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Ebselen exerts antifungal activity by regulating glutathione (GSH) and reactive oxygen species (ROS) production in fungal cells

Shankar Thangamani[1], **Hassan E. Eldesouky**[1], **Haroon Mohammad**[1], **Pete E. Pascuzzi**[2], **Larisa Avramova**[3], **Tony R. Hazbun**[3,4,*], and **Mohamed N. Seleem**[1,5,*]

[1]Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47906, USA

[2]Faculty in Libraries, Purdue University, West Lafayette, Indiana 47906, USA

[3]Bindley Bioscience Center, Purdue University, West Lafayette, Indiana 47906, USA

[4]Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University, West Lafayette, Indiana 47906, USA

[5]Purdue Institute for Inflammation, Immunology, and Infectious Disease, Purdue University, West Lafayette, IN 47907, USA

Abstract

Background—Ebselen, an organoselenium compound and a clinically safe molecule has been reported to possess potent antifungal activity, but its antifungal mechanism of action and *in vivo* antifungal activity remains unclear.

Methods—The antifungal effect of ebselen was tested against *Candida albicans, C. glabrata, C. tropicalis, C. parapsilosis, Cryptococcus neoformans,* and *C. gattii* clinical isolates. Chemogenomic profiling and biochemical assays were employed to identify the antifungal target of ebselen. Ebselen's antifungal activity *in vivo* was investigated in a *Caenorhabditis elegans* animal model.

Results—Ebselen exhibits potent antifungal activity against both *Candida* spp. and *Cryptococcus* spp, at concentrations ranging from 0.5 – 2 μg/ml. Ebselen rapidly eradicates a high fungal inoculum within two hours of treatment. Investigation of the drug's antifungal mechanism of action indicates that ebselen depletes intracellular glutathione (GSH) levels, leading to increased production of reactive oxygen species (ROS), and thereby disturbs the redox homeostasis in fungal

*Correspondence: thazbun@purdue.edu, mseleem@purdue.edu (lead).

Conflicts of interest: None to declare.

Author contributions: S.T, H.E and L.A performed the experiments. P.P analyzed the sequencing results. S.T, T.H and M.S designed the study, analyzed the data and interpreted the results. S.T, T.H, H.M and M.S wrote the manuscript. All authors reviewed and discussed the results.

cells. Examination of ebselen's *in vivo* antifungal activity in two *Caenorhabditis elegans* models of infection demonstrate that ebselen is superior to conventional antifungal drugs (fluconazole, flucytosine and amphotericin) in reducing *Candida* and *Cryptococcus* fungal load.

Conclusion—Ebselen possesses potent antifungal activity against clinically relevant isolates of both *Candida* and *Cryptococcus* by regulating GSH and ROS production. The potent *in vivo* antifungal activity of ebselen supports further investigation for repurposing it for use as an antifungal agent.

General significance—The present study shows that ebselen targets glutathione and also support that glutathione as a potential target for antifungal drug development.

Keywords

ebselen; antifungal activity; glutathione; ROS production*C. elegans*

1. Introduction

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is an organoselenium compound that is known to possess anti-atherosclerotic, anti-inflammatory, antioxidative, cytoprotective, anti-mutagenic and anti-lipoperoxidative properties [1–4]. Several studies have demonstrated that ebselen, due to its highly electrophilic nature, interacts with cysteine rich proteins (such as thioredoxin) and non-proteins (thiols) [2,5–11]. Ebselen specifically interacts with free thiols such as glutathione (GSH) to form ebselen selenenyl sulfide; this intermediate catalyzes reactive oxygen species (ROS) formation. Interestingly, ebselen selenenyl sulfide can be reduced by GSH to form ebselen selenol. This particular intermediate functions as a ROS scavenger, and thereby protects the cell from free radical damage [2,6,7]. As a clinically safe molecule, ebselen has been investigated for the treatment of various ailments such as arthritis, stroke, cardiovascular disease and cancer [2,12–15].

In addition to the beneficial properties of ebselen in mammalian cells, ebselen has also been investigated for its antimicrobial activity against multidrug-resistant Gram positive pathogens, including *Staphylococcus aureus* and *Enterococcus spp.* [16–21]. Recently, we demonstrated that ebselen exerts its antibacterial activity through the inhibition of protein synthesis in bacteria [20,21]. Ebselen has also been shown to possess potent antifungal activity, though different molecular targets have been proposed [22–24]. Studies by Billack et.al and Chan et.al demonstrated that ebselen inhibits the plasma membrane H(+)-ATPase pump (Pma1p) in yeast [23,24]. Azad et.al proposed that ebselen activates the DNA damage response and alters nuclear proteins in yeast [25]. A follow-up study by their research group also proposed that ebselen inhibits glutamate dehydrogenase (GDH3) and induces ROS production in yeast [26]. The studies above highlight that the antifungal mechanism of action of ebselen is challenging to elucidate and currently remains unresolved.

Given the tremendous pressure imposed by the emergence of resistance to antifungal agents currently utilized in the clinic, identifying new classes of antifungal drugs remains an unmet challenge [27–30]. However, the traditional pathway for drug discovery is an arduous process that yields few approved new antimicrobials annually. An alternative approach

steadily gaining support is utilizing drug repurposing to identify promising new anti-infective agents and expedite their regulatory approval [27,31].

Based upon the preliminary data presented in literature, ebselen is a promising drug to repurpose as a novel antifungal agent. However, additional research is necessary to elucidate ebselen's antifungal mechanism of action. Thus, the objectives of our study were to examine ebselen's spectrum of activity against an array of fungal clinical isolates, to deduce ebselen's antifungal mechanism of action, and to confirm the drug's *in vivo* efficacy in two *Caenorhabditis elegans* animal models of fungal infection.

