-Yong Sohn [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] Department of Food and Nutrition, Andong National University, Andong 760-749, Republic of Korea

ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form 28 November 2012
Accepted 13 December 2012
Available online 21 December 2012

Keywords:
Dioscin
Dioscorea nipponica
Antifungal activity
Mechanism

ABSTRACT

Dioscin is a kind of steroidal saponin isolated from the root bark of wild yam *Dioscorea nipponica*. We investigated the antifungal effect of dioscin against different fungal strains and its antifungal mechanism(s) in *Candida albicans* cells. Using the propidium iodide assay and calcein-leakage measurement, we confirmed that dioscin caused fungal membrane damage. Furthermore, we evaluated the ability of dioscin to disrupt the plasma membrane potential, using 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] and bis-(1,3-dibarbituric acid)-trimethine oxanol [$DiBAC_4(3)$]. Cells stained with the dyes had a significant increase in fluorescent intensity after exposure to dioscin, indicating that dioscin has an effect on the membrane potential. To visualize the effect of dioscin on the cell membrane, we synthesized rhodamine-labeled giant unilamellar vesicles (GUVs) mimicking the outer leaflet of the plasma membrane of *C. albicans*. As seen in the result, the membrane disruptive action of dioscin caused morphological change and rhodamine leakage of the GUVs. In three-dimensional contour-plot analysis using flow cytometry, we observed a decrease in cell size, which is in agreement with our result from the GUV assay. These results suggest that dioscin exerts a considerable antifungal activity by disrupting the structure in membrane after invading into the fungal membrane, resulting in fungal cell death.

1. Introduction

According to historic records, natural products such as mineral, plant and animal products have been used as traditional medicines and as the main source for drugs [1]. Medicinal therapies using plants have been widely used to treat ailments ranging from coughs and colds to parasitic infections and inflammation. Especially, the medicinal value of plants lies in the active chemical substances called phytochemicals. Phytochemicals are an important component of the plant defense and have antimicrobial effects [2,3]. Thus, they can be used as new sources of alternative therapies to treat infectious diseases [4]. Therefore, a number of researchers who have studied alternative therapies and the therapeutic use of natural products are focusing on phytochemicals isolated from fruits and vegetables.

Dioscorea nipponica, a perennial herb growing in mountainous areas, has been used as a folk medicine for asthma, rheumatoid arthritis, bronchitis, and other disease [5]. It has been reported that dioscin derivatives among the steroidal saponins isolated from *D. nipponica* possess various therapeutic properties, including anticancer [6], anti-viral [7], anti-fungal [8], anti-obesity [9], and anti-neoplastic [10]. Especially, diosgenin, a steroidal aglycone, can be converted into steroidal saponin dioscin by combining with glucoses (Fig. 1), and this aglycone induces apoptosis in colon cancer cell lines and cell cycle arrest in osteosarcoma cells. Diosgenin is used as an important source of corticosteroid hormones in the pharmaceutical industry.

In this study, we confirmed the antifungal activity of the phytochemical dioscin isolated from the root bark of wild yam *D. nipponica* against various fungal strains. Furthermore, by investigating its antifungal mechanism(s) in *Candida albicans* cells, we show that dioscin is a potential candidate as an alternative antifungal agent through its membrane-active mechanism.

2. Materials and methods

2.1. Isolation of dioscin from the root bark of D. nipponica

The root bark of *D. nipponica* was collected in Kyungpook, Korea in the summer of 2009. A voucher specimen was deposited at the Department of Food and Nutrition, Andong National University, Korea. The procedure for the isolation of dioscin was previously described [9].

Abbreviations: Dioscin, [3-O-α-L-rhamnopyranosyl-(1,4)-[α-L-rhamnopyranosyl-(1,2)]-β-D-glucopyranosyl-diosgenin]; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; LUVs, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; $DiSC_3(5)$, 3,3′-dipropylthiacarbocyanine; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; GUV, giant unilamellar vesicle; ITO, indium tin oxide

* Corresponding author. Tel.: +82 54 820 5491; fax: +82 54 820 7804.
** Corresponding author. Tel.: +82 53 950 5373; fax: +82 53 955 5522.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2012.12.010

The root barks (3 kg) were extracted three times with 5 l of methanol. After filtration, the extract was evaporated *in vacuo* to yield 110 g of dry sample. The evaporated samples were loaded onto a silica gel column (90×6 cm, silica gel 900 g, Merck 7734, Darmstadt, Germany) and eluted with chloroform/methanol/water (7:3:1 v/v/v). Sixty minutes after the start of the elution, eight 2 l fractions were collected. The 5th fraction was further fractionated by silica gel chromatography eluted with ethylacetate/methanol (98:2→95:5). Sixty 0.5 l fractions were collected. The fractions from the 38th to the 40th were collected and those fractions yielding the dioscin were analyzed by IR spectroscopy (Perkin-Elmer 283B, Shelton, CT, USA) and NMR spectroscopy (Burker AMX300, Rheinstetten, Germany). The purity of the dioscin was above 98.5% based on HPLC analysis.

