Electronic Supplementary Information

Target Discovery of Ebselen with a Biotinylated Probe

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1. Synthesis of Biotin-Ebselen

General Information. All reagents used for chemical synthesis were purchased from Sigma-Aldrich unless otherwise specified, and used without further purification. N,N-Dimethylformamide (DMF), dichloromethane, methanol, triethylamine, sodium sulfate were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The silica gel for the flash chromatography was from Qingdao Haiyang Chemical Co. (China). Sartorius ultrapure water (18.2 MΩ cm) was purified with a Sartorius Arium 611 VF system (Sartorius AG, Germany). High-resolution mass spectral analyses for chemical synthesis were performed on a Bruker maXis ultrahigh resolution-TOF MS system. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker Advance 400 MHz spectrometers (Bruker, Germany).

N-(4-aminophenyl)-2-iodobenzamide

To a solution of 1,4-Diaminobenzene (1.08g, 10.0 mmol) and triethylamine (1.039 mL, 7.5 mmol) in anhydrous CH_2Cl_2 (30 mL) was added a solution of 2-iodobenzoyl chloride (5 mmol in 10 mL CH_2Cl_2) dropwise. The mixture was stirred for 12 h at room temperature. Then water was added, the organic layer was separated and the aqueous layer was extracted with CH**2**Cl**2**. Standard work-up gave a residue, which was submitted to column chromatography. The N-(4-aminophenyl)-2-iodobenzamide (0.884 g, 52.3%), eluted with CH_2Cl_2:CH_3OH =40:1, was a light yellow crystal. HRMS (ESI): calculated for $C_{13}H_{11}IN_2O$ (M + H^+) 338.9988, found 338.9969.

1.2 Compound 2

tert-butyl (6-((4-(2-iodobenzamido)phenyl)amino)-6-oxohexyl)carbamate

6-((tert-butoxycarbonyl)amino)hexanoic acid (508 mg, 2.2 mmol) was dissolved in 20 mL of CH_2Cl_2, HOBt (327 mg, 2.42mmol), EDCI·HCl (464 mg, 2.42 mmol), and triethylamine (369 uL, 2.66mmol) were added. The solution was cooled at 0 °C for 1 h, then N-(4-aminophenyl) -2-iodobenzamide (676 mg, 2.0 mmol) a were added. After being stirred for 24 h at room temperature, the white solid was obtained by suction filtration and was washed with CH_2Cl_2 several times. The excess solvent was removed in vacuum to give white solid (973mg, 88.22%). The solid was applied to the next step directly without further purification. HRMS (ESI): calculated for $C_{24}H_{30}IN_3O_4$ (M + Na^+) 574.1173, found 574.1155.

1.3 Compound 3

N-(4-(3-oxobenzo[d][1,2]selenazol-2(3H)-yl)phenyl)-6-((pivaloyloxy)amino)hexanamide

Synthesis of Compound 3 was carried out by following similar procedure as described.[1] In brief, copper iodide (76.0 mg, 0.4mmol) and 1,10-phenanthroline (72.0 mg, 0.4 mmol) in 3 mL DMF were mixed and stirred in a single neck flask. Resulted brownish solution was stirred for 15 min and then Compound 2 (1102.8 mg, 2.0 mmol), selenium powder (189.6 mg, 1.2 mmol), and potassium carbonate (414.0 mg, 3 mmol) were added sequentially to the reaction flask. The brown colored reaction mixture was refluxed at 110 °C using refluxing condenser under nitrogen atmosphere for 24 h. After this, the reaction mixture was poured into brine solution (60 mL) and stirred for another 3 h. Product was precipitated as brown solid which was collected by filtration over Buchner funnel, dried in air, dissolved in ethyl acetate, concentrated over rotary evaporator, and the brown solid product was purified by column chromatography using CH_2Cl_2/ CH_3OH (25:1) over silica gel. Yield 0.84 g (83.6%). HRMS (ESI): calculated for $C_{24}H_{29}N_3O_4Se$ (M + Na^+)

526.1216, found 526.1233.

