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Identification of ebsulfur analogues with broad-spectrum antifungal activity

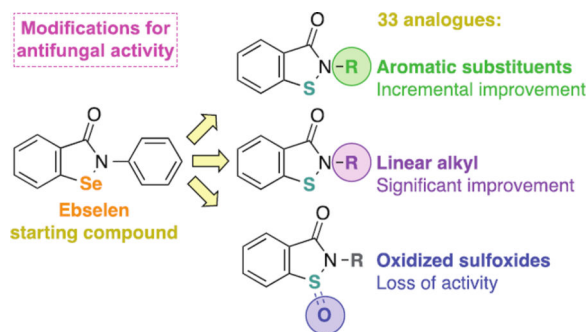
Huy X. Ngo^a, Dr. Sanjib K. Shrestha^a, and Sylvie Garneau-Tsodikova^{a,*} [Prof.]

^aDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, KY, 40536-0596, USA

Abstract

Invasive fungal infections are on the rise due to an increased population of critically ill patients as a result of HIV infections, chemotherapies, and organ transplantations. Current antifungal drugs are helpful, but insufficient in addressing the problem of drug-resistant fungal infections. Thus, there is a growing need for novel antimycotics that are safe and effective. The ebselen scaffold has been evaluated in clinical trials and has been shown to be safe in humans. This makes ebselen an attractive scaffold for facile translation from bench to bedside. We have recently reported a library of ebselen-inspired ebsulfur analogues with antibacterial properties, but their antifungal activity has not been characterized. Herein, we repurposed ebselen, ebsulfur, and 32 additional ebsulfur analogues as antifungal agents by evaluating their antifungal activity against a panel of 13 clinically relevant fungal strains. The effect of induction of reactive oxygen species (ROS) by three of these compounds was evaluated. Their hemolytic and cytotoxicity activities were also determined using mouse erythrocytes and mammalian cells. The MIC values of these compounds were in the ranges of 0.02–12.5 µg/mL against the fungal strains tested. Notably, yeast cells treated with our compounds showed the accumulation of ROS, which may further contribute to the growth inhibitory effect against fungi. This study provides new lead compounds for the development of antimycotic agents.

Graphical Abstract



*Corresponding Author. Sylvie Garneau-Tsodikova: sylviegttsodikova@uky.edu.

ASSOCIATED CONTENT

Supporting Information. The supporting information includes experimental procedures for all assays performed. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

Promising antifungals: The ebselen compound was previously shown to be safe during phase I (U.S.A) and phase III (Japan) clinical trials. Herein, ebselen, ebsulfur, and 32 ebsulfur derivatives were found to display potent antifungal activity against a panel of clinically relevant fungal strains. SAR analysis was done to identify analogues with activity equivalent or better than those of the clinically used antifungal drugs. They were then evaluated and found to be acceptable in terms of cytotoxicity against mammalian cells.

Keywords

Aspergillus; Benzisothiazolinone; *Candida*; Ebselen; ROS production

INTRODUCTION

Fungal infections have become an emerging public health threat mainly due to the increasing size of the immunocompromised patient population.¹ This population includes patients with AIDS, primary immune deficiency, and those who are immunocompromised due to chemotherapy or organ and bone marrow transplantation. Globally, *Candida* species are the predominant causes of invasive systemic fungal infections with the prevalence reported at 6.9 cases per 1000 patients.² In the United States, *Candida* infections rank fourth among all hospital-acquired systemic infections in intensive care units.³ In most population-based studies, *Candida* infections represent the seventh to tenth most common bloodstream infections.⁴ Additionally, many patients are now infected with other fungal species including *Aspergillus fumigatus*,⁵ *Aspergillus nidulans*,⁶ and *Cryptococcus neoformans*.⁷

Common therapeutic classes used to treat systemic fungal infections include azoles (*e.g.*, fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR)), polyenes (*e.g.*, amphotericin B (AmB), nystatin (NYS), and candicidin (CAN)), and echinocandins (*e.g.*, micafungin, caspofungin, and anidulafungin).⁴ These drugs function by different mechanisms of action: (i) inhibition of the cytochrome P450 enzyme 14 α -demethylase (azoles),⁸ (ii) introduction of transmembrane channel leading to monovalent ion leakage (polyenes),^{9, 10} and (iii) inhibition of synthesis of glucan in the fungal cell wall *via* the enzyme 1,3- β -glucan synthase (echinocandins).¹¹

Due to improper usage of these antifungal agents, more drug-resistant fungal strains have evolved.^{12, 13} Specifically, these improper usages include insufficient dosages and durations of treatment.¹⁴ Additionally, new evidence suggests that antibacterials also contribute to this development of fungal resistance.¹⁵ Overall, fungal resistance is still relatively uncommon, but this problem is on the rise and expected to become a major healthcare problem. Thus, we have a critical need for the development of novel antifungal compounds.

Currently, three strategies to overcome antifungal drug resistance have been employed. The first strategy is the development of compounds with novel mechanisms of action distinct from previous antifungal agents. For instance, compound E1210 was discovered as a novel first-in-class antifungal compound by the Tsukuba Research Laboratories of Eisai Co., Ltd. This compound was discovered to inhibit fungal glycosylphosphatidylinositol (GPI) biosynthesis and validated in murine models of candidiasis, aspergillosis, and fusariosis.¹⁶

The second strategy is the combination of two antifungal agents. In the literature, there have been plenty of examples using two compounds in conjunction to produce synergistic antifungal activity and reduce resistance as well as toxicity.^{17, 18} Specifically, in patients diagnosed with cryptococcal meningitis, the combination therapy of flucytosine and AmB was shown to be essential for successful clinical outcomes.¹⁹ Recently, it was also found that a combination of azoles and analogues of the aminoglycoside antibiotics tobramycin and kanamycin B resulted in favorable synergistic effects against drug-resistant *Candida albicans* strains.^{20, 21} Lastly, the third strategy is the repurposing of existing compounds for new applications. For example, the decongestant drug octodrine was identified as a broad-spectrum antifungal compound.²² In this study, we employed a combination of the first and third strategy to address the problem of antifungal drug resistance. We originally attempted to utilize all three strategies, but found that our novel compounds did not display synergy with currently used antifungal agents.

Ebselen (**1**, Fig. 1) is an organoseleno compound, which has completed phase I clinical trial for general safety in human use. Ebselen (**1**) has very diverse therapeutic applications and has been studied in several clinical trials.²³ During the phase I study, up to 1,600 mg of ebselen (**1**) was dosed orally and found to be very well tolerated compared to placebo in 32 healthy male and female subjects.²⁴ Ebselen (**1**) is currently in phase II clinical trials for the treatment of chemotherapy-induced hearing loss and Meniere's disease (<http://clinicaltrials.gov>). Furthermore, ebselen (**1**) completed a 300-patient phase III clinical trial for cerebral ischemia in Japan.²⁵ The ebsulfur or 1,2-benzisothiazol-3(2H)-one scaffold has been demonstrated to have a very narrow spectrum of antibacterial activity (only really being active against methicillin-resistant *Staphylococcus aureus* (MRSA)), in our previous work.²⁶ We hypothesized that ebsulfur (**2a**, Fig. 1) would have a similar safety profile compared to that of ebselen (**1**). This scaffold is interesting because it is structurally very similar to ebselen (**1**, Fig. 1). Therefore, we hypothesized that ebsulfur (**2a**, Fig. 1) and its analogues would have a safety profile comparable to that of ebselen (**1**). In the literature, the antifungal activity of the ebsulfur scaffold had not been well characterized. Herein, we explored the antifungal properties of ebselen (**1**) as well as ebsulfur (**2a**) and 32 of its analogues (**2b–4n**, Fig. 1) by using a combination of minimum inhibitory concentration (MIC) study, time-kill study, and reactive oxygen species (ROS) assays. The safety of these compounds was also assessed and compared to ebselen (**1**) *via* mammalian cytotoxicity and hemolytic assays. Our study provides us with a better understanding of the structure-activity-relationship (SAR) of ebselen (**1**) and the 1,2-benzisothiazol-3(2H)-one scaffold as well as their potential as a new class of antifungal agents.

