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Curcumin potentiates the fungicidal effect of dodecanol by inhibiting drug efflux in wild-type budding yeast

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Running headline: Curcumin inhibits drug efflux

Significance and Impact of the Study:

Drug resistance is common in immunocompromised patients with fungal infections. Curcumin, isolated from *Curcuma longa*, inhibits drug efflux in non-pathogenic budding yeast *Saccharomyces cerevisiae* cells overexpressing ABC transporters *S. cerevisiae* Pdr5p and pathogenic *Candida albicans* Cdr1p and Cdr2p. We examined the effects of curcumin on multidrug resistance in a wild-type strain of the budding yeast with an intrinsic expression system of multidrug-efflux-related genes. Curcumin directly inhibited drug efflux and also suppressed *PDR5* expression, thereby enhancing antifungal effects. Thus, curcumin potentially promotes the efficacy of antifungals via its effects on ABC transporters in wild-type fungal strains.

Abstract

Drug resistance commonly occurs when treating immunocompromised patients who have fungal infections. Curcumin is a compound isolated from *Curcuma longa*. It has been reported to inhibit drug efflux in several human cell lines and non-pathogenic budding yeast *Saccharomyces cerevisiae* cells that overexpress the ATP-binding cassette transporters *S. cerevisiae* Pdr5p and pathogenic *Candida albicans* Cdr1p and Cdr2p. The

aim of this study was to examine the effects of curcumin on multidrug resistance in a wild-type strain of the budding yeast with an intrinsic expression system of multidrug-efflux-related genes. The antifungal activity of dodecanol alone was temporary against *S. cerevisiae*; however, restoration of cell viability was completely inhibited when the cells were co-treated with dodecanol and curcumin. Furthermore, restriction of rhodamine 6G (R6G) efflux from the cells and intracellular accumulation of R6G were observed with curcumin treatment. Reverse transcription-polymerase chain reaction analysis revealed that curcumin reduced dodecanol-induced overexpression of the ABC transporter-related genes *PDR1*, *PDR3*, and *PDR5* to their control levels in untreated cells. Curcumin can directly restrict glucose-induced drug efflux and inhibit the expression of the ABC transporter gene *PDR5,* and can thereby probably inhibit the efflux of dodecanol from *S. cerevisiae* cells. Curcumin is effective in potentiating the efficacy of antifungal drugs via its effects on ABC transporters.

Keywords

Curcumin, antifungal, *S. cerevisiae*, ABC transporter, multidrug resistance

52 **Introduction**

53 Immunocompromised patients usually develop deep-seated mycoses because of
54 opportunistic invasive fungal infections (Miceli *et al*. 2011). As fungi and humans are
55 eukaryotes, they are similar in cellular structure and metabolism. The primary targets of
56 antifungal drugs are ergosterol, the fungal cell wall, and cytosine deaminase.
57 Consequently, the efficacy of antifungal agents is limited due to their similar mechanisms
58 of action (Fairlamb *et al*. 2016). Therefore, it is difficult to develop antifungals with few
59 adverse effects and new modes of action.

60 Clinical isolates are reported to show resistance to antifungals, particularly
61 azoles, which include fluconazole (Masiá Canuto and Gutiérrez Rodero 2002), and 5-
62 fluorocytosine (Polak and Hartman 1991). An opportunistic pathogenic *Candida* species
63 with lower susceptibility to echinocandins has been isolated from humans (Gonçalves *et*
64 *al*. 2016). Therefore, strategies for overcoming drug resistance should be developed to
65 improve antifungal chemotherapy.

66 The mechanisms by which resistance occurs include enzymatic degradation or
67 modification of antifungals, inability of antifungals to bind targets sites due to mutation
68 of target site genes, and efflux of antifungals into the extracellular space (Ghannoum and

69 Rice 1999). Fungi can develop various multidrug efflux pumps, such as ATP-dependent
70 transporters (e.g., ATP-binding cassette (ABC) transporters), which transport drugs out
71 of the fungal cells (Cannon *et al*. 2009; Li and Nikaido 2009; Paul and Moye-Rowley
72 2014).

