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Target Discovery of Ebselen with a Biotinylated Probe†

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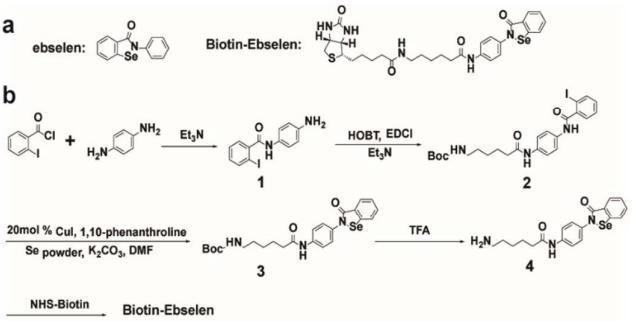
Despite numerous studies on ebselen over the past decade, its cellular targets remain obscure. Here we synthesized a biotinylated ebselen probe (Biotin-Ebselen) and characterized ebselen-binding proteins via an efficient activity-based protein profiling method (ABPP), which allowed for the robust identification of 462 targeted proteins in Hela cells. This first work of global target profiling of ebselen will be helpful to re-design the ebselen-based therapy appropriately in clinical trials.

Ebselen, an interesting organoselenium compound with immunomodulatory effects, is currently used for treatment of various disorders such as cardiovascular diseases, arthritis, and cancer.[1-3] However, ebselen also modulates several biological processes and imparts a lot of toxic effects on cells, for instance, alteration in histone modifications, depression of ribonucleotide reductases and apoptotic cell death.[4-6] Although our traditional recognition of ebselen is mainly based on its glutathione peroxidase activity (GPx) mimic,[7] ebselen is also a potent modulator for proteins that require cysteine for normal function. As a result, there still exists an urgent necessity in deciphering the cellular mechanism of ebselen to fully identify its functions.

Based on the established pathways that reactive selenenyl amide moiety (Se-N) of ebselen reacts with the cysteines to form selenenosulfide (Se-S) bonds, various techniques have been developed to characterize this ebselen-dependent modification.[8,9] The first experiment used radiolabeled ebselen (^{14}C) to show that it was bound by Se-S bonds to reactive thiols in serum albumin.[10] This was followed by demonstration of covalent linkage of the drug with mammalian 15-lipoxygenases in vitro.[11] In another research, ebselen was identified as a potent inhibitor of the Mycobacterium tuberculosis Ag85 complex, and mass spectrometry data show that ebselen binds covalently to a cysteine residue (C209) located near the active site.[12] Lately, proteomic analysis demonstrated that the ebselen derivative could crosslink with proteins through a specific cysteine at the active site of glutamate dehydrogenase (GDH) and thioredoxin reductase (TrxR).[13] A recent exciting work established that ebselen forms a covalent bond with SOD1 at Cys111 and facilitates effective SOD1 maturation.[14] More recently, it is proved that ebselen-based inhibitor could bind to metallo-b-lactamase-1 (NDM-1) by forming a S–Se bond with Cys221 through MALDI-TOF analysis.[15]

We also used mass spectrometry to discover intact protein β-lactoglobulin A derivatization by ebselen, and one or three selenium tag was observed without TCEP or with TCEP pretreatment.[16] Since up to now, only 20 proteins were reported to be targeted covalently and modified upon ebselen treatment and a systemic target profiling of ebselen have not been fully characterized, herein, we employed an activity-based protein profiling (ABPP) method for ebselen-targeted proteins, based on a biotinylated ebselen probe (Biotin-Ebselen, Scheme 1).



Scheme 1 (a) Structures of the ebselen and Biotin-Ebselen. (b) Route for chemical synthesis of Biotin-Ebselen.

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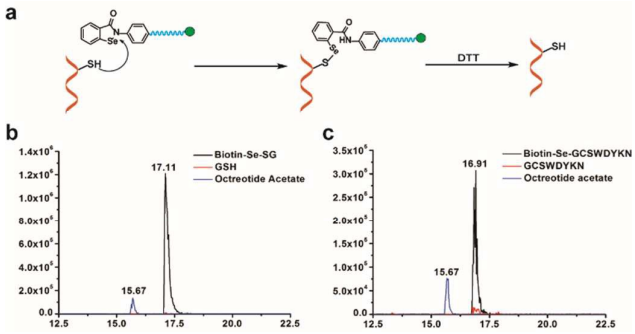

Fig. 1 Reaction between Biotin-Ebselen (50 μM) and GSH or GCSWDYKN (20 μM) were monitored by LC-MS and their major products were detected respectively. (a) Reaction of Biotin-Ebselen with peptides, and DTT reduction. (b) Extracted MS1 chromatograms of Biotin-Se-SG and GSH. (c) Extracted MS1 chromatograms of Biotin-Se-GCSWDYKN and GCSWDYKN. Internal standard: octreotide acetate.

