Supplementary Materials for

Structural basis for benzothiazinone-mediated killing of *Mycobacterium tuberculosis*

João Neres, Florence Pojer, Elisabetta Molteni, Laurent R. Chiarelli, Neeraj Dhar, Stefanie Boy-Röttger, Silvia Buroni, Elizabeth Fullam, Giulia Degiacomi, Anna Paola Lucarelli, Randy J. Read, Giuseppe Zanoni, Dale E. Edmondson, Edda De Rossi, Maria Rosalia Pasca, John D. McKinney, Paul J. Dyson, Giovanna Riccardi, Andrea Mattevi, Stewart T. Cole,* Claudia Binda*

*To whom correspondence should be addressed: claudia.binda@unipv.it or stewart.cole@epfl.ch

SUPPLEMENTARY METHODS

Expression and purification of *M. tuberculosis* DprE1

Countless attempts to produce and purify *M. tuberculosis* DprE1 were made, using multiple different constructs, tags, expression systems and growth conditions. We obtained significant expression of DprE1 from the pMAL c4x (maltose-binding protein fusion) and from the pET32b constructs (thioredoxin-His_6 fusion). However, cleavage of the tag led to substantial protein loss in the first case, whereas with the pET32b construct the protein always co-purified with *E. coli* GroEL (Fig. S2). When pure protein was obtained in the latter case, it showed no activity.

Expression and purification of *M. smegmatis* DprE1 and mutants from the pET 32b construct

The *M. smegmatis* protein was expressed with an N-terminal His_6-thioredoxin tag in E. coli BL21-DE3 pLysS, in ZYP-5052 auto-inducing medium at 37°C for 3 hours and then at 16°C overnight. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 8.0, 500 mM

NaCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, 1% Triton X-100, 1 mM phenylmethanesulfonylfluoride (20 g of cells/100 mL buffer containing 2 tablets of complete EDTA-free protease inhibitors cocktail, Roche). Cell disruption was carried out by sonication and the clear lysate was obtained by centrifugation at 16000 rpm for 1 h at 4°C. Protein purification was carried out on an Akta Purifier FPLC system. The clear lysate was loaded onto a 5 mL HisTrap column (GE Healthcare), followed by elution of unspecifically-bound proteins with 50 mM imidazole. The protein was eluted with 250 mM imidazole, and the fractions containing DprE1 were pooled and dialyzed overnight in presence of Turbo 3C protease (LuBioScience) against 50 mM Tris-HCl, 250 mM NaCl, 1 mM $MgCl_2$. The NaCl concentration was adjusted to 500 mM and the cleaved tag and protease were removed by running the protein solution through a 5 mL HisTrap column. The protein solution was concentrated and further purified by size-exclusion chromatography on a Superdex 75 column (GE Healthcare), eluting with 50 mM Tris-HCl, 250 mM NaCl, 1 mM $MgCl_2$. Fractions containing pure protein were pooled, concentrated and flash-frozen in liquid nitrogen for storage at -80°C. SDS-PAGE gels showing the protein purity at the different stages are shown in Fig. S3a.

Docking studies

DNB1 and VI-9376 in their reduced hydroxylamine forms were docked to the active site of DprE1 using GOLD *(42)* and the DprE1-BTZ043 structure. The docking site was defined based on the BTZ043 position in the crystal structure. A covalent bond was defined between the sulfur atom in Cys394 and the nitrogen atom of the reduced nitro group in the two compounds, in order to generate the semimercaptal adduct. Docking was performed using the default settings for high-efficiency docking, using the GoldScore scoring function. The three highest scoring poses were kept and analyzed. The highest scoring pose for each compound is shown in Fig. S5.

Mass spectrometry analysis

DprE1 (approximately 20 µM) was incubated for 2h at 37°C in presence of 25 µM FAD, 50 µM BTZ043 and 200 µM FPR, in 20 mM Tris pH 7.5, 100 µM NaCl and 1 mM $MgCl_2$. The protein was concentrated to a minimum volume and the buffer exchanged to 15 mM ammonium acetate buffer, pH 7.5 on an Amicon centrifugal device (10,000 MWCO, Millipore). Mass spectrometry analysis was performed at the Mass Spectrometry facility at ISIC, EPFL, on a Q-TOF Ultima (Waters).

