Ebselen inhibits mycobacterial ESX-1 secretion through modification of EccA1

Morwan M. Osman[a,e], Malte L. Pinckert[a,c,e], Sew Peak-Chew[b], Mark A. Troll[a], William H. Conrad[a,d,*], Lalita Ramakrishnan[a*]

[a]Molecular Immunity Unit, Department of Medicine, University of Cambridge, MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK.

[b]MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge CB2 0QH, UK

[c]Present address: Division of Virology, Department of Pathology, Addenbrooke's Hospital, University of Cambridge, Cambridge, CB2 0QQ, UK

[d]Present address: Department of Chemistry, Lake Forest College, Lake Forest, Illinois 60045, USA

[e]M.M.O. and M.L.P. Contributed equally to this work.

[*]Correspondence: Lalita Ramakrishnan, lr404@cam.ac.uk; William Conrad, conrad@lfc.edu

Author contributions: M.M.O., W.H.C., and L.R. designed the research; M.M.O., M.L.P., S.P.C., and W.H.C. performed research; M.M.O., M.L.P., S.P.C., M.A.T., and W.H.C. analyzed data; M.M.O., M.L.P., and L.R. wrote the paper.

Abstract

The type VII secretion system ESX-1 (ESAT-6 secretion system 1) is required for virulence in *Mycobacterium tuberculosis* and *M. marinum*. Here, we identify the clinical drug candidate ebselen as an inhibitor of ESX-1 secretion in both organisms. We find that ebselen inhibits the activity of the ESX-1 AAA+ ATPase EccA1, which is required for full ESX-1 secretion and virulence. EccA1 ATPase activity is inhibited through covalent modification of an N-terminal cysteine residue that is outside its ATPase domain. Our findings suggest the potential of ebselen as an anti-virulence drug complement to standard antituberculous therapy regimens, particularly for drug resistant infection. Our findings also provide new insight into the function of the EccA1 protein domains in its ATPase activity and ESX-1 secretion.

Introduction

Mycobacterium tuberculosis and its close genetic relative *Mycobacterium marinum* require their type VII secretion system ESX-1 (ESAT-6 Secretion System 1) for virulence(1–3). ESX-1 was first identified as a virulence determinant when it was shown that a 9.4 kb deletion (ΔRD1) in its locus was the primary cause of attenuation for the live attenuated vaccine BCG (1, 3–6). ESX-1 is required for multiple stages of infection in both *M. tuberculosis* and *M. marinum*, with mutants attenuated in intramacrophage growth (2, 7), phagosomal permeabilization (8, 9), and granuloma formation (2, 10).

In vitro, ESX-1 mediates membrane disruption of infected lung epithelial cells (11), cultured macrophages (11), and red blood cells (RBCs) (12–14). Previously, ESX-1 membranolytic activity had been ascribed to its secreted substrate ESAT-6 forming pores in host membranes(11, 13, 15). In 2017, we found that the pore-forming activity ascribed to ESAT-6 was due to residual detergent contamination of ESAT-6 preparations(16). Moreover, we found that ESX-1 membrane disruption was exclusively contact-dependent and caused gross membrane disruptions as opposed to distinct pores (16).

In this paper, we report studies that began with our speculation that ESX-1 might mediate membrane disruptions through peroxidation of host membrane lipids. To test this hypothesis, we screened antioxidants for their ability to inhibit *M. marinum's* contact-dependent hemolytic activity and identified the clinical compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) as an inhibitor of ESX-1 mediated hemolysis. However, we found that ebselen inhibited hemolysis independent of its antioxidant activity, through inhibition of ESX-1-dependent secretion. We showed its target to be the ESX-1 AAA+ (ATPases Associated with various cellular Activities) ATPase EccA1, the activity of which it inhibited through covalent modification of a critical cysteine residue.

Results

Ebselen inhibits ESX-1 secretion in *M. marinum* and *M. tuberculosis*

To test the hypothesis that ESX-1-dependent membrane disruptions could occur through peroxidation of host lipids, we screened antioxidants for their ability to inhibit *M. marinum's* contact-dependent hemolytic activity. Of the three tested, only ebselen inhibited *M. marinum's* hemolytic activity with a half maximal inhibitory concentration (IC_{50}) of 9.3 μM (Fig. 1A). These findings indicated that ebselen's effects on RBC hemolysis was independent of its antioxidant effect, suggesting that lipid peroxidation was not the mechanism behind ESX-1 membrane disruption.

We pursued how ebselen might inhibit hemolysis independent of its antioxidant effect. As ebselen has been reported to have antimicrobial activities against *M. tuberculosis (17),* we set to determine if bacterial killing was responsible for ebselen's effect on *M. marinum's* hemolytic activity. We plated *M. marinum* that had been exposed to ebselen for the duration of the hemolysis assay and found that even the highest concentration used, 62.5 μM, did not affect viability (Fig. 1A and 1B). Ebselen's drug-like activities are mediated through its selenium moiety, which enables it to covalently modify free thiols, such as free cysteine residues (18, 19). Consistent with this, we found that an ebselen analog lacking the selenium moiety, 2-(2,6-dimethylphenyl)-1-isoindolinone (DIME) failed to inhibit hemolysis (Fig. 1C and D). Because ebselen inhibited ESX-1-dependent hemolysis (14, 16), we asked if its primary action was on ESX-1 secretion. ESX-1 substrates are secreted in a mutually dependent manner (20, 21), so we measured the secretion of two substrates, ESAT-6 and CFP-10, as a proxy for overall ESX-1 secretion. Ebselen treatment inhibited both ESAT-6 and CFP-10 secretion, while DIME had no effect (Fig. 1E).

We next asked if ebselen's ESX-1 inhibitory effect extended to *M. tuberculosis*. *M. marinum* and *M. tuberculosis* ESX-1 are functionally equivalent, with complementation of the *M.*

tuberculosis ESX-1 into an ESX-1-deficient *M. marinum* RD1 deletion (ΔRD1) restoring hemolysis, secretion, and virulence (16). Ebselen exhibited similar dose-dependent inhibition of sRBC hemolysis by this ESX-1 complemented strain (ΔRD1::ESX-1_{Mtb}) and wildtype *M. marinum* (Fig. 1F). To determine if ebselen also inhibits ESX-1 secretion in *M. tuberculosis*, we used *M. tuberculosis* $mc^2$6206, which is auxotrophic for leucine and pantothenic acid but retains an intact ESX-1 locus (22). Ebselen treatment also reduced ESAT-6 secretion in *M. tuberculosis* (Fig 1G). Altogether, these results show that ebselen inhibits ESX-1 secretion in *M. marinum* and *M. tuberculosis* and suggest that the inhibition is through cysteine modification.