RESULTS AND DISCUSSION

Antifungal activity

Compounds **1–3a–o** and **4e–n** were evaluated for whole-cell activity against a panel of clinically relevant fungal strains (Table 1). Our library of compounds featured ebselen (**1**) and ebsulfur (**2a**) as the main scaffolds. From the ebsulfur scaffold, the library was further organized into three sub-series: analogues with aromatic substituents (**2** series, **2a–o**), analogues with aliphatic substituents (**3** series, **3a–o**), and oxidized sulfoxide analogues (**4**

series, **4e**, **4f**, and **4n**). Series **2** contained aromatic substituents such as mono- and disubstituted phenyl rings (**2a–k**), naphthyl (**2l**), and nitrogen-containing aromatic heterocycles (**2m–o**). Series **3** contained analogues with substituents such as linear alkyl chains (**3a–d**), branched alkyl chains (**3e–g**), alkyl with terminal phenyl ring (**3h–j**), aliphatic rings (**3k–n**), and adamantyl (**3o**). In our previous work, we have verified that all the compounds tested were at least 95% pure by NMR and HRMS.²⁶ We used the commercially available AmB, FLC, ITC, POS, and VOR as positive controls. The MIC values listed for the controls were either tested herein or acquired from some of our previously published manuscripts on unrelated antifungal agents.²⁷ For the controls, AmB, as expected, was the most active against both *Candida* and *Aspergillus* strains with MIC values ranging from 0.98–15.6 µg/mL. Despite its potent antifungal activity, it should be noted that AmB, even with the liposomal formulations, has been well known for its severe and potentially lethal side effects such as nephrotoxicity and hypokalemia.^{28, 29} FLC, the most popular and well-tolerated FDA approved antifungal agent, was fairly inactive against our panel of fungal strains with MIC values mostly from >31.2–>125 µg/mL (except against *Candida parapsilosis* ATCC 22019 (strain **J**), MIC = 1.95 µg/mL). ITC, POS, and VOR displayed similar activity against our strains with MIC values mostly ranging from <0.03–31.2 µg/mL. The azole compounds, however, are potent inhibitors of human cytochrome P450 enzymes, which somewhat limit their applications due to drug-drug interactions with co-administered drugs.³⁰ ITC and VOR are also generally not as well tolerated as FLC.³¹ To effectively evaluate the activities of our compounds, we define poor, good, very good, and excellent activity as 12.5 µg/mL, 1.56–6.25 µg/mL, 0.39–0.78 µg/mL, and 0.10 µg/mL, respectively. In our broth dilution assays, the concentration of DMSO used is less than 1%, which was previously experimentally determined to not cause any DMSO-related cytotoxicity effect.

We first tested ebselen (**1**) against our panel of *Candida* strains (**A–J**). Ebselen (**1**) displayed good activity against *C. albicans* ATCC 90819(R) (strain **D**), *C. albicans* ATCC MYA-2310(S) (strain **E**), *C. glabrata* ATCC 2001 (strain **H**), *C. krusei* (strain **I**), and *C. parapsilosis* ATCC 22019 (strain **J**) (1.56–6.25 µg/mL) and poor activity against strains *C. albicans* ATCC 10231 (strain **A**), *C. albicans* ATCC 64124 (strain **B**), *C. albicans* ATCC MYA-2876(S) (strain **C**), *C. albicans* ATCC MYA-1237(R) (strain **F**), and *C. albicans* ATCC MYA-1003(R) (strain **G**) (12.5 µg/mL). When compared to the controls, these MIC values were generally better than the MIC values of the azoles (except against strains **A** and **H–J**), but were worse than those of AmB. Next, we evaluated ebsulfur (**2a**) to gain insight into the importance of the sulfur atom for antifungal activity. Ebsulfur (**2a**) displayed a very similar anti-*Candida* profile to that of ebselen (**1**). In fact, we noticed that in general, they displayed good and poor activity against the same *Candida* strains. With the exception of strain **J**, **2a** was active against strains **D**, **E**, **H**, and **I** (1.56–6.25 µg/mL) and poorly active against strains **A**, **B**, **C**, **F**, **G**, and **J** (12.5 µg/mL). This finding demonstrates that replacing the Se atom with the S atom does not compromise antifungal activity.

In search for a chemical modification that would increase the activity of the parent scaffolds, we first decided to assess our analogues with substituents at the *para*- and *meta*-positions of the phenyl ring adjacent to the 1,2-benzisothiazol-3(2H)-one (ebsulfur) core. Compounds

2b–d were systematically prepared to contain *p*-substituted halogen atoms that increased in bulkiness with F < Cl < Br. The SAR comparison for these compounds, however, was flat with all three compounds generally displaying MIC values from 1.56–6.25 µg/mL. We then tested the *p*-substituted isopropyl analogue (**2e**), which was previously found to be among the best ebsulfur antibacterial analogues.²⁶ Compound **2e** displayed good MIC values (3.13–6.25 µg/mL) similarly to those of **2b–d**. Lastly, we tested the *p*-ethinyl analogue (**2f**) and found that **2f** displayed mostly poor activity against *Candida* strains (12.5 µg/mL). We speculated that substitution at the *para*-position was not well tolerated due to steric hindrance with the putative target(s) and that a *meta*-substitution might show a different pattern of activities.

Based on this assumption, we next examined the *m*-monosubstituted analogues (**2g,h**) and the 3,5-disubstituted analogues (**2i–k**). While the *m*-Br substitution in **2g** was not beneficial at all (12.5 µg/mL) against *Candida* strains, the *m*-iPr (**2h**), *m,m*-di-Br (**2i**), *m,m*-di-Me (**2j**), and *m,m*-di-OMe (**2k**) analogues were overall better tolerated with good to moderate MIC values (3.13–12.5 µg/mL). By comparing the *p*-substituted analogues **2d,e** and their *m*-substituted counterparts **2g,h**, we noticed that switching from *p*-Br (**2d**) to *m*-Br (**2g**) led to loss of activity, whereas switching from *p*-iPr (**2e**) and *m*-iPr (**2h**) led to compounds which displayed very similar MIC values. Overall, the activity of these compounds appeared to weakly correlate with the number or the positions of the substituents on the phenyl ring.

Moving away from the substituted phenyl strategy, we next explored the analogues with more complex aromatic rings such as the naphthyl (**2l**), pyridyl (**2m,n**), and quinolinyl (**2o**). We found that these relatively bulkier rings were not as well tolerated. Compounds **2l,m** displayed good to poor activity (6.25–12.5 µg/mL), while compound **2n** was poorly active (12.5 µg/mL) and **2o** could not be evaluated due to solubility issues in our RPMI 1640 medium. In conclusion, the chemical strategy of installing flat aromatic moieties to the core scaffold of the **2** series was able to generate many analogues with mostly good MIC values that are comparable to the parent ebsulfur (**2a**). We identified compounds **2d**, **2e**, **2h**, and **2i** that displayed incrementally improved MIC values when compared to those of ebsulfur (**2a**), but these improvements were still insufficient. Next, we pondered whether modifications with substituents possessing more geometric freedom and flexibility would be able to generate more substantially potent analogues.

Inspired by the observation that coupling linear alkyl chains to aminoglycoside antibiotics resulted in a significant improvement of their antifungal activity,^{21, 27, 32–34} we systematically generated and examined the antifungal activities of ebsulfur analogues with linear alkyl chains of 5–12 carbons (C₅, C₆, C₈, and C₁₂, **3a–d**). Based on our previous work with aminoglycosides where tobramycin and kanamycin analogues with C₁₂ and C₁₄ alkyl chains displayed the best antifungal activity, we hypothesized that our longer C₁₂ ebsulfur analogue (**3d**) would be the most active. Surprisingly, we observed an opposite trend than that displayed by the aminoglycosides; we found that our shorter C₅ (**3a**) and C₆ (**3b**) analogues were remarkably effective with very good to good MIC values against all *Candida* strains (0.39–1.56 µg/mL). The C₈ analogue (**3c**) was slightly worse when compared to the C₅ (**3a**) and C₆ (**3b**) analogues (specifically against strains **A** and **C**), and our C₁₂ analogue

(**3d**) displayed poor MIC values (>12.5 µg/mL), which was the worst amongst the analogues.

