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Clioquinol induces G_2/M cell cycle arrest through the up-regulation of TDH3 in *Saccharomyces cerevisiae*

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ABSTRACT

Clioquinol (CQ) has been used as a classical antimicrobial agent for many years. However, its mode of action is still unclear. In our study, the growth of *Candida albicans* and *Saccharomyces cerevisiae* was inhibited by CQ. It did not kill yeast cells, but shortened G1 phase and arrested cell cycle at G2/M phase. By using two-dimensional electrophoresis based proteomic approach, six proteins were found to be significantly affected by CQ. Among them, four (PDC1, ADH1, TDH3, IPP1) were up-regulated and the other two (TDH1 and PGK1) were down-regulated. According to the *Saccharomyces* Genome Database (SGD), these proteins were involved in various biological processes including glycolytic fermentation, gluconeogenesis, glycolytic process, amino acid catabolism, redox reaction and reactive oxygen species metabolic process. It was noted that there was a link between TDH3 and cell cycle. The overexpression of *TDH3* phenocopied CQ treatment and arrested the cell cycle at G2/M phase. RT-PCR analysis showed that the mRNA levels of *CLN3* and *CDC28*, critical genes for passage through G1 phase, were up-regulated after the treatment of CQ as well as the overexpression of *TDH3*. It demonstrates that CQ inhibits the growth of yeast by up-regulating the expression of TDH3 to influence the cell cycle. It is expected to provide new insights for the antimicrobial mechanism of CQ.

1. Introduction

Clioquinol (5-chloro-7-iodoquinolin-8-ol, CQ, Fig. 1), a quinoline compound, was an antimicrobial medication widely used as topical cream for the therapy of various skin infections from the beginning (Ogunniran et al., 2007; Yassin et al., 2000). In the 1950s-1970s, it was used as an oral anti-parasitic agent for the treatment and prevention of intestinal amebiasis. However, oral consumption was banned due to subacute myelo-optic neuropathy (SMON) in Japanese patients in the 1970s (Bareggi and Cornelli, 2012). Recently, clioquinol becomes attractive again for the treatment of non-infectious diseases including malignancy (Daniel et al., 2005; Ding et al., 2005; Chen et al., 2007; Mao et al., 2009) and Alzheimer's disease (Cherny et al., 2001; Mao et al., 2009; Ritchie et al., 2003). Even so, topical formulations of clioquinol are still available for the treatment of topical fungal and parasitic infections so far.

It is therefore confusing how CQ acts as an antibiotic and also fights neurodegenerative diseases and cancers. Its mechanism of action is worth exploring. *Saccharomyces cerevisiae* presents a convenient system for antifungal drugs (Emerson et al., 2002; Li et al., 2010). In addition, it is one of the most intensively studied model organisms for understanding the regulation of eukaryotic cells. Compared to humans, the whole genome sequences of *S. cerevisiae* are very clear and its genes can be easily manipulated, providing a considerable amount of useful information for understanding the molecular basis of drugs. Thus, we used *S. cerevisiae* as our cellular model to study the mechanism of CQ.

In this study, we innovatively took advantage of two-dimensional polyacrylamide gel electrophoresis (2-DE) based proteomic approach to investigate the differential expression of proteins influenced by CQ in *S. cerevisiae* and explored that the cell cycle arrest was one of the mechanisms by which CQ inhibited the growth of yeast.

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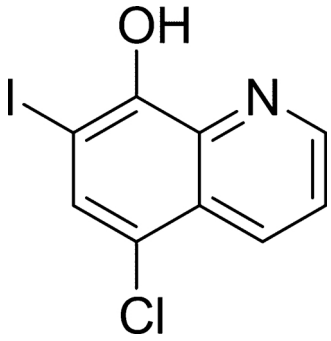

Fig. 1. Chemical structure of Clioquinol (CQ).

Table 1
Yeast strains and plasmids used in this study.

Strains or plasmids	Description	Source
BY4741a	*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Invitrogen
TDH3Δ	*Tdh3Δ: KanMx4* derivative of BY4741a	Invitrogen
TDH1-GFP	*TDH1:KAR2-GFP* derivative of BY4741a	Invitrogen
TDH3-GFP	*TDH3:KAR2-GFP* derivative of BY4741a	Invitrogen
Plasmids		
YEplca 195	Centromeric *E. coli/yeast* shuttle vector with *URA3*	Invitrogen
YEplac 195-TDH3	Centromeric *E. coli/yeast* shuttle vector containing *TDH3* with *URA3*	Invitrogen

2. Materials and methods

2.1. Strains, chemicals and growth conditions

Candida albicans (CMCC96001) and *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. Clioquinol was purchased from Tokyo Kasei Kogyo Co Ltd (Japan). Alpha-factor was bought from EZ-Biolab (USA). *Saccharomyces cerevisiae* and *Candida albicans* were grown to exponential phase in YPD medium (yeast extract 1%, peptone2%, glucose2%) and cells affected by CQ grew in synthetic complete (SC) medium that either contained or lacked uracil (SC-Ura). For making solid-agar plates, 2% agar was added to SC or SC-Ura. Cells of *C. albicans* and yeast grew with continuous shacking (180 rpm) at 30 °C and 37 °C, respectively.