2.2. Fungal strains and antifungal susceptibility testing

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). *C. albicans* (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Amphotericin B was purchased from Sigma-Aldrich Co. and used as a positive control in this study.

Actively growing fungal cells (2×10^6 cells/ml) were inoculated into YPD broth (Difco) and dispensed into microtiter plates with 0.1 ml/well. The minimum inhibitory concentration (MIC) was determined using a standard microdilution method and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. After an overnight incubation at 28 °C, the growth was assayed with a microtiter ELISA reader by monitoring the absorption at 580 nm. MIC values were determined by three independent assays [11].

2.3. Propidium iodide influx analysis

C. albicans cells (2×10^6 cells/ml) were incubated at the log phase and resuspended in YPD medium. Cells were incubated with various compounds (22.5 μg/ml) for 2 h at 28 °C with constant shaking (140 rpm). After incubation, the cells were harvested and resuspended in PBS. Subsequently, cells were treated with propidium iodide (9 μM, final concentration) for 5 min [12]. Flow cytometry was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.4. Calcein leakage measurement

Calcein-encapsulating large unilamellar vesicles (LUVs), composed of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w), were prepared by vortexing the 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 for over 11 cycles and extruded through polycarbonate filters (two stacked 200-nm pore-size filters) with a LiposoFast extruder (Avestin). Untrapped calcein was removed by a gel filtration process on a Sephadex G-50 column. The release of calcein was monitored by measuring fluorescent intensity at wavelengths ($\lambda_{ex}=490$ nm, $\lambda_{em}=520$ nm) with an RF-5301PC spectrofluorophotometer (Shimadzu, Japan). The measurements were conducted at 28 °C. To determine 100% dye leakage, 20 μl of 10% Triton X-100 was added to dissolve the vesicles. The percentage of dye leakage was calculated as follows: % dye leakage $=100\times(F-F_0)/(F_t-F_0)$, where F represents the fluorescence intensity after the addition of the compounds, and F_0 as well as F_t represent the fluorescent intensities without the compounds and with Triton X-100, respectively [13,14]. P values were calculated and analyzed by t-test in SPSS.

Table 1
The antifungal activity of dioscin and amphotericin B.

Fungal strains	MIC (μg/ml)	
	Dioscin	Amphotericin B
C. albicans ATCC90028	22.5 ± 9.2	11.3 ± 4.6
C. parapsilosis ATCC22019	11.3 ± 4.6	8.45 ± 2.3
T. beigelii KCTC7707	11.3 ± 4.6	8.45 ± 2.3
M. furfur KCTC7744	22.5 ± 9.2	11.3 ± 4.6

2.5. Membrane depolarization assay

Changes in the cytoplasmic membrane potential were measured using a membrane potential-sensitive probe, $DiSC_3(5)$ and $DiBAC_4(3)$. $DiSC_3(5)$ was purchased from Sigma Chemical Co. (USA) and $DiBAC_4(3)$ was purchased Molecular Probes, Inc. (USA). *C. albicans* (2×10^6 cells/ml) were cultured aerobically at 28 °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, $DiSC_3(5)$ and $DiBAC_4(3)$. Changes in the fluorescence induced by the collapse of the $\Delta\Psi$ by the various compounds (22.5 μg/ml) were continuously monitored using a spectrofluorophotometer at an excitation wavelength of 490 nm [$DiBAC_4(3)$] or 622 nm [$DiSC_3(5)$]. The measurements were repeated two times under each condition to ensure reproducibility [15].

2.6. Preparation of giant unilamellar vesicles (GUVs)

GUVs were prepared with indium tin oxide (ITO) glasses. Lipids [PC/PE/rhodamine-conjugated PE/PI/ergosterol (5:3.9:0.1:1:2, w/w/

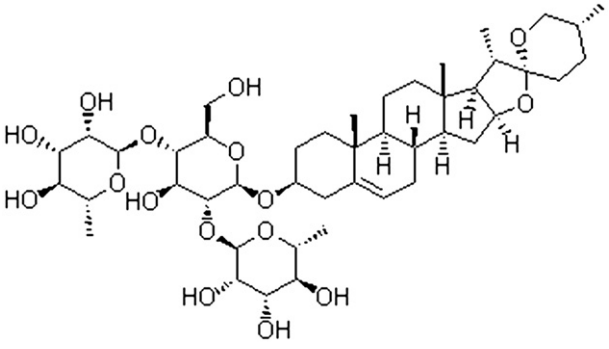

Fig. 1. Chemical structures of dioscin [3-O-α-L-rhamnopyranosyl-(1,4)-[α-L-rhamnopyranosyl-(1,2)]-β-D-glucopyranosyl-diosgenin].