73 *trans*-Anethole is a phenylpropanoid (Fig. 1) and a principal constituent of
74 anise oil. It shows a synergistic antifungal effect against the budding yeast
75 *Saccharomyces cerevisiae* in combination with other antifungal agents by inhibiting the
76 gene expression of multidrug efflux pumps, mainly the ABC transporter Pdr5p (Fujita *et*
77 *al*. 2017). In a preliminary structure-activity relationship study on synergistic antifungal
78 activities, phenylpropanoids were found to inhibit drug efflux (data not shown). Therefore,
79 polyphenols are also expected to show this effect since they have a phenylpropanoid-like
80 structure.

81 Curcumin (Fig. 1) is a polyphenol and a main constituent of turmeric. It is
82 isolated from the rhizomes of *Curcuma longa*, which is part of the ginger family
83 (Zingiberaceae). Curcumin has been reported to reverse multidrug resistance in human
84 colon carcinoma, human gastric carcinoma, and human osteosarcoma cell lines (Tang *et*
85 *al*. 2005; Lu *et al*. 2013; Si *et al*. 2013). Furthermore, it modulates drug efflux in *S.*

cerevisiae cells that overexpress *S. cerevisiae* Pdr5p and the *C. albicans* ABC transporters Cdr1p and Cdr2p (Sharma *et al*. 2009).

In the present study, we investigated the combined effects of curcumin and the antifungal model agent dodecanol on multidrug resistance in a wild-type strain of *S. cerevisiae,* which has an endogenous expression system of multidrug-efflux-related genes. Namely, the study was performed without genetically manipulating the strain. Dodecanol was used because it shows a transient fungicidal action due to its efflux from fungal cells (Fujita *et al*. 2017)

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Results and discussion

Effect of curcumin on the antifungal action of dodecanol against *S. cerevisiae*

It has been reported that curcumin exhibits antifungal activity against *Cryptococcus neoformans*, *C. albicans, Rhizoctonia solani*, *Phytophthora infestans,* and *Erysiphe graminis*, but that its potency is quite weaker than that of antifungal agents on the market (Moghadamtousi *et al*. 2014). Moreover, details of its mechanism of antifungal action are poorly understood. Conversely, dodecanol is reported to show a rapid but temporal fungicidal effect on *S. cerevisiae* (Fujita *et al*. 2017). Our results confirmed the effects of

103 curcumin, dodecanol, and their combination on the growth of a wild-type strain of *S.*
104 *cerevisiae* based on measurements of colony forming units (CFU)(Fig. 2).
105 The minimum growth inhibitory concentration (MIC) of curcumin against *S.*
106 *cerevisiae* ATCC7754 could not be determined; that is, we could not perform the MIC
107 assay at concentrations more than 1000 μM because of the limited aqueous solubility of
108 curcumin. The MIC of dodecanol against *S. cerevisiae* was 40 μM after exposing the
109 fungus to the drug for 24 h; however, no antifungal activity was noted (MIC > 2000 μM)
110 when the exposure period was increased for a further 24 h. The results of the time-kill
111 assay showed that 313 μM curcumin did not affect proliferation of the yeast cells (Fig.
112 2). Furthermore, rapid reduction and restoration of cell viability were observed within 24
113 h of exposure to 156 μM dodecanol, indicating a transient fungicidal activity of the
114 alcohol. However, after 48 h of incubation, cell viability was restored to the control level.
115 These results suggest that curcumin and dodecanol as individual treatments do not
116 completely inhibit yeast growth for long periods. However, restoration of cell viability
117 was completely inhibited for 72 h when the cells were treated with 313 μM curcumin and
118 156 μM dodecanol concurrently. This suggests that curcumin sustained the temporary
119 fungicidal effect of dodecanol on *S. cerevisiae.*

120 Dodecanol was previously found to be resistant to gene deletion strains of
121 *PDR*3Δ and *PDR*5Δ (Fujita *et al*. 2017). Pdr5p is a major multidrug efflux pump and
122 Pdr3p is its transcription factor (MacPherson *et al*. 2006; Sipos and Kuchler 2006;
123 Yibmantasiri *et al*. 2014). This suggests that the intracellular dodecanol level was mainly
124 depleted by Pdr5p. However, this drug efflux system is possibly inhibited by curcumin to
125 maintain the intracellular dodecanol level, thereby inhibiting the growth of yeast cells.
126 Therefore, we examined the effect of curcumin on the activity of multidrug efflux pumps.
127
128 **Curcumin inhibits the efflux of R6G from *S. cerevisiae* cells**
129 Generally, the fluorescent dye rhodamine 6G (R6G) is passively incorporated into cells.
130 It is reported that Pdr5p is mainly responsible for the efflux of intracellular R6G (Egner
131 *et al*. 1998). In order to examine the effect of curcumin on the activity of multidrug efflux
132 pumps in R6G-prestained cells, the fluorescence derived from R6G in the supernatant of
133 the cell suspension was measured after the cells were treated with or without 312.5 μmol
134 l^{-1} curcumin.
135 It was noted that the fluorescent spectra of curcumin and R6G overlapped (data
136 not shown). Therefore, it is difficult to quantify R6G in the presence of curcumin. R6G