2. Materials and Methods

2.1. Fungal strains and reagents

Candida albicans, C. glabrata, C. tropicalis, C. parapsilosis, Cryptococcus neoformans, and *C. gattii strains* used in this study are presented in Table 1. RPMI powder, MOPS and L-glutathione (reduced) were purchased from Sigma-Aldrich (St. Louis, MO). Yeast peptone dextrose agar (YPD) (BD Biosciences, San Jose, CA), fluconazole (Acros Organics, New Jersey), flucytosine and ebselen (TCI chemicals, Tokyo, Japan) were purchased from commercial vendors.

2.2. Antifungal susceptibility testing

Antifungal susceptibility testing was done as per the National Committee for Clinical Laboratory Standards M-27A3 (NCCLS) guidelines [32]. Briefly, five colonies from 24-hour old cultures of *Candida* spp. or 48-hour old cultures of *Cryptococcus* were transferred to 5 ml of sterile Phosphate buffered saline (PBS). After adjusting to reach a McFarland standard 0.5, fungal suspensions were diluted 1:2000 in RPMI 1640 buffered to pH 7.0 with 0.165 M MOPS (RPMI-MOPS). The drugs (ebselen, fluconazole, flucytosine and amphotericin) were serially diluted and the minimum inhibitory concentration (MIC) was determined as follows: (i) For fluconazole and flucytosine, the MIC was classified as a significant decrease (approximately 50%) in visible growth compared to untreated controls; (ii) For ebselen and amphotericin B, the MIC was categorized as the lowest concentration that produced no visible fungal growth. All experiments were carried out in triplicate wells and repeated at least twice.

2.3. Time kill assay

Cultures of *Candida albicans* and *Cryptococcus neoformans* at a dilution of 5×10^5 CFU/ml were treated with 5 × MICs of ebselen, fluconazole, flucytosine and amphotericin B (in triplicate) in RPMI-MOPS, at 35°C. At specific time points, aliquots were collected, serially diluted in PBS, and plated onto YPD agar plates. After incubation at 35°C for 24–48 hours the fungal colony forming units (CFU) were obtained, as described elsewhere [33].

2.4. Chemogenomics profiling of *Saccharomyces cerevisiae*

The initial testing of *Saccharomyces cerevisiae* response to ebselen was performed with the wild type BY4743 diploid strain, the isogenic parent to the heterozygous diploid deletion collection. BY4743 was grown in YPD in 96-well plates with 1% DMSO or ebselen in

concentrations ranging from 10 to 200 μM to determine a suitable level of growth inhibition. Ebselen (25 μM) was used for haploinsufficiency profiling because it delayed growth by 30% compared to the no drug control half-maximal optical density (OD). All experiments were performed at 30°C and cultures were shaken at 300 rpm. A frozen aliquot (200 μL) of the heterozygous deletion pool (Thermo Fisher Scientific, Waltham, MA) was thawed and used to inoculate 2 mL of YPD broth and grown for 9 hours to reach an OD_{600} of 4.0. The culture was diluted to an OD_{600} of 0.13 and either 1% DMSO or 25 μM ebselen was added (three replicates each) and grown for 7 hours. The cultures were grown again by diluting to an OD_{600} of 0.13 in 1 mL YPD broth with DMSO or 25 μM ebselen and grown for 8 hours, harvested and genomic DNA extracted using the YeaStar Genomic DNA kit (Zymo Research, Irvine, CA). The UPTAGs were amplified by PCR with Phusion Hot Start II High-Fidelity DNA polymerase at 0.02 U/μL (Thermo Fisher Scientific, Waltham, MA) using 0.5 ng/μL genomic DNA. The 267 bp PCR product was electrophoresed on an agarose gel and the DNA extracted using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified DNA was measured using a Qubit instrument and samples were normalized and mixed to a final concentration of 10 nM. Strains were grown and maintained on media using standard practices [34].

The pooled PCR products were sequenced using standard Illumina sequencing in a HiSeq 2500 instrument. The reads were separated based on a 5 base multiplex tag unique for each experiment and an average of 5 million reads per replicate was obtained. The UPTAG barcodes in each experimental sample were separated based on a reference database of recharacterized barcode sequences [35].

The resulting strain counts were imported into R and analyzed with edgeR [36]. Sequencing library sizes were normalized using default parameters. Only strains with one or more counts in three or more samples were analyzed further. Differential representation of strains was determined using the quantile-adjusted conditional maximum likelihood (qCML) method. False discovery rates were determined to control for multiple testing.

2.5. *Saccharomyces* deletion strain haploinsufficiency validation

Overnight grown saturated cultures of yeast cells were diluted to 1 to 10 and further to 1 to 5000 before treating with indicated concentration of ebselen. After 24 hours of incubation, yeast growth was monitored using a spectrophotometer (OD_{600}) and the results were expressed as percent growth rate for each strain compared to the untreated control group, as described elsewhere [37].

2.6. Determining fungal growth with L- glutathione supplementation

In experiments with L- glutathione supplementation, indicated concentration of glutathione was added to the fungal cultures (with or without ebselen) and the percent growth rate or MIC was determined as described above.

2.7. Glutathione assay

The assay was conducted as per the manufacturer's instructions (Glutathione assay kit from Cayman chemicals, Michigan, USA). Briefly, saturated cultures of wild type and deletion

strains of *S. cerevisiae* cells were diluted to 1:5 in YPD broth and treated with ebselen (20 μg/ml) for 2.5 hours. After treatment, tubes were centrifuged. The cells were subsequently washed once with cold water and followed by 1 × GSH MES buffer (supplied by the manufacturer). After washing, cells were re-suspended in 250 μL of 1 × GSH MES buffer and sonicated for 45 seconds. Tubes were centrifuged and the supernatant was collected for the assay. An aliquot (50 μL) of cell supernatant was added to each well in a 96-well plate and then 150 μL of the assay cocktail, prepared per the manufacturer's guidelines, was added. After two minutes of incubation, the intensity of yellow color produced was measured using a spectrophotometer (OD_{410}). The results are expressed either as absorbance per ml or percent glutathione production relative to untreated control groups.

