Supporting Information for:

Screening for dimethylarginine dimethylaminohydrolase inhibitors reveals ebselen as a bioavailable inactivator

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MATERIALS Unless otherwise noted, all chemicals and buffers were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid and HEPES were purchased from Themo Fisher Scientific (Waltham, MA, USA). All buffer solutions were filtered by 0.22 µm Express PLUS filters (Millipore, Billerica, MA, USA). The NIH Clinical Collection (BioFocus, South San Francisco, CA) and Spectrum Collection (Microsource Discovery Systems, Inc. Gaylordsville, CT) libraries were obtained from the inventory of the Automation and High Throughput Screening Facility at the Texas Institute for Drug and Diagnostics Development (TI-3D, The University of Texas at Austin).

EXPRESSION AND PURIFICATION OF DDAH Expression and purification of DDAH from *Pseudomonas aeruginosa* was carried out as previously described using *Echerichia coli* BL21 (DE3) electrocompetent cells.[1] Protein purity was verified by SDS-PAGE and the expected mass was confirmed by electrospray ionization mass spectrometry (ESI-MS, Analytical Core Facility, College of Pharmacy, University of Texas at Austin, USA). To determine protein concentration, purified *P. aeruginosa* DDAH was added to Denaturing Buffer (guanidinium hydrochloride (6 M), sodium phosphate (20 mM), pH 6.6). The absorbance of the sample at 280 nm was determined using a Cary 50 UV-vis spectrophotometer (Varian, Walnut Creek, CA, USA). The *P. aeruginosa* DDAH extinction coefficient (17,210 $M^{-1}cm^{-1}$) was calculated (http://workbench.sdsc.edu) based on amino acid sequence. Expression and purification of human DDAH-1 was completed using a previously published method and *E. coli* BL21 (DE3) cells.[2] Protein purity and expected mass were verified by SDS-PAGE and ESI-MS, respectively. Protein concentration was determined as described above, using a calculated

extinction coefficient of 8,400 $M^{-1}cm^{-1}$.

HTS ASSAY A 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 5 mM) stock solution was prepared in HEPES buffer (10 mM) and potassium chloride (100 mM) at pH 6.5, and stored at 4 ºC. For assay validation experiments, *P. aeruginosa* DDAH (50 nM) was added to Reaction Buffer (HEPES (250 mM), potassium chloride (250 mM), bovine serum albumin (BSA, 0.2 mg/mL) and ethylenediaminetetraacetic acid (EDTA, 5 mM), pH 7.3). Addition of BSA and EDTA were required to achieve linear kinetics over incubation times typically used in microcuvette-based assays. BSA was used to prevent enzyme adsorption to the walls of the plate, and EDTA was necessary to chelate contaminating divalent metals present in the BSA stock solutions, because Zn^{2+} is a potent DDAH inhibitor.[2,3] Control reactions without enzyme were also prepared for all HTS validation experiments. This mixture (45 μL) was then added to each well in a clear polystyrene 384-well plate (Corning Inc., Corning, NY, USA). To initiate the reaction, 45 μL of substrate solution containing *S*-methyl-L-thiocitrulline (SMTC, 60 μM) and DTNB (1 mM) was added to each well. Absorbance at 405 nm was measured approximately every 20 s for 10 min using a Victor Wallac 1420 plate reader (PerkinElmer, Waltham, MA, USA). A path length of 0.75 cm and a published extinction coefficient of 14,150 $M^{-1}cm^{-1}$ were used to determine product concentrations.[4] Rates were determined based on the slope of the product concentration plotted against time. For each plate, rates for all wells were converted to normalized percent inhibition by: $\%\text{inhibition} = 100 \times \left(1 - \frac{v_{pos} - v_0}{v_{pos} - v_{neg}}\right)$.