We were intrigued to identify the C₅ (**3a**) and C₆ (**3b**) linear alkyl analogues as the best anti-*Candida* agents in our library, thus far. Compared to FLC, **3a** was 20- to 320-fold more potent in MIC values (except against strain **J**). When compared to AmB, **3a** was 1.25- to 10-fold more active. Since the C₅ and C₆ alkyl chains were extremely well tolerated, we speculated whether our putative target(s) could also tolerate branched alkyl chains with similar chain lengths. We went ahead and evaluated the *iso*-butyl (**3e**) and *iso*-amyl (**3f**) analogues. Interestingly, both **3e** and **3f** were equally as effective as **3a** and **3b** (0.39–1.56 µg/mL). We then examined the final branched analogue in our library, the *tert*-butyl compound **3g**. Compound **3g** was also as effective as **3a–f** (within 2-fold dilution, 0.78–3.13 µg/mL). Against *Candida* strains, analogues with aliphatic alkyl chains (linear or branched) were found to be very beneficial, which could possibly be attributed to the added rotational flexibility.

As we found that additional methylene groups were highly favorable, we hypothesized that adding methylene linkers (C₁ (**3h**), C₂ (**3i**), and C₃ (**3j**)) in between the 1,2-benzisothiazol-3(2H)-one core and the phenyl ring could provide the added rotational flexibility needed to generate analogues with improved MIC values when compared to the parent compound (**2a**), which was flat and rigid. The C₁ and C₂-linker analogues (**3h,i**) indeed had better MIC values comparing to **2a** (0.39–1.56 µg/mL). However, the C₃-linker analogue (**3j**) was not as potent (1.56–62.5 µg/mL). From these observations, we noticed that addition of flexible methylene linkers were well tolerated up to two carbons.

To further understand the correlation between the flexibility of the R group and activity, we tested our non-aromatic analogues (**3k–o**) as non-aromatic rings are considerably more flexible than their aromatic counterparts. We first tested the cyclopentyl analogue (**3k**) and found it to be just as active (0.39–1.56 µg/mL) as some of our best analogues **3a–c** described above. On the other hand, the cyclohexyl analogue (**3l**) still displayed very good to good activity (0.78–3.13 µg/mL), but overall was slightly worse relative to **3k**. The cycloheptyl (**3m**) and cyclooctyl (**3n**) analogues were also not as good as **3k,l**. We also attempted to test whether an ultra-bulky ring such as the adamantane could still be accommodated, but our adamantyl analogue (**3o**) was not soluble in the RPMI 1640 medium that we used for determination of MIC values. Collectively, the SAR showed a modest preference for smaller size ring, as systematically expanding the ring size resulted in a gradual loss in activity.

Lastly, we tested the oxidized analogues **4e**, **4f**, and **4n**. Oxidizing the sulfur atom to sulfoxide completely abolish antifungal activity. This finding was in accord with our previous report of these compounds as antibacterials and with other reports in the literature that the biological activity of ebselen (**1**) and ebsulfur (**2a**) was highly dependent on the Se-N or S-N bonds.^{26, 35} Ebselen (**1**) has been reported to utilize the electrophilic Se-N bond to covalently bind to cysteine residues of multiple enzyme targets.²³

Invasive aspergillosis is highly correlated with fulminant development and poor prognosis.³⁶ Compounds with potent anti-*Aspergillus* activity are considered to be of great valuable. To

evaluate the antifungal spectrum of our compounds, we tested them against freshly harvested spores of three *Aspergillus* strains: *A. flavus* ATCC MYA-3631 (strain **K**), *A. nidulans* ATCC 38163 (strain **L**), and *A. terreus* ATCC MYA-3633 (strain **M**). Overall, our compounds were mostly active against *Aspergillus* strains and the SAR trends observed from our study with *Candida* strains were highly translatable to *Aspergillus* strains. Aromatic analogues (**2a–o**) remained to have either good to poor activity against strains **K–M** (1.56–12.5 µg/mL). We were especially intrigued to observe that our linear-chain C₅, C₆, and C₈ analogues (**3a–c**) displayed excellent activity at ng/mL concentrations (0.02–0.20 µg/mL). These results were equivalent or slightly better when comparing them to VOR (0.03–0.24 µg/mL), the gold standard for the treatment of invasive aspergillosis.³⁷ Other analogues (**3e–m**) displayed very good activity (0.10–0.78 µg/mL), but they were not as effective as **3a–c**. We were also surprised to find that our cyclooctyl analogue (**3n**) displayed excellent activity against *Aspergillus* strains (0.02–0.05 µg/mL). These values were equivalent to our best anti-*Aspergillus* analogues **3a–c**. We realized that many of our potent analogues were lipophilic. Thus, we investigated the log P values of all the compounds by two log P calculators (ChemDraw and molinspiration) (Table S1). In general, we observed that compounds with extremely high or very low lipophilicity did not display good antifungal activity. However, we found that increasing lipophilicity, to a certain extent, generally correlated with increase in antifungal activity.

Time-kill curves

To gain insight for the rate of fungicidal activity of our compounds, we performed time-kill assays with ebsulfur (**2a**) and our most potent analogue **3a** (Fig. 2). We then compared the results of our compounds to ebselen (**1**) and the clinically potent and widely used antifungal agent AmB, which also served as our positive control. We selected ebsulfur (**2a**) because we previously observed that this compound was bacteriostatic and we pondered whether a similar fungistatic effect would be observed.²⁶ We commenced our study by dosing all of our tested compounds (ebselen (**1**), ebsulfur (**2a**), **3a**, and AmB) at 1× their respective MIC values (Fig. 2A). For ebsulfur (**2a**), although the MIC value for ebsulfur (**2a**) against strain **B** was greater than 12.5 µg/mL, we decided to test this compound at 12.5 µg/mL because we were concerned that higher concentration may lead to precipitation of the compounds. We observed that ebselen (**1**) (at 12.5 µg/mL) displayed potent fungicidal activity leading to complete fungal cell death at the 6-h mark, which was even quicker than AmB (at 3.9 µg/mL). Our ebsulfur (**2a**) (at 12.5 µg/mL) and **3a** (at 0.39 µg/mL) displayed fungistatic effects. However, at their 1× MIC, ebsulfur (**2a**) and **3a** were not able to completely inhibit fungal re-growth even after 24 h incubation. Hence, we decided to double the doses of our compounds for our additional time-kill analysis experiments.

At 2× MIC (Fig. 2B), Ebselen (**1**) (at 25 µg/mL) and AmB (at 7.8 µg/mL) were completely fungicidal from 3 and 6 h, respectively. At the higher concentration, ebsulfur (**2a**) (25 µg/mL) interestingly became fungicidal. Conversely, compound **3a** (at 0.78 µg/mL) remained fungistatic with a 4-log reduction of fungal cells at approximately the 24-h mark. We were very intrigued by this result and pondered whether fungicidal and fungistatic effects could be concentration dependent. Thus, we further increase the concentration of compound **3a** to 4× its MIC value with the hope that **3a** would also switch to the fungicidal

mode. We performed the assay and actually found that **3a** at 4× MIC (at 1.56 µg/mL) still remained fungistatic (Fig. 2C). These findings suggested that in order for ebsulfur (**2a**) and compound **3a** to be effective antifungal agents, they would have to be dosed at 2× their respective MIC values while albeit at high concentration, ebselen (**1**) could still be effective at 1× MIC.

Based on the time-kill curves, we observed that ebselen (**1**) and ebsulfur (**2a**) were fungicidal at high concentrations (12.5 and 25 µg/mL or 45.6 and 110 µM, respectively) and our best compound from the MIC determination assays, **3a** was fungistatic at 0.78 and 1.56 µg/mL (3.5 and 7.0 µM), which were much lower than the fungicidal concentrations of the other compounds. Fungistatic property does not necessarily mean that compound **3a** is not as good as ebselen (**1**) and ebsulfur (**2a**) as an antifungal agent because the most popular antifungal compound (FLC) is also fungistatic. This data, however, gave us hints that compound **3a** may potentially not be suitable for some specific fungal infections that absolutely require fungicidal effect for clinical efficacy such as cryptococcal meningitis. Additionally, the fact that these benzisothiazolinone compounds exhibit fungicidal effect at high concentrations may mean that they target a different fungal enzymatic pathway when the concentrations are high.

