

Dehydrozingerone Exhibits Synergistic Antifungal Activities in Combination with Dodecanol against Budding Yeast via the Restriction of Multidrug Resistance



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ABSTRACT

Drug resistance in fungal infections has been a more frequent occurrence with the increasing number of immunocompromised patients. In efforts to overcome the problem of fungal drug resistance, we focused on the phenolic compound dehydrozingerone, which is isolated from *Zingiber officinale*. The effectiveness of this compound on the model yeast *Saccharomyces cerevisiae* has not been reported. In our study, dehydrozingerone showed a weak antifungal activity against the yeast, but demonstrated a synergistic effect in combination with dodecanol, which typically only restricts cell growth transiently. Efflux of rhodamine 6G through the multidrug efflux pumps was significantly restricted by dehydrozingerone. The transcription level of *PDR5*, encoding a primary multidrug efflux pump in *S. cerevisiae*, was enhanced with dodecanol treatment, whereas the level was reduced by dehydrozingerone. These results suggest that dehydrozingerone may be effective for potentiating antifungal activity of other drugs that are expelled from fungi by multidrug transporters like Pdr5p.

Abbreviations

ABC	ATP-binding cassette
CFU	colony forming unit
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein diacetate
DMF	<i>N,N</i> -dimethylformamide
MFC	minimum fungicidal concentration

MIC	minimum growth inhibitory concentration
qRT-PCR	real-time quantitative reverse transcriptase-polymerase chain reaction
R6G	rhodamine 6G
RNA	ribonucleic acid
ROS	reactive oxygen species
YPD	yeast extract peptone dextrose

Introduction

With the increasing number of immunocompromised patients, fatal deep-seated mycoses frequently occur due to opportunistic invasive fungal infections [1]. Fungi and humans are both eukaryotic organisms. Because both are eukaryotes, the number of targets for antifungal drugs like ergosterol, the fungal cell wall, and cytosine deaminase has been limited due to their common or similar metabolisms and cellular structures [2]. Therefore, it is difficult to develop antifungals with fewer adverse effects and new modes of action.

Adding to the problem is the development of drug resistance. Clinical isolates have been reported to show resistance to antifungals such as azoles, including fluconazole [3] and 5-fluorocytosine [4]. The occurrence of pathogenic *Candida* clinical isolates with lower susceptibility to echinocandins has also been reported [5]. Therefore, strategies for overcoming drug resistance are needed in order to improve antifungal chemotherapy.

Mechanisms of drug resistance are generally classified as enzymatic degradation of the antifungals, the inability of the antifungals to bind to target sites due to mutation of their genes, or expulsion of antifungals to the extracellular space [6]. Among these, fungi have particularly developed various multidrug efflux pumps such as ATP-dependent ABC transporters that expel harmful drugs from the fungi [7–9].

The polyphenol curcumin (► Fig. 1) is a main constituent of turmeric isolated from the rhizomes of *Curcuma longa* L., a member of the ginger family (Zingiberaceae). Curcumin is reported to reverse the effects of multidrug resistance in human colon carcinoma, human gastric carcinoma, and human osteosarcoma cell lines [10–12]. Furthermore, curcumin is reported to modulate drug efflux in the nonpathogenic model yeast *Saccharomyces cerevisiae* cells overexpressing the ABC transporter Pdr5p, and the *Candida albicans* ABC transporters Cdr1p and Cdr2p [13]. Even with the assistance of surfactants, curcumin is difficult to dissolve in water-based solutions because of its high hydrophobicity. Thus, this compound seems to be unsuitable for use in clinical applications in addition to food protection.