HTS was carried out at the TI-3D Automation Facility (University of Texas, Austin, TX). Library compounds were dissolved to 10 mM in DMSO, stored in polypropylene 384-well plates at -20 ºC and thawed prior to use. Unless otherwise noted, all subsequent reactions were done at room temperature. Into each well of a 384-well Dilution Plate, 45 μL Assay Buffer (HEPES (250 mM), potassium chloride

(250 mM), pH 7.3) was dispensed using a Microflo Select (BioTek Instruments Inc., Winooski, VT, USA). Compounds were diluted 10:1 in the Dilution Plate using a Janus liquid handling workstation (PerkinElmer, Waltham, MA, USA). To a 384-well clear polystyrene Assay Plate (Corning Inc., Corning, NY, USA), Reaction Buffer (36 µL) containing HEPES (250 mM), potassium chloride (250 mM), BSA (0.2 mg/mL), EDTA (5 mM) and *P. aeruginosa* DDAH (50 nM) at pH 7.3 was dispensed to each well using the Janus workstation. Columns 1, 2, 23, and 24 were reserved as controls, with columns 1 and 2 containing no inhibitor and columns 23 and 24 containing no enzyme and no inhibitor (Figure S1). The diluted library compounds (9 uL aliquots) were then mixed with the reaction buffer using the Janus workstation, centrifuged for one minute at 1,000 rpm to remove any bubbles formed during mixing and incubated for at least 10 min to allow for equilibration of any time-dependent inhibitors. To initiate the reaction, Substrate Solution (45 µL) containing SMTC (60 µM) and DTNB (1 mM) in water was dispensed into each well using the Microflo Select liquid handling instrument. After additional centrifugation for one min to remove any bubbles, the plate was loaded into an EnVision Microplate Reader (PerkinElmer, Waltham, MA, USA) and absorbance at 405 nm of all wells was read each minute for 10 min. The slope of the resulting plot of absorbance over time was used as the observed rate. Each library compound was screened in duplicate. All of the HTS reactions were performed in less than 4 h.

HIT VALIDATION Ebselen, cefatrizine (Sequoia Research Products, Pangbourne, UK), and disulfiram were each purchased, dissolved to 20 mM in DMSO, and stored at -20 ºC. *P. aeruginosa* DDAH (50 nM) was incubated in 96-well plates with HEPES (250 mM), potassium chloride (250 mM), BSA (0.1 mg/mL), EDTA (2 mM) and inhibitor (30 µM) for 10 minutes. Dimethylarginine (200 µM) was then added to initiate the reaction, which was incubated at room temperature for 30 minutes. The concentration of the product citrulline was then measured using an established discontinuous derivatization assay.[5]

COMPUTATIONAL DOCKING Computational docking was carried out using Autodock 4.2.[6] The energy grid was prepared with spacing of 0.1 Å, and a Lamarckian genetic algorithm was used with a population size of 300, mutation rate of 0.02, and crossover rate of 0.8. Simulations were run for a maximum of 25,000,000 energy evaluations. Each ligand was docked 10 times, and the lowest energy conformation was used.

CONCENTRATION DEPENDENCE OF INHITIBION The concentration dependence of ebselen and cefatrizine inhibition of *P. aeruginosa* DDAH activity was carried out in Reaction Buffer (potassium phosphate (125 mM), potassium chloride (125 mM), Tween-20 (0.02% v/v) and EDTA (2 mM) at pH 7.3, room temperature). The final concentration of DMSO in all reactions was 1 %. After incubating enzyme (50 nM) in Reaction Buffer with each inhibitor (0.6 nM - 1 mM) for 10 min, the reaction was initiated upon addition of dimethylarginine (1 mM) and allowed to run for approximately 4 h. Reactions were then quenched and analyzed as described.[5] Positive control reactions containing enzyme but no inhibitor and negative control reactions with no enzyme and no inhibitor were used to determine the background rates.

TIME-DEPENDENT INACTIVATION KINETICS Time-dependent inactivation of DDAH by ebselen was measured as follows. Dimethylarginine (1 - 6 mM) was mixed with *P. aeruginosa* DDAH (129 - 200 nM) in potassium phosphate (82 mM), potassium chloride (82 mM), Tween-20 (0.02%) and EDTA (2 mM) at pH 7.3 at room temperature. After approximately 30 s of incubation to ensure the reaction had reached the steady state, ebselen (0.64 - 5.70 μM) was added. Control reactions were carried out without ebselen or enzyme. At each time point, a 60 μL aliquot of the above reaction was removed and the reaction was quenched by addition of trifluoroacetic acid to a final concentration of 1 M. Samples were stored at room temperature until the inactivation experiment was complete

(approximately 1 h) and were analyzed for citrulline content by derivatization.[5]