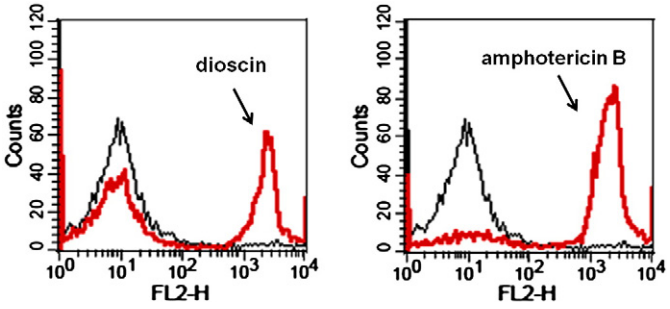

Fig. 2. Effect of dioscin on membrane permeabilization was confirmed by propidium iodide uptake staining in *C. albicans*. Fungal cells (2×10^6 cells/ml) were incubated with 22.5 μg/ml of dioscin and amphotericin B. The change in fluorescent intensity in cells treated with dioscin was monitored by flow cytometer.

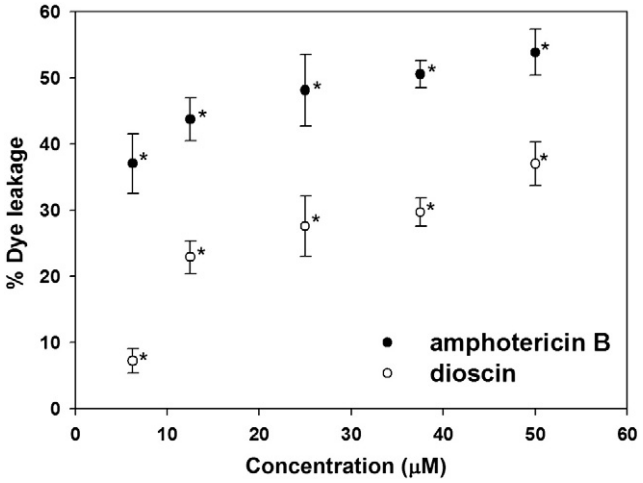

Fig. 3. Percent leakage of fluorescent dye from calcein-entrapped LUVs, composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), in the presence of dioscin (○) or amphotericin B (●). The error bars represent the SD values for three independent experiments. Differences were statistically considered significant at a P value of <0.01 (*).

w/w/w)] were prepared at a concentration of 3.75 mg/ml in chloroform. The lipid solutions (100 μl) were spread onto ITO (indium tin oxide) coated glass in a spin coater (Spin Coater, ACE-1020 Series) at 500 rpm for 5 min. The lipid-coated ITO glass was evaporated under a vacuum for 2 h. Both lipid-coated and uncoated glasses were separated by a distance of 2 mm with a thin Teflon spacer. The chamber was filled with 10 mM HEPES buffer (pH 7.2) through a hole in the silicon spacer. Immediately, the application of 1.7 V (peak-to-peak, sine wave) and 10 Hz to the ITO electrodes was made with a sweep function generator (Protek, SWEEP FUNCTION GENERATOR 9205C) for 2 h. GUVs from the ITO glass were then detached with the following conditions 4 V (peak-to-peak) at 4 Hz for 10 min. The compounds were treated and the changes in the GUVs were observed with an inverted fluorescence phase contrast microscope (Leica, DFC420C) [16–18].

2.7. Flow cytometric analysis of the morphological changes in C. albicans

Cells treated with the various compounds (22.5 μg/ml) were incubated for 2 h at 28 °C with constant shaking (140 rpm). After incubation, the cells were harvested by centrifugation and suspended in PBS. Cells were illuminated by 480 nm light from an argon ionic laser, and forward light scatter (FSC) and side scatter (SSC) were determined. The morphological changes were measured by a flow cytometer [12,19].

3. Results

3.1. Antifungal activity of dioscin

The antifungal activity of dioscin on various fungal strains was investigated and described with the MIC. Amphotericin B was used as a positive control, which is an antifungal agent widely used in treating serious systemic infections [20–22]. The results showed that dioscin had an antifungal effect against various fungal strains (Table 1). Fungal strains such as *C. albicans*, *C. parapsilosis*, *T. beigelii*, and *M. furfur* exist in humans as commensals and are superficial contaminants that can cause a variety of serious infections. Dioscin, with an MIC range of 11.3 ± 4.6–22.5 ± 9.2 μg/ml, showed significant antifungal activity; however, the antifungal activity was less potent than amphotericin B with MIC values between 8.45 ± 2.3 and 11.3 ± 4.6 μg/ml.

3.2. The change in fungal membrane permeability

To elucidate the mode of antifungal action of dioscin, we investigated the effect of dioscin on the membranes of *C. albicans*. The effect of dioscin on the integrity of fungal membranes was investigated by analyzing the propidium iodide influx (Fig. 2). Propidium iodide, a membrane impermeable dye, only enters cells that have damaged membranes, and thereafter, the fluorescence of the probe is enhanced 20 to 30-fold [23]. In cells exposed to dioscin, an increased fluorescent intensity (59.2 ± 11.1%) was detected by flow cytometry compared to control cells (13.2 ± 1.1%), indicating that dioscin has an effect on the membrane structure.

