Supporting information

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Materials and instrumentation. The antifungal agents amphotericin B (AmB), fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR) were obtained from AK Scientific (Union City, CA, USA) and used without further purification. AmB, FLC, ITC, POS, and VOR were dissolved in DMSO at a final concentration of 5 mg/mL. All these antifungal agent stocks were stored at -20 °C. Fungal MIC determination experiments were performed using untreated 96-well plates (Corning). Cells were counted either by using a hemocytometer (Hausser Scientific, Horsham, PA, USA) or by measuring optical density at attenuance of 600 nm (OD_{600}) by using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Spectrophotometric and colorimetric measurements in 96-well plates were performed using a SpectraMax M5 spectrometer (Molecular Devices, Sunnyvale, CA, USA).

Fungal strains. The yeast strains *Candida albicans* ATCC 10231 (strain **A**), *C. albicans* ATCC 64124 (strain **B**), and *C. albicans* ATCC MYA-2876(S) (strain **C**) were kindly provided by Dr. Jon Y. Takemoto (Utah State University, Logan, UT, USA). *C. albicans* ATCC MYA-90819(R) (strain **D**), *C. albicans* ATCC MYA-2310(S) (strain **E**), *C. albicans* ATCC 1237(R) (strain **F**), *C. albicans* ATCC MYA-1003(R) (strain **G**), *Candida glabrata* ATCC 2001 (strain **H**), *Candida krusei* ATCC 6258 (strain **I**), and *Candida parapsilosis* ATCC 22019 (strain **J**) were obtained

from the American Type Culture Collection (ATCC, Manasas, VA, USA). The (S) and (R) indicate that ATCC reports these strains to be susceptible (S) and resistant (R) to ITC and FLC. The filamentous fungal strains *Aspergillus flavus* ATCC MYA-3631 (strain **K**), and *Aspergillus terreus* ATCC MYA-3633 (strain **M**) were also obtained from the American Type Culture Collection (ATCC, Manasas, VA, USA). *Aspergillus nidulans* ATCC 38163 (strain **L**) was kindly provided by Dr. Jon S. Thorson (University of Kentucky, Lexington, KY, USA), respectively. Yeast strains were cultured at 35 °C. Filamentous fungal strains were cultured at 25 °C and the spores were harvested. All fungal strains were cultured in RPMI 1640 medium (catalog # R6504, Sigma-Aldrich Chemical Co., St. Louis, Mo.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma-Aldrich Chemical Co.).

Mammalian cell lines. The human embryonic kidney cell line HEK-293 (ATCC CRL-1573) and the murine macrophage cell line J774A.1 (ATCC TIB-67) were kindly provided by Dr. Matthew S. Gentry and Dr. David J. Feola (University of Kentucky, Lexington, KY, USA), respectively. The HEK-293 cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manasas, VA, USA) with 10% fetal bovine serum (FBS) (ATCC, Manasas, VA, USA) and 1% Pen/Strep (ATCC, Manasas, VA, USA). The J774A.1 cell line was grown under the same conditions, except that the medium used was a different type of DMEM (catalog# 30-2002, ATCC, Manasas, VA, USA). The HEK-293 cell line was passaged by trypsinization with 0.05%-trypsin-0.53 mM EDTA (ATCC, Manasas, VA, USA). The J774A.1 cell line was passaged mechanically by cell scrapers (ATCC, Manasas, VA, USA). Cell confluency was observed by using a Nikon Eclipse TS100 microscope (Minato, Tokyo, Japan).

Determination of MIC values against fungal strains. The MIC values against fungal strains were determined based on a previously published protocol.[1] MIC values for ebsulfur (**2a**) analogues against fungal cells were evaluated in 96-well plates as described in the CLSI document M27-A3 with minor modifications.[2] Some of the fungal strains, such as *C. albicans* ATCC 64124 (strain **B**) tend to produce pseudohyphae (filaments) in RPMI 1640 medium, which was found to hinder cell counting when using a hemocytometer. Therefore, potato dextrose broth (PDB) was used to grow the yeast inocula of all strains tested, which were later diluted in RPMI 1640 medium to perform determination of MIC values. Minor modifications included growing