Inactivation rates were determined by fitting the product vs. time curves to the single exponential equation $[P] = \frac{v_0}{A[I]}\left(1 - e^{-A[I]t}\right)$[6], where [P] is the concentration of product at a particular time t, v_0 is the initial reaction rate, A is the apparent rate of inactivation and [I] is the concentration of inhibitor. [I] and v remained constant as the substrate concentration was varied. An apparent rate constant A was determined for each substrate concentration tested. These observed rate constants were then plotted against substrate concentration, and fit to the equation previously derived for non-complexing inhibitors in the presence of substrate $A = \frac{k_E K_M + k_{ES}[S]}{K_M + [S]}$[6], where k_E is the rate of inactivation of free enzyme, k_{ES} is the rate of inactivation of substrate-bound enzyme and A is the apparent rate constant at the substrate concentration [S] determined as above. In this way, the effect of competing substrate on the rate of inhibition can be quantified. All inactivation reactions were carried out in triplicate, and average A values were computed for each reaction. Most enzyme will have substrate bound in the presence of substrate in excess of K_M. Based on the relationships $K_M = \frac{[E]\cdot[S]}{[ES]}$ and $[E]_t = [E] + [ES]$, where E_t is the total enzyme concentration, E is the concentration of free enzyme, and ES is the concentration of the Michaelis complex, the amount of free enzyme was therefore estimated as $[E] = \frac{[E]_t}{1 + \frac{[S]}{K_M}}$.

MASS SPECTRUM ANALYSIS OF COVALENTLY MODIFIED DDAH To characterize the covalent modification of DDAH by ebselen, incubations of DDAH were prepared as follows. Human DDAH-1 or *P. aeruginosa* DDAH (30 μM) was incubated 1 min with and without ebselen (30 μM) in HEPES (10 mM), potassium chloride (100 mM), and glycerol (5% v/v) at pH 7.3, 25 °C, and

immediately subjected to gel filtration using G-25 Superfine Sephadex resin equilibrated with the same buffer described above. Samples were then desalted on a protein trap (Protein MicroTrap, Michrom, Auburn, CA, USA) and analyzed by ESI-MS on a ThermoFinnigan LCQ (San Jose, CA, USA) ion trap mass spectrometer.

DATA ANALYSIS AND STATISTICS Linear fits for absorbance vs. time were calculated using the SciPy python module (http://www.scipy.org). Further calculations were done in spreadsheets using OpenOffice Calc (http://www.openoffice.org/). Nonlinear regression was performed using the open source suite QtiPlot (http://soft.proindependent.com/). Z and Z' factors were computed as previously described.[7]

EXAMINATION OF EBSELEN-MEDIATED HUMAN DDAH-1 INHIBITION IN CELLS

Cultured HEK 293T cells were seeded in a 12-well polystyrene plate using complete growth medium containing DMEM with 10% FBS (Invitrogen, Carlsbad, CA) and grown to 80 % confluency. pEF6a-hDDAH-1[8] was transfected into HEK 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 h, spent medium was removed and cell was washed with 1 × 1mL PBS at pH 7.2 (Invitrogen, Carlsbad, CA). Ebselen stock solutions or neat DMSO controls were diluted 1 : 200 into 1 mL complete growth medium in the cultures (500 µL) to give final concentrations of 1.25, 2.5, 5, 10, 25, 30, and 0 µM ebselen. These were subsequently incubated for 30 min at 37 ºC in an atmosphere of 5% CO_2 before addition of the activity probe *N*-but-3-ynyl-2-chloroacetamidine (154 µM), followed by an additional 90 min incubation. After treatment, cells were washed three times with PBS (1 mL) and harvested by 500 µL PBS followed by centrifugation at 1000 rpm for 5 min at 4 ºC. Cell pellets were stored at – 80 ºC. Frozen cell pellets were lysed and labeled with biotin-PEO_3-azide as described earlier.[8] Two-color Western blot detection was used to detect the expression levels of DDAH-1 and the response to the biotin tag as described previously.[8] Images were scanned using an Odyssey

Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) at the Core DNA Facility (University of Texas, Austin). Fluorescence intensities for both 680 nm and 800 nm channels were integrated. The 680 nm value (the response to myc tag, displayed in red) was used for normalization. The resulting fluorescence intensities for the response to biotin at 800 nm (displayed in green) were converted to percent relative fluorescence for each ebselen concentration and were subsequently fit to the following equation, % Relative Fluorescence Intensity = 100% - (100% / (1+ (IC_{50}/[ebselen])^{h})) to determine " in cell" IC_{50} values for ebselen, with *h* serving as the Hill coefficient.