3.3. Activity of dioscin on model membranes

In order to confirm the ability of dioscin to disrupt fungal plasma membranes, we used calcein-entrapped LUVs composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), mimicking the membranes of *C. albicans* cells [24]. After the LUVs were treated with dioscin, the percentage of calcein leakage was measured (Fig. 3). As the dioscin concentration increased, the fluorescent intensity also increased, due to the release of calcein in the LUVs. This calcein leakage from the LUVs means that the structure of the liposome was significantly disrupted. In this assay, dioscin exhibited a relatively lower activity compared to amphotericin B, in agreement with the results from the antifungal susceptibility testing and propidium iodide influx assay.

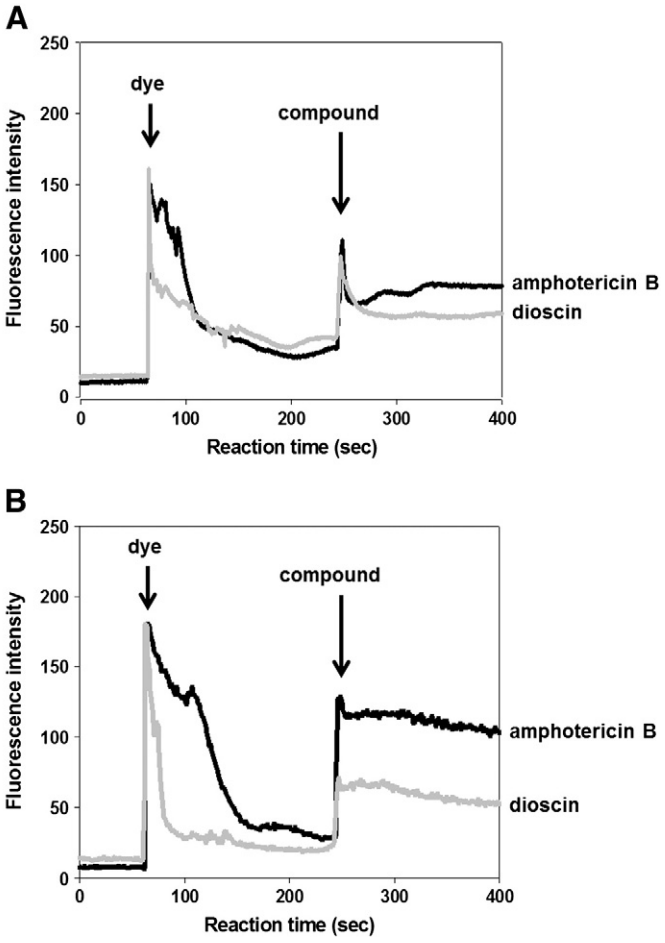

Fig. 4. Effects of dioscin and amphotericin B on the membrane potential of *C. albicans*. (A) Membrane depolarization of *C. albicans* cells by various compounds using $DiSC_3(5)$ staining. (B) Time course investigation of $DiBAC_4(3)$ fluorescence in cells treated with various compounds.

3.4. Membrane depolarization in C. albicans

The structural damage of cell membranes may cause the dissipation of the membrane potential [25]. To confirm the change in the plasma membrane potential by dioscin, we used lipophilic potentiometric indicator dyes $DiSC_3(5)$ and $DiBAC_4(3)$ (Fig. 4). After addition of the dyes into the cells (at 60 s), we monitored the fluorescent changes in real time to achieve a stable baseline of fluorescent intensity (at 60 s). After internalization of the probe, the cells were treated with dioscin and amphotericin B (at 240 s). As a result, the fluorescent intensities of $DiSC_3(5)$ and $DiBAC_4(3)$ increased, suggesting the induction of membrane depolarization.

3.5. Visualization of the fungal membrane-disruptive action of dioscin, using a rhodamine-conjugated single GUV

GUVs, composed of PC/PE/rhodamine-conjugated PE/PI/ergosterol (5:3.9:0.1:1:2, w/w/w/w/w) [26], mimicking the membranes of *C. albicans*, were prepared by using the electroformation method [24]. GUVs have become a useful model to use when imitating biological membranes [27]. With fluorescence phase-contrast microscopy, we observed the morphological changes of the GUVs in response to damage by the various compounds. When treated with dioscin and amphotericin B, the diameter of the GUV rapidly diminished with a concomitant reduction over time (Fig. 5A). We also confirmed the decrease in the fluorescent intensity of the GUV membrane by measuring rhodamine fluorescent level (Fig. 5B). The whole response of the GUV to dioscin and amphotericin B was gradually processed over 3 min from start to finish. However, the fluorescent intensity and the size of the control GUV were unchanged.

