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

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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 remain 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 to 2 μg/ml. Ebselen rapidly eradicates a high fungal inoculum within 2 h 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 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.

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

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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

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