Synthesis of BTZ-TAMRA (Fig. S6)

***tert*-Butyl (2-(2-(2-((8-nitro-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-2-yl)amino) ethoxy)ethoxy)ethyl)carbamate (3).** 2-(Methylthio)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3] thiazin-4-one (**1**) (*43*) (70 mg, 0.22 mmol) was suspended in 10 ml EtOH. *tert*-Butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (**2**, 54 mg, 0.22 mmol) were added to the suspension, which was then heated to 60°C with stirring and under N_2 for 2 h. The solvent was removed under vacuum and the residue purified by flash chromatography (DCM/2% MeOH), affording the title compound (150 mg, 93%) as a clear oil: R_f 0.26 (DCM/2% MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 9H), 3.28-3.31 (m, 2H), 3.55-3.71 (m, 8H), 3.88 (br s, 2H), 8.72 (s, 1H), 9.06 (s, 1H). HRMS (ESI+) calcd for $C_{20}H_{26}F_3N_4O_7S$ $[M + H]^+$, 523.1469; found 523.1475 (error 1.1 ppm).

2-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)-5-((2-(2-(2-((8-nitro-4-oxo-6-(trifluoromethyl)-4*H*-benzo[*e*][1,3]thiazin-2-yl)amino)ethoxy)ethoxy)ethyl)carbamoyl) benzoate (BTZ-TAMRA). Compound 3 (60 mg, 0.12 mmol) was dissolved in DCM/TFA solution (1:1, 4 ml) and the resulting solution was stirred at rt for 2 h. The solvent was evaporated and the residue dried under high-vacuum, affording 2-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-8-nitro-6-(trifluoromethyl)-4*H*-benzo[*e*][1,3]thiazin-4-one as a thick oil, which was used without further purification for the next step.

To 2-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-8-nitro-6-(trifluoromethyl)-4*H*-benzo[*e*][1,3]thiazin-4-one (7.2 mg, 0.017 mmol) in 2 ml anhydrous DMF was added triethylamine (10 µl, 0.072 mmol) and the solution was stirred under N_2 for 15 min. The NHS ester of 5-carboxytetramethylrhodamine (ChemPep; 7 mg, 0.013 mmol) was added to the solution and stirring continued under N_2 at room temperature for 6 h. The solvent was removed under vacuum and the residue dried overnight under high-vacuum. Purification by column chromatography [gradient of DCM/(MeOH containing 3.5% ammonia)] afforded the title product (9 mg, 83%) as dark purple solid: R_f 0.12 (DCM//(MeOH containing 3.5% ammonia) 9:1); ^{1}H NMR (400 MHz, MeOD) δ 3.27 (s, 12H), 3.64 (t, *J*=5.3 Hz, 2H), 3.65-3.76 (m, 10H), 6.85 (d, *J*=2.4 Hz, 2H), 6.98 (dd, *J*=2.4, 5.5 Hz, 2H), 7.23 (d, *J*=9.4 Hz, 2H), 7.37 (d, *J*=7.9 Hz, 1H), 8.05 (dd, *J*=1.5, 7.9 Hz, 1H), 8.52 (d, *J*=1.5 Hz, 1H), 8.82 (d, *J*=1.9 Hz, 1H), 8.85(d, *J*=1.9 Hz, 1H). HRMS (ESI+) calcd for $C_{40}H_{37}F_3N_6O_9S$ $[M + H]^+$, 835.2368; found 835.2373 (error 0.6 ppm).