1.4 Compound 4

6-amino-N-(4-(3-oxobenzo[d][1,2]selenazol-2(3H)-yl)phenyl)hexanamide

Compound 3 (502.47 mg, 1.0 mmol) were dissolved in CH_2Cl_2/ CH_3OH (10:1, 10mL) with ice bath. A solution of trifluoroacetic acid (2 mL, 27mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise. Upon complete addition of the trifluoroacetic acid, the ice bath was removed and the reaction mixture was stirred at room temperature for 12h. The reaction was quenched with saturated $NaHCO_3$(aq) solution and the organic layer was washed with brine. The organic layer was dried over Na_2SO_4 and concentrated to give crude Compound 4, which was purified chromatographically over silica gel. Yield 384 mg (95.6%)

1.5 NHS-Biotin：*N*-hydroxysuccinimido biotin[2]

To a solution of biotin (489mg, 2.0 mmol) and *N*-hydroxysuccinimide (255 mg, 2.2 mmol in 25.0 mL DMF) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (460 mg, 2.4 mmol). After being stirred for 24 h at room temperature, the reaction solution was concentrated to give white solid, which was washed by methanol several times, and excess solvent was removed in vacuum to give white product, and it was applied to the next step directly without further purification.

1.6 Biotin-Ebselen.

To a solution of *N*-hydroxysuccinimido biotin (376 mg, 1.1 mmol) and 6-amino-N-(4-(3-oxobenzo [d][1,2]selenazol-2(3H)-yl)phenyl)hexanamide (402 mg, 1.0 mmol) in DMF (10.0 mL) was added 305 μL of triethylamine (223 mg, 2.0 mmol). After stirring for 24 h at room temperature, the reaction solution was concentrated in vacuum and the resulting residues was washed by ethyl acetate (10 mL × 3), dried in air, dissolved in DMF, and recrystallized. Yield 567 mg (90.2%) ^{1}H NMR (400 MHz, DMSO) δ 10.00 (s, 1H), 8.08 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 7.5 Hz, 1H), 7.77 (s, 1H), 7.66 (d, J = 8.6 Hz, 2H), 7.60 – 7.40 (m, 3H), 6.41 (d, J = 27.8 Hz, 2H), 4.29 (s, 1H), 4.12 (s, 1H), 3.20 – 2.94 (m, 3H), 2.81 (dd, J = 11.5, 4.4 Hz, 1H), 2.57 (d, J = 12.9 Hz, 1H), 2.40 – 2.24 (m, 2H), 2.04 (t, J = 6.4 Hz, 2H), 1.59 (d, J = 6.5 Hz, 3H), 1.46 (dd, J = 26.1, 5.5 Hz, 5H), 1.26 (d, J = 29.1 Hz, 5H). ^{13}C NMR (400 MHz, DMSO) δ 172.27, 171.69, 165.38, 163.18, 139.35, 137.6, 134.89, 132.60, 128.91, 128.35, 126.69, 126.29, 125.67, 120.00, 61.50, 59.65, 55.92, 38.76, 36.80, 35.69, 29.49, 28.69, 28.50, 26.59, 25.72, 25.33. HRMS (ESI): calculated for $C_{29}H_{35}N_5O_4SSe$ ($M + H^+$) 630.1648, found 630.1637.