3.6. Morphological changes induced by dioscin

Morphological changes were observed by three dimensional flow cytometric dot plot analysis (Fig. 6). FSC (x-axis) is an indicator of cell size and SSC (90° scattering, SS log, y-axis) is an indicator of cell granularity, respectively [28]. Through the change in cell population, it was possible to confirm the effect of dioscin on the fungal cytoplasmic membrane. In cells exposed to dioscin ($9.3 \pm 0.1\%$) and amphotericin B ($9.0 \pm 0.1\%$), there was a notable decrease in FSC compared to the untreated cells ($44.5 \pm 2.2\%$). This result indicated that dioscin permeabilized the fungal membranes and reduced the size of the cells due to the membrane damage.

4. Discussion

Plants produce several secondary metabolites in response to attacks by microbial invasion or other elicitors [29]. These secondary metabolites consist of various chemical compounds called phytochemicals that are synthesized naturally in plants. Because these phytochemicals have relatively low cytotoxicity and significant antifungal properties, they have received much attention as novel antifungal agents and a number of medical phytochemicals have been isolated from several plants [30]. Dioscin, isolated from the root bark of the wild yam *D. nipponica,* is a kind of steroidal saponin, which is isolated as antifungal constituents from medicinal plants. The aim of the present study was to assess the antifungal effects of

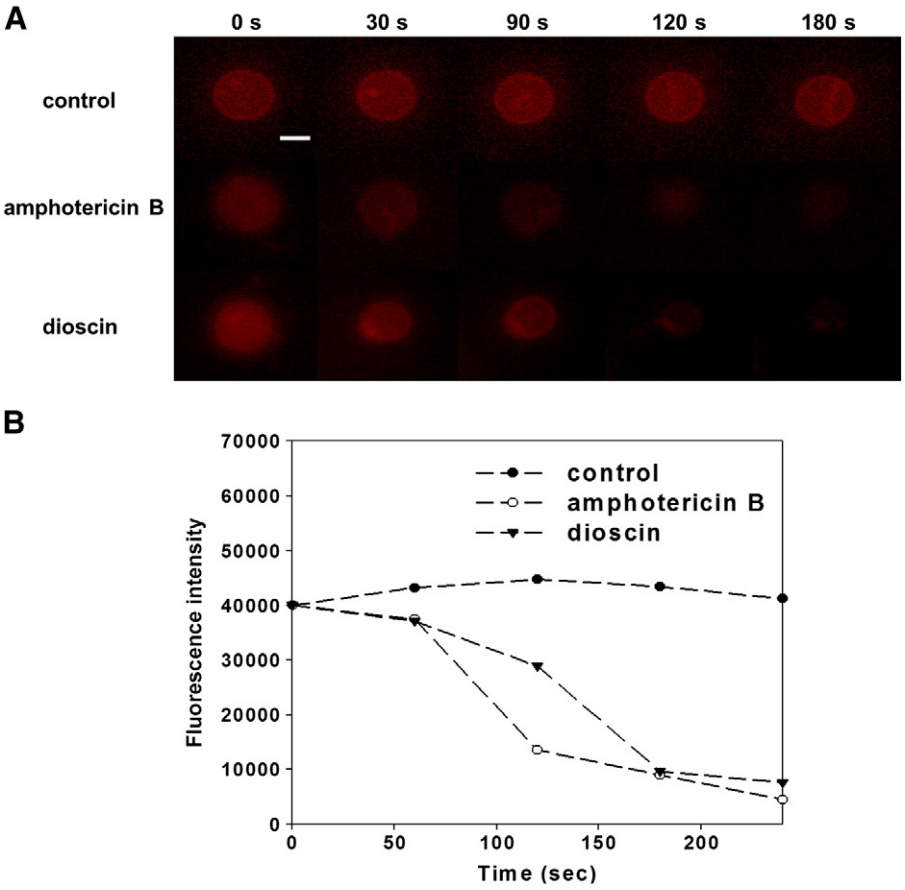

Fig. 5. The response of a single GUV composed of PC/PE/rhodamine-conjugated PE/PI/ergosterol (5:3.9:0.1:1:2, w/w/w/w/w) in the presence of various compounds. (A) The times above each image show the time after the addition of the compounds. The scale bar represents 10 μm. (B) The fluorescent intensities of GUV treated with amphotericin B or diosicin are decreased as time goes on in comparison with a control GUV.