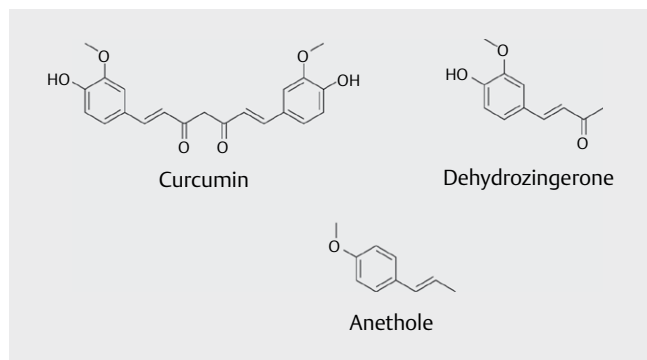
In order to improve drug solubility in water, we selected, as a promising new candidate, the phenolic compound dehydrozingerone [(E)-4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one] (synonyms, feruloylmethane, and vanillylideneacetone; ► Fig. 1). Dehy-

drozingerone is a water-soluble and half structural analog of curcumin (► Fig. 1), and a pungent constituent in the rhizomes of ginger *Zingiber officinale* Roscoe (Zingiberaceae) [14]. On the other hand, the phenylpropanoid *trans*-anethole (► Fig. 1), a principal constituent of anise oil, demonstrates synergistic and durable antifungal effects against *S. cerevisiae* via its induced inhibition of gene expression of the fungal multidrug efflux pumps, primarily Pdr5p [15]. Therefore, phenols, including dehydrozingerone, in addition to curcumin, in which phenylpropanoid-like structures are involved, were also expected to show such an effect. Dehydrozingerone has been reported to show various biological activities, including antitumor, antioxidative, anti-amyloid, and anti-inflammatory activities [14, 16]. In addition, its antifungal activities against *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Fusarium oxysporum*, and *Penicillium chrysogenum* have also been reported [17]. However, its antifungal potency against these fungi were weaker than the antifungal agents that were already available on the market.

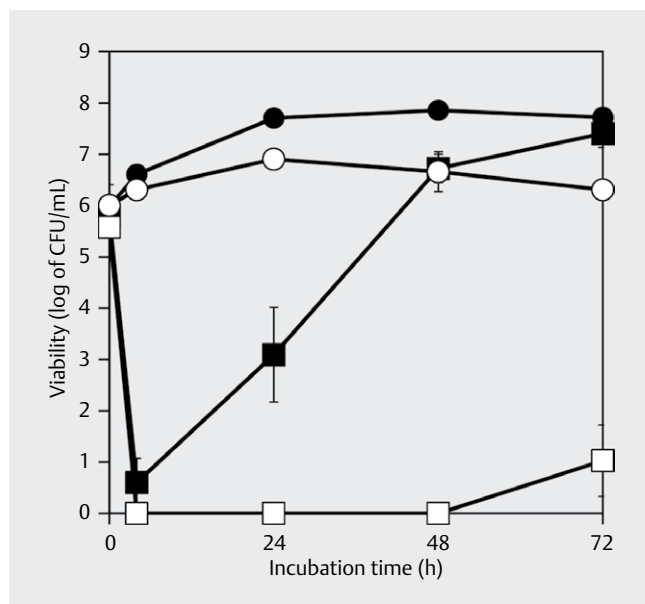
Since it did show some antifungal activity, dehydrozingerone was expected to show synergistic and potentially durable antifungal activity when combined with other drugs due to its ability to restrict drug efflux from the fungi. In the current study, we evaluated the effects of dehydrozingerone with the antifungal action of the model antifungal agent *n*-dodecanol, which transiently restricts fungal growth [15]. In addition, we investigated the function of drug efflux, and the gene expression of *PDR5*, a primary multidrug efflux pump in *S. cerevisiae*, which is upregulated by dodecanol [15]. Findings from these studies and the structure-activity relationships of dehydrozingerone as a lead compound may be beneficial in developing new strategies for overcoming drug resistance and improving antifungal chemotherapy.

