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Dodecyltriphenylphosphonium inhibits multiple drug resistance in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

Multiple drug resistance pumps are potential drug targets. Here we asked whether the lipophilic cation dodecyltriphenylphosphonium (C_{12}TPP) can interfere with their functioning. First, we found that suppression of ABC transporter gene *PDR5* increases the toxicity of C_{12}TPP in yeast. Second, C_{12}TPP appeared to prevent the efflux of rhodamine 6G – a fluorescent substrate of Pdr5p. Moreover, C_{12}TPP increased the cytostatic effects of some other known Pdr5p substrates. The chemical nature of C_{12}TPP suggests that after Pdr5p-driven extrusion the molecules return to the plasma membrane and then into the cytosol, thus effectively competing with other substrates of the pump.

1. Introduction

As in many cases, pathogenic fungi possess a robust MDR system [1,2], MDR pumps are potential targets of antimycotic mixtures. We decided to test whether dodecyltriphenylphosphonium (C_{12}TPP) and its plastoquinone derivative SkQ1, [3], the nontoxic penetrating lipophilic cations (Fig. 1A), can be used to suppress MDR in yeast cells. Two lines of evidence were pointing in this direction.

First, C_{12}TPP and SkQ1 are very likely to be substrates of MDR pumps in the plasma membrane. Indeed, amphiphilic cations are thought to be preferable substrates of mammalian P-glycoprotein and bacterial ATP binding cassette (ABC) proteins [4]. In particular, it was shown that triphenylmethylphosphonium and tetraphenylphosphonium are substrates of mammalian P-glycoprotein [5]. The fluorescent amphiphilic cation rhodamine 6G was found to be a substrate of yeast Pdr5 – a multidrug pump [6]. The antiarrhythmic drug amiodarone, which is able to inhibit MDR in human cell lines [7], was found to enhance C_{12}TPP-stimulated respiration in MDR-positive but not in MDR-negative yeast cells [8]. Moreover, it was shown that the inhibitor of multidrug efflux pluronic L61 induces accumulation of C_{12}TPP-based antioxidants SkQ1 in K562 myeloid leukemia cells [9]. Not surprisingly, the antioxidant SkQ1 was shown to protect the MDR-negative cells but not the MDR-positive ones against pro-oxidant treatments [10].

Second, being both charged and hydrophobic, the cations are highly membranophilic, i.e. they tend to accumulate at the lipid/water interface. Thus we reasoned that the molecules extruded from the cell may then become immediately trapped in the outer leaflet of the plasma membrane and then move back into the inner one. The reverse transport is expected to be facilitated by the charge: in fungi the outer membranes typically maintain electric potential of up to the 200 mV [11], which acts to attract the penetrating cations into the cells. Therefore, such futile cycling seemed likely to compete with other substrates of MDR pumps.

In this work, using *Saccharomyces cerevisiae* as a model cell system, we found that the ABC-transporter Pdr5p protects the cells from the toxic effects of C_{12}TPP. Consistent with this, we show that C_{12}TPP augments the toxic effects of Pdr5p substrates cycloheximide D and clotrimazole and also inhibits rhodamine 6G efflux.

2. Material and methods

2.1. Strains and growth conditions

In this work we used *W303-1A S. cerevisiae* strains and its derivatives: *AD1-8* with deletions of eight MDR genes [*W303-1A*,

Abbreviations: C_{12}TPP, dodecyltriphenylphosphonium; R6G, rhodamine 6G; MDR, multiple drug resistance; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; ABC, ATP binding cassette.

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yor1::hisG, snq2::hisG, pdr5::hisG, pdr10::hisG, pdr11::hisG, ycf1::hisG, pdr3::hisG, pdr15::hisG] [12], P_{GAL}-*PDR5* [*W303-1A HIS3::*P_{GAL}-*PDR5*], P_{GAL}-*SNQ2* [*W303-1A HIS3::*P_{GAL}-*SNQ2*], and P_{GAL}-*YOR1* [*W303-1A HIS3::*P_{GAL}-*YOR1*]. Cells were grown in YPD medium (2% glucose, 1% bacto-peptone, 1% yeast extract) or in YPGal (2% galactose, 1% bacto-peptone, 1% yeast extract). For genetic screening and maintaining of strains with conditionally expressed genes, synthetic drop-out media YNB-Leu or YNB-His were used according to Sherman 2000 [13]. The growth rates were measured by increase in light scattering (λ = 550 nm) in liquid yeast culture.