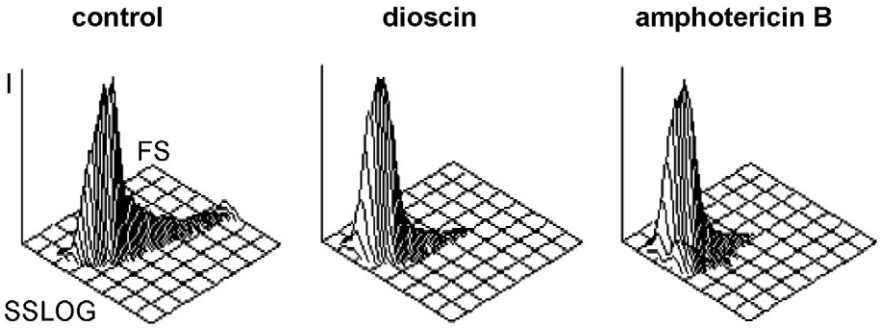

Fig. 6. Three-dimensional flow cytometric contour-plot analysis of *C. albicans* treated with 22.5 μg/ml dioscin and amphotericin B. FSC (y-axis) is an indicator of size and SSC (90° scattering, SS-log, x-axis) is an indicator of granularity. The z-axis represents the cellular population intensity.

dioscin and its mode of action for the antifungal activity against various fungal strains.

In the MIC test, we selected four fungal strains sush as *C. albicans, C. parapsilosis, T. beigelii, and M. furfur.* They are representative strains which cause various infectious diseases in human. Especially, *C. albicans* is a fungus that causes severe opportunistic infections in humans. It causes candidiasis which is the fourth most common cause of hospital acquired infections and is the cause of significant morbidity and mortality in immunocompromised patients [42]. And the importance of the *C. albicans* in human medicine is increasing because treatment is not easy, so we selected *C. albicans* for the elucidation of the antifungal activity of dioscin. Although the antifungal activity of dioscin was less potent than that of amphotericin B, we confirmed that dioscin had significant antifungal activity against various fungal strains. In order to understand the antifungal mechanism of dioscin, we investigated its effect on the cell membranes of *C. albicans*.

Phytochemicals possess a membrane-active mechanism that causes significant membrane damage, due to the destruction of the membrane integrity [31,32]. Hence, the effect of dioscin on the fungal membrane permeability was investigated by monitoring the influx of propidium iodide, which is a DNA-staining fluorescent probe. Propidium iodide only enters membrane-compromised cells and then, intercalates between the bases with cytosine and guanine pairs or with a stoichiometry of one dye per 4–5 base pairs [33]. If the cell membranes were disrupted by dioscin, propidium iodide could permeate into the cytoplasm and bind to the DNA, showing an increased fluorescent intensity. Through the propidium iodide influx assay, we confirmed that dioscin affects fungal cells by injuring their membranes and then, increasing their membrane permeability.

To further understand the membrane-active mechanism of dioscin, we made calcein-entrapped LUVs and rhodamine-conjugated single GUVs, which resemble the phospholipid bilayer of *C. albicans*. Because the extent of fluorescence dye leakage is related to the disruption of the membrane integrity, these experiments using liposomes are also powerful tools to investigate the effects of dioscin on model lipid membranes. GUVs with an average diameter greater than 10 μm can be easily observed under a fluorescent or confocal microscope and provide information on changes in the structural and physical properties, such as the elastic properties of the phospholipid membranes, shape change of the vesicles, interaction of the cytoskeleton proteins with the membranes, membrane fusion, and reconstitution of the artificial cells during interaction with antimicrobial agents [34,35]. Through these results, we confirmed the morphological changes of the GUV from the damage in the membrane structure and the release of the fluorescent dyes from LUVs from the membrane-disruptive mechanism of dioscin.

The perturbation of the membrane lipid bilayers can cause the formation of pores or dissipation of the electrical potential of the membrane [36]. Through the preceding real-time assay using $DiSC_3(5)$ and $DiBAC_4(3)$, a change in the membrane potential by dioscin was observed. $DiSC_3(5)$ is accumulated on hyperpolarized membranes and translocated into the lipid bilayer according to the electrical potential gradient of the membrane [37], and it can detect the cytoplasmic membrane depolarization as a membrane potential-sensitive dye [38]. $DiBAC_4(3)$ is an anionic lipophilic dye, sensitive to the membrane potential and enters only depolarized cells according to the Nernst equation, where it binds reversibly to lipid-rich intracellular components [25,39]. After treating the fungal cells with dioscin, the fluorescent quantum yield and emission increased, indicating that dioscin disrupted the cytoplasmic membrane and that this action resulted in the depolarization of the plasma membrane potential.

The morphological changes from the structural and functional damages of the membranes were investigated by flow cytometry analysis. The cell population exposed to dioscin appeared in the three dimensional density plots with a smaller size (decreased FSC) compared to the non-treated cell population, and this change indicates membrane damage [40]. To sum up, it can be construed that dioscin causes the increased permeability of cell membrane and disrupts the membrane potential. Furthermore, it is thought that those changes in osmolarity led to the cell shrinkage of *C. albicans*, resulting in cell death [41].

In conclusion, dioscin exhibited potent antifungal effects on various fungal strains via membrane-disruptive mechanism(s). Therefore, we conclude that dioscin has considerable antifungal activity, deserving further investigation for clinical applications.

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (no. 2012-0000639) and by the Kyungpook National University Research Fund, 2012.