Results

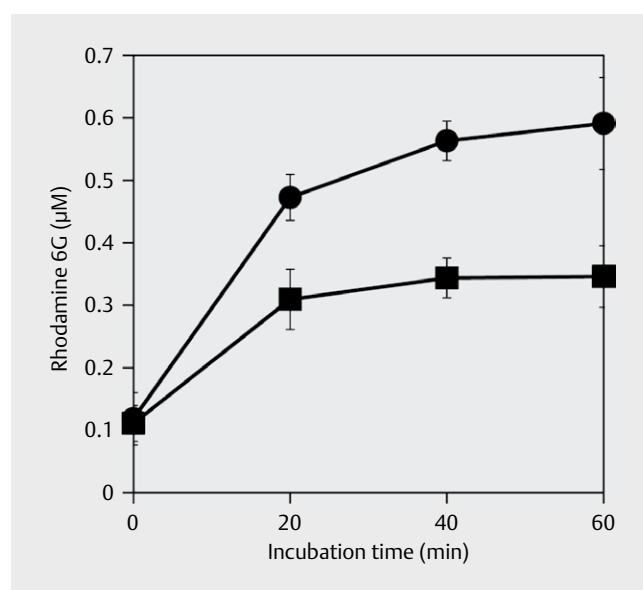
We here examined the effects of dehydrozingerone and dodecanol, separately and in combination, on the growth of *S. cerevisiae* based on CFU measurements (► Fig. 2). The MIC of dehydrozingerone was greater than 4000 μ M against *S. cerevisiae* (► Table 1). Its efficacy was less than that of the phenylpropanoid *trans*-anethole (► Table 1). Treatment with 2000 μ M dehydrozingerone failed to show any definitive fungicidal activity against the yeast cells, and demonstrated only weak inhibitory effects on fungal growth in a time-kill assay (► Fig. 2). On the other hand, dodecanol is reported to show a rapid fungicidal effect against *S. cerevisiae*, but its effect is not long lasting, with only transient activity [15]. The MIC of dodecanol was 40 μ M against *S. cerevisiae* at 24 h post-drug exposure. At post-drug exposure times beyond 24 h, the antifungal activity of dodecanol was not observed (> 2000 μ M; ► Table 1). Rapid reduction and restoration of cell viability were observed within 24 h of drug exposure with 156 μ M dodecanol, indicating the transient fungicidal activity induced by the fatty alcohol (► Fig. 2). However, beyond 48 h incubation, viability was recovered to levels seen in the control cells. These results suggested that dodecanol was unable to induce a fungicidal effect following a long period after drug exposure. However, cell viability was almost completely inhibited 72 h after drug exposure when the cells were treated with 2000 μ M dehydrozingerone in



► Fig. 1 Chemical structures of curcumin, dehydrozingerone, and *trans*-anethole.



► **Fig. 2** Effect of dehydrozingerone on dodecanol-induced temporary cell death of *S. cerevisiae* BY4741. Yeast cells were grown in YPD medium at 30 °C. Drugs were added to the culture as follows: none (closed circle), 156 µM dodecanol (closed square), 2000 µM dehydrozingerone (open circle), or 156 µM dodecanol + 2000 µM dehydrozingerone (open square). Data are means ± standard deviations of 3 independent experiments.



► **Fig. 3** Effect of dehydrozingerone on the drug efflux of R6G. Yeast cells were incubated without shaking at 30 °C in PBS containing 10 mM glucose with 2000 µM dehydrozingerone (closed square) or without dehydrozingerone (closed circle). The fluorescence intensity of the supernatant was measured using a GENios microplate reader with 485 nm excitation and 535 nm emission filters. Data are means ± standard deviations of 3 independent experiments.

► **Table 1** MICs of anethole, dodecanol, and dehydrozingerone in *S. cerevisiae*.