137 and curcumin were separated by HPLC as shown in Fig. 3. R6G fluorescence in the
138 supernatants was measured every 20 min after adding glucose to measure the total activity
139 of ATP-dependent transporters, primarily Pdr5p (Mamnun *et al*. 2004; Paul and Moye-
140 Rowley 2014). When the yeast cells were not treated with curcumin, the fluorescence
141 intensity of R6G increased linearly as incubation time was increased up to 60 min.
142 Conversely, when the cells were treated with 313 μmol l^{-1} curcumin, increase in
143 fluorescence intensity was apparently reduced (Fig. 4, left). This phenomenon is possibly
144 caused by a decrease in the total amount of intracellular R6G dependent on the
145 degradation of R6G. Therefore, we confirmed the intracellular R6G levels in the cells
146 treated with or without 313 μmol l^{-1} curcumin. The intracellular R6G level in the
147 curcumin-treated cells was 38% of that in untreated cells (Fig. 4, right), indicating the
148 intracellular accumulation of R6G induced by curcumin. These results suggest that
149 curcumin inhibits the total activity of multidrug efflux pumps.
150
151 **Curcumin inhibits the expression of genes related to efflux pumps in the presence of**
152 **dodecanol**
153 Curcumin suppresses the overexpression and function of the human multidrug resistance

154 (MDR1) gene (P-glycoprotein), thereby reversing the multidrug-resistant phenotype
155 (Anuchapreeda *et al*. 2006; Choi *et al*. 2008; Neerati *et al*. 2013). Moreover, it dose-
156 dependently reduces MDR1-mediated drug efflux in multidrug-resistant cervical
157 carcinoma cells via direct interaction with MDR1 proteins (Anuchapreeda *et al*. 2002).
158 In addition, curcumin has been reported to regulate the mRNA expression of MDR1 by
159 inhibiting several signalling pathways involving phosphatidylinositol-4, 5-bisphosphate
160 3-kinase, Akt, and nuclear factor-kappa B (Choi *et al*. 2008; Rodrigues *et al*. 2016).
161 Human MDR1 proteins are ABC transporter proteins (Riordan *et al*. 1985;
162 Roninson *et al*. 1986; Gulshan and Moye-Rowley 2007). In contrast, the multidrug-
163 resistant phenotype of *S. cerevisiae* is responsible for pleiotropic resistance (Balzi and
164 Goffeau 1995). *S. cerevisiae* was reported to possess at least 16 ABC multidrug transport
165 proteins (Chinen *et al*. 2011). Although curcumin modulates drug efflux in *S. cerevisiae*
166 cells overexpressing the ABC transporters Cdr1p, Cdr2p, and Pdr5p (Sharma *et al*. 2009),
167 no synergistic antifungal effects against wild-type fungal strains without genetic
168 manipulation, such as a stress-inducible overexpression of multidrug efflux pump-related
169 genes, have been reported.
170 Among the seven principal ABC transporters (*PDR5*, *PDR10*, *PDR11*, *PDR15*,

171 *SNQ2*, *YOR1*, and *YCF1*) in *S. cerevisiae*, *PDR*5Δ was found to be hypersensitive to
172 dodecanol (Fujita *et al*. 2017). Thus, we measured the relative gene expression of *PDR*5
173 and its transcription factors *PDR1* and *PDR3* (Salin *et al*. 2008) in the cells treated with
174 dodecanol and curcumin or only curcumin. Pdr1p encoded by *PDR1* responds to
175 intracellular stress signals, after which it promotes the transcription of *PDR3* (Sipos and
176 Kuchler 2006; Ma and Liu 2010). Conversely, Pdr3p encoded by *PDR3* regulates its
177 transcription and that of *PDR5* (Sipos and Kuchler 2006; Ma and Liu 2010). It was noted
178 that the expression levels of *PDR1*, *PDR3*, and *PDR5* were unaffected by curcumin (Fig.
179 5). Conversely, the expression levels of *PDR1*, *PDR3*, and *PDR5* in the cells were
180 approximately 3.0-, 3.1-, and 6.3-fold, respectively, higher after treatment with 32 μM
181 dodecanol than they were without drug treatment. However, as a combined treatment,
182 curcumin and dodecanol reduced the expression levels of the genes compared to their
183 respective control levels. These results suggest that curcumin prevents dodecanol-
184 induced overexpression of *PDR1*, *PDR3*, and *PDR5*. This indicates that curcumin
185 possibly maintains the accumulation of dodecanol in the cells, thereby preventing the
186 restoration of cell viability. However, it is unclear whether curcumin directly affects the
187 transcription of *PDR1*, *PDR3*, and *PDR5,* or other genes.