Ebselen inhibits *M. tuberculosis* EccA1 ATPase activity and covalently modifies its cysteine residues

Ebselen has been shown to inhibit the activity of prokaryotic and eukaryotic enzymes, including the yeast plasma membrane H+-ATPase (23). These inhibitory functions occur through ebselen's reactivity with free thiols, including free cysteine residues (19). ESX-1-mediated secretion is dependent on two ATPases within the locus: the FtsK/SpoIIIE ATPase EccC1, encoded by the adjacent genes EccCa1 and EccCb1, which powers the transport of ESX-1 substrates(7, 24, 25), and the AAA+ ATPase EccA1, which is thought to act as a chaperone for ESX-1 substrates (20, 24, 26). Both enzymes contain multiple free cysteines, making them potential targets for ebselen. The ATPase activity of purified *M. tuberculosis* EccA1 protein has been demonstrated (27) whereas there are no published data on the activity of purified *M. tuberculosis* EccC1. We attempted to purify both EccA1 and EccC1 (as an EccCa1/Cb1 fusion) and only succeeded in purifying EccA1. We found that ebselen inhibited the ATPase activity of purified EccA1 in a dose-dependent manner, starting at 1μM and plateauing at 8μM with an IC_{50} of 2.5 μM (Fig. 2A).

To determine if ebselen's inhibition of EccA1 was due to covalent cysteine modification, mass spectrometry analyses were performed on the ebselen-treated aliquots in Fig. 2A. Analysis of tryptic peptides from these samples revealed ebselen modification of cysteines 204 and 531. Representative MS/MS spectra of these peptides from the 8 μM ebselen treatment are depicted for Cys204 and Cys531 (Fig. 2B and 2C). We found that these modifications occurred in a two-step manner: at ebselen concentrations up to 4 μM we observed only Cys204 ebselen-modified peptides, and at higher concentrations we observed ebselen-modified peptides for both Cys204 and Cys531 (Fig 2D). Based on this result, we hypothesized that ebselen's inhibition of EccA1 was a two-step process, where ebselen modification of Cys204 and Cys531 each contributed to inhibition of ATPase activity. If so, the inhibition curve would incorporate two curves, reflecting the contribution of each modification. We fit two logistic functions to the curve: a two curve model corresponding to a two-step inhibition (full model) or a single-curve model corresponding to a single step inhibition (reduced model) (Fig S1), and we found that the two-curve model was a better fit (p=0.021, nested model F-test), supporting the hypothesis that ebselen inhibition of EccA1 was a two-step process.

EccA1 Cys204 is located in a hydrophobic pocket essential for ATPase activity

In addition to the ATPase domain that mediates EccA1's assembly and enzymatic activity(27), EccA1 has a large N-terminal domain that is comprised of six tetratricopeptide repeat (TPR) motifs and a β-finger insert (28), and this domain is dispensable for its *in vitro* ATPase activity(27). As TPR motifs mediate protein-protein interactions(29), this domain is predicted to mediate the EccA1 chaperone's substrate recognition (28). Of the two residues modified by ebselen, Cys531 is located in the C-terminal ATPase domain whereas Cys204 is within N-terminal TPR domain (Fig. 3A). The modification of Cys531 provides an obvious explanation for ebselen's inhibitory effect. However, we found that Ebselen's binding to Cys204 was also linked to inhibition of activity, suggesting that conservation of the TPR domain is

required for full-length EccA1's ATPase activity. The two-step inhibition curve along with the MS analysis of ebselen-treated EccA1 indicated that ebselen modification of Cys204 preceded that of Cys531. Moreover, sequence alignment revealed that of the two cysteine residues, only Cys204 is conserved across mycobacterial EccA1 homologs (Fig. 3B), and while *M. marinum* EccA1 lacks Cys531, yet ESX-1 secretion was inhibited by ebselen. These results suggested that Ebselen's binding to EccA1 Cys204 mediates in substantial measure its inhibition of ESX-1 secretion.

Examination of the crystal structure of EccA1's TPR domain (PDB: 4F3V) revealed that Cys204 is located in the TPR domain's hydrophobic core (Fig. 3C) (28). To confirm Cys204's role in ebselen's activity, we engineered an EccA1 where the Cys204 had been changed to a valine (EccA1 C204V) or to the more hydrophilic residue serine (EccA1 C204S). We saw that EccA1 C204V had similar ATPase activity to the wildtype enzyme (Fig. 3D), while EccA1 C204S was showed only 14% of wildtype EccA1 (Fig. 3F). These findings suggested that while Cys204 is dispensable for enzymatic activity, conservation of its hydrophobic environment is essential for EccA1's ATPase function.

Ebselen allosterically inhibits EccA1 through modification of Cys204

If ebselen's ATPase inhibitory activity was primarily due to modification of Cys204, then EccA1 C204V should not be inhibited by ebselen treatment. When we treated both wildtype and EccA1 C204V with ebselen, we found modification of EccA1 Cys204 was essential for ebselen inhibition: when treated with 8 μM ebselen, EccA1 C204V retained 95% of its ATPase activity vs ~60% for WT EccA1 (Fig. 3F). We expected to see an intermediate level of inhibition, as we identified Cys531 modified peptides in wildtype EccA1 treated with 8 μM ebselen. Instead, EccA1 C204V's insensitivity indicates that Cys204 modification must precede Cys531 modification, consistent with the two-step binding we saw, or that Cys531 modification does not

contribute substantially to ebselen's inhibitory effect. Together, these results show EccA1 Cys204 is essential for ebselen's allosteric inhibition of EccA1 ATPase activity.

Discussion

In searching for the mechanism of mycobacterial ESX-1-mediated membrane disruption, we serendipitously identified ebselen as an allosteric inhibitor of ESX-1 secretion. Our findings suggest that ebselen's effects on ESX-1 secretion are mediated in substantial measure by inhibiting EccA1's ATPase activity. The nature of the allosteric mechanism through which ebselen inhibits EccA1's ATPase activity remains to be determined, but our observation of sequential Cys204 and Cys531 modification by ebselen provides a hint. Ebselen modification of Cys204 may induce conformational flexibility in the ATPase domain allowing Cys531 to become accessible. This additional flexibility could influence multimer assembly or coordination of the ATP hydrolysis cycle mediated by ATPase domains of AAA+ ATPases (27, 30), resulting in decreased ATPase activity.