SUPPLEMENTARY FIGURES

Figure S1. Sequence alignment of the DprE1 protein sequences from *M. smegmatis* (MSMEG_6382) and *M. tuberculosis* (Rv3790). The starting point of the two constructs of the *M. smegmatis* DprE1 expressed in this work (fl-DprE1 and Δ6-DprE1) are shown. The fl-DprE1 sequence contains two extra aminoacids (shown in red) at the N-terminus. The cysteine that forms a covalent adduct with BTZ043 is highlighted in yellow and the other residues that were mutated in this work (Q343A and K425A) are highlighted in green.

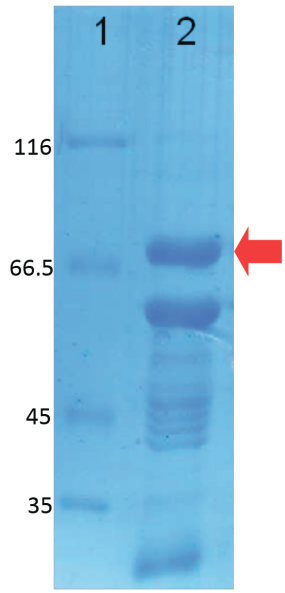

Figure S2. SDS-PAGE showing *E. coli* expression of *M. tuberculosis* DprE1 cloned in a modified version of pET32b vector. Lane 1, molecular weight markers (masses in kDa indicated in the gel); lane 2, elution from Ni-NTA resin of *M. tuberculosis* DprE1 fused to the thioredoxin-His_6 tag (red arrow). The band below was shown to correspond to the monomeric subunit of bacterial GroEL, which co-eluted with DprE1 in all purification steps. The *M. smegmatis* ortholog was expressed in much larger amounts and without GroEL contamination (Fig. S3), which led us to carry out the structural and biochemical studies on *M. smegmatis* DprE1.