yeast cells in PDB for 24-48 h at 35 °C at 200 rpm, diluting in RPMI 1640 medium to a concentration of 1×10^6 cells/mL (as determined by using a hemocytometer or an OD_{600} of 0.12) and using a final inoculum size of 5×10^3 CFU/mL for all the assays. The tested compounds (10 mg/mL) were diluted to the working stocks (500 μg/mL) by addition of DMSO. Two-fold serial dilution of the working stocks was prepared by addition of RPMI 1640 medium (100 μL) and cell suspension (100 μL) to 96-well microtiter plate to achieve final drug and inoculum concentrations ranging from 12.5-0.02 μg/mL and 5×10^3 CFU/mL, respectively. Plates were incubated for 48 h at 35 °C. The MIC values for all tested compounds studied were defined as the lowest drug concentration that inhibits the visible growth of fungal strains after a 48-h incubation period. MIC assays for the spore-forming filamentous fungi, such as strain *A. flavus* ATCC MYA-3631 (strain **K**), were performed in a similar fashion. The filamentous fungal strains were first cultured at 25 °C on potato dextrose agar (PDA) plates for 3-5 days or until confluent. We collected the spores by washing the surface of the agar plates with ddH_2O (5 mL) and then isolated the spores by gravity filtration (the spores are H_2O soluble). The spores were then counted by using a hemocytometer and added to the MIC assays to achieve a final concentration of 5×10^3 cells/mL. Researchers working with spores should wear a facemask to prevent spore inhalation. These MIC data are presented in Tables 1.

Time-kill curves. The efficiency of the compounds to kill *C. albicans* ATCC 64124 (strain **B**) was monitored using a previously published protocol.[1] The cell suspensions were prepared to achieve an inoculum of approximately $1\text{-}4 \times 10^5$ CFU/mL in RPMI 1640 medium at 35 °C. 100 μL of cell suspension was added to 900 μL of sterile ddH_2O (control) or to sterile ddH_2O with ebselen, ebsulfur (**2a**), and **3a** at concentrations of 1×, 2×, and 4× their respective MIC values. After fungal cell addition, at 0, 3, 6, 9, 12, and 24 h, the tubes were vortexed and 100 μL aliquots were removed from each solution, spread onto PDA plates, and incubated at 35 °C. Colony counts were determined after 24 h of incubation. The experiments were performed in duplicate (Fig. 2).

Determination of hemolytic activity. Hemolytic activity was determined as previously described with minor modifications (Fig. 3).[1] Murine red blood cells (mRBCs) (1 mL) were

suspended in 9 mL of phosphate buffer saline (PBS; 10 mM, pH 7.2) and then centrifuged (1,200 rpm) for 10 min at room temperature. mRBCs were washed with PBS 4 times and then resuspended in fresh PBS (5 mL) to achieve the final concentration of (1×10^7 mRBCs/mL). Compounds were serially diluted in Eppendorf tubes containing H_2O (100 μL). The mRBC suspension was then added to achieve final concentrations ranging from 31.2-0.24 μg/mL and 5×10^6 mRBCs/mL of tested compounds and mRBCs, respectively. The tubes were then incubated for 1 h at 37 °C. Tubes containing ddH_2O (200 μL) and triton X-100® (1% *v*/*v*, 2 μL) served as negative (blank) and positive (100%) control, respectively. The percent hemolysis was calculated using the following equation: % hemolysis = [(absorbance of sample) – (absorbance of blank)]×100/(absorbance of positive control). Fifty percent hemolysis (HC_{50}) values were defined as the concentrations of compounds required to lyse 50% of the mRBCs.

Mammalian cytotoxicity assays. Mammalian cytotoxicity assays were performed as previously described with minor modifications (Fig. 4).[1] The HEK-293 and J774A.1 cell lines were grown in various Dulbecco's Modified Eagle's Medium (DMEM) (see mammalian cell lines section above) with 10% fetal bovine serum (FBS) and 1% Pen/Strep at 37 °C with 5% CO_2. The confluent cells were either trypsinized with 0.05%-trypsin-0.53 mM EDTA (HEK-293 cell line) or mechanically removed by cell scrapers (J774A.1 cell line). The cells were transferred into 96-well microtiter plates at a density of 1×10^4 cells/mL (HEK-293 cell line) or 2×10^4 cells/mL (J774A.1 cell line) and were grown for 16 h overnight. The following day, the media were replaced by fresh media (100 μL) containing no compound (negative control), triton-X 100® (positive control) (1%, *v*/*v*), and serially diluted ebselen (**1**), **3a**, **3b**, and **3g** at final concentrations of 10-0.02 μg/mL. Every well contained 0.1% DMSO, which is not toxic against these mammalian cell lines. The cells were incubated with tested compounds for another 24 h at 37 °C with 5% CO_2. Cell survival was assessed by resazurin assay. Each well was treated with resazurin (10 μL of a 25 mg/L solution) for 6 h. Live cells produced the highly fluorescent pink dye resorufin, which was detected at λ_{560} absorption and λ_{590} emission by a SpectraMax M5 plate reader. Dead cells remain purple/blue. The percentage of survival rate was calculated by using the following formula: [(test value)/(control value) × 100]. The control value is obtained from the wells, which have cells and resazurin, but no tested compounds.