Compounds	MIC (µM)
Anethole	1250
Dodecanol	>2000
Dehydrozingerone	>4000

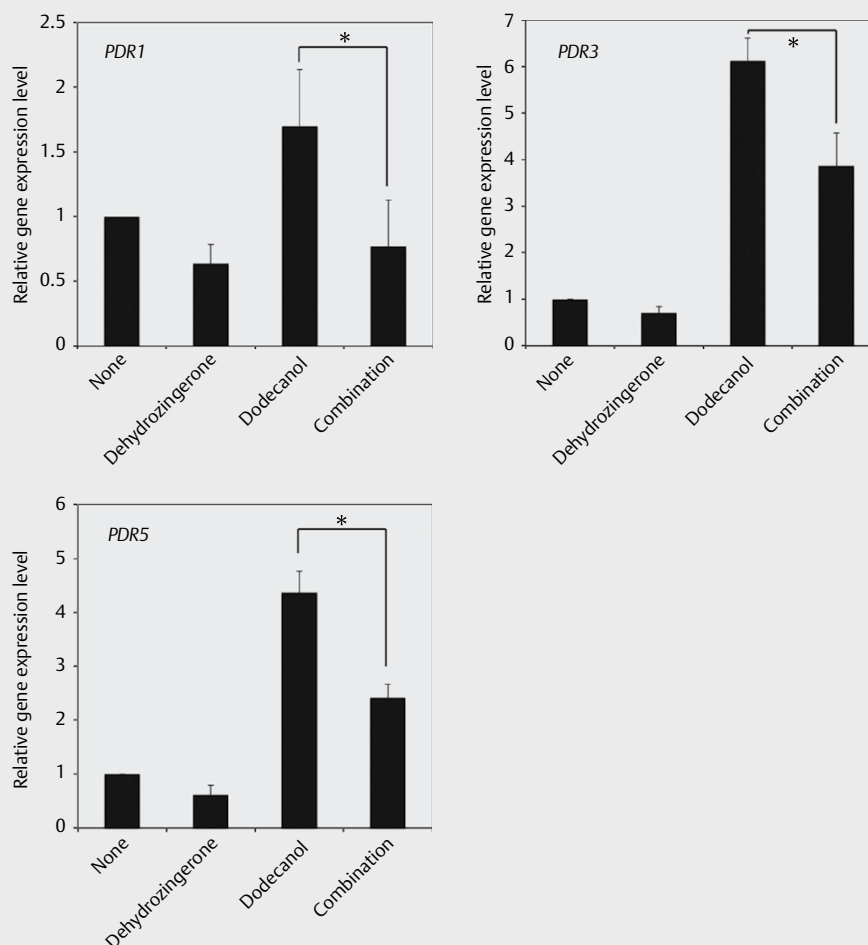
combination with 156 µM dodecanol (► **Fig. 2**). In addition, synergistic MFCs were also observed at 31.25 µM dodecanol and 500 µM dehydrozingerone. These results suggested that the dehydrozingerone sustained the transient fungicidal effect on *S. cerevisiae* from dodecanol alone.

We examined whether dehydrozingerone could restrict glucose-induced R6G efflux in *S. cerevisiae*. When the cells were treated without dehydrozingerone, the fluorescent intensity derived from R6G exhausted into supernatants was elevated linearly with increased incubation times (► **Fig. 3**). In comparison, when the yeast was treated with 2000 µM dehydrozingerone, the level of fluorescence decreased by 58.5 % compared with the control cells that were not exposed to the drug. These results suggested that dehydrozingerone significantly restricted the drug efflux, possibly being depend on glucose-stimulated multidrug efflux pumps. Therefore, we next examined the effects of dehydrozingerone on the expression of genes related to the multidrug efflux pumps.

The expression of *PDR5*, which encodes a primary multidrug efflux pump in *S. cerevisiae*, resulted in dodecanol being removed from the cell [15]. On the other hand, *PDR5* may be suppressed by

dehydrozingerone in a fashion similar to that observed with curcumin treatment. Based on qRT-PCR analysis, when the cells were treated with 2000 µM dehydrozingerone, the transcription levels of *PDR5* were similar to those seen in control cells not treated with dehydrozingerone (► **Fig. 4**). When the cells were treated with 156 µM dodecanol, the *PDR5* transcription levels were elevated approximately 4.5-fold above that observed in non-treated cells. However, in treatment of the yeast with the combination of 156 µM dodecanol and 2000 µM dehydrozingerone, a 45 % reduction was observed in the transcription level compared with the dodecanol only treatment. These results suggested that dehydrozingerone counteracted the activity of the drug efflux pumps, thereby reducing the intracellular levels of dodecanol by also suppressing the increased expression of *PDR5* that dodecanol stimulated. As a result, the lack of recovery in cell viability was probably maintained long term after the exposure to dodecanol.