Our findings raise the question as to why a moderate reduction in EccA1's *in vitro* ATPase activity by ebselen (~60% activity at 8 μM ebselen) results in a drastic reduction of ESX-1 secretion. EccA1 is a member of the CbxX/CfxQ family of multimeric chaperones, which transform ATP hydrolysis into remodeling of their bound substrates (31). Cys204's hydrophobic groove directly mediates protein-protein interactions in TPR proteins, and may mediate EccA1's recognition of its substrates required for its chaperone role(28, 29). Thus, modification of Cys204 may inhibit EccA1's substrate binding and ATPase activities. This dual effect on EccA1's *in vivo* functions would explain why what we observe as a partial reduction of EccA1's *in vitro* ATPase activity may result in a drastic reduction of its *in vivo* ESX-1 secretory function.

Three other inhibitors of *M. tuberculosis* ESX-1 secretion have been identified: Ethoxzolamide (ETZ), an FDA- approved drug, inhibits ESX-1 secretion in *M. tuberculosis* via suppression of PhoPR signaling (32), BTP15, which inhibits the MprB histidine kinase, and

BBH7 which affects metal ion stasis (33). We demonstrate here that ebselen inhibits secretion of ESX-1 substrates, but there remains the possibility that ebselen could have a broader effect on mycobacterial secretion.

Ebselen is a promiscuous compound, inhibiting human enzymes including inositol monophosphatase (34) and indoleamine 2,3-dioxygenase (35). Ebselen has been demonstrated to show antimicrobial activity in *Helicobacter pylori* and *M. tuberculosis* (36). The antimicrobial effects on *M. tuberculosis* have been attributed to covalent modification of the essential Antigen 85 complex (Ag85C) by ebselen(37). Ag85C synthesizes the lipid trehalose dimycolate from trehalose monomycolate, and ebselen treatment abrogates this activity (37). However, ebselen's antimicrobial and lipid synthesis activities are not observed at concentrations below 73 µM *M. tuberculosis* (37), while we observe inhibition of *M. tuberculosis* ESX-1 secretion at 8 µM. Thus, ebselen's effect on ESX-1 secretion is separate from its effects on the Antigen 85 complex.

Ebselen has been demonstrated to be clinically safe and well tolerated in humans (38), suggesting its use an anti-virulence drug complement to standard anti-tuberculous chemotherapy. It is currently undergoing clinical trials for preventing aminoglycoside-induced ototoxicity (NCT: 02819856). Aminoglycosides remain essential components of MDR-TB treatment, but cause difficult to predict ototoxicity leading to hearing loss (39). Ebselen's potential otoprotective and anti-virulence properties may combine to make it particularly attractive in aminoglycoside containing tuberculosis treatment regimens.

Materials and Methods

Bacterial strains and methods Strains used are listed in Supplementary Table 2. All *M. marinum* strains were derived from wild-type *M. marinum* purchased from American Type Culture Collection (strain M, ATCC #BAA-535). Wildtype and ΔRD1::ESX-1_{Mtb} strains were generated and maintained as described previously(16). *M. tuberculosis* $mc^2$6206 was cultured in 7H9 complete media containing 0.05% tween 80 supplemented with pantothenic acid (12 µg/mL) and leucine (25 µg /mL).

Hemolysis assay Hemolytic activity was assessed as described previously(16). Briefly, 100 μL of sheep red blood cells (sRBCs) were transferred to each condition and 100 μL of PBS or bacterial suspension were added on top and incubated for two hours at 33°C. 100% lysis with 0.1% Tx100 (Sigma). Drugs stocks were made at 200x concentrations and 0.5% DMSO was used as a vehicle control.

Secretion Assays Secretion assays were conducted as described previously with minor modifications (16). Briefly, *M. tuberculosis* $mc^2$6206 and *M. marinum* were grown to mid to late log stage and washed with PBS before being resuspended to a final OD_{600} of 0.8. *M. tuberculosis* $mc^2$6206 was resuspended in 50 mL 7H9 media supplemented with 10% DC (2% dextrose, 145 mM NaCl, 30 µg/mL bovine catalase), pantothenic acid, (12 µg/mL) and leucine (25 µg /mL). Cultures were incubated for 4 days at 37°C in the presence of drug or 0.5% DMSO. *M. marinum* was resuspended in 50 mL Sauton's Media and incubated for 4 hours at 33°C in the presence of drug or 0.5% DMSO. Culture filtrate (CF) and cell pellet (CP) fractions were prepared as described previously (16). 10 µg of CP and 30 µg of CF were loaded per well for SDS-PAGE, and presence of ESAT-6, CFP-10 and GroEL2 were determined by western blotting with mouse anti-ESAT-6 clone 11G4 (1:1,000; Thermo Fisher, HYB-076-08-02), rabbit

anti-CFP-10 (1:500; BEI, product NR13801), or mouse anti-GroEL2 clone IT-56 (1:1,000; BEI, product NR-13655).