Hemolytic assay

Although we were excited to discover new analogues with improved antifungal activity relatively to ebselen (**1**), we pondered whether this cytotoxic property could be more selective towards fungal cells than mammalian cells. We were cautiously optimistic that our analogues would still retain some of the good tolerability properties that were highly desirable in the original ebselen (**1**) scaffold. Previously, our lab studied aminoglycoside analogues with linear alkyl chains and reported that aminoglycoside analogues with linear alkyl chains could potentially be toxic to red blood cells (RBCs) as these RBCs have ultra thin cell membrane and thus, are prone to hemolysis.²¹ Therefore, we decided to evaluate some of our compounds with linear alkyl chains, the C₅ analogue **3a** and the C₈ analogue **3c** against murine red blood cells (mRBCs) and compare their results to ebselen (**1**) (Fig. 3). Although the C₆ analogue **3b** was also one of our top analogues, we did not test this analogue because its chain length (C₆) was extremely similar structurally to the C₅ compound **3a**. We also excluded the C₁₂ analogue **3d** because it was completely inactive against fungal strains. Ebsulfur (**2a**) was chosen because we were interested to verify that the linear alkyl chain would be a required feature for hemolysis. Most of the compounds (ebselen (**1**), ebsulfur (**2a**), and compound **3c**) tested did not show any significant hemolytic activity until 15.6 µg/mL (56.9 µM for ebselen (**1**), 68.6 µM for ebsulfur (**2a**), and 59.2 µM for **3c**). At first glance, compound **3a** appeared to be very hemolytic at approximately 3.9 µg/mL (17.6 µM). However, it should be noted that this compound showed remarkable potency against fungal cells. The MIC values of compound **3a** were at least 5- to 195-fold lower than the hemolytic concentrations for *Candida* and *Aspergillus* strains, respectively. Thus, we could observe that there was still some cytotoxic selectivity towards fungal cells. Initially, we expected the C₈ analogue **3c** to be more hemolytic because analogues with longer linear alkyl chains tend to perforate cell membranes easily. Thus, it was unexpected to find the C₅ analogue **3a** to be hemolytic. This observation led us to speculate that the

hemolytic activity was not due to disruption of the cell membrane of the mRBCs, but was simply an artifact of general mammalian cytotoxicity. This prompted us to evaluate our three best compounds **3a**, **3b**, and **3g** (in terms of their overall antifungal activity against both *Candida* and *Aspergillus* strains) against two different mammalian cell lines, which have normal cell membranes and are not susceptible to membrane-lytic compounds.

Mammalian cytotoxicity assay

Compounds **3a**, **3b**, and **3g** were evaluated for their cytotoxicity against HEK293 and J774 cell lines using a resazurin assay.²⁰ The concentration of DMSO used in the cytotoxicity assay is 0.1%, which was ensured to not demonstrate any DMSO-related toxicity to the cells. We compared the percentage of surviving cells treated with our analogues *versus* the percentage of surviving cells treated with ebselen (**1**) (Fig. 4). Against the HEK293 cell line (Fig. 4A), we noticed that all of our analogues (**3a**, **3b**, and **3g**) were slightly more toxic but overall, quite comparable to ebselen (**1**) at all concentrations tested. Overall, for all the tested compounds (ebselen (**1**), **3a**, **3b**, and **3g**) the IC₅₀ values were greater than 10 µg/mL (Fig. 4A). This corresponds to IC₅₀ values of 36.5 µM for ebselen (**1**), 45.2 µM for **3a**, 42.5 µM for **3b**, and 48.2 µM for **3g**. We verified that the cytotoxicity data of ebselen (**1**) found in our study was fairly consistent with other reported *in vitro* mammalian cytotoxicity studies of ebselen (**1**).^{38, 39} Given the good tolerability of ebselen (**1**) during clinical trials, it was perplexing to us ebselen (**1**) displayed some *in vitro* cytotoxicity. The HEK293 cell line was chosen because we were interested to determine whether our compounds could potentially cause kidney injury. The kidney is a highly perfused organ and comes in contact with many compounds due to renal excretion. Thus, many compounds such as AmB are highly nephrotoxic and cause great burden to patients with compromised renal function.

Next, we evaluated our compounds against J774 (Fig. 4B), a murine macrophage cell line. This cell line was selected because macrophages are the first-line of defense against fungal infection and we were hopeful that our compounds would not interfere with the survival of host macrophages. Against the J774 cell line, we observed a trend similar to the HEK293 cell line and found that our analogues (**3a** and **3b**) were slightly more toxic but still comparable to ebselen (**1**) with IC₅₀ values approximately at 10 µg/mL. It was interesting that compounds **3g** actually did not show any toxicity at all up to 5 µg/mL (24.1 µM). Typically, the difference between the toxic dose in mammalian cells and the fungal MIC value should be at least 10-fold. The fact that this was the case for our most potent antifungal compounds is highly encouraging.

We acknowledge that there are concerns in the literature regarding the highly reactive benzisothiazolinone moiety of the ebsulfur (**2a**) scaffold, which possibly explains the toxicity effects observed against mammalian cell lines.⁴⁰ This concern is valid considering that the parent compound ebselen (**1**) has also been found to target different proteins.²³ However, we argue that this scaffold still merits further consideration as a potential antifungal candidate based on two particular reasons. First, since many potent antifungal compounds are only available intravenously, there is currently a dire clinical need for orally active antifungals to assist the azoles as an alternative option for step-down therapy.⁴¹ These azoles often complicate drug dosing due to interactions with the metabolism of many drugs

and have experienced an increased rate of resistance.^{42, 43, 44} The ebsulfur analogues would most likely be orally active due to its similarity to ebselen (**1**), which was successfully administered orally.^{24, 45} Secondly, while we are also concerned about the high reactivity of the benzisothiazolinone moiety towards non-specific cysteine residues, ebselen (**1**) with the benzoselenazolinone moiety has been shown to be well-tolerated during clinical trials. There are also examples of other clinically successful small-molecule drugs with highly reactive pharmacophores within the FDA-approved chemical space. Some of these compounds are penicillin, fosfomycin, or bendamustine.⁴⁶ Compounds **3a** and **3b** displayed MIC values against *Candida* strains at 780 ng/mL and *Aspergillus* strains at 20 ng/mL, which are much lower than their IC₅₀ values against mammalian cells. This could potentially be due to the fact that **3a** and **3b** may have a fungal-specific mechanism of action at lower concentrations. To gain insights on the mechanism of action, we decided to first look at ROS induction of these compounds.

ROS production

Recently, we showed that ebselen (**1**) and our ebsulfur analogues with antibacterial activity were highly correlated with ROS production in MRSA bacterial cells.²⁶ Another group independently reported that ebselen (**1**) induced ROS-mediated cytotoxicity in *Saccharomyces cerevisiae* via inhibition of glutamate dehydrogenase.⁴⁷ Thus, we sought to determine whether our analogues would also induce ROS against *C. albicans* via inhibition of the *C. albicans* glutamate dehydrogenase. Ebselen (**1**), ebsulfur (**2a**), and compound **3a** were tested against *C. albicans* ATCC 10231 cells (strain **A**) at 1× and 2× their respective MIC values. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was then used to detect and visualize ROS production (Fig. 5). As a positive control, we treated cells with H₂O₂, which is an inducer of hydroxyl radical formation. After 1-h treatment, we found that all the compounds tested and the positive control were highly fluorescent, which indicated ROS induction. We also compared the samples that were treated with different doses of compounds (1× and 2× their respective MIC values) and observed that the amount ROS induction could potentially be concentration dependent. It is certainly possible that this ROS induction in *C. albicans* spp. is due to inhibition of *C. albicans* glutamate dehydrogenase as we hypothesized. However, this finding still does not rule out other potential drug targets such as fungal enzymes responsible for ROS regulation or it is simply a downstream secondary effect as the ebselen (**1**) and ebsulfur (**2a**) scaffolds inhibit enzymes that are unrelated to ROS generation. Assays to determine the specific molecular target(s) of our best compound, **3a**, are ongoing and will be the subject of future reports.

