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Antibacterial activity of CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine) generating reactive oxygen species

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ABSTRACT

CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine) is an antifungal and chemosensitizing agent that induces oxidative stress in yeast and filamentous fungi and enhances the cytotoxic activity of 5-fluorocytosine and azole antimycotics. This study reports the effect of CTBT on bacterial cells. CTBT inhibited the growth of both Gram-positive and Gram-negative bacterial species. The action of CTBT was bactericidal. In *Escherichia coli*, CTBT induced an increased formation of reactive oxygen species (ROS), as determined with a ROS specific probe 2′,7′-dichlorodihydrofluorescein diacetate. In zone inhibition assays, bacterial cells were more sensitive to CTBT compared with paraquat, menadione and hydrogen peroxide. The deletion of oxidative stress related genes resulted in increased susceptibility of *E. coli* mutant strains to CTBT treatment. Exogenous antioxidants such as ascorbic acid, cysteine and glutathione exhibited a protective effect against the growth inhibition induced by CTBT. CTBT may be a useful tool in the studies of ROS generation, oxidant sensing and oxidative stress response in different bacterial species.

1. Introduction

The emergence of dangerous bacteria that have developed resistance to drugs used in the treatment of infection is a worldwide clinical threat. To combat antibiotic resistance, the better understanding of molecular mechanisms leading to the emergence of bacterial drug resistance and virulence as well as the development of novel therapeutic agents are needed (Aleksun and Levy 2007; Fischbach and Walsh 2009). Recent studies revealed that some antibiotics induce changes in bacterial metabolism promoting the formation of reactive oxygen species (ROS), which play a role both in the antibiotic action and resistance (Albesa et al. 2004; Dwyer et al. 2007; Kohanski et al. 2007).

ROS are generated via intracellular single-electron reduction of molecular oxygen leading to superoxide anion radical ($O_2^{\bullet -}$) formation. Superoxide is dismutated to H_2O_2 by superoxide dismutases. Via Fenton and Haber-Weiss reactions H_2O_2 is able to generate the highly toxic hydroxyl radical ($OH^{\bullet}$). The increased formation of ROS induces oxidative stress and damage to DNA, proteins and lipids, leading to the loss of cell viability (Imlay 2003; Toledano et al. 2007; Herrero et al. 2008).

Recently, we have identified a novel antifungal agent, named CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine) (Fig. 1), which sensitizes yeast (Cernicka et al. 2007) and filamentous fungi (Culakova et al. 2012) to antimycotics. CTBT induced superoxide generation and oxidative stress resulting in cell death. The genome-wide studies revealed at least 169 non-essential genes whose deletion lead to increased CTBT sensitivity (Batova et al. 2010) and 3 genes, *YAP1*, *PDE2* and *STB3*, that upon over-expression enhanced the CTBT tolerance in yeast cells (Drobna et al. 2012). Among yeast deletion mutants hypersensitive to CTBT mainly genes encoding proteins involved in mitochondrial functions, DNA repair, transcription and oxidative stress response were identified. Transcriptome analysis of cells grown in the presence of CTBT identified the over-expression of 314 genes that were under the control of the main transcription factors required for the oxidative and general stress response in yeast. The CTBT-induced superoxide generation in yeast mitochondria *in vivo* required molecular oxygen and at least functional NADH dehydrogenases (Batova et al. 2010). Since the mitochondria have evolved from the prokaryotic cells during the evolution, we were interested to know how bacteria respond to CTBT action.

In this study, we assessed the antibacterial activity of CTBT. We determined the bacterial susceptibility, viability as well as the ROS

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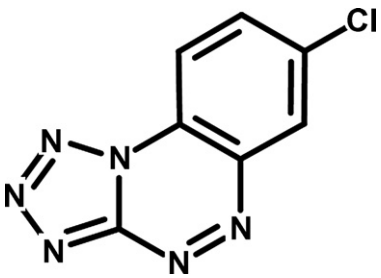

Fig. 1. Structure of CTBT.

generation and the protective effect of some low molecular antioxidants in bacterial cells treated with CTBT.

2. Materials and methods

2.1. Strains and culture conditions

The list of bacterial species used is in Table 1. In addition, the mutant strains Δ*soxS* (*E. coli* JW 4023), Δ*oxyR* (*E. coli* JW 3933), Δ*fur* (*E. coli* JW 0669) and their parental strain *E. coli* K-12 BW25113 (Baba et al. 2006) were used. Strains originated from the Czech Culture Collection (CCM), Brno, Czech Republic, the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Keio collection (National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan). Bacteria were grown in Luria-Bertani (LB), Mueller-Hinton (MH) broth (Biolife Italiana S.r.l., Milano, Italia) or in M9 minimal medium containing 2% glycerol at 37 °C with vigorous shaking. The media were solidified with agar (20 g/L). Lactobacilli were grown in/on MRS medium (Biolife Italiana S.r.l., Milano, Italia).