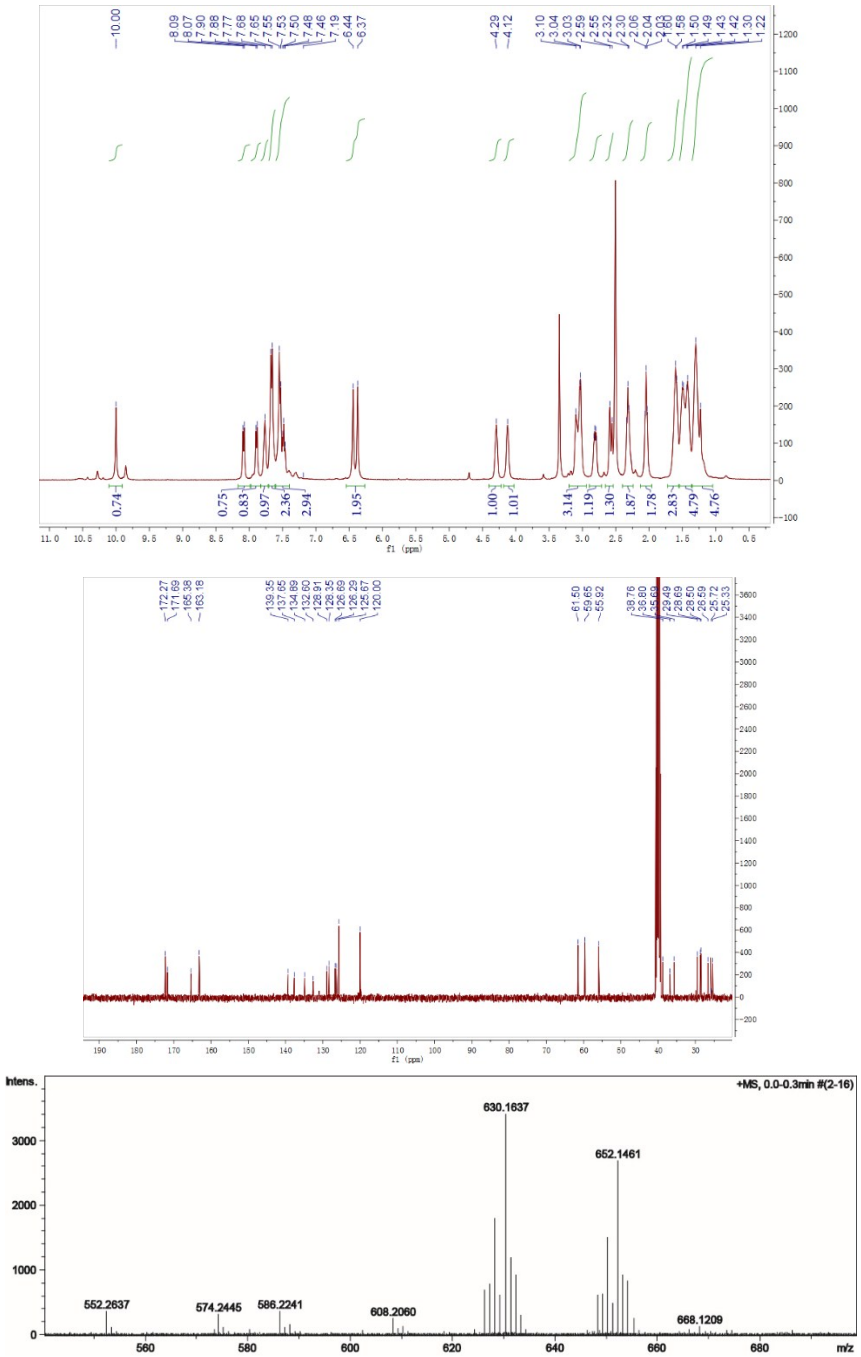


Fig. S1 ^{1}H NMR, ^{13}C NMR and mass spectra of Biotin-Ebselen

2. Biological Methods

Reagents and antibodies. Iodoacetamide (IAA), Glutathione (GSH), DL-Dithiothreitol (DTT) were purchased from Sigma-Aldrich unless otherwise specified. GCSWDYKN (Gly-Cys-Ser-Trp-Asp-Tyr-Lys-Asn) was synthesized from GL Biochem. Fetal bovine serum (Thermo Fisher Scientific), Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific), Penicillin-streptomycin (Thermo Fisher Scientific), *β*-lactoglobulin A (Sigma-Aldrich),
Octreotide Acetate (Beyotime Biotechnology), Pierce Streptavidin Agarose Resins (Thermo scientific), HRP-labeled Streptavidin (Thermo scientific), Actin antibody (Beyotime Biotechnology), HRP-labeled Goat Anti-Mouse lgG(H+L)(Beyotime Biotechnology), and other chemical or biological reagents were obtained from commercial suppliers without anymanipulation.

2.1 Cell Culture and Preparation of Cell Lysates. HeLa cells were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured at 37 °C under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin. For Western blots and mass spectrometry experiments, cells are grown to 80-85% confluence and were collected by trypsinization and pelleted by centrifugation at 1 000 rpm for 3 min at 25 °C, followed by washing with PBS (1 mL) three times and the cell pellets were flash frozen in liquid nitrogen and stored in -80 °C for use. Cell pellets were then resuspended followed by ultrasonication and centrifugation at 4 °C for 30 min at 20 000 g. The resulting supernatant (soluble cell lysate) was collected and protein concentration was determined via BCA assay (Pierce, Thermo Scientific).