REFERENCES

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(6) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *J Comput Chem* **2009**, *30*, 2785-2791.

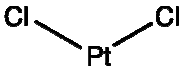
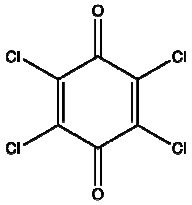
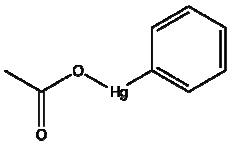
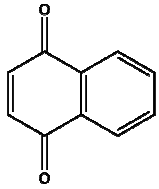
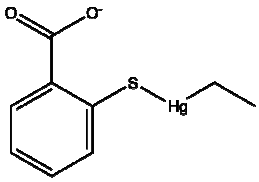
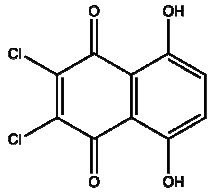
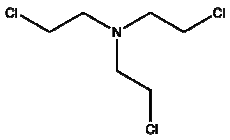
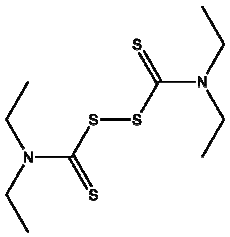
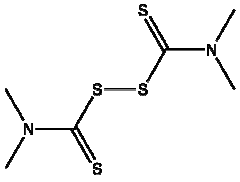
(7) Zhang; Chung; Oldenburg A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **1999**, *4*, 67-73.

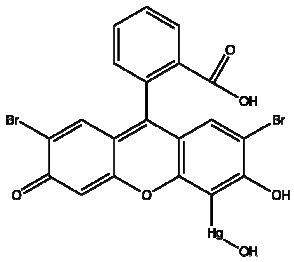
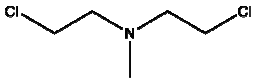
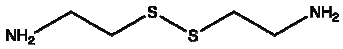
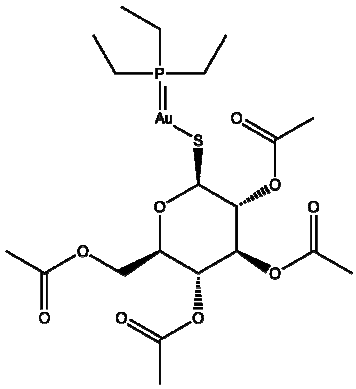
(8) Wang, Y.; Hu, S.; Fast, W. A Click Chemistry Mediated in vivo Activity Probe for Dimethylarginine Dimethylaminohydrolase. *J Am Chem Soc* **2009**, *131*, 15096-15097.