Purification of EccA1 Purification constructs were expressed in *E. coli* C43 (DE3) (Lucigen). Single colonies were used to inoculate 100 mL of Lysogeny Broth (LB) media containing 50 *μ*g/mL Kanamycin and incubated overnight (O/N) at 37°C. 1L of Terrific Broth (TB) was inoculated at an OD_{600} of 0.015 and grown at 37°C to an OD_{600} between 0.6-0.8, and cooled to 20°C and induced with 1 mM IPTG O/N. Pelleted cells were resuspended in ice-cold Lysis Buffer (2.5xPBS; 10 mM Imidazole) with SIGMAFAST™ Protease Inhibitor tablets (Sigma). Cells were lysed in three passages through an Avestin Emulsiflex C3 at 15,000 psi. Debris and unbroken cells were removed by centrifuging at 18,000 x g for 20m. ATP and $MgCl_2$ were added to lysate to a final concentration of 5 and 20 mM respectively, and lysate was bound to a HisTrap HP column (GE Life Sciences). Column was washed 3x with 10 Column Volumes (CV) of Wash Buffer [2.5 x PBS; 40 mM Imidazole] and eluted with 12 CV of Elution Buffer [2.5x PBS; 500 mM Imidazole]. Eluted fractions were concentrated to 2 mL with 30 kDa cutoff concentrators (Amicon) and diluted 1:10 in IEX1 buffer [20mM Tris pH 8.0 (RT) ; 15mM NaCl]. ATP and $MgCl_2$ were then added to a final concentration of 1 and 20 mM and then applied to a HiTrap Q column (GE Life Sciences). Separation occurred over an 18 CV gradient from 100% IEX1 to 100% IEX2 buffers [20mM Tris pH 8.0 (RT) ; 500mM NaCl]. Peak fractions were concentrated with 30 kDa cutoff concentrators (Amicon) to ~10 mg/ml and loaded onto a S200 Increase 10/300 GL (GE Life Sciences) in SEC Buffer [20mM Tris, pH 8.0 (RT); 100mM NaCl]. Peak fractions were pooled and concentrated, and aliquots were flash frozen and stored at -70°C.

ATPase Assays ATPase activity of EccA1 and EccA1 point mutants was measured with an ATPase/GTPase kit (Sigma) adapted for 384 well-plates. Experiments were conducted for 180 minutes with a final EccA1 concentration of 1 μM. For ebselen treatments, samples were incubated on ice with drug for 20 min prior to assay. Background activity was determined by conducting reactions in the absence of added ATP.

Mass Spectrometry Protein samples were alkylated with 10 mM iodoacetamide in the dark at 37°C for 30m and subsequently digested with trypsin (Promega, 50 ng) overnight at 37°C. Digested peptide mixtures were then acidified, partially dried down in a SpeedVac (Savant) and desalted using a home-made C18 (3M Empore) stage tip filled with 0.4μl of poros R3 (Applied Biosystems) resin. Bound peptides were eluted with 30-80% acetonitrile in 0.1% Trifluoroacetic acid and partially dried to prepare for LC-MS/MS. Liquid chromatography was performed on a fully automated Ultimate 3000 RSLC nano System (Thermo Scientific) fitted with a 100 μm x 2 cm PepMap100 C18 Nano Trap column and a 75 µm×25 cm reverse phase C18 nano column (Aclaim PepMap, Thermo Scientific). Samples were separated using a binary gradient consisting of buffer A (2% MeCN, 0.1% formic acid) and buffer B (80% MeCN, 0.1% formic acid), with a flow rate of 300 nL/min. The HPLC system was coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific) equipped with a nanospray ion source. The mass spectrometer was operated in standard data dependent mode, performed MS full-scan at 350-1600 m/z range, with a resolution of 70000. This was followed by MS2 acquisitions of the 15 most intense ions with a resolution of 17500 and NCE of 27%. MS target values of 1e6 and MS2 target values of 1e5 were used. Isolation window of precursor was set at 1.5 Da and dynamic exclusion of sequenced peptides was enabled for 30s. The acquired MS/MS raw files were searched using Sequest (Proteome Discoverer v2.1) search engine. MS/MS spectra were searched against the *M. tuberculosis* EccA1 sequence including a contaminant database. A list

of EccA1 peptides were selected from the result for Parallel Reaction Monitoring (PRM) experiment. For PRM, the mass spectrometer performed a MS full-scan at 400-1600 m/z range, with a resolution of 35000. This was followed by one MS2 acquisitions with a resolution of 35000. MS target values of 3e6 and MS2 target values of 5e5 were used. Isolation window of precursor was set at 0.7 Da.

Generation of plasmids and mutants Primers and plasmids are listed in Supplementary Tables 1 and 3. *M. tuberculosis* EccA1 was amplified from the pRD1-2F9 cosmid (Kind gift from Roland Brosch, Institut Pasteur), and cloned into the pH3c-LIC backbone (PSI:Biology-Materials Repository). EccA1 C204S and C204V point mutants were generated via site directed mutagenesis of the resulting pH3c-LIC EccA1 plasmid.

Stepwise inhibition model The models for the stepwise inhibition were based on sums of logistic functions. The single logistic function was the reduced model, nested in the stepwise inhibition model which was the sum of two logistic functions. The general formulas, as well as the final values based on Excel Solver fitting can be seen in Fig. S1. Nested models are required for the F-test to be appropriate. Calculations were performed in Excel (Microsoft).

Acknowledgements

We would like to thank Ramanujan Hegde for advice and discussion, and Paul Edelstein for his comments on the manuscript. The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: monoclonal anti-*Mycobacterium tuberculosis* GroEL2 (gene Rv0440), clone CS-44 (produced *in vitro*), NR-13813. *M. tuberculosis* $mc^2$6206 was a kind gift from William Jacobs.

This work was supported in part by a Wellcome Trust Principal Research Fellowship, the National Institutes of Health (NIH) (Grant R37AI054503), and the National Institute of Health Research Cambridge Biomedical Research Centre (L.R.)

References:

1. A. S. Pym, P. Brodin, R. Brosch, M. Huerre, S. T. Cole, Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. *Molecular Microbiology* **46**, 709–717 (2002).

2. H. E. Volkman, *et al.*, Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *PLoS biology* **2**, e367 (2004).

3. K. N. Lewis, *et al.*, Deletion of RD1 from Mycobacterium tuberculosis Mimics Bacille Calmette-Guérin Attenuation. *The Journal of Infectious Diseases* **187**, 117–123 (2003).

4. M. A. Behr, *et al.*, Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science (New York, N.Y.)* **284**, 1520–3 (1999).

5. G. G. Mahairas, P. J. Sabo, M. J. Hickey, D. C. Singh, C. K. Stover, Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. *Journal of bacteriology* **178**, 1274–82 (1996).

6. K. M. Guinn, *et al.*, Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. *Molecular microbiology* **51**, 359–70 (2004).

7. S. a Stanley, S. Raghavan, W. W. Hwang, J. S. Cox, Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proceedings of the National Academy of Sciences* **100**, 13001–13006 (2003).

8. N. van der Wel, *et al.*, M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* **129**, 1287–98 (2007).

9. R. Simeone, *et al.*, Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS pathogens* **8**, e1002507`` (2012).

10. V. R. Parasa, *et al.*, Modeling Mycobacterium tuberculosis early granuloma formation in experimental human lung tissue. *Disease Models & Mechanisms* **7**, 281–288 (2014).