2.2 Labeling of Cysteine-containing Peptides. Peptides containing cysteines, GSH and GCSWDYKN were used to evaluate the reactivity of Biotin-Ebselen toward thiol molecules. Equal volume of peptides (40 μM, water) and Biotin-Ebselen (100 μM, 1:1 methanol/water) were mixed, and the solution was vortexed for 10 s. Then octreotide acetate (20 μM) was added as an internal standard before analysis by LC-MS.

2.3 Labeling of Purified Proteins. *β*-lactoglobulin A was dissolved in 1 mL of PBS to get a final concentration of 50 μM. Then 1 μL of 100 mM Biotin-Ebselen (DMSO) was added, and the mixture was incubated at 25 °C for 5 min. After that, the solution was divided into two parts equally, and 1 μL of 500 mM DTT was added in one part. The mixture was allowed to react for 30 min at room temperature. To keep the concentrations consistent, 1 μL water was added to the other part. Then the proteins were filtered with NAP-5 columns (GE Healthcare) to remove small molecule species and diluted to 25 μM for further mass analysis. 50 μL of *β*-lactoglobulin (25 μM) was desalted with Zeba Spin Desalting column (Thermo Fisher Scientific) for intact mass analysis.

2.4 Western Blotting. Protein concentration was adjusted to 2 mg/mL with PBS before use. Assuring that the added probe volume is equal, different concentrations of probes in DMSO were mixed individually with cell lysates to a final volume of 100 μL and incubated at 25 °C for 1 h to observe the effect of concentrations on labeling. To visualize the rate of labeling, cell lysates were incubated with the probe (100 μM) for different lengths of time. Furthermore, to explore the influence of small thiol molecules on probe-binding in proteomes, small molecule species in cell

lysates were removed by NAP-5 columns and then the same procedure on labeling were performed.

For comparison experiments, equal volumes of Biotin-Ebselen (100 μM) or DMSO were added to two equally divided cell lysates, before incubation at 25 °C for 1 hour. To investigate the competitive effect of ebselen, equal volumes of ebselen (250 μM) or DMSO were added to equally divided cell lysates respectively, and incubated for 1 hour at 25 °C, which was followed by same amount of probes (100 μM) incubation for another 1 hour. As for the IAA competition experiments, two equally divided cell lysates were added with equal volumes of IAA (20 mM) or water, respectively, with subsequent incubation for 1 hour at 25 °C, which was followed by same amount of probes (100 μM) incubation for another 1 hour.

Then SDS-PAGE and Western blotting was conducted following the standard experimental steps. HRP-labeled Streptavidin was used at a 1:10 000 dilution according to the manufacturer's protocol. Blotting was imaged using a ChemiDoc™ Touch Imaging System (Bio-Rad).

2.5 Biotin Enrichment. Cell lysates (1mL, 2mg/mL) were labeled with Biotin-Ebselen (100 μM), DMSO or Ebselen (250 μM) according to the needs of experimental objectives. In the enrichment experiments (Probe/DMSO), we added equal volumes of probe or DMSO to same amounts of cell lysates, respectively, and allowed incubation at 25 °C for 1 hour. While in the competition experiments (Probe/Competitor), two equally divided cell lysates were added with equal volumes of ebselen or DMSO, respectively, and incubated for 1 hour at 25 °C, which was followed by probe incubation. Then sequential addition of isopyknic PBS and 2.5 volumes of MeOH/CHCl3 (4:1,v/v) were added into the cell lysates. Precipitated proteins were centrifuged at 6500g for 4 min at 4 °C and washed two times with 1mL of ice-cold MeOH, with resuspension of the pellet each time. The pellet was then air-dried for 10 min. To capture the biotinylated proteins by streptavidin beads, the air-dried protein pellet was resuspended in 1 mL of resuspension buffer (1.2%SDS/PBS) by bath sonication. Then transfer the solution to a 15 mL conical tube and dilute to 0.2% SDS with 5 mL of PBS. Streptavidin beads (100μL of slurry) were washed with PBS (3*5ml) and added into each sample. The mixtures were incubated at room temperature for 3h with rotation. The beads were then washed with 1*5mL of 0.2% SDS/PBS, 3*5ml of PBS and 3*5ml of water. Beads were resuspended in 1 mL water , transferred to screw-top tubes, and pelleted by centrifugation (1400 g for 3 min).

