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Ebselen induces reactive oxygen species (ROS)-mediated cytotoxicity in *Saccharomyces cerevisiae* with inhibition of glutamate dehydrogenase being a target☆

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ABSTRACT

Ebselen is a synthetic, lipid-soluble seleno-organic compound. The high electrophilicity of ebselen enables it to react with multiple cysteine residues of various proteins. Despite extensive research on ebselen, its target molecules and mechanism of action remains less understood. We performed biochemical as well as *in vivo* experiments employing budding yeast as a model organism to understand the mode of action of ebselen. The growth curve analysis and FACS (florescence activated cell sorting) assays revealed that ebselen exerts growth inhibitory effects on yeast cells by causing a delay in cell cycle progression. We observed that ebselen exposure causes an increase in intracellular ROS levels and mitochondrial membrane potential, and that these effects were reversed by addition of antioxidants such as reduced glutathione (GSH) or N-acetyl-L-cysteine (NAC). Interestingly, a significant increase in ROS levels was noticed in *gdh3*-deleted cells compared to wild-type cells. Furthermore, we showed that ebselen inhibits GDH function by interacting with its cysteine residues, leading to the formation of inactive hexameric GDH. Two-dimensional gel electrophoresis revealed protein targets of ebselen including CPR1, the yeast homolog of Cyclophilin A. Additionally, ebselen treatment leads to the inhibition of yeast sporulation. These results indicate a novel direct connection between ebselen and redox homeostasis.

Introduction

A number of cellular defense mechanisms exist to quench free radicals and prevent intracellular damage by reducing the harmful effects of reactive oxygen species (ROS). These mechanisms or factors include low-molecular-weight antioxidants such as ascorbic acid and glutathione, and antioxidant enzymes such as thioredoxins, superoxide dismutase (SOD), catalase, and glutathione peroxidase [1–3]. These activities play a key role in minimizing the physiological levels of ROS. However, with continuous elevation of the levels of ROS, the defense systems can be exhausted, resulting in cellular damage. Normally functioning cells can sustain and tolerate background levels of damage, but if an imbalance occurs, cellular damage will be increased. ROS are reactive in nature and may cause damage to key cellular components including DNA, proteins, and lipids [4,5]. ROS, including hydrogen peroxide, the superoxide anion, and the hydroxyl radical are highly toxic oxidants which are inevitably produced in response to multiple stimuli [5–9]. Therefore, cells possess a complex system to neutralize the deleterious effects of ROS [10–13]. Because ROS are principal mediators of the cellular damage, compounds that regulate the fate of such species may be of great importance.

Ebselen is a synthetic, lipid-soluble seleno-organic compound having potent antioxidant capacity. It is also a novel anti-inflammatory agent having glutathione peroxidase-like activity [14–18]. It has therapeutic activity in neurological disorders, acute pancreatitis, noise-induced hearing loss, and cardiotoxicity. It also exhibits antiatherosclerotic, antithrombotic, and cytoprotective properties [15,19–21]. However, excessive amounts of ebselen are toxic to the cells. Ebselen is genotoxic above a concentration of 10 μM in V79 cells [22]. It induces apoptosis in HepG2 cells through a rapid depletion of intracellular thiols [23]. At high concentrations, it stimulates Ca^{2+} release from mitochondria via an NAD^{+} hydrolysis-dependent mechanism, and accelerates mitochondrial respiration and swelling, which are indicative of deterioration of the mitochondrial function [24]. A previous study from our laboratory documented the activation of DNA repair genes in yeast cells exposed to Ebselen [25].

Seleno-organic compounds exhibit strong electrophilic activity and are therefore capable of forming selenenyl-sulfide bonds with the

Abbreviations: CypA, Cyclophilin A; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; FACS, florescence activated cell sorting; GDH, glutamate dehydrogenase; GSH, glutathione; NAC, N-acetyl-L-cysteine; Ni-NTA, nickel-nitrilotriacetic acid; ROS, reactive oxygen species; SOD, superoxide dismutase.

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cysteine residues in proteins [26–28]. The ability of ebselen to covalently react with proteins' cysteine residues is thought to explain why the selenazal drugs modulate the activity of various inflammation-related enzymes, including lipoxygenase, nitric oxide synthase, and NADPH oxidase [29–31]. Various antioxidant enzymes have evolved to regulate the cellular levels of ROS. Glutathione (GSH), which is the most abundant peptide in cells, fulfills several functions, including directly scavenging of $HO^{\cdot}$ and singlet oxygen [32]. Glutamate, which is required for the biosynthesis of GSH, is synthesized by GDH. The 2 isofunctional NADP-GDH of *Saccharomyces cerevisiae* (GDH1 and GDH3) [33–35] are involved in the synthesis of GSH, and the normal functioning of these enzymes is required for the regulation of ROS levels [34]. Multiple cysteine residues are seen in the primary structure of GDH3. Hence, selenazal drugs may modulate its activity, resulting in its defective functioning.

In this study, we report that ebselen potently inhibits chicken GDH by reacting with the enzyme's cysteine residues, leading to its inhibition. Ebselen exposure induces high intracellular ROS levels, and the deletion of yeast *gdh3* potentiates ROS production, indicating that GDH3 is an *in vivo* target of this drug. Taken together, our results depicts GDH as a novel target of ebselen, and these observations can be used to design ebselen-based molecular therapeutics for the regulation of ROS levels under various conditions.