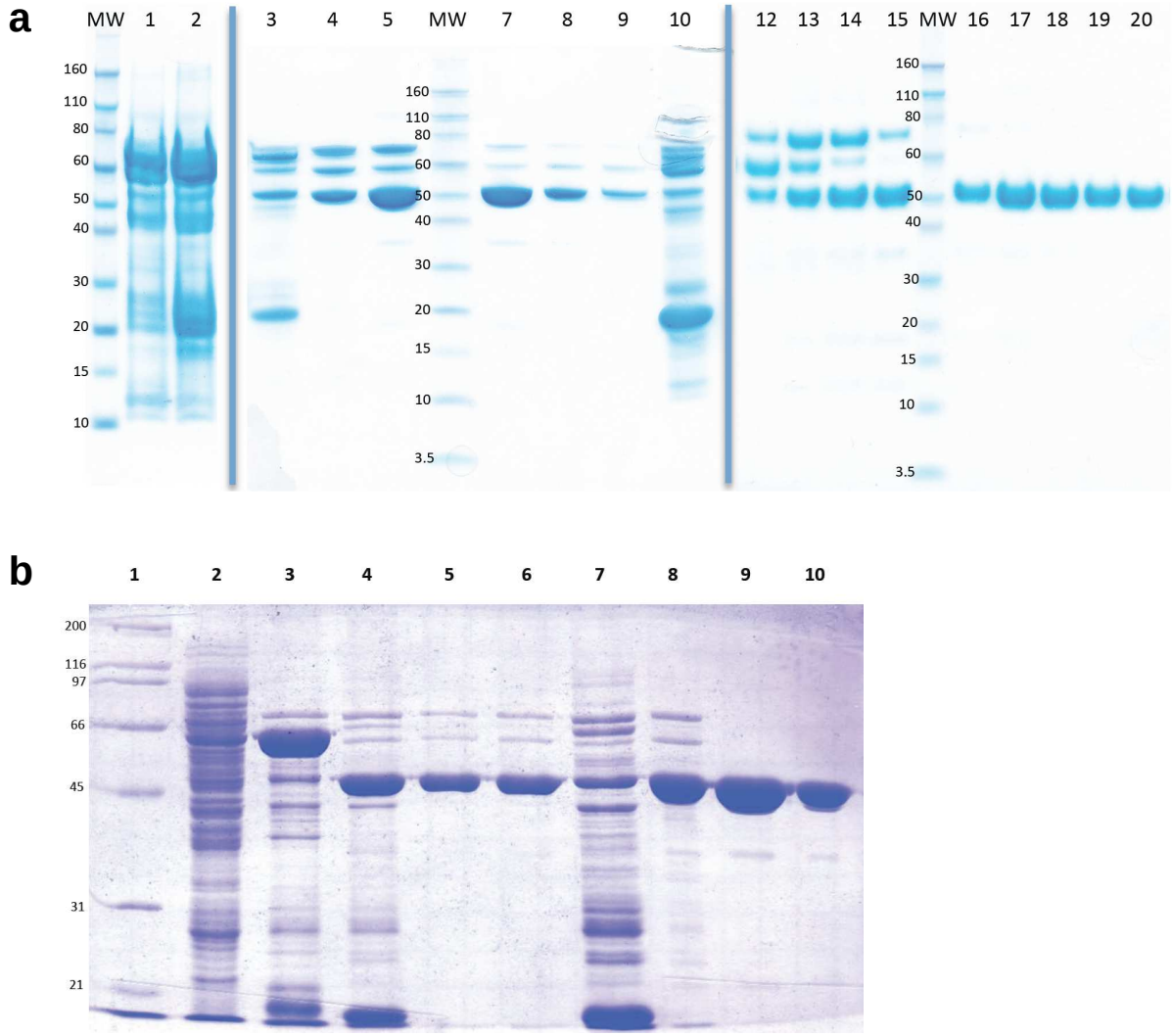

Figure S3. SDS-PAGE analysis of purified *M. smegmatis* DprE1. **(a)** DprE1-fl construct cloned in pET32b. Lanes with molecular weight markers are labeled with "MW" (masses in kDa indicated in the gel); lanes 1 and 2, HisTrap Trx-His_6-DprE1-fl elutions from HisTrap (200 mM imidazole); lane 3: Trx-His_6 tag-cleaved DprE1 sample loaded onto a second HisTrap column; lanes 4-9, flowthrough fractions of the second HisTrap column containing DprE1-fl; lane 10, HisTrap wash by 200 mM imidazole; lanes 12-20, Superdex 75 elution fractions (fractions 16-20 contained pure DprE1). **(b)** DprE1-Δ6 construct cloned in pET SUMO. Lane 1, molecular weight markers (mass in kDa indicated in the gel); lane 2, HisTrap column wash; lane 3, His_6-SUMO-DprE1-Δ6 elution from HisTrap (100 mM imidazole); lane 4, His_6-SUMO tag-cleaved DprE1 sample loaded onto a second HisTrap column; lanes 5-6, flowthrough fractions of the second HisTrap column containing DprE1-Δ6; lane 7, HisTrap wash by 100 mM imidazole; lane 8, concentrated flowthrough loaded onto MonoQ column; lane 9, concentrated MonoQ elution fractions loaded onto Superdex200; 10, final concentrated sample eluted from Superdex200.

