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Triclabendazole protects yeast and mammalian cells from oxidative stress: Identification of a potential neuroprotective compound

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ABSTRACT

The Prestwick and NIH chemical libraries were screened for drugs that protect baker's yeast from sugar-induced cell death (SICD). SICD is triggered when stationary-phase yeast cells are transferred from spent rich medium into water with 2% glucose and no other nutrients. The rapid, apoptotic cell death occurs because reactive oxygen species (ROS) accumulate. We found that triclabendazole, which is used to treat liver flukes in cattle and man, partially protects against SICD. Characterization of triclabendazole revealed that it also protects yeast cells from death induced by the Parkinson's disease-related protein alpha-synuclein (α-syn), which is known to induce the accumulation of ROS.

1. Introduction

Baker's yeast (*Saccharomyces cerevisiae*) is used to model numerous human diseases, from asthma to various neurodegenerative diseases [1–3]. The yeast system enables the discovery of both genes and drugs that modify the toxicity of disease-related proteins. We recently used *S. cerevisiae* to screen approximately 1500 compounds from the Prestwick and NIH libraries of FDA-approved drugs for drugs that block sugar-induced cell death (SICD) [4] and identified two drugs—antimycin A and 5-chloro-6-(2,3-dichlorophenoxy)-2-(methylthio)-1*H*-benzimidazole (TCBZ, triclabendazole)—that block SICD. Antimycin A inhibits the mitochondrial respiratory enzyme complex III. Blocking respiration, either with antimycin A or with various mutations in Krebs cycle genes, partially protects yeast from ROS accumulation and thus SICD [5]. Evidence is presented here that TCBZ protects both yeast and mammalian cells from oxidative stress, possibly by inducing stress-responsive genes.

TCBZ is used in many parts of the world to treat cattle and humans for liver fluke (*Fasciola hepatica*) infections [6–8] (Fig. 1). It is approved for veterinary purposes in the USA. TCBZ is thought to inhibit β-tubulin from *F. hepatica* [9]. Note that two structurally related benzimidazole antihelminthics, albendazol and flubendazol, which were components of the libraries, failed to protect yeast cells from SICD. The microtubule-depolymerizing drug benomyl [10] was used as a control in the experiments described herein.

2. Materials and methods

2.1. Strains, media and reagents

The wild-type yeast strain BY4741 (*MAT***a**, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and the rat pheochromocytoma (PC12) cell line were purchased from American Type Culture Collection (Manassas, VA). Liquid rich medium (YPD) contained yeast extract (1%), peptone (2%) and dextrose (2%), and YPD plates were supplemented with agar (2%) (Difco, Detroit, MI). Synthetic complete (SC) medium contained 0.67% yeast nitrogen base, 0.16% yeast drop-out mix (Sigma–Aldrich, St-Louis, MO) and 2% glucose was supplemented with 0.016% each of histidine, leucine, methionine and uracil. PC12 cells were grown in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum and were maintained in a humid incubator (37 °C, 5% CO_2). Triclabendazole (TCBZ), albendazole (ALBZ), and benomyl (BEN) were purchased from Sigma–Aldrich. TCBZ was 99.8% pure, based on integration of a high performance liquid chromatogram by the supplier, and was used without further purification. Drugs were dissolved in DMSO to a concentration of 5 mM. The description of the construction of a yeast strain with chromosomally integrated α-syn is given in the Supplementary data.

2.2. SICD, chronological aging and stress resistance assays

For the SICD assay, wild-type yeast cells were pre-grown in 4 ml of YPD medium in glass tubes with shaking for 2 days at 30 °C to a density of 5–6 × 10^8 cells/ml [11]. Cells were washed and resuspended in 2 ml of water, aliquots were transferred to 3 ml of water,

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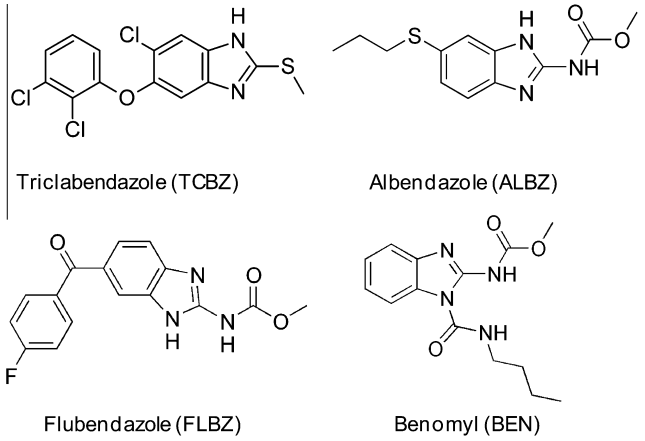