Biotin-Ebselen was designed to mimic ebselen as closely as possible, and the installed biotin was positioned to avoid interference with its normal function. As illustrated in Scheme 1, amidation and Cu(I) catalysis procedures were utilized as previously described to synthesize and obtain Biotin-Ebselen.[17] NMR and MS data confirmed the structure of desired probe (ESI, Fig. S1†).

To test that the character of Se-N bond in Biotin-Ebselen we synthesized is well retained as that in ebselen, we monitored the reaction between the probe and cysteine-containing peptides, glutathione (GSH) and GCSWDYKN by LC-MS, and plotted MS1 chromatograms of their products over time, respectively. As shown in Fig. 1, both two peptides were completely consumed by excessive probe, and the peak of Biotin-Se-SG (m/z=937.25), labeling product of GSH, appeared with a retention time of 17.11 min (Fig. 1a), while Biotin-Se-GCSWDYKN (m/z=1601.54), labeling product of GCSWDYKN (m/z=972.38) appeared with a retention time of 16.91 min (Fig. 1b). Octreotide acetate (m/z=1019.45) was utilized as an internal standard (Fig. 1 and Fig. S2†), which contains a disulfide bond and remained the same after addition of the probe, proving that disulfide does not interfere with the reaction between the probe and the two peptides. The reversibility of the thiol derivatization reaction was further investigated using dithiothreitol (DTT) as a reductant. The MS1 chromatogram revealed the recovery of GCSWDYKN (Fig. S3†) 10 min after adding DTT (500 μM) to the reaction mixture that contains Biotin-Se-GCSWDYKN, and this phenomenon was identical with the previous study.[16] The same result was also obtained with Biotin-Se-SG (data not shown).

To go a step further, Biotin-Ebselen was used for identification of free cysteine residues in proteins. β-lactoglobulin A, a representative thiol protein which contains

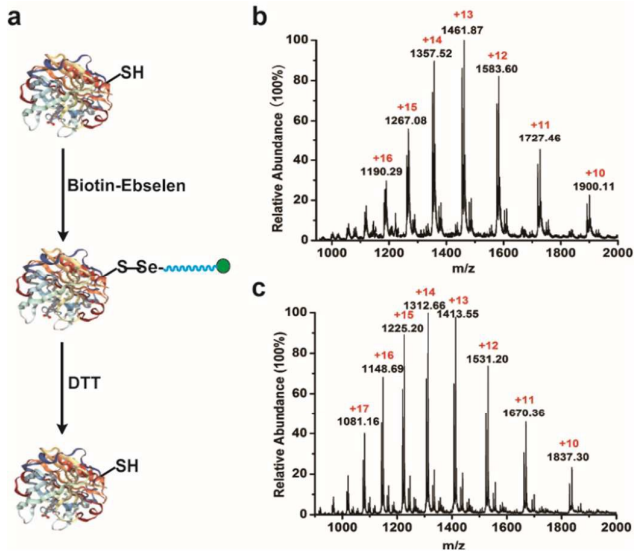

Fig. 2 Chemoselective labeling of cysteines on purified proteins. (a) Scheme of purified proteins induced with the probe to generate probe-labeled proteins, and released through reduction of DTT to the primal proteins. (b) Full mass-spectra showing the β-lactoglobulin A (50μM) labeled by Biotin-Ebselen (100μM, charge numbers are marked). (c) Full mass-spectra showing the released β-lactoglobulin A 30 min after mixing the labeled proteins with DTT (1 mM).