2.8. Measuring ROS production in yeast cells

The Image-iT™ LIVE Green Reactive Oxygen Species (ROS) detection kit (Molecular Probes, Inc. Eugene, OR) was utilized and the ROS production was measured as per the manufacturer's instructions. Briefly, saturated cultures of wild type and deletion strains of *S. cerevisiae* cells were diluted to 1:5 in YPD broth and treated with ebselen (20 μg/ml) for 2.5 hours. Then 10mM of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) dye was added at a dilution of 1:500. After 2 hours of incubation, the cells were washed with PBS and the intensity of fluorescence produced was measured using spectrophotometer or imaged by Leica confocal laser scanning microscopy.

2. 9. Caenorhabditis elegans (C. elegans) infection study

C. elegans AU37 (sek-1; glp-4) strain (glp-4(bn2) was used to investigate the antifungal efficacy of ebselen, as described elsewhere [20,38–41]. Briefly, L4-stage worms were infected either with *Cryptococcus neoformans* NR-41292 or *Candida albicans* ATCC 10232 for three hours at room temperature. After infection, worms were washed with M9 buffer and treated for 24 hours either with DMSO or drugs (ebselen, amphotericin B, fluconazole, and flucytosine), at indicated concentrations. Post-treatment, worms were washed, disrupted using silicon carbide particles [20,39–41], and the resulting suspensions were serially diluted and transferred to YPD agar plates containing ampicillin (100 μg/ml), streptomycin (100 μg/ml) and kanamycin (45 μg/ml). Plates were incubated for 24–48 hours at 35°C before the colony forming unit (CFU) per worm was determined.

2.10. Statistical analyses

Statistical analyses were done using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). *P* values ≤ were calculated using the Student *t* test. *P* values 0.05 were deemed significant.

3. Results

3.1. Antifungal activity and killing kinetics of ebselen

Ebselen's antifungal activity was examined against numerous clinical isolates of *Candida* and *Cryptococcus*. Ebselen inhibited isolates of *Candida albicans, C. glabrata, C. tropicalis* and *C. parapsilosis* at concentrations ranging from 0.5 to 2 μg/ml (Table 1). Ebselen retained its potent antifungal activity against *Cryptococcus neoformans* and *Cryptococcus gattii*, as

the drug inhibited growth of these fungal species at concentrations ranging from 0.5 to 1 μg/ml (Table 1).

In order to investigate the killing kinetics of ebselen against both *C. albicans* and *C. neoformans*, a time-kill assay was conducted. Unlike fluconazole and flucytosine, ebselen (at 5 × MIC) completely eradicated *C. albicans* ATCC 10231 and *C. neoformans* NR-41291 within two hours of treatment (Figure 1). Ebselen's fungicidal activity was superior to amphotericin which required at least four hours to completely eliminate fungal cells (Figure 1).

3.2. Glutathione as a potential target of ebselen

After verifying ebselen's potent antifungal activity, we proceeded to investigate the antifungal mechanism of ebselen. Chemogenomic profiling, using drug-induced haploinsufficiency, was utilized due to its nature as a highly-specific technique to deduce the molecular mechanism of unknown compounds [42–44]. Haploinsufficiency profiling (HIP) allows for the simultaneous assessment of the sensitivity of the pooled genome-wide set of heterozygous deletion strains due to the fact that each strain possesses a unique synthetic DNA barcode. The method is an unbiased approach to find strains exhibiting the most sensitivity to ebselen. After determining the concentration that reduced wild-type growth by 30%, we used 25 μM of drug to profile the pooled heterozygous strains in the biological samples. PCR was used to amplify the unique UPTAG DNA barcodes located at the gene deletion site and the barcode abundance was tracked using Illumina sequencing. The resulting counts were normalized and visualized using EdgeR (Supplementary figure).

We identified 33 heterozygous deletion strains that were under-represented based on a FDR less than 0.02, when comparing ebselen treatment to DMSO. These strains were enriched for glutathione metabolic process (p-value = 0.003). In addition, we also included additional heterozygous deletion strains based on previous chemogenomic profiling of ebselen using heterozygous and homozygous diploid strain pools: *pma1Δ* which was the 35th ranked strain from a heterozygous screen [45]*; glr1Δ, gsh1Δ, gsh2Δ* hits from a homozygous screen [44]; *ubx4Δ*, *gsh1Δ, trp2Δ* from a homozygous screen [44]; and *rad4Δ* from a heterozygous screen [44]. In addition, a cluster of the deletion strains are associated by adjacent chromosomal locations and these may share a background mutation rendering them sensitive to multiple compounds as documented previously [44]. Strains with deletions corresponding to this previously identified strain cluster (*YLR274W* to *YLR321C*) are likely not specific to ebselen. A full description and rationale for including additional strains are available in supplementary information (supplementary excel file). It is important to note that some of these additional strains were derived from homozygous deletion strain profiling but we tested the heterozygous counterparts.