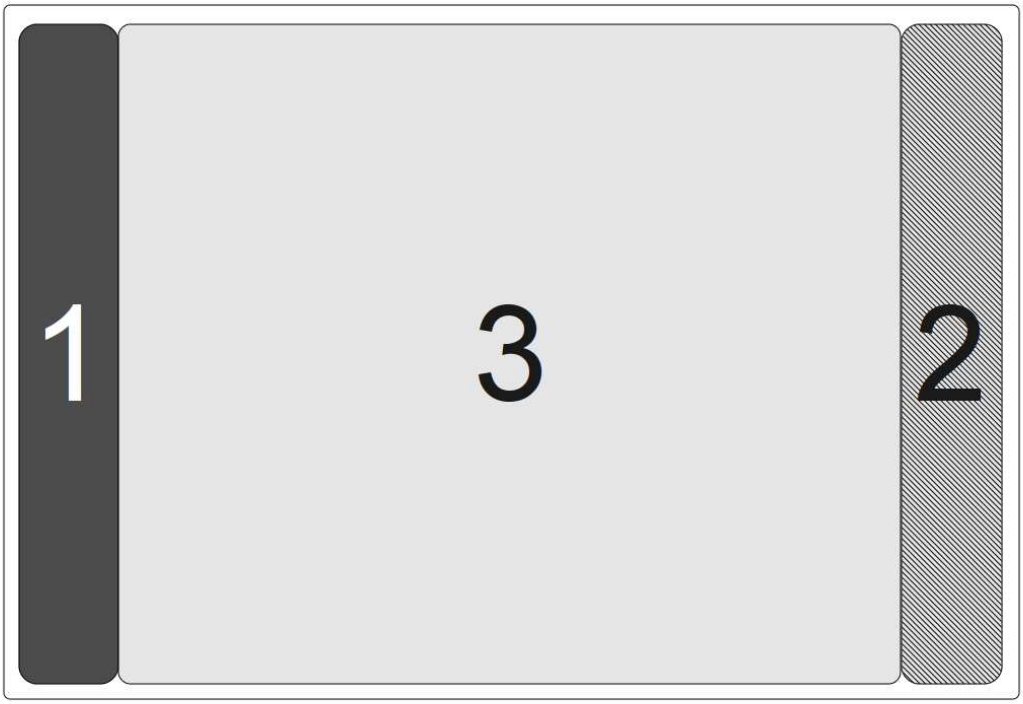
Table S1. Per-plate high throughput screening statistics: v_0/[E] rates for positive controls containing enzyme and no inhibitor (+Control) and for negative controls containing no enzyme and no inhibitor (-Control), signal to noise ratio (SNR $= \frac{\mu_{pos}}{\sigma_{pos}}$ for positive controls), window (window $= \frac{\mu_{pos}}{\mu_{neg}}$), coefficient of variation (%CV $= 100 \times \frac{\sigma_{pos}}{\mu_{pos}}$), Z' factor (Z' factor $= 1 - \frac{3 \cdot \left(\sigma_{pos} + \sigma_{neg}\right)}{\left|\mu_{pos} - \mu_{neg}\right|}$), and Z factor (Z factor $= 1 - \frac{3 \cdot \left(\sigma_{sample} + \sigma_{neg}\right)}{\left|\mu_{sample} - \mu_{neg}\right|}$), where μ_{pos}, μ_{neg}, and μ_{sample} are the mean rates of the positive controls, negative controls, and samples, σ_{pos}, σ_{neg}, and σ_{sample}, are the standard deviations of the positive controls, negative controls, and samples.

Plate	+Control (min^{-1})	-Control (min^{-1})	SNR	Window	% CV	Z' factor	Z factor
1	30.27 ± 0.95	0.32 ± 0.39	29.16	27.29	3.14	**0.87**	**0.83**
2	31.88 ± 1.14	0.23 ± 0.27	26.95	23.98	3.59	**0.87**	**0.81**
3	31.35 ± 0.94	0.29 ± 0.17	32.58	29.56	2.99	**0.89**	**0.85**
4	32.56 ± 1.16	0.32 ± 0.16	27.51	24.36	3.56	**0.88**	**0.82**
5	31.38 ± 1.04	0.21 ± 0.12	29.81	26.66	3.31	**0.89**	**0.74**
6	33.26 ± 1.29	0.34 ± 0.13	25.42	22.25	3.88	**0.87**	**0.71**
7	31.46 ± 1.11	0.45 ± 0.26	27.16	24.19	3.53	**0.87**	**0.62**
8	33.33 ± 0.97	0.41 ± 0.13	33.52	30.42	2.92	**0.90**	**0.61**
9	31.60 ± 1.19	0.32 ± 0.19	25.89	22.74	3.77	**0.87**	**0.86**
10	34.19 ± 1.24	0.40 ± 0.24	26.69	23.60	3.64	**0.87**	**0.83**
11	31.88 ± 1.32	0.30 ± 0.13	23.86	20.68	4.13	**0.86**	**0.60**
12	33.79 ± 1.05	0.43 ± 0.17	31.31	28.24	3.11	**0.89**	**0.55**
13	34.41 ± 0.88	0.50 ± 0.17	37.88	35.00	2.55	**0.91**	**0.67**
14	30.99 ± 1.22	0.44 ± 0.48	23.33	20.90	3.93	**0.83**	**0.78**
15	31.74 ± 1.25	-0.05 ± 0.33	24.51	21.55	3.95	**0.85**	**0.74**
16	31.83 ± 1.84	0.06 ± 0.18	17.18	13.97	5.78	**0.81**	**0.76**
17	31.89 ± 1.66	-0.03 ± 0.32	18.91	15.68	5.20	**0.81**	**0.67**
18	29.88 ± 2.02	-0.04 ± 0.21	14.75	11.52	6.75	**0.78**	**0.66**

Table S2. Spectrum Collection hits.