2.2. Microscopy

Cells stained with R6G were visualized with an upright Olympus BX2 microscope and U-MNG2 filter set (excitation 530–550 nm, 570 nm beamsplitter filter, emission >590 nm).

2.3. Genetic screening

Yeast mutants of *W303* strain of *S. cerevisiae* carrying multicopy plasmid YEp13 with inserts of 8–10 Kb at BamHI restriction site were constructed by transformation. Three cycles of enrichment of the mutant collection for C_{12}TPP-resistant strains were performed. During each cycle the mutants at logarithmic stage of growth on YNB-Leu media were treated with 18 μM C_{12}TPP for 3 h, then washed, diluted, and grown overnight on fresh solid YNB-Leu. After the third cycle, the cells were transferred onto solid YNB-Leu media. C_{12}TPP resistance of separated colonies was compared with a wild type. To identify the genes carried by the multicopy plasmid, the genomic DNAs of the selected strains were transformed in *E. coli*. Loci of insertion were determined by sequencing the selected YEp13-insertion plasmids with primers YEp13-DIR 5′-cgctatatgcgttgatgc YEp13-REV 5′-cctgccaccatacccacg.

2.4. Rhodamine 6G efflux

To measure the relative rate of rhodamine 6G efflux, we used the fluorometric assay described by Kolaczkowski et al. [6] with a few modifications. Cells were grown overnight in 40 ml in liquid YPD to the density of 0.5–1 × 10^7 cells/ml, washed twice with cold sterile water, and resuspended in 10 ml phosphate buffer saline supplemented with 5 mM 2-deoxyglucose and 2.5 mM 2,4-dinitrophenol. The cell suspension was incubated for 45 min on a rotatory shaker, and then the inhibitors were removed by two cycles of centrifugation/resuspension in cold water. The energy-deprived cells were resuspended in 10 ml of PBS and then stained with R6G (10 μM) for 40 min. Then the cell suspension was pelleted, resuspended in an equal volume of PBS, and stored on ice for 1–5 h. The efflux was measured with a FluoroMax-3 fluorometer system with excitation wavelength set to 480 nm, and emission wavelength set to 560 nm. The efflux of R6G was initiated by addition of 1% glucose; cell density in the fluorometric cuvette was 10^6 cells/ml.

2.5. Survival assay

Exponentially growing cells were taken and treated with indicated amounts of C_{12}TPP or SKQ1 for 3 h. Then the cell suspensions were plated on solid YPD medium and incubated for 48 h, and the number of formed colonies was counted. 100% refers to the number of colony forming units (CFU) in the yeast suspension at the beginning of the experiment.