188 The inhibition of R6G efflux was first observed after 20 min of incubation with
189 curcumin (Fig. 4, left). Therefore, curcumin possibly inhibits the efflux activity of Pdr5p
190 due to direct interaction with the protein molecules of Pdr5p, degradation of Pdr5p, or
191 abnormality in localisation of Pdr5p, in addition to the restriction of *PDR5* transcription.
192 In the present study, curcumin and dodecanol showed a synergetic antifungal
193 activity against the nonpathogenic fungus *S. cerevisiae*. The human opportunistic
194 pathogen *C. albicans* possesses *CDR1* and *CDR2* genes as its primary multidrug pumps
195 (Sipos and Kuchler 2006). Cdr1p and Cdr2p are homologs of *S. cerevisiae* Pdr5p (Coste
196 *et al*. 2006). Therefore, curcumin might be effective in potentiating the effect of antifungal
197 drugs that undergo efflux by Cdr1p and/or Cdr2p (Sanguinetti *et al*. 2015), which include
198 azoles (e.g., fluconazole).
199 Although curcumin and dodecanol exhibited synergistic antifungal activity
200 against *S. cerevisiae,* curcumin must be further investigated for its clinical application
201 since it has poor aqueous solubility. For instance, it is reported that microencapsulating
202 curcumin improves its stability and solubility, as well as its antimicrobial effects against
203 several foodborne pathogens and spoilage microbes such as *Escherichia coli*, *Yersinia*

204 *enterocolitica*, *Staphylococcus aureus*, *Bacillus subtilis*, *B. cereus, Aspergillus niger*,
205 *Penicillum notatum*, and *S. cerevisiae* (Wang *et al*. 2009).
206 In, conclusion, curcumin directly inhibited drug efflux and also restricted *PDR5*
207 expression, thereby enhancing antifungal effects. Thus, curcumin potentially promotes
208 the efficacy of antifungals via its effects on ABC transporters in wild-type fungal strains.
209
210 **Materials and methods**
211 **Strain and culture conditions**
212 *S. cerevisiae* BY4741 and ATCC7754 were obtained from Yeast Knockout Collection
213 (Thermo Scientific Open Biosystems, Waltham, MA, USA) and American Type Culture
214 Collection (Manassas, VA, USA), respectively. The yeast cells were grown in 2.5% malt
215 extract broth (Oriental Yeast, Tokyo, Japan) for 16 h at 30°C without shaking prior to
216 performing the experiments.
217 **Chemicals**
218 *n*-Dodecanol was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Curcumin
219 and *N,N*-dimethylformamide (DMF) were purchased from Wako Pure Chemicals (Osaka,
220 Japan). R6G was purchased from Sigma-Aldrich (St. Louis, MO, USA). *n*-Dodecanol and

221 curcumin were diluted with DMF before use, whereas R6G was diluted with ethanol.

222 **Antifungal assay**

223 An antifungal assay was performed as previously described (Fujita and Kubo 2002; Nihei
224 *et al*. 2004). Serial two-fold dilutions of the tested compounds, curcumin and dodecanol,
225 were prepared in DMF, after which 30 µl of a 100-fold concentrated solution was added
226 to 3 ml of 2.5% malt extract broth in a test tube (diameter, 10 mm). The yeast cells were
227 inoculated into the medium to obtain a final inoculum size of 10^6 CFU ml^{-1}. The cultures
228 were incubated without shaking for 48 h, after which MIC was determined. MIC was
229 defined as the lowest concentration of a test compound that allowed for no visible
230 growth. After determining the MIC, an aliquot was withdrawn from each culture and
231 diluted 100-fold with 2.5% malt extract broth. After 48 h of incubation, the minimum
232 fungicidal concentration was determined as the lowest concentration of a test compound
233 that did not allow for any recovery of yeast cells.