CONCLUSIONS

In summary, we expanded our knowledge of this scaffold in terms of the antifungal activity against a panel of clinically relevant *Candida* and *Aspergillus* strains. In light of our SAR analysis, we identified that the addition of flexible chemical moieties to the 1,2-benzisothiazol-3(2H)-one scaffold is a viable strategy to generate analogues with potent antifungal activities. Interestingly, the majority of our compounds displayed comparable or, in most cases, enhanced antifungal activities against all fungal strains when compared to ebselen (**1**) and the reference drugs used in this study. Although, our best compound (**3a**)

exhibited some hemolytic activity, its effect on nucleated mammalian cells was found to be in the acceptable range considering their antifungal efficacies. Finally, our preliminary study on mechanism of action indicated that the growth inhibitory effect of fungi by these compounds might be due to the elevating concentration of ROS in the yeast cells. However, the biological activities of the ebselen (**1**) and ebsulfur (**2a**) scaffolds are complex due to their general electrophilicity. An extensive study is required to identify the specific mode of action against fungal strains. Once the mechanism of action is established, we feel that this scaffold would have merit for further evaluation in a fungal infected murine model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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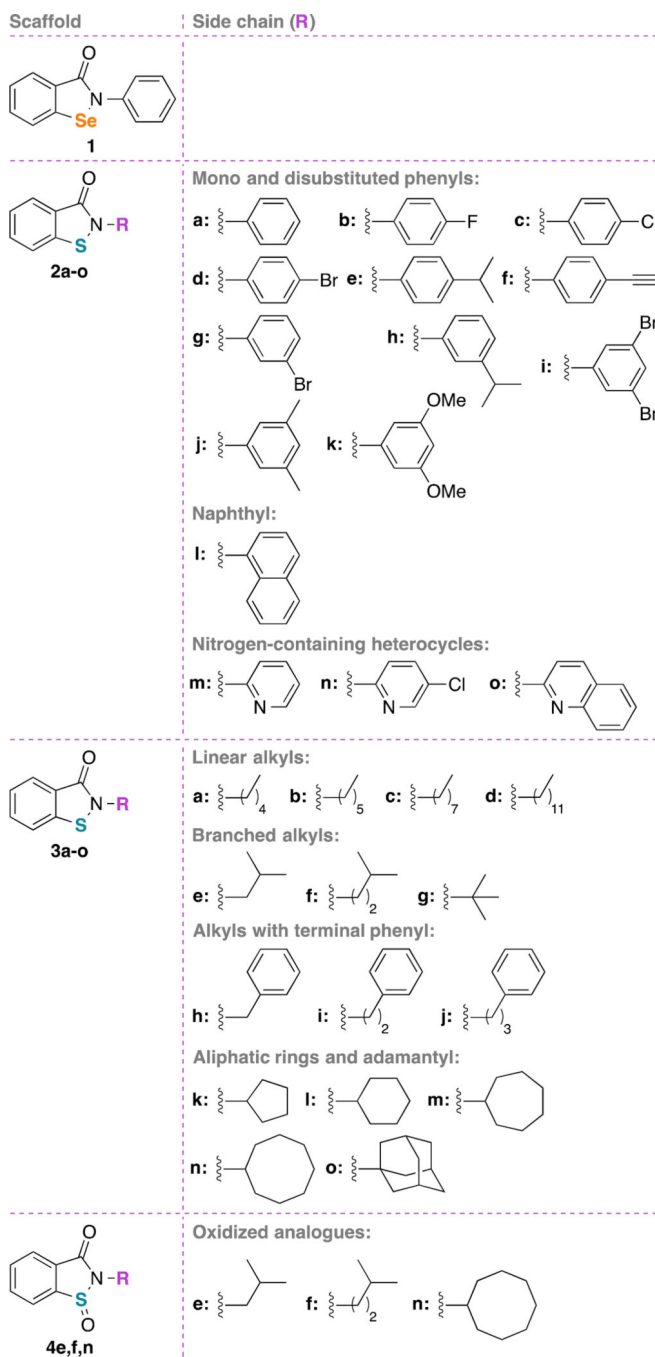


Figure 1. Chemical structures of our library featuring ebselen (**1**), ebsulfur (**2a**), and 32 ebsulfur analogues.

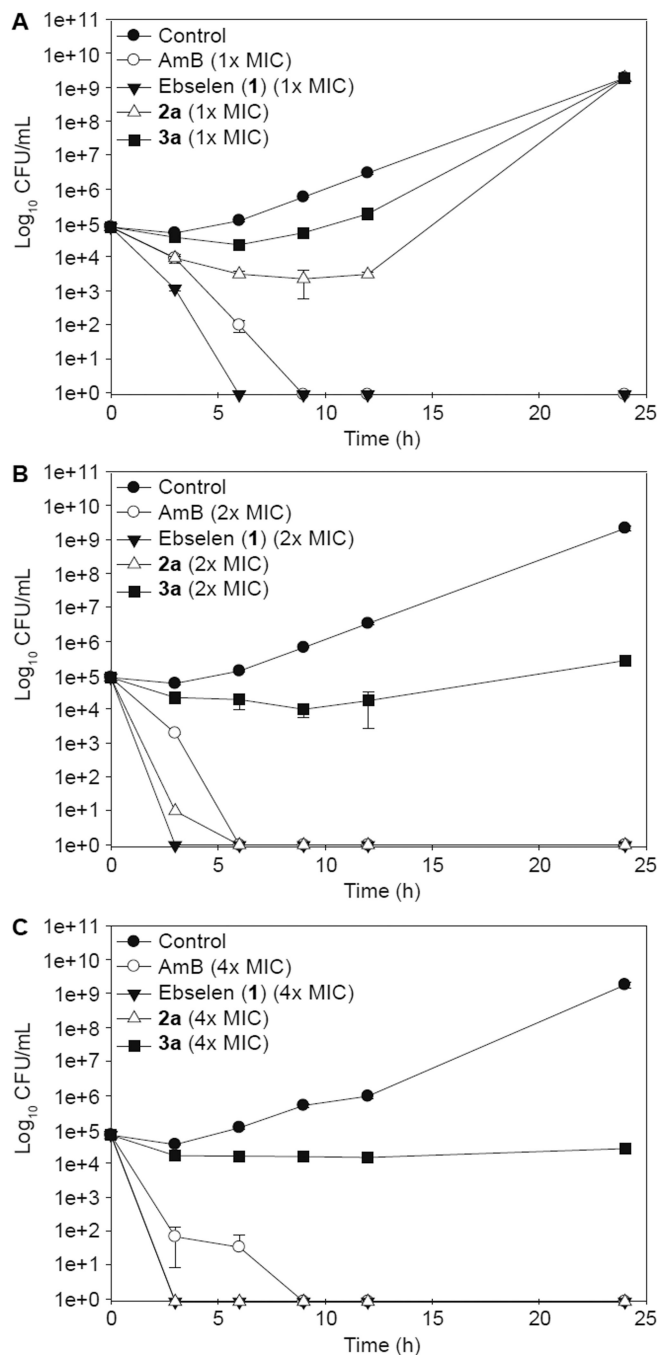


Figure 2. Time-kill analysis of ebselen (**1**) (black inverted triangles), ebsulfur (**2a**) (white triangle), compound **3a** (black squares) at 0, 3, 6, 9, 12, and 24 h. **A.** Cultures were exposed to compounds at 1× their respective MIC values. **B.** Cultures were exposed to compounds at 2× their respective MIC values. **C.** Cultures were exposed to compounds at 4× their respective MIC values. Untreated culture (black circles) was used as the negative control and AmB (white circles) was used the positive control. Data was combined from two independent experiments. The first experiment was conducted with the standard 1× and 2× respective

MIC values. The second experiment was needed to supplement the analysis and was performed at 4× their respective MIC values. Each data points were collected in duplicates. The error bars were reported as \pm standard deviations.

