Vacuole Disruption as the Primary Fungicidal Mechanism of Action of Polygodial, a Sesquiterpene Dialdehyde

Takuya Kondo1, Yusuke Takaochi1, Yoshihiro Yamaguchi1,2, Akira Ogita1,3, Ken-ichi Fujita1, Toshio Tanaka1
1 Graduate School of Science, Osaka City University, Osaka, Japan
2 OCU Advanced Research Institute for Natural Science and Technology, Osaka City University, Osaka, Japan
3 Research Center for Urban Health and Sports, Osaka City University, Osaka, Japan

Abstract

The fungicidal mechanism of action of polygodial was examined in malt extract medium in which lethality against Saccharomyces cerevisiae was achieved at a much lower dose than in glucose-containing nutrient medium. Vacuolar membrane disruption, rather than cellular oxidative stress induction and damage to the plasma membrane, was considered the most likely lethal event underlying these observations. Vacuolar membrane disruption and polygodial-mediated lethality were similarly observed against the pathogenic fungi Candida albicans and Aspergillus niger.

Key words

polygodial · fungicidal activity · vacuole · Saccharomyces cerevisiae

The fungicidal activity of amphotericin B (AmB; Fig. 1A), a polyene macrolide, is attributed mainly to its ability to form a molecular complex with plasma membrane-embedded ergosterol [1]. This complex functions as an ion channel, enhancing the leakage of intracellular K+ and other ionic substances. AmB-mediated lethality is alternatively attributed to the induction of oxidative stress via the enhanced generation of superoxide anions by this macrolide, as well as to its ability to extract ergosterol from the plasma membrane phospholipid bilayer [2, 3]. In previous studies, for the first time, we found that AmB is incorporated across the plasma membrane of fungal cells, resulting in vacuolar membrane disruption with lethal effects [4–6]. Such vacuole-targeting fungicidal activity is also exerted by niphymycin from Streptomyces sp., as well as by polymyxin B from Bacillus polymyxa, with both selective and effective lethal effects against fungal cells [4]. To date, however, vacuole-disrupting activity has been reported only for microbial metabolites, not for plant-derived compounds.

Polygodial (Fig. 1B), a bicyclic sesquiterpene dialdehyde, was first isolated as a pungent principle from the sprout of Polygonum hydropiper L. (Polygonaceae), known as “tade”, which is used as a food spice in Japan [7, 8]. The biological activity of polygodial has been reported to include antifungal effects [9–12]. The biological mechanism of action of polygodial involves structural and functional damage to the plasma membrane, which is accompanied by the leakage of intracellular components. Polygodial-induced plasma membrane damage is attributed to the enhanced induction of cellular oxidative stress by this compound, as reflected by a marked increase in the level of mitochondrial reactive oxygen species (ROS) production [13]. It is still not known whether polygodial-induced lethality is dependent on plasma membrane damage and ROS production.

In this study, we examined the fungicidal activity of polygodial under conditions in which cells were protected from oxidative stress and plasma membrane damage. Vacuole disruption was identified as the most likely mechanism by which polygodial exerts lethal effects against Saccharomyces cerevisiae and other pathogenic fungal strains.

We first examined the relationship between lethality and ROS production by polygodial treatment of S. cerevisiae cells in 2.5% malt extract medium. As shown in Fig. 2A, polygodial was not lethal to S. cerevisiae cells at a concentration of 2 µM; however, as is the case with AmB, this sesquiterpene dialdehyde was found to markedly reduce the viable cell number to around 0.1% of the original level at 10 µM over the course of a 2-h incubation. The lethality of polygodial was far lower in nutrient-rich medium consisting of 2.0% (w/v) glucose, 2.0% (w/v) peptone, and 1.0% (w/v) yeast extract (YPD); in the latter, the viable cell number was reduced to 15% of the original level, even at a polygodial concentration of 100 µM, during 2 h of incubation [13]. In YPD medium, polygodial, at a concentration of 100 µM, reduced the viable cell number via an increase in mitochondrial ROS production in cells of the parent strain, but not in the respiration-deficient mutant with polygodial-resistant phenotype [13]. We therefore examined the level of ROS production in polygodial-treated cells in malt extract medium. In agreement with a previous finding, polygodial, at a concentration of 100 µM, was found to markedly increase the level of ROS production in the medium. The observed increase was comparable to that induced by an isoprenoid farnesol due to hyperpolarization of the mitochondrial transmembrane potential [14], as shown in Fig. 2B. At a concentration of 10 µM, polygodial did not elicit an elevation in cellular ROS production levels; however, lethal effects were observed at this concentration, suggesting that polygodial exhibits lethal activity against fungal cells via modes of action other than the induction of oxidative stress.

We therefore examined the possibility that polygodial damages the vacuole, a recently identified fungicidal target, resulting in...
the disruption of vacuolar membrane architecture. As shown in Fig. 2C, the yeast vacuole was observed to possess a swollen, rounded architecture in untreated cells. In addition, no structural alteration was observed in cells treated with polygodial at the nonlethal concentration of 2 µM. However, even under nonlethal conditions, the entire luminal space exhibited abnormal staining with the fluorescent dye FM4–64, suggesting the impairment of normal transport function across the vacuolar membrane (Fig. 2C). In cells treated with polygodial at the lethal concentration of 10 µM, the dye was found to be scattered throughout the cytoplasm, in which normal vacuolar membrane architecture could not be observed clearly, reflecting the disruption of the vacuolar membrane into smaller fragments. The observed mode of vacuolar membrane disruption by polygodial appeared to be similar to that of AmB-treated cells (Fig. 2C).