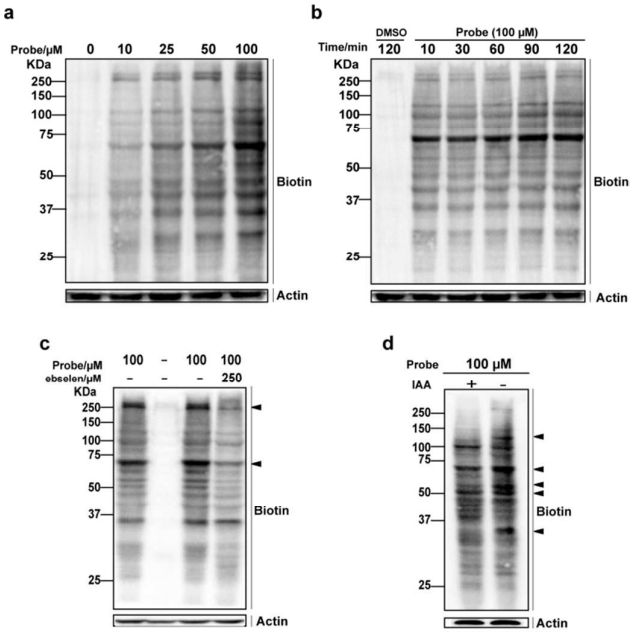

Fig. 3 Characterization of Biotin-Ebselen in HeLa proteomes. (a) HeLa cell lysates were treated with indicated concentrations of Biotin-Ebselen for 1 h at 25 °C and visualized by western blotting. (b) HeLa cell lysates were treated with Biotin-Ebselen (100 μM) for indicated lengths of time and visualized by western blotting. (c) Western blotting showing the labeling of probe and competition by 2.5-fold ebselen in HeLa proteomes. Two significant competition bands are indicated by black arrows. (d) Western blotting showing the labeling of probe and competition by 20 mM IAA in HeLa proteomes. Five significant competition bands are indicated by black arrows.

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only one free thiol group at Cys121 and two disulfide bridges at Cys66-Cys160 and Cys106-Cys119, was utilized as a substrate to react with the probe and for subsequent reduction (Fig. 2a and Fig. S4†). Fig. 2b shows the ESI-MS spectrum of the labeled β-lactoglobulin A by Biotin-Ebselen, while Fig. 2c demonstrates the reduction of the labeled protein by DTT (1 mM), respectively. In the deconvoluted spectra, the mass difference between tagged and reduced protein ions is 629 Da, indicating the addition of a Biotin-Se-tag to the sole free cysteine site of β-lactoglobulin A. The results were in well agreement with those reported previously.[18] Hence, on the basis of the revealed characters of Biotin-Ebselen, it's well established that the character of Se-N bond in the probe remains the same with that in ebselen.

We next attempted to characterize Biotin-Ebselen labeling in HeLa proteomes using cell lysates. Western blotting showed a notable dose-dependent increase of the probe from 10 to 100 μM (Fig. 3a). The probe also demonstrated a fast labeling for targeted proteins in as little as 10 min and a stable signal in 2 h (Fig. 3b). On the other hand, we explored the influence of small thiol molecules on probe-binding with proteomes and NAP-5 columns were utilized to remove small molecule species. Western blotting demonstrated that there was no significant difference whether NAP-5 columns is used or not (Fig. S5†), which excluded the interference of small thiol molecules. Next, to test whether Biotin-Ebselen treatment resulted in the labeling of the same proteins that are bound by ebselen, HeLa cell lysates were treated with the probe (100 μM) in the presence of a 2.5-fold concentration of ebselen (250 μM) for 2 h before analysis by western blotting. Co-treatment with ebselen resulted in an approximately 60% reduction in labeling signal (Fig. 3c), suggesting that Biotin-Ebselen largely modifies the same proteins as ebselen does. In addition, iodoacetamide (IAA), a common alkylation reagent, whose chemistry is more selective toward cysteine, was also investigated as a competition reagent for labeling.[19] As shown in Fig. 3d, western blotting of probe labeling and competition by 20 mM IAA in HeLa proteomes resulted in appearance of significant competition bands, which further proved that the probe can target proteins containing free cysteine residues.