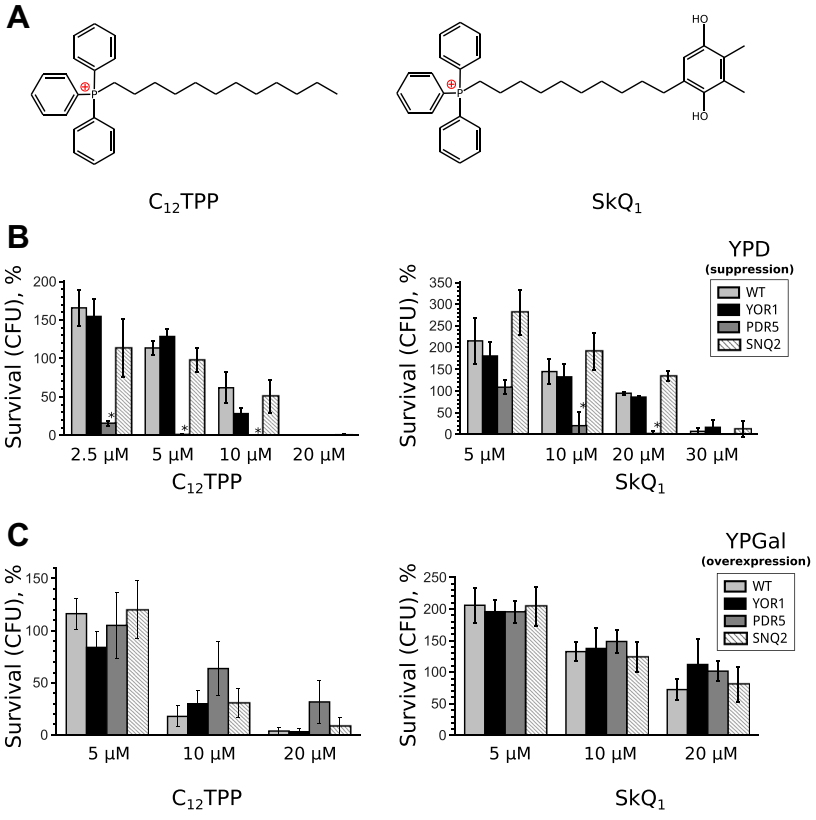

Fig. 1. Expression levels of Pdr5 affect resistances to C_{12}TPP. (A) Chemical structure of dodecyltriphenylphosphonium C_{12}TPP and its plastoquinone derivative (SkQ1) used in this study. Glucose-grown (B) or galactose-grown (C) cells treated with indicated concentration of C_{12}TPP or SkQ1. *$P < 0.05$ compared to untreated wild type (WT) according to Wilcoxon signed-ranked unpaired test.

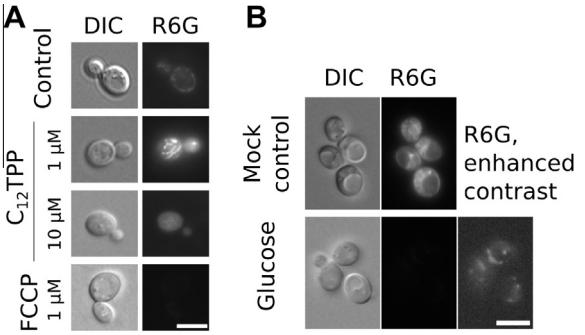

Fig. 2. C_{12} TPP or energy deprivation enhances Rhodamine 6G accumulation in yeast cells. (A) R6G staining of yeast cells is enhanced by low concentrations of C_{12}TPP but not of FCCP. Exponentially grown yeast cells were stained with 500 nM R6G in the presence of the indicated concentrations of C_{12}TPP or FCCP. (B) Addition of glucose results in a decrease in total fluorescence. Weak signals are observed from R6G retained in mitochondria (enhanced contrast). Representative photographs. Bars, 5 μm.

2.6. Statistics

Wilcoxon signed-ranked tests (n = 3–11) and the slopes of the fluorometric curves were calculated using the R software package. Error bars on figures represent standard errors of the mean.

3. Results and discussion

To see whether C_{12}TPP can inhibit MDR pump activity, we first decided to find the particular pump that extrudes it from *S. cerevisiae* cells. We performed genetic screening using yeast *S. cerevisiae* with a yeast genome library on a multicopy plasmid (see Section 2). As a result, we found that a plasmid with chromosome II fragment (coordinates from 216287 to 222344) harboring two genes, *LDB7* and *PDR3*, provided a significant increase in resistance to 20 μM C_{12}TPP. Pdr3p is a transcription factor responsible for upregulation of a set of ABC-transporters including three unspecific MDR pump genes: *PDR5*, *SNQ2*, and *YOR1* [14–16]. These data strongly suggested that C_{12}TPP is a substrate of one of these pumps. To find the specific pump responsible for C_{12}TPP detoxication, we produced a set of mutant strains with corresponding genes under the control of galactose-inducible, glucose-repressible *GAL* promoters (see Section 2).

It appeared that expression of *PDR5* is critical for resistance of cells to C_{12}TPP, whereas repression of *SNQ2* or *YOR1* did not show a statistically significant effect (Fig. 1B). Accordingly, overexpression of *PDR5* provided moderate protection to high concentrations of C_{12}TPP (Fig. 1C). We did not observe any effect of MDR overexpression in the case of SkQ1. Possibly, basal levels of expression of the pumps that extrude SkQ_1 are sufficient to prevent its toxicity.