234 **Time-kill assay**

235 Yeast cells were grown overnight in 2.5% malt extract broth and diluted with the same
236 broth to obtain 1×10^6 cells ml^{-1}. The cell suspensions were incubated at 30°C without
237 shaking in 2.5% malt extract broth containing dodecanol, curcumin, or their combination.

238 Thereafter, the number of viable cells in each suspension was determined as CFU, using
239 1.5% agar plates containing 1% yeast extract, 2% polypeptone, and 2% glucose. The agar
240 plates were incubated at 30℃ for 48 h prior to counting CFU.

241 **RNA extraction**

242 Total RNA fractions were extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany)
243 according to the manufacturer's instructions. The yeast cells treated with dodecanol
244 and/or curcumin were collected by centrifugation at 5,000 × *g* for 10 min, prior to cell
245 lysis with zymolyase. RNA was filtered out of each suspension using an RNA column
246 and treated with DNase. The RNA fractions were reverse-transcribed into cDNA using
247 ReverTra Ace (TOYOBO, Osaka, Japan).

248 **Reverse transcription-polymerase chain reaction (RT-PCR) analysis**

249 Gene expression was relatively quantified by RT-PCR in BY4741 cells treated with
250 dodecanol and/or curcumin in 2.5% malt extract broth with shaking at 30°C for 4 h. Total
251 RNA was isolated from the cells using the RNeasy Mini Kit and 0.5–5.0 μg of it was used
252 for cDNA synthesis using ReverTra Ace. RT-PCR was conducted using Taq polymerase
253 (BioLabs, Ipswich, MA, USA), cDNA, and a thermal cycler (Applied Biosystems 2720;
254 Thermo Fisher Scientific, Waltham, MA, USA). The cycling parameters were 2 min at

94°C and then 23 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72 °C, and then 5 min at 72 °C. The relative expression levels of *PDR1, PDR3,* and *PDR5* genes were normalised against those of the housekeeping gene *ACT1*. The primers used in this study are listed in Table S1.

Each amplified DNA sample was electrophoresed on 1% agarose gel, stained with GelRed (Biotium, Inc., Hayward, CA, USA), and visualised under UV light. The relative expression levels of each gene were quantified using Fujifilm Multi Gauge Version 2.1. Data have been expressed as mean ± standard deviation of triplicate determinations.

Efflux of R6G

Yeast cells from an overnight culture in 2.5% malt extract broth were centrifuged at 9,600 × *g* for 5 min at 27°C. Next, the cells were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. Thereafter, the cell suspension was incubated with shaking at 30°C for 12 h, centrifuged at 9,600 × *g* for 5 min at 27°C, and resuspended in PBS to obtain a cell density of 5×10^8 cells ml^{-1}. R6G (10 µmol l^{-1}) was added to the suspension, after which the cells were incubated for 60 min at 30°C, washed, and resuspended in PBS at 7.5×10^7 cells ml^{-1}. Curcumin and 10 mmol l^{-1} glucose were

272 then added to the suspension. Aliquots (1 ml) of the suspension were withdrawn at
273 predetermined times and centrifuged at 2,000 × *g* for 30 s at 27°C to obtain supernatant
274 for the assay of R6G efflux. After 60-min incubation with or without curcumin, the cells
275 were harvested by centrifugation, and lysed in 70% ethanol by 10 cycles of 6 s with 0.5-
276 mm acid-washed glass beads using a bead beater (Bio Medical Science, Tokyo, Japan).
277 The suspensions were centrifuged and the cell-free extracts were then obtained for
278 determination of intracellular R6G level.

279 The fluorescence intensity of R6G in the supernatant and the cell-free extracts
280 was measured by high-performance liquid chromatography (HPLC) using an ODS
281 column (5C_{18}-MS-II; Nacalai Tesque, Kyoto, Japan). Isocratic elution was performed at
282 30°C with 50% acetonitrile containing 0.1% formic acid. The flow rate of the mobile
283 phase was set at 1.0 ml min^{-1}. Detection was performed using a fluorescence detector (FP-
284 1520S; JASCO, Tokyo, Japan) at excitation and emission wavelengths of 485 and 535
285 nm, respectively. A calibration curve was plotted for calculating the concentration of R6G
286 from its fluorescence intensity.