11. T. Hsu, *et al.*, The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12420–5 (2003).

12. C. H. King, S. Mundayoor, J. T. Crawford, T. M. Shinnick, Expression of contact-dependent cytolytic activity by Mycobacterium tuberculosis and isolation of the genomic locus that encodes the activity. *Infection and Immunity* **61**, 2708–2712 (1993).

13. J. Smith, *et al.*, Evidence for Pore Formation in Host Cell Membranes by ESX-1-Secreted ESAT-6 and Its Role in Mycobacterium marinum Escape from the Vacuole. *Infection and Immunity* **76**, 5478–5487 (2008).

14. L.-Y. Gao, *et al.*, A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Molecular microbiology* **53**, 1677–93 (2004).

15. M. I. de Jonge, *et al.*, ESAT-6 from Mycobacterium tuberculosis Dissociates from Its Putative Chaperone CFP-10 under Acidic Conditions and Exhibits Membrane-Lysing Activity. *J. Bacteriol.* **189**, 6028–6034 (2007).

16. W. H. Conrad, *et al.*, Mycobacterial ESX-1 secretion system mediates host cell lysis through bacterium contact-dependent gross membrane disruptions. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 1371–1376 (2017).

17. J. Lu, *et al.*, Inhibition of bacterial thioredoxin reductase: an antibiotic mechanism targeting bacteria lacking glutathione. *The FASEB Journal* **27**, 1394–1403 (2013).

18. K. Xu, *et al.*, Study of Highly Selective and Efficient Thiol Derivatization Using Selenium Reagents by Mass Spectrometry. *Anal. Chem.* **82**, 6926–6932 (2010).

19. G. K. Azad, R. S. Tomar, Ebselen, a promising antioxidant drug: mechanisms of action and targets of biological pathways. *Molecular biology reports* **41**, 4865–79 (2014).

20. M. M. Champion, E. A. Williams, R. S. Pinapati, P. A. D. Champion, Correlation of Phenotypic Profiles Using Targeted Proteomics Identifies Mycobacterial Esx-1 Substrates. *Journal of proteome research* **13**, 5151–5164 (2014).

21. S. M. Fortune, *et al.*, Mutually dependent secretion of proteins required for mycobacterial virulence. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 10676–81 (2005).

22. S. L. Sampson, *et al.*, Protection elicited by a double leucine and pantothenate auxotroph of Mycobacterium tuberculosis in guinea pigs. *Infection and immunity* **72**, 3031–7 (2004).

23. G. Chan, D. Hardej, M. Santoro, C. Lau-Cam, B. Billack, Evaluation of the antimicrobial activity of ebselen: Role of the yeast plasma membrane H+-ATPase. *Journal of Biochemical and Molecular Toxicology* **21**, 252–264 (2007).

24. W. Bitter, *et al.*, Systematic genetic nomenclature for type VII secretion systems. *PLoS pathogens* **5**, e1000507 (2009).

25. O. S. S. Rosenberg, *et al.*, Substrates Control Multimerization and Activation of the Multi-Domain ATPase Motor of Type VII Secretion. *Cell*, 1–12 (2015).

26. P. Brodin, *et al.*, Dissection of ESAT-6 System 1 of Mycobacterium tuberculosis and Impact on Immunogenicity and Virulence. *Infection and Immunity* **74**, 88–98 (2006).

27. A. Luthra, A. Mahmood, A. Arora, R. Ramachandran, Characterization of Rv3868, an Essential Hypothetical Protein of the ESX-1 Secretion System in Mycobacterium tuberculosis. *J. Biol. Chem.* **283**, 36532–36541 (2008).

28. J. M. Wagner, T. J. Evans, K. V. Korotkov, Crystal structure of the N-terminal domain of EccA1 ATPase from the ESX-1 secretion system of Mycobacterium tuberculosis. *Proteins: Structure, Function and Bioinformatics* **82**, 159–163 (2014).

29. L. D. D'Andrea, L. Regan, TPR proteins: the versatile helix. *Trends in Biochemical Sciences* **28**, 655–662 (2003).

30. T. A. Sysoeva, Assessing heterogeneity in oligomeric AAA+ machines. *Cell. Mol. Life Sci.* **74**, 1001–1018 (2017).

31. J. Y. Bhat, G. Thieulin-Pardo, F. U. Hartl, M. Hayer-Hartl, Rubisco Activases: AAA+ Chaperones Adapted to Enzyme Repair. *Front. Mol. Biosci.* **4** (2017).

32. B. K. Johnson, *et al.*, The Carbonic Anhydrase Inhibitor Ethoxzolamide Inhibits the Mycobacterium tuberculosis PhoPR Regulon and Esx-1 Secretion and Attenuates Virulence. *Antimicrobial agents and chemotherapy* **59**, 4436–45 (2015).

33. J. Rybniker, *et al.*, Anticytolytic Screen Identifies Inhibitors of Mycobacterial Virulence Protein Secretion. *Cell Host & Microbe* **16**, 538–548 (2014).

34. N. Singh, *et al.*, A safe lithium mimetic for bipolar disorder. *Nature Communications* **4**, 1332 (2013).

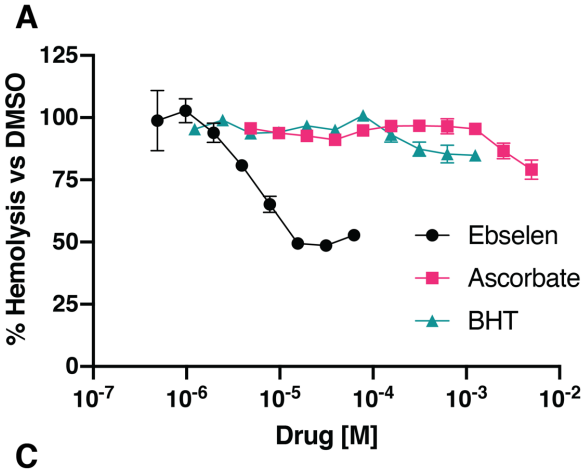
35. A. C. Terentis, *et al.*, The selenazal drug ebselen potently inhibits indoleamine 2,3-dioxygenase by targeting enzyme cysteine residues. *Biochemistry* **49**, 591–600 (2010).