Fig. 1. The antihelminthics triclabendazole (TCBZ), albendazole (ALBZ) and flubendazole (FLBZ). Benomyl (BEN) depolymerizes microtubules.

glucose/water, or glucose/water with drugs to yield 2.0×10^7 cells/ml, and cultures were incubated with shaking at 37 °C. For the viability assay, aliquots were taken at the indicated times, diluted, and plated on YPD plates. The plates were incubated for 3 days at 30 °C, and then colony-forming units (cfu) were counted. The cfu value at the end of day 2 was the zero time point and represented 100% survival.

For the chronological aging assay, wild-type yeast cells were pre-grown in 4 ml of YPD medium in glass tubes with shaking for 2 days at 30 °C to a density of 5–6 $\times 10^8$ cells/ml [11,12]. Cells were washed and resuspended in 4 ml of water, and 10 μl of the culture was inoculated into 5 ml SC-glucose media or the SC-glucose media with added drug (5 μM TCBZ, ALBZ, or BEN) and incubated on a rotating roller drum such that the tubes were vertically tilted ~15° from horizontal and rotating at ~50 rpm to maintain the cells in suspension. The roller drum was positioned within an incubator set at 30 °C. Diluted cultures were incubated until stationary phase was reached (48 h), and then the chronological aging assay was started. Viability was monitored by cfu assay as described above. The cfu value after 48 h was the zero time point and represents 100% survival.

For the stress resistance assay in yeast, yeast cells were diluted to a low optical density in synthetic complete media with added drug and cultured until stationary phase was reached (48 h at 30 °C). Aliquots (10 μl) were removed and diluted with phosphate-buffered saline (PBS) buffer and then incubated with H_2O_2 (10 mM for 1 h). Cell viability assay was then determined by the cfu assay. For the stress resistance assay in rat PC12 cells, PC12 cells were seeded at a density of 1×10^4 cells/well in a flat-bottomed 96-well plate with poly D-lysine (0.2 μg/ml). The next day, cells were pre-treated with drug (50 μM TCBZ, ALBZ, or DMSO) for 3 h and then treated with 1 mM H_2O_2 for 21 h with at least 6 replicates for each treatment. After this incubation, 20 μl of the CellTiter 96® AQ_{ueous} One Solution reagent (Promega, Madison, WI) was added to each well and the plates were incubated for 3 h. Cell viability was determined by measuring the optical density (OD) at 490 nm using a Wallac 1420 Multilabel Counter (Perkin -Elmer Life Science). H_2O_2 concentration was determined using horseradish peroxidase and sodium iodide [13].

2.3. Reactive oxygen species (ROS) assay

The ROS assay using the 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) dye was performed as described [11]. Stationary-phase yeast cells (5 days old) were stained with DCFH-DA (10 μg/ml) for 1 h at 30 °C and then the cells washed with phosphate buffered saline. An Olympus AX70 microscope, equipped with an Olympus UPlanFI 100×/1.35 NA objective and a Roper CoolSNAP HQ CCD camera, was used to detect 2′-7′-dichlorodihydrofluorescein (DCF).

3. Results and discussion

S. cerevisiae cells grown in glucose-containing liquid media ferment glucose and produce ethanol. When glucose becomes limiting, the cells utilize ethanol and switch to a respiratory mode for energy production. After these two carbon sources are consumed, cells stop dividing and enter a quiescent or stationary state. Stationary-phase yeast cells maintained in spent liquid medium, or that are even washed and resuspended in pure water, can live for weeks, whereas the same cells when washed and resuspended in water with 2% glucose and no other nutrients die within hours. SICD is an apoptotic form of cell death that occurs because of the accumulation of ROS [14].

3.1. TCBZ partially protects yeast against SICD

In the SICD experiments, yeast cells were diluted to a low optical density in rich media and then cultured until stationary phase was reached (48 h at 30 °C) (Fig. 2A). Cells were then washed and resuspended in glucose/water with added drug. This was the zero time point for the SICD experiment. Cells were periodically removed to determine the number of viable cells in culture. A survival curve gives the percentage of viable cells over time, and the mean survival time, $t_{1/2}$, occurs when 50% of the cells are dead. TCBZ partially protected yeast from SICD, increasing the mean survival time ($t_{1/2}$) by 200% (0.3 ± 0.1→0.9 ± 0.3 days) (Fig. 2B), whereas albendazole and benomyl had no effect.