Next, we measured the transcription levels of *PDR1* and *PDR3*, which are transcription factors of *PDR5* [18], in the cells treated with dodecanol and/or dehydrozingerone. Pdr1p encoded by *PDR1* responds to intracellular stress signals, after which it promotes the transcription of *PDR3* [19]. Conversely, Pdr3p encoded by *PDR3* regulates its transcription and that of *PDR5* [19, 20]. It was noted that the expression levels of *PDR1*, *PDR3*, and *PDR5* were unaffected by dehydrozingerone (► **Fig. 4**). Conversely, the expression levels of *PDR1* and *PDR3* in the cells were approximately 1.7- and 6.1-fold, respectively, higher after treatment with 156 µM dodecanol than they were without drug treatment. However, the combination of dehydrozingerone and dodecanol reduced the expression levels of



► **Fig. 4** Relative gene expression levels for *PDR1*, *PDR3*, and *PDR5* normalized to *ACT1*. *S. cerevisiae* cells were incubated in YPD medium containing 2000 μ M dehydrozingerone, 156 μ M dodecanol, or both prior to total RNA extraction and qRT-PCR analysis. Data are means \pm standard deviations of 3 independent experiments (* $p < 0.05$).

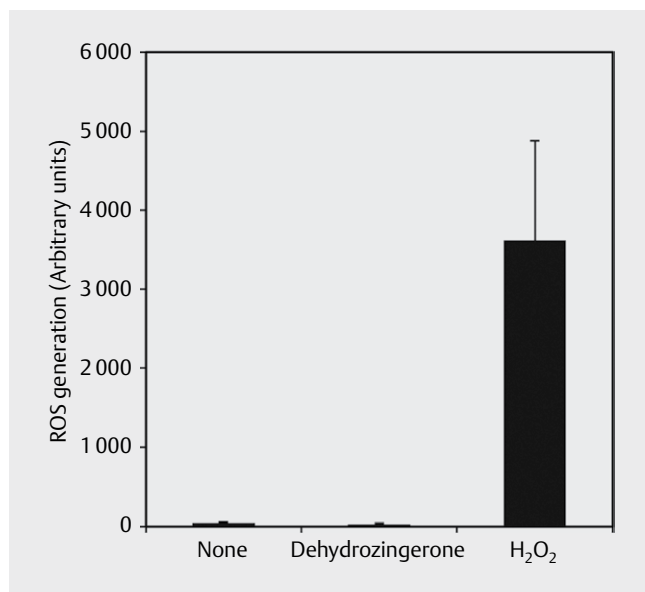
PDR1 and *PDR3* by 45 and 63 % compared to dodecanol-treated cells, respectively. These results suggest that dehydrozingerone prevents dodecanol-induced overexpression of *PDR1* and *PDR3* in addition to *PDR5*. This indicates that dehydrozingerone possibly maintains the accumulation of dodecanol in the cells, thereby preventing the restoration of cell viability. However, it is unclear whether dehydrozingerone directly affects the transcription of *PDR1*, *PDR3*, and *PDR5* or other genes.

The phenylpropanoid *trans*-anethole, a principal component of anise oil, is reported to exhibit synergistic antifungal activity against *S. cerevisiae* and *C. albicans* when combined with dodecanol and fluconazole, respectively [15]. This synergistic activity also is involved in the attenuation of gene expression related to multidrug efflux pumps [15]. Anethole is reported to induce the production of ROS against a nonpathogenic strain of *S. cerevisiae* and a strain of *Aspergillus fumigatus* that is pathogenic in humans [21]. In addition, dehydrozingerone induces cell-cycle arrest at the G2/M phase, accompanying the accumulation of cellular ROS in the human colon cancer cell line HT-29 [22]. If dehydrozingerone induces cellular ROS pro-

duction against fungi, including *S. cerevisiae*, the produced ROS may affect the expression of genes related to multidrug efflux. Therefore, we examined whether dehydrozingerone induced the production of cellular ROS. Hydrogen peroxide at a concentration of 2000 μ M greatly induced cellular ROS production (► **Fig. 5**). On the other hand, when the cells were treated with 2000 μ M dehydrozingerone, ROS production was not detected. This result suggested that decreased levels of gene expression, and R6G efflux caused by dehydrozingerone, were not dependent on ROS production.