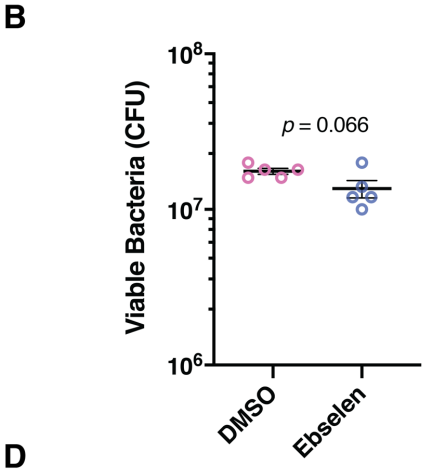
36. J. Lu, *et al.*, Inhibition of bacterial thioredoxin reductase: an antibiotic mechanism targeting bacteria lacking glutathione. *The FASEB Journal* **27**, 1394–1403 (2012).

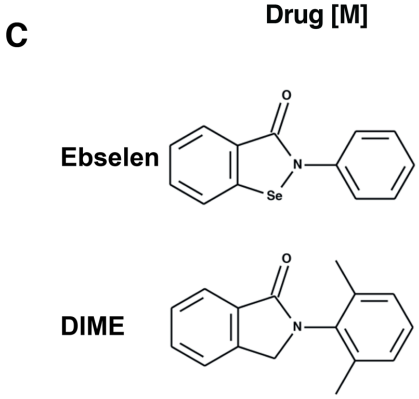
37. L. Favrot, *et al.*, Mechanism of inhibition of Mycobacterium tuberculosis antigen 85 by ebselen. *Nature Communications* **4**, 1–10 (2013).

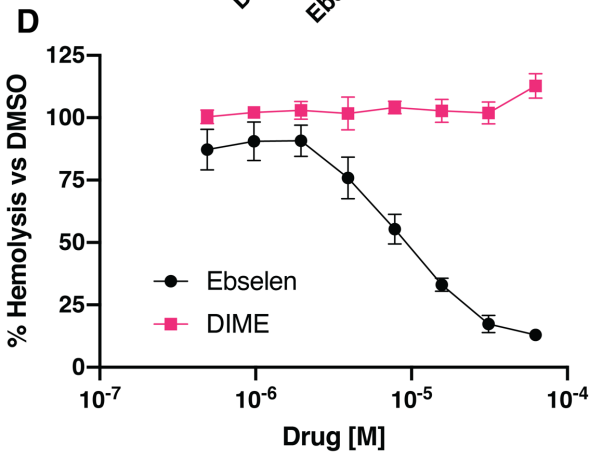
38. J. Kil, *et al.*, Safety and efficacy of ebselen for the prevention of noise-induced hearing loss: A randomised, double-blind, placebo-controlled, phase 2 trial. *The Lancet* **390**, 969–979 (2017).

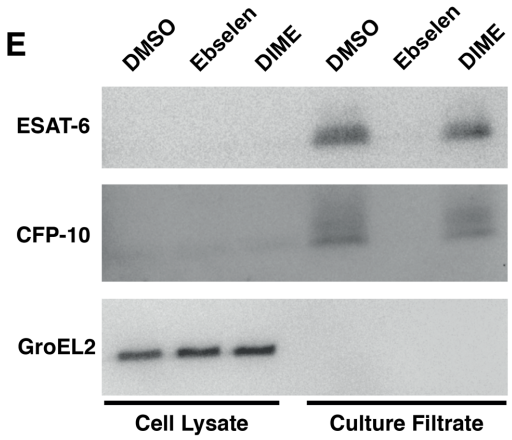
39. J. A. Seddon, *et al.*, Hearing loss in patients on treatment for drug-resistant tuberculosis. *European Respiratory Journal* **40**, 1277–1286 (2012).

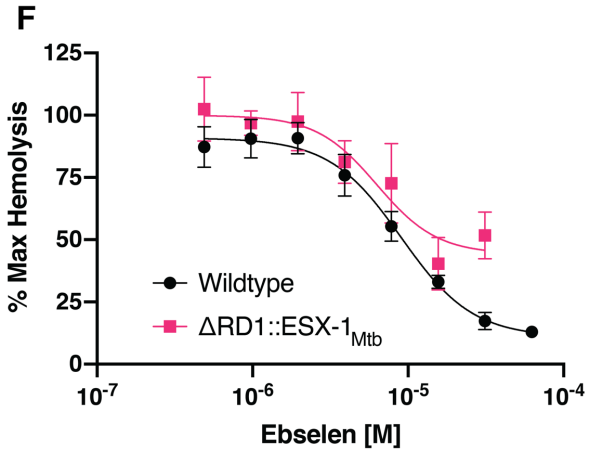

A
% Hemolysis vs DMSO
125
100
75
50
25
0
10-7
10-6
10-5
10-4
10-3
10-2
Drug [M]
Ebselen
Ascorbate
BHT


B
Viable Bacteria (CFU)
108
107
106
p = 0.066
DMSO
Ebselen


C
Ebselen
O
N
Se
DIME
O
N


D
% Hemolysis vs DMSO
125
100
75
50
25
0
10-7
10-6
10-5
10-4
Drug [M]
Ebselen
DIME


E
DMSO
Ebselen
DIME
DMSO
Ebselen
DIME
ESAT-6
CFP-10
GroEL2
Cell Lysate
Culture Filtrate


F
% Max Hemolysis
125
100
75
50
25
0
10-7
10-6
10-5
10-4
Ebselen [M]
Wildtype
ΔRD1::ESX-1Mtb

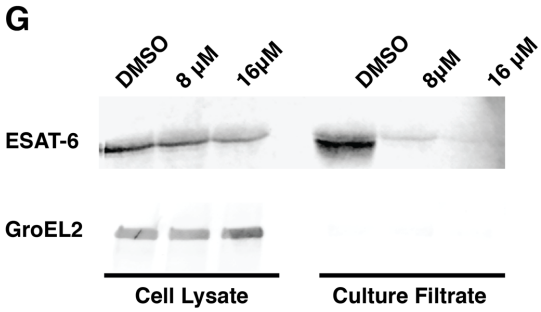
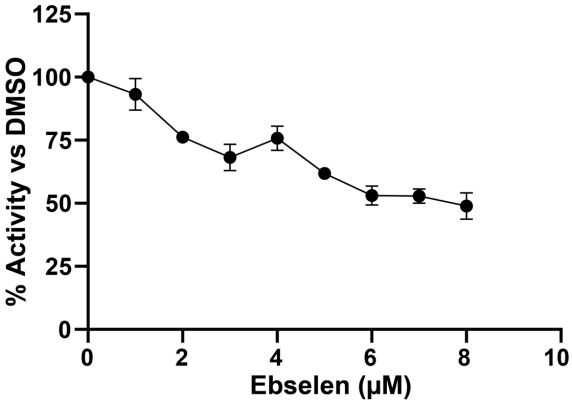