We next set out to apply the probe to characterize ebselen-binding proteins in complex cellular proteomes using a quantitative, MS-based chemical proteomic platform, activity-based protein profiling (ABPP, Fig. 4a and 4b).[20] To obtain potential ebselen-binding proteins with high confidence, two biological replicates of the probe vs control (DMSO), and the probe vs competitor (ebselen) treated HeLa proteomes were included to overcome biological and experimental variations. As shown in Fig. 4a, Hela cell lysates treated with the probe (100 μM) or DMSO for 1 h were enriched by streptavidin beads, then DTT was added to release the targeted proteins with subsequent IAA addition to block all free thiols (reduced from Se-S bond or S-S bond). Afterwards the targeted proteomes were proteolytically digested by trypsin. The tryptic

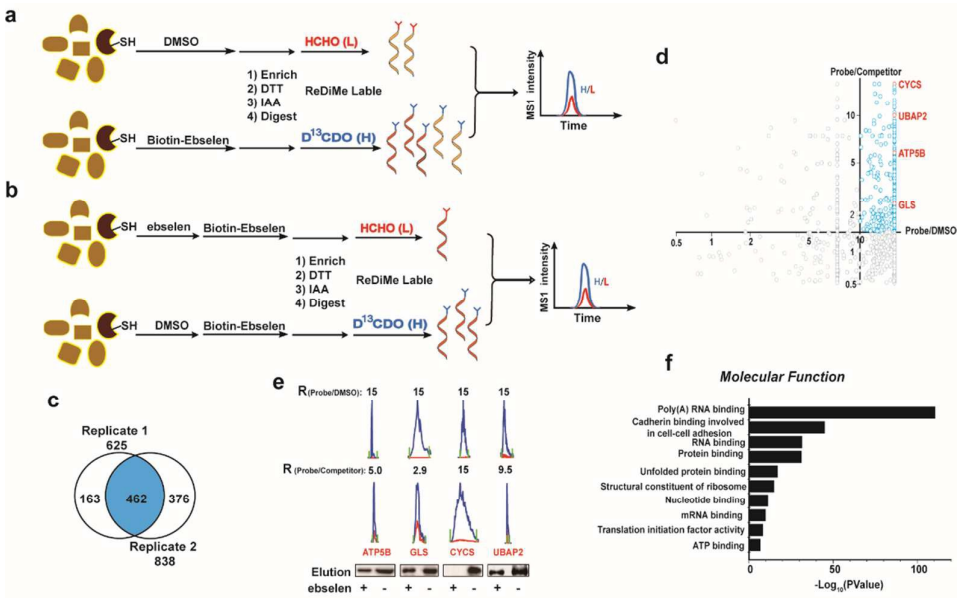

Fig. 4 Quantitative MS-based profiling of ebselen-binding proteins in HeLa proteomes with Biotin-Ebselen. (a) Scheme for MS-based profiling of ebselen-binding proteins that includes probe *vs* control (DMSO). (b) Scheme for MS-based profiling of ebselen-binding proteins that includes probe *vs* competitor (ebselen). The potential ebselen-binding proteins are quantified with stable isotope dimethyl labeling method. (c) Venn diagram showing the overlap of candidate ebselen-binding proteins quantified in both Probe *vs* DMSO and Probe *vs* Competitor experiments across two biological replicates. (d) Mean ratio plot for all proteins identified across two biological replicates. The *x*-axis is the ratio quantified in Probe *vs* DMSO experiment. The *y*-axis is the ratio quantified in Probe *vs* Competitor experiment. The proteins colored in cyan ($R_{Probe/DMSO}$ ≥10 and $R_{Probe/Competitor}$ ≥1.5) are candidate ebselen-binding proteins. Four representative proteins are colored in red. (e) Extracted MS1 chromatograms of representative tryptic peptides with enrichment ratios shown and western blotting verification. (f) Bioinformatic analysis of ebselen-binding proteins based on their molecular functions.

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peptides were then isotopically differentiated by reductive dimethylation (ReDiMe), combined pairwise and fractionated by high-pH HPLC for LC-MS/MS analysis. Meanwhile, as shown in Fig. 4b, further competitor-confirmation procedures were also performed, and HeLa cell lysates were treated with ebselen (250 μM) or DMSO before Biotin-Ebselen labeling. These experiments enable us to discriminate specific protein targets from endogenously biotinylated and non-specific proteins, hence increasing the confidence of identifying specific targets while minimizing experimental errors.