Materials and methods

Reagents and yeast strains

All reagents, unless otherwise stated, were purchased from Sigma–Aldrich (USA). Yeast strains were grown in SC (synthetic complete) medium. All experiments were performed on wild type strain W1588-4c (MATa ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1, ura3-1, RAD5+), BY4743 (MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) gdh1, gdh2 and gdh3 KO generated in BY4743 background were purchased from Open Biosystem. For sporulation experiment USY61 (MATa/MATalpha ura3D0/ura3D0 his3D1/his3D1 CAN1/can1::Ste2::spHis5 flo8D0/flo8D0) yeast diploid strain was used, we got this strain as a kind gift from Ulrich Schlecht. Ebselen was dissolved in DMSO. Concentration of DMSO was kept below 0.1% in all experiments.

Growth sensitivity and methylene blue assays

To investigate the effect of ebselen on the growth of yeast mutants, wild type yeast strains were inoculated into YPD liquid medium and grown to saturation by incubating cultures at 30 °C and 200 rpm. Yeast saturated cultures were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) in 1.0 ml of sterile double distilled water. 3 μl of cultures were spotted onto SC agar plates containing ebselen (2.5, 5.0, 7.5 and 10 μM) or DMSO. Plates were incubated at 30 °C and growth of the yeast strains were recorded at time intervals of 24, 48 and 72 h by scanning (HP scanjet G2410).

Wild type yeast cells were grown in YPD medium till log phase (OD_{600} equals to 0.6–0.8) and treated with ebselen at different concentrations (DMSO, 5, 10, 20, 30 and 50 μM) for 6 h. After treatment OD_{600} was recorded at regular intervals for growth curve analysis. Methylene blue assay was performed as described earlier [36,37] after 3 h of ebselen treatment, cells were stained with 100 μg/ml methylene blue to differentiate between live (unstained) and dead/metabolically inactive (dark blue colored) cells. Cells were observed under the bright field microscope by using LAS EZ-V1.7.0 software (LEICA DM500).

FACS analysis of yeast cells

Yeast cells in exponential phase were treated with alpha factor to synchronize cells in G1 phase. Cells were released in DMSO (control) or 25 μM ebselen containing media for 6 h. Samples were collected at regular intervals and harvested by centrifugation. Ethanol was added to cell pellets, with vigorous vortexing. Cells were collected by centrifugation and washed once with 50 mM sodium citrate buffer (pH 7.0). RNase A was added to the samples and incubated at 37 °C for 1 h. RNase A-treated samples were transferred to BD FACS flow containing 20 mg/ml propidium iodide (Sigma). Cellular DNA was detected by a BD FACS Aria III with BD FACS Diva software.

Detection of cellular ROS levels and assays for mitochondrial membrane potential ($\Delta\Psi$)

To measure ROS production we used 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, D6883). DCFH-DA is membrane-permeable and is trapped intracellularly following deacetylation. The resulting compound, DCFH, reacts with ROS (primarily H_2O_2 and hydroxyl radicals) to produce the oxidized fluorescent form 2,7-dichlorofluorescein (DCF). ROS analysis using DCFH-DA was performed as follows. Yeast cells were treated with 10 μM DCFH-DA in culture media for 1 h prior to harvesting. Cells were washed twice in ice-cold PBS (phosphate buffer saline), resuspended in same buffer and immediately observed under fluorescence microscope (AXIOVERT 4.0) using FITC filter. The membrane potential-dependent stain MitoTracker (Molecular Probes-Invitrogen) was used to assess the mitochondrial membrane potential of yeast cells. After treatment with drug approximately 1×10^7 yeast cells were harvested and washed with ice-cold PBS. Cells were resuspended in 100 μl of PBS followed by staining with MitoTracker. After staining cells were visualized under fluorescence microscope (AXIOVERT 4.0) using Rhodamine filter or by FACS. For analyzing the effect of reduced glutathione (GSH – 10 mM), or N-acetyl-L-cysteine (NAC – 20 mM) supplementation on ROS levels and mitochondrial membrane potential, they were added in exponential yeast culture 30 min prior to addition of ebselen. Cells were further grown for 3 h followed by staining with DCF-DA or MitoTracker and immediately analyzed by FACS.

Glutathione measurement assays

Glutathione levels were measured using the method described by Wu et al. [38]. Briefly, cells were grown to exponential phase and treated with DMSO (control) or ebselen for 3 h, washed with ice cold water, and resuspended in 250 μl of cold 1% 5-sulfosalicylic acid. Cells were broken by vigorous vortexing with glass beads and incubated at 4 °C for 15 min. The extract was centrifuged and supernatants were used to determine glutathione levels. Total glutathione was determined by adding 10 μl of lysate to 150 μl of assay mixture (0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 0.03 mg/ml 5,5′-dithiobis(2-nitrobenzoic acid), 0.12 unit of glutathione reductase). The samples were mixed and incubated for 5 min at room temperature followed by addition of 50 μl of NADPH (0.16 mg/ml). The formation of thiobis (2-nitrobenzoic acid) was measured spectrophotometrically at 420 nm over a 5-min period. Standard curves were generated for each experiment using 0–0.5 nmol of glutathione in 1% 5-sulfosalicylic acid. To measure GSSG alone, 100 μl lysate samples were derivatized by adding 2 μl of 97% 2-vinylpyridine, and the pH was adjusted by adding 2 μl of 25% triethanolamine followed by incubation at room temperature for 60 min. The samples were then assayed as described above for total GSSG. GSSG standards (0–0.1 nmol) were also treated with 2-vinylpyridine in an identical manner to the samples. Subtraction of the amount of GSSG in the lysate from the total glutathione concentration allowed a determination of GSH levels