2.6 On-Bead Trypsinolysis and Peptide Stable Isotope Dimethyl Labeling. The beads in screw-top tubes were then denatured in 6 M urea/TEAB (100mM), reduced with 10 mM dithiothreitol (DTT) at 37 °C for 30min and alkylated with 20mM IAA at 35 °C for 30 min in dark. The redundant IAA in mixtures was quenched with isometric DTT at 37 °C for 10 min. To this bead-mixture was added just the right amount of TEAB(100mM) to dilute solution to 2M urea/TEAB and 4 μL of trypsin (Promega, 20μg reconstituted in 40 μL of the TEAB buffer) and incubated at 37 °C for 16 h.

To the bead-mixture was added 4 μL (per 100 μL of sample) of 4% Heavy or Light formaldhyde (CH_2O, 37 % stock solution, Sigma, and $^{13}CD_2O$, 20 % stock solution, Sigma) respectively, and followed by addition of 4 μL (per 100 μL of sample) of freshly prepared sodium cyanoborohydride (0.6 M in water) and incubation at room temperature for 1 h. Then 16 μL (per 100 μL of sample) of 1% ammoina (28% stock solution, Sigma) and 8 μL (per 100 μL of sample) of 5% formic acid (Sigma) was added to the bead-mixture, sequentially. Beads were pelleted by

centrifugation at 1400g for 3 min and the supernatant was transferred to a new Eppendorf tube. The equivoluminal solution of stable isotope dimethyl-labeled peptide were mixed to a new Eppendorf tube.

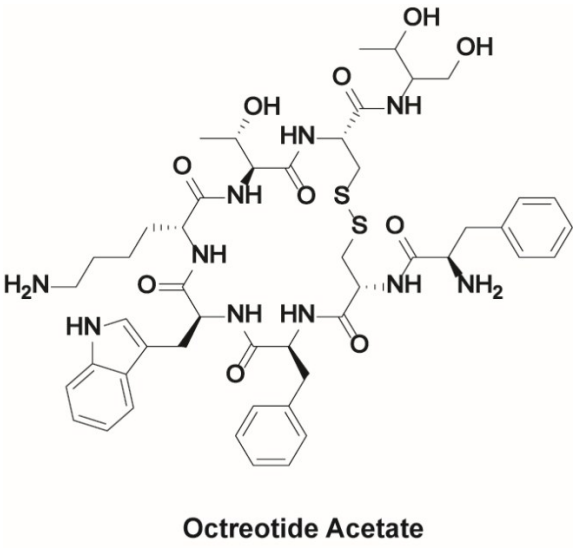
2.7 LC-MS/MS Analysis, Peptide Identification and Quantification. The dimethyl labeled peptide samples were dried under vacuum and resuspended into 100μL of water. Then the samples were centrifuged (100,000g, 10min, 4°C) and fractionated with a fast sequencing workflow by dual reverse phase high performance liquid chromatography (RP-HPLC). The first dimension of high pH RP chromatography was performed on an Agilent 1260 infinity quaternary LC by using a durashell RP column (5μM, 150Å, 250mm * 4.6mm i.d., Agela). Mobile phase A (2% acetonitrile, adjusted pH to 10.0 using NH3•H2O) and B (98% acetonitrile, adjusted pH to 10.0 using NH3•H2O) were used to develop a gradient. The solvent gradient was set as follows:

Time/min	A%	B%
0.10	95.0	5.0
2.00	92.0	8.0
13.00	82.0	18.0
22.00	68.0	32.0
23.00	5.0	95.0
24.00	5.0	95.0
26.00	85.0	15.0
27.00	85.0	15.0