Two groups of the proteomic results across two biological replicates were obtained and summarized (ESI, Fig. S6† and Table S1†). We regarded the targets that satisfy the quantitative criteria ($R_{Probe/DMSO \geq 10}$ and $R_{Probe/Competitor} \geq 1.5$) are candidate ebselen-binding proteins, and there are 625 and 838 candidate ebselen-binding proteins in two biological replicates respectively. Ultimately, we concluded that there are 462 candidate ebselen-binding proteins in common with these two biological replicates (Fig. 4c, 4d and Table S2†). We elected four candidate proteins, ATP5B, GLS, CYCS and UBAP2 to show their distribution on mean ratio plot and extracted MS1 chromatograms of representative tryptic peptides respectively (Fig. 4d and 4e). In these four candidate proteins, there are known ebselen-binding proteins (ATP5B, GLS)[2] and newly identified ebselen-binding proteins (CYCS, UBAP2). What's more, the enrichment of these four candidate proteins by affinity purification and immunoblotting (Fig. 4e and Fig. S7†) verified the results are consistent with those obtained by mass spectrometry, especially for CYCS, with a $R_{Probe/Competitor}$ =15 and an obvious competition by ebselen on the western blot.

Gene ontology analysis by DAVID (Database for Annotation, Visualization and Integrated Discovery) revealed that the ebselen-binding protein targets were enriched in important biological processes such as poly(A) RNA binding (Cytoskeleton- associated protein 4; Protein diaphanous homolog 1), cadherin binding involved in cell-cell adhesion (Ras GTPase-activating-like protein; Ras-related protein Rab-1A) as well as RNA binding (Splicing factor 3A subunit 3; Glutamine--tRNA ligase) (Fig. 4f and Table S3†). Besides, several of the core histone proteins were also in our list of identified proteins, including H2A and H2B, which indicated the potential importance of ebselen in transcriptional regulation.

In summary, we employed an efficient activity-based protein profiling (ABPP) method for ebselen-targeted proteins, based on a biotinylated ebselen probe Biotin-Ebselen. This first work of global profiling of target proteins of ebselen allowed the potential identification of ebselen-binding proteins in different cell types besides HeLa cells, which is crucial for a complete understanding of the role of ebselen in various biological circumstances and is also helpful for re-design ebselen-related therapy.

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Conflicts of interest

There are no conflicts to declare.

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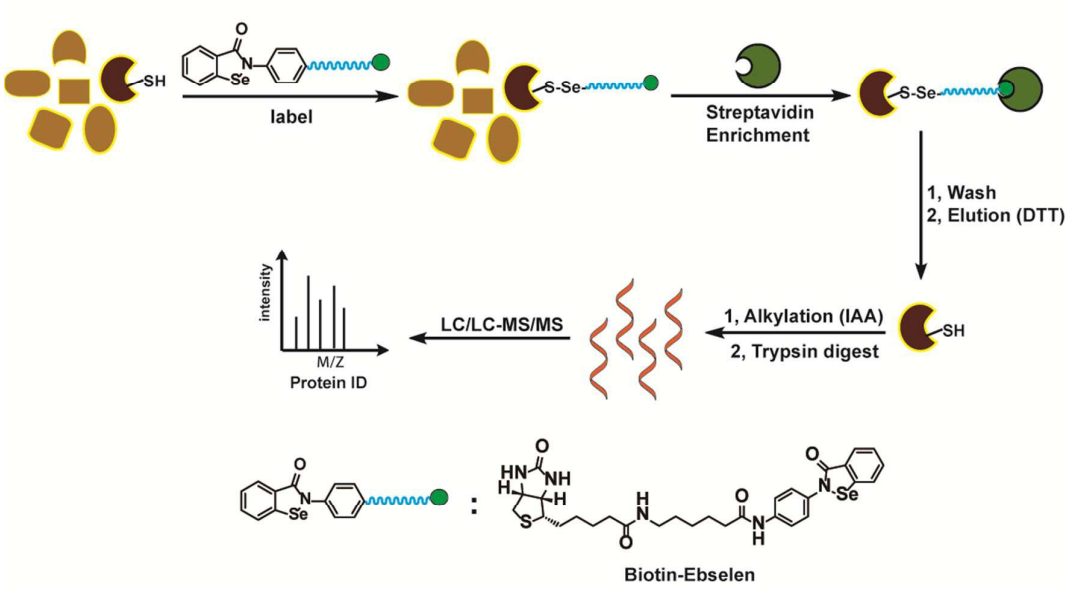

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