Molecule	Name	% Inhibition
	Cisplatin	102
	Chloranil	102
	Phenylmercuric Acetate	101
	1,4-Napthoquinone	92
	Thimerosal	91
	2,3-dichloro-5,8-dihydroxynapthoquinone	90
	Trichlormethine	90
	Disulfiram	82
	Thiram	78

Molecule	Name	% Inhibition
	Merbromin	77
	Mechlorethamine	76
not shown	Coenzyme B12	69
	Cystamine	60
	Auranofin	56



Group	Description	# Wells	DDAH	Compound
1	0% inhibition controls	32	Yes	DMSO
2	100% inhibition controls	32	No	DMSO
3	Samples	320	Yes	Library compound

Figure S1. Plate layout for high throughput screening.

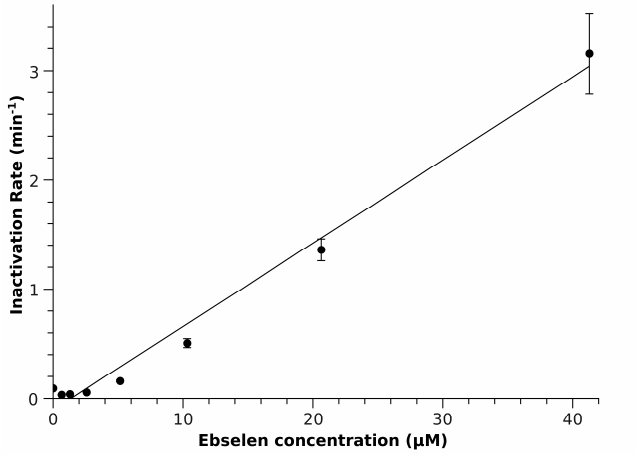


Figure S2. Dependence of observed inactivation rate of *P. aeruginosa* DDAH on ebselen concentration. The data fits to a line with slope 44,000 ± 2,400 $M^{-1}s^{-1}$.