Discussion

The MIC values of dehydrozingerone have been reported to be from 755 to 911 μ M against *A. ochraceus*, *A. niger*, *A. flavus*, *A. oryzae*, *P. chrysogenum*, and *F. oxysporum* [17]. The MFC values of this phenolic compound are from 880 to 1041 μ M against the same fungi described above [17]. Based on morphological alterations in hyphae and the subsequent cell lysis, the fungicidal activities may be a result of cell wall fragility [17]. Our study demonstrated a weak antifungal



► **Fig. 5** Effect of dehydrozingerone on ROS generation in *S. cerevisiae* cells. Exponentially growing *S. cerevisiae* cells were inoculated into 3 mL of YPD medium with or without 2000 μ M dehydrozingerone at 30 °C. Hydrogen peroxide (H₂O₂; 2 mM) was used as a positive control. Data are means \pm standard deviations of 3 independent experiments.

activity of dehydrozingerone against the yeast *S. cerevisiae*, consistent with the findings for the filamentous fungi. The antifungal activity against *S. cerevisiae* (MIC > 4000 μ M; ► **Table 1**) was weaker than that against other fungi previously reported [17]. Although we could not determine the distinct mechanism of the fungicidal activity, long-term exposure of dehydrozingerone in a nutrient medium induced a slight reduction of the cell viability that gradually increased following the cultivation of the cells in the presence of the drug (► **Fig. 2**). Generally, antimicrobials that affect the cell wall, and/or its metabolism, are accompanied with a decrease in cell viability and culture turbidity as a result of cell lysis [23, 24]. This may explain the harmful effect of dehydrozingerone on the cellular architecture of *S. cerevisiae*.

Dodecanol is a model drug in that it induces rapid and complete reduction in cell viability of *S. cerevisiae*, based on CFU analysis [15, 25]. However, the reduction is only temporary. During a temporal analysis of treatment, the cell viability is gradually restored to that of cells not treated with drugs due to drug efflux [15]. When the *S. cerevisiae* cells were co-treated with dehydrozingerone, the transient fungicidal activity of dodecanol was converted to a longer lasting one (► **Fig. 2**). Curcumin is reported to inhibit drug efflux based on analyses using R6G assays in *S. cerevisiae* cells overexpressing Pdr5p and *C. albicans* ABC transporters Cdr1p and Cdr2p [13]. In addition, curcumin, in a dose-dependent fashion, reduced MDR1-mediated drug efflux in cervical carcinoma cells showing multidrug resistance via direct interaction between curcumin and MDR1 proteins, a primary human multidrug efflux pump [26]. Dehydrozingerone at 2000 μ M, inducing a feeble growth inhibition (► **Fig. 2**), also inhibited R6G efflux (► **Fig. 3**), indicating that a half portion of curcumin was at least needed for the restriction of the drug efflux.

If dehydrozingerone decreases cytosolic levels of ATP as a result of its induced growth inhibition, R6G efflux, via ABC transporters driven by ATP, may be indirectly restricted.

Dodecanol is hypersensitive to a *S. cerevisiae* strain deficient in *PDR5* [15], a primary ABC transporter in *S. cerevisiae* for drug efflux [19]. Curcumin has been reported to regulate the mRNA expression of *MDR1* through inhibition of several signaling pathways related to phosphatidylinositol-4,5-bisphosphate 3-kinase, the serine/threonine protein kinase AKT, and nuclear factor-kappa B [27]. However, it is poorly understood whether curcumin directly binds to these proteins. Dehydrozingerone also restricted dodecanol-induced overexpression of *PDR1*, *PDR3*, and *PDR5* (► **Fig. 4**), indicating that the inhibition of drug efflux caused by dehydrozingerone at least occurs at the level of gene transcription. The reduction of the expression levels in *PDR1*, *PDR3*, and *PDR5* could not be explained only by cellular ATP depletion described above, as the expression levels of *ACT1*, encoding the single essential gene for a cytoskeletal protein actin, were not affected by the treatment of dodecanol and/or dehydrozingerone (data not shown).