2.2. Drug susceptibility testing

The zone inhibition assay on MH, LB, M9 and MRS agars was used for determination of susceptibility of bacteria to CTBT and other drugs. Briefly, cells were grown to $OD_{600} \sim 0.5$, diluted to the 0.5 McFarland turbidity and spread on plates. After air drying, drugs were applied to sterile cellulose discs (diameter, 6 mm) previously placed at the plates which were then incubated overnight at 37 °C. Minimum inhibitory concentrations (MIC) of drugs in liquid media were based on triplicate assays in the microtitre plates and defined as the lowest concentrations where no bacterial growth was visible.

2.3. Detection of ROS

To determine intracellular ROS, the oxidant sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes) was used. Cells of *Escherichia coli* CCM 3954 grown in MH medium ($OD_{600} \sim 0.5$) were centrifuged at 3000 × *g* for 5 min, washed with 10 mM phosphate-buffered saline (PBS) pH 7.2 and treated in MSE sonicator on ice for 30 s with 30 s allowed for cooling. The total sonication time was 2 min. Then, CTBT (10–30 μg/mL) and H_2DCFDA (10 μM) were added to sonicated cells and incubated in the dark at 37 °C. At indicated time 100 μL samples were loaded in duplicate in 96-well plates. The fluorescence intensity was determined and normalized to the value for time = 0 using a Tecan Saphir II TM spectrofluorimeter (Tecan Austria GmbH, Grödig/Salzburg, Austria) with excitation and emission wavelengths of 485 nm and 520 nm, respectively.

3. Results

The antibacterial activity of CTBT was assessed by agar diffusion method using 13 different bacterial species. Most of them, both Gram-positive and Gram-negative, were sensitive to CTBT action. CTBT (10 μg) applied on cellulose disc induced growth inhibition zones, varying in diameter from 7 mm for *Enterococcus faecium* to 21 mm for *Bacillus subtilis*. *Enterococcus faecalis* and all the lactobacilli strains tested were resistant to CTBT (Table 1). The inability of CTBT to inhibit growth of lactobacilli on MRS medium was not caused by its composition that is optimal for growth of these bacterial species (De Man et al. 1960). Under the same experimental conditions, *B. subtilis* and *Staphylococcus aureus* were even more sensitive to CTBT (10 μg/disc) on MRS medium than on the MH agar, and displayed growth inhibition zones with diameter of 25 mm and 32 mm, respectively (Fig. 2A). When the MRS agar was supplemented with 5 mM EDTA, *Lactobacillus fermentum* became highly sensitive to CTBT indicating that Mg^{2+} plays a role in the control of its susceptibility to CTBT (Fig. 2B). Under the same conditions, EDTA (1 and 5 mM) present in MRS agar did not sensitize *Lactobacillus amylovorus* and *Lactobacillus casei* to CTBT. Although 1 mM EDTA had no effect, its 5 mM concentration already prevented growth of *Lactobacillus acidophilus* even in the absence of CTBT.

The antibacterial activity of CTBT was quite comparable with that of other antibacterial drugs such as ampicillin and chloramphenicol. However, compared with ofloxacine, the activity of CTBT was lower (Table 2). On solid or in liquid medium, the Gram-positive *B. subtilis* was more sensitive to CTBT then the Gram-negative *E. coli*. Minimum inhibitory concentrations (MIC) of CTBT in MH broth for *B. subtilus* and *E. coli* were 4 μg/mL and 10 μg/mL, respectively. When cells of *E. coli* were exposed to CTBT (20 μg/mL)

Table 1
CTBT inhibits the growth of Gram-positive (G^+) and Gram-negative (G^-) bacteria. Lactobacilli were grown on MRS agar at 37 °C for 24 h. Other bacterial strains were grown on MH agar at 37 °C for 24 h. Disks with drugs had the diameter of 6 mm.

Strain	G^+/G^-	Diameter of the growth inhibition zone (mm)	
		CTBT (10 μg/disc)	CTBT (20 μg/disc)
Bacillus subtilis CCM 2216	+	21	25
Enterococcus faecalis CCM 7000	+	6	6
Enterococcus faecium CCM 71687	+	7	13
Escherichia coli CCM 3954	–	14	21
Klebsiela pneumoniae CCM 4985	–	19	23
Lactobacillus acidophilus CCM 4388	+	6	6
Lactobacillus amylovorus CCM 4380	+	6	6
Lactobacillus casei CCM 7088	+	6	6
Lactobacillus fermentum CCM 91	+	6	6
Pseudomonas aeruginosa ATCC 3955	–	15	19
Salmonella enterica serovar *Typhimurium TA100* ATCC 3812	–	19	25
Serracia marcescens CCM 303	–	13	22
Staphylococcus aureus CCM 885	+	20	24