287 **Statistical analysis**

288 Statistical evaluation was performed using Student's *t*-test. *P* values < 0.05 indicated

289 statistical significance.

290

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294

295 **Conflict of interest**

296 The authors have no conflict of interest to declare.

297

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444

445 **Supporting information**

446 Additional Supporting Information may be found in the online version of this article:

447

448 **Table S1**. Primer sets for RT-PCR analysis

449

450 **Figure legends**

451 **Figure 1** Chemical structures of curcumin, *trans*-anethole, and *n*-dodecanol.

452

453 **Figure 2** Effect of curcumin on dodecanol-induced temporary death of *S. cerevisiae*
454 ATCC7754.

455 The yeast cells were grown in 2.5% malt extract broth at 30°C. The following drugs were
456 then added to the culture: 156 μmol l^{-1} dodecanol (■), 312.5 μmol l^{-1} curcumin (○), and
457 156 μmol l^{-1} dodecanol + 312.5 μmol l^{-1} curcumin (□). The closed circle (●) denotes no
458 drug treatment. Data are expressed as mean ± standard deviation (n = 3).

459

460 **Figure 3** Separation of R6G from curcumin by HPLC.

461 HPLC was performed using the ODS column 5C_{18}-MS-Ⅱ. Isocratic elution was

462 performed at 30°C using H_2O:acetonitrile (1:1, v/v) containing 0.1% formic acid as the

463 mobile phase. The flow rate of the mobile phase was set at 1.0 ml min^{-1}. Detection was

464 carried out at excitation and emission wavelengths of 485 and 535 nm, respectively.

465

466 **Figure 4** Effect of curcumin on R6G efflux and intracellular level of R6G.

467 R6G efflux (left). *S. cerevisiae* ATCC7754 cells were incubated without shaking at 30°C

468 in PBS containing 10 mmol l^{-1} glucose with (■) or without (●) 312.5 μmol l^{-1} curcumin.

469 Fluorescence intensity of the supernatant was determined by HPLC. Data have been

470 expressed as mean ± standard deviation (n = 3). Intracellular level of R6G (right). *S.*

471 *cerevisiae* ATCC7754 cells were incubated without shaking at 30°C for 60 min in PBS

472 containing 10 mmol l^{-1} glucose with or without 312.5 μmol l^{-1} curcumin. After incubation,

473 fluorescence intensity in cell-free extracts was determined by HPLC. Data are expressed

474 as mean ± standard deviation (n = 3).

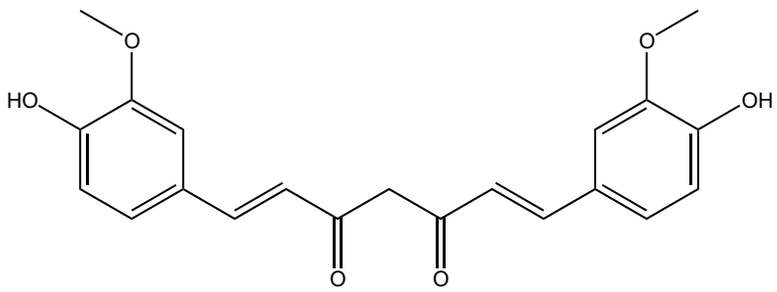
475

476 **Figure 5** Expression levels of *PDR1*, *PDR3*, and *PDR5* relative to that of *ACT1*.

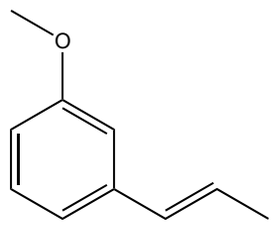
477 *S. cerevisiae* BY4741 cells were incubated in 2.5% malt extract broth containing 312.5

478 μmol l^{-1} curcumin and/or 32 μmol l^{-1} dodecanol. Total RNA was extracted for RT-PCR

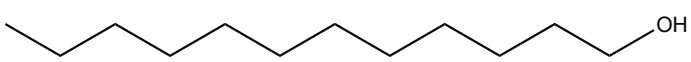
479 analysis. Data are expressed as mean ± standard deviation (n = 3). * indicates $p < 0.05$.



Curcumin



trans-Anethole



n-Dodecanol

Figure 1. Yamawaki et al.