In our previous study showing synergistic antifungal activity, anethole inhibited drug efflux via transcriptional repression of several efflux pump genes [15]. In addition, anethole induces cellular ROS production [21]. Therefore, transcriptional repression may be involved in the ROS production. Dehydrozingerone did not induce ROS production (► **Fig. 5**), suggesting that the transcriptional repression was probably not triggered by ROS production. These results may be applicable to the case of curcumin in regards to its inhibition of drug efflux.

Curcumin is undesirable for use in clinical applications because it is difficult to dissolve in water-based solutions. On the other hand, dehydrozingerone, a water-soluble compound, is a potential candidate for the suppression of fungal proliferation in combination with other drugs in which the drug efficacy is tempered or abolished by multidrug resistance. However, high concentrations of dehydrozingerone (at least 2000 μ M) were needed to exert such effects against *S. cerevisiae*. Therefore, it is difficult for dehydrozingerone to be directly applied to clinical use. Further studies on the structure-activity relationships of dehydrozingerone as a lead compound are needed for the enhancement of synergistic antifungal activities dependent on the restriction of multidrug resistance.

Materials and Methods

Strains and culture conditions

A parental strain *S. cerevisiae* BY4741 and its *pdr5* knockout mutant were obtained from the Yeast Knockout Strain Collection (Thermo Scientific Open Biosystems). Yeast cells were grown in YPD medium containing 1 % Bacto-yeast extract (Difco Laboratories), 2 % Bacto-peptone (Difco Laboratories), and 2 % D-glucose for 16 h at 30 °C without shaking in preparation for experimental use.

Chemicals

n-Dodecanol (purity 97 % by GC) was purchased from Kishida Chemical. *trans*-Anethole (purity > 98.0 % by GC) and dehydrozingerone (purity > 98.0 % by GC) were purchased from Tokyo Chemical Industry. DMF (purity > 99.0 % by GC) was purchased from Wako pure

chemicals. R6G (purity 99.0%) and DCFH-DA (purity >97.0%) were purchased from Sigma-Aldrich. Anethole, dodecanol, and dehydrozingerone were diluted with DMF, and R6G and DCFH-DA were diluted with ethanol prior to use.

Antifungal assay

Antifungal assays were performed using previously described methods [28, 29]. In general, serial 2-fold dilutions of the tested compounds were prepared in DMF, and 30 μ L of the 100-fold diluted concentration were added to 3 mL of YPD medium in a test tube (diameter 10 mm). The yeast cells were inoculated into the medium to give a final concentration of 10^6 CFU/mL. The cultures were incubated without shaking for 48 h at 30 °C. The MIC was determined as the lowest concentration of test compound at which no visible fungal growth was detected. After determining the MIC, an aliquot was withdrawn from each culture and diluted 100-fold using YPD medium. Following incubation for 48 h at 30 °C, the MFC was determined as the lowest concentration of the test compounds in which no recovery of the yeast cells was observed.

Time-kill assay

The yeast cells grown overnight in YPD medium were diluted into YPD medium to a concentration of 1×10^6 cells/mL. Dodecanol, dehydrozingerone, or combinations of the drugs were added to the yeast cultures. The treated cell suspensions were incubated at 30 °C without shaking within 72 h. The measurement of viable cell numbers, based on CFU, was determined after cultivation for 48 h at 30 °C on YPD agar plates.