The peptides were separated at an eluent flow rate of 1.5 ml/min and monitored by UV at 214 nm. The temperature of column oven was set as 45°C. Eluents were collected every minute. The samples were dried under vacuum, combined into 10 fractions and reconstituted in 10 μL of 0.1% (v/v) formic acid in water. The second dimension of low pH RP chromatography was performed on an Ultimate 3000 LC system coupled with a Q-Exactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific) for MS/MS analysis. Each fraction from the first dimension was separated on a C18 column. Mobile phase A consisted of 0.1% formic acid in water solution, and mobile phase B consisted of 0.1% formic acid in acetonitrile solution; according to the hydrophobicity of each fraction, an adjusted linear gradient was applied with a flow rate of 350nL/min. The MS conditions are as the followings: Under the positive-ion mode, full-scan mass spectra were acquired over the m/z range from 350 to 1800 using the Orbitrap mass analyzer with a resolution of 70,000. MS/MS fragmentation was performed in the data-dependent mode, in which the 20 most intense ions were selected from each full-scan mass spectrum for fragmentation by high-energy collision induced dissociation (HCD). MS/MS spectra were acquired with a resolution of 17,500 using the Orbitrap analyzer. Some other parameters in the centroid format: isolation window, 2.0 m/z units; default charge, 2+; normalized collision energy, 28%; maximum IT, 50 ms; dynamic exclusion, 20.0 s. The MS/MS spectra were extracted from the raw file using RAW Xtractor into a ms2 format, which were searched using the ProLuCID algorithm using a reverse concatenated, nonredundant variant of the Human UniProt database (release-2012_11).3 Further detailed procedures for LC-MS/MS analysis, peptide identification and quantification

were carried out by following similar procedure as described.[4]

3. Structure of Octreotide Acetate



Octreotide Acetate

Fig. S2 Structure of octreotide acetate. The sequence of octreotide acetate is D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol and structure of it contains one disulfide bond.

4. Extracted MS1 Chromatograms of Biotin-Se-GCSWDYKN and GCSWDYKN

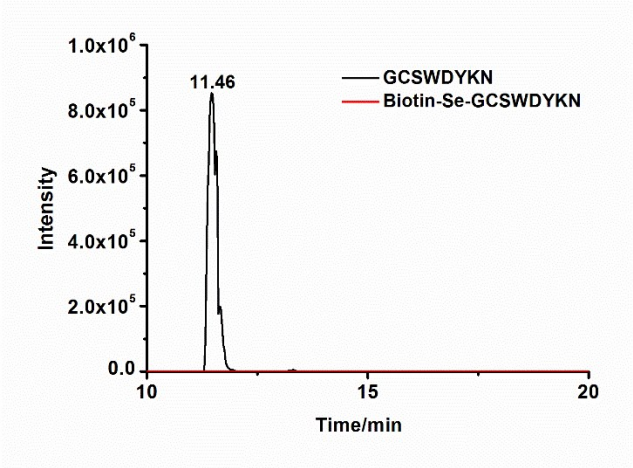


Fig. S3 Extracted MS1 chromatograms of Biotin-Se-GCSWDYKN and GCSWDYKN. It reveals the recovery of GCSWDYKN 10 min after adding DTT (500 μM) to the reaction mixture that contains Biotin-Se-GCSWDYKN.

5. Full Mass-spectra of β-lactoglobulin A

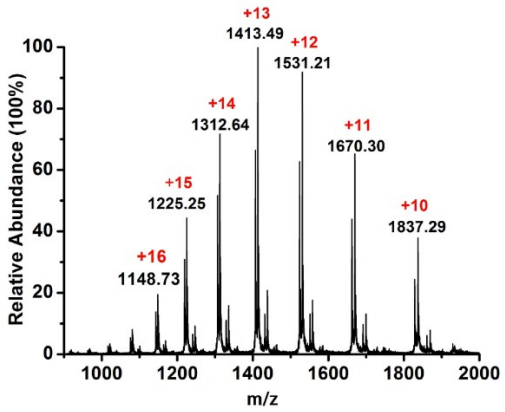


Fig. S4 Full mass-spectra of *β*-lactoglobulin A.