The heterozygous strains including *gsh1Δ*, *gsh2Δ*, *glr1Δ*, *trr1Δ*, *trr2Δ, fks1Δ, ylr287cΔ, ylr282cΔ, guf1Δ, ylr296wΔ, est2Δ, rrf1Δ* and *ycr006cΔ* experienced a significant reduction in growth when exposed to ebselen (Figure 2A). Importantly, two heterozygous deletion strains (*gsh1Δ* and *gsh2Δ*) encoding genes involved in glutathione (GSH) synthesis were the most sensitive to ebselen. A haploid set of these two deletion strains (*gsh1Δ* and *gsh2Δ*) was also tested. These haploid deletion strains (*gsh1Δ* and *gsh2Δ*) were not resistant to ebselen

and exhibited increased sensitivity to ebselen when compared to the diploid strains (Figure 2B). The results indicate that ebselen most likely does not directly target the proteins (Gsh1 and Gsh2) involved in glutathione synthesis but likely impinges on glutathione metabolism and exhibits antifungal activity. Based on the fact that ebselen binds directly to GSH and depletes GSH levels, leading to apoptosis in mammalian cells [10,46], we hypothesized ebselen exhibits a similar mode of action in yeast. Glutathione levels in wild type (BY4743 and BY4741), heterozygous and homozygous deletion strains (*gsh1Δ* and *gsh2Δ*) were quantified using a glutathione assay kit. Results indicate that all deletion strains experienced a significant reduction in GSH levels compared to their respective wild-type strains (Figure 2C). Homozygous deletion strains have a very low presence of GSH compared to their heterozygous strain counterpart (Figure 2C). However, treatment of homozygous deletion strains with ebselen further reduced GSH levels (approximately by 40%) compared to untreated control groups. On the other hand, the wild type and heterozygous deletion strains (*gsh1Δ* and *gsh2Δ*) showed no considerable decrease in GSH levels when treated with ebselen at this concentration (Figure 2D). These results suggest that ebselen depletes intracellular glutathione levels in yeast cells.

3.3. Depletion of glutathione by ebselen leads to increased ROS production

Glutathione plays a central role in maintaining redox-homeostasis in yeast [47,48]. Significant decreases in GSH levels might lead to dysregulation of redox homeostasis and in turn increase ROS production [10,46–48]. Given that ebselen was shown to deplete GSH levels in yeast cells, we investigated the effect of ebselen on ROS production. Basal level of ROS production in wild-type and GSH deletion strains were quantified. As expected, homozygous deletion strains (*gsh1Δ* and *gsh2Δ*) displayed a considerable increase in ROS levels compared to both the wild-type and heterozygous deletion strains (Figure 3A). However, when exposed to ebselen treatment, all strains (except the *gsh2Δ* heterozygous deletion strain) experienced a significant increase in ROS production (Figure 3B). As expected, *gsh1Δ* and *gsh2Δ* homozygous deletion strains exhibited the largest increase in ROS production (almost two-fold increase) compared to untreated control groups (Figure 3B). These results were confirmed using confocal microscopy. As presented in Figure 3C, ROS production was prominently noticed only in the *gsh1Δ* and *gsh2Δ* homozygous deletion strains. Collectively, the results support the notion that ebselen exerts its antifungal activity by causing a sharp decrease in GSH levels that subsequently leads to increased ROS production in yeast cells.

3.4. Supplementation of L-glutathione restored the cell growth

Based upon the above result, we hypothesized that GSH supplementation would reverse the inhibitory effect in yeast caused by ebselen. As expected, supplementation with L-glutathione restored cell growth and reversed the inhibition caused by ebselen, in a concentration-dependent manner (Figure 4). GSH, at 25 μg/ml, completely restored the cell growth (Figure 4). In addition, the effect of GSH supplementation on susceptibility of *Candida* and *Cryptococcus* strains to ebselen were also examined. Interestingly, with GSH supplementation (0.25 mg/ml), all tested fungal strains including *C. albicans, C. glabrata, C. tropicalis*, *C. parapsilosis, C. neoformans* and *C. gattii* become resistant to ebselen (MIC >128 μg/ml) (Table 2). On the other hand, the MIC of control antifungal drugs (fluconazole

and flucytosine amphotericin) was not altered with GSH supplementation (Table 2). These results suggest a mode of action of ebselen that is specifically reversed by elevated GSH levels.

3.5. *In vivo* efficacy of ebselen in infected *C. elegans* model

To investigate if the *in vitro* antifungal activity of ebselen translates *in vivo*, the antifungal efficacy of ebselen was tested in a *C. albicans* and *C. neoformans*-infected *C. elegans* animal model. As shown in Figure 5, treatment of infected *C. elegans* with amphotericin, fluconazole, flucytosine and ebselen at 4 and 8 μg/ml, produced a significant reduction ($P \leq 0.05$) in mean fungal load when compared to the untreated control groups. *C. elegans* treated with ebselen (8μg/ml) completely eradicated *C. albicans*, Amphotericin (8 μg/ml) produced a 1.53±0.08 $\log_{10}$ CFU reduction which was nearly identical to ebselen at 4 μg/ml (1.52±0.14 $\log_{10}$). Fluconazole, at 8 μg/ml, reduced the burden of *C. albicans* by 1.36±0.07 $\log_{10}$ followed by amphotericin (4 μg/ml) (1.05±0.16 $\log_{10}$), flucytosine (8 μg/ml) (0.79±0.09 $\log_{10}$), flucytosine (4 μg/ml) (0.62±0.08 $\log_{10}$) and fluconazole (4 μg/ml) (0.55±0.09 $\log_{10}$).

Treatment of *C. neoformans*-infected *C. elegans* with ebselen (8 μg/ml) also generated the highest reduction in CFU count (2.31±0.02 $\log_{10}$), followed by amphotericin (8 μg/ml) (1.98±0.13 $\log_{10}$), (1.97±0.09 $\log_{10}$), ebselen at 4 μg/ml (1.97±0.09 $\log_{10}$), amphotericin (4 μg/ml) (1.46±0.03 $\log_{10}$), fluconazole (8 μg/ml) (1.32±0.04$\log_{10}$), flucytosine (8 μg/ml) (0.75±0.02 $\log_{10}$), fluconazole (4 μg/ml) (0.66±0.02 $\log_{10}$) and flucytosine (4 μg/ml) (0.58±0.11 $\log_{10}$).