Ribonucleic acid extraction and real-time quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the yeast cells using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Briefly, the yeast cells treated with dodecanol, dehydrozingerone, or a combination of the drugs as described above were collected by centrifugation at $5000 \times g$ for 10 min, and then lysed with zymolyase. The RNA samples were purified using the columns provided in the kit, and then DNase treated. The RNA samples were reverse transcribed to generate complementary DNA (cDNA) using ReverTra Ace (TOYOBO). The qRT-PCR was conducted using SsoAdvanced Universal SYBR Green Supermix (Bio Rad), using the cDNA as a template with a CFX Connect Real-Time PCR Detection Systems (Bio Rad). The 20 μ L qPCR reaction mixtures contained 10 μ L of 2 \times Supermix,

0.1 μ L of template, and 5 pmol/ μ L of both forward and reverse primers. The cycling profile was 3 min at 95 °C, followed by 40 sequential cycles of 10 s at 95 °C, 30 s at 55 °C. The relative expression levels of *PDR1*, *PDR3*, and *PDR5* were normalized against the expression of housekeeping gene *ACT1* as an internal positive control. The primers used in this study are listed in ► Table 2.

Efflux of rhodamine 6G

R6G efflux from the yeast cells was measured by the method of Niimi et al. [30] with slight modifications. Namely, the yeast cells from an overnight culture in YPD medium were centrifuged at $9600 \times g$ for 5 min at 27 °C. The harvested cells were washed twice with PBS, and resuspended in PBS. The cells were incubated with shaking at 30 °C for 12 h. The cells suspension was centrifuged at $9600 \times g$ for 5 min at 27 °C and resuspended to a cell density of 5×10^8 cells/mL in PBS. R6G was added to a concentration of 10 μ M, and the yeast cells were incubated at 30 °C for 60 min to allow uptake of the R6G dye. The R6G-treated cells were washed, and then resuspended at 7.5×10^7 cells/mL in PBS. The test compounds and 10 mM glucose were added to the suspension. One mL aliquots of the suspensions were collected at the indicated times and centrifuged at $2000 \times g$ for 30 s at 27 °C. The fluorescence intensity in the supernatant was measured with a Tecan GENios microplate reader using 485 nm excitation and 535 nm emission filters.

Measurement of reactive oxygen species generation

Cellular ROS generation was measured using a method dependent on intracellular deacetylation and oxidation of DCFH-DA to produce the fluorescent compound DCF, as described previously [21]. As reported, DCFH-DA as a probe is highly reactive with hydrogen peroxide (H_2O_2) and may be used in evaluating ROS generation in yeast cells [21]. After preincubation of the yeast cells (1×10^7 CFU/mL) in YPD medium with 40 μ M/mL of DCFH-DA at 30 °C for 60 min, 3.0 mL aliquots of the cell suspensions were treated with the test compounds for 60 min at 30 °C, and then were washed and resuspended in 350 μ L of PBS buffer. The fluorescence intensity of the cell suspension was determined with the Tecan GENios microplate reader (excitation at 480 nm and emission at 530 nm). The arbitrary units assigned to the results were based on the fluorescence intensity of 10^7 cells. H_2O_2 was used as a positive control.

Statistical methods

Statistical evaluation was performed using Student's t-test to compare the results between experimental groups. A *p*-value < 0.05 was considered statistically significant.

Acknowledgements

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Conflict of Interest

No conflict of interest has been declared by the authors.

► Table 2 Primer sets for qRT-PCR analysis.

Gene	Sequence (5' → 3')
<i>ACT1</i> -F	CGTCTGGATTGGTGGTCTATC
<i>ACT1</i> -R	GGACCACTTTCGTCGATTCTT
<i>PDR1</i> -F	GGAGCGAAGCTTTTGACAAC
<i>PDR1</i> -R	CTGCAGAAATGGTGCTCGTA
<i>PDR3</i> -F	GTTTGGGCATGTTGGACTT
<i>PDR3</i> -R	CCCGGTCAACTCTTTCAA
<i>PDR5</i> -F	ATTCACCAACCTATGCTATTT
<i>PDR5</i> -R	TTACAACCTTCGCCCAAGTC

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