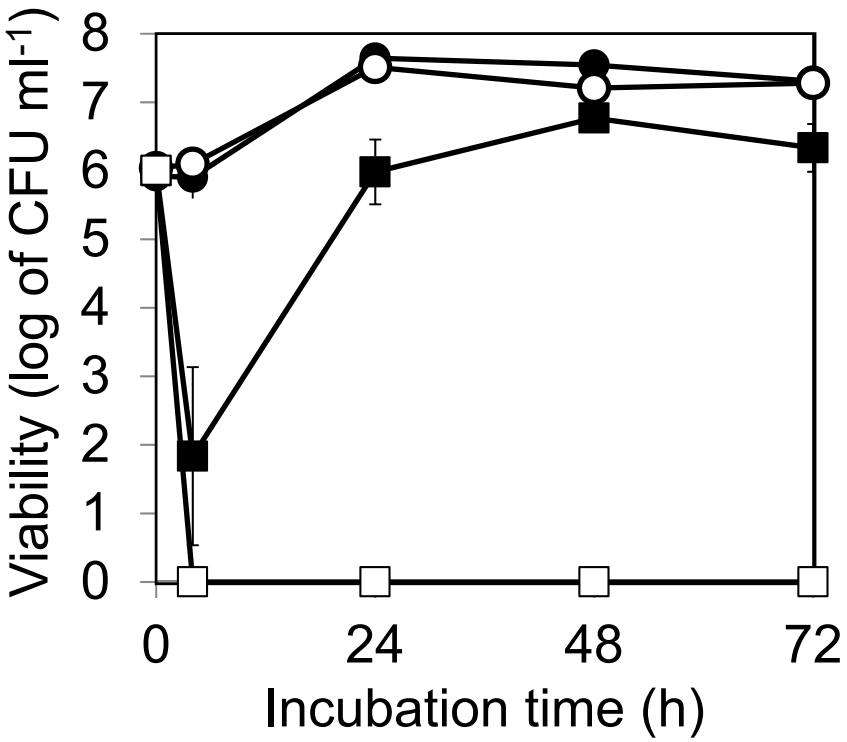


Figure 2. Yamawaki et al.

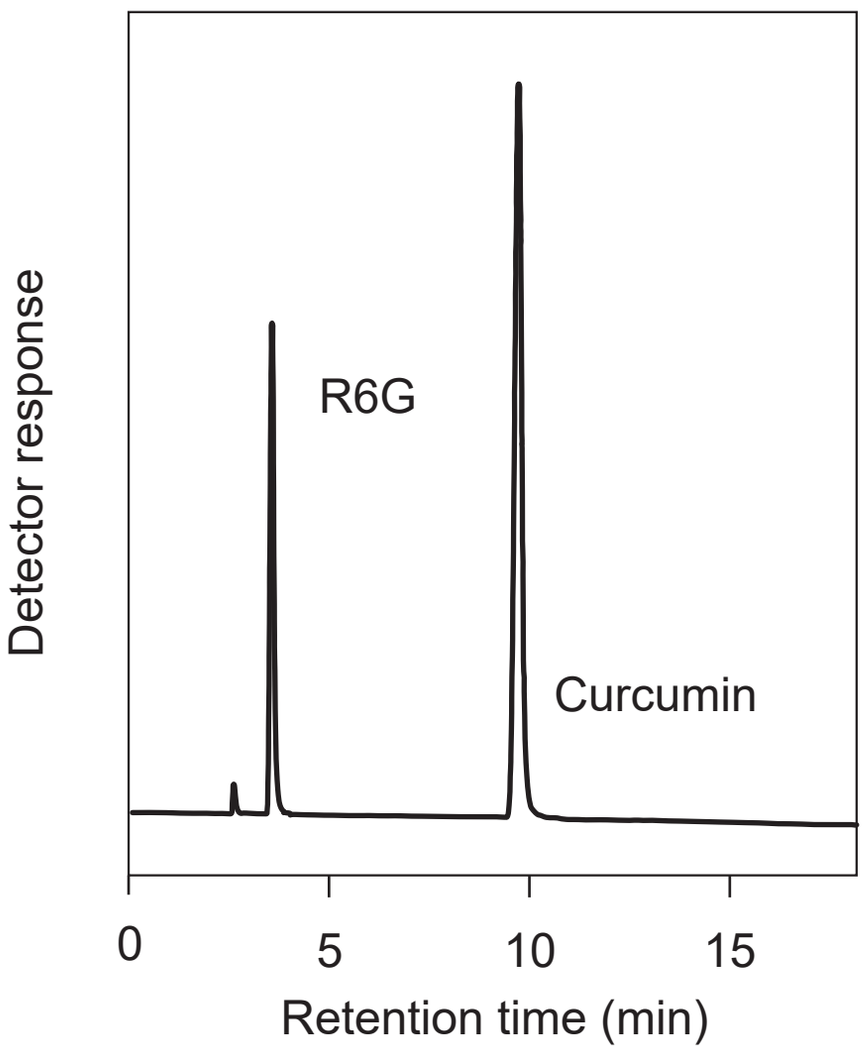


Figure 3. Yamawaki et al.