3.2. TCBZ protects yeast from cell death induced by the Parkinson's disease-related protein alpha-synuclein (α-syn)

Given that TCBZ partially protects cells from SICD, we sought to determine whether this drug could protect yeast cells from death induced by the human Parkinson's disease-related protein α-syn, which is known to induce reactive oxygen species, such as hydrogen peroxide and superoxide, to accumulate in a variety of cells, including yeast [15,16]. The yeast strain we used for this analysis contained three chromosomally integrated copies of human α-syn.

The ability of TCBZ to protect yeast cells from α-syn-induced death was conducted in a chronological aging experiment. The chronological lifespan (CLS) refers to the length of time stationary-phase cells that can survive in culture. In the CLS experiments, yeast cells were diluted to a low optical density in synthetic complete media with added drug and cultured until stationary phase was reached (48 h at 30 °C); this was the zero time for the CLS

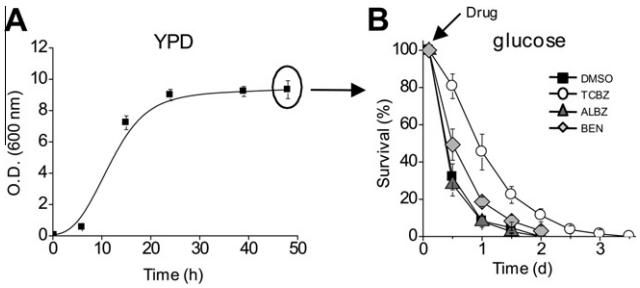

Fig. 2. TCBZ protects yeast from SICD. (A) Growth curve of yeast in rich medium (YPD). Cells were pre-incubated YPD until stationary phase (48 h). (B) SICD. The stationary-phase cells were resuspended in 2% glucose/water with the indicated drug (5 μM) or vehicle (DMSO). Cell viability as a function of time is plotted. Data points are the mean ± S.E. of the three independent experiments.

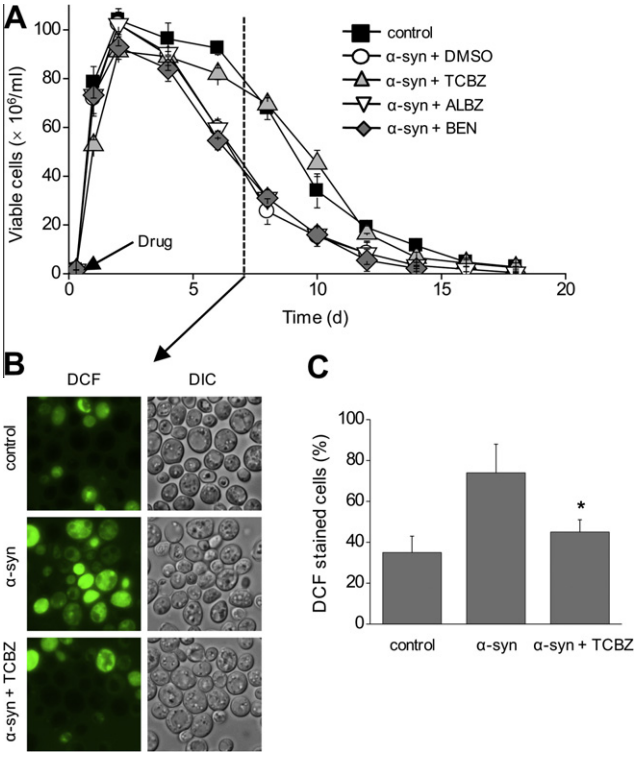

Fig. 3. TCBZ protects cells from α-syn-induced cell death. (A) Plot of viability as a function of time for cells (±α-syn) with indicated drug (5 μM). Cells were in the stationary phase after 2 days in culture, which was considered the time zero point for the aging experiment. (B) ROS assay. Five day-stationary-phase cells were removed from culture, stained with DCFH (10 μg/ml) and then visualized by fluorescence (DCF) and differential interference contrast (DIC) microscopy. (C) Plot of the percentage of cells staining for DCF. Values were obtained from two independent experiments, where the total number of cells counted was 500. Error bars are ± S.E. $^*P < 0.01$ (two-tailed Student's *t* test, versus α-syn).