G
DMSO
8 μM
16μM
DMSO
8μM
16 μM
ESAT-6
GroEL2
Cell Lysate
Culture Filtrate

Figure 1: Ebselen treatment inhibits ESX-1 Mediated Membrane Disruption & Secretion A: Percent sRBC hemolysis by *M. marinum* relative to DMSO treatment in the presence of increasing concentrations of antioxidants. Data are presented in biological triplicate. **B:** Structures of ebselen and analogue 2-(2,6-dimethylphenyl)-1-isoindolinone (DIME) **C:** Colony forming units of *M. marinum* following 4 hours of exposure to 62.5 μM Ebselen or 0.5% DMSO. Data representative of 3 independent experiments. Student's t-test. **D:** Percent maximum hemolysis by wildtype *M. marinum* in response to ebselen or DIME treatment. Data presented in biological triplicate **E:** Western blot of lysed *M. marinum* cell pellet and culture filtrate fractions after 4 hours incubation with 32 μM ebselen, DIME, or 0.5 %DMSO. anti-ESAT-6; anti-CFP-10 and anti-GroEL2 (loading control). Blot is representative of 3 independent experiments. **F:** Percent maximum hemolysis by wildtype and ΔRD1::ESX-1$_{Mtb}$ complement *M. marinum* in response to increasing doses of ebselen. Data presented in biological triplicate. **G:** Western blots of lysed *M. tuberculosis* cell pellet and culture filtrate fractions after 96 hours of incubation with 0.5% DMSO, 8 µM Ebselen, or 16 µM Ebselen. Blot representative of three independent experiments. anti-ESAT-6 and anti-GroEL2 (loading control). All error bars represent SEM.

A

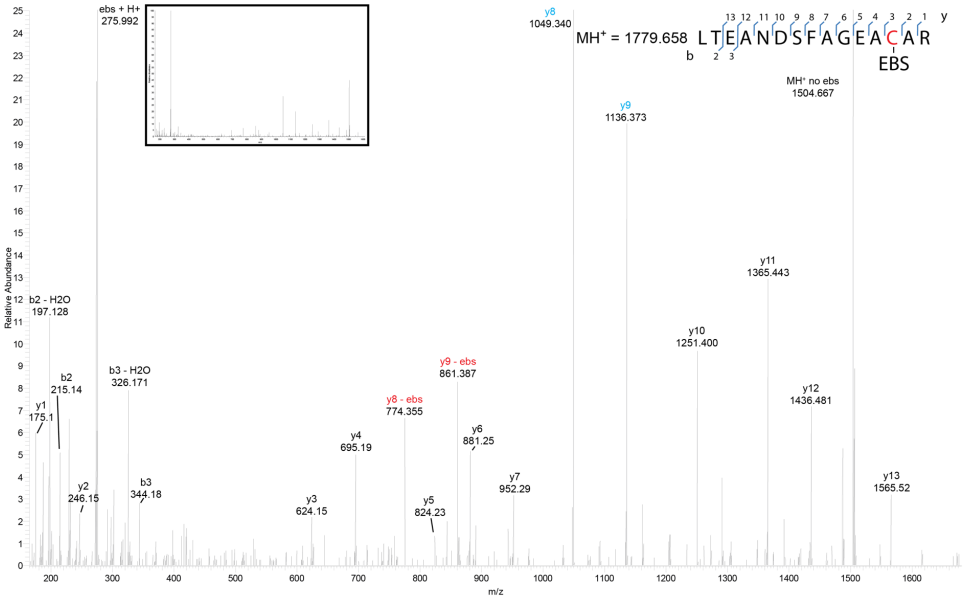


D

		[Ebselen] μM							
Cysteine	Peptide	0	1	2	3	4	5	6	7	8
204	LTEANDSPAGEACAR	-	+	+	+	+	+	+	+	+
531	QLVEASEQCR	-	-	-	-	+	+	+	+	+