References

[1] A. De Pasquale, Pharmacognosy: the oldest modern science, J. Ethnopharmacol. 11 (1984) 1–16.
[2] G.F.N. Gislene, L. Juliana, C.F. Paulo, L.S. Giuliana, Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria, Braz. J. Microbiol. 31 (2000) 247–256.
[3] Z.S. Khan, S. Nasreen, Phytochemical analysis, antifungal activity and mode of action of methanol extracts from plants against pathogens, J. Agric. Technol. 6 (2010) 793–805.
[4] R.A. Dixon, Natural products and plant disease resistance, Nature 411 (2001) 843–847.
[5] M.L. Ho, Y.S. Hsieh, J.Y. Chen, K.S. Chen, J.J. Chen, W.H. Kuo, S.J. Lin, P.N. Chen, Antimetastatic potentials of *Dioscorea nipponica* on melanoma *in vitro* and *in vivo*, Evid. Based Complement. Alternat. Med. 2011 (2011) 507920.
[6] X. He, A. Qiao, X. Wang, B. Liu, M. Jiang, L. Su, X. Yao, Structural identification of methyl protodioscin metabolites in rats' urine and their antiproliferative activities against human tumor cell lines, Steroids 71 (2006) 828–833.
[7] R. Aquino, C. Conti, F. De Simone, N. Orsi, C. Pizza, M.L. Stein, Antiviral activity of constituents of *Tamus communis*, J. Chemother. 3 (1991) 305–309.
[8] M. Sautour, A.C. Mitaine-Offer, T. Miyamoto, A. Dongmo, M.A. Lacaille-Dubois, Antifungal steroid saponins from *Dioscorea cayenensis*, Planta Med. 70 (2004) 90–92.
[9] C.S. Kwon, H.Y. Sohn, S.H. Kim, J.H. Kim, K.H. Son, J.S. Lee, J.K. Lim, J.S. Kim, Anti-obesity effect of *Dioscorea nipponica* Makino with lipase-inhibitory activity in rodents, Biosci. Biotechnol. Biochem. 67 (2003) 1451–1456.