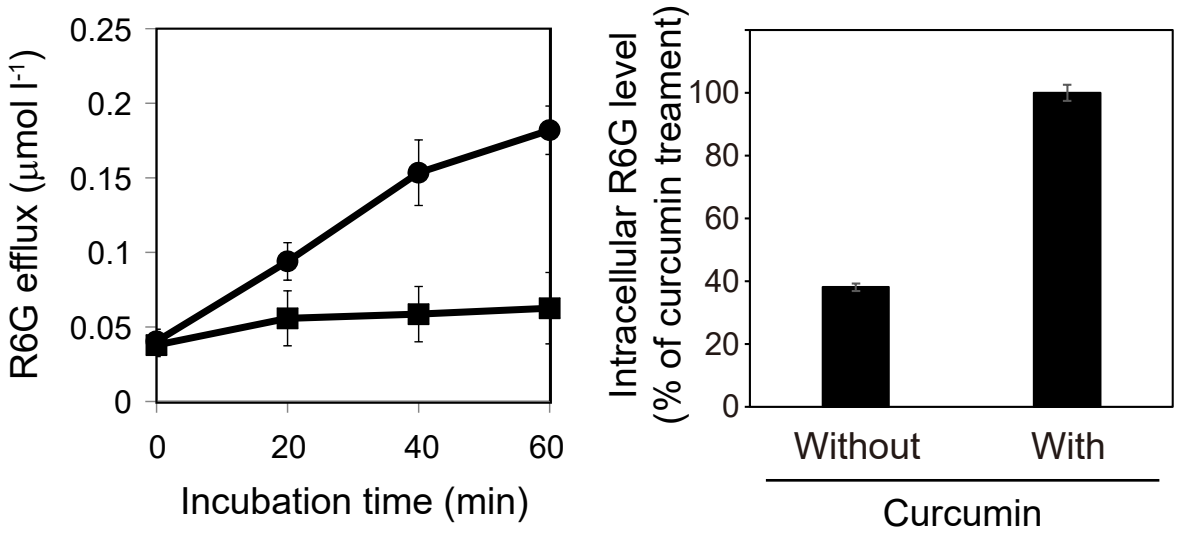


Figure 4. Yamawaki et al.

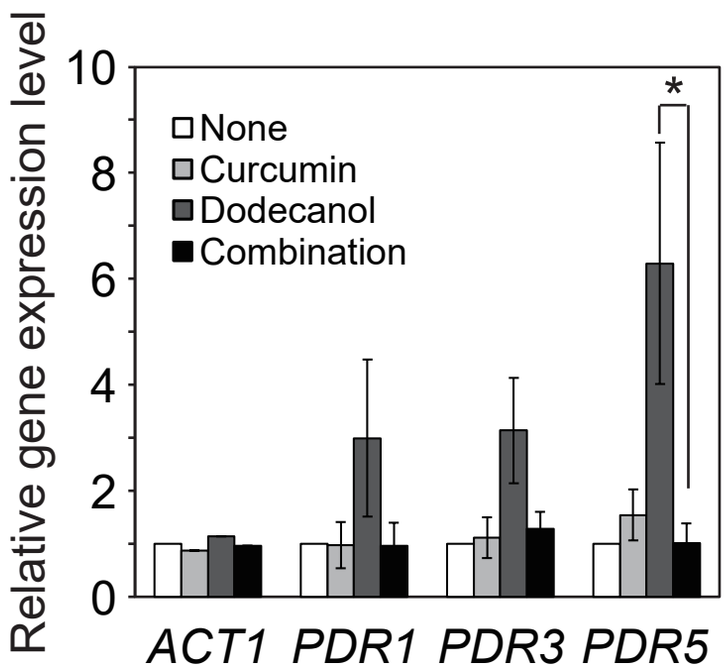


Figure 5. Yamawaki et al.