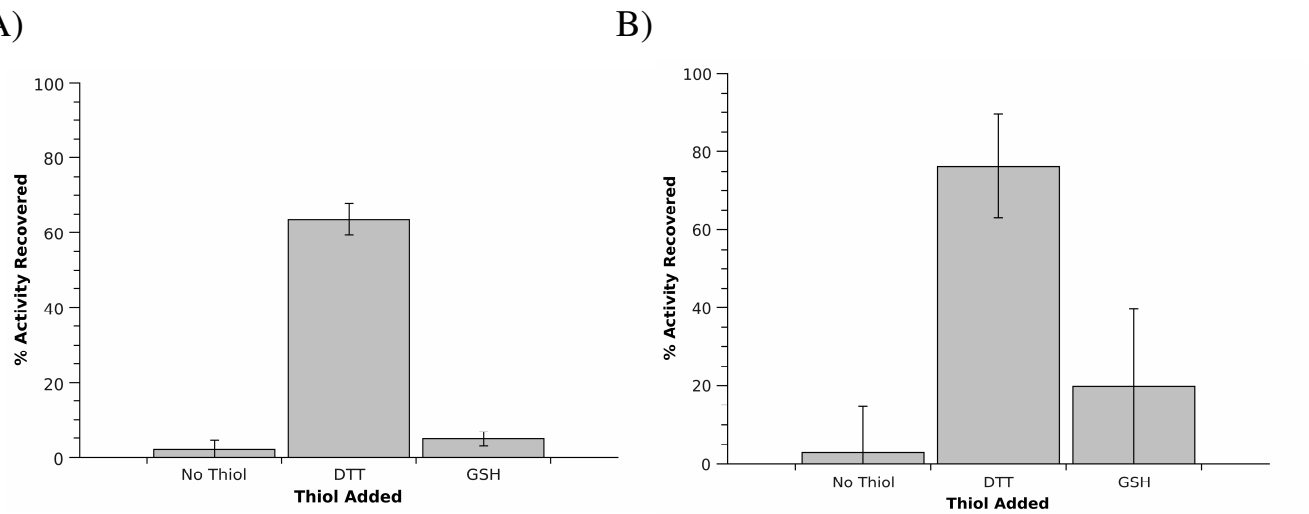


Figure S3. Recovery of A) *P. aeruginosa* DDAH and B) human DDAH-1 activity upon addition of dithiothreitol (DTT, 1 mM) or reduced glutathione (GSH, 1 mM) after inactivation with ebselen (80 μM) for 1 min at room temperature in HEPES (125 mM), potassium chloride (125 mM), BSA (0.1 mg/mL), and EDTA (2 mM) at pH 7.3. To attempt to free the adduct, samples were then incubated with N^{ω}-methyl-L-arginine (1 mM) for 90 min at room temperature and citrulline production was derivitized and quantified using the colorimetric procedure described above.