2.2. Growth curve assay

Growth curve analysis was performed as described earlier (Dai et al., 2017). In brief, exponentially growing cells of *C. albicans* and yeast were treated with different concentrations of CQ (0, 0.5 μM, 1 μM, 1.5 μM, 2 μM for *C. Albicans*; 0, 0.1 μM, 0.2 μM, 0.4 μM for yeast) in SC liquid medium. The initial optical density at 600 nm (OD_{600}) of yeast was 0.1. And the growth was measured spectrophotometrically for 48 h by measuring the OD_{600} after diluted 10-fold with sterile water. Cells grew with continuous shacking (180 rpm) at 30 °C or 37 °C.

2.3. Spot assay

Yeast in exponential growth phase was harvested by centrifugation, washed 3 times and resuspended in sterile water. The cell density was normalized to 1×10^7 cells/ml. A ten-fold serial dilution was made and 5 μl of each dilution was spotted onto SC or SC-Ura plates. Placed the plates at 30 °C for 2-5 days.

2.4. Protein extraction and western blot

10 OD cells were harvested by centrifugation and washed three times with PBS. Resuspended the cells in lysis buffer composed of 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, Protease inhibitor mix, and 60 mM DTT. Then the cell debris was removed by centrifugation at 13000 rpm for 15 min after sonication (45 W; 2 s/2 s; 0 °C) for 30 min on ice. The protein concentration was determined by Bradford with BSA as a standard. The extractive at an extracellular protein concentration of 50 μg were separated by 8% SDS-PAGE before transferred to PVDF membranes. The primary antibody was used green fluorescent protein (GFP) (Sangon Biotech, China) followed by incubation with goat anti-rabbit IgG HRP secondary antibodies (Sinbio, China). These proteins were detected by using a chemiluminescense-based kit (Tian Gen Biotech, China). Signal density was detected by using Tanon 6200 (Biotano, China).

2.5. 2-DE and protein identification

For the first dimension, an amount of 150 μg of protein after 2-D clean-up kit(GE Healthcare Life Sciences, USA) processed was loaded on a 7 cm Immobiline Dry-Strip pH 3–10 NL (BIO-RAD,USA) in 150 μl sample buffer containing 7 M urea, 4% (w/v) CHAPS, 2 M thiourea, 60 mM DTT, and 0.5% (v/v) IPG Buffer, pH 3–10 nonlinear, and protease inhibitors. Second dimension SDS-PAGE was performed on 10% polyacrylamide gels. After the gel coloma bright blue dyed, PD Quest was processed to acquire the different points. Mass spectra of the spot were acquired by 4800 Plus MALDI TOF/TOFTM Analyzer (Applied Biosystems, USA). Then the data were analyzed using the matrix-Science Mascot bioinformatics database search engine for peptide mass fingerprint (PMF) matching against peptides from known protein sequences entered in publicly available NCBI databases. The classification of proteins refers to *Saccharomyces* Genome Database (SGD).

2.6. RT-PCR

RNA was isolated by using the Yeast RNAiso Plus (Takara, China), and reverse transcription was processed with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, China). PCR was carried out with

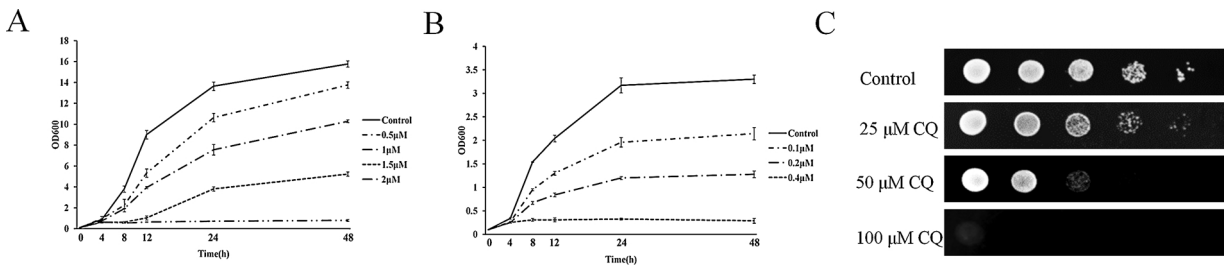

Fig. 2. Wild type strain BY4741 was sensitive to CQ. (A) The influence of CQ of different concentrations on *Candida albicans* in liquid medium. The IC_{50} value was obtained by Prism software. (B) The influence of CQ of different concentrations on BY4741 in liquid medium. (C) Confirmation of the repression on the solid medium. BY4741 were grown for 2–5 days on SC plates with or without the addition of CQ, 10-fold serially diluted, and then spotted on plates. Experiments were performed in at least triplicates and repeated three times or more.