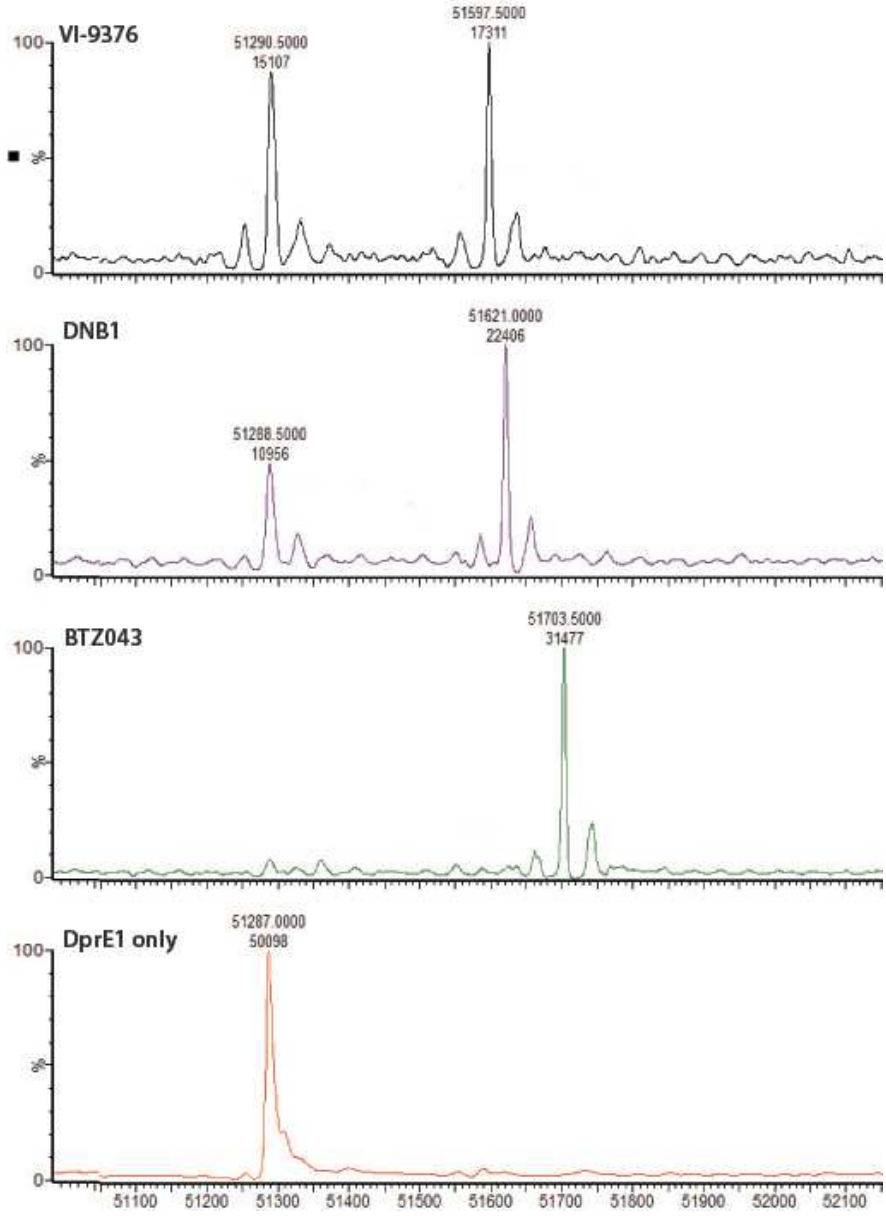

Figure S4. Mass spectrometry analysis of the covalent DprE1-inhibitor adducts, namely BTZ043, DNB1 and VI-9376, and a DprE1 control. The expected masses are 51288 (free protein), 51703 (BTZ043-labeled DprE1), 51633 (DNB1-labeled DprE1) and 51616 (VI-9376-labeled DprE1). The expected mass was observed for the BTZ043 adduct. The masses observed for the DNB1- and VI-9376-protein adducts were different from those expected based solely on the formation of the semimercaptal product, indicating that other reactions took place in these cases.