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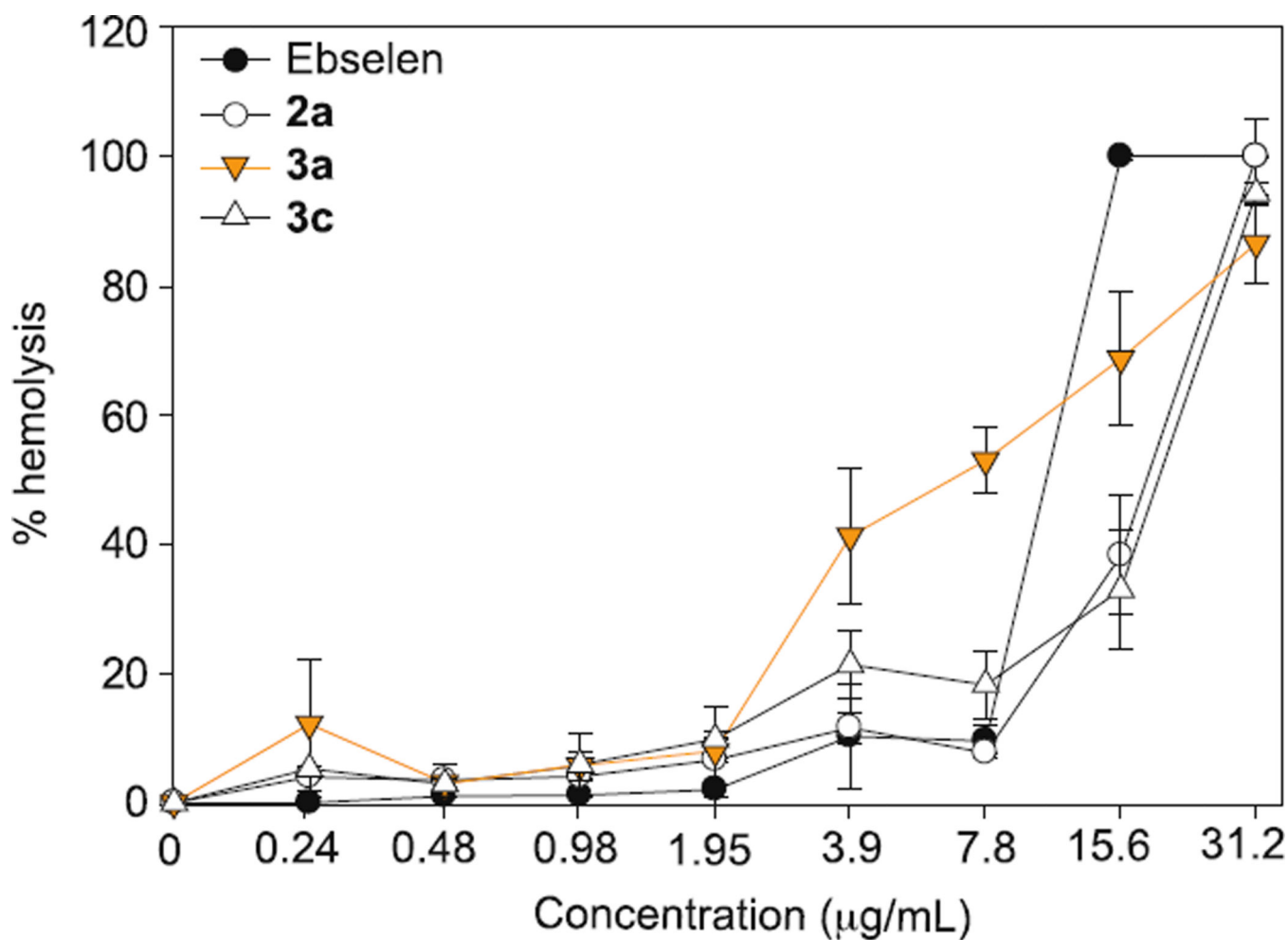


Figure 3.

Hemolytic assays of ebselen (**1**), ebsulfur (**2a**), compound **3a**, and compound **3c** against murine red blood cells (mRBCs). Ebselen (**1**), ebsulfur (**2a**), compound **3a**, and compound **3c** are represented as black circles, white circles, inverted orange triangle, and upright white triangle, respectively. The data points of ebselen (**1**), ebsulfur (**2a**), and compound **3c** were previously present in one of our publications and are used here for comparison.²⁶ Each data point was collected in duplicates. The error bars were reported as \pm standard deviations.

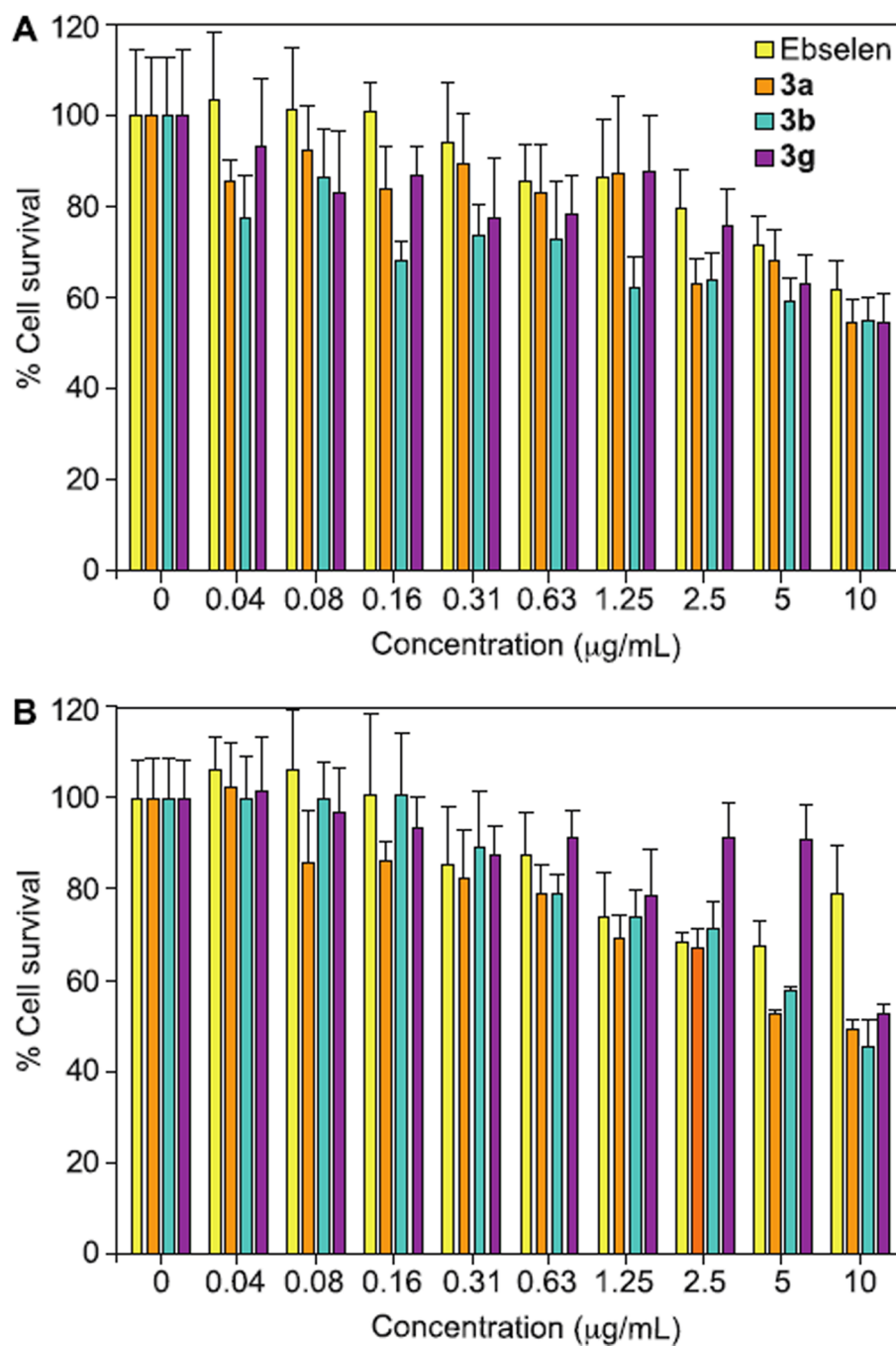


Figure 4. Mammalian cell cytotoxicity of ebselen (**1**) (yellow bars), and compounds **3a** (orange bars), **3b** (turquoise bars), and **3g** (purple bars) against **A.** HEK 293 cell line and **B.** J774 cell line. Triton-X 100® (1%, *v/v*) was used as the positive control (data not shown). Each data point was collected in quadruplicates. The error bars were reported as \pm standard deviations.