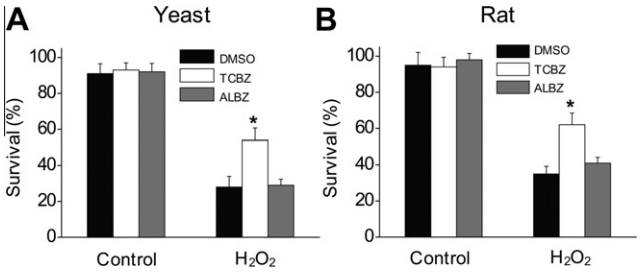

Fig. 4. TCBZ protects cells from hydrogen peroxide-induced cell death. (A) Effect of the indicated drug (5 μM) on the survival of stationary-phase cells incubated with 10 mM H_2O_2 for 1 h at room temperature. (B) Effect of the indicated drug (50 μM) on the survival of rat PC12 cells incubated with 1 mM H_2O_2 for 21 h at 37 °C. Values are the mean ± S.E. of three independent experiments. $^*P < 0.005$ (two-tailed Student's *t* test, versus DMSO).

experiment. Cells were periodically removed to determine the number of viable cells in culture. Previous studies have shown that α-syn significantly shortens the CLS of yeast [17] and induces ROS to accumulate [18].

Yeast cells expressing α-syn exhibited decreased survival ($t_{1/2}$ = 5.0 ± 0.4 days) compared to cells without α-syn expression ($t_{1/2}$ = 8.2 ± 0.1 days) (Fig. 3A). Notably, TCBZ reversed the effect of α-syn, i.e., for cells expressing α-syn, TCBZ increased mean survival by 94% (5.0 days → 9.7 days), whereas ALBZ and BEN had no effect.

To test whether TCBZ decreases the ROS burden in cells expressing α-syn, stationary-phase cells expressing α-syn were incubated with the ROS-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is a non-fluorescent, cell permeant dye that becomes trapped inside cells once it is oxidized by cytoplasmic ROS to the polar, fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCF). TCBZ-treated cells expressing α-syn showed significantly fewer cells (45 ± 6%) staining for ROS compared with the same cells without drug (74 ± 14%) (Fig. 3B and C). Note that no further decrease in ROS occurred when TCBZ was added to stationary-phase cells. This suggests that the first addition of TCBZ, to exponential phase cells, may up-regulate genes that protect cells from oxidative stress, and that subsequent additions cannot enhance the response anymore. TCBZ inhibits α-syn-induced ROS, thereby increasing the CLS.

3.3. TCBZ protects yeast and rat cells from hydrogen peroxide

We also tested whether TCBZ protects cells from an exogenous oxidant (hydrogen peroxide). Fig. 4A shows the effect of various drugs (5 μM) on the survival of stationary-phase yeast cells incubated with H_2O_2 (10 mM) for 1 h at room temperature. In this experiment, yeast cells were first diluted to a low optical density in synthetic complete media with added drug and cultured until stationary phase was reached (48 h at 30 °C). H_2O_2 was then added for 1 h, and viability was measured. TCBZ protected cells from death induced by such a high concentration of H_2O_2, i.e., 54 ± 7% of the TCBZ-treated cells survived whereas only ~30% of DMSO or ALBZ-treated cells survived. Fig. 4B shows the effect of various drugs (50 μM) on the survival of rat PC12 cells incubated with H_2O_2 (1 mM) for 21 h at 37 °C. In this experiment, PC12 cells were seeded at a density of 1 × 10^4 cells/well in a flat-bottomed 96-well plate with poly D-lysine (0.2 μg/ml). The next day, cells were pretreated with drug (50 μM TCBZ, ALBZ, or DMSO) for 3 h, incubated with 1 mM H_2O_2 for 21 h and then viability was measured. TCBZ protected cells from H_2O_2-induced death, i.e., 62 ± 6%% of the TCBZ-treated cells survived, whereas only 35–41% of the DMSO or ALBZ-treated cells survived. No evidence was found that any of these drugs decompose H_2O_2. The results indicate that TCBZ protects yeast and rat cells from H_2O_2-induced death.

The results show that TCBZ protects yeast cells from SICD and death induced by α-syn or H_2O_2, and at a 10-fold higher concentration TCBZ also protects rat PC12 cells from H_2O_2-induced death. The results raise the possibility that TCBZ protects cells by up-regulating stress-responsive genes. TCBZ may have use for neurodegenerative diseases that involve oxidative stress such as Parkinson's disease.

Disclosure statement

Yong Joo Lee, Shaoxiao Wang and Stephan N. Witt have a provisional patent application regarding the use of triclabendazole in the treatment of neurodegenerative diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.057.

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