4. Discussion

Fungal infections are a significant healthcare challenge particularly in immunocompromised individuals, such as HIV patients [49,50]. *Candida albicans* is the fourth leading cause of bloodstream infections in the United States and has been associated with a high mortality rate (50%) [51,52]. In addition to infections caused by *C. albicans*, Cryptococci, particularly *C. neoformans*, are a major source of infections in immunocompromised individuals. Cryptococcal meningitis is a significant cause of mortality in HIV patients with an estimated 1 million cases each year, resulting in nearly 625,000 deaths [50,53]. The immune system in these immunocompromised patients is not capable of eradicating these fungal pathogens. Thus treatment is highly dependent on antifungal drugs successfully resolving the fungal infection [27]. Unfortunately, antifungal agents have limited effectiveness in treating some invasive fungal infections and suffer from restrictions in spectrum of activity, route of administration, and crossing blood brain barrier [30,54]. Further compounding this problem, the number of antifungal drug classes currently available to clinicians is limited. This issue is exacerbated by the fact that resistance to antifungal agents is increasing and many current antifungal agents exhibit unusual toxicities thus further restricting their use [27–30]. This highlights the pressing need to identify new antifungal drugs to combat these dangerous pathogens. The traditional route of antifungal innovation and regulatory approval is a time-consuming, expensive venture. This has led researchers to explore alternative approaches, such as drug repurposing, to expedite anti-infective drug development [27,31,55–58].

Ebselen is an organoselenium compound that is currently undergoing clinical trials for the prevention and treatment of cardiovascular disease, arthritis, stroke, atherosclerosis, and cancer [2,12–15]. In an intensive search for non-antifungal drugs exhibiting antifungal activity, we and others [22–24] demonstrated that ebselen possesses potent broad-spectrum fungicidal activity against *Candida* and *Cryptococcus spp* with the MIC values ranging from 0.5 to 2 μg/ml. Although, its antifungal activity has been reported before, the antifungal mechanism of action and *in vivo* efficacy of ebselen remains unclear with several potential targets proposed [17,22–24]. In the present study, we demonstrated that ebselen reduces GSH concentration in yeast cells leading to dysregulation of redox homeostasis. These results correlate with studies conducted by Yang *et.al* and Shi *et.al* that reported ebselen depletes GSH levels in mammalian cells, ultimately leading to apoptosis [10,46].

Although, ebselen has been shown to have an antioxidant effect and protects cells from free radical damage, it has also been shown to cause apoptosis by reducing thiol levels in mammalian cells [10,46]. The present study indicates that ebselen also exhibits a similar mode of action in yeast cells. Decreased GSH levels subsequently leads to increased ROS production thereby placing cells under oxidative stress. This finding is in agreement with a recent study by Ngo *et. al* that demonstrated ebselen treatment induces ROS in *Candida albicans* [22]. In addition, Azad *et. al* proposed that ebselen increases ROS levels in yeast by inhibiting the Gdh3 enzyme involved in glutathione synthesis [26]. However, we found that *gdh3Δ* heterozygous and haploid deletion strains were not susceptible to ebselen compared to both the *gsh1Δ* and *gsh2Δ* deletion strains. Studies conducted by Billack *et. al* and Chan *et. al* proposed that the plasma membrane H(+)-ATPase pump (Pma1) is the potential target of ebselen in yeast [23,24]. However, we also confirmed that the *pma1Δ* heterozygous deletion strain does not experience significant growth impairment when exposed to ebselen relative to the *gsh1Δ* and *gsh2Δ* deletion strains. Many of the proposed targets for ebselen have been demonstrated via biochemical based assays in which it is difficult to assess the specificity of ebselen for the protein target compared to other targets especially because of the reactivity of the molecule for cysteines. Collectively, results from our study demonstrate that ebselen reduces intracellular GSH concentration leading to dysregulation of redox homeostasis and that deficiency in glutathione biosynthesis exacerbates this mode of action.

Glutathione is an essential metabolite required to protect yeast from oxidative stress [47,48,59–62]. *S. cerevisiae* lacking c-glutamyl cysteine synthase (Gsh1), the first enzyme in glutathione biosynthesis, leads to glutathione autotrophy in which the cells dependent on exogenous GSH for its growth and survival [63,64]. In the absence of endogenous GSH, yeast has the ability to uptake GSH from an environment through high-affinity glutathione transporters such as Hgt1 [63–65]. In the present study, we also demonstrated that *gsh1Δ* and *gsh2Δ* homozygous deletion strains has relatively low amount of basal GSH when compared to wild type and the counterpart heterozygous deletion strains. The GSH observed in the *gsh1Δ* and *gsh2Δ* homozygous deletion strains is likely derived from growth medium. The presence of low amount of GSH in these deletion strains was further depleted by ebselen treatment and in turn places cells in oxidative stress. Importance and essentiality of GSH has also been demonstrated in other fungi species including *Candida* [60,65], suggesting that glutathione might form an attractive novel target for the development of new

antifungal drugs [66–68]. Future studies are required to delineate the interaction between ebselen and yeast GSH, and also the affinity of ebselen towards mammalian GSH.

Chemogenomic profiling was employed in this study, and it identified 33 heterozygous deletion strains sensitive to ebselen that were under-represented based on an FDR less than 0.02. However, the hits recovered did not include the *GSH1* or *GSH2* genes. It is also interesting to note that these strains were also not identified as hits in other heterozygous chemogenomic profiling screens employed by two other groups [44,45]. Potential hits obtained using this technique greatly depend on (i) the concentration of the drug/compound used to test the deletion pool (ii) Many technical factors such as the PCR quality and number of reads. These factors should be taken into consideration when utilizing chemogenomic profiling to identify the mode of action of unknown compounds.