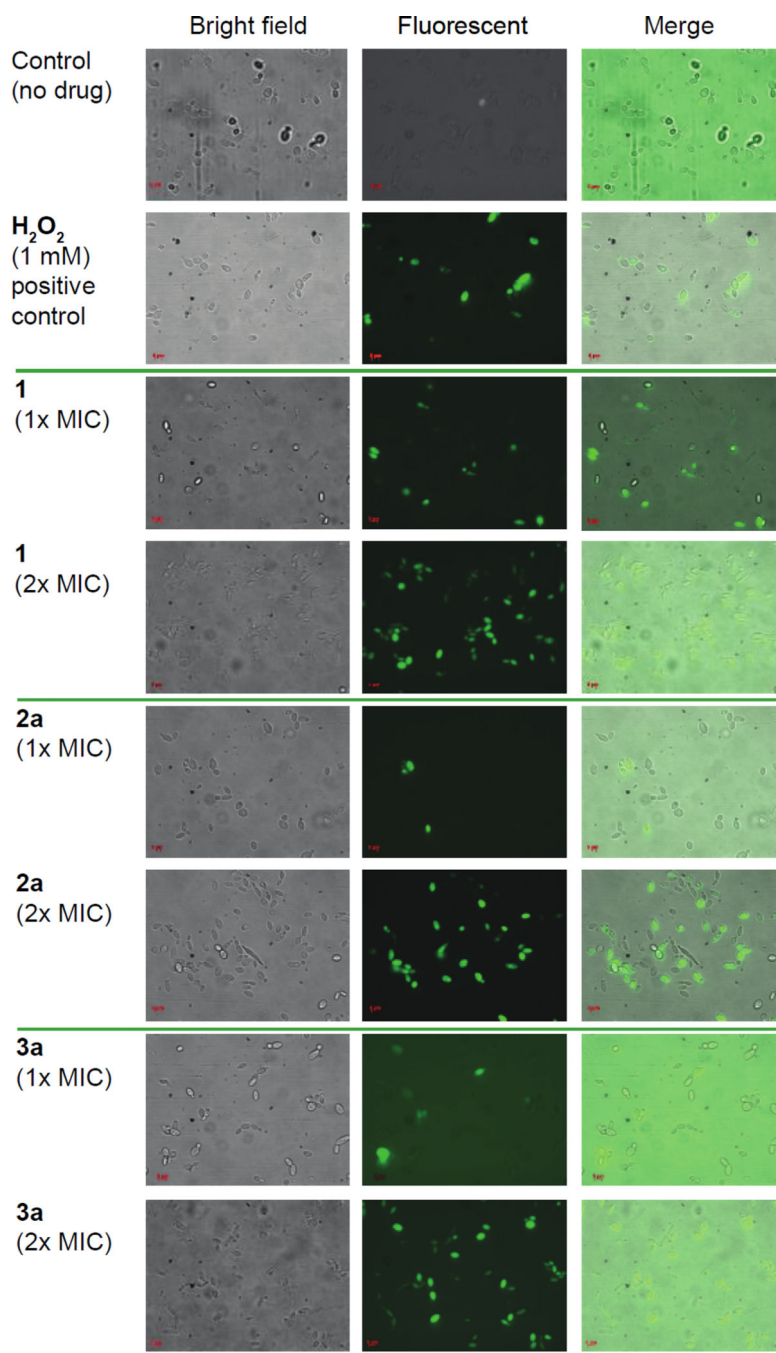


Figure 5. ROS induction assay of ebselen (**1**), ebsulfur (**2a**), and compound **3a** against *C. albicans* ATCC 10231 (strain A). *Candida* cells were treated with no drug (negative control), 1 mM of H₂O₂ (positive control), or ebselen, **2a**, and **3a** at their 1× and 2× respective MIC values for 1 h at 37 °C. DCFH-DA (40 µg/mL) was added to detect ROS and the samples were analyzed using a Zeiss Axovert 200M fluorescence microscope.

Table 1
***In vitro* susceptibility of *Candida* and *Aspergillus* species to ebselelen (1) and compounds 2a–4n**

MIC values^{a,b} (in µg/mL) (and µM in parentheses)^c determined for compounds 1–4n and for five control antifungal agents (AmB, FLC, ITC, POS, and VOR) against various yeast strains and filamentous fungi.

Cpd #	Yeast strains										Filamentous fungi				
	A	B	C	D	E	F	G	H	I	J	K	L	M		
1	12.5 (45.6)	12.5 (45.6)	12.5 (45.6)	1.56 (5.69)	3.13 (11.4)	>12.5 (45.6)	12.5 (45.6)	1.56 (45.6)	1.56 (45.6)	6.25 (22.8)	6.25 (22.8)	1.56 (5.69)	1.56 (5.69)		
2a	12.5 (55.0)	>12.5 (55.0)	12.5 (55.0)	3.13 (13.8)	6.25 (27.5)	>12.5 (55.0)	>12.5 (55.0)	6.25 (27.5)	3.13 (13.8)	12.5 (55.0)	6.25 (27.5)	6.25 (27.5)	6.25 (27.5)		
2b	3.13 (12.8)	6.25 (25.5)	3.13 (12.8)	3.13 (12.8)	3.13 (12.8)	6.25 (25.5)	1.56 (6.36)	6.25 (25.5)	3.13 (12.8)	12.5 (12.8)	6.25 (25.5)	12.5 (51.0)	12.5 (51.0)		
2c	3.13 (12.0)	>12.5 (47.8)	12.5 (47.8)	6.25 (23.9)	6.25 (23.9)	6.25 (23.9)	12.5 (47.8)	6.25 (23.9)	3.13 (12.0)	12.5 (47.8)	6.25 (23.9)	6.25 (23.9)	6.25 (23.9)		
2d	3.13 (10.2)	3.13 (10.2)	1.56 (5.10)	3.13 (10.2)	0.78 (2.55)	1.56 (5.10)	3.13 (10.2)	6.25 (20.4)	3.13 (10.2)	6.25 (20.4)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)		
2e	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	6.25 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	6.25 (23.2)	6.25 (23.2)	6.25 (23.2)		
2f	12.5 (49.7)	12.5 (49.7)	>12.5 (49.7)	12.5 (49.7)	>12.5 (49.7)	12.5 (49.7)	12.5 (49.7)	6.25 (49.7)	6.25 (49.7)	12.5 (49.7)	12.5 (49.7)	12.5 (49.7)	12.5 (49.7)		
2g	>12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	>12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)	12.5 (40.8)		
2h	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	3.13 (11.6)	6.25 (23.2)	12.5 (46.4)	12.5 (46.4)		
2i	3.13 (8.13)	6.25 (16.2)	6.25 (16.2)	6.25 (16.2)	6.25 (16.2)	3.13 (8.13)	6.25 (16.2)	6.25 (16.2)	6.25 (16.2)	6.25 (16.2)	12.5 (32.5)	3.13 (8.13)	12.5 (32.5)		
2j	3.13 (12.3)	6.25 (24.5)	1.56 (6.11)	12.5 (49.0)	0.78 (3.05)	3.13 (12.3)	3.13 (12.3)	12.5 (49.0)	12.5 (49.0)	12.5 (49.0)	12.5 (49.0)	12.5 (49.0)	12.5 (49.0)		
2k	12.5 (43.5)	12.5 (43.5)	3.13 (10.9)	3.13 (10.9)	1.56 (5.43)	12.5 (43.5)	12.5 (43.5)	6.25 (21.8)	6.25 (21.8)	6.25 (21.8)	6.25 (21.8)	6.25 (21.8)	6.25 (21.8)		
2l	6.25 (22.5)	12.5 (45.1)	>12.5 (45.1)	3.13(11.3)	>12.5 (45.1)	>12.5 (45.1)	6.25 (22.5)	12.5 (45.1)	6.25 (22.5)	12.5 (45.1)	12.5 (45.1)	12.5 (45.1)	12.5 (45.1)		
2m	6.25 (27.4)	6.25 (27.4)	12.5 (54.8)	6.25 (27.4)	6.25 (27.4)	6.25 (27.4)	6.25 (27.4)	12.5 (54.8)	6.25 (27.4)	12.5 (54.8)	12.5 (54.8)	12.5 (54.8)	12.5 (54.8)		
2n	12.5 (47.6)	12.5 (47.6)	12.5 (47.6)	>12.5 (47.6)	12.5 (47.6)	12.5 (47.6)	12.5 (47.6)	>12.5 (47.6)	>12.5 (47.6)	>12.5 (47.6)	>12.5 (47.6)	>12.5 (47.6)	>12.5 (47.6)		