A possible explanation for these results is that C_{12}TPP is extruded by Pdr5 from yeast cells, and that C_{12}TPP competes with other Pdr5 substrates. Indeed, low concentrations of C_{12}TPP enhance the staining of yeast cells with the positively charged fluorescent dye rhodamine 6G (Fig. 2A), which is likely to be a Pdr5p substrate [6]. Accordingly, in energy-deprived cells R6G accumulates in high concentration, and after the addition of glucose the fluorescence inside cells is decreased and remains mostly in polarized mitochondria (Fig. 2B). We measured the efflux of R6G from

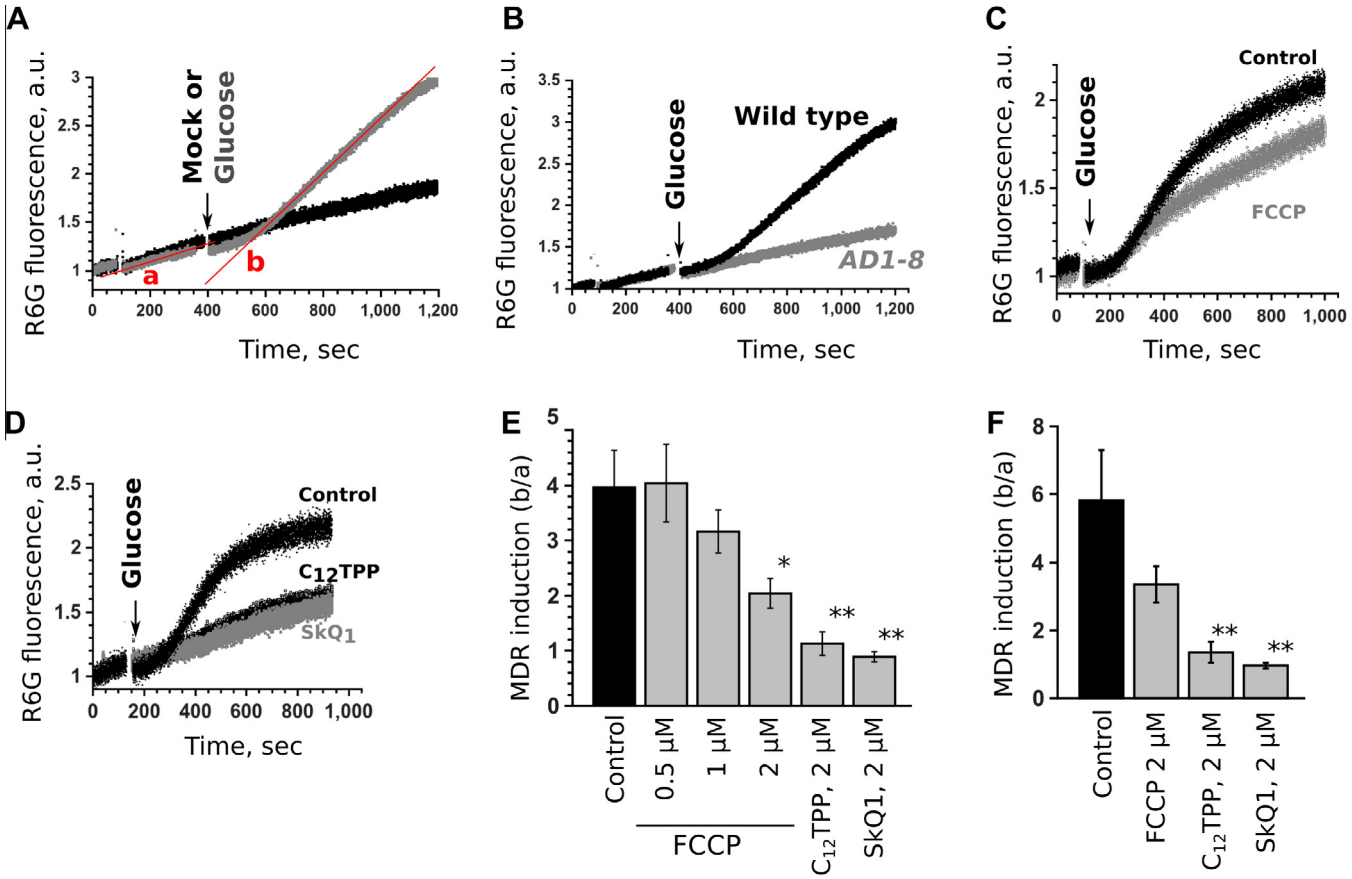

Fig. 3. C_{12}TPP and SkQ1 prevent R6G efflux from yeast cells. (A) Indirect measurements of glucose-induced R6G efflux from yeast cells. (B) Glucose-induced R6G efflux is negligible from *AD1-8* (MDR-negative) cells. (C and D) FCCP (2 μM), C_{12}TPP (2 μM), or SkQ1 (2 μM) inhibits glucose-induced R6G efflux from yeast cells. (E) Quantification of the results. The *Y*-axis shows the ratios of slopes in a and b (see Section 2). (F) FCCP (2 μM), C_{12}TPP (2 μM), or SkQ1 (2 μM) inhibits glucose-induced R6G efflux from yeast cells pretreated with 10 mM NaN_3. $^*P < 0.05$, $^{**}P < 0.01$ compared with untreated WT according to Wilcoxon signed-ranked unpaired test.