Assay for reactive oxygen species (ROS) production. ROS production assay was performed as previously described with minor modifications.[3, 4] The 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe was used to measure the production of ROS in fungal cells after treatment of cells with ebselen, ebsulfur (**2a**), and **3a**. Once entering the cells, the DCFH-DA probe is first hydrolyzed to the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH) by cellular esterases. After that, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by intracellular ROS. A colony of *C. albicans* ATCC 10231 (strain **A**) was used to inoculate 5 mL of PDB in a Falcon tube and grown overnight at 35 °C at 200 rpm. In the morning, we diluted the culture by addition of fungal cells (200 μL) to RPMI 1640 medium (800 μL). After that, we added the newly diluted cell suspension (100 μL) to the RPMI 1640 medium (900 μL) containing no drug (negative control) or ebselen, **2a**, and **3a**, at their 1x and 2x MIC values and incubated for 1 h at 37 °C. Glass slides (with 10-15 of each mixture) were prepared and observed in bright field and fluorescence modes (FITC filter set, λ_{ex} = 488 nm and λ_{em} = 512 nm excitation) using a Zeiss Axovert 200M fluorescence microscope (Fig. 5).

References:

[1] Shrestha, S. K., Fosso, M. Y., and Garneau-Tsodikova, S. (2015) A combination approach to treating fungal infections, *Sci. Rep. 5*, 17070.

[2] Clinical and Laboratory Standards Institute. (2008) *Reference method for broth dilution antifungal susceptibility testing of yeasts - Approved standard. CLSI document M27-A3. Wayne, PA.*

[3] Ngo, H. X., Shrestha, S. K., Green, K. D., and Garneau-Tsodikova, S. (2016) Development of ebsulfur analogues as potent antibacterials against methicillin-resistant *Staphylococcus aureus*, *Bioorg Med Chem*.

[4] Li, Y., Chang, W., Zhang, M., Li, X., Jiao, Y., and Lou, H. (2015) Diorcinol D exerts fungicidal action against *Candida albicans* through cytoplasm membrane destruction and ROS accumulation, *PloS one 10*, e0128693.

Table S1: log P[a] values for all compounds studied.			
Cpd #	**log P**	**Cpd #**	**log P**
1	2.84 (2.92)	**3b**	N/A[b] (4.33)

2a	3.46 (3.16)	**3c**	N/A[b] (5.34)
2b	3.62 (3.32)	**3d**	N/A[b] (7.37)
2c	4.02 (3.84)	**3e**	N/A[b] (3.01)
2d	4.29 (3.97)	**3f**	N/A[b] (3.54)
2e	4.70 (4.67)	**3g**	N/A[b] (3.07)
2f	3.62 (2.92)	**3h**	3.53 (3.48)
2g	4.29 (4.16)	**3i**	N/A[b] (3.69)
2h	4.70 (4.86)	**3j**	N/A[b] (4.21)
2i	5.12 (5.13)	**3k**	N/A[b] (3.29)
2j	4.44 (4.41)	**3l**	N/A[b] (3.79)
2k	3.21 (3.62)	**3m**	N/A[b] (4.30)
2l	4.46 (4.53)	**3n**	N/A[b] (4.80)
2m	2.84 (2.68)	**3o**	N/A[b] (4.80)
2n	3.40 (3.53)	**4e**	N/A[b] (2.16)
2o	4.26 (3.93)	**4f**	N/A[b] (2.69)
3a	N/A[b] (3.83)	**4n**	N/A[b] (3.95)

[a] Calculated from ChemDraw and from Molinspiration (in parenthesis). [b] Indicates that ChemDraw did not provide a log P value.