The final segment of this study investigated the *in vivo* antifungal efficacy of ebselen in a *C. albicans* and *C. neoformans*-infected *C. elegans* animal model. Ebselen, at 8 μg/ml, completely eradicated the *C. albicans* load and produced a more than two- $\log_{10}$ reduction in *C. neoformans* CFU load. Ebselen's antifungal activity was found to be superior to currently approved antifungal drugs including amphotericin, fluconazole and flucytosine in reducing the fungal load in the *C. elegans* animal model. These results lay a strong foundation for future studies to test the antifungal efficacy of ebselen in appropriate mice models of fungal infection. Ebselen is also known to be capable of crossing the blood brain barrier [15]. This quality provides an added advantage to investigate the potential use of this drug for the treatment of *Cryptococcal* meningitis infections particularly in HIV patients [15,50].

In conclusion, the present study confirms ebselen, with its unique mechanism of action and potent *in vivo* antifungal activity, is a promising clinical molecule that necessitates further investigation for repurposing as a novel antifungal agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Ebselen exhibits potent antifungal activity against *Candida* and *Cryptococcus* spp.
- Ebselen rapidly kills fungi within two hours of treatment
- Ebselen induces cell death by regulating GSH and ROS production
- Ebselen shows potent *in vivo* antifungal activity in *C. elegans* models of infection

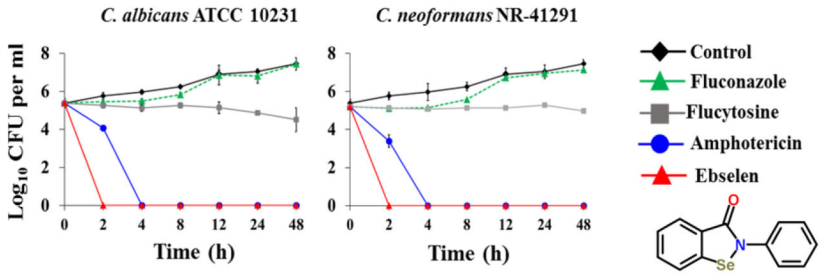

Figure 1.

Killing kinetics of ebselen. An overnight culture of *C. albicans* ATCC 10231 and *C. neoformans* NR-41291 were treated with 5 × of ebselen, fluconazole, flucytosine and amphotericin (in triplicate) in RPMI-MOPS and incubated at 35°C. Samples were collected at indicated time points and plated onto YPD plates. Plates were incubated for 24–48 h prior to counting the colony forming units (CFU).

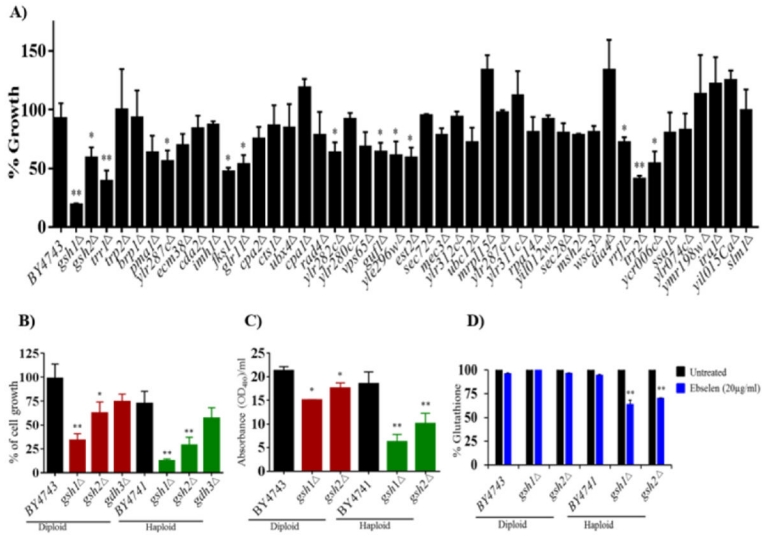

Figure 2.

Glutathione as a potential target of ebselen. (A and B) The percent growth of yeast cells (OD_{600} after 24 h) incubated with ebselen (2μg/ml) in YPD broth was determined in relation to the DMSO treatment. The results are presented as means ± SD (n = 3). (C) Saturated cultures of yeast cells were diluted to 1:5 and grown for 2.5 h. The cells were sonicated and amount of glutathione was determined using glutathione assay kit. The absorbance measured using spectrophotometer indicates the glutathione production in each strain. (D) Yeast cells were treated with ebselen (20μg/ml) for 2.5 h and the glutathione concentration was measured as indicated above. The results are expressed as percent glutathione production relative to untreated control groups. Statistical analysis was calculated using the two-tailed Student's *t* test. *P* values of (* $P \leq 0.05$) (** $P \leq 0.01$) are considered as significant.