Cpd #	Yeast strains										Filamentous fungi			
	A	B	C	D	E	F	G	H	I	J	K	L	M	
2o	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3a	1.56 (7.05)	0.39 (1.76)	0.78 (3.52)	1.56 (7.05)	0.39 (1.76)	0.78 (3.52)	0.78 (3.52)	1.56 (7.05)	0.78 (3.52)	0.78 (3.52)	0.78 (3.52)	0.02 (0.09)	0.02 (0.09)	0.02 (0.09)
3b	1.56 (6.63)	1.56 (6.63)	0.78 (3.31)	1.56 (6.63)	0.78 (3.31)	1.56 (6.63)	0.78 (3.31)	1.56 (6.63)	0.78 (3.31)	0.78 (3.31)	0.78 (3.31)	0.02 (0.08)	0.02 (0.08)	0.02 (0.08)
3c	6.25 (23.7)	1.56 (5.92)	6.25 (23.7)	3.13 (11.9)	1.56 (5.92)	1.56 (5.92)	1.56 (5.92)	1.56 (5.92)	1.56 (5.92)	0.78 (2.96)	0.78 (2.96)	0.02 (0.08)	0.02 (0.08)	0.02 (0.08)
3d	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)	>12.5 (39.1)
3e	0.78 (3.76)	1.56 (7.53)	0.78 (3.76)	0.78 (3.76)	0.78 (3.76)	0.78 (3.76)	0.78 (3.76)	1.56 (7.53)	0.39 (1.88)	0.78 (3.76)	0.78 (3.76)	0.02 (0.10)	0.02 (0.10)	0.10 (0.48)
3f	1.56 (7.05)	0.78 (3.52)	1.56 (7.05)	0.78 (3.52)	0.78 (3.52)	0.78 (3.52)	1.56 (7.05)	1.56 (7.05)	0.39 (1.76)	1.56 (7.05)	0.20 (0.90)	0.02 (0.09)	0.02 (0.09)	0.10 (0.45)
3g	0.78 (3.76)	1.56 (7.53)	3.13 (15.1)	1.56 (7.53)	1.56 (7.53)	1.56 (7.53)	1.56 (7.53)	1.56 (7.53)	0.78 (3.76)	1.56 (7.53)	0.10 (0.48)	0.10 (0.48)	0.10 (0.48)	0.10 (0.48)
3h	0.39 (1.62)	1.56 (6.46)	1.56 (6.46)	1.56 (6.46)	1.56 (6.46)	1.56 (6.46)	0.78 (3.23)	1.56 (6.46)	0.39 (1.62)	0.78 (3.23)	0.78 (3.23)	0.39 (1.62)	0.39 (1.62)	0.39 (1.62)
3i	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	1.56 (6.11)	0.78 (3.05)	0.20 (0.78)	0.20 (0.78)	0.20 (0.78)
3j	6.25 (23.2)	3.13 (11.6)	6.25 (23.2)	3.13 (11.6)	6.25 (23.2)	6.25 (23.2)	6.25 (23.2)	1.56 (5.79)	1.56 (5.79)	6.25 (23.2)	0.78 (2.90)	0.20 (0.74)	0.20 (0.74)	0.20 (0.74)
3k	0.39 (1.78)	1.56 (7.11)	0.78 (3.56)	0.39 (1.78)	0.39 (1.78)	0.39 (1.78)	0.78 (3.56)	0.78 (3.56)	0.39 (1.78)	1.56 (7.11)	0.20 (0.91)	0.10 (0.46)	0.10 (0.46)	0.10 (0.46)
3l	0.78 (3.34)	1.56 (6.69)	3.13 (13.41)	0.78 (3.34)	1.56 (6.69)	0.78 (3.34)	1.56 (6.69)	1.56 (6.69)	0.78 (3.34)	1.56 (6.69)	0.20 (0.86)	0.20 (0.86)	0.20 (0.86)	0.20 (0.86)
3m	1.56 (6.31)	1.56 (6.31)	3.13 (12.7)	1.56 (6.31)	3.13 (12.7)	1.56 (6.31)	3.13 (12.7)	3.13 (12.7)	0.39 (1.58)	3.13 (12.7)	0.10 (0.40)	0.20 (0.81)	0.20 (0.81)	0.20 (0.81)
3n	3.13 (11.2)	3.13 (11.2)	6.25 (23.9)	6.25 (23.9)	6.25 (23.9)	6.25 (23.9)	6.25 (23.9)	3.13 (11.2)	0.39 (1.49)	3.13 (11.2)	0.02 (0.08)	0.05 (0.19)	0.02 (0.08)	0.02 (0.08)
3o	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4e	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)	>12.5 (56.0)
4f	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)	>12.5 (52.7)
4n	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)	>12.5 (45.1)

Cpd #	Yeast strains										Filamentous fungi		
	A	B	C	D	E	F	G	H	I	J	K	L	M
Amb	3.9 (4.22)	3.9 (4.22)	1.95 (2.11)	0.98 (1.06)	1.95 (2.11)	3.9 (4.22)	3.9 (4.22)	1.95 (2.11)	3.9 (4.22)	1.95 (2.11)	15.6 (16.9)	15.6 (16.9)	3.9 (4.22)
FLC	62.5 (204) ^a	>125 (408) ^a	15.6 (51.0) ^a	>125 (408) ^a	>125 (408) ^a	62.5 (204) ^a	62.5 (204) ^a	>31.2 (102)	>31.2 (102)	1.95 (6.37)	62.5 (204)	62.5 (204) ^a	62.5 (204)
ITC	0.5 (0.71) ^a	>62.5 (88.6) ^a	7.8 (11.1) ^a	31.2 (44.2) ^a	31.2 (44.2) ^a	31.2 (44.2) ^a	31.2 (44.2) ^a	7.8 (11.1)	0.48 (0.68)	0.12 (0.17)	0.48 (0.68)	0.195 (0.28) ^a	0.975 (1.38)
POS	0.5 (0.71) ^a	>62.5 (89.3) ^a	7.8 (11.1) ^a	31.2 (44.5) ^a	31.2 (44.5) ^a	15.6 (22.3) ^a	15.6 (22.3) ^a	0.12 (0.17)	0.06 (0.09)	<0.03 (0.04)	0.24 (0.34)	0.195 (0.28) ^a	0.48 (0.68)
VOR	7.8 (22.3)	>31.2 (89.3)	0.975 (2.79)	1.95 (5.58)	1.95 (5.58)	0.975 (2.79)	7.8 (22.3)	0.06 (0.17)	0.12 (0.34)	<0.03 (0.09)	0.24 (0.69)	0.03 (0.09)	0.12 (0.34)

Yeast strains: A = *Candida albicans* ATCC 10231, B = *C. albicans* ATCC 64124, C = *C. albicans* ATCC MYA-2876(S), D = *C. albicans* ATCC 90819(R), E = *C. albicans* ATCC MYA-2310(S), F = *C. albicans* ATCC MYA-1237(R), G = *C. albicans* ATCC MYA-1003(R), H = *Candida glabrata* ATCC 2001, I = *Candida parapsilosis* ATCC 6258, J = *Candida parapsilosis* ATCC 22019. NOTE: Here, the (S) and (R) indicate that ATCC reports these strains to be susceptible (S) and resistant (R) to ITC and FLC.

Filamentous fungi: K = *Aspergillus flavus* ATCC MYA-3631, L = *Aspergillus nidulans* ATCC 38163, M = *Aspergillus terreus* ATCC MYA-3633.

Known antifungal agents: Amb = amphotericin B, FLC = fluconazole, ITC = itraconazole, POS = posaconazole, and VOR = voriconazole.

^aThese values were previously reported in ref #27.

^bFor yeast strains: MIC-0 values are reported for compounds 1-4n and Amb, whereas MIC-2 values are reported for azoles. For filamentous fungi, MIC-0 values are reported for all compounds.

^cValues in μM are presented so that the reader can visualize the potential impact of the varied molecular weights of the compounds.

ND indicates that MIC values were not determined due to solubility issues with the compound.