The simultaneous addition of K⁺ and Mg²⁺ is known to protect S. cerevisiae cells against AmB lethality, suggesting their maintenance effects against the loss of viability of polygodial-treated cells. As shown in Fig. 3C, D, polygodial was found to exert a marked lethal effect with serious vacuolar membrane disruptive damage similarly in the absence as well as in the presence of both ions. The result suggests that the vacuole-targeting action of polygodial contributed to cell death more significantly than that of AmB in the malt extract medium.

We finally examined whether polygodial elicits disruption of the vacuole membrane in the pathogenic fungal strains Candida albicans and Aspergillus niger. The vacuole was found to exhibit a swollen, spherical architecture in untreated C. albicans cells; however, the organelles were observed as small discrete dots in the cytoplasm of cells treated with polygodial at a lethal concentration (Fig. 4A, B). Polygodial was also found to cause vacuole disruption in the filamentous cells of A. niger at the higher concentration of 150 µM (Fig. 4C). This concentration is equivalent to the value at which fungal spore germination is inhibited (data not shown).

Vacuoles are organelles that are involved in osmoregulation, ion and pH homeostasis, metabolite accumulation, and macromolecular degradation in fungal cells [17]. Vacuoles are additionally involved in autophagic processes required for the degradation of cytoplasmic components as well as the cellular stress response [18,19]. Therefore, structural and functional damage to this organelle is considered to trigger cell death [4–6]. The antifungal property of polygodial has been attributed to its disruptive effects against the phospholipid bilayers of the plasma membrane, which is thought to occur via the induction of cellular oxidative stress. Our findings suggest that the vacuole-targeting fungicidal activity of polygodial may represent a novel mechanism of action of the compound, which is distinct from those previously reported. Our findings are additionally considered to be of potential therapeutic significance: the selective enhancement of the vacuole-targeting fungicidal activity of polygodial, via the structural modification of this compound, should enable its application in clinical settings.

Fig. 2 Effects of polygodial on cell viability, ROS production, and vacuole disruption. Cells were incubated in 2.5% malt extract medium containing polygodial at the indicated concentration, at 30°C, with vigorous shaking for measurement of the viable cell number (A) and cellular ROS production (B). Data are shown as means ± SD of triplicate experiments (A, B). Cells were additionally used for microscopic observation of vacuole morphology (C); bar, 2 µm. AmB (A, C) and farnesol (B) were used as positive controls. (Color figure available online only.)
Materials and Methods

Measurement of cell growth and viability
S. cerevisiae W303-1A and C. albicans IFO 1061 cells were grown overnight in malt extract medium (2.5% malt extract, pH 5.0) at 30°C with vigorous shaking. Then, cells were inoculated to 1×10⁷ cells/mL in freshly prepared malt extract medium (S. cerevisiae) or RPMI 1640 medium (C. albicans) containing polygodial or AmB at various concentrations. Cell suspensions were then incubated with vigorous shaking at 30°C, and plated on YPD medium containing 1.8% (w/v) agar. Cell viability was determined as the number of colonies formed after a 48-h incubation at 30°C.

Vacuole staining
Vacuoles were stained with the fluorescent probe FM4–64 [N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)hexatrienyli]pyridinium dibromide] according to previously described methods [20, 21], with some modifications, depending on the fungal strain used, as follows. In the case of S. cerevisiae and C. albicans, cells were grown overnight in malt extract medium and harvested by centrifugation. Cells were then incubated with vigorous shaking at 30°C, and plated on YPD medium containing 1.8% (w/v) agar. Cell viability was determined as the number of colonies formed after a 48-h incubation at 30°C.

Assay of cellular ROS production
The effect of polygodial on cellular ROS production was assayed using a previously described method [22, 23]. Cells from an overnight culture were inoculated into freshly prepared YPD medium to a density of approximately 1×10⁷ cells/mL. Cells were then incubated with 40 μM 2,7’-dichlorodihydrofluorescein diacetate (DCHF-DA) at 30°C for 60 min, collected by centrifugation, and suspended in an equal volume of 2.5% malt extract medium. Cell suspensions were then washed and resus-
Pended in 50 mM succinate buffer (pH 6.0). Fluorescence from the cells was then measured with the excitation at 485 nm and emission at 530 nm.

Chemicals
Polygodial (97% purity) and farnesol (89% purity) were purchased from Wako Pure Chemical Industries, Ltd. AmB (80% purity) and FM4–64 was obtained from Sigma-Aldrich. DCFH-DA was purchased from Molecular Probes. All other reagents were of analytical grade. Malt extract medium was purchased from Oriental Yeasts Co.

Statistical analysis
Statistical evaluation was performed using Student’s t-test; p < 0.05 was considered to represent statistical significance.

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Conflicts of Interest
The authors declare no conflicts of interest.

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