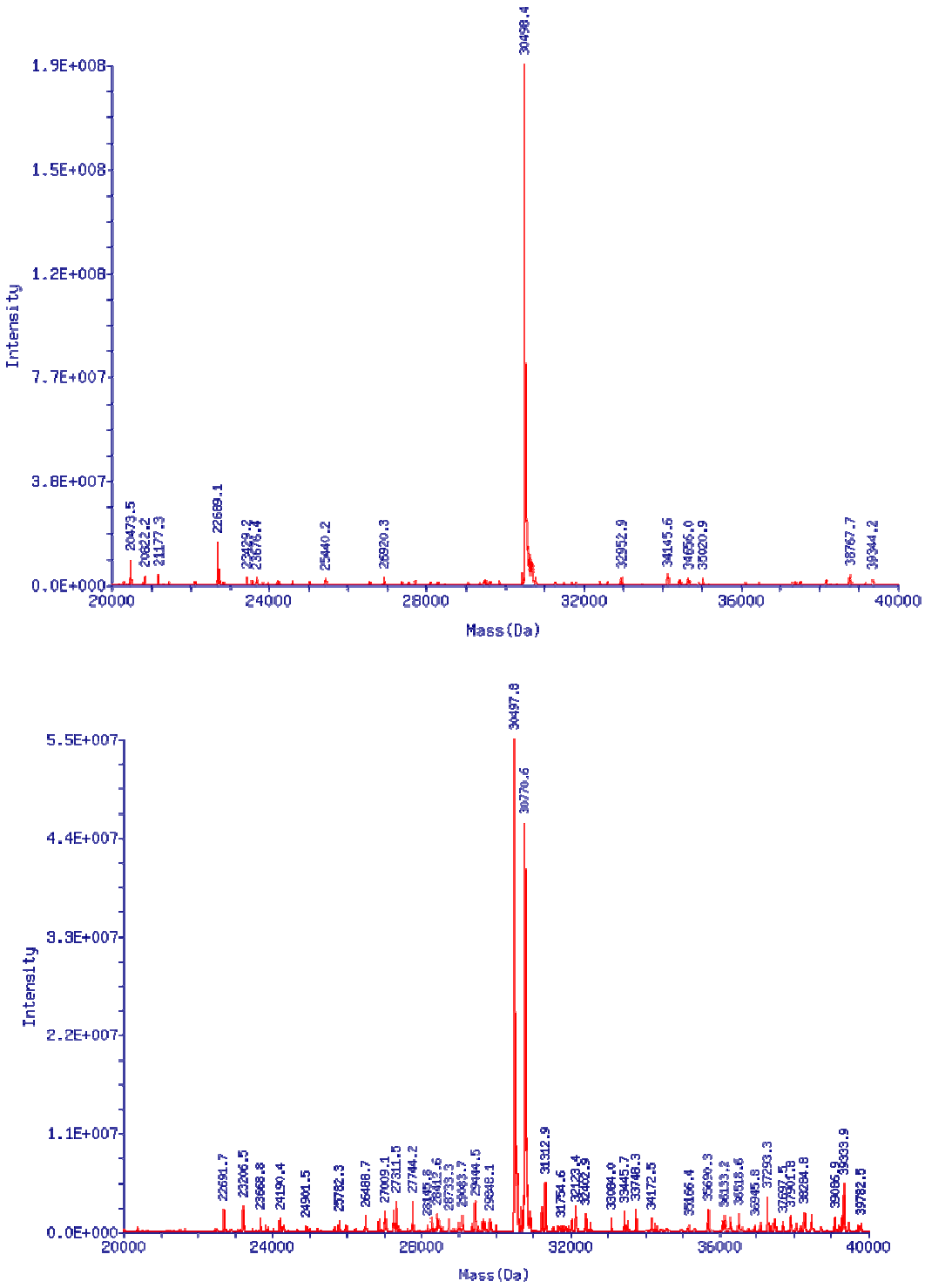


Figure S4. ESI-MS of *P. aeruginosa* DDAH (25 µM) A) incubated without ebselen and B) incubated with ebselen (40 µM). Both samples were incubated for one minute prior to gel filtration.

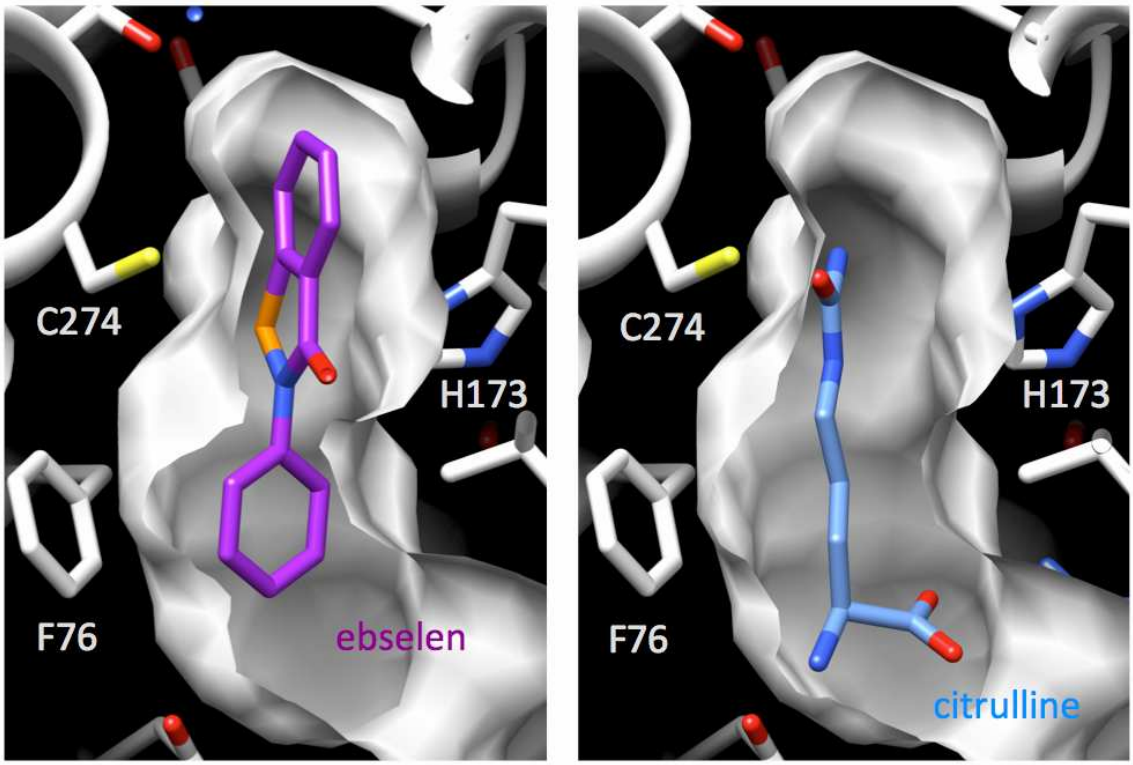


Figure S5. Left: Structure of the lowest-energy pose of ebselen (purple) docked with human DDAH-1 (Protein Data Bank accession code 2JAI, with citrulline removed). Right: Structure of citrulline complexed with human DDAH-1. Oxygen, nitrogen, sulfur and selenium are color coded red, blue, yellow and orange, respectively. In both panels, the active-site Cys274 is labeled and the surface of the binding pocket is colored in grey. The sulfur of Cys274 is placed approximately 3.2 Å from the selenium atom in ebselen.