6. Influence of Small Thiol Molecules on Probe-binding with Proteomes

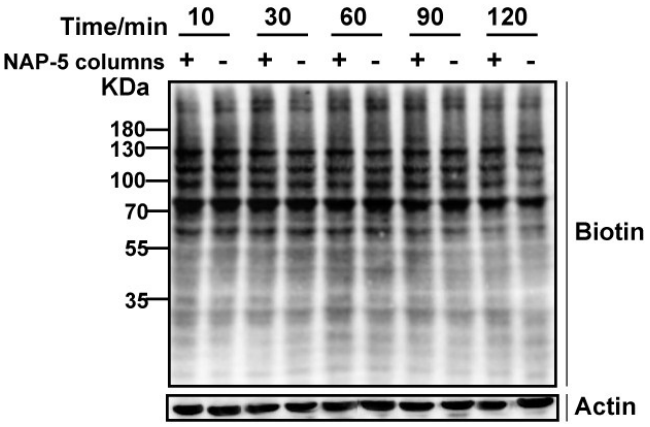


Fig. S5 Western blotting showing the labelling of Biotin-Ebselen in HeLa proteomes with or without small molecule species. HeLa proteomes were treated with Biotin-Ebselen (100 μM) for the indicated lengths of time at 25 °C.

7. Venn Diagram of Identified Ebselen-binding Proteins

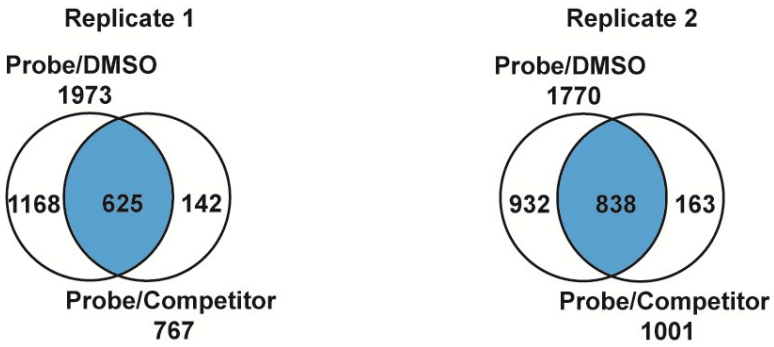


Fig. S6 Analysis of potential ebselen-binding proteins identified in the quantitative MS-based profiling experiments. Venn diagram showing the overlap of identified ebselen-binding proteins between Probe *vs* DMSO and Probe *vs* Competitor experiments in two biological replicates, respectively.

8. Verification of the Enrichment of Selected Known and New Proteins and the Competition by Ebselen

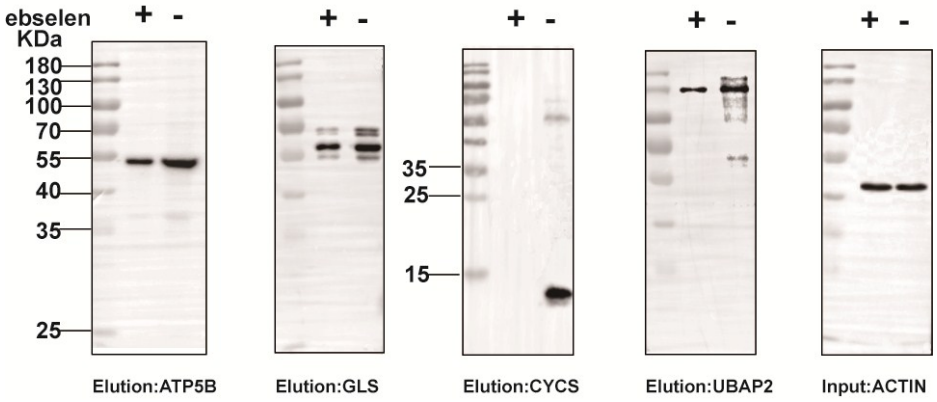


Fig. S7 Western blots confirming the enrichment of selected known and new proteins, ATP5B, GLS, CYCS, UBAP2, and their competition by ebselen.

9. Table of Identified Biotin-Ebselen Labeling Proteins in HeLa Proteomes

Table S1 List of identified Biotin-Ebselen labeling proteins from HeLa proteomes in two biological replicate experiments

Table S2 List of identified ebselen-bonding proteins from HeLa proteomes

Table S3 List of bioinformatic analysis of potential Ebselen-binding proteins based on their molecular functions

10. Reference

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