intact yeast cells using a fluorometric method. The efflux was visualized by fluorescence spectroscopy: rhodamine 6G is self-quenched in cells, and therefore R6G release results in a detectable increase in total fluorescence of the dye (Fig. 3A). Importantly, R6G release is completely abrogated in MDR-negative cells lacking all major *PDR* genes (Fig. 3B).

The addition of the uncoupler FCCP was able to partially prevent R6G efflux (Fig. 3C), while C_{12}TPP and SkQ1 appeared to be much more efficient in this respect (Fig. 3D and E). Apparently, this effect of lipophilic cations could be due either to a direct inhibition of MDR pumps or be a result of mitochondrial uncoupling causing a decrease in ATP concentration. However, the latter seemed unlikely because FCCP is a much more potent uncoupler than C_{12}TPP [17]. Moreover, this possible effect of C_{12}TPP can be ruled out because it was also able to inhibit R6G efflux in cells pretreated with an excess of NaN_3 (Fig. 3F). NaN_3 inhibits both the respiratory chain [18] and mitochondrial ATP synthase [19]. At the same time, in contrast to another ATP synthase inhibitor, oligomycin, it is not as efficient in inhibiting MDR activity in yeast [6]. Therefore, in the presence of 10 mM NaN_3, the contribution of mitochondria to the ATP supply appears to be minimal, and the effects of C_{12}TPP and SkQ1 are mainly due to the repression of MDR.

Is it possible to induce the uptake of other Pdr5 substrates by addition of C_{12}TPP? To test this possibility, we measured the growth rate of yeast cells in the presence of the protein synthesis inhibitor cycloheximide D (ChD), which is well-known substrate of Pdr5 [20]. We found that 1 μM C_{12}TPP significantly enhances the inhibitory effect of ChD (Fig. 4A). Moreover, C_{12}TPP augments the action of the antifungal clotrimazole (Fig. 4B), which was also previously reported to be a substrate of yeast MDR pumps [21]. Importantly, we did not detect any significant stimulation of the toxicity for another antifungal – amphotericin B, which acts in the plasma membrane and is not attributed to ABC pumps substrates.

To conclude, together with previous observation, our data show that C_{12}TPP inhibits multidrug resistance and suggest that the interference is due to a futile cycle of its extrusion followed by returning back into the cells. Therefore, it can be used to increase cellular uptake of other ABC-transporter substrates (including other amphiphilic compounds). Remarkably, earlier it was shown that C_{12}TPP facilitates the transport of anionic molecules across membranes: the fluorescent dye fluorescein [22], fatty acids [23], and anionic uncouplers [17]. Thus, C_{12}TPP appears to be a universal plasma membrane permeabilizer. Therefore, our findings make it a promising supplement for antimycotic drugs to prevent their efflux from cells.

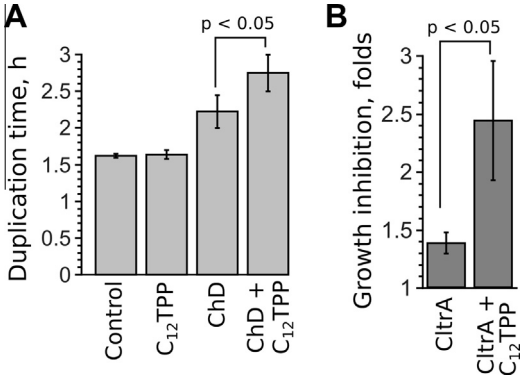

Fig. 4. C_{12}TPP enhances the effects of Pdr5 substrates cycloheximide D and clotrimazole. (A) Duplication times of yeast cells grown in the presence of ethanol (mock control), C_{12}TPP (1 μM), Cycloheximide D (ChD, 0.05 μM), or both chemicals. (B) C_{12}TPP (1 μM) increases the inhibitory effect of Clotrimazole (CltrA, 60 μM). *P*-value of Wilcoxon signed-ranked paired test is shown.

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