[10] K. Hu, A. Dong, X. Yao, H. Kobayashi, S. Iwasaki, Antineoplastic agents; I. Three spirostanol glycosides from rhizomes of *Dioscorea collettii* var. *hypoglauca*, Planta Med. 62 (1996) 573–575.
[11] K.J. Kim, W.S. Sung, S.K. Moon, J.S. Choi, J.G. Kim, D.G. Lee, Antifungal effect of silver nanoparticles on dermatophytes, J. Microbiol. Biotechnol. 18 (2008) 1482–1484.
[12] C. Park, D.G. Lee, Fungicidal effect of antimicrobial peptide arenicin-1, Biochim. Biophys. Acta 1788 (2009) 1790–1796.
[13] J. Lee, J.S. Hwang, B. Hwang, J.K. Kim, S.R. Kim, Y. Kim, D.G. Lee, Influence of the papiliocin peptide derived from *Papilio xuthus* on the perturbation of fungal cell membranes, FEMS Microbiol. Lett. 311 (2010) 70–75.
[14] W.S. Sung, D.G. Lee, The combination effect of Korean red ginseng saponins with kanamycin and cefotaxime against methicillin-resistant *Staphylococcus aureus*, Biol. Pharm. Bull. 31 (2008) 1614–1617.
[15] S.C. Park, M.H. Kim, M.A. Hossain, S.Y. Shin, Y. Kim, L. Stella, J.D. Wade, Y. Park, K.S. Hahm, Amphipathic α-helical peptide, HP (2–20), and its analogues derived from *Helicobacter pylori*: pore formation mechanism in various lipid compositions, Biochim. Biophys. Acta 1778 (2008) 229–241.
[16] M.I. Angelova, D.S. Dimitrov, Liposome electroformation, Faraday Discuss. Chem. Soc. 81 (1986) 303–311.
[17] M.I. Angelova, S. Sloeau, P. Meleard, J.F. Faucon, P. Bothorel, Preparation of giant vesicles by external fields. Kinetics and application, Prog. Colloid Polym. Sci. 89 (1992) 127–131.
[18] S.C. Park, J.Y. Kim, C. Jeong, S. Yoo, K.S. Hahm, Y. Park, A plausible mode of action of pseudin-2, an antimicrobial peptide from *Pseudis paradoxa*, Biochim. Biophys. Acta 1808 (2011) 171–182.
[19] S. Herman, N. Zurgil, M. Deutsch, Low dose methotrexate induces apoptosis with reactive oxygen species involvement in T lymphocytic cell lines to a greater extent than in monocytic lines, Inflamm. Res. 54 (2005) 273–280.
[20] A.A. Volmer, A.M. Szpilman, E.M. Carreira, Synthesis and biological evaluation of amphotericin B derivatives, Nat. Prod. Rep. 27 (2010) 1329–1349.
[21] R.D. Santo, Natural products as antifungal agents against clinically relevant pathogens, Nat. Prod. Rep. 27 (2010) 1084–1098.
[22] F.C. Odds, H.V. Bossche, Antifungal activity of itraconazole compared with hydroxy-itraconazole *in vitro*, J. Antimicrob. Chemother. 45 (2000) 371–373.
[23] C. Pina-Vaz, F. Sansonetty, A.G. Rodrigues, Cytometric approach for a rapid evaluation of susceptibility of *Candida* strains to antifungals, Clin. Microbiol. Infect. 7 (2001) 609–618.
[24] A. Makovitzki, Y. Shai, pH-dependent antifungal lipopeptides and their plausible mode of action, Biochemistry 44 (2005) 9775–9784.
[25] M. Tanaka, H. Kanatsuka, B.H. Ong, T. Tanikawa, A. Uruno, T. Komaru, R. Koshida, K. Shirato, Cytochrome P-450 metabolites but not NO, PGI2, and H_2O_2 contribute to ACh-induced hyperpolarization of pressurized canine coronary microvessels, Am. J. Physiol. Heart Circ. Physiol. 285 (2003) 1939–1948.
[26] H. Choi, J. Cho, Q. Jin, E.R. Woo, D.G. Lee, Antifungal property of dihydrodehydrodiconiferyl alcohol 9′-O-β-D-glucoside and its pore-forming action in plasma membrane of *Candida albicans*, Biochim. Biophys. Acta 1818 (2012) 1648–1655.
[27] O. Wesołowska, K. Michalak, J. Maniewska, A.B. Hendrich, Giant unilamellar vesicles — a perfect tool to visualize phase separation and lipid rafts in model systems, Acta Biochim. Pol. 56 (2009) 33–39.
[28] I. Zelezetsky, S. Pacor, U. Pag, N. Papo, Y. Shai, H.G. Sahl, A. Tossi, Controlled alteration of the shape and conformational stability of alpha-helical cell-lytic peptides: effect on mode of action and cell specificity, Biochem. J. 390 (2005) 177–188.
[29] M.A. Gordon, E.W. Lapa, M.S. Fitter, Susceptibility of zoopathogenic fungi to phytoalexins, Antimicrob. Agents Chemother. 17 (1980) 120–123.
[30] R.J. Grayer, J.B. Harborne, A survey of antifungal compounds from higher plants, 1982–1993, Phytochemistry 37 (1994) 19–42.
[31] W.S. Sung, I.S. Lee, D.G. Lee, Damage to the cytoplasmic membrane and cell death caused by lycopene in *Candida albicans*, J. Microbiol. Biotechnol. 17 (2007) 1797–1804.
[32] M.J. Abad, M. Ansuategui, P. Bermejo, Active antifungal substances from natural sources, ARKIVOC 7 (2007) 116–145.
[33] T. Suzuki, K. Fujikura, T. Higashiyama, K. Takata, DNA staining for fluorescence and laser confocal microscopy, J. Histochem. Cytochem. 45 (1997) 49–53.
[34] Y. Tamba, H. Ariyama, V. Levadny, M. Yamazaki, Kinetic pathway of antimicrobial peptide magainin 2-induced pore formation in lipid membranes, J. Phys. Chem. B 114 (2010) 12018–12026.
[35] Y. Tamba, T. Tanaka, T. Yahagi, Y. Yamashita, M. Yamazaki, Stability of giant unilamellar vesicles and large unilamellar vesicles of liquid-ordered phase membranes in the presence of Triton X-100, Biochim. Biophys. Acta 1667 (2004) 1–6.
[36] M. Wu, R.E. Hancock, Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane, J. Biol. Chem. 274 (1999) 29–35.
[37] A.L.A. Ruissen, J. Groenink, E.J. Helmerhorst, E. Walgreen-Weterings, W.V. Hof, E.C.I. Veerman, A.V.N. Amerongen, Effects of histatin 5 and derived peptides on *Candida albicans*, Biochem. J. 356 (2001) 361–368.
[38] P.J. Sims, A.S. Waggoner, C.H. Wang, J.F. Hoffman, Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles, Biochemistry 13 (1974) 3315–3330.
[39] R.S. Liao, R.P. Rennie, J.A. Talbot, Assessment of the effect of amphotericin B on the vitality of *Candida albicans*, Antimicrob. Agents Chemother. 43 (1999) 1034–1041.
[40] S. Pacor, A. Giangaspero, M. Bacac, G. Sava, A. Tossi, Analysis of the cytotoxicity of synthetic antimicrobial peptides on mouse leucocytes: implications for systemic use, J. Antimicrob. Chemother. 50 (2002) 339–348.
[41] M.L. Nelson, M.C. Grier, S.E. Barbaro, M.Y. Ismail, Polyfunctional antibiotics affecting bacterial membrane dynamics, Antiinfect. Agents Med. Chem. 8 (2009) 3–16.
[42] R.S. Klein, C.A. Harris, C.B. Small, B. Moll, M. Lesser, G.H. Friedland, Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome, N. Engl. J. Med. 311 (1984) 354–358.