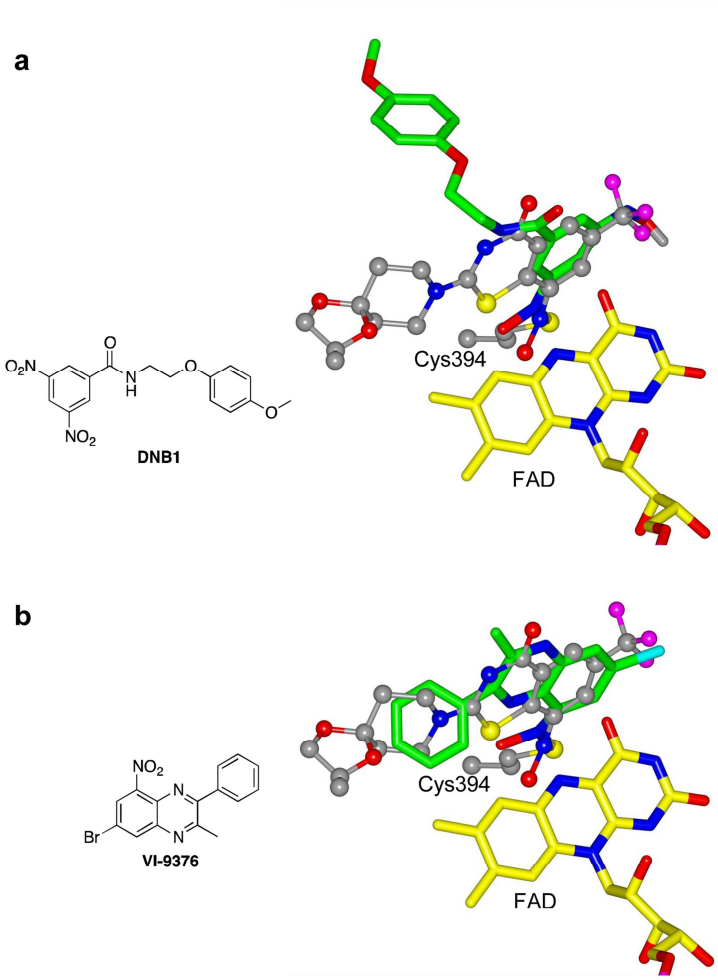

Figure S5. Highest-scoring docked poses of DNB1 and VI-9376, following covalent docking using GOLD, in the active site of DprE1. **(a)** DNB1; **(b)** VI-9376. The docked structures are shown superposed with the crystallographic BTZ043 structure. Carbon atoms are colored in yellow in the FAD cofactor and in green in the docked compound structures. The crystallographic conformation of BTZ043 is in grey ball and stick representation. In all molecules nitrogen, oxygen, and sulfur atoms are colored in blue, red, and yellow, respectively. Fluoride and bromine atoms are colored in magenta and cyan, respectively.

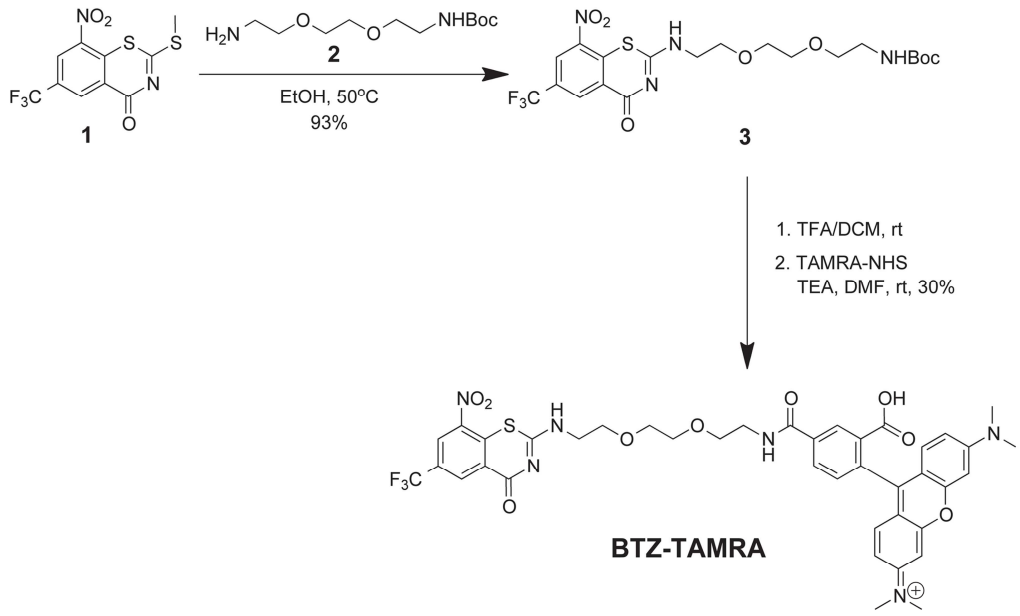

Figure S6. Synthesis of **BTZ-TAMRA**.