B

Cys204 + Ebselen



C

Cys531 + Ebselen

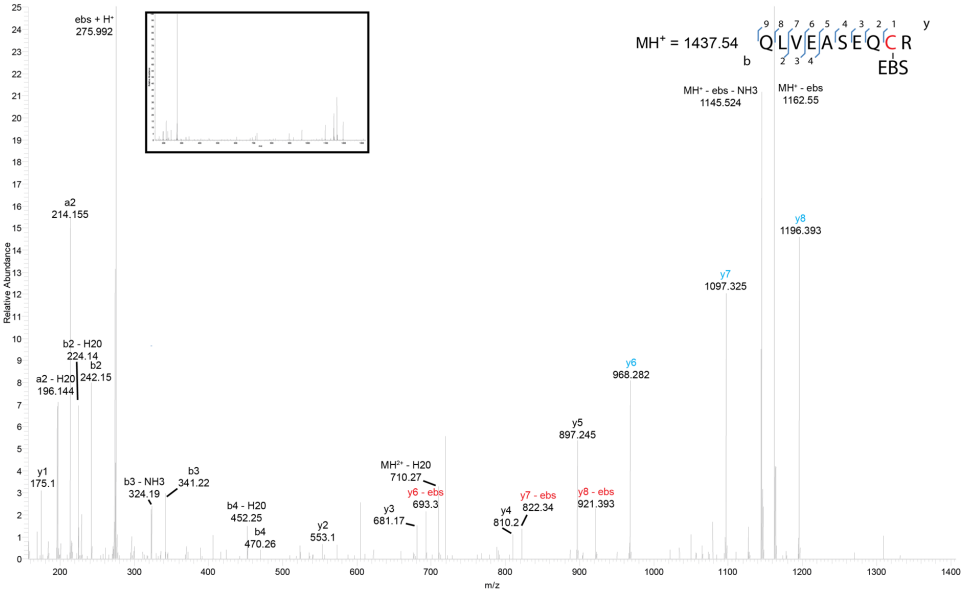


Figure 2: Ebselen inhibits EccA1 through covalent modification of Cys204 and Cys531. A: Dose-response curve of EccA1 treated with ebselen. ATPase activity is normalized to EccA1 treated with DMSO. Data are biological triplicate. Error bars show SEM. **B**: Qualitative representation of ebselen modified peptides containing Cys204 and Cys531 detected by MS at increasing concentrations of ebselen. + symbols indicate concentrations at which ebselen modified peptides were observed. **C and D:** MS/MS spectra of ebselen bound Cys204 and Cys531 generated from EccA1 treated with 8 µM ebselen. Fragmentation map is included in the top right corner. Spectra are scaled for readability, unscaled are inset. Masses of different b/y ions are displayed. y-ions include mass of ebselen, unless otherwise stated. Ionized ebselen is visible at 275.992 m/z, as some ebselen-cysteine bonds are broken during peptide fragmentation. As a result, fragments are observed with (cyan) and without (red) bound ebselen, separated by ebselen's nominal mass (275).

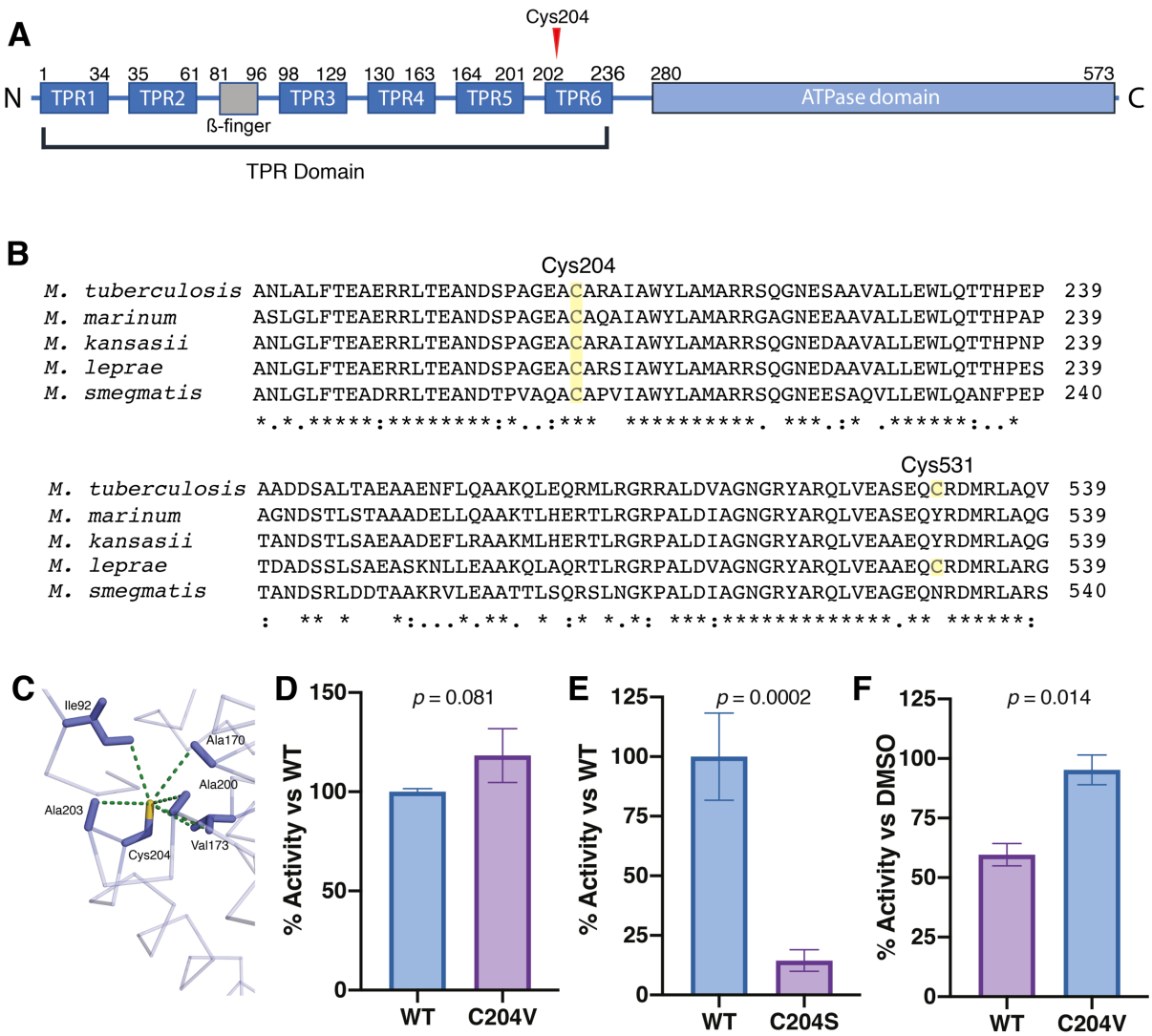


Figure 3: Cys204 is crucial for *M. tuberculosis* EccA1's ATPase activity. Diagram of *M. tuberculosis* EccA1's N-terminal TPR domain including β-finger insertion, and its C-terminal AAA+ ATPase domain. Redrawn from (28).**B:** Clustal Omega multiple sequence alignment of EccA1 homologs with Cys204 and Cys531 annotated. * indicates positions with fully conserved residues. (:)indicates conservation between groups of strongly similar properties (> 0.5 in Gonnet PAM 250 matrix). (.) indicates conservation between groups of weakly similar properties (≤0.5 Gonnet PAM 250 matrix). **C**: Model of Cys204 of EccA1 (PDB accession code 4F3V) and residues located within 5 angstroms, with dashes measuring distance. **D:** ATPase activity of EccA1 C204V point mutant (C204V) vs wildtype EccA1 (WT). Activity normalized to WT. Data from two independent experiments. ATPase activity is normalized to wildtype. Student's t-test. **E**: ATPase activity of EccA1 C204S point mutant (C204S) compared to wildtype EccA1 (WT). ATPase activity is normalized to wildtype. Data from five independent experiments. Student's t-test. **F:** Relative ATPase activity of EccA1 C204V and wildtype EccA1 (WT) treated with 8 µM ebselen. ATPase activity is normalized to DMSO treatment for each protein. Data from three independent experiments. Student's t-test. Error bars show SEM, p values are indicated.