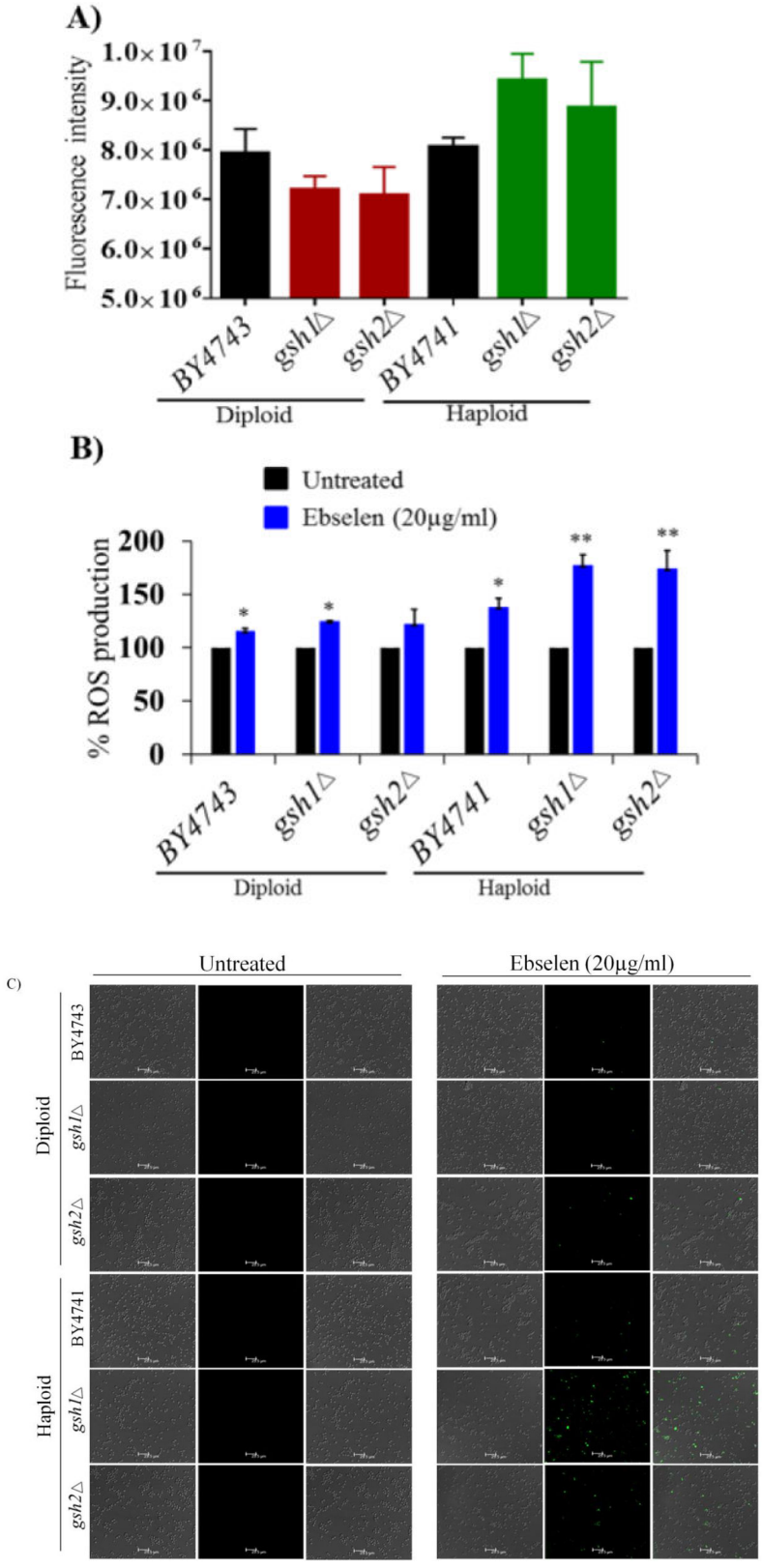

Figure 3.

Depletion of glutathione by ebselen leads to ROS production in yeast cells. (A)Wild type and deletion strains of *S. cerevisiae* cells were grown in the presence of carboxy-H_2DCFDA dye and the intensity of fluorescence produced was measured using spectrophotometer. (B and C) Yeast cells were grown in the presence of ebselen (20μg/ml) for 2.5 h and incubated with carboxy-H_2DCFDA dye to determine the glutathione production by spectrophotometer or Leica confocal laser scanning microscopy. The results are expressed as percent glutathione in ebselen treated cells in relative to untreated control groups (B). Green fluorescence indicates the ROS production in yeast cells (C). Statistical analysis was calculated using the two-tailed Student's *t* test. *P* values of (* $P \leq 0.05$) (** $P \leq 0.01$) are considered as significant.

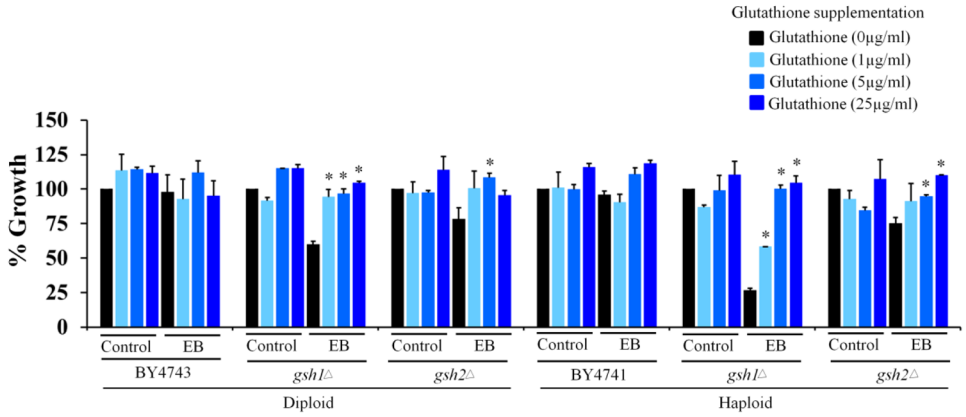

Figure 4.

Supplementation of L- glutathione restored the cell growth. Wild type and deletion strains of *S. cerevisiae* cells were grown and in the absence (or) presence of indicated concentration of ebselen and glutathione and the percent growth rate (OD_{600} after 24 h) was determined by using spectrophotometer. Statistical analysis was calculated using the two-tailed Student's *t* test. *P* values of (* $P \leq 0.05$) (** $P \leq 0.01$) are considered as significant.

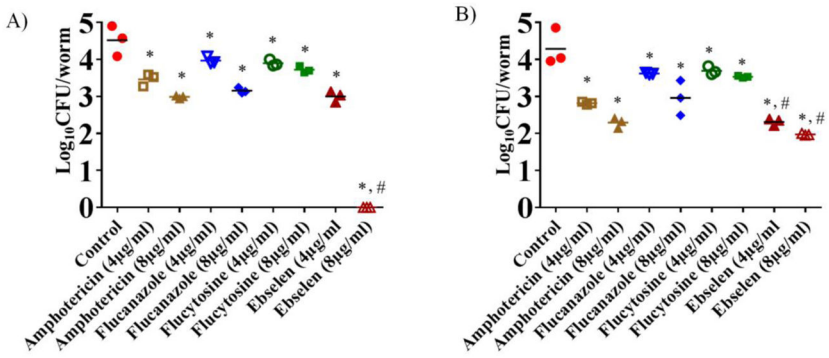

Figure 5.

Efficacy of ebselen in *C. albicans* (or) *C. neoformans*-infected *C. elegans.* L4-stage worms were infected with *C. albicans* or *C. neoformans* and treated with ebselen, fluconazole, flucytosine and amphotericin at a concentrations of 4 and 8 μg/ml. After 24 h, worms were lysed and plated onto YPD plates to determine the CFU per worm. Each dot represents average fungal load in each worm (n=10) per well. The results are presented as means ± SD for three wells. Statistical analysis was calculated using the two-tailed Student's *t* test. *P* value of (** $P \leq 0.01$) are considered as significant.

Table 1

MIC of ebselen and control antifungal drugs against *Candida* and *Cryptococcus* strains

Strains	Fluconazole (μg/ml)	Flucytosine (μg/ml)	Amphotericin (μg/ml)	Ebselen (μg/ml)
C. albicans NR 29434	4	0.125	1	1
C. albicans ATCC 10231	2	0.25	0.5	2
C. albicans NR 29449	2	4	1	2
C. albicans NR 29435	4	0.0625	0.5	2
C. albicans NR 29448	>64	0.0625	1	2
C. albicans NR 29437	2	0.0625	1	2
C. albicans NR 29446	>64	0.25	0.5	1
C. albicans NR 29453	2	0.0625	0.5	2
C. albicans NR 29438	2	0.0625	1	2
C. albicans ATCC 26790	2	0.0625	1	2
C. albicans ATCC 24433	4	1	1	2
C. albicans ATCC 14053	4	0.125	1	2
C. albicans ATCC 90028	4	1	1	2
C. albicans NR 29366	>64	0.0625	1	4
C. albicans NR 29367	>64	0.0625	1	2
C. glabrata ATCC MYA-2950	4	0.0625	1	0.5
C. glabrata ATCC 66032	2	0.0625	2	0.5
C. tropicalis ATCC 13803	2	0.125	1	2
C. tropicalis ATCC 1369	1	0.25	1	2
C. parapsilosis ATCC 22019	1	0.25	1	1
C. neoformans NR-41291	1	0.5	1	1
C. neoformans NR-41292	1	0.5	0.5	0.25
C. neoformans NR-41296	2	0.5	0.5	0.5
C. neoformans NR-41295	2	0.5	0.5	0.5
C. neoformans NR-41294	4	2	0.5	0.5
C. neoformans NR-41297	8	4	0.5	1
C. neoformans NR-41298	4	2	0.5	1
C. neoformans NR-41299	4	2	1	1
Cryptococcus gattii - CBS1930	2	2	0.5	0.5
Cryptococcus gattii - R265	1	1	0.5	0.5
Cryptococcus gattii - Alg40	2	0.5	0.5	0.5
Cryptococcus gattii - Alg75	8	8	0.5	2
Cryptococcus gattii - Alg81	8	4	0.5	2
Cryptococcus gattii - Alg99	8	4	1	2
Cryptococcus gattii - Alg114	8	4	1	2
Cryptococcus gattii - Alg115	8	4	1	2
Cryptococcus gattii - Alg127	4	4	1	2

Table 2

MIC of ebselen and control antifungal drugs against Candida and Cryptococcus strains with L- glutathione supplementation

Strains	Fluconazole (μg/ml)		Flucytosine (μg/ml)		Amphotericin (μg/ml)		Ebselen (μg/ml)	
	GSH (−)	GSH (+)	GSH (−)	GSH (+)	GSH (−)	GSH (+)	GSH (−)	GSH (+)
C. albicans ATCC 10231	2	2	0.25	0.25	0.5	0.5	2	>128
C. albicans - 18E	2	1	0.0625	0.0625	1	1	1	>128
C. tropicalis ATCC 1369	1	1	0.25	0.25	1	1	2	>128
C. tropicalis ATCC 13803	2	2	0.125	0.125	1	1	2	>128
C. parapsilosis ATCC 22019	1	1	0.25	0.25	1	1	1	>128
C. glabrata ATCC MYA-2950	4	4	0.0625	0.0625	1	1	0.5	>128
C. glabrata LRA 85.10.75	0.5	0.5	0.0625	0.0625	2	2	1	>128
C. gattii - R265	1	1	1	1	0.5	0.5	0.5	>128
C. gattii - CBS1930	2	2	2	2	0.5	0.5	0.5	>128
C. neoformans NR-41297	8	8	4	4	0.5	0.5	1	>128
C. neoformans NR-41299	4	4	1	2	1	1	1	>128

(−) indicates no supplementation and (+) indicates supplementation of L- GSH (0.25mg/ml) to the growth medium